Regulation and control of L-arabinose catabolism in *Aspergillus niger*

Marco de Groot

| Stellingen

1. Het regulatie mechanisme van de L-arabinose katabole route en de arabinan afbrekende enzymen in *Aspergillus niger* bestaat uit tenminste twee componenten en wedijvert met het XLNR regulatie systeem met betrekking tot koolstof kataboliet repressie. (dit proefschrift)

2. Door gebruik te maken van klassieke genetica kunnen nieuwe *Aspergillus niger* stammen gegenereerd worden welke waardevol zijn voor het moderne genomics en proteomics onderzoek. (dit proefschrift)

3. Binnen een proteomics experiment is de kwaliteit van het monster en daarmee de keuze van de monstervoorbewerking van cruciaal belang en zal een klassieke fractionatiemethode niet altijd uitkomst bieden. (Lopez & Melov, 2002; Huber *et al.*, 2003; Prokisch *et al.*, 2004)

4. Het gebruik van schimmels in de strijd tegen malaria toont aan dat ook agrotechnologische technieken humaan gerelateerde epidemiologische problemen kunnen helpen oplossen. (Scholte *et al.*, 2005)

5. Wetenschap is als werken met computers; zonder geduld kom je nergens.

6. Wantrouw de promovendus zonder stress.

7. Door te stellen dat een terroristische aanval "not if but when" zal plaatsvinden is een "self fulfilling prophecy".

Stellingen behorende bij het proefschrift:

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| Chapter 1

Introduction

Introduction

Plant cell walls

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They make up 90% of the plant cell wall leaving the remaining 10% for proteins. The polysaccharides can be divided into three groups: cellulose, hemicellulose, and pectin (McNeill *et al.*, 1984). In this thesis we concentrate on L-arabinan degradation and L-arabinose catabolism in *Aspergillus*. L-arabinan/L-arabinose residues can be found in hemicellulose and in the hairy regions of pectin. L-arabinose can for instance be attached to the xylose residues of xyloglucans wich consists of a β-1,4-linked D-glucose backbone substituted by D-xylose. The xyloglucans interact with cellulose microfibrils by the formation of hydrogen bonds thereby contributing to the structural integrity of the cellulose network (Carpita & Gibeaut, 1993). Cereal xylans contain large quantities of L-arabinose and are therefore often referred to as arabinoxylans. Arabinose is connected to the backbone of xylan via an α-1,2- or α-1,3-linkage either as single residues or as short side chains. These L-arabinose side chains may also contain galactose or xylose residues. The xylan backbone may also contain acetyl (O-2, O-3 linked) or glucuronic acid (α-1,2-linked) residues attached to xylose. At the terminal arabinose residues aromatic residues may be found (feruloyl and *p*-coumaroyl) attached to the O-5 (Saulnier *et al.*, 1995; Smith & Hartley, 1983; Wende & Fry, 1997).

Initially it was believed that pectins consists of a backbone of α-1,4-linked D-galacturonic acid residues. In this model the galacturonic acid backbone is interrupted by α-1,2-linked L-rhamnose residues in specific "hairy" regions. Long side chains of pectin consisting mainly of L-arabinose and D-galactose residues can be attached to these rhamnose residues. The arabinan chains consist of a main chain of α-1,5-linked L-arabinose residues that may be substituted by α-1,3-linked L-arabinose and by feruloyl residues attached terminally to O-2 of the arabinose residues. (Colquhoun *et al.*, 1994; Guillon & Thibault, 1989). In view of more recent experimental proof a new model of the structure of pectin has been proposed in which the rhamnogalacturonan is the backbone of which the homogalacturonan is a side chain (Vincken *et al.*, 2003).

The black *Aspergillus*

Saprophytes and phytopathogens degrade polysaccharides using enzymes. The genus *Aspergillus* is one group of species that has this capability. The first record of this fungus can be found in Micheli's *Nova Plantarum Genera* (Micheli, 1729). It took up to 1926 until another effort was made to classify the *Aspergilli*. The first classification of these fungi was proposed describing 11 groups within the genus

(Thom & Church, 1926). In 1945 this was revised resulting in 14 groups (Thom & Raper, 1945). A number of these fungi is pathogenic (e.g., *A. fumigatus*, *A. flavus*, and *A. parasiticus*). However, the black *Aspergilli* harbor species which are saprophytic and very important for industrial applications (e.g. *A. niger* and *A. oryzae*). In addition to the morphological techniques traditionally applied, new molecular and biochemical techniques have been used in the reclassification of this group of *Aspergilli* (Hamari *et al.*, 1997; Kusters-van Someren *et al.*, 1991; Megnegneau *et al.*, 1993; Nikkuni *et al.*, 1996; Parenicová *et al.*, 1997; Varga *et al.*, 1994). These analyses resulted in the clear distinction of eight groups of black *Aspergilli* (Parenicová *et al.*, 1997).

A major advantage of these species in industrial applications is that products of several species have obtained a GRAS (Generally Regarded As Safe) status. This allows for use in food and feed applications. Besides the GRAS (Schuster *et al.*, 2002) status the black *Aspergilli* have some other characteristics which make them ideal organisms for industrial applications, such as good fermentation capabilities as for citric acid production (Karaffa & Kubicek, 2003) and high levels of protein secretion. The wide variation of plant cell wall degrading enzymes produced by *Aspergillus* is of major importance for the food and feed industry such as soy sauce production. Recently, several *Aspergillus* spp. have received increased interest as hosts for heterologous protein production (Dean & Timberlake, 1989).

Arabinan degradation

As mentioned above *Aspergilli* can degrade the complex structure of the plant cell wall and for this they have a large number of enzymes available (De Vries & Visser, 2001). One part of this degradation process concerns the release of L-arabinose residues present in hemicelluloses and pectin. *Aspergillus* has a number of enzymes available each having a distinct role in these processes. Two types of enzymes removing L-arabinose residues are the α-L-arabinofuranosidases and arabinoxylan arabinofuranohydrolases (EC 3.2.1.55). These enzymes are not specific for *Aspergilli* and their respective genes can be found in many different microorganisms.

Several arabinofuranosidases and arabinoxylan arabinofuranohydrolases have been purified from *Aspergillus* spp. and studied with respect to their activity on polymeric and oligomeric substrates (Beldman *et al.*, 1993; Gielkens *et al.*, 1997; Gunata *et al.*, 1990; Kaneko *et al.*, 1993; Kimura *et al.*, 1995; Kormelink *et al.*, 1991; Luonteri *et al.*, 1995; Muzakhar *et al.*, 1999; Ramon *et al.*, 1993; Rombouts *et al.*, 1988; Tagawa & Kaji, 1988; Van der Veen *et al.*, 1991; Wood & McCrae, 1996).

As noted these enzymes have a very distinct substrate specificity. The *A. niger* arabinofuranosidase purified by Kaneko et al. (Kaneko *et al.*, 1993) was able to release only terminal α-1,3-linked arabinose residues, whereas

arabinofuranosidase B from *A. niger* was able to release terminal α-1,2-, α-1,3- and α-1,5-linked arabinose residues (Beldman *et al.*, 1993).

Two biochemically characterised arabinofuranosidases of *A. niger* are ABFA and ABFB and the latter is able to release L-arabinose from terminal singly substituted residues from the intact polysaccharide as well as from xylo-oligosaccharides. ABFA was able to release arabinose from both terminal and nonterminal singly substituted xylose residues from xylooligosaccharides. Arabinoxylan arbinofuranohydrolase (AXH) is specifically involved in arabinoxylan degradation while ABFA and ABFB are more general arabinose-releasing enzymes (Kormelink *et al.*, 1993). Arabinan polysaccharides having an α-1,5-linkage, found as side chains of pectin, can be hydrolysed as well by endoarabinanases (EC 3.2.1.99). The degradation patterns of linear (1–5)-α-L-arabino-oligosaccharides using *A. niger* endoarabinanase demonstrated that the enzyme is not (or is hardly) able to release terminal residues but preferentially acts on internal linkages (Dunkel & Amado, 1995). Although arabinofuranosidases are able to hydrolyze polymeric arabinan, endoarabinanases strongly enhance the efficiency of arabinan degradation and positively influence the action of arabinofuranosidases. So far, no biochemical indications have been obtained for the presence of more than one endoarabinase in any *Aspergillus* sp.

The genes encoding these enzymes have been cloned, being *abfA* (Flipphi *et al.*, 1993c), *abfB* (Flipphi *et al.*, 1993b), *abnA* (Flipphi *et al.*, 1993a), and *axhA* (Gielkens *et al.*, 1997).

The difference between arabinoxylan arabinofuranohydrolases and arabinofuranosidases is also apparent from the assignment to the glycosidase families. Arabinofuranosidases are assigned to families 51 and 54, which both have a retaining mechanism, whereas arabinoxylan arabinofuranohydrolases belong to family 62 (Table 1). ABFA from *A. niger* is assigned to a different family than ABFB, which might reflect the differences in the substrate specificity of the enzymes. Both enzymes are able to release arabinose from arabinan and sugar beet pulp, but only ABFB is able to release arabinose from xylan. Only one endoarabinanase-encoding gene has been described in *Aspergillus* sp. Based on the sequence of its gene, ABNA was assigned to family 43 of the glycosidases and has an inverting mechanism (Table 1).

Table 1. *Aspergillus* arabinofuranosidase (ABF), endoarabinanase (ABN) and arabinoxylan arabinofuranohydrolase (AXH) genes and their family assignment.

Species	Enzyme	Gene & accession number	Glycosidase family	Reference
A. nidulans	ABFB	*abfB* (Y13759)	54	(Gielkens *et al.*, 1999b)
A. niger	ABFA	*abfA* (L29005)	51	(Flipphi *et al.*, 1993c)
A. niger	ABFB	*abfB* (X74777)	54	(Flipphi *et al.*, 1993b)
A. niger	ABNA	*abnA* (L23430)	43	(Flipphi *et al.*, 1993a)
A. niger	AXHA	*axhA* (Z78011)	62	(Gielkens *et al.*, 1997)

L-arabinose catabolism

Once arabinan is degraded to L-arabinose, *Aspergillus* is able to take up and catabolise this pentose using its L-arabinose catabolic pathway (Witteveen *et al.*, 1989). This pathway is shared in part with D-xylose degradation. D-xylose is released from xylan by the xylanolytic system. The L-arabinose catabolic pathway consists of a series of oxidizing and reducing reactions. L-arabinose is reduced by L-arabinose reductase (EC 1.1.1.21) (ARDA) resulting in L-arabitol, which is then converted to L-xylulose by L-arabitol dehydrogenase (EC 1.1.1.12) (LADA). L-xylulose on its turn is reduced to xylitol by L-xylulose reductase (EC 1.1.1.10) (LXRA). This enzyme is also referred to as the NADP-dependent xylitol dehydrogenase. At this point D-xylose also enters the pathway after conversion to xylitol catalysed by D-xylose reductase (EC 1.1.1.21) (XYRA). Xylitol is then converted to D-xylulose by NAD-dependent xylitol dehydrogenase (EC 1.1.1.9) (XDHA). This enzyme is also referred to as D-xylulose reductase. As a last step D-xylulose is phosphorylated by D-xylulose kinase (EC 2.7.1.17), and xylulose 5-phosphate then enters the pentose phosphate pathway for further metabolism (Figure 1).

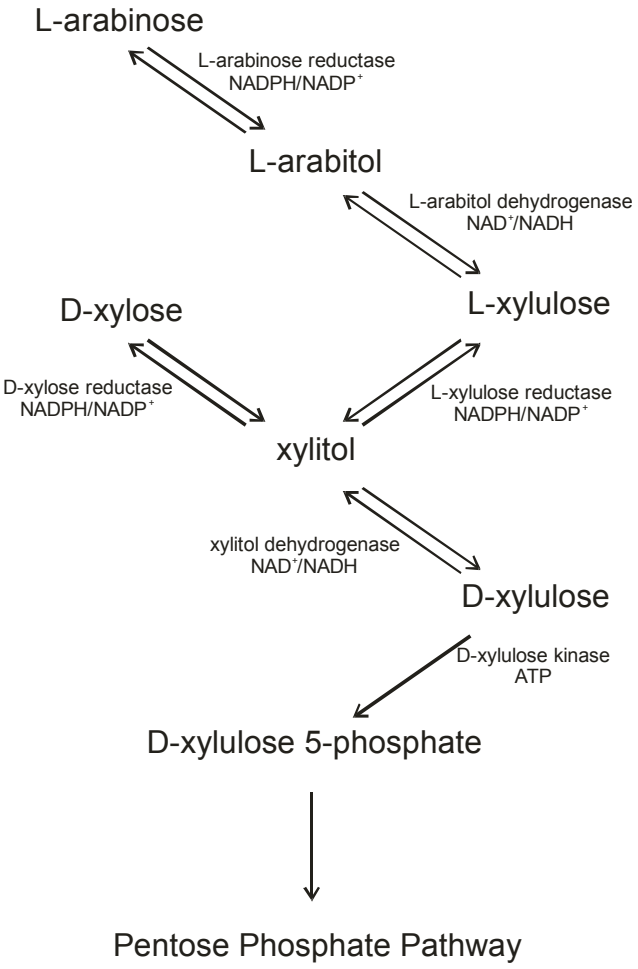

Figure 1. The L-arabinose/D-xylose catabolic pathway.

For *A. niger*, so far only genes encoding D-xylose reductase (*xyrA*) (Hasper *et al.*, 2000) and D-xylulokinase (*xkiA*) (Chapter 4 & VanKuyk *et al.*, 2001) have been described. In *Trichoderma reesei*, genes encoding L-arabitol dehydrogenase (*lad1*) (Richard *et al.*, 2001) and xylitol dehydrogenase (*xdh1*) (Seiboth *et al.*, 2003) have been identified.

Regulation of gene expression

Relatively little is known about the regulation of gene expression of the arabinolytic system. Induction of the extracellular enzymes was the first aspect to be elucidated. Production of arabinofuranosidases was observed on arabinoxylan (Kaneko *et al.*, 1993), sugar beet pulp (Flipphi *et al.*, 1993b; Van der Veen *et al.*, 1991), and L-arabinose and L-arabitol (Ramon *et al.*, 1993; Van der Veen *et al.*, 1993). Arabinoxylan arabinofuranohydrolases are produced when *Aspergillus* is grown on oat straw (Kormelink *et al.*, 1991) and birchwood xylan (Gielkens *et al.*, 1997). The production of endoarabinanases by *Aspergillus* spp. was observed on sugar beet pulp (Van der Veen *et al.*, 1991) and on L-arabinose and L-arabitol

(Ramon *et al.*, 1993; Van der Veen *et al.*, 1993). In *A. niger*, induction of *abnA* seems to occur simultaneously with the induction of *abfA* and *abfB* (Flipphi *et al.*, 1994).

Intracellular arabitol is believed to be the low molecular weight inducer of the arabinanase system. When the intracellular arabitol concentration is high like in a D-xylulose kinase defective mutant (Witteveen *et al.*, 1989) also a high arabinanase production is observed (De Vries *et al.*, 1994). In overexpression strains of the individual arabinanases, expression of the arabinanase related genes which are not overexpressed is decreased. This has been proposed to be due to the titration of a specific transcriptional activator binding to the promoter of the multiple copies of the overxepressed gene (Flipphi *et al.*, 1994). As the genes encoding components of the L-arabinose catabolic pathway have not been identified we do not know under which conditions these are expressed. However, studies on enzyme levels showed that the intracellular enzymes L-arabinose reductase, L-arabitol dehydrogenase, L-xylulose reductase, xylitol dehydrogenase and D-xylulose kinase are induced when L-arabinose is present in the growth medium (Witteveen *et al.*, 1989). Besides this direct effect on the pathway by its substrate, the enzyme production is also influenced by other metabolites via carbon catabolite repression (ccr) mediated by CREA. CREA mutants show derepression of the arabinase system when both D-glucose and L-arabinose are present as growth substrates (Ruijter *et al.*, 1997). In figure 2 a model overview is given of all the components involved in the coordinated expression of the *A. niger* arabinanase system.

A related metabolic system concerns the xylan degrading enzymes. In fact this system shares the lower part of the L-arabinose catabolic pathway for the metabolisation of D-xylose. In *A. niger* the xylanase system is regulated via the transcriptional activator XLNR which was first identified as a xylanase regulator (Van Peij *et al.*, 1998b), but was later shown to be also involved in the expression regulation of other hemicellulases and cellulases, but not of the arabinanase system (Gielkens *et al.*, 1999a; Van Peij *et al.*, 1998a). Interestingly XLNR also acts on the expression of the gene encoding the D-xylose reductase, however no significant control on the remaining enzymes in the pathway was observed (Hasper *et al.*, 2000).

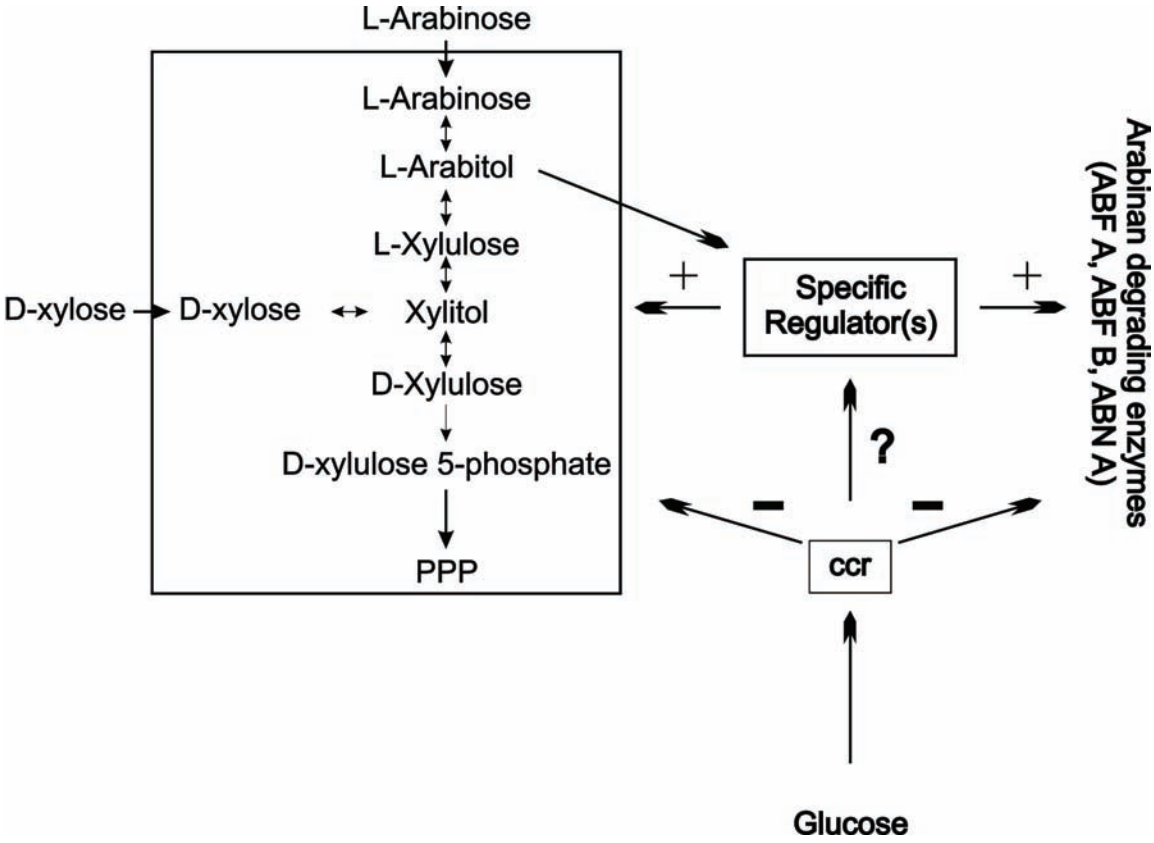

Figure 2. Schematic overview of the L-arabinose/D-xylose catabolic pathway and arabinan degrading enzymes appear to be regulated via specific regulators and repressed by carbon catabolite repression mediated by CREA. L-arabitol is believed to be the key metabolite in the induction pathway of the arabinan degrading enzyme system.

Metabolic control analysis

A metabolic pathway can be engineered for a certain purpose like increasing its flux or changing an important metabolite concentration. In order to increase a flux through a pathway one can increase all its enzymes by overexpression, however this may not be necessary as not all components have an equal influence on the flux. In order to predict which enzymes of a pathway are determining the flux, metabolic control analysis (MCA) can help. MCA is a quantitative framework for analysing the steady state behaviour of metabolic pathways. It quantifies the dependence of the system variables (like fluxes and concentrations) on the parameters (like enzyme concentrations and kinetic properties), which determine the steady state of the system.

In MCA flux control is quantified in terms of the flux control coefficients ($C_i^{J_j}$), which specify the sensitivity of the flux (J_j) to changes in the individual enzyme concentrations or the rate of the individual reaction steps (v_i) in the pathway, i.e.

$$C_i^{J_j} = \frac{\frac{\partial J_j}{J_j}}{\frac{\partial v_i}{v_i}} = \frac{v_i}{J_j}\frac{\partial J_j}{\partial v_i}$$

The concentration control coefficients ($C_i^{M_j}$) are defined by the sensitivity of a metabolite concentration M_j to changes in the individual enzyme concentrations or the rate of the individual reaction steps in the pathway i.e.

$$C_i^{M_j} = \frac{\frac{\partial M_j}{M_j}}{\frac{\partial v_i}{v_i}} = \frac{v_i}{M_j}\frac{\partial M_j}{\partial v_i}$$

By using these concepts one can construct a mathematical model which can aid in the discovery of highly flux or metabolite controlling enzymes in a pathway, thereby guiding a researcher in successfully engineering a pathway (Fell, 1992; Heinrich & Rapoport, 1974; Liao & Delgado, 1993; Westerhoff & Chen, 1984).

Uses and potentials of *A. niger* in industry

The use of *A. niger* enzymes is particularly attractive in the food industry because they are generally regarded as safe (Archer, 2000). However, the search for new hemicellulose modifying activities has prompted studies of other organisms such as *Trichoderma reesei* and *Phanerochaete chrysosporium* (Castanares *et al.*, 1995; Margolles-Clark *et al.*, 1996). *A. niger* has a number of industrially interesting applications such as the production of organic acids and, due to its natural ability for high level extracellular protein secretion, the production of a variety of enzymes and proteins (Gouka *et al.*, 1997; Maras *et al.*, 1999). Enzyme preparations find a wide application in different industries, including agriculture, textile, detergent, pulp and paper, and food and feed and beverage industries (Duarte *et al.*, 1999; Farrel & Skerker, 1992; Grassin & Fauquembergue, 1996; Maat *et al.*, 1992; Nissen *et al.*, 1992).

Aim and outline of the thesis

The aim of this thesis is to further characterize the L-arabinose catabolic pathway enzyme kinetics and the regulation of expression of the genes encoding the catabolic enzymes and the arabinan degrading enzyme system of *A. niger.*

In chapters two and three the isolation of *A. niger* mutants which are impaired in the induction of the L-arabinose catabolic pathway and the arabinanase enzymes

is described. Chapters four and five focus on the isolation of several genes encoding the enzymes of the L-arabinose catabolic pathway, L-arabitol dehydrogenase, xylitol dehydrogenase and xylulose kinase, and on characterisation of these enzymes. Regulation of gene expression is investigated as well as substrate specificity of the enzymes in light of their physiological role. In chapter six the kinetic parameters of the enzymes of the L-arabinose catabolic pathway are used to perform metabolic control analysis focusing on the in silico manipulation of metabolic flux and intracellular L-arabitol levels.

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| Chapter 2

A versatile selection method to obtain catabolic and regulatory mutations in *Aspergillus niger* pentose metabolism

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Summary

Aspergillus niger is known for its biotechnological applications, such as the use of xylan degrading enzymes. Unravelling the regulation of the production of hemicellulose modifying enzymes is of interest for food technology, but also for industrial conversion of xylan to pentoses for the production of fuel ethanol by recombinant yeast. The aim of this study was to develop a new mutant selection system that identifies genes involved in pentose catabolism and its regulation. An *A. niger* strain carrying the *xkiA1* mutation lacks D-xylulose kinase and therefore cannot grow on certain pentoses, such as D-xylose and L-arabinose, but these pentoses still repress the use of other carbon sources such as D-gluconate. This genetic background was used to select for pentose-derepressed mutants on xylitol, L-arabinose and D-xylose. Genetic and biochemical characterisation revealed that these new mutants were affected in several aspects of pentose metabolism (pentitol uptake and pentose catabolism, pentose-mediated carbon catabolite repression). Detailed investigation of a xylitol-derepressed mutant showed its xylitol transport to be severely affected. This mutant selection method is a sensitive tool to isolate new mutants in which pentose metabolism is altered.

Introduction

Aspergillus is a genus of mainly saprophytic fungi known for their biotechnological applications. Many of these applications are related to hemicellulose degradation, such as the use of xylanases and arabinanases. The identification of new hemicellulose-modifying enzymes and their regulation has been the subject of recent studies (reviewed by (De Vries & Visser, 2001)). The use of *A. niger* enzymes is particularly attractive in the food industry because they are generally regarded as safe (Archer, 2000). The search for new hemicellulose-modifying activities has prompted studies of other organisms such as *Trichoderma reesei* and *Phanerochaete chrysosporium* (Margolles-Clark *et al.*, 1996) (Castanares *et al.*, 1995). Another interest in this research field is related to the production of fuel ethanol from lignocellulose by recombinant yeast strains. Wild type *Saccharomyces cerevisiae* does not metabolise D-xylose, but the introduction of pentose-catabolising activities and hemicellulose-degrading capabilities may greatly enhance ethanol production from xylan and L-arabinose by transgenic yeast (Jeppsson *et al.*, 2002; La Grange *et al.*, 2001).

In most organisms, the hemicellulose-degrading enzymes can be induced by monomeric sugars and combinations thereof (De Vries, 2003). The transcriptional regulator *xlnR*, affecting most xylanolytic functions, was identified in *A. niger* by (Van Peij *et al.*, 1998). (Hasper *et al.*, 2000) found the regulation of intracellular

xylose metabolism to be linked to the regulation of extracellular xylanases via this regulator. Recently, (Chapter 3 & De Groot *et al.*, 2003) have described the effect of two arabinanase regulatory mutations upon intracellular pentose metabolism and hemicellulase production in *A. niger*. Moreover, arabinanase regulation was demonstrated to be antagonistic to xylanase regulation. (De Vries *et al.*, 1999) found that increasing concentrations of D-xylose result in increased carbon catabolite repression (CCR), mediated via *creA*. The *araA* and *araB* mutations described by (Chapter 3 & De Groot *et al.*, 2003) not only affect arabinan metabolism but apparently also play a role in L-arabinose-dependent CCR. These findings have led to a complex model, which described the various interactions between the different inducing and repressing systems. However, both the nature of these interactions and the components of the signalling pathways involved remain obscure. Despite the availability of new tools such as proteomics and genome-wide transcription analysis, it will be difficult to identify these components because of their low abundance and because they have not been described in any other organism yet. Selection of new mutants in these regulation systems and signalling pathways is required to identify more of its components. In this study we investigated whether the mutant selection system used by (Chapter 3 & De Groot *et al.*, 2003) can be more broadly applied to identify new genes involved in regulating pentose metabolism and hemicellulose catabolism. We show this selection system to be capable of selecting new mutants affected in L-arabinose, D-xylose and xylitol-mediated CCR. Detailed investigation of a xylitol-derepressed mutant shows it to be severely hampered in xylitol transport.

Materials and methods

Strains and growth conditions

All *A. niger* strains used for this study were derived from *A. niger* N400 (CBS 120.49) and are described in Table 1.

NW315 was used for mutagenesis and N402 was used as a reference strain. Mycelium cultures were grown at pH 6 in minimal medium (MM) containing per litre 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 10 mg EDTA, 4.4 mg $ZnSO_4.7H_2O$, 1.0 mg $MnCl_2.4H_2O$, 0.32 mg $CoCl_2.6H2O$, 0.32 mg $CuSO_4.5H_2O$, 0.22 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.47 mg $CaCl_2.2H_2O$ and 1.0 mg $FeSO_4.7H_2O$ and carbon sources as indicated in the text. Culturing was done in a rotary shaker at 250 rpm and 30°C or in a 2.5 l jacketed Applicon bioreactor with pH controlled at 5.0 and dissolved oxygen tension controlled at a minimum of 30%. For growth of strains with auxotrophic mutations, the necessary supplements were added to the

medium. For growth on plates, 1.5% (w/v) agar was added to the medium before autoclaving.

Table 1. *A. niger* strains used in this study.

Strain	Genotype	Reference
N402	*cspA1*	Bos *et al.*, 1988
N572	*cspA1; xkiA1; nicA1*	Witteveen *et al.*, 1989
NW148	*hisD4; lysA7 bioA1 cspA1; leuA1; metB10 argB15; pabA1; cnxC5; trpB2*	Chapter 3 & De Groot *et al.*, 2003
NW315	*fwnA1; pyrA6 cspA1; xkiA1; nicA1*	Witteveen *et al.*, 1989
689.1	*fwnA1; pyrA6 cspA1; xkiA1; nicA1; xtlA36*	This study
689.2	*fwnA1; pyrA6 cspA1; xkiA1; nicA1; xtlA46*	This study
689.3	*fwnA1; pyrA6 cspA1; xkiA1; nicA1; xtlA55*	This study
NW324	*lysA7 bioA1 cspA1; leuA1; nicA1; xtlA36*	This study
NW316	*fwnA1; pyrA6 cspA1; xkiA1; nicA1 araA4*	Chapter 3 & De Groot *et al.*, 2003
NW318	*fwnA1; pyrA6 cspA1 araB3; xkiA1; nicA1*	Chapter 3 & De Groot *et al.*, 2003
NW320	*fwnA1; lysA7 bioA1 cspA1; nicA1 araA4*	Chapter 3 & De Groot *et al.*, 2003
786.19	*lysA7 bioA1 araB3 cspA1; leuA1; metB10 argI15*	Chapter 3 & De Groot *et al.*, 2003
761.3	*fwnA1; pyrA6 cspA1; xkiA1 araC33; nicA1; xtlA36*	This study
808.9	*fwnA1; pyrA6 cspA1 araD5; xkiA1; nicA1*	This study
761.10	*fwnA1; pyrA6 cspA1; xkiA1; nicA1 araA4; xyl-1*	This study
761.12	*fwnA1; pyrA6 cspA1; xkiA1; nicA1; xyl-3*	This study
761.13	*fwnA1; pyrA6 cspA1; xkiA1; nicA1; xylA4*	This study

Selection and genetic analysis of mutants

The selection of mutants was done similarly to the method described by (Chapter 3 & De Groot *et al.*, 2003); in addition to the use of L-arabinose as the repressing pentose, we also used xylitol and D-xylose. Conidiospores were irradiated with UV light and 10^5 spores were spread on solid media plates containing 50 mM xylitol, D-xylose or L-arabinose in combination with 50 mM sodium D-gluconate. The plates were incubated for 3 days at 30 °C and colonies were purified on the same selective medium. The mutants were tested by replica plating for growth on D-xylose and combinations of xylitol, D-xylose or L-arabinose with either D-

gluconate or L-alanine. Mutants that remained unable to grow on D-xylose, but showed growth on any of the pentose + D-gluconate combinations were analysed in more detail. Genetic localisation of the pentose-derepressed mutations was determined by mitotic recombination using master strain NW148 (Bos *et al.*, 1988). Complementation of the xylitol-derepressed mutations was tested using heterokaryons of the original mutants with strain NW324 containing the *xtlA36* mutation. In the complementation tests of the *araA4* and *araB*3 mutants with *araC33* we used diploids heterozygous for *xkiA1*, testing for growth on L-arabinose. Complementation of the *xylA4* strain with the *xyl-3* strain was tested using diploids homozygous for *xkiA1*, looking at D-xylose repression of D-gluconate utilisation.

Consumption and excretion experiments

NW324 and N402 were pre-cultured on MM containing 2% xylose. After 16 hours mycelium was harvested and washed using 30 °C MM by suction over a filter. Aliquots of 33-44 g wet-weight were transferred to 2.2 l MM containing xylitol. Samples used for dry-weight and polyol analyses were not washed. N572 and 689.1 were pre-grown using MM containing 2% fructose. After 16 hours mycelium was harvested, washed and transferred to MM containing 2 % xylose followed by incubation for 4 hours in order to induce the D-xylose catabolism. After induction, the biomass was transferred to xylitol containing medium. Samples were taken as described above. The biomass remained constant at 0.66 ± 0.02 g/l for 689.1 and 0.62 ± 0.02 g/l for N572.

Statistical Analysis

Statistical analysis of the polyol and enzyme levels was carried out using the Student's standard *t*-test with a reliability interval of 95%.

Polyol extraction and determination

The extraction of intracellular polyols was carried out as described previously (Witteveen *et al.*, 1994). Polyol and xylose concentrations were measured by HPAEC (Dionex) with a Carbopac MA1 column using isocratic elution with 0.48 M NaOH.

Results

Isolation of *A. niger* mutants

We adapted the selection method described by (Chapter 3 & De Groot *et al.*, 2003) to obtain *A. niger* mutants altered in xylitol, D-xylose and L-arabinose mediated carbon catabolite repression (CCR). The selection system uses a parental strain

containing the *xkiA1* mutation, which lacks D-xylulose kinase activity. This strain is able to take up L-arabinose, L-arabitol, xylitol and D-xylose, but unable to metabolise it beyond D-xylulose, leading to accumulation of catabolites (Witteveen *et al.*, 1989). This accumulation may increase CCR by the pentoses. D-xylose, L-arabinose and xylitol are able to repress the use of poorer carbon sources such as D-gluconate and L-alanine, preventing an *xkiA1* strain from growing on a combination of these pentoses and D-gluconate. By selecting for mutants capable of growth on D-gluconate in the presence of D-xylose, L-arabinose or xylitol, we obtained several mutants (Table 2).

Table 2. Growth of parent strain and a selection of the isolated mutants on various carbon source mixtures.
Growth is indicated from poor growth (0) to good growth (4).

Strain	Relevant Genotype	D-xylose + D-gluconate	L-arabinose + D-gluconate	xylitol + D-gluconate
NW315	*xkiA1*	0	0	0
689.1	*xkiA1 xtlA36*	0	0	4
NW316	*xkiA1 araA4*	0	2	0
NW318	*xkiA1 araB3*	0	2	0
761.3	*xkiA1 araC33 xtlA36*	0	2	1
808.9	*xkiA1 araD5*	1	2	3
761.10	*xkiA1 xyl-1 araA4*	2	3	1
761.12	*xkiA1 xyl-3*	2	0	1
761.13	*xkiA1 xylA4*	2	0	4

We were unable to select for L-arabitol derepressed mutants because L-arabitol does not sufficiently repress D-gluconate and L-alanine utilisation. In order to avoid selection of mutants affected only in D-gluconate or L-alanine catabolism, we considered only those mutants derepressed for the utilization of both.

Selection of xylitol-derepressed mutants

Xylitol-derepressed mutants were selected in two petri dishes containing MM xylitol + D-gluconate, one containing 10^5 and the other containing 10^6 irradiated spores of NW315. From the plate with 10^5 spores we isolated three xylitol-derepressed mutants, 689.1, 689.2 and 689.3. All three mutants were derepressed for both D-gluconate and L-alanine utilisation on combinations with xylitol only (Table 2). Strain 689.1 was subjected to mitotic recombination with tester strain NW148 for three purposes: first, to gather information on the genetic localisation of the *xtlA36* mutation, second to remove the *xkiA1* background from the *xtlA36* recombinants in order to determine the phenotype of the *xtlA36* mutation. The

third purpose was to isolate an *xtlA36* recombinant with a different auxotrophic marker, which could then be used in complementation tests with the other *xtl* mutants.

Some of the 53 haploid recombinants we isolated from diploid 689.1//NW148 were unable to grow on xylitol, whereas growth on L-arabinose, L-arabitol and D-xylose was not affected (Table 3). This phenotype, apparently resulting from the *xtlA36* mutation, enabled us to determine the presence of the *xtlA36* mutation in all 53 progeny, not just in the *xkiA1*- carrying ones. Recombination frequencies of the genetic markers on chromosome IV with *xtlA36* were 33 % for *leuA1* and 17% for *xkiA1*. The heterozygous diploid strain, derived from 689.1 and NW148, grew well on xylitol, demonstrating the *xtlA36* mutation to be recessive.

Table 3. Linkage data and phenotype of several mutations.

Mutation	Lowest recombination frequencies	Reduced growth on substrate
xtlA36	17 % with *xkiA1* on IV	xylitol
araA4	28 % with *argB13* on V	L-arabinose, L-arabitol
araB3	38% with *pyrA6* and *bioA1* on III	L-arabinose, L-arabitol
araC33	24% with *leuA1* on IV	none
araD5	29% with *pyrA6* and *bioA1* on III	none
xyl-3	4% with *trpB2* on VIII	none
xylA4	7% with *trpB2* on VIII	none

This allowed us to perform complementation tests with the other two *xtl* mutations. Recombinant strain NW324 (Table 1) was forced to form a heterokaryon with 689.2 (*xtlA46*) and 689.3 (*xtlA55*). Both heterokaryons grew on glucose, but not on xylitol, indicating that they are allelic. Because they were isolated from the same petri dish it is not certain that these *xtlA* mutations are truly independent.

Selection of L-arabinose-derepressed mutants

Because in the *xkiA1* background the phenotype of mutant 808.9 (*araD5*) was clearly different from that of the *araA4*, *araB*3 and *araC33* mutants, the mutants containing *araC33* and *araD5* were subjected to mitotic recombination with tester strain NW148. Unfortunately, among the *xkiA+* recombinants no phenotype of the putative *araC33* or *araD5* strains could be observed. For the *xkiA1* recombinants, a weak linkage was found between *araC33* and markers on chromosome IV and between *araD5* and markers on chromosome III (Table 3). The *araC33* is located on linkage group IV, just like the $creA^d$ mutations reported by (Ruijter *et al.*, 1997), which also result in a derepressed phenotype. It is unlikely, however, that this mutation is allelic with *creA* because the $creA^d$

mutations were found to be more tightly linked to *leuA* than *araC33* (5 and 12% recombination compared to 24%). Because the crossed-out mutation *araC33* does not lead to reduced growth on L-arabinose, the complementation tests do not exclude *araC33* to be a weak allelic mutation in *araA* or *araB*. However, its location on chromosome IV, different from that of *araA4* and *araB3*, suggests it to affect another gene. In contrast, the *araD5* mutation is located on the same chromosome as *araB3*. Although no complementation tests have been done, the clear difference in phenotype between the *araD5* and the *araB3* mutant (Table 2) suggests different genes to be affected.

Selection of D-xylose-derepressed mutants

D-xylose-derepressed mutants were selected on 11 petri dishes, each containing 10^6 irradiated spores of NW315. In addition, we used the arabinose-derepressed strain NW316 for the selection of mutants dependent on the *araA4* mutation. This was done on 4 petri dishes each with 10^6 irradiated spores of NW316. After a few rounds of selection we ended up with a total of 5 mutants that were consistently derepressed on D-xylose for the use of D-gluconate and, to a lesser extent, the use of L-alanine. Mutant 761.10 (*xyl-1*) originated from NW316 (Table 2) and was derepressed on D-xylose as well as on L-arabinose, possibly caused by the separate effects of the *xyl-1* (D-xylose-derepressed) and the *araA4* (L-arabinose-derepressed) mutations. Of the other four mutants, two were derepressed on D-xylose only (such as 761.12), while two others were also derepressed on xylitol (such as 761.13, see Table 2). Two phenotypically different mutants, 761.12 (*xyl-3*) and 761.13 (*xylA4*), were analysed by mitotic recombination with tester strain NW148. Looking at the recombinants containing *xkiA1*, we found that both mutations were located on chromosome VIII, with a small difference in recombination frequency with *trpB2* (Table 3). In both cases, D-xylose or xylitol utilisation was not altered among the *xkiA+* recombinants, which means that both the *xyl-3* and *xylA4* mutation do not play a key role in xylose or xylitol catabolism. Therefore, we tested the complementation of *xyl-3* with *xylA4* in diploid 761.3//786.19, which was homozygous for *xkiA1*. Unfortunately, the diploid exhibited derepression for D-gluconate catabolism on D-xylose, indicating that the mutations do not complement each other. We did not test whether the mutations are recessive or dominant in a homozygous *xkiA1* background, so a slight possibility remains that the two *xyl* mutations affect different genes.

Biochemical analysis of the *xtlA36* mutation

Since the *xtlA36* mutation had a clear phenotype, we decided to study this particular mutant in more detail. To test whether or not xylitol was taken up, we conducted a consumption assay (Figure 1).

During the time course of 80 hours, the biomass of the reference strain increased from 0.65 ± 0.03 to 0.75 ± 0.04 g/l. The biomass of the *xtlA36* strain remained constant at 0.70 ± 0.05 g/l. In the first 18 hours there was a rapid decrease of the xylitol concentration in all 4 cultures, after which the xylitol consumption levelled off. Looking at the initial xylitol consumption rate over these 18 hours, there was a clear difference between the reference strain and the *xtlA36* mutant. Starting at a high concentration of 60 mM xylitol, the initial xylitol consumption of the reference strain was 0.78 mmol/ g DW/h. The *xtlA36* mutant consumed only 0.46 mmol/g DW/h over the first 18 hours. At a lower concentration of 6 mM xylitol there was also a clear difference between the two strains. The initial xylitol consumption rate of the reference strain was 0.13 mmol xylitol/g DW/h whereas that of the *xtlA36* mutant was only 0.08 mmol xylitol/g DW/h.

To investigate whether or not the *xtlA36* mutation would also affect the excretion of xylitol, we used the *xkiA1* background. Using D-xylose as the carbon source in this background causes the accumulation of xylitol and arabitol (Witteveen *et al.*, 1989) and Figure 2). We observed excretion of up to 2 mM of xylitol in the *xkiA1* mutant N572, but no significant excretion in the *xkiA1 xtlA36* strain 689.1 (Figure 3). In the last two time samples, the arabitol excretion was higher in the *xkiA1 xtlA36* strain than in the *xkiA1* strain. The polyol accumulation (Figure 2) in this experiment was comparable between the two strains.

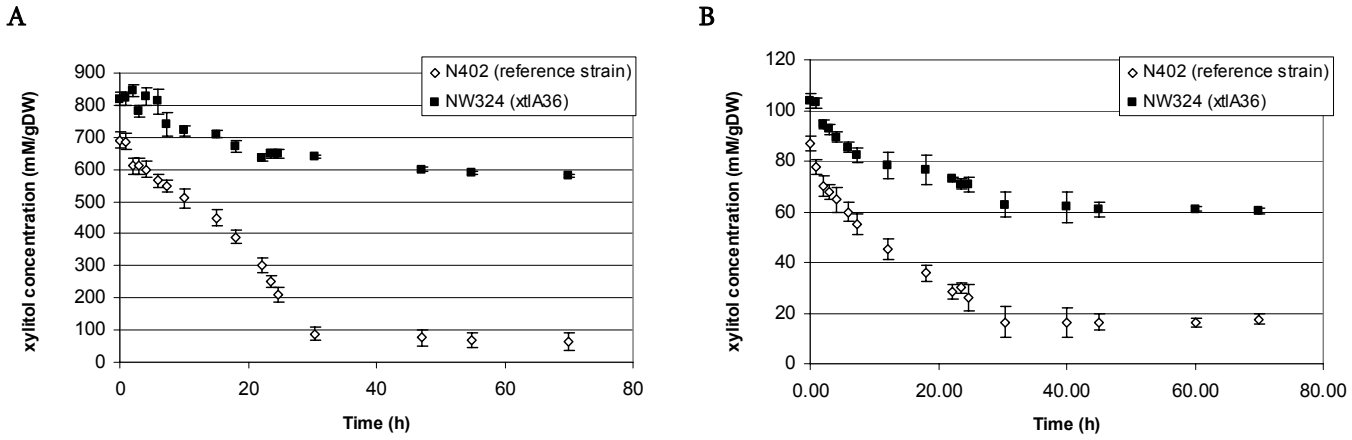

Figure 1. Xylitol consumption in strains N402 (◊, reference strain) and NW324 (■, *xtlA36*) at initial concentrations of 60 (A) and 6 (B) mM. The values are means of three independent experiments.

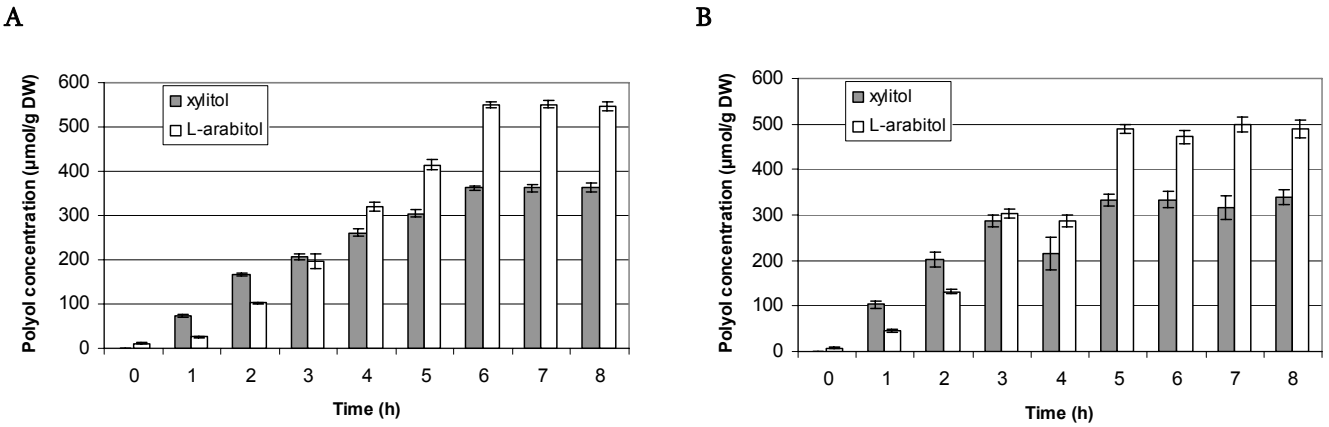

Figure 2. Intracellular polyol accumulation in strains N572 (*xkiA1*) (A) and 689.1 (*xkiA1*, *xtlA36*) (B) on 2% D-xylose.

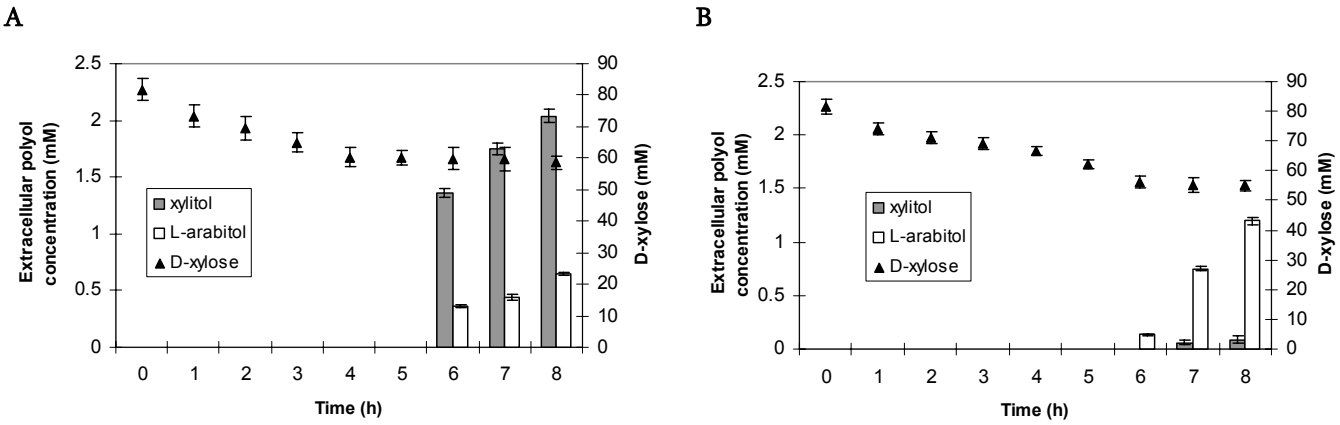

Figure 3. D-xylose consumption and polyol excretion by strains N572 (*xkiA1*) (A) and 689.1 (*xkiA1*, *xtlA36*) (B) using a initial concentration of 2% D-xylose. The values are means of three independent experiments.

Discussion

The selection method described in this report proves useful for identifying new genes involved in pentose metabolism and pentose-mediated Carbon catabolite repression (CCR). We identified new mutants affected in CCR exerted by the pentoses L-arabinose and D-xylose, and in the transport of xylitol. The *xtlA36* mutation causes a xylitol non-utilising phenotype, while the *araC33* and *araD5* mutations do not result in an aberrant phenotype on any of the pentoses or pentitols tested. Also, two other phenotypically different mutations, *xylA4* and *xyl-3*, did not show altered pentose or pentitol utilisation in a *xkiA+* background. For the *araC33*, *araD5*, *xylA4* and *xyl-3* mutations we only found a phenotype in the *xkiA1* background, viz. derepression on L-arabinose or D-xylose, often in

combination with derepression on xylitol (Table 2). The mutations with a clear phenotype of their own were further characterised: the *araA* and *araB* mutations by (Chapter 3 & De Groot *et al.*, 2003) and the *xtlA36* mutation in this study.
In order to understand why certain types of mutants are isolated with our selection system, it is useful to look at current knowledge of glucose repression in *Aspergillus nidulans*. In the case of glucose repression, mutants no longer capable of phosphorylating glucose have reduced CCR (Flipphi *et al.*, 2003), indicating that a glucose metabolite is involved in this pathway. Many mutants impaired in glucose repression turned out to be mutated in the *creA* gene (Arst & Cove, 1973), the product of which is directly involved in repressing gene transcription by its DNA binding activity (Kulmburg *et al.*, 1993). Other mutants found were *creB* and *creC* mutants, exhibiting pleiotropic effects on several carbon sources (Hynes & Kelly, 1977). In our study we looked at pentose-mediated CCR, but we do not know which compound or metabolite is the key signal to pentose-mediated CCR. The types of mutants one can expect to find using our selection method affect pentose uptake and catabolism of these pentoses to the final metabolite(s) that influence the signalling pathway to pentose-mediated CCR, as well as compounds of the signalling pathway itself. We indeed identified a mutation, *xtlA36*, affecting xylitol transport. As D-xylose is taken up by different transporters (VanKuyk *et al.*, 2004) a mutant with a single mutation in one of the transporters would probably not lead to D-xylose derepression. This selection system also resulted in isolation of signalling mutants, *araA4* and *araB*3, which are surprisingly diverse in their phenotype (Chapter 3 & De Groot *et al.*, 2003). The mutations with only the derepression phenotypes might influence metabolite levels, without completely blocking catabolic pathways, or they might be involved in CCR signalling transduction specific for L-arabinose or D-xylose but some also affect xylitol repression.
Mutants we did not find are those impaired in specific steps of pentose catabolism. This is probably due to low substrate specificities of the enzymes in pentose catabolism, causing redundancy for some of the enzymes. For example, L-arabinose reductase and D-xylose reductase have overlapping activities, both converting L-arabinose and D-xylose to L-arabitol and xylitol, respectively. The gene encoding D-xylose reductase (*xyrA*) was cloned and knocked-out by (Hasper *et al.*, 2000). The use of this *xyrA* disruption in the *xkiA1* background should enable the selection of L-arabinose reductase mutants on L-arabinose plus D-gluconate. A different mutation selection technique, filtration enrichment, has resulted only in the selection of the xylulose kinase loss-of-function mutation *xkiA1* (Witteveen *et al.*, 1989). Also here the redundancy of metabolic functions in pentose metabolism has probably prevented the isolation of other mutations than that of the key function xylulose kinase. Using the *xyrA* disruption strain

would probably enable the direct isolation of a L-arabinose reductase mutant with this mutant selection technique.

The difference in consumption between the *xtlA36* mutant and the reference strain clearly shows that the transport of xylitol into the cell is dramatically impaired. The remaining consumption of xylitol by the *xtlA36* mutant can be explained either by another transporter having a low affinity for xylitol or by adherence of xylitol to the biomass, which lowers the xylitol concentration in the medium. The polyol excretion experiment shows a marked difference between the excretion of xylitol by the *xkiA1* reference strain and the *xkiA1*, *xtlA36* double mutant. Because of the *xkiA1* mutation, these strains cannot metabolise any of the substrates from the L-arabinose/D-xylose metabolism. However, the metabolites can be taken up and converted, causing an extreme accumulation of the polyols arabitol and xylitol (Witteveen *et al.*, 1989). It appears that the *xtlA36* mutation prevents the fungus from excreting the high amounts of xylitol produced but does not reduce the excretion of arabitol compared to the reference strain, while maintaining a similar intracellular level of both polyols as in the reference strain. Altogether these data lead to the conclusion that the *xtlA36* mutation leads to a severe reduction in xylitol transport and therefore probably is located in the gene encoding the xylitol transporter. This transporter would be solely responsible for both the import and export of xylitol as by the single *xtlA36* mutation both consumption and excretion are impaired. Also in yeast bi-directional transporters are found: glucose transport (Jansen *et al.*, 2002) and glycerol transport (Karlgren *et al.*, 2004).

In conclusion, we demonstrated a mutant selection system revealing new functions in pentose metabolism. The *xtlA* gene encodes a gene involved in xylitol transport. The *araA* and *araB* genes are components of the regulatory system involved in the expression of arabinanase-encoding genes and genes encoding the L-arabinose catabolic pathway (Chapter 3 & De Groot *et al.*, 2003). The use of this novel selection method can help us understand the complex interaction of pentose-mediated CCR, pentose metabolism and hemicellulase gene expression.

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| Chapter 3

Isolation and characterisation of two specific regulatory *Aspergillus niger* mutants shows antagonistic regulation of arabinan and xylan metabolism

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Summary

In this study we describe two *Aspergillus niger* mutants (*araA* and *araB*) specifically disturbed in the regulation of the arabinanase system in response to the presence of L-arabinose. Expression of the three known L-arabinose-induced arabinanolytic genes, *abfA*, *abfB* and *abnA*, was substantially decreased or absent in the *araA* and *araB* strains compared to the wild-type when incubated in the presence of L-arabinose or L-arabitol. In addition, the intracellular enzyme activities of L-arabitol dehydrogenase and L-arabinose reductase, involved in L-arabinose catabolism, were decreased in the *araA* and *araB* strains. Finally, the data shows that the gene encoding D-xylulose kinase, *xkiA*, is also under control of the arabinanolytic regulatory system. L-arabitol, most likely the true inducer of the arabinanolytic and L-arabinose catabolic genes, accumulated to a high intracellular concentration in the *araA* and *araB* mutants. This indicates that the decrease of expression of the arabinolytic genes was not due to lack of inducer accumulation. Therefore, we propose that *araA* and *araB* are mutations in positively acting components of the regulatory system involved in the expression of the arabinanase encoding genes and the genes encoding the L-arabinose catabolic pathway.

Introduction

Degradation of the plant cell wall polysaccharide xylan *Aspergillus niger* has been studied in detail in *Aspergillus niger*. Regulation of the xylanolytic system is mediated by the transcriptional activator XLNR (Van Peij *et al.*, 1998b). XLNR regulates the expression of a number of genes involved in xylan degradation, such as those encoding β-xylosidase (*xlnD*), arabinoxylan arabinofuranohydrolase (*axhA*), α-glucuronidase (*aguA*) and D-xylose reductase (*xyrA)* (Hasper *et al.*, 2000; Van Peij *et al.*, 1998a).

Arabinan is another polysaccharide found in plant cell wall heteropolysaccharides as a side chain of pectin (De Vries & Visser, 2001). *A. niger* is able to degrade arabinan to the pentose L-arabinose using its arabinanolytic system. To date three arabinanases have been characterised: two α-L-arabinofuranosidases (ABFA and ABFB) and an arabinan 1,5-α-L-arabinanase (ABNA) (Van der Veen *et al.*, 1991). L-arabinose can be used by the fungus as a carbon source via the L-arabinose catabolic pathway. This pathway converts L-arabinose to D-xylulose 5-phosphate which is further metabolised via the non-oxidative pentose phosphate pathway. Two *A. niger* genes involved in pentose metabolism have previously been cloned and characterised: x*yrA* encoding D-xylose reductase (Hasper *et al.*, 2000) and *xkiA* encoding D-xylulose kinase (Chapter 4 & VanKuyk *et al.*, 2001).

Little is known about the regulation of the arabinanase system. Arabinanonlytic genes are specifically induced when *A. niger* is grown on arabinan-containing substrates or the monomeric compounds L-arabinose and L-arabitol (Flipphi *et al.*, 1994; Gielkens *et al.*, 1997; Gielkens *et al.*, 1999; Ramon *et al.*, 1993; Van der Veen *et al.*, 1991; Van der Veen *et al.*, 1993). One of the intermediates of the L-arabinose catabolic pathway, L-arabitol, is believed to be the low molecular mass inducer of the system (Van der Veen *et al.*, 1993). In *Aspergillus nidulans* it has been shown that increased intracellular arabitol accumulation correlates with higher production of the enzymes involved in arabinan breakdown (De Vries *et al.*, 1994), suggesting that L-arabitol is the true inducer of this system. The genes encoding three arabinan degrading enzymes, *abfA*, *abfB* and *abnA*, have been cloned and characterised (Flipphi *et al.*, 1993a; Flipphi *et al.*, 1993b; Flipphi *et al.*, 1993c). Expression analysis of these genes showed that they are co-ordinately expressed. Moreover, insertion of additional copies of one of the arabinanase genes in *A. niger* resulted in lower expression of the other two genes, suggesting a a titration effect at the level of a single specific transcriptional activator (Flipphi *et al.*, 1994).

The aim of this study was to find genetic evidence for a positive-acting specific regulatory system involved in the induction of arabinanases in *A. niger* and to assess whether the same system regulates induction of the intracellular enzymes of the L-arabinose catabolic pathway, and to investigate interaction between the regulation of arabinanase and xylanase systems.

Materials and Methods

Strains and growth conditions

All *A. niger* strains used for this study were derived from *A. niger* N400 (CBS120.49) and are described in Table 1.

NW315 was used for mutagenesis and N423 was used as a reference strain. Mycelium cultures were grown at pH 6 in minimal medium (MM) containing per litre: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 1 ml of trace elements solution (Vishniac & Santer, 1957) and carbon sources as indicated in the text. Culturing was done in a rotary shaker at 250 rpm and 30°C. For growth of strains with auxotrophic mutations, the necessary supplements were added to the medium. For growth on plates 1.5% (mass/vol) agar was added to the medium before autoclaving. In transfer experiments strains were pre-grown in MM containing 1% (mass/vol) D-fructose; 0.5 % (mass/vol) yeast extract; and 0.2% (mass/vol) casamino acids. After 16 h mycelium was harvested by suction over a filter, washed with MM without carbon source, and aliquots of 1.5 g (wet weight) were transferred to 50 ml

of MM containing carbon sources as indicated in the text. The mycelium was harvested after 2, 4 or 8 hours, dried between paper and frozen in liquid nitrogen. Mycelium used for the partial purification of intracellular enzymes was washed in 4 °C 10 mM potassium phosphate (pH 7.0) during harversting. The mycelium samples were stored at -70°C.

Table 1. *A. niger* strains used in this study.

Strain	Genotype	Reference
N402	*cspA1*	(Bos *et al.*, 1988)
N423	*cspA1; nicA1*	(Bos *et al.*, 1988)
NW148	*cspA1; hisD4; bioA1 lysA7; leuA1; metB10 argB15; pabA1; cnxC5; trpB2*	(Bos *et al.*, 1988)
NW199	*cspA1; fwnA1; goxC17; pyrA6 xlnRΔ::pIM240; leuA1*	(Hasper *et al.*, 2000)
NW315	*cspA1; fwnA1; pyrA6; xkiA1; nicA1*	(Witteveen *et al.*, 1989)
NW316	*cspA1; fwnA1; pyrA6; xkiA1; nicA1 araA4*	This study
NW318	*cspA1; fwnA6; pyrA6 araB3; xkiA1; nicA1*	This study
NW319	*cspA1; fwnA6; pyrA6; xkiA1; nicA1 araA2*	This study
NW320	*cspA1; fwnA6; lysA7 bioA1; nicA1 araA4*	This study
NW321	*cspA1; fwnA6; leuA1; araA4*	This study
NW322	*cspA1; bioA1 lysA7 araB3; nicA1*	This study

NW315 was used for mutagenesis and N423 was used as a reference strain. Mycelium cultures were grown at pH 6 in minimal medium (MM) containing per litre: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 1 ml of trace elements solution (Vishniac & Santer, 1957) and carbon sources as indicated in the text. Culturing was done in a rotary shaker at 250 rpm and 30°C. For growth of strains with auxotrophic mutations, the necessary supplements were added to the medium. For growth on plates 1.5% (mass/vol) agar was added to the medium before autoclaving. In transfer experiments strains were pre-grown in MM containing 1% (mass/vol) D-fructose; 0.5 % (mass/vol) yeast extract; and 0.2% (mass/vol) casamino acids. After 16 h mycelium was harvested by suction over a filter, washed with MM without carbon source, and aliquots of 1.5 g (wet weight) were transferred to 50 ml of MM containing carbon sources as indicated in the text. The mycelium was harvested after 2, 4 or 8 hours, dried between paper and frozen in liquid nitrogen. Mycelium used for the partial purification of intracellular enzymes was washed in 4 °C 10 mM potassium phosphate (pH 7.0) during harversting. The mycelium samples were stored at -70°C.

Selection and genetic analysis of mutants

Conidiospores were irradiated as described by Witteveen *et al.* (1989). Subsequently, 10^5 spores were spread on solid media plates containing 50 mM L-arabinose and 50 mM sodium D-gluconate. The plates were incubated for 3 days at 30 °C and colonies were purified on the same medium. The mutants were tested by replica plating for growth on D-xylose and combinations of xylitol, D-xylose or L-arabinose with either D-gluconate or L-alanine. Mutants that were still unable to grow on L-arabinose, but showed increased growth on L-arabinose + D-gluconate and L-arabinose + L-alanine were analysed in more detail.

Genetic localisation of the *araA4* and *araB2* mutations was determined by somatic recombination using test strain NW148 as described by Bos *et al.* (Bos *et al.*, 1988). Complementation tests of the *araA4* mutation with other mutations were performed using heterokaryons. Strain NW320 was isolated from somatic recombination experiment using strains NW148//NW316. Heterokaryons were selected from NW320 with some of the other *ara* mutants isolated from NW315. Balanced heterokaryons, recognisable by a good mixture of fawn-coloured and black conidiospores, were tested for growth on minimal medium with nicotinamide and L-arabinose, and minimal medium with nicotinamide and D-glucose. NW320 and NW322 were used as negative control and N423 as positive control.

Preparation of cell extracts and protein determination

Mycelium was harvested and disrupted as described previously (Witteveen *et al.*, 1989). Cell extracts were obtained by suspending disrupted frozen mycelium in extraction buffer (10 mM Bis-Tris, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5 mM EDTA pH 6.3) followed by centrifugation at 15000-20000 *g* in order to remove cell debris. The entire procedure was performed at 0 to +4°C. After denaturation with sodium deoxycholate and precipitation of protein with trichloroacetic acid (Bensadoun & Weinstein, 1976) protein concentrations were determined using the Bicinchoninic acid method as described by the manufacturer (Sigma Chemical Company).

Fractionation of cell extracts

Cell extracts were fractionated by anion exchange chromatography. Two ml of cell extract was loaded on a ResourceQ column with a bed volume of 1 ml (Pharmacia Biotech). The column was washed with 8 ml of extraction buffer. Elution was started with a gradient of 0-0.5 M NaCl over 18 column volumes. The flow rate used was 3 ml/min. Fractions of 0.5 ml were collected during this gradient and kept at on ice until they were used in the enzyme activity measurements.

Enzyme assays

All enzyme assays were performed at 30°C. L-arabitol dehydrogenase and xylitol dehydrogenase activities were determined using 100 mM glycine pH 9.6, 0.2 mM NAD^+ and 40 mM L-arabitol or xylitol, respectively. L-arabinose reductase and D-xylose reductase activities were determined using 50 mM sodium phosphate pH 7.8, 0.2 mM NADPH and 40 mM L-arabinose or D-xylose, respectively. Absorbance changes at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) were measured using a Cobas Bio autoanalyser (Roche) or an UV-2501PC spectrophotometer (Shimadzu Scientific Instruments).

Extracellular hydrolytic activities were assayed as follows using p-nitrophenyl-α-L-arabinofuranoside (pNP-A) or p-nitrophenyl-β-D-xylopyranoside (pNP-X) as substrates: 0.01 % pNP-substrate, 20-40 µl sample and 25 mM sodium acetate pH 5.0 were used in a total volume of 100 µl , incubated for one hour at 30°C (pNP-A) or 50 °C (pNP-X) and the reaction was subsequently stopped by adding 100 µl 0.25 M Na_2CO_3. Absorbance was measured at 405 nm in a microtiter platereader (Molecular Devices). The activity was calculated using a standard curve ranging from 0 – 80 nmol p-nitrophenol (pNP) per assay volume.

Polyol extraction and determination

The extraction of intracellular polyols was carried out as described previously (Witteveen *et al.*, 1994). Polyol concentrations were measured by HPAEC (Dionex) with a Carbopac MA1 column using isocratic elution with 0.48 M NaOH.

Statistical Analysis

Statistical analysis of the polyol and enzyme levels was carried out using the Student's standard *t*-test with a reliability interval of 95%.

Expression analysis

Total RNA was isolated from powdered mycelium using TRIzol Reagent (Life Technologies), according to the supplier's instructions. For northern blot analysis 3 µg total RNA was incubated with 3.3 µl 6 M glyoxal, 10 µl DMSO and 2 µl 0.1 M sodium phosphate pH 7 in a total volume of 20 µl for 1 h at 50°C to denature the RNA. RNA electrophoresis was performed on a 1.5% agarose gel using 0.01 M sodium phosphate buffer pH 7 and transferred to Hybond-N filters (Amersham) by capillary blotting. Filters were hybridised at 42°C in a solution of 50% (vol/vol) formamide, 10% (mass/vol) dextran sulphate, 0.9 M NaCl, 90 mM Na_3-citrate, 0.2% (mass/vol) ficoll, 0.2% (mass/vol) polyvinylpyrolidone, 0.2% (mass/vol) bovine serum albumin, 0.1% (mass/vol) SDS and 100 µg/ml single stranded herring sperm DNA. Washing was performed under stringent conditions to 30 mM NaCl, 3 mM Na_3-citrate and 0.5% (mass/vol) SDS at 68°C. Probes used were: a 2.0 kb *Pst*I fragment of *abfA* (Flipphi *et al.*, 1994); a 2.8 kb *Pst*I fragment of *abfB* (Flipphi *et al.*,

1994); a 3.1 kb *Hind*III fragment of *abnA* (Flipphi *et al.*, 1994); a 1.2 kb *Eco*RI/*Xho*I fragment of *axhA* (Gielkens *et al.*, 1997); a 0.5 kb *Eco*RV/*Xho*I fragment of *aguA* (De Vries *et al.*, 2002); a 2.8 kb *Pst*I/*Nsi*I fragment of *xlnD* (Van Peij *et al.*, 1997); a 0.3 kb *Sal*I/*Xho*I fragment of *xyrA* (Hasper *et al.*, 2000); a 4.0 kb *Sst*I fragment of *xkiA* (Chapter 4 & VanKuyk *et al.*, 2001) and a 0.7 kb *Eco*RI fragment from the gene encoding the 18S rRNA subunit (Melchers *et al.*, 1994). The 18S probe was used as an RNA loading control.

Results

Isolation of *A. niger* mutants

We developed a selection method to obtain *A. niger* mutants altered in L-arabinose catabolism making use of a parent strain containing the *xkiA1* mutation. The *xkA1* containing strain lacks D-xylulose kinase activity and is therefore unable to use L-arabinose, L-arabitol, xylitol or D-xylose as a carbon and energy source (Witteveen *et al.*, 1989). Since L-arabinose is able to repress the use of poorer carbon sources such as D-gluconate and L-alanine (Ruijter & Visser, 1997), an *xkiA1* strain fails to grow on a combination of L-arabinose and D-gluconate. By selecting for mutants capable of growth on a combination of L-arabinose and D-gluconate we obtained mutants disturbed in L-arabinose metabolism.

Out of 10^5 spores of the *xkiA1* strain NW315 which were irradiated, 20 colonies were purified. First, we tested these mutants for growth on D-xylose to exclude reversion of the *xkiA1* mutation. Subsequently, derepression of growth on D-gluconate or L-alanine in the presence of L-arabinose, D-xylose or xylitol was tested. Three mutants (NW316, NW318 and NW319) showed increased growth on L-alanine or D-gluconate with L-arabinose and not with D-xylose or xylitol.

Genetic analysis of the *ara* mutations

Two *ara* mutants (NW316 and NW318) were subjected to a somatic recombination experiment with tester strain NW148 for several purposes. Firstly, to gather information on the genetic localisation of the *ara* mutations and secondly to remove the *xkiA1* mutation. From the somatic recombination of NW316 (*araA4*) and NW148, 85 recombinants were analysed. Recombination frequencies of the genetic markers on chromosome V with *araA4* were 28 % for *argB13*, 31% for *nicA1* and 31% for *metB10*. Recombination frequencies between *araA4* and markers on the other seven chromosomes ranged from 37% (*trpB2*) to 62% (*fwnA1*). Recombination frequencies higher than 25% are not considered to be absolute evidence for linkage, but since none of the markers on the other chromosomes showed a better linkage, we concluded that the *araA4* mutation is located on chromosome V. From the somatic recombination of NW318 (*araB3*)

and NW148, 109 recombinants were analysed. No convincing linkage was found with any of the markers used. Best linkage of *araB3* was found with *pyrA6* and *bioA1*, both located on chromosome III, with 38% recombination. Since recombination percentages with the markers on the other chromosomes ranged from 43% to 57%, *araB3* is probably located on chromosome III.

Complementation of the *araA4* mutation with 2 other *ara* mutations was tested. Heterokaryons of strains NW318 (*araB3*) and NW319 (*araA2*) with NW320 (*araA4*) were isolated and tested for growth on L-arabinose and D-glucose. NW318 was found to complement the *araA4* defect. NW319 did not complement *araA4*, indicating that the mutations designated *araA2* and *araA4* are allelic. NW315 and the mutants derived from it are pentose non-utilising due to the *xkiA1* mutation. Growth tests of *xkiA+* recombinants of the original mutants showed that the *araA* and *araB* mutations resulted in strongly reduced growth on L-arabinose and L-arabitol and a slight decrease in growth on xylitol, whereas growth on D-xylose was not affected (Table 2).

Table 2. Growth on plates of the *A. niger* wild type, *araA2*,*araA4*, *araB3* and *xlnRΔ strains* and *araA//araB* heterokaryons, grown for 3 days on solid minimal media and 50 mM carbon source at 30°C. +++ good, ++ mediocre, + poor, -no growth and ND = Not Determined.

Strains	D-glucose	D-xylose	xylitol	L-arabinose	L-arabitol
N423	+++	+++	++	+++	++
NW321	+++	+++	+	-	-
NW322	+++	+++	+	-	-
NW199	+++	++	++	+++	++
NW315//NW148	+++	ND	ND	+++	ND
NW320//NW318	+++	ND	ND	+++	ND
NW320//NW319	+++	ND	ND	-	ND

L-arabitol dehydrogenase and L-arabinose reductase activities are strongly reduced in *araA* and *araB* mutants.

Under inducing conditions (D-xylose and L-arabinose) L-arabitol dehydrogenase activity in NW321 (*araA*) and NW322 (*araB*) was 5-fold lower on D-xylose and 15-fold lower on L-arabinose compared to the wild type (N423) and the *ΔxlnR* strain (NW199) (Figure 1A). L-arabitol dehydrogenase activity in the wild type and *ΔxlnR* strains were comparable. L-arabitol dehydrogenase activity was higher during induction on L-arabinose than on D-xylose.

Xylitol dehydrogenase activity (Figure 1B) appeared not to be significantly different in the *araA* and *araB* and wild type strains on either L-arabinose and D-

xylose. Xylitol dehydrogenase activity was affected by the disruption of *xlnR*, resulting in a lower activity on D-xylose. Unexpectedly, the xylitol dehydrogenase activity in the *xlnR* disruption strain is higher on L-arabinose than in the wild type strain.

In order to visualise the relative level of L-arabinose reductase and D-xylose reductase a ratio of the activities was calculated (Figure 1C). Both these enzymes are able to reduce L-arabinose and D-xylose, and they elute at almost the same salt concentration during the fractionation of cell extracts. It appeared that a ratio of L-arabinose reductase/D-xylose reductase activity of 1.9 to 2.2 represents a situation in which L-arabinose reductase is predominantly present and a ratio of 0.5 to 0.6 represents a situation in which D-xylose reductase is mostly present. In the wild-type strain L-arabinose reductase is induced on L-arabinose and D-xylose reductase on D-xylose. In the *ΔxlnR* strain the L-arabinose reductase/D-xylose reductase ratio was 2 on both L-arabinose and D-xylose which is consistent with the absence of expression of *xyrA* encoding D-xylose reductase (Hasper *et al.*, 2000). The L-arabinose reductase/D-xylose reductase ratio in the *araA* and *araB* strains after incubation on L-arabinose and D-xylose resembled that of the wild-type grown on D-xylose. In other words only D-xylose reductase can be detected and L-arabinose reductase was not present in the *ara* mutant strains.

α-L-arabinofuranosidase is absent in the *araA* and *araB* strains

Extracellular α-L-arabinofuranosidase activity levels (Figure 2A) were strongly reduced in the *araA* and *araB* strains compared to the wild-type and *ΔxlnR* strains when grown on L-arabinose. There also appeared to be some production of α-L-arabinofuranosidase by the wild type when grown on D-xylose although not to the same level as when grown on L-arabinose. In NW321 (*araA*), NW322 (*araB*) and NW199 (*ΔxlnR*) there is no production of α-L-arabinofuranosidase on D-xylose apart from the basal level observed on D-glucose.

β-xylosidase activity (Figure 2B) was induced in the wild-type strain on D-xylose, but not on L-arabinose. There was no production of this activity in the *ΔxlnR* strain on either carbon source. With the *araA* strain there was a normal level of β-xylosidase activity on D-xylose and an elevated production on L-arabinose compared to the wild-type strain. In the *araB* strain the production of β-xylosidase activity was low on D-xylose and approximately 2-fold on L-arabinose compared to the basal level in the wild type strain.

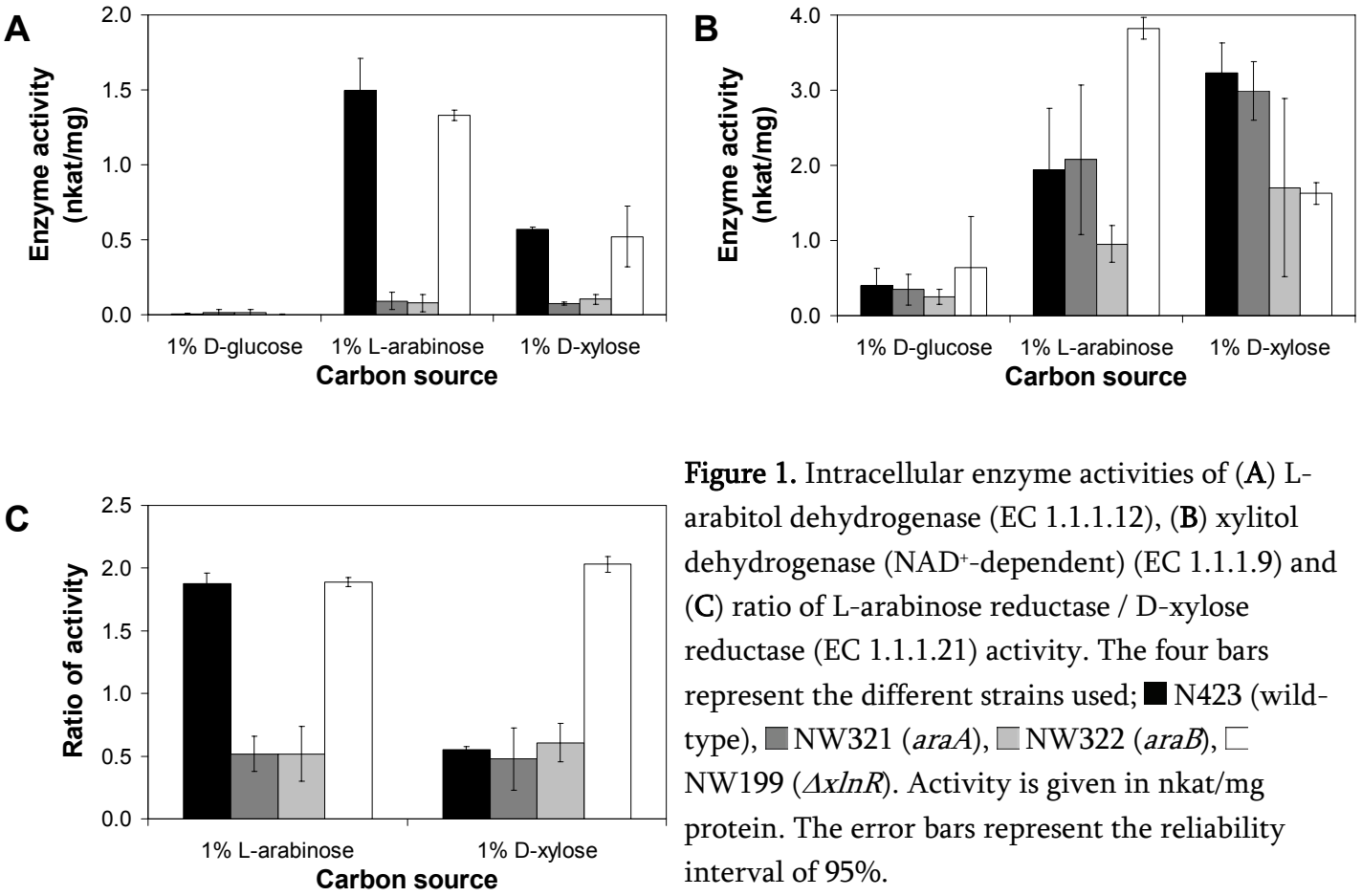

Figure 1. Intracellular enzyme activities of (**A**) L-arabitol dehydrogenase (EC 1.1.1.12), (**B**) xylitol dehydrogenase (NAD$^+$-dependent) (EC 1.1.1.9) and (**C**) ratio of L-arabinose reductase / D-xylose reductase (EC 1.1.1.21) activity. The four bars represent the different strains used; ■ N423 (wild-type), ■ NW321 (*araA*), ■ NW322 (*araB*), □ NW199 (*ΔxlnR*). Activity is given in nkat/mg protein. The error bars represent the reliability interval of 95%.

The *araA* and *araB* strains accumulate arabitol

Intracellular arabitol levels (Figure 3A) were at least 5-fold higher in the *araA* and *araB* strains grown on L-arabinose than in the wild type and *ΔxlnR* strains. Arabitol was present at a low level in all four strains on D-xylose and D-glucose. The arabitol produced during growth on D-glucose is most likely D-arabitol produced from an intermediate of the pentose phosphate pathway. Xylitol levels (Figure 3B) were lower in the *araA* and *araB* mutants compared to the wild type strain on L-arabinose, but comparable to the level in the wild type on D-xylose. Xylitol was not detected in mycelium grown on D-glucose in any of the four strains.

Levels of other polyols, such as glycerol and erythritol, appeared not to be affected by disruption of *xlnR* or the *araA* and *araB* mutations under the conditions tested (data not shown).

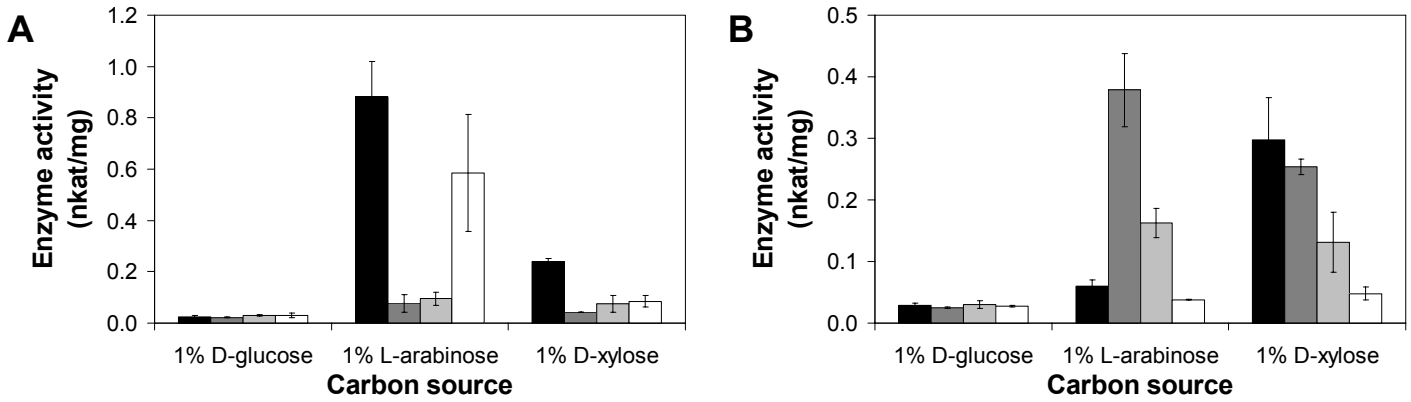

Figure 2. Extracellular enzyme activities of (**A**) α-L-arabinofuranosidase (EC 3.2.1.55) and (**B**) 1,4-β-xylosidase activity (EC 3.2.1.37). As above the four bars represent the different strains used; ■ N423 (wild-type), ■ NW321 (*araA*), ■ NW322 (*araB*), □ NW199 (*ΔxlnR*). Activity is given in nkat/mg dry weight. The error bars represent the reliability interval of 95%.

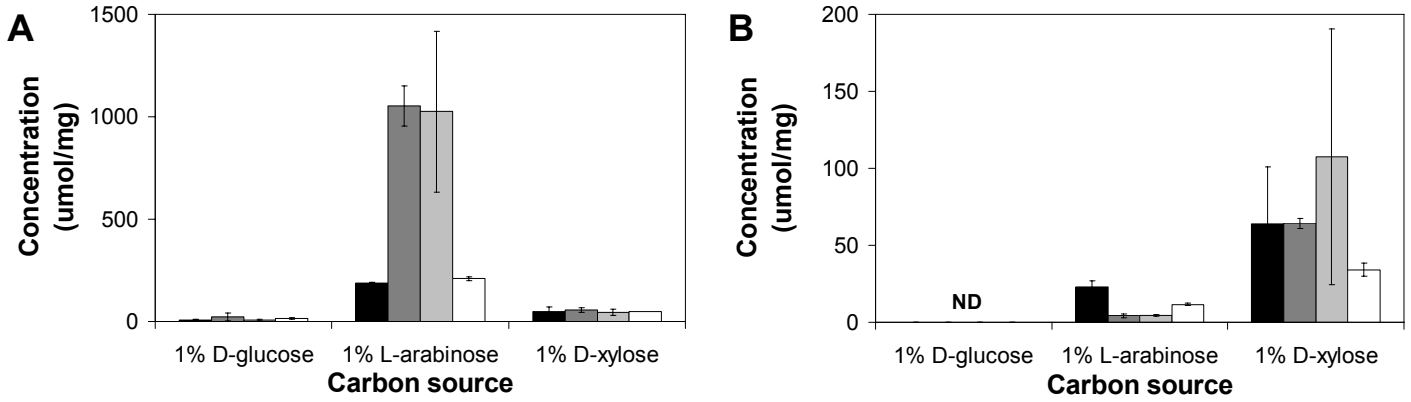

Figure 3. Intracellular (A) arabitol and (B) xylitol accumulation. The four bars represent the different strains used; ■ N423 (wild-type), ■ NW321 (*araA*), ■ NW322 (*araB*), □ NW199 (*ΔxlnR*). The error bars represent the reliability interval of 95%. ND = not detected.

Expression of genes of the arabinanase system is decreased in the *ara* mutants

Expression of the known L-arabinose induced genes (Figure 4), abf*A* and *abfB*, was severely decreased or completely absent in the *araA* and *araB* strains on L-arabinose or L-arabitol compared to the expression in the wild-type. The data shows that *xkiA* is regulated by both L-arabinose and D-xylose induction. Expression of *xkiA* is normal in the *araA* and *araB* strains on D-xylose. In the *ΔxlnR* strain a higher expression level of the arabinanase encoding genes and *xkiA* was detected. Conversely, the expression of the XLNR regulated genes, *xlnD, aguA* and *xyrA*, was decreased or absent in the *ΔxlnR* strain and elevated in the two *ara* mutants. Expression of *xyrA* in the *ΔxlnR* strain is less than in the wild-type strain but not zero.

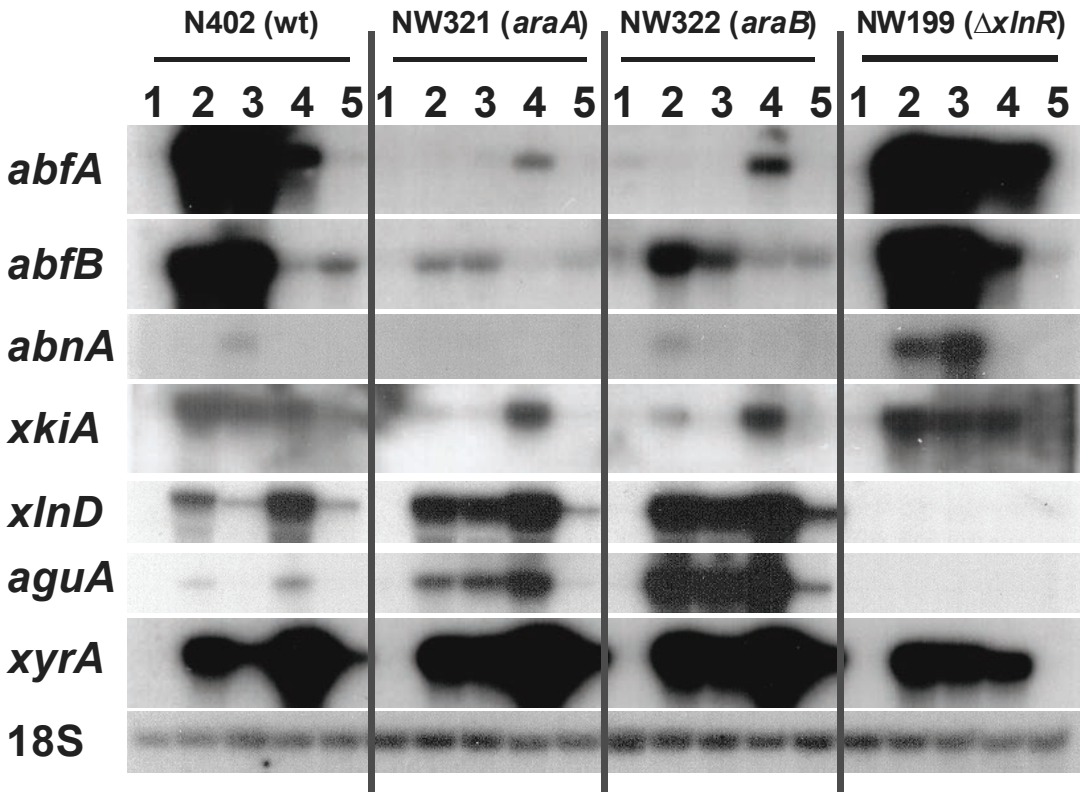

Figure 4. Northerns showing the expression of *abfA*, *abfB*, *abnA*, *xkiA*, *xlnD*, *aguA*, *xyrA* and 18S (loading control) in the different strains used; N423 (wild-type), NW321 (*araA*), NW322 (*araB*) and NW199 (*ΔxlnR*). The numbers represent the following carbon sources at a concentration of 15 mM : (1) D-fructose, (2) L-arabinose, (3) L-arabitol, (4) D-xylose, (5) xylitol.

Discussion

In this report we describe the *A. niger araA* and *araB* mutations which give rise to a decrease in the expression and production of the arabinanases. In addition, these mutations affect intracellular catabolism, since L-arabinose reductase, L-arabitol dehydrogenase activities, and D-xylulose kinase expression are strongly decreased.
L-arabitol, the most potent inducer of arabinanase biosynthesis and L-arabinose catabolism, accumulates to much higher levels in the *araA* and *araB* strains than in the wild type, indicating that the lack of expression and production of the arabinanase system is not due to lack of inducer accumulation. Therefore, we propose that *araA* and *araB* are mutations in components of the arabinanoliytic regulatory system. Whether one of these mutations is located in the actual transcriptional whereas the other one may, for instance, be involved in modifying this activator, e.g. by phosphorylation/dephosphorylation, is not clear at this point.
The intracellular accumulation of arabitol in the *araA* and *araB* mutants in the presence of L-arabinose can be explained by the ability of D-xylose reductase to reduce L-arabinose to L-arabitol and the low L-arabitol dehydrogenase activity. This also shows that the fungus can still take up L-arabinose even though induction of the L-arabinose catabolic pathway is disturbed. L-arabinose uptake seems not to be strictly regulated by *araA* or *araB*. Whether a specific L-arabinose uptake system exists and whether such uptake system is under the control of *araA* and/or *araB* is not clear. If such a specific uptake system controlled by *araA* and/or *araB* would exist, the uptake of L-arabinose in the mutants requires the presence of another transporter having affinity towards L-arabinose.
The expression of *xkiA*, encoding D-xylulose kinase, was previously shown not to be controlled by XLNR (Chapter 4 & VanKuyk *et al.*, 2001), even though expression was observed on both L-arabinose and D-xylose. This suggests that expression of *xkiA* on L-arabinose is induced by the regulatory system that also activates the expression of the arabinolytic genes.
Genes of the xylanolytic system, such as *xlnD* and *aguA* were not expressed in the *xlnR* disruptant (Figure 3 *xlnD, aguA*) which is in agreement with previous results (Van Peij *et al.*, 1998a). The expression of *xyrA*, encoding D-xylose reductase, was previously reported to be controlled by XLNR (Hasper *et al.*, 2000) and absence of expression of *xyrA* in a *xlnR* disruption strain was reported in that study. In our investigation, however, expression of *xyrA* the same *xlnR* disruption strain was reduced compared to wild type but not absent. XLNR control of *xyrA* expression is therefore not absolute. The differences in expression level between the two studies in the *xlnR* disruption strain can be explained by the different D-xylose concentrations used. It was demonstrated previously (De Vries *et al.*, 1999) that apart from being an inducer of xylanolytic gene expression mediated by XLNR, D-xylose also represses the expression of these genes. This repression is stronger at

higher xylose concentrations. In our study we used 15 mM D-xylose whereas Hasper et al. 2000 used 50 mM (Hasper *et al.*, 2000) resulting in lower expression levels for *xyrA*.

Expression of *xlnD* and *aguA* was increased in the *araA* and *araB* mutants, especially on L-arabinose, compared to the expression in wild type. Conversely expression of *abfA*, *abfB*, *abnA* and *xkiA* was higher in the *xlnR* disruptant compared to wild type. This suggests an interaction between the *xlnR* and the *ara* system and may be explained by a relief of repression when either regulatory system is not functional or by a change in intracellular inducer concentration. The latter was observed for L-arabitol, however the L-arabitol concentration was much higher in the *araA* and *araB* mutants compared to wild-type which suggests a high level of induction if the regulatory system was present van (De Vries *et al.*, 1994; Van der Veen *et al.*, 1993). The intracellular concentration of D-xylose, which is believed to be the inducer of the XLNR system, cannot be measured accurately. This supports a model (Figure 5) in which the repression caused by the presence of D-xylose or L-arabinose is relieved and that XLNR and the arabinose regulatory system are involved in the signaling of this repression. In the presence of L-arabinose, *araA* and *araB* play a role in the activation of carbon catabolite repression of other catabolic systems, e.g xylanolytic functions. Mutations in *araA* or *araB* relieve this repression. This model explains how the mutants have been selected, i.e. selecting for a relief of repression of L-arabinose towards D-gluconate or L-alanine. This catabolite repression can either be regulated directly mediated by the *ara* or XLNR system or by the changes in metabolite levels.

It is well known that D-glucose in *Aspergilli* is the most repressing carbon source (Ruijter & Visser, 1997). We found a clear order of repression using combinations of pentoses with other carbon sources. The order in degree of repression of the carbon sources tested is D-xylose, L-arabinose, xylitol, L-arabitol, D-gluconate and L-alanine. The *araA* and *araB* mutants show reduced carbon catabolite repression specifically for L-arabinose on the repressible carbon sources D-gluconate and L-alanine. This implies that the *araA* and *araB* mutations are located in genes that are involved, not only in a pathway leading to arabinanase induction, but also have an impact on carbon catabolite repression by L-arabinose. It was demonstrated quite clearly that the simultaneous induction and repression of hemicellulases by D-xylose is regulated by XLNR and CREA (De Vries *et al.*, 1999), but the role of XLNR in signaling the presence of D-xylose, and resulting in carbon catabolite repression by CREA in the presence od D-xylose was not investigated. Such a role, however, could at least partially explain the elevated induction of the L-arabinose system in the *xlnR* disruptant.

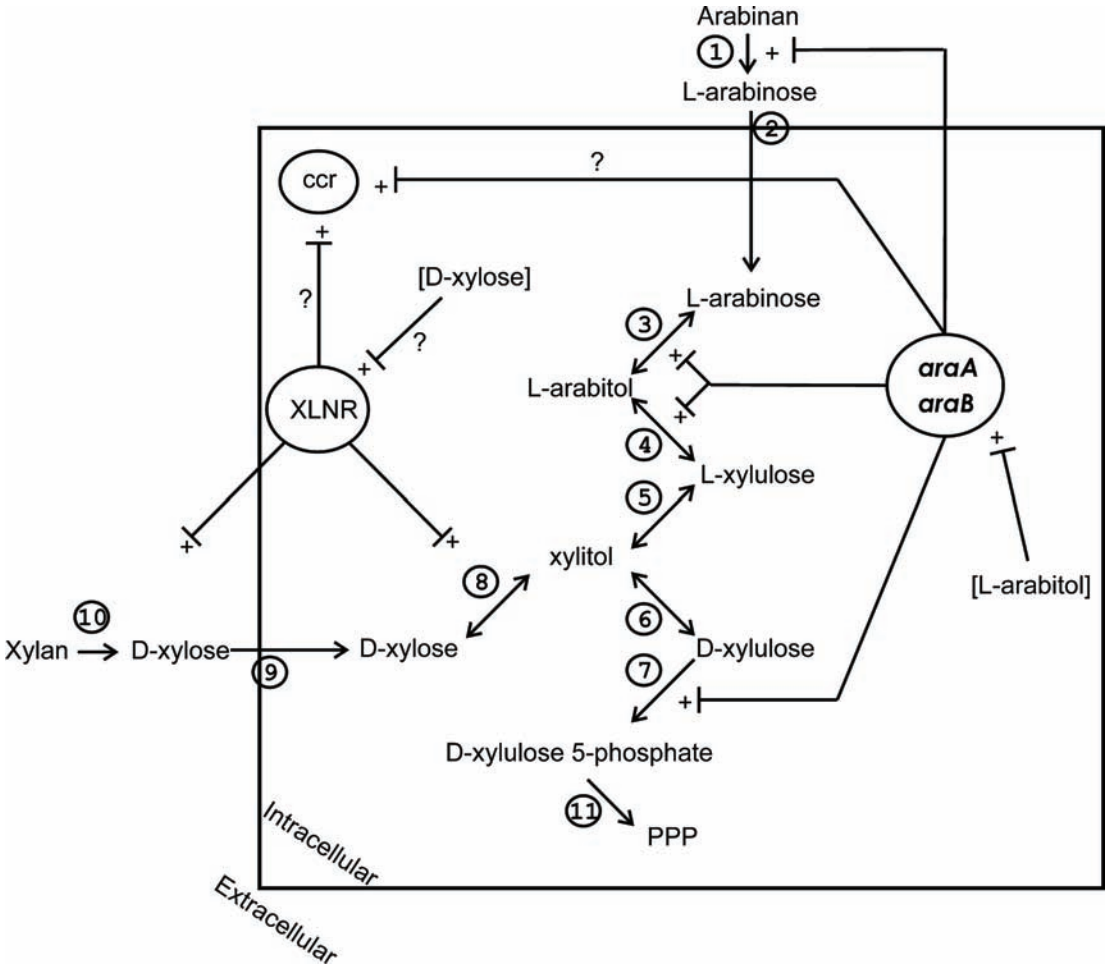

Figure 5. Model shows the regulation, by XLNR and the proteins encoded by the *araA* and *araB*, of the extracellular and intracellular components of the arabinan and xylan degrading systems. The question marks represent steps/features of which the mechanism is unknown. The circled numbers represent the following enzymes/steps: (1) α-L-arabinofuranosidases (EC 3.2.1.55) (ABFA, ABFB, AXHA) and arabinan endo-1,5-α-L-arabinosidase (EC 3.2.1.99) (ABNA); (2) transport of L-arabinose; (3) L-arabinose reductase (EC 1.1.1.21); (4) L-arabitol dehydrogenase (EC 1.1.1.12); (5) L-xylulose reductase (EC 1.1.1.10); (6) D-xylulose reductase (EC 1.1.1.9); (7) D-xylulokinase (EC 2.7.1.17) (XKIA); (8) D-xylose reductase (EC 1.1.1.21) (XYRA); (9) transport of D-xylose. (10) endo-1,4-β-xylanase (EC 3.2.1.8) (XYN1) and xylan 1,4-β-xylosidase (XLND); (11) transketolase (EC 2.2.1.1) and ribulose-phosphate 3-epimerase (EC 5.1.3.1). PPP = non-oxidative pentose phosphate pathway. ccr=carbon catabolite repression. + is a positive regulation effect. – is a negative regulation effect.

The function of the two specific inducers (L-arabitol and D-xylose), of the *ara* and XLNR systems respectively, as both an inducer for its own targets, and as carbon catabolite repressors mediated by CREA or by a more specific system, enable *A. niger* to respond specifically to the presence of different pentoses and

hemicelluloses. Since the occurrence of these two pentoses in hemicellulose is never completely separate, such a system would function to fine-tune the regulation of the arabinanase and xylanase encoding genes. The metabolism of L-arabitol and D-xylose is linked. Both by enzymatic equilibrium and by broad substrate specificity of the enzymes involved, and always leads to induction of both the arabinanan and xylan degrading systems. The bias created by the induction-repression system proposed reduces the production of unnecessary enzymes and is therefore beneficial to the organism.

Acknowledgements

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| Chapter 4

The *Aspergillus niger* D-xylulose kinase gene is co-expressed with genes encoding arabinan degrading enzymes, and is essential for growth on D-xylose and L-arabinose.

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Summary

The *Aspergillus niger* D-xylulose kinase encoding gene has been cloned by complementation of a strain deficient in D-xylulose kinase activity. Expression of *xkiA* was observed in the presence of L-arabinose, L-arabitol, and D-xylose. Expression of *xkiA* is not mediated by XLNR, the xylose-dependent positively acting xylanolytic regulator. Although the expression of *xkiA* is subject to carbon catabolite repression, the wide domain regulator CREA is not directly involved. The *A. niger* D-xylulose kinase was purified to homogeneity, and the molecular mass determined using electrospray ionization mass spectrometry concurred with the calculated molecular mass of 62816.6 Da. The activity of XKIA is highly specific for D-xylulose. Kinetic parameters were determined as K_m(D-xylulose) = 0.76 mM and K_m(ATP) = 0.061 mM. Increased transcript levels of the genes encoding arabinan and xylan degrading enzymes, observed in the xylulose kinase deficient strain, correlates with increased accumulation of L-arabitol and xylitol, respectively. This result supports the suggestion that L-arabitol may be the specific low molecular weight inducer of the genes involved in arabinan degradation. It also suggests a possible role for xylitol in the induction of xylanolytic genes. Conversely, overproduction of XKIA did not reduce the size of the intracellular arabitol and xylitol pools, and therefore had no effect on expression of genes encoding xylan and arabinan degrading enzymes nor on the activity of the enzymes of the catabolic pathway.

Introduction

Due to their utility in industrial applications, many of the genes whose products are involved in the degradation of plant cell wall polysaccharides have been cloned and characterised in detail from *Aspergillus* (De Vries & Visser, 2001) and other fungal species. Arabinanase activity is induced by sugar beet pulp, L-arabinose, and L-arabitol, and it is has been suggested that L-arabitol is the actual low molecular weight inducer (Van der Veen *et al.*, 1993). It has been shown in *A. nidulans* that increasing intracellular accumulation of arabitol enhances expression of enzymes from L-arabinose catabolism (De Vries *et al.*, 1994), further supporting the claim of L-arabitol as true inducer. Two α-L-arabinofuranosidase encoding genes, *abfA* and *abfB* (Flipphi *et al.*, 1993b; Flipphi *et al.*, 1993c), and one endoarabinanase encoding gene, *abnA* (Flipphi *et al.*, 1993a), from *A. niger* have been cloned and characterised. Expression analysis of these genes (Flipphi *et al.*, 1994) indicated that they are co-ordinately regulated. Increasing the copy number of individual arabinanase encoding genes was an initial attempt to increase the yield of arabinanases. Unfortunately this resulted in a decrease in the expression of the other arabinanase encoding genes (Flipphi *et al.*, 1994), indicating titration of a

positively acting transcriptional regulator. An alternative strategy to increase the yield of arabinanases is to increase the concentration of the positively acting regulatory protein, either directly or by manipulating the concentration of the low molecular weight inducer which is believed to be L-arabitol (De Vries *et al.*, 1994; Flipphi *et al.*, 1994).

The pathway for L-arabinose and D-xylose utilisation in *A. niger* was previously elucidated (Witteveen *et al.*, 1989). The D-xylose catabolic pathway joins the L-arabinose catabolic pathway, and both proceed to the formation of D-xylulose-5-phosphate, which feeds into the pentose phosphate pathway (Figure 1). This final step, the phosphorylation of D-xylulose, is catalysed by D-xylulose kinase. With the exception of two xylitol dehydrogenases that have been partially purified and characterised (Witteveen *et al.*, 1994), the *A. niger* enzymes from the L-arabinose/D-xylose catabolic pathway have not been purified and studied in detail. Previous studies, using an *A. niger* strain deficient in D-xylulose kinase activity, have shown that the loss of D-xylulose kinase activity results in an increase in intracellular L-arabitol and xylitol levels (Witteveen *et al.*, 1989). A correlation between intracellular arabitol levels and arabinanase activity was observed in this strain (Van der Veen *et al.*, 1993). The aims of this study were to analyze in detail the effects of the absence (in the *xkiA* mutant) or the increase (in multicopy transformants) of XKIA activity on the production of the other arabinose catabolic enzymes, the intracellular polyol concentrations, and the expression of arabinanolytic and xylanolytic genes. In addition, XKIA was characterized biochemically, and the expression of *xkiA* was studied in different genetic backgrounds and compared to the expression of arabinanolytic and xylanolytic genes and the gene encoding xylose reductase (*xyrA*), to determine whether *xkiA* is co-regulated with any of these genes.

A thorough understanding of the biochemical properties of the enzymes of the catabolic pathway, in addition to an understanding of their regulation, will contribute to determining strategies for the manipulation of steps of this pathway that result in improved production levels of arabinanases.

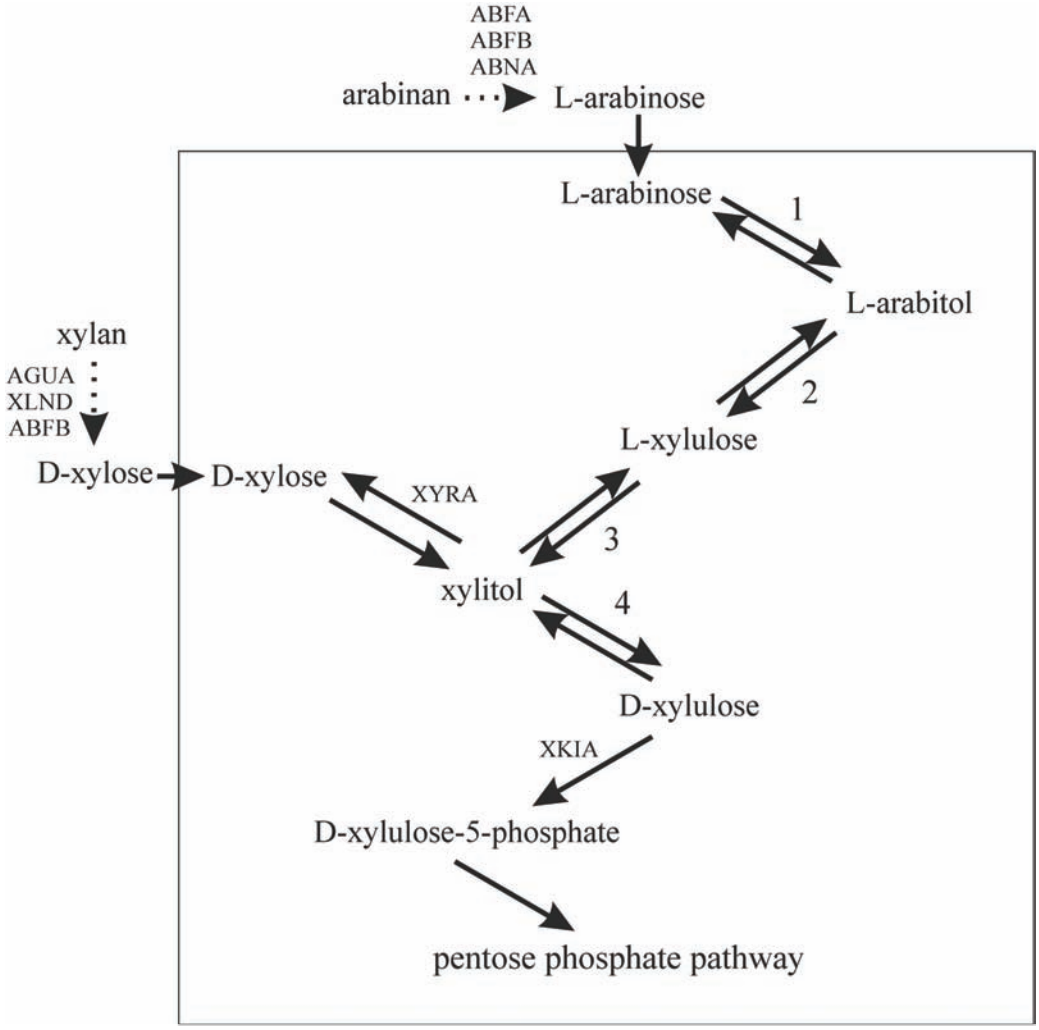

Figure 1. *A. niger* pentose catabolism. The box encloses the intracellular steps involved in pentose catabolism. Dashed arrows indicate degradation of polysaccharides, which involves many enzymes. Only those enzymes examined in this study, at the level of protein or mRNA, are shown. ABFA, ABFB and ABNA are involved in the degradation of arabinan, which ultimately results in the liberation of L-arabinose. ABFB, AGUA and XLND are involved in the degradation of xylan, which results in the production of D-xylose, amongst other monosaccharides. The enzymatic steps 1 - 4 of the pathway for which the genes have not been cloned are: 1 L-arabinose reductase (EC 1.1.1.21); 2 L-arabitol dehydrogenase (EC 1.1.1.12), 3 L-xylulose reductase (EC 1.1.1.10), and 4 D-xylulose reductase (EC 1.1.1.9) also known as NAD^+-dependent xylitol dehydrogenase. XKIA = D-xylulose kinase (EC 2.7.1.17), XYRA = D-xylose reductase (EC 1.1.1.21), ABFA = α-L-arabinofuranosidase A (EC 3.2.1.55), ABFB = α-L-arabinofuranosidase B (EC 3.2.1.55), ABNA = endo-1, 5-α-L-arabinanase (EC 3.2.1.99), AGUA = α-glucuronidase A (EC 3.2.1.139), and XLND = β-xylosidase (EC 3.2.1.37).

Materials and methods

Strains and growth conditions

All *A. niger* strains used were derived from *A. niger* N400 (CBS 120.49) and are described in Table 1. *Escherichia coli* DH5αF' was used for routine plasmid propagation. *E. coli* LE392 was used as a host for phage λEMBL3. pUC19 was used for subcloning (Yanisch-Perron *et al.*, 1985). *Aspergillus* co-transformations were performed as described by Kusters-van Someren (Kusters-van Someren *et al.*, 1991) using the *pyr*A gene as a selection marker.

Table 1. *A. niger* strains used in this study.

Strain	Genotype	Reference
N402	*cspA1*	(Bos *et al.*, 1988)
NW283	*fwnA1*; *cspA1*; *lysA7 pyrA6*; *creAd4*	(Ruijter *et al.*, 1997)
N902::pIM230::25.12	*fwnA1*; *cspA1*; *argB15*; *metB10* (20 copies *xlnR*)	(Van Peij *et al.*, 1998a)
NW199	*fwnA1*; *cspA1*; *goxC17*; *pyrA6* *xlnRΔ::pIM240*; *leuA1*	(Hasper *et al.*, 2000)
N572	*cspA1*; *fwnA1*; *pyrA6*; *xkiA1*; *nicA1*	(Witteveen *et al.*, 1989)
N755	*cspA1*; *pyrA6*; *nicA1*	This study
N755::pIM635	N755 + pGW635	This study.
N755::pIM4901(S2)	N755 + pGW635 + pIM4901 (10-15 copies *xkiA*)	This study.
N755::pIM4901(S6)	N755 + pGW635 + pIM4901 (25-30 copies *xkiA*)	This study.

The *A. niger* gene encoding D-xylulose kinase was cloned by complementation of the *xki*A1 mutation (strain N572). Co-transformation was performed using a genomic library of *A. niger* (Harmsen *et al.*, 1990) and the autonomously replicating plasmid pHELP1 (Gems & Clutterbuck, 1994) as described previously (Van Peij *et al.*, 1998b).

Cultures were grown in minimal medium (MM) containing per litre; 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, trace elements (Vishniac & Santer, 1957), pH 6.0 with carbon sources as indicated in the text, in a rotary shaker at 250 rpm and 30°C. For the growth of strains with auxotrophic mutations, the necessary supplements were added to the medium. In transfer experiments strains were pre-grown in MM containing: 1% (mass/vol) D-fructose as a carbon source; 0.5 % w/v yeast extract; and 0.2% casamino acids. After 16 h mycelium was harvested by suction over a filter, washed with MM without carbon source, and aliquots of 1.5 g (wet weight) were transferred to 50 ml of MM containing different carbon sources and incubated as indicated in the text. After two hours the mycelium was harvested,

dried between paper and frozen in liquid nitrogen. Mycelium used for the partial purification of intracellular enzymes was washed in 10mM potassium phosphate (pH 7.0). The mycelium samples were stored at -70° C.

Cloning and molecular characterisation of *xkiA*

Standard methods were used for DNA manipulations, such as Southern analysis, subcloning, DNA digestions, and plasmid DNA isolations (Sambrook *et al.*, 1989). Chromosomal DNA was isolated as previously described (Lee & Taylor, 1990). Sequence analysis was performed at EuroSequence Gene Service (Génopole, Evry, France) on an ABI 377 sequencer using ABI PRISM ™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (PE Applied Biosystems). Nucleotide sequences were analysed with computer programs based on Devereux et al. (Devereux *et al.*, 1984). Contour-clamped homogeneous electric field (CHEF) Southern analysis was performed as described previously (Verdoes *et al.*, 1994) using modifications as described by van den Hombergh et al. (Van den Hombergh *et al.*, 1996).

Total RNA was isolated from powdered mycelium using TRIzol Reagent (Life Technologies), according to the supplier's instructions. For northern blot analysis 3 µg total RNA was incubated with 3.3 µl 6 M glyoxal, 10 µl DMSO and 2 µl 0.1 M sodium phosphate buffer pH 7 in a total volume of 20 µl for 1 h at 50°C to denature the RNA. The RNA samples were separated on a 1.5% agarose gel using 0.01 M sodium phosphate buffer pH 5 and transferred to Hybond-N filters (Amersham) by capillary blotting. Filters were hybridised at 42°C in a solution of: 50% (vol/vol) formamide, 10% (mass/vol) dextran sulphate, 0.9 M NaCl, 90 mM Na_3-citrate, 0.2% (mass/vol) ficoll, 0.2% (mass/vol) polyvinylpyrolidone, 0.2% (mass/vol) bovine serum albumin, 0.1% (mass/vol) SDS and 100 µg/ml single stranded herring sperm DNA. Washing was performed under stringent conditions to 30 mM NaCl, 3 mM Na_3-citrate and 0.5% (mass/vol) SDS at 68°C. The probes used were: a 2.0 kb *Pst*I fragment from *abfA* (Flipphi *et al.*, 1994); a 2.8 kb *Pst*I fragment from *abfB* (Flipphi *et al.*, 1994); a 3.1 kb *Hind*III fragment from *abnA* (Flipphi *et al.*, 1994); a 0.5 kb *Eco*RV/*Xho*I fragment from *aguA* (De Vries *et al.*, 2002); a 2.8 kb *Pst*I/*Nsi*I fragment from *xlnD* (Van Peij *et al.*, 1997); a 0.3 kb *Sal*I/*Xho*I fragment from *xyrA* (Hasper *et al.*, 2000); a 4.0 kb *Sst*I fragment containing *xkiA*; a 0.7 kb *Eco*RI fragment from the gene encoding the 18S rRNA subunit (Melchers *et al.*, 1994) was used as an RNA loading control. The *xkiA* nucleotide accession number is AJ305311.

Preparation of cell extracts and protein determination

Mycelium was harvested and disrupted as described previously (Hondmann & Visser, 1990). Cell extracts were obtained by suspending disrupted frozen mycelium in extraction buffer (10 mM Bis-Tris, 5 mM MgCl, 1 mM 2-mercaptoethanol, 0.5 mM EDTA pH 6.3) followed by centrifugation at 15000-20000 x *g* in order to remove cell debris. The entire procedure was performed at 0 to 4°C. Protein concentrations were determined after denaturation and precipitation of protein, with sodium deoxycholate and trichloroacetic acid respectively (Bensadoun & Weinstein, 1976), using the bichinchoninic acid method as described by the manufacturer (Sigma Chemical Company).

Purification and kinetic characterisation of D-xylulose kinase

D-xylulose kinase was purified from *xki*A transformant N755::pIM4901(S6), which overproduces the enzyme approximately 66-fold when harvested after an eight hours transfer to MM containing 1% (w/v) D-xylose. The purification procedure was carried out at 4°C up to the Resource Q step which was carried out at room temperature. Extract was prepared with 3 g of frozen mycelium powder per 15 ml extraction buffer and ammonium was added to 60% saturation. Following 10 min centrifugation at 14 000 *g* the resulting supernatant, containing the D-xylulose kinase activity, was brought to 80% ammonium sulphate saturation and centrifuged. The pellet resulting from this step was dissolved in 1 M ammonium sulphate and applied to a Phenyl-Sepharose CL4-B column (Pharmacia Biotech 16 mm i.d. x 84 mm, flow rate 6-8 ml/min). Bound protein was eluted by applying a gradient from 1 M to 0 M ammonium sulphate. Fractions containing D-xylulose kinase activity were pooled, and dialysed overnight against 5 mM Bis-Tris pH 6.3 and applied to a Resource Q column (Pharmacia Biotech 6.4 mm i.d. x 30 mm, flow rate: 4 ml/min). Protein was eluted using a gradient from 0 M to 0.5 M NaCl in extraction buffer. Fractions containing XKIA activity were stored at 4°C.

Kinetic measurements were performed under the conditions mentioned below. Kinetics were studied by varying the concentration of one substrate while maintaining a fixed concentration of the other substrate. Initial rate data were fitted to the Michaelis-Menten equation using EnzFitter (Elsevier). Secondary plots were used to obtain the kinetic constants. K_i values were determined by replotting the slopes and intercepts of the double reciprocal plots against the inhibitor concentration (K_{ii} for the intercept effect, K_{is} for the slope effect).

Enzyme assays

All enzyme assays were performed at 30°C. Absorbance changes at 340 nm (ε_{NADPH} = 6.22 mM^{-1} cm^{-1}) were measured using a Cobas Bio (Roche) autoanalyser or an UV-2501PC spectrophotometer (Shimadzu Scientific Instruments).

D-xylulose kinase activity was measured in 50 mM glycylglycine pH 7.8, 5 mM $MgCl_2$, 1 mM ATP, 1.5 mM PEP, 0.2 mM NADH, 5 mM D-xylulose, 1 U lactate dehydrogenase, and 3 U pyruvate kinase. Final assay volume was 1 ml. Inhibition of D-xylulose kinase by ADP was determined using a discontinuous assay. The reaction was performed in 50 mM glycylglycine pH 7.8, 5 mM $MgCl_2$, 0.75 mM D-xylulose and a varying concentration of ATP. 500 µl samples of the reaction mixture were taken at a number of time points, mixed with 50 µl of 1.1 M PCA to stop the reaction, and stored on ice. The samples were neutralised with KOH. ATP was determined using 250 mM triethanolamine pH 7.5, 3 mM $MgCl_2$, 1 mM D-glucose, 0.5 mM NADPH, 3 U hexokinase, 3.5 U glucose 6-phosphate dehydrogenase and 0.5 ml sample containing ATP in a reaction mixture of 1 ml final volume.

L-arabitol dehydrogenase and xylitol dehydrogenase activities were determined using 100 mM glycine pH 9.6, 0.2 mM NAD^+ and 40 mM L-arabitol or xylitol respectively. Enzyme preparation was used in amounts containing 2-24 mU/ml (0.02-0.1 nkat).

Polyol extraction and determination

The extraction of intracellular polyols was carried out as described previously (Witteveen *et al.*, 1994). Polyol concentrations were measured by HPAEC (Dionex) with a Carbopac MA1 column using isocratic elution with 0.48 M NaOH (Witteveen *et al.*, 1994).

Statistical analysis

Statistical analysis of the polyol and enzyme levels was carried out using the ANOVA data analysis tool of Excel 97 SR-2 (Microsoft). An alpha value of 0.05 was used (probability $\geq$ 95%).

Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry analyses of the protein were carried out on a Micromass LCT time-of-flight instrument (Micromass UK Ltd., Wythenshawe, Manchester, United Kingdom) equipped with a 'Z-Spray' nanoflow electrospray source using in-house pulled and gold coated borosilicate glass needles. ESI-MS operating parameters were as follows: capillary voltage, 1.8 kV; cone voltage, 150 V; source block temperature, 70°C; TOF analyser pressure 1.4 x 10^{-6} mbar (standard 8.5 x 10^{-7} mbar). Spectra were recorded in the positive ion

mode and the standard mass range of 800-100000 Thompson (Th) was scanned. Samples for EIMS were prepared by desalting pure XKIA into 200 mM ammonium acetate buffer (pH 6.7). The samples contained protein at a concentration of 15 μM.

SDS-PAGE analysis

Denaturing electrophoresis in 10% (mass/vol) polyacrylamide gels containing 0.1% (mass/vol) SDS was performed as described by Laemmli (Laemmli, 1970) in a Mini-V system (Life Technologies). Proteins were detected by staining with Coomassie Brilliant Blue R250 (Weber & Osborn, 1969). SERVA Protein mixture 4 (Boehringer Ingelheim Bioproducts) containing phosphorylase B (92.5 kDa), BSA (68 kDa), ovalbumine (45 kDa) and carbonic anhydrase (29 kDa) was used to determine the molecular mass of denatured D-xylulose kinase.

Results

Cloning and molecular characterisation of *xkiA*

In order to examine the expression of *xkiA* and generate multicopy strains, the *A. niger* D-xylulose kinase encoding gene, *xkiA*, was cloned by complementation of a xylulose kinase deficient mutant isolated previously (Witteveen *et al.*, 1989). Transformants complementing the *xki*A1 mutation were selected directly by plating the transformation mixture onto MM plates containing 50 mM D-xylose as the sole carbon source, and 0.95 M KCl as the osmotic stabilizer. Eleven transformants, that had regained the ability to grow on D-xylose as the sole carbon source, were obtained from 1.6 x 10^8 transformed protoplasts. Plasmid was recovered from three of the eleven transformants. Subcloning and subsequent transformation identified a *Sst*I fragment, of approximately 4.0 kb, as the smallest fragment able to complement the *xki*A1 mutation. This fragment was cloned into pUC19 to generate the plasmid pIM4901.

Southern analysis showed that, when used as a probe, this *Sst*I fragment hybridised to a genomic DNA *Sst*I fragment identical in size (result not shown). A CHEF blot probed with this fragment showed that the complementing DNA hybridised to chromosome IV (result not shown), the same chromosome to which the *xkiA1* mutation has been localised using classical genetic analysis (Witteveen *et al.*, 1989). Sequence analysis showed that the coding region of *xkiA* is interrupted by a single intron, and codes for a protein of 570 amino acids with a calculated molecular mass of 62816.6 Da. At the level of derived amino acid sequence this gene showed homology to a number of (putative) D-xylulose kinase encoding genes including *xuk3* from *Pichia stipitis* (GenPept accession no. AF127802.1) (41% identical aa); *xks1p* from *S. cerevisiae* (Rodriguez-Pena *et al.*,

1998) (40% identical aa); xylulokinase from *Homo sapiens* (Tamari *et al.*, 1998) (36% identical aa); and xylulose kinase from *Arabidopsis thaliana* (Sato *et al.*, 2000) (36% identical aa). Based on the derived amino acid sequence the SOSUI (Hirokawa *et al.*, 1998) program predicted XKIA to be a soluble protein. Both ProDom (Corpet *et al.*, 2000) and Pfam (Bateman *et al.*, 2000) analysis indicated the presence of amino acid motifs common to the FGGY family of carbohydrate kinases (Hurley *et al.*, 1993). Prosite (Hofmann *et al.*, 1999) detected a Myc-type, "helix-loop-helix" dimerization domain which has also been detected in a number of eukaryotic DNA-binding proteins.

Located within a 961 bp region, 5' to the start codon of *xkiA*, are a number of putative regulatory elements. There are two putative binding sites for XLNR (GGCTAA), the regulator of (hemi-)cellulolytic genes in *A. niger* (Van Peij *et al.*, 1998b), located at positions –617 and –594 bp relative to the translational start of *xkiA*. One putative CCAAT box, previously shown to play a role in gene activation (Papagiannopoulos *et al.*, 1996), at position –335 bp was also found upstream of the *xkiA* start codon. There are no sites containing the consensus sequence for CREA (SYGGRG), the wide domain regulator responsible for carbon catabolite repression in *Aspergillus* (Kulmburg *et al.*, 1993).

Transcriptional regulation of *xkiA*

The expression of *xkiA* has been examined in a wild type strain, a *creA* derepressed mutant, a *xlnRΔ* strain and a *xlnR* multicopy strain of *A. niger* (Figure 2). No *xkiA* transcript was detected in the preculture samples (results not shown). In the wild type strain *xkiA* mRNA was detected in mycelium 2 hours after transfer to media containing D-xylose, L-arabinose, or L-arabitol as the sole carbon source, indicating that these three carbon sources are able to induce *xkiA* expression. Xylitol was unable to induce detectable levels of expression of *xkiA*. Repression by D-glucose was observed in the cultures containing D-glucose in addition to D-xylose, L-arabinose or L-arabitol, where expression of *xkiA* is lower than the comparable cultures without D-glucose. A pattern identical to wild type, with no significant alteration in the levels of expression, was observed for the *creAd4* strain. These results show that although the presence of D-glucose affects *xkiA* expression, CREA does not mediate this response.

The expression of *xkiA* in a *xlnR* multicopy or *xlnRΔ* strain did not differ from that observed in the wild type strain. This indicates that *xkiA*, although induced by D-xylose, is not under the control of XLNR. XLNR-regulated genes such as *xyrA*, the gene encoding D-xylose reductase, are not expressed in a *xlnRΔ* strain, and increased transcript levels are observed in *xlnR* multicopy strains (Hasper *et al.*, 2000). Unlike the other three strains, the *xlnR* multicopy strain also produced detectable levels of *xkiA* mRNA when xylitol was the sole carbon source. An

increase in the expression of *xkiA* is also observed when the *xlnRΔ* strain is grown on D-xylose. A similar increase in expression of a gene encoding a plant cell wall degrading enzyme, *abfB*, that is not XLNR regulated was observed in a *xlnR* loss of function mutant cultured on xylose (Van Peij *et al.*, 1998a).

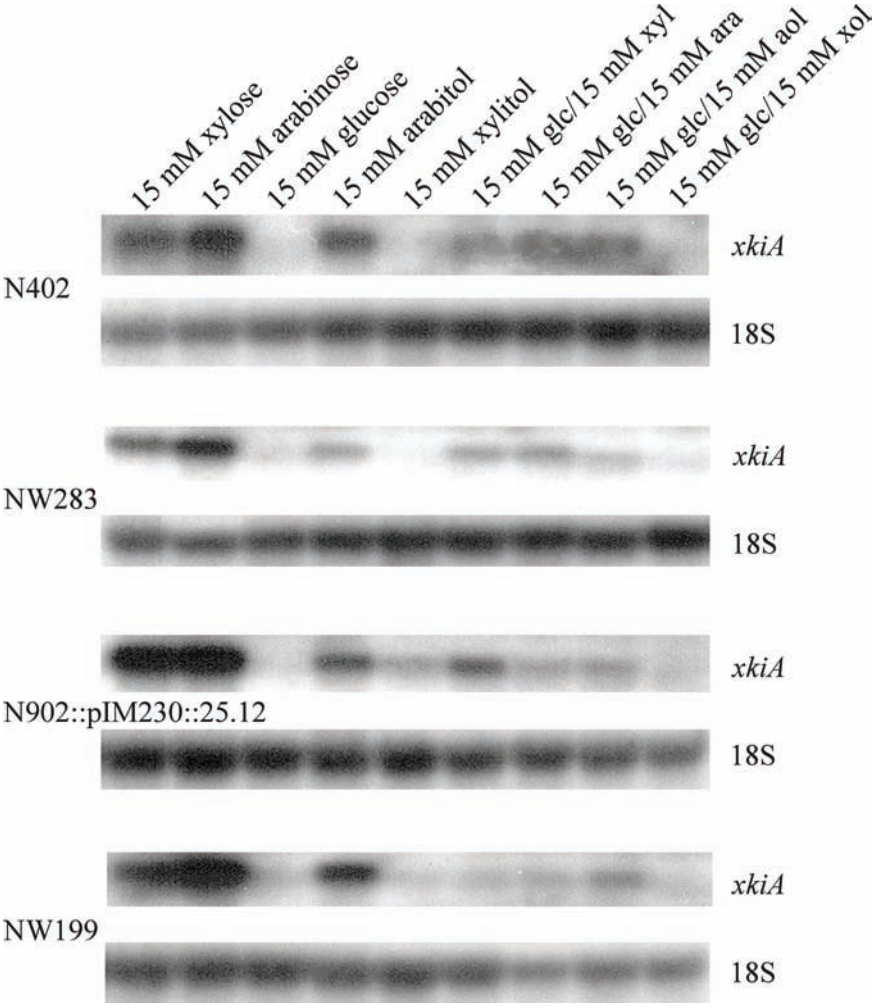

Figure 2. Regulation of the *A. niger xkiA* gene. The expression levels of *xkiA* were determined in cultures harvested 2 hours after transfer to a variety of carbon sources. Expression of *xkiA* was examined in N402 (a wild type strain), NW283 (*creAd4*), N902::pIM230::25.12 (*xlnR* multicopy), and NW199 (*xlnRΔ*). glc = D-glucose, xyl = D-xylose, ara = L-arabinose, aol = L-arabitol, and xol = xylitol. This result is representative for an experiment done in duplicate.

Purification and enzymatic properties of *A. niger* D-xylulose kinase

The *A. niger* D-xylulose kinase was purified by ammonium sulphate precipitation, Phenyl-Sepharose and ResourceQ chromatography, resulting in an apparently homogeneous XKIA preparation (Figure 3) suitable for use in determining the biochemical properties of this enzyme. After the final purification step 31% of the total XKIA activity was recovered (Table 2).

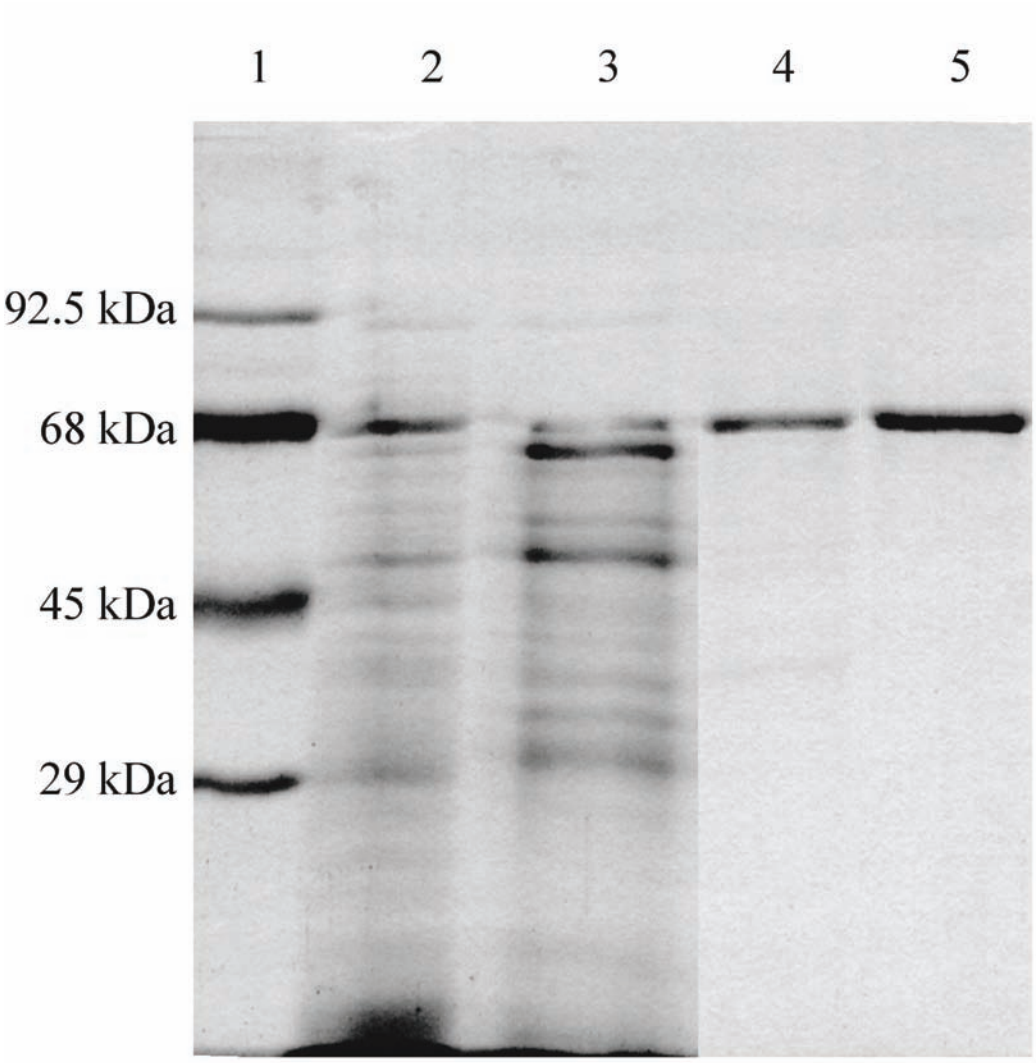

Figure 3. SDS-PAGE of *A. niger* D-xylulose kinase at various stages during purification. Lane 1, marker proteins with indicated molecular masses; lane 2, crude extract (100 µg); lane 3, sample after 80% ammonium sulphate precipitation (5 µg); lane 4, sample obtained from Phenyl Sepharose CL-4B chromatography (5 µg); lane 5, final sample after ResourceQ chromatography (5 µg).

Table 2. Purification of D-xylulose kinase from *A. niger* transformant N755::pIM4901(S6).

Step	Volume ml	Total activity µkat	Specific activity µkat/mg	Recovery %
Extract	40	67.9	0.7	100
Ammonium Sulphate Precipitiation (60-80%)	15	52.9	2.2	78
HIC	35	26.6	5.4	39
ResourceQ	3	21.0	29.7	31

From SDS-PAGE a molecular mass of 67 kDa was estimated for the denatured protein. The molecular mass of 62815.2 Da was calculated for XKIA using electrospray ionization mass spectrometry (ESI MS). A protein of 125.6 kDa, the size expected for a XKIA dimer, was also detected (data not shown). ESI MS analysis showed a dimer/monomer ratio of approximately 1:10.

A. niger D-xylulose kinase has a broad pH optimum from pH 7.0 to pH 8.5 (data not shown). As the pH of the cytosol of *A. niger* is pH 7.8 (Hesse *et al.*, 2002) the enzyme kinetics were studied in detail with excess Mg^{2+} at this pH. Hyperbolic kinetics were observed for both ATP and D-xylulose as substrates (Figure 4). No deterioration of activity was observed after several months of storage at 4°C. The K_m values for D-xylulose and ATP were 0.76 and 0.061 mM, respectively. A V_{max} of 33.6 μkat/mg was determined. From the substrates tested only D-xylulose acted as a substrate for this enzyme. No activity was detected when using L-xylulose, xylitol, D-xylose, L-xylose, L-arabitol, D-arabitol, L-arabinose, D-arabinose, D-glucose, or glycerol as substrates at 5, 10, and 20 mM (results not shown). In comparison to other D-xylulose kinases (Bateman *et al.*, 2000; Dills *et al.*, 1994; Flanagan & Waites, 1992; Hickman & Ashwell, 1958; Mortlock *et al.*, 1965; Neuberger *et al.*, 1981; Simpson, 1966) the *A. niger* XKIA is highly specific for D-xylulose and has favourable kinetic properties (high affinity for both D-xylulose and ATP).

Only a few compounds appeared to have an inhibiting effect on D-xylulose kinase. AMP and ADP showed strong inhibition (Table 3).

Table 3. Kinetic parameters of inhibition by ADP and AMP. Determination of inhibition patterns and K_i values was performed as described in Materials and Methods. Inhibition patterns: C = competitive; NC = non-competitive; ND = not detected.

Inhibitor	Variable substrate	Constant substrate	Inhibition pattern	K_{ii}	K_{is}
ADP	ATP	D-xylulose (0.75 mM)	*C*	ND	0.09
AMP	ATP	D-xylulose (0.75 mM)	*C*	12.7	0.72
AMP	D-xylulose	ATP (0.06 mM)	*NC*	1.3	0.97

Surprisingly, D-xylulose 5-phosphate (10 mM) did not have an inhibiting effect on the activity of D-xylulose kinase (results not shown). Besides the adenonucleotides, erythrose 4-phosphate had a minor negative effect on D-xylulose kinase activity (10% inhibition at 1 mM erythrose 4-phosphate, results not shown). No inhibition was observed for 0.5, 1, 2, or 5 mM fructose 6-

phosphate, glucose 1-phosphate, glucose 6-phosphate, mannitol 1-phosphate, ribose 5-phosphate, fructose 1,6-bisphosphate, glycerate 3-phosphate, glycerol 3-phosphate, gluconate 6-phosphate, ribulose 5-phosphate and cyclic AMP using ATP and D-xylulose as the substrates (results not shown).

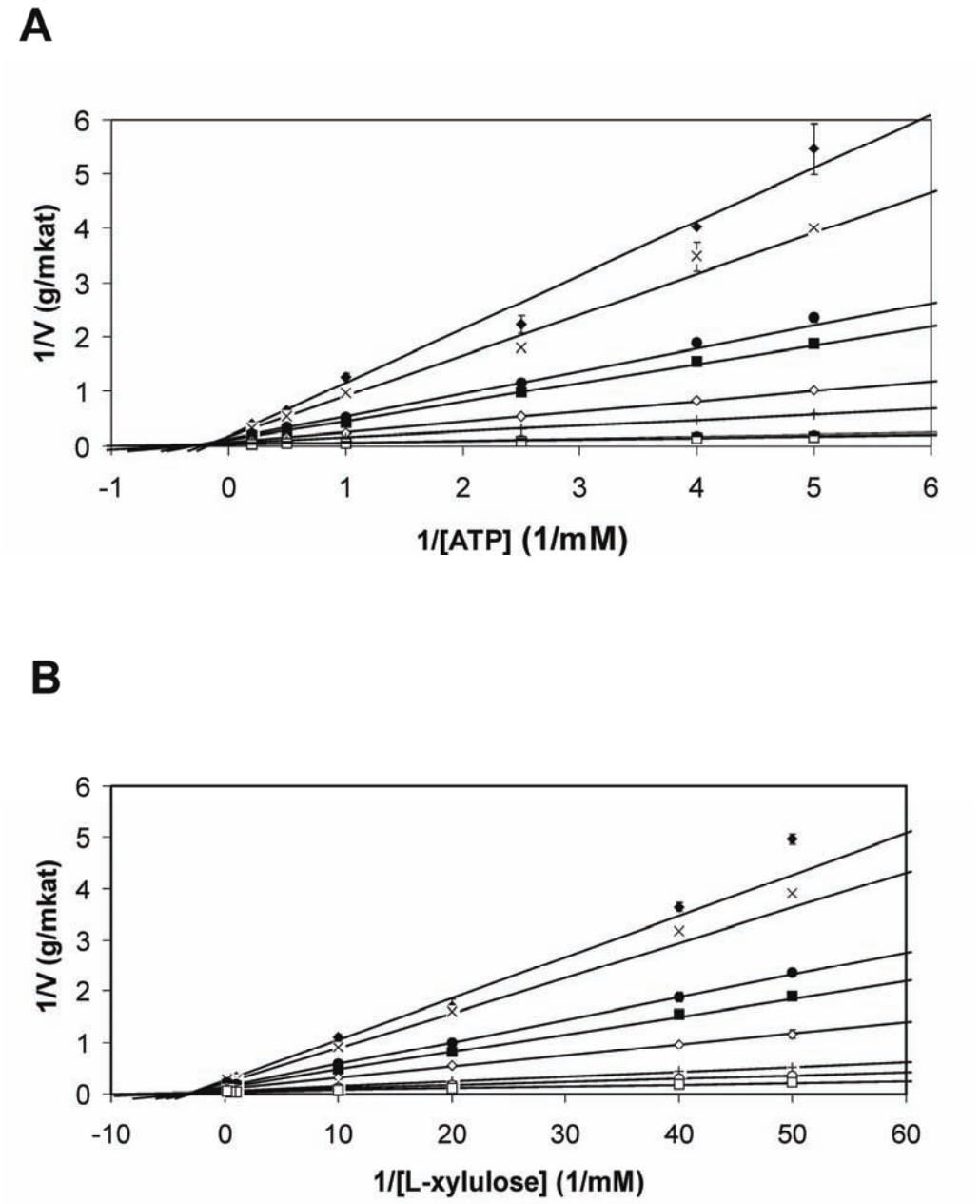

Figure 4. Double-reciprocal plots of D-xylulose kinase activity versus the concentration of D-xylulose and ATP. (A) shows the effect of the D-xylulose concentration at the following ATP concentrations: 0.01 mM (♦), 0.0125 mM (x), 0.02 mM (●), 0.025 mM (■), 0.05 mM (◊), 0.1 mM (+), 1 mM (○), 2 mM (□) and 5 mM (-). (B) shows the effect of the ATP concentration at a number of fixed D-xylulose concentrations: 0.1 mM (♦), 0.125 mM (x), 0.2 mM (●), 0.25 mM (■), 0.4 mM (◊), 1 mM (+), 2 mM (○) and 5 mM (□). Data points are the average of 2-4 measurements, the standard deviation is shown by error bars.

Comparison of *A. niger* wild type, *xkiA*1, and *xkiA* multicopy strains

In order to study the effects of overexpressing XKIA, multicopy strains of *A. niger* were constructed by co-transformation of strain N755 with pGW635, a plasmid carrying the *A. niger pyrA* gene as selectable marker (GenBank X96734), and pIM4901, a plasmid which contains the coding region for *xkiA* and 248 bp of the promoter. Southern analysis was used to determine *xkiA* copy number, and enzyme assays of XKIA activity were performed in order to determine XKIA activity levels in the transformants and the parental strain (results not shown). On the basis of these results two multicopy strains were selected to examine the effect of alterations in the level of XKIA on the pentose metabolic pathway. N755::pIM4901(S2) and N755::pIM4901(S6) have 10-15 and 25-30 copies of *xkiA*, respectively, and produced approximately 9.5 and 66 times the XKIA activity of the parental strain, respectively.

To examine the effects of manipulating the level of XKIA activity on the intracellular L-arabitol and xylitol pools the D-xylulose kinase deficient mutant and the two *xkiA* multicopy strains were compared to N755::pGW635. The expression of a number of arabinanase and xylanase encoding genes, intracellular polyol levels, and the activity of L-arabitol dehydrogenase and xylitol dehydrogenase, as representatives of the intracellular enzymes involved in L-arabinose and D-xylose catabolism, have been examined.

As can be seen in Figure 5, increased levels of *xkiA* transcript are produced by the *xkiA* multicopy strains N755::pIM4901(S2) and N755::pIM4901(S6). No dramatic difference in the transcript levels were observed between the wild type (N755::pGW635) and *xkiA* multicopy strains for any of the other genes tested (see Figure 1 for their relevance to D-xylose and L-arabinose catabolism). There was no significant difference ($F \leq 12.81$, $F_{crit} = 18.51$), between the wild type (N755::pGW635) and *xkiA* multicopy strains, in the level of polyol pathway intermediates (Table 4), with the exception of arabitol in xylose grown N755 compared to N755::pIM4901(S2) ($F = 20.35$, $F_{crit} = 18.51$). Neither of these strains differed significantly from N755::pIM4901(S6) cultured under the same conditions ($F \leq 12.81$, $F_{crit} = 18.51$).

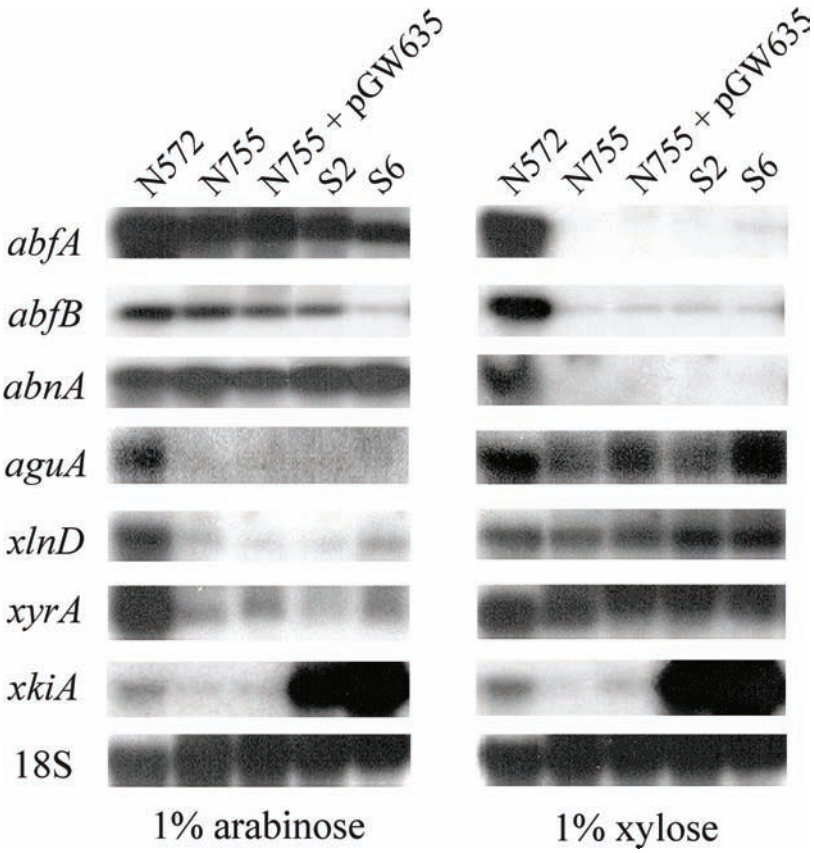

Figure 5. The effects of altered XKIA levels on the expression of a number of genes induced by L-arabinose or D-xylose. The steady state mRNA levels of the genes were determined in cultures harvested 4 hours after transfer to L-arabinose or D-xylose. Expression was determined in strains N572 (a D-xylulose kinase deficient mutant), N755 (parent of multicopy strains), N755 + pGW635 (parent transformed with *pyrA*$^{+}$), S2 (10-15 copies of *xkiA*), and S6 (25-30 copies of *xkiA*). The results shown are representative of an experiment done in duplicate.

In the strain deficient in D-xylulose kinase activity (N572) increased levels of transcript were detected for *xkiA* and the genes involved in arabinan degradation (*abfA*, *abfB*, *abnA*) in the presence of 1% D-xylose. The transcript levels of *xkiA* and the xylanolytic genes (*aguA*, *xlnD*, *xyrA*) were higher in the *xkiA* deficient strain in the presence of L-arabinose. This increase in expression levels correlates with an accumulation of arabitol that was observed when this strain was grown on D-xylose, and an increase in intracellular xylitol which occurred when this strain was grown on L-arabinose (Table 4, (Witteveen *et al.*, 1989)). The activity of L-arabitol dehydrogenase also correlates with the intracellular level of arabitol, with the multicopy strains having activity comparable to the wild type strain, while significantly higher levels were detected in the strain deficient in D-xylulose kinase activity. The exception is the levels of L-arabitol dehydrogenase detected in samples obtained from N755::pIM4901(S6) which were significantly lower than those detected for N755::pIM4901(S2) transferred to D-xylose or L-arabinose ($F = 20.50$, $F_{crit} = 18.51$, and $F = 431.05$, $F_{crit} = 18.51$, respectively), and N755::pGW635 transferred to either D-xylose or L-arabinose ($F = 371.83$, $F_{crit} = 18.51$, and $F = 110.51$, $F_{crit} = 18.51$ respectively). The level of xylitol dehydrogenase activity detected did not correlate with the intracellular concentration of either of the polyol intermediates from this pathway. In comparison to the wild type strain N755::pGW635, the level of xylitol dehydrogenase activity detected in the multicopy strain N755::pIM4901(S6), which expressed very high levels of XKIA,

Table 4. The effects of altered D-xylulose kinase levels on intracellular polyol concentrations and the levels of enzyme activity of L-arabitol dehydrogenase, xylitol dehydrogenase and D-xylulose kinase. Strains were transferred to L-arabinose or D-xylose containing media for 4 hours. Results shown are the average of two independent experiments with standard deviation. Intracellular xylose (in L-arabinose grown mycelia) and arabinose (in D-xylose grown mycelia) were not detected.

		Strain							
		N572		N755::pIM635		N755::pIM4901(S2)		N755::pIM4901(S6)	
		L-arabinose	D-xylose	L-arabinose	D-xylose	L-arabinose	D-xylose	L-arabinose	D-xylose
Polyol levels (μmol/g dry weight)	arabitol	300 ± 18	311 ± 48	130 ± 14	39 ± 2	153 ± 13	19± 4	197 ± 23	31 ± 1
	xylitol	259 ± 16	242 ± 24	15 ± 1	48 ± 5	13 ± 3	40 ± 3	6 ± 6	47 ± 10
Enzyme activity (mkat/mg protein)	L-arabitol dehydrogenase	3.8 ± 0.12	4.8 ± 0.07	2.9 ± 0.10	2.2 ± 0.05	2.8 ± 0.05	1.9 ± 0.20	1.8 ± 0.004	1.0 ± 0.02
	xylitol dehydrogenase	10.5 ± 0.88	11.2 ± 0.12	11.9 ± 0.97	11.3 ± 1.04	10.2 ± 0.23	10.8 ± 1.43	7.2 ± 0.42	6.0 ± 0.06
	D-xylulose kinase	0.05 ± 0.067	0.11 ± 0.150	10.1 ± 2.35	11.1 ± 0.38	289 ± 155.2	239 ± 100.8	1110 ± 406.2	1012 ± 103.4

was significantly lower in cultures grown on either D-xylose or L-arabinose ($F = 25.70$, $F_{crit} = 18.51$, and $F = 19.99$, $F_{crit} = 18.51$ respectively). Significantly less xylitol dehydrogenase was detected in mycelium obtained from N755::pIM4901(S6) transferred to L-arabinose than the equivalent samples from N755::pIM4901(S2) ($F = 38.46$, $F_{crit} = 18.51$).

Discussion

We have shown that *A. niger* XKIA is, without any doubt, a D-xylulose kinase as it is not active on any of the other substrates tested. Absence of D-xylulose kinase activity renders *A. niger* unable to grow on either L-arabinose or D-xylose (or the corresponding polyol pathway intermediates) as sole carbon source (Witteveen *et al.*, 1989).

Based on its amino acid sequence XKIA belongs to the FGGY family of carbohydrate kinases (Hurley *et al.*, 1993). This family consists of L-fucolokinases (EC 2.7.1.51), gluconokinases (EC 2.7.1.12), glycerokinases (EC 2.7.1.30), L-xylulose kinases (EC 2.7.1.53), and D-xylulose kinases (EC 2.7.1.17), suggesting that the genes encoding these enzymes are derived from a common ancestor. The only other eukaryotic D-xylulose kinase in this family for which functional studies have been performed is XKS1 from *S. cerevisiae* (Bateman *et al.*, 2000). However, it is debatable that the physiological function of this enzyme is the phosphorylation of D-xylulose as it is generally accepted that this organism is not able to utilise D-xylose as a sole carbon source (Walfridsson *et al.*, 1997). It is more likely that the primary function of *S. cerevisiae* XKS1 *in vivo* is the phosphorylation of another carbohydrate. A second argument for this hypothesis is that *S. cerevisiae* D-xylulose kinase is also able to use D-ribulose as a substrate, while D-xylulose kinases characterised from organisms which can utilize D-xylose, such as *A. niger*, exhibit high specificity for D-xylulose.

As mentioned in the results, after purification of XKIA two proteins were detected using EIMS indicating that a proportion of the protein in the sample is present as a dimer under the conditions used. It is therefore possible that the Myc-type, "helix-loop-helix" dimerization domain detected by Prosite (Hofmann *et al.*, 1999) is functional, and plays a role in XKIA dimer formation. It remains to be determined if the active form of XKIA is a monomer, a dimer, or that both forms are active. It has been reported that D-xylulose kinase purified from bovine liver exists solely as a monomer (Dills *et al.*, 1994), whilst the D-xylulose kinase, purified from *Aerobacter aerogenes* (Mortlock *et al.*, 1965), *P. stipitis* (Flanagan & Waites, 1992), and *Klebsiella aerogenes* (Neuberger *et al.*, 1981) have been detected as a dimer using gel filtration.

To date the only other gene cloned from the *A. niger* L-arabinose/D-xylose catabolic pathway is the D-xylose reductase encoding gene, *xyrA* (Hasper *et al.*, 2000). It is interesting to note that these two genes are regulated by different systems. The expression of *xyrA* is under the control of the xylanolytic activator XLNR (Hasper *et al.*, 2000). Although expression of *xkiA* is induced by D-xylose, apparently XLNR does not mediate this response even though the promoter contains putative XLNR binding sites. A similar report has been published for the endo-β-1,4-glucanase A gene of *A. nidulans* which also contains putative XLNR binding sites in its promoter but is not regulated by XLNR (Chikamatsu *et al.*, 1999).

In both *xkiA* multicopy strains (N755::pIM4901(S2) and N755::pIM490(S6)) expression of *xkiA* is repressed by D-glucose indicating that the cis-acting regulatory elements required for carbon catabolite repression and induction by L-arabinose/L-arabitol and D-xylose are contained within the 248 bp promoter included in the construct pIM4901. Expression of *xkiA* is not detected in the presence of D-glucose, this may be due to carbon catabolite repression that is not mediated by CREA. Alternatively this effect may be due to inducer exclusion, where the presence of D-glucose prevents uptake of L-arabinose/L-arabitol.

There are two possible explanations for the increase in expression of *xkiA* and the xylanolytic genes (*aguA*, *xlnD*, *xyrA*) observed for the *xkiA* deficient strain in the presence of L-arabinose. A small amount (< 0.1 %) of D-xylose is present in commercially available L-arabinose (De Vries *et al.*, 1998), and this level can induce XLNR regulated genes at short time points after transfer (ie. 2 hrs). Induction, of XLNR regulated genes is not observed at 4 or 8 hrs after transfer of strains able to metabolise D-xylose (de Vries, unpublished results). The lack of expression of XLNR regulated genes in the N755 series of strains is due to the D-xylose having been taken up and metabolised preferentially (over the L-arbinose), and therefore no longer being present by the 4 hr time point. Induction by D-xylose is not observed in these strains. The *xkiA* deficient strain, N572, is unable to grow on D-xylose or L-arabinose. It is probable that this strain also takes up D-xylose preferentially, but as it cannot metabolise either sugar past D-xylulose (see figure 1), pathway intermediates form and their intracellular concentrations reach a state of equilibrium. Consequentially we expect D-xylose to be present intracellularly. Intracellular xylose was undetectable in this strain, indicating that the intracellular concentration must be below 50μM (the detection limit). The intracellular concentration required to trigger induction is unknown. An alternative explanation is that xylitol may play a role in induction of the xylanase encoding genes.

Although increased levels of xylitol in the N755 strain correlates with increased expression of the arabinase encoding genes, such a correlation (between xylitol

and expression of arabinase encoding genes) does not occur for the other *A. niger* strains used in this study. This indicates that intracellular xylitol levels play no role in inducing the expression of the genes encoding the arabinases.

Transcription of *xkiA* is induced by L-arabinose and L-arabitol, as well as by D-xylose. Apart from *xkiA*, L-arabinose and L-arabitol also induce the production of the other L-arabinose catabolic enzymes as well as the expression of the genes encoding the extracellular arabinan degrading enzymes, suggesting that all these functions may be co-regulated. L-arabitol is believed to be the true low molecular weight inducer of the arabinanase encoding genes and it is therefore most probably the inducer of (at least some of) the genes encoding enzymes involved in L-arabinose catabolism. The correlation, between intracellular L-arabitol levels with both the expression levels of genes encoding extracellular arabinan degrading enzymes and, L-arabitol dehydrogenase activity, is consistent for all strains studied and supports this hypothesis. The activity of xylitol dehydrogenase does not correlate with intracellular L-arabitol levels, suggesting that production of this enzyme is regulated in a different manner. The effect of very high overproduction of XKIA on the levels of both L-arabitol and xylitol dehydrogenases could be due to specific effects such as competition by the respective genes for a common positively acting regulator, or down regulation of L-arabitol dehydrogenase production in order to maintain the intracellular L-arabitol pool above a certain concentration. Alternatively, a general phenomenon may occur in which the high level of XKIA production may result in a decrease in the production of other proteins.

In this work we have endeavoured to characterise the *A. niger* D-xylulose kinase and conduct a preliminary analysis of the factors regulating its transcription. Although increased D-xylulose kinase activity had no apparent effect on the expression of arabinanase or xylanase encoding genes, loss of this activity resulted in increased expression. These results further indicate that L-arabitol is likely to be the actual low molecular weight inducer of the arabinanase encoding genes.

A thorough characterisation of the other L-arabinose/D-xylose catabolic pathway enzymes will be crucial in determining the best strategy by which either the intracellular arabitol or xylitol pool can be manipulated. Manipulation of the intracellular arabitol pool is a viable strategy for increasing production of the arabinan-degrading enzymes. The mechanism(s) by which *xkiA* expression is induced by D-xylose, L-arabinose and L-arabitol, and repressed by D-glucose remains to be elucidated.

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| Chapter 5

Regulation of the pentose catabolic pathway of *Aspergillus niger*

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Abstract

The aim of this study was to obtain a better understanding of the pentose catabolism in *Aspergillus niger* and the regulatory systems that affect it. To this end, we have cloned and characterised the genes encoding *A. niger* L-arabitol dehydrogenase (*ladA*) and xylitol dehydrogenase (*xdhA*), and compared the regulation of these genes to other genes of the pentose catabolic pathway. This resulted in a model in which an L-arabinose specific regulator activates the expression of all genes required for the conversion of L-arabinose to D-xylulose-5-phosphate. In addition, XLNR regulates the first step and, to a lesser extent, the other steps of the conversion of D-xylose into D-xylulose-5-phosphate.

Introduction

Aspergillus niger is a filamentous ascomycete that predominantly grows on dead or decaying plant material that mostly consists of polysaccharides (cellulose, xylan, pectin, xyloglucan, galactomannan). L-arabinose and/or D-xylose are major components of xylan, pectin and xyloglucan (De Vries *et al.*, 2002a), reflecting the importance of the catabolic pathway related to these carbon sources for *A. niger*. The pentose catabolic pathway of *A. niger* consists of a number of reversible reduction and oxidation steps leading to D-xylulose, which is irreversibly converted into D-xylulose-5-phosphate (Figure 1) (Witteveen *et al.*, 1989), from where it enters the pentose phosphate pathway. L-arabinose reductase and D-xylose reductase from *A. niger* are both active on L-arabinose and D-xylose, but with different affinities (Chapter 6 & De Groot *et al*, submitted). Conversely, the L-arabitol and xylitol dehydrogenases appear to be highly specific for their own substrate (Witteveen *et al.*, 1994). For *A. niger*, so far only the genes encoding D-xylose reductase (*xyrA*) (Hasper *et al.*, 2000) and D-xylulokinase (*xkiA*) (Chapter 4 & VanKuyk *et al.*, 2001) have been described. In *Trichoderma reesei*, genes encoding L-arabitol dehydrogenase (*lad1*) (Richard *et al.*, 2001) and xylitol dehydrogenase (*xdh1*) (Seiboth *et al.*, 2003) have been identified. Recently, *Magnaporthe grisea* L-arabinose reductase and D-xylose reductase encoding genes and their corresponding proteins have been characterised (De Vries *et al.*, personal communication).

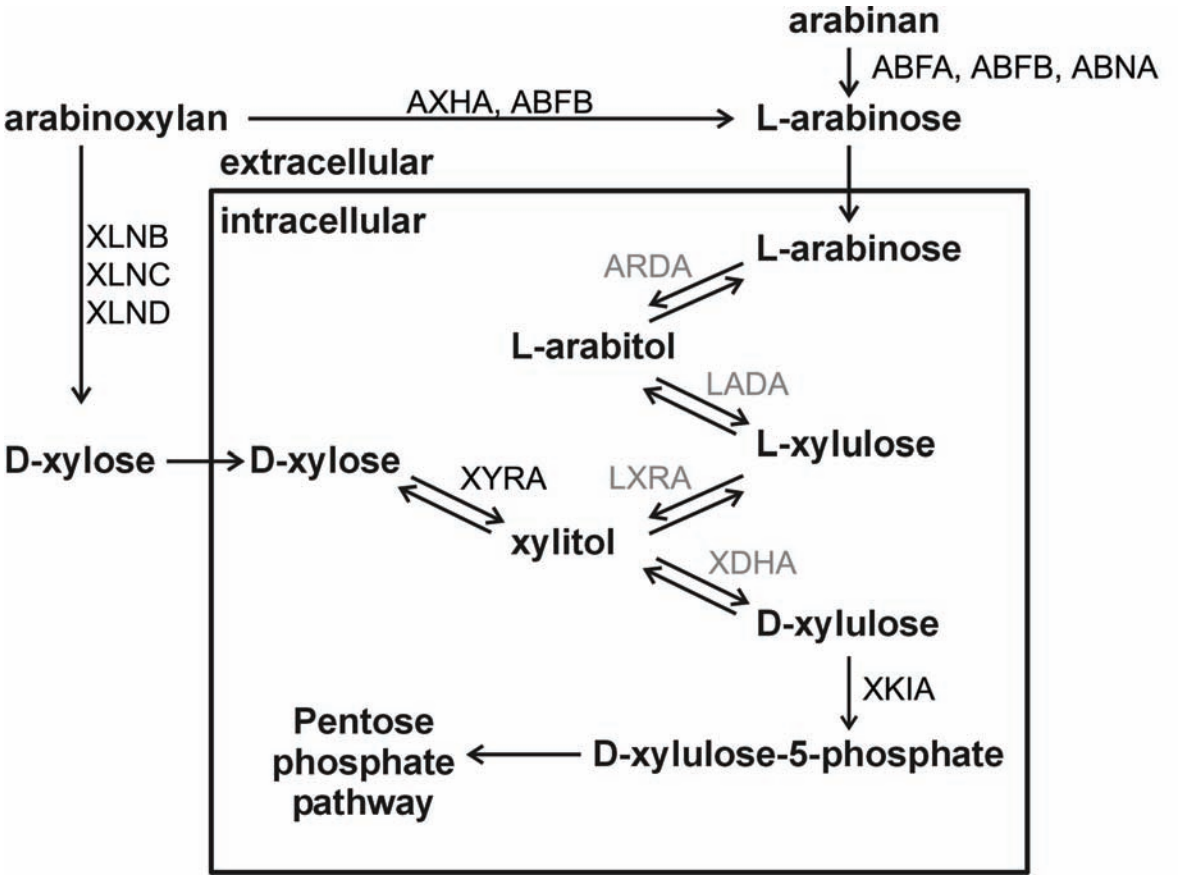

Figure 1. Pentose catabolism in *Aspergillus niger.* ARDA = L-arabinose reductase; LADA = L-arabitol dehydrogenase; LXRA = L-xylulose reductase; XDHA = xylitol dehydrogenase; XYRA = D-xylose reductase; XKIA = D-xylulose kinase; ABFA, ABFB = α-L-arabinofuranosidase A and B; ABNA = endoarabinanase A; AXHA = arabinoxylan arabinofuranohydrolase A; XLNB, XLNC = endoxylanase B and C; XLND = β-xylosidase. Abbreviations in grey indicate that the corresponding gene has not yet been identified. ARDA, LXRA and XYRA are NADPH/$NADP^+$-dependent. LADA and XDHA are NADH/NAD^+ dependent.

Induction of the pentose catabolic pathway in *Aspergillus* occurs during growth in the presence of L-arabinose or D-xylose (Witteveen *et al.*, 1989). The presence of D-xylose results in the activation of the xylanolytic transcriptional activator XLNR (Van Peij *et al.*, 1998) that regulates the expression of genes encoding extracellular polysaccharide degrading enzymes, as well as the expression of *xyrA* (reviewed in De Vries, 2003). XLNR is not responsible for the L-arabinose induction of the pentose catabolic pathway. Analysis of an L-arabitol dehydrogenase mutant of *A. nidulans* demonstrated that the L-arabinose catabolic pathway and the production of the extracellular arabinanolytic enzymes (α-L-arabinofuranosidase and endoarabinanase) are co-induced and that L-arabitol is most likely the small molecular weight inducer (De Vries *et al.*, 1994). Analysis of a D-xylulokinase

mutant of *A. niger* and the corresponding gene (*xkiA*) supported the inducer function of L-arabitol (Chapter 4 & VanKuyk *et al.*, 2001). Recently, two *A. niger* mutants have been described that are specifically disturbed in the regulation of the L-arabinose catabolic pathway and the production of extracellular arabinolytic enzymes (Chapter 3 & De Groot *et al.*, 2003). These mutants confirmed the co-regulation of extracellular arabinolytic functions and the L-arabinose catabolic enzymes, and showed an antagonistic effect between XLNR and the L-arabinose/L-arabitol responsive regulator.

In this paper we report the characterisation and regulation of the *A. niger* genes encoding L-arabitol dehydrogenase (*ladA*) and xylitol dehydrogenase (*xdhA*). The *ladA* gene is the first identified *A. niger* gene of the pentose catabolic pathway that is required for L-arabinose utilisation, but not for D-xylose utilisation. A comparison of the regulation of *ladA*, *xdhA*, *xyrA*, and *xkiA* facilitated a detailed analysis of the contribution of XLNR and the arabinolytic regulator (AraR) to the overall regulation of the pathway. Comparison of the amino acid sequence of the dehydrogenase enzymes of this pathway to sorbitol dehydrogenases enabled us to determine which enzyme classes are most closely related.

Materials and methods

Strains, libraries and growth conditions

All *A. niger* strains were derived from *A. niger* N400 (CBS 120.49) and are described in Table 1.

Table 1. *Aspergillus niger* strains used in this study.

Strain no.	Phenotype	Genotype	Reference
N402	reference	*cspA1*	(Bos *et al.*, 1988)
NW176	*creA* mutant	*fwnA1, cspA1, hisD4, lysA7, creAd2, nicA1, pabA1*	(Ruijter *et al.*, 1997)
NW199	*xlnR* disruptant	*cspA1, fwnA1, goxC17, pyrA6, xlnRΔ::pIM240, leuA1*	(Hasper *et al.*, 2000)
NW321	*araA* mutant	*cspA1, fwnA6, leuA1, araA4*	(Chapter 3 & De Groot *et al.*, 2003)
NW322	*araB* mutant	*cspA1, bioA1, lysA7, araB3, nicA1*	(Chapter 3 & De Groot *et al.*, 2003)

Escherichia coli DH5αF', LE392 and M15[pREP4] were used for routine plasmid propagation, as a host for amplification of the cDNA libraries, and for production of LADA and XDHA, respectively. Subcloning was performed using pBluescript SK^+(Short *et al.*, 1988), pGEM-T easy (Promega) and pQE32 (Qiagen). A genomic library of *A. niger* was constructed in a similar manner as was described previously (Harmsen *et al.*, 1990). The L-arabinose specific cDNA library has been previously

described (VanKuyk *et al.*, 2004). For the D-xylose specific cDNA library, *A. niger* NW176 was grown for 16 h in complete medium (CM) (De Vries *et al.*, 2004) with 2% D-fructose. The mycelium was harvested, washed with minimal medium (MM) (De Vries *et al.*, 2004) without carbon source, and aliquots of 3.0 g (wet weight) were transferred to 100 mL of MM containing 50 mM D-xylose. Cultures were harvested at 2, 4, and 8 hours after transfer. Total RNA was isolated as described previously (De Vries *et al.*, 2002b). Isolation of PolyA$^+$ mRNA and construction of the library was performed in the same manner as was previously published for the L-arabinose-specific library (VanKuyk *et al.*, 2004).
Liquid cultures were inoculated with 10^6 spores/ml, and incubated at 30°C in an orbital shaker at 250 rpm. For the growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.
Transfer experiments were performed by pre-growing the strains for 16 h in CM containing 2% (w/v) D-fructose as carbon source, after which the mycelium was harvested and washed with MM without carbon source. Aliquots (1.5 g) of wet mycelium were then transferred to 50 ml MM containing carbon sources as indicated in the text. After 2 h of incubation in a rotary shaker at 250 rpm and 30°C, mycelium was harvested, dried between tissue paper, frozen in liquid nitrogen and stored at -70°C.

Molecular biology methods

Standard methods were used for DNA manipulations, such as subcloning, DNA digestions, and plasmid DNA isolations (Sambrook *et al.*, 1989). Chromosomal DNA was isolated as previously described (De Graaff *et al.*, 1988). Sequence analysis was performed using the Big Dye Terminator kit, Version 1.1 (Applied Biosystems, Foster City, CA) according to the supplier's instructions. The reactions were analysed with an ABI 310 (Applied Biosystems) or on an ABI 377 (Applied Biosystems) in which case Longranger Single Packs (Cambrex Bio Science, Rockland, Inc., Rockland, ME) were used. Northern analysis was performed as described previously (De Vries *et al.*, 2002b). Per sample, 5 µg of total RNA was loaded on the gel.
The *A. niger ladA* and *xdhA* accession numbers are AJ854040 and AJ854041, respectively.

Sequence analysis

Nucleotide sequences were analysed with computer programs based on those of Devereux *et al.* (Devereux *et al.*, 1984). Sequence alignments were performed by using the Blast programs (Altschul *et al.*, 1990) at the server of the National Center for Biotechnology Information, Bethesda, Md., USA (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignment and NJ tree construction was performed using ClustalW (Thompson *et al.*, 1994) version 1.8 at the server of the Bioinformatics Center at the Institute of Chemical Research, Kyoto University, Japan (http://clustalw.genome.jp/).

Production of recombinant LADA and XDHA

Based on the genomic sequence of *ladA* and *xdhA* oligonucleotides were designed to allow cloning of the cDNAs of these genes into *E. coli* expression vector pQE32 (Qiagen). One oligonucleotide was designed just behind the ATG of the genes and contained a *Bam*HI site for *ladA* (5'-GGATCCCTACCGCAACTGTTCTCG-3') and a *Bgl*II site for *xdhA* (5'-AGATCTGCACCCAGAACACCAACG-3'). The other was designed based on the sequence behind the STOP codon of the genes and contained a *Kpn*I site for *ladA* (5'-GGTACCACTGTTTAAATCTTCTGACC-3') and a *Pst*I site for *xdhA* (5'-CTGCAGAATTCTATGAATCGACACC-3'). PCRs were performed with these oligonucleotides using the L-arabinose/L-arabitol specific *A. niger* cDNA library (VanKuyk *et al.*, 2004) and the D-xylose specific *A. niger* cDNA library as template for *ladA* and *xdhA*, respectively. PCR fragments were cloned in pGEM-T easy (Promega) and confirmed by sequence analysis. Fragments were isolated from these constructs using the restriction enzymes mentioned above and cloned into pQE32. The resulting constructs were transformed to *E. coli* M13 cells as indicated by the supplier (Qiagen). Purification of the recombinant proteins was performed according to the supplier's recommendations.

Enzyme assays

All enzyme assays were performed at 20°C. Dehydrogenase activities were determined using 100 mM glycine pH 9.6, 0.4 mM NAD^+ and 100 mM substrate. Reductase activities were determined using 50 mM sodium phosphate pH 7.6, 0.2 mM NADH and 100 mM substrate. Absorbance changes at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) were measured on a Unicam UV-1 spectrophotometer (Spectronic Unicam, Rochester, NY).

Accession numbers

The sequences of *ladA* and *xdhA* have been submitted to EMBL and their accession numbers are AJ854040 and AJ854041, respectively

Results

Cloning and characterisation of *ladA* and *xdhA*

The amino acid sequence of *T. reesei* L-arabitol dehydrogenase and xylitol dehydrogenase were used to identify their paralogues in the *A. niger* genome sequence (DSM, Delft, The Netherlands). The *A. niger* L-arabitol dehydrogenase encoding gene (*ladA*) consists of 1228 bp and contains one intron. The gene encodes a protein of 386 amino acids with a predicted molecular mass of 41350 Da and a predicted pI of 5.99. The *A. niger* xylitol dehydrogenase encoding gene (*xdhA*) consists of 1262 bp and contains two introns. The gene encodes a protein

of 357 amino acids with a predicted molecular mass of 38836 Da and a predicted pI of 9.05.
Several putative regulatory binding sites were detected in the promoters of *ladA* and *xdhA*. The promoter of *ladA* contains 1 CCAAT box (Papagiannopoulos *et al.*, 1996), 4 putative CREA binding sites (Kulmburg *et al.*, 1993), 2 putative PACC binding sites(Tilburn *et al.*, 1995), two putative AREA binding sites (Omichinski *et al.*, 1993), a conserved sequence present in the promoters of pectinolytic genes (CCCTGA) (Benen *et al.*, 1996), and a sequence conserved in the promoters of arabinolytic genes (YGACRT), modified from (Flipphi *et al.*, 1994) based on analysis of the promoters of the arabinanolytic and L-arabinose catabolic genes. The *xdhA* promoter contains 8 CCAAT sites, 2 putative XLNR binding sites (De Vries *et al.*, 2002b), 2 putative CREA binding sites, 1 putative AREA binding site, and 2 copies of a conserved promoter sequence of arabinolytic genes (YGACRT).

Regulation of the expression of *ladA* and *xdhA*

The expression of *ladA* and *xdhA* was studied in a transfer experiment (see materials and methods) using a wild type, two arabinolytic mutants (Chapter 3 & De Groot *et al.*, 2003) and an *xlnR* disruptant strain (Hasper *et al.*, 2000) and compared to the previously determined expression of *xkiA*, *xyrA*, and *abfB* (encoding α-L-arabinofuranosidase B) (Chapter 3 & De Groot *et al.*, 2003). High expression of *ladA* was observed in the wild type and the *xlnR* disruptant on L-arabinose and L-arabitol, while expression on D-xylose was very low in all strains except for the *xlnR* disruptant (Figure 2). In the arabinolytic mutants, very low *ladA* expression on these three carbon sources was detected. For *xdhA*, expression was highest on L-arabinose and L-arabitol in the wild type, but significant expression levels were also detected on D-xylose (Figure 2). Expression of *xdhA* on L-arabitol and L-arabinose in the arabinolytic mutants was decreased, while a small increase was observed on D-xylose. In the *xlnR* disruptant, increased *xdhA* expression on L-arabinose and L-arabitol was observed. No expression was detected for any of the genes tested in the presence of xylitol.

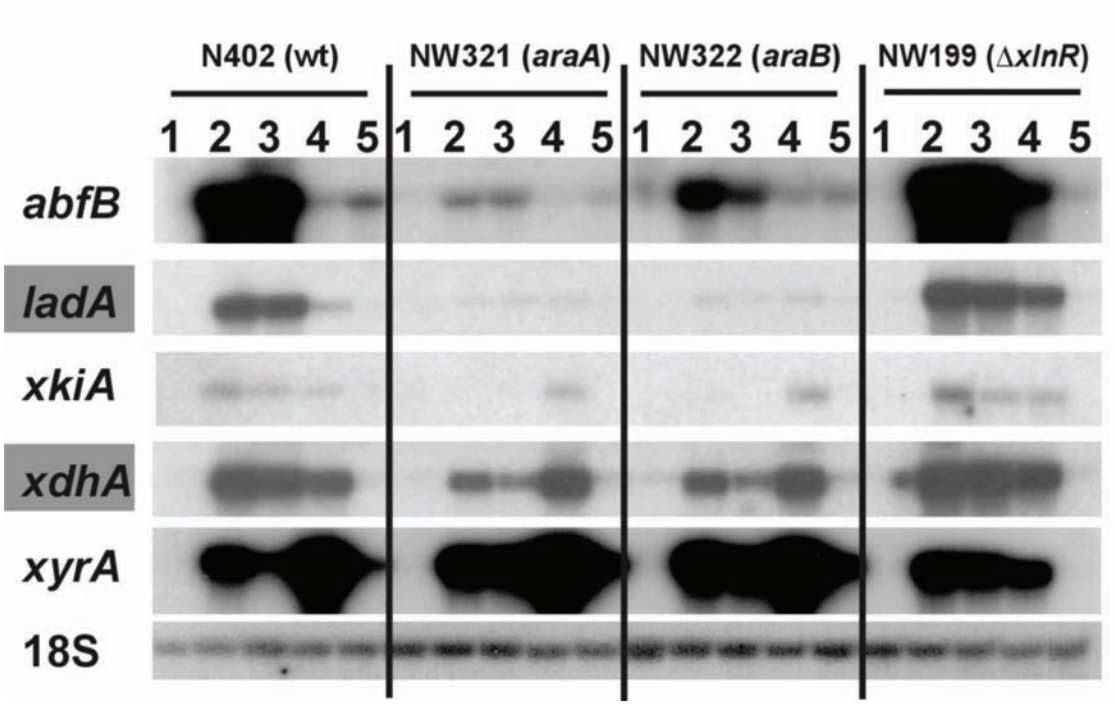

Figure 2. Comparison of the expression of *ladA* and *xdhA* to other pentose catabolic genes and *abfB*.
1 = D-fructose, 2 = L-arabinose, 3 = L-arabitol, 4 = D-xylose, 5 = xylitol.

Characterisation of recombinant LADA and XDHA

Recombinant LADA and XDHA were purified from *E. coli* and substrate specificities were determined for both enzymes. L-arabitol dehydrogenase (LADA) could only use NAD^+ as a cofactor and had the highest dehydrogenase activity on L-arabitol (100%), while the activity on xylitol, D-sorbitol and galactitol are 41%, 6%, and 4%, respectively, of the L-arabitol activity (Table 2). LADA had reductase activity on L-xylulose, D-xylulose, and D-fructose and activities were 283%, 143%, and 1.5%, respectively, relative to the dehydrogenase activity on L-arabitol. Xylitol dehydrogenase (XDHA) was also specific for NAD^+ and had the highest dehydrogenase activity on xylitol (100%), while the activity on D-sorbitol and L-arabitol was 57% and 3%, respectively and no activity was detected on Galactitol. XDHA had reductase activity on D-xylulose and D-fructose, and activities were 170% and 7%, respectively, relative to the dehydrogenase activity on xylitol. No activity for either enzyme was found using D-arabitol, D-mannitol, glycerol, ethanol, L- or D-arabinose, L- or D-xylose, D-glucose, D-galactose, D-ribose, D-mannose, L-rhamnose, L- or D-sorbose, L- or D-fucose, or D-tagatose as substrates.

Table 2. Specific activity of recombinant L-arabitol dehydrogenase and xylitol dehydrogenase. ND = not detected.

Enzyme Substrate	LADA	XDHA
	Specific activity (U/mg)	
L-arabitol	211 ± 25	12 ± 3
xylitol	86 ± 6	380 ± 8
D-sorbitol	2.3 ± 0.3	218 ± 3
D-galactitol	9 ± 3	ND
L-xylulose	597 ± 9	ND
D-xylulose	302 ± 2	650 ± 6
D-fructose	3.1 ± 0.1	28 ± 6

Fungal xylitol dehydrogenases and L-arabitol dehydrogenases are unequally related to sorbitol dehydrogenases of higher eukaryotes

In a recent paper it was shown that the L-arabitol dehydrogenase from *Trichoderma reesei* has homology with sorbitol dehydrogenases of higher eukaryotes and it was hypothesized that the L-arabitol dehydrogenase may be the fungal orthologue of sorbitol dehydrogenases from higher eukaryotes (Pail *et al.*, 2004). To evaluate this in more detail for both L-arabitol dehydrogenase and xylitol dehydrogenase, we have compared the amino acid sequences of characterised and putative L-arabitol and xylitol dehydrogenases of eight fungi to the sorbitol dehydrogenases of ten eukaryotes. A representative of 10 phylogenetic trees (Figure 3) shows that the sorbitol dehydrogenases of mammals and plants split into two groups reflecting the kingdoms, with the *D. melanogaster* and *C. elegans* sorbitol dehydrogenases branching off the mammalian group. The fungal L-arabitol and xylitol dehydrogenases also split into separate groups reflecting function, with the xylitol dehydrogenases being more closely related to the sorbitol dehydrogenases.

Discussion

Previously it was shown that two regulatory systems are involved in the pentose catabolic pathway in *A. niger*, responding to either D-xylose (XLNR) or L-arabinose/L-arabitol (unidentified arabinolytic regulator ARAR), and that they act in an antagonistic fashion (Chapter 3 & De Groot *et al.*, 2003). XLNR appeared to regulate D-xylose reductase (*xyrA*) (Hasper *et al.*, 2000), but not D-xylulokinase (*xkiA*) (Chapter 4 & VanKuyk *et al.*, 2001), while the putative arabinolytic regulator activates *xkiA*, but not *xyrA* (Chapter 3 & De Groot *et al.*, 2003). The effect of these regulators on the other members of the catabolic pathway could only be studied using enzyme activities, as the genes have not yet been identified.

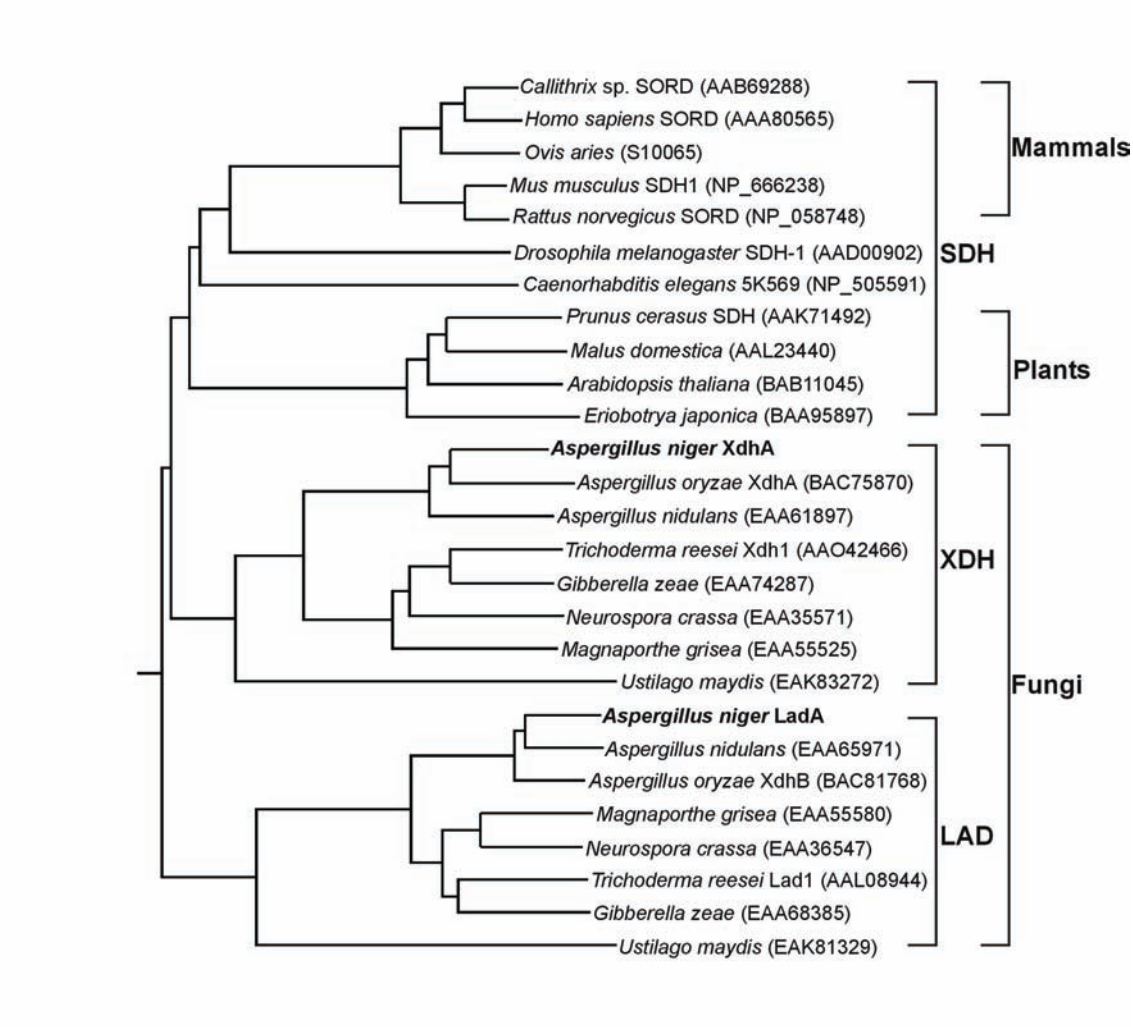

Figure 3. Phylogenetic tree of sorbitol, L-arabitol and xylitol dehydrogenases.
The two *A. niger* enzymes are shown in bold.
Accession numbers of the protein sequences are indicated in brackets.
The tree is a representative of 10 NJ trees with branch lengths.

This was complicated due to the fact that L-arabinose reductase and D-xylose reductase are active on both L-arabinose and D-xylose. The analysis of the expression of *ladA* and *xdhA*, and re-analysis of the expression of *xkiA* and *xyrA* performed in this study clearly shows the influence of the two regulatory systems on the pentose catabolic pathway in *A. niger*. Expression of *ladA* on L-arabinose and L-arabitol is strongly reduced in the arabinolytic mutants, while significant expression on D-xylose is only detected in the *xlnR* disruptant. Taking into account the antagonistic action of the two regulatory systems (Chapter 3 & De Groot *et al.*, 2003), this indicates that *ladA* is predominantly regulated by the arabinolytic system, while XLNR has no or very little influence on *ladA* expression. The expression of *xkiA* and *xdhA* is more complex. They have reduced expression on L-arabinose and L-arabitol in the arabinolytic mutants and

increased expression on D-xylose in the *xlnR* disruptant, indicating that they are under the control of the arabinolytic regulator. However, they also have increased expression on D-xylose in the arabinolytic mutants and on L-arabinose and L-arabitol in the *xlnR* disruptant, indicating regulation by XLNR. Based on the changes in expression levels, the effect of XLNR on the expression of these genes appears to be smaller than the effect of the arabinolytic regulator. Expression of *xyrA* is increased on L-arabinose and L-arabitol in the arabinolytic mutants and reduced on D-xylose in the *xlnR* disruptant, demonstrating that XLNR regulates this gene. Contrary to what was demonstrated for a number of other XLNR-regulated genes (Van Peij *et al.*, 1998) and what was stated previously (Hasper *et al.*, 2000) the expression of *xyrA* on D-xylose in the *xlnR* disruptant is not completely absent. This indicates that XLNR is not the only factor affecting *xyrA* expression. The expression of *xyrA* on L-arabinose does not decrease in the arabinolytic mutants, suggesting that the arabinolytic regulator is not involved in *xyrA* expression. In fact, *xyrA* expression on L-arabinose could be the result of the small amount of D-xylose present in the L-arabinose preparation used in these studies (De Vries *et al.*, 1998). Taking all the data together (Figure 4), we propose that there is an L-arabinose/L-arabitol responsive positively acting regulatory system in *A. niger* (ARAR) that regulates the expression of the genes involved in the conversion of L-arabinose to D-xylulose-5-phosphate. In addition, XLNR is the major regulatory system that regulates the first step (*xyrA*) of the D-xylose catabolism, but this protein has a less pronounced effect on the steps that are required for both L-arabinose and D-xylose catabolism (*xdhA*, *xkiA*) than ARAR. This model is also supported by the results of the promoter analysis of these genes. In the promoters of *xdhA*, and *xkiA* putative binding sites for XLNR have been detected as well as a sequence commonly found in genes that are influenced by ARAR.

However, the promoter of *ladA* only contains this conserved arabinolytic promoter sequence, while the promoter of *xyrA* only contains putative XLNR binding sites. Such a system is explicable from a fungal perspective as it ensures expression of all the relevant genes in the presence of either L-arabinose or D-xylose. The effects observed are not due to starvation or other stress factors, as all strains can grow on the carbon sources used for the study (Chapter 3 & De Groot *et al.*, 2003).

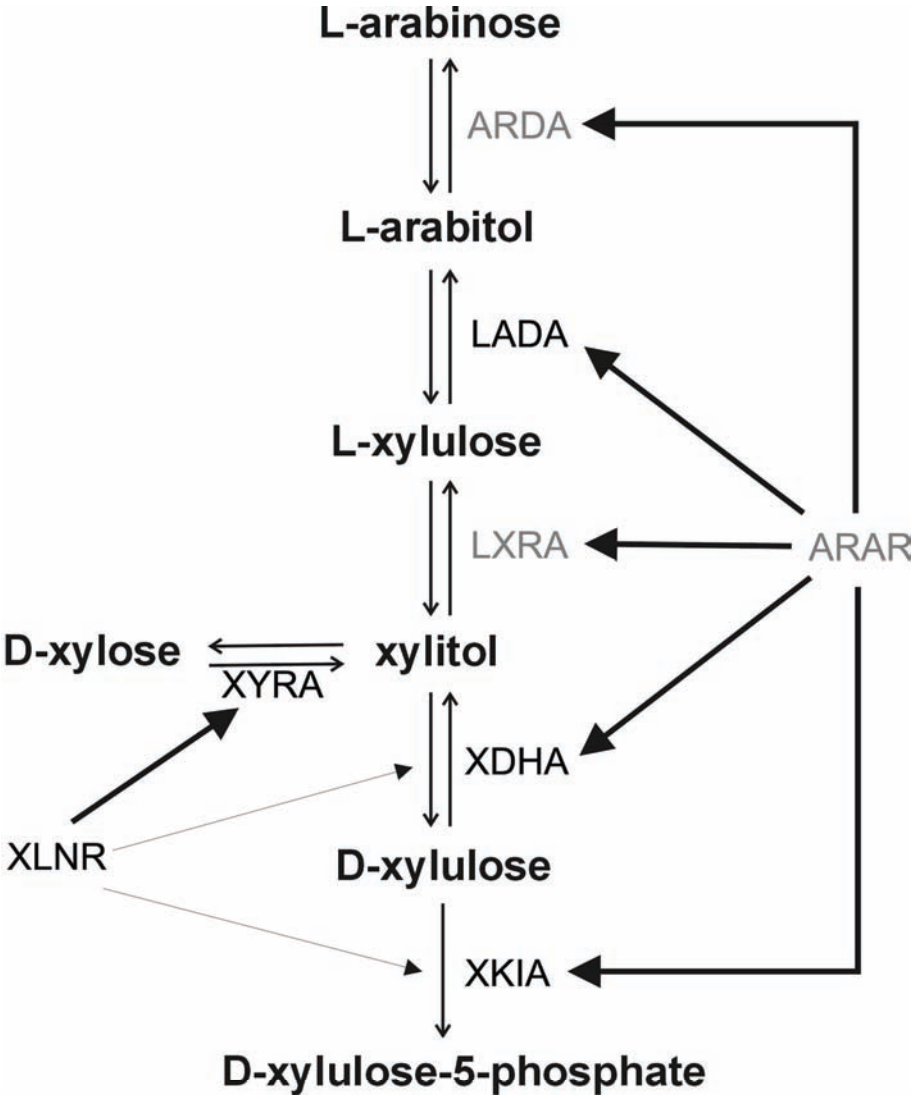

Figure 4. Model for the induction of the pentose catabolic pathway in *A. niger*. ARDA = L-arabinose reductase; LADA = L-arabitol dehydrogenase; LXRA = L-xylulose reductase; XDHA = xylitol dehydrogenase; XYRA = D-xylose reductase; XKIA = D-xylulose kinase; XLNR = xylanolytic transcriptional activator; ARAR = L-arabinose specific transcriptional activator. Abbreviations in grey indicate that the corresponding gene has not yet been cloned. The thickness of the arrows indicates the relative contribution of the two regulators.

Comparison of the deduced amino acid sequences of LADA and XDHA to other L-arabitol, xylitol and sorbitol dehydrogenases, demonstrated strong amino acid conservation of the three types of dehydrogenases. Previously it was suggested that L-arabitol dehydrogenase might be the fungal orthologue of sorbitol dehydrogenase of higher eukaryotes (Pail *et al.*, 2004). However, the data in this study indicates that fungal xylitol dehydrogenases are more closely related to the sorbitol dehydrogenases than fungal L-arabitol dehydrogenases.

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| Chapter 6

Metabolic control analysis of *Aspergillus niger* L-arabinose catabolism

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Summary

A mathematical model of the L-arabinose/D-xylose catabolic pathway of *Aspergillus niger* was constructed based on the kinetic properties of the enzymes. For this purpose L-arabinose reductase, L-arabitol dehydrogenase and D-xylose reductase were purified using dye-affinity chromatography and their kinetic properties characterised. For the other enzymes of the pathway the kinetic data were available from the literature. The metabolic model was used to analyse flux and metabolite concentration control of the L-arabinose catabolic pathway. The model demonstrated that flux control does not reside at the enzyme following the intermediate with the highest concentration, L-arabitol, but is distributed over the first three steps in the pathway, preceding and following L-arabitol. Flux control appeared to be strongly dependent on the intracellular L-arabinose concentration. At 5 mM intracellular L-arabinose, a level that resulted in realistic intermediate concentrations in the model, flux control coefficients for L-arabinose reductase, L-arabitol dehydrogenase and L-xylulose reductase were 0.68, 0.17 and 0.14 respectively. The analysis can be used as a guide to identify targets for metabolic engineering aiming at either flux or metabolite level optimisation of the L-arabinose catabolic pathway of *A. niger.* Faster L-arabinose utilisation may enhance utilisation of readily available organic waste containing hemicelluloses to be converted into industrially interesting metabolites or valuable enzymes or proteins.

Introduction

Arabinan is a polysaccharide found in plant cell wall heteropolysaccharides as a side chain of pectin (De Vries & Visser, 2001). *Aspergillus niger* is able to degrade arabinan extracellularly to the pentose L-arabinose using its arabinanolytic system. To date three arabinanases have been characterised: two α-L-arabinofuranosidases (ABFA and ABFB) and an arabinan 1,5-α-L-arabinanase (ABNA) (Van der Veen *et al.*, 1991). L-arabinose can be used by the fungus as a carbon source via the L-arabinose catabolic pathway. This pathway (Figure 1) converts L-arabinose to D-xylulose 5-phosphate, which is further metabolised via the non-oxidative pentose phosphate pathway. Of the L-arabinose/D-xylose catabolic pathway the genes encoding D-xylulose kinase (*xkiA*) and D-xylose reductase (*xyrA*) have previously been cloned in *A. niger* (Chapter 4 & VanKuyk *et al.*, 2001) (Hasper *et al.*, 2000). Of the L-arabinose specific part of the pathway recently the L-arabitol dehydrogenase (LAD) and L-xylulose reductase (LXR) encoding genes were cloned and characterised in *Trichoderma reesii* (Richard *et al.*, 2001; Richard *et al.*, 2002).

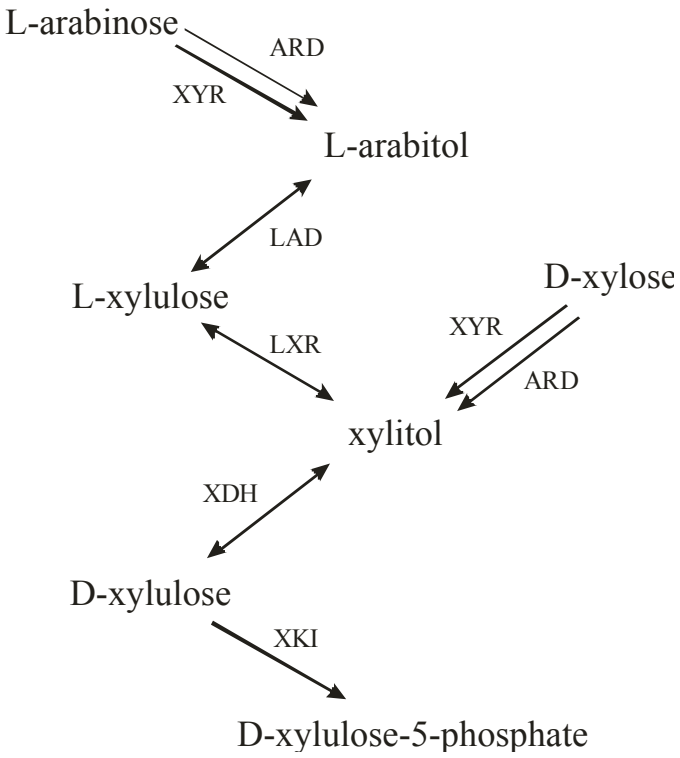

Figure 1. The L-arabinose/D-xylose catabolic pathway. The arrows represent the directions of the rate equations used in the model. ARD = L-arabinose reductase, XYR = D-xylose reductase, LAD = L-arabitol dehydrogenase, LXR = L-xylulose reductase, XDH = xylitol dehydrogenase and XKI = D-xylulose kinase.

The enzyme kinetics in *A. niger* were elucidated for D-xylulose kinase (XKI) (Chapter 4 & VanKuyk *et al.*, 2001), xylitol dehydrogenase (XDH, D-xylulose forming) and L-xylulose reductase (LXR) (Witteveen *et al.*, 1994).
Little is known about the regulation of the arabinanase system. Recently we showed that at least 2 genetic loci are involved in the positive regulation of both the catabolic and extracellular components in arabinan breakdown and catabolism (Chapter 3 & De Groot *et al.*, 2003).
The arabinananolytic genes are specifically induced when *A. niger* is growing on arabinan-containing substrates or on the monomeric compounds L-arabinose and L-arabitol (Flipphi *et al.*, 1994; Gielkens *et al.*, 1997; Gielkens *et al.*, 1999; Ramon *et al.*, 1993; Van der Veen *et al.*, 1991; Van der Veen *et al.*, 1993). The genes encoding three arabinan degrading enzymes, *abfA*, *abfB* and *abnA*, have been cloned and characterised (Flipphi *et al.*, 1993a; Flipphi *et al.*, 1993b; Flipphi *et al.*, 1993c). Expression analysis of these genes showed that they are co-ordinately expressed. One of the intermediates of the L-arabinose catabolic pathway, L-arabitol, is believed to be the low molecular mass inducer of the system (Van der Veen *et al.*, 1993). In *Aspergillus nidulans* it has been shown that increased

intracellular arabitol accumulation correlates with higher production of the enzymes involved in arabinan breakdown (De Vries *et al.*, 1994), suggesting that L-arabitol is the true inducer of this system.

These polysaccharide degrading systems enable *A. niger* to grow on complex media containing arabinan and xylan. Such substrates are widely available in the organic waste which is generated in the form of leftovers from forestry and agriculture. These leftovers could therefore be an alternative energy source being cheap and readily available (Hinman *et al.*, 1989).

A. niger has a number of industrially interesting applications such as citric acid production and, due to its natural ability for high level extracellular protein secretion, the production of a variety of enzymes and proteins (Gouka *et al.*, 1997; Maras *et al.*, 1999).

Since L-arabinose is not as good a carbon source as e.g. glucose, one of the interests regarding the L-arabinose catabolic pathway would be to optimise the flux through the pathway and thereby increasing growth or the capacity to produce certain metabolites like citric acid or proteins. Another interesting question is whether it would be possible to engineer the pathway in such a way that the accumulation of the inducer L-arabitol is increased. Combined with overexpression of the regulatory components this might result in higher production levels of arabinanases.

In order to assess the engineering target(s) and to get a better understanding of the pathway and its regulation we constructed a mathematical model of the L-arabinose/D-xylose catabolic pathway and performed metabolic control analysis (MCA). MCA indicates which enzymes in the catabolic pathway have control on the flux through the pathway and on the intermediate metabolite levels such as L-arabitol. The control of an enzyme is defined as the relative effect of changes in the enzyme level on the variable of interest (such as the flux or a metabolite level). To construct such a model we need the kinetic properties of the enzymes. As not all the enzymes were purified and characterised before (Chapter 4 & VanKuyk *et al.*, 2001) (Witteveen *et al.*, 1994), L-arabinose reductase (ARD), D-xylose reductase (XYR) and L-arabitol dehydrogenase (LAD) were purified and their properties investigated.

Materials and methods

Strains and growth conditions

All *A. niger* strains used for this study were derived from *A. niger* N400 (CBS120.49); N402 *cspA1* (Bos *et al.*, 1988) and NW280 *cspA1 nicA1 leuA1* Δ *xyrA*::PYRA+ (Hasper *et al.*, 2000). Mycelium cultures were grown at pH 6 in

minimal medium (MM) containing per litre: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 1 ml of trace elements solution (Vishniac & Santer, 1957) and 2 % carbon sources. Culturing was done in a rotary shaker at 250 rpm and 30°C. For growth of strains with auxotrophic mutations, the necessary supplements were added to the medium. For growth on plates 1.5% (mass/vol) agar was added to the medium before autoclaving. In transfer experiments strains were pre-grown in MM containing 1% (mass/vol) D-glucose or D-fructose; 0.5 % (mass/vol) yeast extract; and 0.2% (mass/vol) casamino acids (preculture). After 16 h mycelium was harvested by suction over a filter, washed with MM without carbon source and transferred to MM containing carbon sources (D-glucose, D-fructose, L-arabinose, D-xylose, L-arabitol or xylitol). The mycelium was harvested after 8 h of induction. Mycelium used for purification of intracellular enzymes was washed in 4 °C 10 mM potassium phosphate (pH 7.0) during harvesting and frozen in liquid nitrogen. The mycelium samples were stored at -70°C.

Preparation of cell extracts and protein determination

Mycelium was harvested and disrupted as described previously (Witteveen *et al.*, 1989). Cell free extracts were obtained by suspending disrupted frozen mycelium in extraction buffer (10 mM Bis-Tris, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5 mM EDTA pH 6.0) followed by centrifugation at 15000-20000 *g* in order to remove cell debris. The entire procedure was performed between 0 and 4°C. Protein concentrations were determined after denaturation using sodium deoxycholate and precipitation of protein with trichloroacetic acid (Bensadoun & Weinstein, 1976) using the Bicinchoninic acid method as described by the manufacturer (Sigma Chemical Company).

Separation of enzyme activities from cell extracts by anion exchange chromatography.

Cell free extracts were fractionated by anion exchange chromatography in order to separate the enzyme activities. Two ml of cell extract was loaded on a ResourceQ column with a bed volume of 1 ml (Pharmacia Biotech 6.4 mm i.d x 30 mm). The column was washed with 8 ml of extraction buffer. Elution was started with a gradient of 0-0.5 M NaCl over 18 ml. The flow rate used was 3 ml/min. Fractions of 0.5 ml were collected and kept on ice until the enzyme activity measurements.

Purification of enzymes

XYR was purified using *A. niger* N402 after an 8 h transfer of preculture to MM containing 1% D-xylose. The purification procedure was carried out at 4 °C up to the ResourceQ steps, wich were carried out at room temperature. Extract was prepared with 3 g of frozen mycelium powder per 15 ml extraction buffer. As a first purification step we used a dye-affinity screening to select a suitable dye-

Sepharose matrix for the purification of XYR, ARD and LAD (Hondmann & Visser, 1990).

For XYR dye Procion Olive P-7G (ICI Ltd.) Sepharose CL-4B (20 mg dye/g Sepharose) was a suitable matrix using elution with 0.5 mM NADPH in extraction buffer. The fractions containing XYR activity were applied to a ResourceQ column at pH 6.0. After washing with extraction buffer (pH 6.0) XYR was eluted using extraction buffer at pH 4.8. Other reductases were retained on the column under these conditions. This preparation was brought to pH 6.0 using 0.1 M NaOH and again applied to a ResourceQ column. Elution was achieved with a gradient of 0-0.5 M NaCl over 18 ml yielding an apparently pure protein.

For ARD the same dye as used for the purification of XYR (Procion Olive P-7G) linked to Sepharose CL-4B was suitable. We used a *xyrA* disruptant (NW280) to circumvent co-purification of XYR activity. The strain was induced for 8 h by transfer of a preculture to MM containing 1% L-arabinose. The ARD was eluted from the dye-Sepharose column material using 0.5 mM NADPH in extraction buffer. The fractions containing ARD activity were applied to a ResourceQ column at pH 6.0 and elution was achieved with a gradient of 0-0.5 M NaCl over 18 ml yielding an apparently pure protein.

For LAD another dye was selected and biomass was obtained as fot the ARD purification. Cibacron Brilliant Gelb 3G-E (Ciba Geigy) linked to Sepharose CL-4B at 20 mg dye/g Sepharose was used. Elution was achieved using 0.5 mM NAD^+ in extraction buffer. Further purification appeared to be difficult as LAD activity was rapidly lost when applying the fractions eluted from the dye-Sepharose onto any other separation method tested (anion/cation exchange, hydrophobicity). Therefore, a partially purified LAD preparation was used for characterisation of this enzyme.

Enzyme assays

All enzyme assays were performed at 30°C. LAD and XDH activities were determined using 100 mM glycine buffer pH 9.6, 0.2 mM NAD^+ and 40 mM L-arabitol or xylitol, respectively. ARD and XYR activities were determined using 50 mM sodium phosphate pH 7.6 (intracellular pH at L-arabinose/D-xylose grown conditions (Hesse *et al.*, 2002)), 0.2 mM NADPH and 40 mM L-arabinose or D-xylose, respectively. Absorbance changes at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) were measured using a Cobas Bio autoanalyser (Roche) or an UV-2501PC spectrophotometer (Shimadzu Scientific Instruments). When screening the dye-affinity 96 well matrices the absorbance was measured at 340 nm in a microtiter platereader (Molecular Devices). When measuring kinetics the same buffers and equipment was used varying the substrate concentrations. Enzyme levels used in the model were analysed using 50 mM sodium phosphate pH 7.6.

Metabolic control analysis

A metabolic model of the L-arabinose/D-xylose catabolic pathway (Figure 1) was constructed for SCAMP (Sauro, 1993). With this software package both steady-state analysis and metabolic control analysis (MCA) of the pathway can be performed.

In MCA flux control is quantified in terms of the flux control coefficients (FCCs), which specify the sensitivity of the flux (*J*) to changes in the individual enzyme concentrations or the rate of the individual reaction steps (*v*) in the pathway, i.e.

$$C_i^{J_j} = \frac{v_i}{J_j}\frac{\partial J_j}{\partial v_i}$$

The concentration control coefficients (CCC's) are defined by the sensitivity of a metabolite concentration (*M*) to changes in the individual enzyme concentrations or the rate of the individual reaction steps in the pathway i.e.

$$C_i^{M_j} = \frac{v_i}{M_j}\frac{\partial M_j}{\partial v_i}$$

Within the model we used the following rate equation 1 for two substrate Michaelis-Menten kinetics (Cleland, 1963a; Cleland, 1963b; Cleland, 1963c).

Rate equation 1.

$$v = \frac{r_{\max}\cdot[A]\cdot[B]}{([A]\cdot[B]+[A]\cdot K_{m,B}\cdot\prod_1^n(1+\frac{[I_n]}{K_{is,B-I_n}})+[B]\cdot K_{m,A}\cdot\prod_1^m(1+\frac{[I_m]}{K_{is,A-I_m}})+K_{m,AB})\cdot\prod_1^m(1+\frac{[I_m]}{K_{ii,A-I_m}})\cdot\prod_1^n(1+\frac{[I_n]}{K_{ii,B-I_n}})}$$

In which r_{max} is the V_{max} corrected by the relative enzyme level and the terms $K_{is,B-I_n}$ and $K_{ii,B-I_n}$ are the inhibition constants for inhibitor I_n for substrate *B*. For example rate equation 2 for XKI.

The kinetic parameters of the enzymes were obtained from literature for XKI (Chapter 4 & VanKuyk *et al.*, 2001), XDH and LXR (Witteveen *et al.*, 1994). The kinetic data of the other enzymes of the pathway were obtained in this study. An overview of all the kinetic data used can be found in Table 1.

Enzyme levels were obtained by measuring their activities after fractionation of cell extracts from L-arabinose-induced *A. niger* N402 mycelium. In *A. niger* xylitol and arabitol concentrations were measured to be 20-30 and 200 μmol (g DW)$^{-1}$, respectively. In *A. nidulans* similar xylitol and arabitol concentrations (35 and 168 μmol (g DW)$^{-1}$, respectively) were present (Van der Veen *et al.*, 1994; Witteveen *et al.*, 1994). This corresponds to xylitol and arabitol concentrations in the mycelium of 8-15 and 68-82 mM, respectively. These values were used to validate the model during fitting with the L-arabinose concentration. Cofactor levels were used from (Führer *et al.*, 1980) and (Van der Veen *et al.*, 1995). The D-

Rate equation 2.

$$v = \frac{r_{\max} \cdot [D-xylulose] \cdot [ATP]}{([D-xylulose] \cdot [ATP] + [D-xylulose] \cdot K_{m,ATP} \cdot (1 + \frac{[ADP]}{K_{is,ATP-ADP}})(1 + \frac{[AMP]}{K_{is,ATP-AMP}}) + [ATP] \cdot K_{m,D-xlu} \cdot (1 + \frac{[AMP]}{K_{is,D-xlu-AMP}})(1 + \frac{[E4P]}{K_{is,D-xlu-E4P}}) + K_{m,D-xlu,ATP}) \cdot (1 + \frac{[AMP]}{K_{ii,d\_xlu-AMP}})(1 + \frac{[E4P]}{K_{ii\_D-xlu-E4P}})(1 + \frac{[AMP]}{K_{ii,ATP-AMP}})}$$

Table 1. Kinetic parameters (as determined for purified enzymes) and relative enzyme levels (cel free extract:purified enzyme) of L-arabinose and D-xylose catabolism used in the model. ND = not detected, NA = not available.

Enzyme	Level	Substrate	co-substrate	V_{max} (U/mg)	K_m,substrate (mM)	K_m, co-substrate (mM)	Km, substrate-cosubstrate (mM^2)	Reference
L-arabinose reductase	16.6	L-arabinose	NADPH	70	70	0.01	0.68	This report
		D-xylose	NADPH	87	231	0.009	2.2	
L-arabitol dehydrogenase	2.32	L-arabitol	NAD	96	89	0.05	27	This report
		L-xylulose	NADH	805	5	0.008	0.71	
D-xylose reductase	0.01	D-xylose	NADPH	75	39	0.008	ND	This report
		L-arabinose	NADPH	57	93	0.008	1.9	
L-xylulose reductase	18.3	L-xylulose	NADPH	200	17	0.03	NA	(Witteveen *et al.*, 1994)
		xylitol	NADP	33	925	0.13	NA	
xylitol dehydrogenase	13.5	xylitol	NAD	270	70	0.3	NA	(Witteveen *et al.*, 1994)
		D-xylulose	NADH	1500	4	0.03	NA	
D-xylulose kinase	70.7	D-xylulose	ATP	2015	0.061	0.76	0.28	(Chapter 4 & VanKuyk *et al.*, 2001)

xylulose 5-phosphate level was 0.23 mM (Poulsen *et al.*, 2005). XKI activity can be strongly inhibited by erythrose 4-phosphate. However, since the erythrose 4-phosphate levels reported by Ruijter et al.(Ruijter & Visser, 1999) was below their detection limit of 25 pmol, we have set the level to 0 in the model. An overview of these data is given in Table 2.

Table 2. Metabolite levels used in the mathematical model. ND=not detected.

Metabolite	Level (mM)	Reference
NADPH	0.03	(Führer *et al.*, 1980)
NADP	0.051	(Führer *et al.*, 1980)
NADH	0.065	(Führer *et al.*, 1980)
NAD	0.62	(Führer *et al.*, 1980)
ATP	0.50	(Van der Veen *et al.*, 1995)
ADP	0.70	(Van der Veen *et al.*, 1995)
AMP	0.27	(Van der Veen *et al.*, 1995)
erythrose 4-phosphate	ND (<25 pM)	(Ruijter & Visser, 1999)
D-xylulose 5-phosphate	0.23	(Poulsen *et al.*, 2005)

Results and discussion

Purification and kinetic characterisation of L-arabinose catabolic enzymes

In order to be able to construct a kinetic model of the L-arabinose/D-xylose catabolic pathway the kinetic parameters of the individual enzymes of the pathway are required. As for some of the enzymes these were not available we determined those kinetic parameters in this study. Firstly, we purified the enzymes using dye-affinity chromatography (Hondmann & Visser, 1990). Both XYR and ARD could be purified using Procion Olive P-7G linked to Sepharose CL-4B followed by anion exchange chromatography. The enzymes were stable during storage for months at 4°C and for at least two years at –70 °C. The LAD could only be partially purified using Cibacron Brilliant Gelb 3G-E linked to Sepharose CL-4B. Attempts to further purify LAD failed. The LAD activity diminished rapidly at 4 °C and freezing and thawing of the enzyme resulted in a complete loss of activity. Preparations of LAD were therefore made fresh prior to measuring kinetics. Figure 2 shows SDS-PAGE patterns of the different enzyme preparations. The two reductases showed only one band at approximately 36-38 kDa, whereas the LAD preparations showed two main bands, one at 35-37 kDa and one at 28 kDa. The recovery of the reductases was highest for ARD (71%), whereas for XYR the highest purification factor (46-fold) was achieved (Table 3).

Table 3. Specific activities and recovery of the purification of D-xylose reductase, L-arabinose reductase and L-arabitol dehydrogenase.

Enzyme		Extract	Dye-affinity	ResouceQ pH 4.8	ResourceQ 6.0	
D-xylose reductase	Activity (U/mg)	0.8	20	23		38
	Purification factor	1	24	28		46
	Recovery	100%	43%	28%		28%
L-arabinose reductase	Activity (U/mg)	0.59	9.0			24
	Purification factor	1	15			41
	Recovery	100%	84%			71%
L-arabitol dehydrogenase	Activity (U/mg)	0.95	37			
	Purification factor	1	39			
	Recovery	100%	74%			

Table 4. Kinetic parameters for L-arabinose reductase, L-arabitol dehydrogenase, and D-xylose reductase from different microorganisms.

Enzyme	V_{max} (U/mg)	K_m,co-factor (mM)	K_m,metabolite (mM)	Organism	Reference
L-arabinose reductase	56.9	0.0032	67.7	*Pichia stipitis* (pentose reductase)	(Rizzi, 1988)
L-arabitol dehydrogenase	-	0.180	40	*Trichoderma reesei*	Richard 2001
D-xylose reductase	56.9	0.0032	67.7	*Pichia stipitis* (pentose reductase)	(Rizzi, 1988)
	23.2	0.009	42	*Pichia stipitis*	(Verduyn *et al.*, 1985)
	0.16	0.036	-	*Candida mogii*	(Sirisansaneeyakul *et al.*, 1995)
	-	0.008	78	*Pichia quercuum*	(Suzuki & Onishi, 1975)
	-	0.016	28	*Candida*	(Scher & Horecker, 1966)

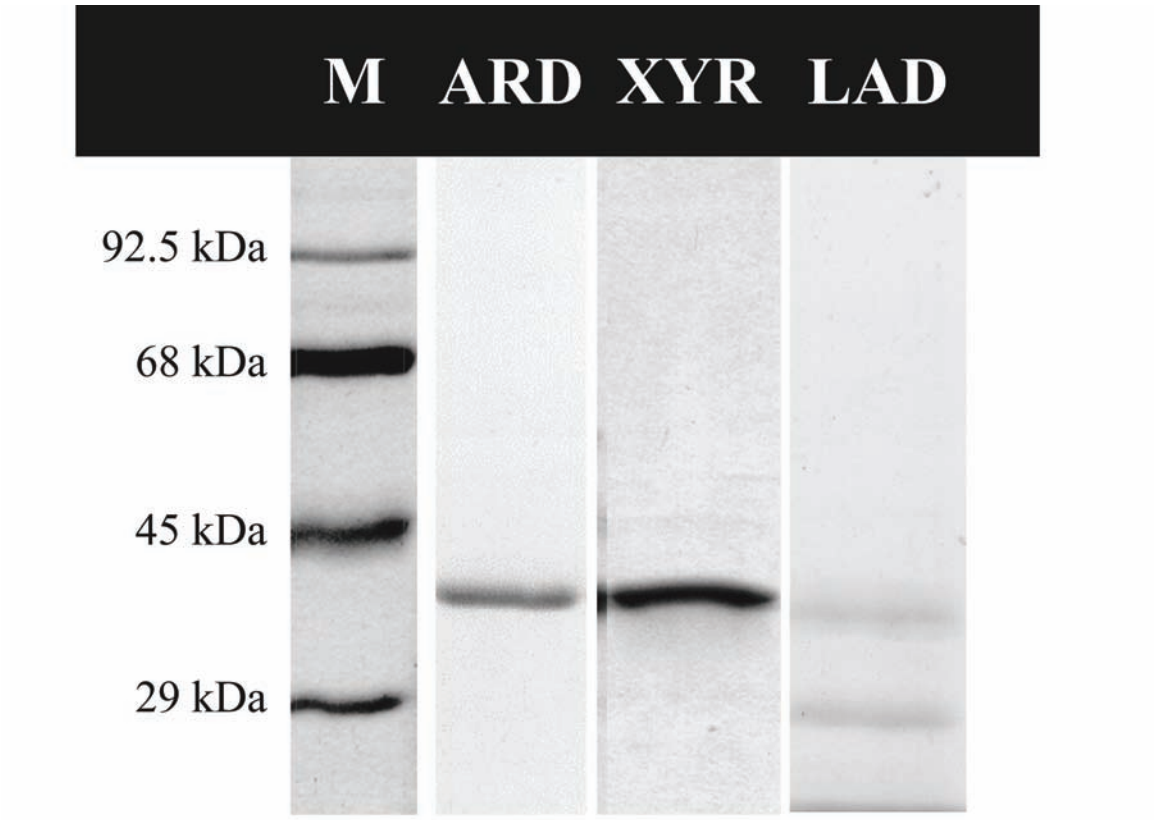

Figure 2. SDS-PAGE of L-arabinose reductase (ARD), D-xylose reductase (XYR) and L-arabitol dehydrogenase (LAD). First lane (M) is the molecular weight marker.

Enzyme kinetics of the reductases were measured at pH 7.6, whereas for LAD pH 9.6 was used. For the two reductases the kinetics for both L-arabinose and D-xylose were determined. All substrates from the pathway were tested (L-xylulose, xylitol, D-xylulose, L-arabinose, D-xylose, D-xyluose 5-phosphate and L-arabitol), but only L-arabinose and D-xylose gave detectable activity. The reverse reaction of the reductases was not observed under the conditions used. When testing all the possible substrates from the pathway only L-arabitol appeared to be a substrate for LAD. When comparing the kinetic parameters found to properties of similar enzymes described in literature they appeared to be within the same order of magnitude (Table 4). Although for recombinant xylose-utilising *Saccharomyces cerevisiae* NADH can also be used by D-xylose reductase we did not find this for the D-xylose reductase of *A. niger* (Eliasson *et al.*, 2001).
Using the kinetic parameters of ARD to calculate the rate on 40 mM L-arabinose and 40 mM D-xylose, the ratio of these two activities equalled 2.0. This is consistent with what was presented in (Chapter 3 & De Groot *et al.*, 2003) that in an extract a ratio of 1.9-2.0 indicates that there is no XYR present, but solely ARD.

To determine the enzyme levels under L-arabinose grown conditions, we prepared cell extracts of *A. niger* N402 (wild type) mycelium induced on L-arabinose. These extracts were fractionated in order to separate the enzyme activities enabling the measurement of the enzyme levels (Table 1).

Construction of the model and metabolic control analysis of L-arabinose metabolism

A mathematical model of L-arabinose metabolism was constructed as outlined in Fig. 1 using rate equations as described under methods and kinetic parameters as shown in Table 1. We used SCAMP (Sauro, 1993) to analyse the model. Since the intracellular L-arabinose concentration is not known and technically difficult to determine we have fit the model with varying L-arabinose concentration to match the xylitol and L-arabitol levels at steady state to values found in literature (8-15 and 68-82 mM respectively). It appeared that a concentration of 5 mM L-arabinose gave 11 mM xylitol and 74 mM L-arabitol, which is within the measured range. This L-arabinose concentration was subsequently used in a number of analyses (see below). The concentrations of cofactors, such as NAD(P)(H), were kept constant in the model. Using the different values obtained in several studies from different growth conditions (Führer *et al.*, 1980; Poulsen *et al.*, 2005; Ruijter *et al.*, 1997) no substantial effect was observed on the control coefficients and fluxes.

In order to assess the effect of the L-arabinose concentration on flux and metabolic control these variables were analysed in a range from 0.1 mM up to 1 M L-arabinose (Figure 3). The flux was clearly affected by the L-arabinose concentration. A steep increase of flux was observed between 0.1 and 100 mM L-arabinose (Figure 3-A). Flux control resided largely at ARD at lower L-arabinose concentrations and shifted towards LAD and LXR at higher L-arabinose concentrations (Figure 3-B). One dogma in pathway analysis states that the enzyme following the metabolite having a high concentration is rate controlling, in this case L-arabitol and xylitol (Table 2). Our model indicates that this is only partially true similar to what was found for D-xylose catabolism in *Aspergillus* (Prathumpai *et al.*, 2003). Assuming 5 mM L-arabinose the flux control resided at the first three enzymes of the pathway, i.e. mainly at the step before L-arabitol (ARD; FCC=0.7) and in part also at the two steps after L-arabitol (LAD; FCC=0.17, LXR; FCC=0.14). Even at extremely high L-arabinose concentrations (up to 1 M) flux control remained shared with LXR (Figure 3-B).

The concentration control of L-arabitol was, however, only slightly affected by changes in the L-arabinose concentration (Figure 3-C). More dramatic changes were found on the concentration control coefficients (CCC's) of xylitol (Figure 3-D). Concentration control of xylitol shifted from ARD towards LAD and LXR similar to the change of flux control (Fig C-B). These findings show that the L-

arabinose concentration in the model had a substantial effect on flux control of the pathway, whereas it did not affect the distribution of L-arabitol concentration control.

The effect of changing enzyme levels

Assuming a concentration of 5 mM for intracellular L-arabinose as explained above we investigated the effect of changing every enzyme level 10-fold upwards and 10-fold downwards. In this way a prediction could be made which enzyme level should be engineered by either over- or reduced expression of their corresponding genes in order to increase the flux and ultimately metabolite or protein production. Alternatively an increase in the L-arabitol level might be achieved yielding a higher induction of the arabinanase system.

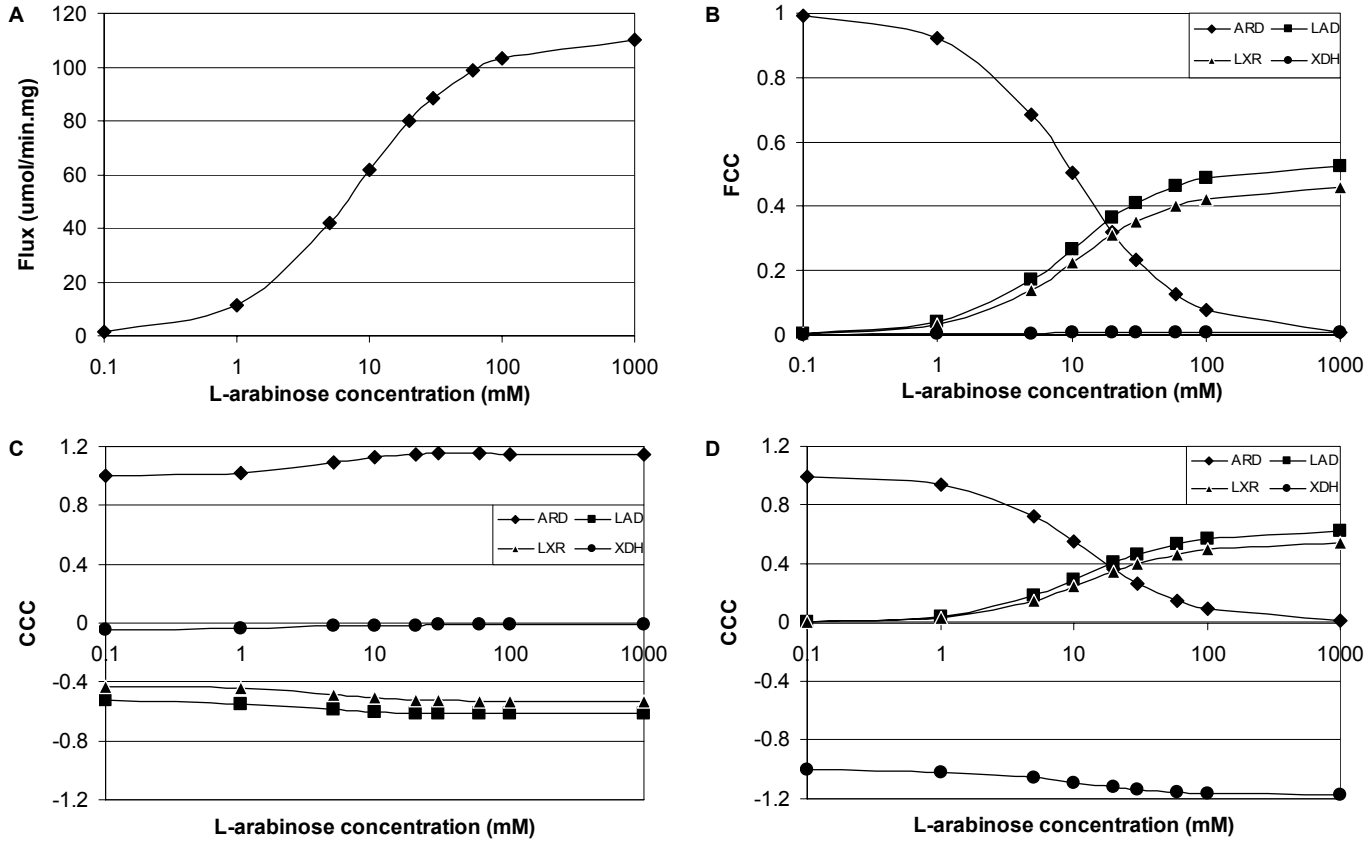

Figure 3. Flux and flux control coeffiecients at steady state as a function of L-arbinose concentration. Enzymes having a flux control coefficient of less then 0.05 are not represented. ARD=L-arabinose reductase, LAD=L-arabitol dehydrogenase, LXR=L-xylulose reductase, XDH=xylitol dehydrogenase.

A. Flux through the L-arabinose catabolic pathway
B. Flux control coefficients (FCC's)
C. L-arabitol concentration control coefficients (CCC's)
D. Xylitol concentration control coefficents (CCC's)

In the basic model the flux control resided in the first three (ARD, LAD and LXR) enzymes of the pathway (Figure 3-B and Figure 4-A). Only for these three enzymes a change of flux control was observed when changes in the individual levels of the enzymes were made. The other enzymes (XKI and XDH) did not have significant flux control (FCC<0.05) and did not gain any when their level was changed 10-fold either upwards or downwards. Increasing the ARD, LAD or LXR levels decreased their flux control coefficient drastically. For ARD the flux control lost was redistributed over LAD and LXR. For LAD and LXR, however, the control was shifted to ARD only. As expected a reduction in the level of the individual enzymes gave the opposite effect. Flux control was gained by the enzyme whose level was decreased at the expense of the control by the other two enzymes (Figure 4-A).

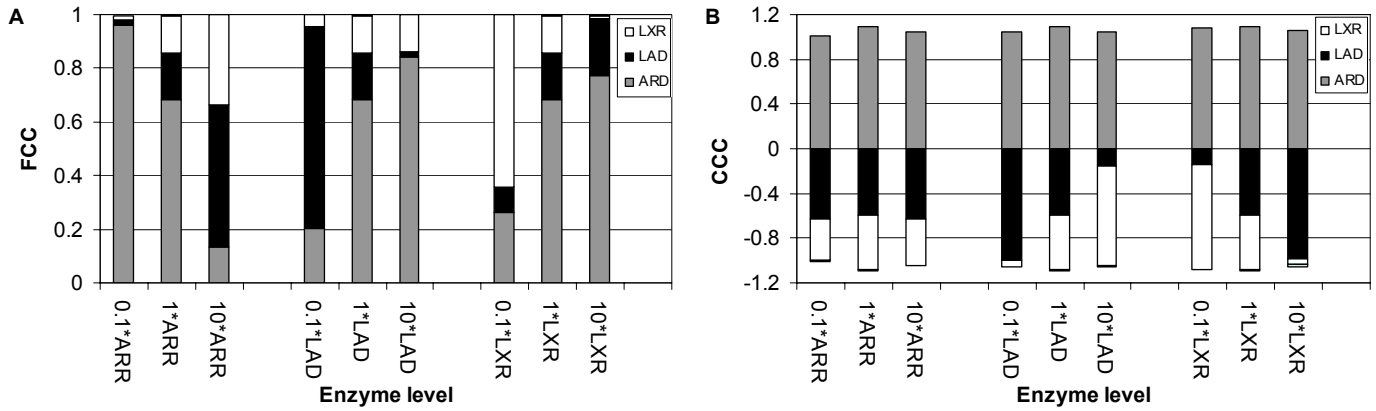

Figure 4. Control coefficients at steady state for different levels of ARD, LAD and LXR. Only one enzyme level was changed at a time. For each enzyme a set of three bars shows distribution of control at three different levels of this enzyme. The middle bar of each set of three depicts the basic situation. Enzymes having a flux control coefficient of less then 0.05 are not represented. Intracellular L-arabinose concentration was 5 mM. ARD=L-arabinose reductase, LAD=L-arabitol dehydrogenase, LXR=L-xylulose reductase.

A. Flux control coefficients (FCC's) of ARD, LAD and LXR at different individual enzyme levels.
B. L-arabitol concentration control coefficients (CCC's) of ARD, LAD and LXR at different individual enzyme levels.

In contrast to the flux control for which ARD levels appeared to be most important, the ARD level hardly affected the distribution of the L-arabitol concentration control. The L-arabitol concentration control hardly changed when either increasing or decreasing the ARD level 10-fold. More drastic changes in L-arabitol concentration control were seen when the LAD and LXR levels were changed. The control of LAD on the L-arabitol concentration was inversely

correlated to the LAD level. Upon an increase in the level of LAD the L-arabitol concentration control was shifted towards LXR. Likewise, increased LXR levels shifted the L-arabitol concentration control towards LAD. Again XDH and XKI had concentration control coefficients towards L-arabitol of less than 0.05.

From these data we can conclude that increasing the ARD level will increase the flux, but flux control will shift to LAD and LXR . Already at a 3-fold increase of ARD the flux control is distributed almost equally over the first three enzymes of the pathway (FCC_{ARD}= 0.36, FCC_{LAD}=0.34, FCC_{LXR}=0.29). This is a confirmation of the notion that flux control is shared among enzymes and moves away from the enzyme whose level is increased (Cornish-Bowden *et al.*, 1995).

The control on the L-arabitol concentration, however, strictly followed the levels of the enzymes converting the metabolite further down the pathway. This implies that a decrease of either or both LAD or LXR can be a target of metabolic engineering in order to increase the intracellular concentration of the arabinanase inducer L-arabitol.

In our study we found that XKI had hardly any control on either the flux or the L-arabitol concentration. Even upon a 700-fold decrease of XKI activity, which is then close to the minimal value at which the model can still reach a steady-state, the enzyme did not have significant control on the flux (FCC_{XKI}=0.005) or the L-arabitol concentration (CCC_{XKI}=0.02). This is consistent with the conclusion by vanKuyk et al (Chapter 4 & VanKuyk *et al.*, 2001) who reported that an increase in XKI activity by over expression of its encoding gene did not affect L-arabitol levels and growth rate.

Concluding we propose that in order to increase the flux through the L-arabinose catabolic pathway one would ultimately need to increase the level of the first three enzymes of the pathway. Since the kinetics of L-arabinose transport is not known it was not included it in our model. However, the dependence of the results on intracellular L-arabinose implies there could be significant control by the transporter. Increased flux might enable *A. niger* to rapidly metabolise L-arabinose released from hemicelluloses and thereby make these substrates accessible for production of industrially interesting metabolites, such as citric acid or proteins. With respect to arabinanase production, higher intracellular L-arabitol levels cause a higher induction of the arabinanase system. An increase could possibly be achieved with decreasing LAD and LXR levels. Since this is opposite to the approach to increase the flux it would therefore be necessary to engineer one strain for optimal arabinanase activity and L-arabinose production and another strain for industrial production of metabolites.

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| Chapter 7

General Discussion

General Discussion

One of the topics in this thesis is regulation of arabinanase production and the L-arabinose catabolic pathway. We isolated several mutants which were affected in regulation of the arabinolytic system. We showed that these mutants (*araA* & *araB*) were no longer capable of sufficiently expressing the extracellular and intracellular components of the arabinolytic system.

The approach which enabled the isolation of the *araA* and *araB* mutants was based on the fact that L-arabinose causes catabolite repression of D-gluconate metabolism (Figure 1) and the inability to grow on L-arabinose in a D-xylulose kinase negative (*xkiA*) background (Ruijter & Visser, 1997; Witteveen *et al.*, 1989). When an *xkiA* strain is offered a mixture of L-arabinose and D-gluconate no growth was observed, due to the lack of D-xylulose kinase activity and the presence of the carbon catabolite repression of gluconate meatabolism by L-arabinose and/or its metabolites .

There are several ways the fungus can circumvent this challenge. Firstly, a mutation can occur enabling metabolisation of L-arabinose via D-xylulose kinase by reversing the mutation used in our selection. Secondly by enabling or derepressing expression of the D-gluconate metabolism or thirdly by relieve of the repression by L-arabinose, either by preventing the repressing metabolite to be formed or by a mutation in the signaling cascade of the repression system.

Initially we aimed at isolating a mutant in the catabolism, specifically the L-arabinose reductase and the L-arabitol dehydrogenase. However the aspecific nature of the reductases in the L-arabinose and D-xylose pathway would require at least a two mutations. One mutation in the D-xylose reductase and one in L-arabinose reductase, in order to find a phenotype as either enzyme can reduce L-arabinose to L-arabitol. As at the time no disruption strain of the *xyrA* gene (Hasper *et al.*, 2000) was available it was unlikely to find an L-arabinose reductase mutant using the *xkiA* mutant.

Another possibility would be to find a mutant unable to transport the repressing metabolite into the cell. For L-arabinose, however, no such mutant was found which suggests that also redundancy in transporters may be present. However, when we used xylitol instead of L-arabinose as a repressing and selecting carbon source we did find a mutant which shows a strong decrease in xylitol transport (chapter two) suggesting that transport of xylitol is catalysed by one transporter.

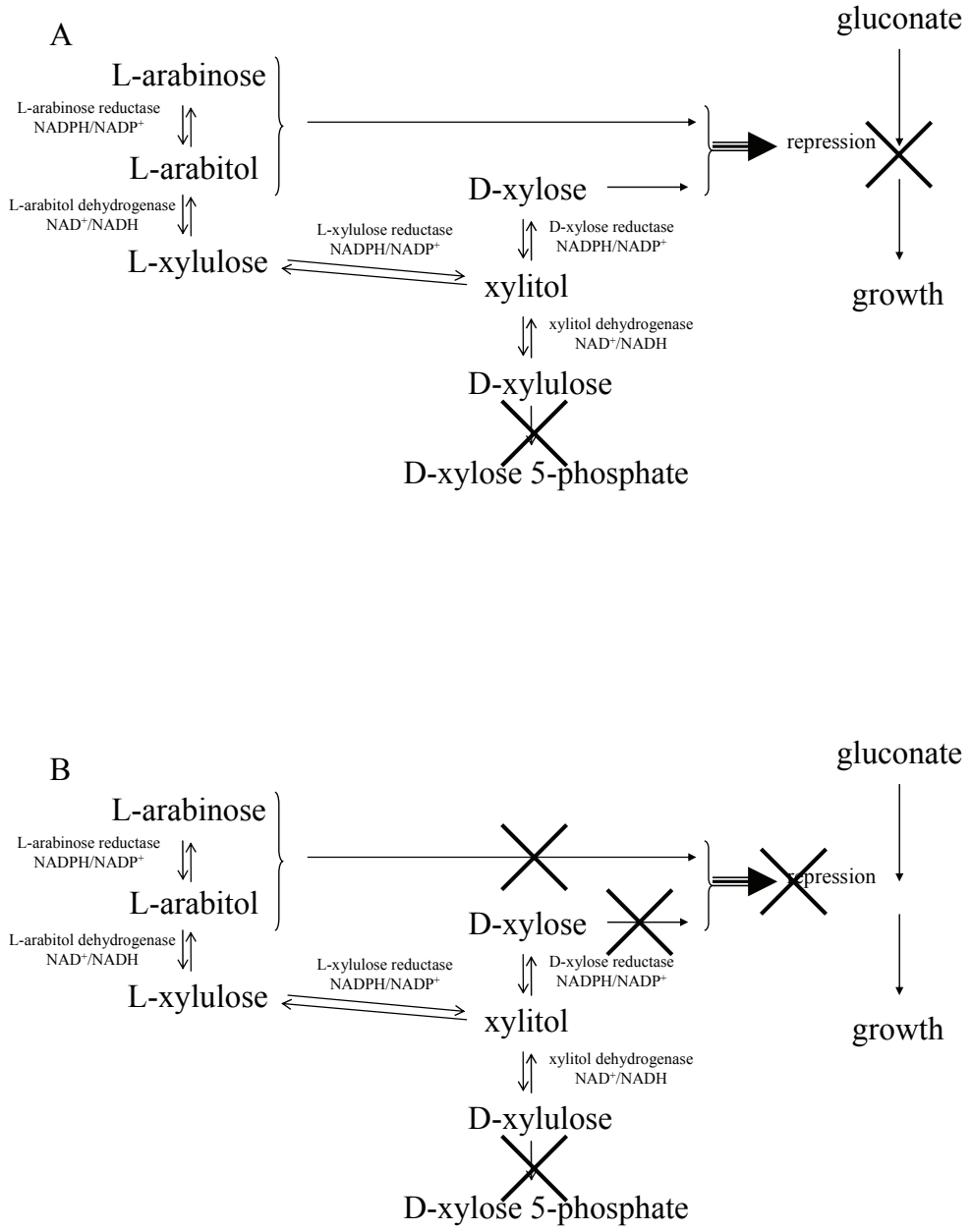

Figure 1. Selection strategy used to isolate *A. niger* mutants affected in the regulation of the arabinolytic system. **A**. Original situation before the selection. L-arabinose catabolism represses the D-gluconate catabolism preventing growth. **B**. When the repression by L-arabinose catabolism is relieved *A. niger* can grow on D-gluconate.

We did, however, find several mutants that were derepressed for growth on D-gluconate. After removing the *xkiA* background from these strains we found that they were severely impaired for growth on L-arabinose and L-arabitol, but not on D-xylose and xylitol. Also induction of the extracellular arbinanases was impaired. Further investigation of these strains showed that the mutations resided in two different loci which we named *araA* and *araB*. We investigated the expression of several genes involved in arabinan degradation and the L-arabinose / D-xylose catabolic pathway and the enzyme activity levels of both the catabolic pathway and the arabinanases. The results from these experiments showed that these mutations specifically prevented the induction of the arabinolytic system from which we conclude that the mutated genes are involved in regulation of the expression of this arabinolytic system. This also implicates that these components are involved in carbon catabolite repression of D-gluconate metabolism in the presence of L-arabinose. It is clear that the L-arabinose regulatory system (ARAR) induces both the L-arabinose catabolic pathway and the arabinolytic enzyme system. Furthermore one could speculate the ARAR system is also responsible for the signaling towards carbon catabolite repression which may also explain the antagonistic effect with the XLNR system.

In chapter three (Chapter 3 & De Groot *et al.*, 2003) it was shown that at least two regulators are involved in the pentose catabolic pathway, responding to either D-xylose (XLNR) or L-arabinose/L-arabitol (unidentified arabinolytic regulator ARAR), and that they act in an antagonistic fashion. XLNR appeared to regulate D-xylose reductase (*xyrA*) (Hasper *et al.*, 2000), but not D-xylulokinase (*xkiA*) (Chapter 4 & VanKuyk *et al.*, 2001), while the putative arabinolytic regulator activates *xkiA*, but not *xyrA* (Chapter 3 & De Groot *et al.*, 2003). Re-analysis of the *xkiA* expression in chapter 5, however, indicated that XLNR is involved in its regulation of expression but only having a minor contribution. Initially the effect of these regulators on the other members of the catabolic pathway could only be studied using enzyme activities, as the genes were not yet identified. These enzyme measurements were complicated by the fact that L-arabinose reductase and D-xylose reductase are active on both L-arabinose and D-xylose.

In chapter five we investigated in detail the L-arabinose catabolic pathway, in particular the L-arabitol and xylitol dehydrogenases. Using the genomic sequences from *A. niger*, which were made available through DSM, we were able to identify and express the genes encoding L-arabitol dehydrogenase (*ladA*) and xylitol dehydrogenase (*xdhA*). The availability of these genes and the *araA, araB* and *xlnR* deficient strains enabled us to further dissect regulation of the expression of the genes encoding the pathway enzymes. Analysis of the expression of *ladA* and *xdhA*, and re-analysis of the expression of *xkiA* and *xyrA* performed in chapter six

clearly showed the influence of the two regulatory systems on the pentose catabolic pathway in *A. niger*. Expression of *ladA* on L-arabinose and L-arabitol is strongly reduced in the arabinolytic mutants, while significant expression on D-xylose is only detected in the *xlnR* disruptant. Taking into account the antagonistic action of the two regulatory systems (Chapter 3 & De Groot *et al.*, 2003), this indicates that *ladA* is predominantly regulated by the arabinolytic system, while XLNR has no or very little influence on *ladA* expression. The expression of *xkiA* and *xdhA* is more complex. They have reduced expression on L-arabinose and L-arabitol in the arabinolytic mutants and increased expression on D-xylose in the *xlnR* disruptant, indicating that they are under the control of the arabinolytic regulator. However, they also have increased expression on D-xylose in the arabinolytic mutants and on L-arabinose and L-arabitol in the *xlnR* disruptant, indicating regulation by XLNR. Based on the changes in expression levels, the effect of XLNR on the expression of these genes appears to be smaller than the effect of the arabinolytic regulator. Expression of *xyrA* is increased on D-xylose in the arabinolytic mutants and reduced in the *xlnR* disruptant, demonstrating that XLNR regulates this gene. Contrary to what was demonstrated for a number of other XLNR-regulated genes (Van Peij *et al.*, 1998), the expression of *xyrA* on D-xylose in the *xlnR* disruptant is not completely absent. This indicates that XLNR is not the only factor affecting *xyrA* expression. The expression of *xyrA* on L-arabinose does not decrease in the arabinolytic mutants, suggesting that the arabinolytic regulator is not involved in *xyrA* expression. In fact, *xyrA* expression on L-arabinose could be the result of the small amount of D-xylose present in the L-arabinose preparation used in these studies (De Vries *et al.*, 1998). The regulation of expression of the genes encoding the L-arabinose pathway in related organisms is studied in much less detail. Expression of *lad1*, encoding L-arabitol dehydrogenase, in *Hypocrea jecorina* (*Trichoderma reesei*) is induced on L-arabinose (Richard *et al.*, 2001; Seiboth *et al.*, 2003). The gene encoding xylitol dehydrogenase is expressed on xylan, D-xylose, L-arabinose and L-arabitol (Seiboth *et al.*, 2003) in *H. jecorina* and on D-sorbitol and xylitol in the dimorphic yeast *Arxula adeninivorans* (Boer *et al.*, 2005). The *alx1* gene encoding an L-xylulose reductase in *Ambrosiozyma monospora* (Verho *et al.*, 2004) is induced on L-arabinose and to a lesser extent on glycerol and D-xylose. These similarities in expression data suggest that also in these species a similarly evolved regulation network exist.

In chapter six the individual enzymatic conversions within the L-arabinose catabolic pathway were investigated and used to construct a metabolic model for metabolic control analysis. The individual enzymes of the catabolic pathway were isolated. The enzyme kinetics and the individual enzyme levels were determined to construct the model. Next the key metabolite levels were determined to

validate the model. From this model we learned that the highest control on the flux having L-arabinose as carbon source resides in the upper part of the pathway and that the L-arabitol level is mainly controlled by L-arabinose reductase and L-arabitol dehydrogenase. Along with the genes cloned in chapter four and five this information enables engineering of strains which have a higher flux through the L-arabinose pathway or give a higher induction of the arabinanases by increasing the intracellular L-arabitol level.

Another interest in this research field is related to the production of fuel ethanol from lignocelluloses by recombinant yeast strains. Such substrates are widely available in the organic waste which is generated in the form of leftovers from forestry and agriculture. These leftovers could therefore be an alternative energy source being cheap and readily available (Hinman *et al.*, 1989). Wild type *Saccharomyces cerevisiae* does not metabolise D-xylose or L-arabinose, but the introduction of pentose catabolising activities and hemicellulose degrading capabilities greatly enhances ethanol production from D-xylose and L-arabinose by transgenic yeast (Jeffries & Jin, 2004; Jeppsson *et al.*, 2002; Karhumaa *et al.*, 2005; Kuyper *et al.*, 2004; La Grange *et al.*, 2001; Richard *et al.*, 2003; Wang *et al.*, 2004). The results presented in this thesis may aid the engineering of yeast in order to improve conversion of L-arabinose to ethanol.

The genome of *A. niger* NRRL3122 has been sequenced by DSM. By searching this genome database one would be able to find genes encoding new putative arabinolytic enzymes or intracellular pathway enzymes based on homology with known sequences from *A. niger* and other microorganisms. In chapter five we have already shown the successful identification of the L-arabitol dehydrogenase and xylitol dehydrogenase encoding genes (*ladA* & *xdhA*) using this strategy.

In order to asses if the genome of *A. niger* contains possible iso-enzymes for the L-arabinose and D-xylose catabolic pathways a TBLASTN search was performed on the DSM genome database. Using the sequences referred to in table 1A and applying an e-value cutoff of $<1.10^{-50}$, 9 genes having similarity to L-arabitol dehydrogenase, 9 genes similar to xylitol dehydrogenase and 2 possible D-xylose (aldose) reductases were be found. For D-xylulose kinase no prologue sequence was found. This suggests that *A. niger* possesses a large number of reductase and dehydrogenase encoding genes which probably have overlapping substrate specificities. One of or more these genes may encode L-arabinose reductase. Using the protein sequence of three enzymes from *Saccharomyces cerevisiae* (GRE3, YPR1, YJ66) which were reported to exhibit L-arabinose reductase activities (Traff *et al.*, 2002) (Table 1B) 14 possible candidates were found for L-arabinose reductase in the *A. niger* genomic database. Two of these candidates overlap between GRE1 and YPR1 resulting in twelve unique candidate sequences.

Table 1. Number of homologues identified in the *A. niger* genome using protein sequences involved in pentose catabolism and arabinan degradation. **A**: Enzymes of the L-arabinose and D-xylose catabolic pathway of *A. niger*. **B**: Enzymes involved in L-arabinose catabolism which have not yet been identified in *A. niger*. **C**: Enzymes involved in arabinan degradation of *A. niger*. All were used in a TBLASTN homology search in the DSM *A. niger* genome database.

A

Enzyme	Protein abbrev.	Source of protein sequence	Accession ID	Number of homologs in *A. niger* $e<10^{-50}$
L-arabitol dehydrogenase	LAD	*Aspergillus niger*	emb|CAH69383.1	9
Xylitol dehydrogenase	XDH	*Aspergillus niger*	emb|CAH69384.1	9
D-xylose reductase	XYR	*Aspergillus niger*	sp|Q9P8R5	2
D-xylulose kinase	XKI	*Aspergillus niger*	emb|CAC83746.1	0

B

Enzyme	Protein abbrev.	Source of protein sequence	Accesion ID	Number of homologs in *A. niger* $e<10^{-50}$
L-xylulose reductase	LXR	*Aspergillus fumigatus*	gb|EAL93840.1	6
		Hypocrea jecorina	gb|Q8NK50	6
		Ambrosiozyma monospora	emb|AE47547.1	1
		Cavia porcellus	sp|Q920N9	0
		Homo sapiens	sp|Q7Z4W1	0
		Mesocricetus auratus	sp|Q91XV4	0
		Mus musculus	sp|Q91X52	0
		Rattus norvegicus	sp|Q920P0	0
L-arabinose reductase	GRE3	*Saccharomyces cerevisiae*	sp|P38715	6
	YPR1	*Saccharomyces cerevisiae*	sp|Q12458	6
	YJ66	*Saccharomyces cerevisiae*	sp|P47137|	2

C

Enzyme	Protein abbrev.	Source of protein sequence	Accession ID	Number of homologs in *A. niger* $e<10^{-50}$
Alpha-N-arabinofuranosidase A	ABFA	*Aspergillus niger*	sp|P42254	1
Alpha-N-arabinofuranosidase B	ABFB	*Aspergillus niger*	sp|P42255	0
Arabinan endo-1,5-alpha-L-arabinosidase	ABNA	*Aspergillus niger*	sp|P42256	3
Arabinoxylan arabinofuranohydrolase	AXHA	*Aspergillus niger*	sp|P79019	0

Eight genes encoding L-xylulose reductase (LXR) have been identified in different species (Table 1B). LXR sequences from *Aspergillus fumigatus* (sequence based on

homology), *H. jecorina* and *A. monospora* gave high identity with several loci in *A. niger*. The highest hit found with both *H. jecorina* and *A. fumigatus* LXR sequences gave 75% and 85% identity and e-values of 1.10^{-120} and 1.10^{-135}, respectively. Another locus having an e-value of 6.10^{-52} and 49% identity was identified when blasting the *A. monospora* sequence. The sequences for LXR from several mammals do not show any homologues in *A. niger*. Clearly these dicarbonyl/L-xylulose reductases are descendents in a different enzyme class having no related sequences in the yeasts and fungi analysed.

Particularly the fact that six out of the seven hits having the highest homology were identical for these three LXR sequences from different species is a good indication that one of these actually is the gene encoding L-xylulose reductase in *A. niger*.

When using the protein sequence of the three known arabinan degrading enzymes (Table 1C) one similar sequence was found for ABFA, none for ABFB and AXHA and three for AbnA again using an e-value cutoff of $<1.10^{-50}$.

Considering this new information it would be interesting to see if and under which conditions these new genes are expressed and whether their expression is under control of the proposed ARAR system. In addition these genes may share the promoter sequence similarities as mentioned in chapter five. The *araA* and *araB* mutants will be a valuable aid in uncovering the true nature of these genes. This will surely lead to an even more complete description of the pathway leading to a better understanding of the coordinated regulation and pathway dynamics.

Furthermore an annotated genome database enables analysis of the proteome or even the extracellular sub proteome (Aebersold & Mann, 2003) of *A. niger* and may give insight in which proteins are actually produced and thereby adding to the functional analysis of the new putative enzymes.

By combining biochemistry, microbiology and bioinformatics we achieved a better understanding of the L-arabinose and D-xylose catabolic pathway, specifically their individual components and the coordinated regulation of both the pathway and the arabinan degrading enzyme system by a common set of regulatory factors. The scheme presented in the introduction (Chapter one, figure 2) has now been expanded to the model presented in figure 2 (modified from chapter three, figure 5 and chapter five figure 4). The findings presented in this thesis can be used to a new line of research using current genomic and proteomic tools, leading to an even better and more detailed understanding of the regulation of the arabinolytic system of *A. niger* and the discovery of new industrial applications.

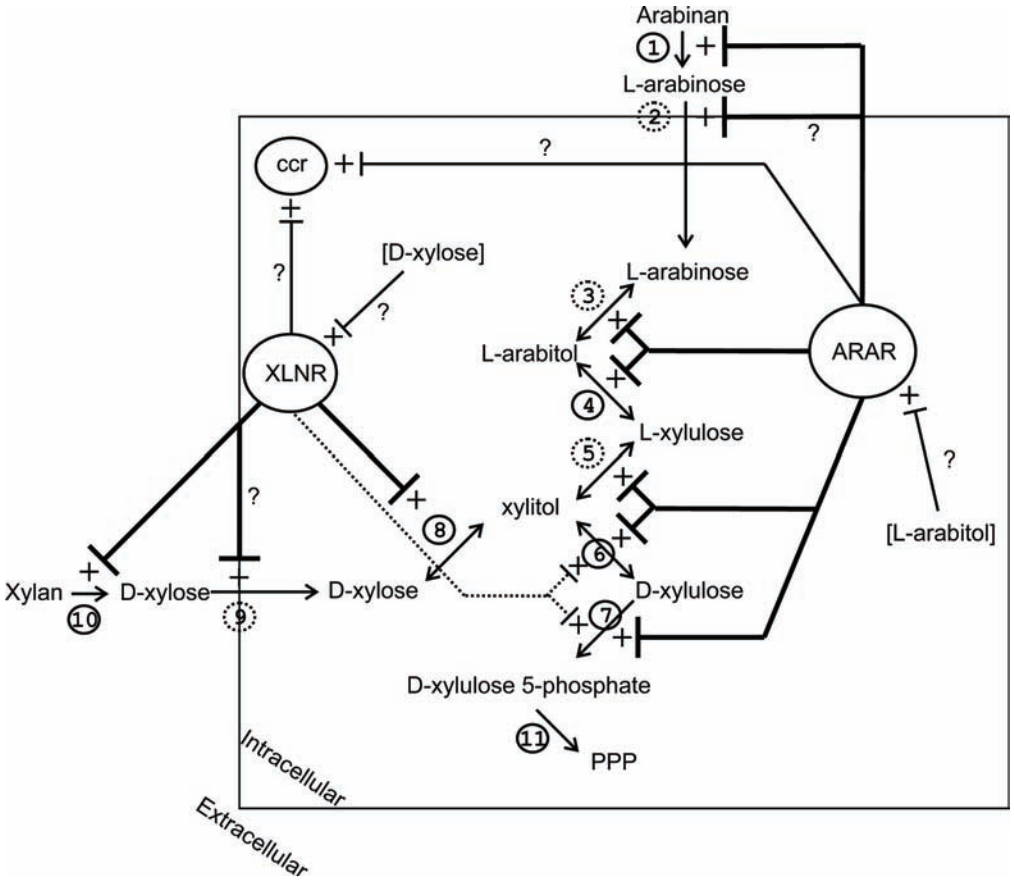

Figure 2. Model of regulation, by ARAR (arabinanolytic transcriptional activator) and XLNR (xylanolytic transcriptional activator), of the extracellular and intracellular components of the arabinan and xylan degrading pathways.
The circled numbers represent the following enzymes: (1) α-L-arabinofuranosidases (EC 3.2.1.55) (ABFA, ABFB, AXHA) and arabinan endo-1,5-α-L-arabinosidase (EC 3.2.1.99) (ABNA); (2) transport of L-arabinose; (3) L-arabinose reductase (EC 1.1.1.21) (ARDA); (4) L-arabitol dehydrogenase (EC 1.1.1.12) (LADA); (5) L-xylulose reductase (EC 1.1.1.10) (LXRA); (6) xylitol dehydrogenase/D-xylulose reductase (EC 1.1.1.9) (XDHA); (7) D-xylulokinase (EC 2.7.1.17) (XKIA); (8) D-xylose reductase (EC 1.1.1.21) (XYRA); (9) transport of D-xylose. (10) endo-1,4-β-xylanase (EC 3.2.1.8) (XYN1) and xylan 1,4-β-xylosidase (XLND); (11) transketolase (EC 2.2.1.1) and ribulose-phosphate 3-epimerase (EC 5.1.3.1). PPP = non-oxidative pentose phosphate pathway. ccr=carbon catabolite repression. + is a positive regulation effect. The question marks represent steps/features of which the mechanism is uncertain or unknown. The numbers encircled with a dotted line indicate that the corresponding gene or genes have not yet been cloned. The thickness of the lines indicates the relative contribution of the two regulators.

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| Summary

This thesis describes studies on the biochemical properties and regulation of L-arabinose metabolism and arabinan degrading enzymes of *Aspergillus niger*. We focused on the investigation of the catabolic pathway, firstly by isolating pathway specific regulatory mutants using a newly developed selection system and, secondly, by purifying the enzymes and characterising their kinetics for use in metabolic control analysis. Finally, by cloning genes encoding these enzymes we were able to analyse expression of these genes.

Using a D-xylulose kinase deficient strain we developed a mutant selection system that identified genes involved in pentose catabolism and their regulation. The *A. niger* strain carrying the *xkiA1* mutation lacks D-xylulose kinase and cannot grow on pentoses, such as D-xylose and L-arabinose, but these sugars still repress the use of other carbon sources such as D-gluconate. We used this genetic background to select for pentose-derepressed mutants on media containing combinations of gluconate with xylitol, L-arabinose or D-xylose. A subset of these mutants was further analysed and turned out to have rather interesting properties as described in chapters two and three.

One of the mutants isolated using this method carried a mutation called *xtlA36* and is described in chapter two. This mutation results in a severe decrease in xylitol consumption, suggesting that *xtlA36* inactivates a xylitol transporter and opens the way of isolation of genes encoding the corresponding genes. Two other *A. niger* mutants, carrying *araA* and *araB* are specifically disturbed in the regulation of the arabinanase system in the presence of L-arabinose. Expression of three arabinanolytic genes, *abfA, abfB* and *abnA*, is substantially decreased or absent in the *araA* and *araB*, strains compared to the wild-type when incubated in the presence of L-arabinose or L-arabitol. In addition, the intracellular enzyme activities of L-arabitol dehydrogenase and L-arabinose reductase, involved in L-arabinose catabolism, were decreased in the *araA* and *araB* strains.

L-arabitol, most likely the true inducer of the arabinanolytic and L-arabinose catabolic genes, accumulates to a high intracellular concentration in the *araA* and *araB* mutants. This indicates that the decreased expression of the arabinolytic genes is not due to lack of inducer accumulation. Therefore, we propose that *araA* and *araB* are mutations in positively acting components of the regulatory system involved in the expression of the arabinanase encoding genes and the genes encoding the L-arabinose catabolic pathway.

Chapter four describes cloning of the *A. niger* D-xylulose kinase encoding gene (*xkiA*) by direct complementation of the strain deficient in D-xylulose kinase activity. This enabled us to investigate the expression of *xkiA* in the presence of L-arabinose, L-arabitol, and D-xylose. Although XKI is part of the D-xylose catabolic pathway, expression of *xkiA* appeared not to be mediated by XLNR, the xylose-dependent positively acting xylanolytic regulator. Expression of *xkiA* is subject to carbon catabolite repression (ccr) but the wide domain regulator CREA is not directly involved. Using the *araA* and *araB* strains described in chapter three, we showed that *xkiA* is under control of the arabinanolytic regulatory system.

Overexpression of *xkiA* enabled us to purify the encoded D-xylulose kinase enzyme. The molecular mass, determined using Electrospray Ionization Mass Spectrometry (ESI-MS) concurred with the calculated molecular mass of 62816.6 Da. The activity of D-xylulose kinase is highly specific for D-xylulose. Kinetic parameters were determined, including K_m(D-xylulose), 0.76 mM and K_m(ATP), 0.061 mM.

In a D-xylulose kinase deficient strain a higher accumulation of intracellular arabitol and xylitol correlated to increased transcript levels of the genes encoding arabinan and xylan degrading enzymes, respectively. This supports the suggestion that L-arabitol may be the specific low molecular weight inducer of the genes involved in arabinan degradation. It also suggests a possible role for xylitol in the induction of xylanolytic genes. Overproduction of XKIA did not reduce the size of the intracellular arabitol and xylitol pools, and had no effect on expression of genes encoding xylan and arabinan degrading enzymes. This suggests that the enzymes preceding D-xylulose kinase in the L-arabinose/D-xylose catabolic pathway probably have more control on the flux through this pathway than D-xylulose kinase itself.

In chapter five, we cloned the genes encoding *A. niger* L-arabitol dehydrogenase (*ladA*) and xylitol dehydrogenase (*xdhA*), and produced the enzymes in *Escherichia coli*. Analysis of the substrate specificity showed that LADA is most active on L-arabitol and also has significant activity on xylitol, but only low activity on D-sorbitol and galactitol. XDHA has the highest activity on xylitol, significant activity on D-sorbitol, but very low activity on L-arabitol. The higher activity on sorbitol for XDHA is in agreement with the amino acid similarity of the different enzyme classes, since a phylogenetic tree of L-arabitol dehydrogenases, xylitol dehydrogenases and sorbitol dehydrogenases (SDH) suggests that xylitol dehydrogenases are more similar to sorbitol dehydrogenases than L-arabitol dehydrogenases. Expression analysis of the pentose catabolic pathway genes confirmed the model in which an arabinose specific regulator activates the expression of all genes required for the conversion of L-arabinose to

D-xylulose-5-phosphate. In addition, XLNR regulates the first step and, to a lesser extent, the other steps of the conversion of D-xylose into D-xylulose-5-phosphate.
Using dye-affinity chromatography we isolated enzymes of the L-arabinose and D-xylose catabolic pathways that had not been described previously. Using the complete set of kinetic parameters a metabolic model was constructed which we used to perform steady state metabolic control analysis (chapter six). The metabolic model was used to analyse flux and metabolite concentration control of the L-arabinose catabolic pathway. The model predicts that flux control does not only reside at the enzyme following the intermediate with the highest concentration, L-arabitol, but is distributed over the first three steps in the pathway, preceding and following L-arabitol. Flux control appeared to be strongly dependent on the intracellular L-arabinose concentration. At 5 mM intracellular L-arabinose, a level that resulted in realistic intermediate concentrations in the model, flux control coefficients for L-arabinose reductase, L-arabitol dehydrogenase and L-xylulose reductase were 0.68, 0.17 and 0.14 respectively. This analysis can be used as a guide to identify targets for metabolic engineering aiming at either flux or metabolite level optimisation of the L-arabinose catabolic pathway of *A. niger*.
In chapter seven the results from chapters two through six are discussed in light of possible engineering applications and recent scientific developments such as genomics.

| Samenvatting

In dit proefschrift wordt onderzoek naar de biochemische eigenschappen en regulatie van het L-arabinose catabolisme en de arabinan afbrekende enzymen van *Aspergillus niger* beschreven. We hebben ons voornamelijk gericht op onderzoek aan de katabole route. Op de eerste plaats hebben we specifieke regulatoire mutanten geïsoleerd, daarbij gebruik makend van een nieuw ontwikkelde selectie strategie. Ten tweede hebben we enzymen van de katabole route geïsoleerd en hun kinetische eigenschappen bepaald en als laatste zijn genen gekloneerd welke coderen voor deze enzymen en is hiervan de expressie onderzocht.
Bij de selectiemethode om mutanten te isoleren in het L-arabinose metabolisme en de regulatie daarvan is gebruik gemaakt van een D-xylulose kinase (*xkiA*) deficiënte stam. Deze stam heeft geen D-xylulose kinase activiteit en kan daardoor niet groeien op pentoses zoals D-xylose en L-arabinose. Echter, deze pentoses kunnen nog wel consumptie van andere koolstofbronnen, zoals D-gluconaat remmen. Pentose gederepresseerde mutanten werden geselecteerd op media met combinaties van gluconaat en xylitol, L-arabinose of D-xylose. Een aantal mutanten is in hoofdstukken twee en drie nader geanalyseerd en blijkt uiterst interessante eigenschappen te hebben.
Één van de geïsoleerde mutanten had de *xtlA36* mutatie welke wordt beschreven in hoofdstuk twee. Deze mutatie resulteert in verminderde xylitol consumptie wat er op duidt dat *xtlA36* het xylitol transport inactiveert. Dit maakt het mogelijk om het corresponderende gen te isoleren. Twee andere *A. niger* mutanten (*araA* en *araB*) hebben een defect in de regulatie van het arabinanase systeem in aanwezigheid van L-arabinose. De expressie van drie bekende genen betrokken bij arabinan afbraak (*abfA*, *abfB* en *abnA*) is substantieel verlaagd of afwezig in deze *araA* en *araB* stammen in vergelijking met het wild-type. Daarnaast zijn onder deze condities ook de intracellulaire enzym activiteiten van L-arabinose reductase en L-arabitol dehydrogenase sterk verlaagd. L-arabitol, hoogst waarschijnlijk het metaboliet dat expressie van arabinolytische systeem induceert, wordt in de *araA* en *araB* mutanten opgehoopt tot een hoge intracellulaire concentratie. Dit geeft aan dat de verminderde expressie van de arabinolytische genen niet veroorzaakt wordt door afwezigheid van de inducer. Wij concluderen dat de mutaties in *araA* en *araB* waarschijnlijk hebben plaatsgevonden in componenten van een positieve regulator van zowel de arabinanase coderende genen als van genen die coderen voor de enzymen betrokken bij het L-arabinose katabolisme.

In hoofdstuk vier wordt de klonering beschreven van het gen dat codeert voor D-xylulose kinase. Dit hebben we gedaan via directe complementatie in de D-xylulose kinase deficiënte stam (*xkiA*). Het maakt analyse van de expressie van dit gen mogelijk. We hebben de expressie bestudeerd in aanwezigheid van L-arabinose, L-arabitol en D-xylose. Hoewel XKIA deel uit maakt van de D-xylose katabole route staat de expressie van het *xkiA* gen niet onder invloed van XLNR (de xylose-afhankelijke xylanolytische activator). Hoewel de expressie van *xkiA* aan koolstof kataboliet repressie onderhevig is, blijkt de "wide domain" regulator CREA hier niet verantwoordelijk voor te zijn. Gebruikmakend van expressie analyse in de *araA* en *araB* stammen beschreven in hoofdstuk drie blijkt dat *xkiA* gereguleerd wordt door het arabinolytisch systeem.
Overexpressie van *xkiA* vergemakkelijkte isolatie van het D-xylulose kinase enzym mogelijk. De moleculaire massa van het eiwit werd bepaald met behulp van Electrospray Ionisatie Massaspectrometrie (ESI-MS) en deze kwam overeen met de berekende moleculaire massa van 62816,6 Da. D-xylulose kinase is uiterst specifiek voor D-xylulose. De kinetische parameters zijn bepaald waaronder de K_m(D-xylulose) 0,76 mM en de K_m(ATP) 0,061 mM.
In een D-xylulose kinase deficiënte stam hopen L-arabitol en xylitol intracellulair op tot zeer hoge concentraties. Deze ophoping correleert met verhoogde expressie van de genen coderend voor arabinan en xylan afbrekende enzymen en ondersteunt de theorie dat L-arabitol de specifieke laag moleculaire inducer is voor de genen betrokken bij arabinan afbraak. Overproductie van D-xylulose kinase had echter nagenoeg geen effect op de concentratie van deze polyolen en had ook geen effect op de expressie van de genen coderend voor de arabinolytische en xylanolytische enzymen. Dit geeft aan dat de stappen in de katabole route vóór XKIA grotere controle hebben op de flux door deze route dan D-xylulose kinase zelf.
In hoofdstuk vijf staat de klonering van genen coderend voor L-arabitol dehydrogenase (*ladA*) en xylitol dehydrogenase (*xdhA*) beschreven. Na productie van de enzymen in *Escherichia coli* is de substraat specificiteit van beide enzymen bepaald. LADA is voornamelijk actief op L-arabitol maar ook op xylitol en heeft geen activiteit met D-sorbitol en galactitol. XDHA heeft de hoogste activiteit met xylitol en significante activiteit op D-sorbitol, maar erg lage activiteit op L-arabitol. De hogere activiteit van XDHA op D-sorbitol en xylitol is in overeenstemming met het feit dat XDHA op aminozuur niveau meer overeenkomst vertoont met sorbitol dehydrogenases dan LADA. Expressie analyse van de genen betrokken bij de pentose katabole route bevestigt het model waarbij een arabinose specifieke regulator de expressie van alle genen welke nodig zijn voor de omzetting van L-arabinose in D-xylulose 5-fosfaat activeert. XLNR

reguleert de eerste stap en in mindere mate de rest van de stappen welke de conversie van D-xylose in D-xylulose 5-fosfaat bewerkstelligen.
Met behulp van kleurstofaffiniteitschromatografie zijn de enzymen van de L-arabinose en D-xylose katabole routes, welke nog niet eerder waren beschreven, gezuiverd en gekarakteriseerd. Met de complete set van kinetische parameters is een computermodel van het metabolisme geconstrueerd waarmee een steady state metabole controle analyse is uitgevoerd (Hoofdstuk zes).
Het computermodel is gebruikt om flux- en metabolietconcentratiecontrole te analyseren binnen de L-arabinose katabole route. Het model voorspelt dat de fluxcontrole zich niet alleen bevindt in de stap net na het metaboliet met de hoogste concentratie (L-arabitol), maar juist verdeeld is over het enzymen ervoor en de enzymen erna. De fluxcontrole blijkt sterk afhankelijk van de L-arabinose concentratie, waarbij 5 mM L-arabinose resulteert in realistische intracellulaire metaboliet concentraties voor L-arabitol en xylitol. De fluxcontrole coëfficiënten van L-arabinose reductase, L-arabitol dehydrogenase en L-xylulose reductase waren respectievelijk 0,68, 0,17 en 0,14. Deze analyse kan worden gebruikt als leidraad tot het bepalen welke stappen in de route aangepast zouden moeten worden om bijvoorbeeld de flux of een metabolietconcentratie te optimaliseren.
In hoofdstuk zeven worden de resultaten uit de hoofdstukken twee tot en met zes bediscussieerd in het licht van recente wetenschappelijke ontwikkelingen op het gebied van onder andere genomics.

| Curriculum Vitae

Marco Jan Lambertus is op 21 mei 1974 in Emmen geboren. In 1992 behaalde ik mijn VWO diploma aan de Scholengemeenschap Lelystad en begon ik aan de studie Scheikunde aan de Universiteit van Amsterdam. In 1997 studeerde ik af in de richting Biochemie na mijn hoofdvakstage binnen de onderzoeksgroep van Karel van Dam over het glucose transport van *Saccharomyces cerrevisiae*. Nog voordat de diploma uitreiking was geweest ben ik gaan werken binnen de groep Moleculaire Genetica van Industriële Micro-organismen van Jaap Visser aan de Wageningen Universiteit. In eerste instantie via uitzendwerk en uiteindelijk als Assistent in Opleiding binnen het Eurofung project aan het L-arabinose katabolisme van *Aspergillus niger*.
Na het beëindigen van mijn praktische werkzaamheden ben ik als Postdoc gaan werken, een jaar bij het Biomedical Primate Research Centrum in Rijswijk in de groep Parasitologie aan een proteomics project binnen het malaria onderzoek. Na dit jaar als Postdoc binnen de groep Biomoleculaire massaspectrometrie van de Universiteit Utrecht waar ik me tot op heden bezig houd met de quantitative proteomics van *S. cerrevisiae.*

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| Nawoord

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