

Fungal strain engineering from understanding towards applications

**Jiali Meng
September 2022**

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PhD thesis, Utrecht University, Utrecht, The Netherlands (2022)

ISBN: 978-94-6423-959-1

Cover design: Jiali Meng

Lay-out and design: Jiali Meng

Printing: ProefschriftMaken || www.proefschriftmaken.nl

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Fungal strain engineering from understanding towards applications

Schimmel stam verbetering van begrip naar toepassingen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 28 september 2022 des middags te 12.15 uur

door

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To my family who always stands by me

The research described in this thesis was performed at the Fungal Physiology Group, Westerdijk Fungal Biodiversity Institute (formerly CBS-KNAW), Utrecht, The Netherlands, and supported by the China Scholarship Council (CSC student number: CSC201907720027) to Jiali Meng.



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Chapter 1

General introduction

This chapter was published in *Advances in Applied Microbiology*

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Molecular engineering to improve lignocellulosic biomass based applications using filamentous fungi

Volume 114, 2021, 73-109

<https://doi.org/10.1016/bs.aambs.2020.09.001>

Abstract

Lignocellulosic biomass is an abundant and renewable resource, and its utilization has become the focus of research and biotechnology applications as a very promising raw material for the production of value-added compounds. Filamentous fungi play an important role in the production of various lignocellulolytic enzymes, while some of them have also been used for the production of important metabolites. However, wild type strains have limited efficiency in enzyme production or metabolic conversion, and therefore many efforts have been made to engineer improved strains. Examples of this are the manipulation of transcriptional regulators and/or promoters of enzyme-encoding genes to increase gene expression, and protein engineering to improve the biochemical characteristics of specific enzymes. This review provides an overview of the applications of filamentous fungi in lignocellulosic biomass based processes and the development and current status of various molecular engineering strategies to improving these processes.

1. Introduction

As demands for energy and various materials and chemicals continue to increase, a growing number of countries have realized the disadvantages of using fossil resources, including their future shortage and the negative effect on our environment. Therefore, much research has focused on low-cost and abundantly available renewable raw materials as substrates for production of biofuels and biochemicals (Fig. 1). A considerable amount of lignocellulosic residues is available around the world, most of which is left unused or burned as waste rather than being recycled in bioindustries. The use of these residues is therefore an attractive alternative for fossil resources [1–3].

Filamentous fungi are ubiquitous in all natural and artificial environments and many species use plant biomass as their main carbon source. In order to degrade plant cell wall polymers to different monomers, fungi produce a wide range of hydrolytic and oxidative enzymes that match the composition of these complex polymers. The resulting monomers are converted into energy and metabolites, needed by the fungal cells for growth and reproduction, through a set of metabolic pathways [4–6]. For the degradation of plant polysaccharides, it has been shown that different transcriptional regulators form a complicated regulatory system, which not only controls the production of extracellular plant polysaccharide-degrading enzymes, but also the metabolic pathways that convert the released monosaccharides [7].

In recent decades, filamentous fungi have been applied as industrial cell factories, because of their excellent abilities to produce relevant metabolites, such as antibiotics, organic acids and active pharmaceutical ingredients. In addition, filamentous fungi play a vital role in the production of enzymes with different activities, which are used for diverse industrial applications, including production of biofuels and biochemicals [8–10]. Still, it remains important to increase production levels of enzymes and improve enzyme cocktails or specific enzymes for different industrial requirements through strain engineering. The most common strategies include mutagenesis and screening, and protein and transcription factor engineering. In addition, metabolic engineering has been successfully employed in filamentous fungi to improve production of biochemicals for biotechnology applications [2,11].

In this review, we introduce recent developments and strategies of production of diverse bioproducts from lignocellulosic biomass by filamentous fungi through molecular engineering. We discuss how the production of biomass degrading enzymes and biochemicals can be improved through the manipulation of transcriptional regulators, and promoter and protein engineering. Furthermore, we present the use of filamentous fungi to produce value-added chemicals from lignocellulosic biomass by metabolic engineering.

2. Lignocellulosic biomass

2.1. The composition of lignocellulosic biomass

The effective utilization of lignocellulosic residues requires a good understanding of their chemical composition. In general, lignocellulosic biomass consists of cellulose, hemicelluloses, pectin and lignin as well as small amounts of extractives and proteins. Cellulose is the major structural component of cell wall polysaccharides and consists of linear chains of β -1,4-linked D-glucose residues [12]. Hemicelluloses are the second most abundant polysaccharide of plant cell wall and have three main types, (arabino- or glucurono-)xylan, xyloglucan and galacto(gluco)mannan, which consists of various monosaccharides, such as D-xylose, L-arabinose, D-mannose, D-galactose, D-glucose and D-glucuronic acid [13,14]. Pectin is a structural acidic heteropolysaccharide, which is mainly composed of α -1,4-linked D-galacturonic acid. Four distinct polysaccharides belong to the pectin group: homogalacturonan, xylogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II [15,16]. Lignin is a complex aromatic polymer build from *p*-coumaryl alcohol, coniferyl alcohol and

Lignocellulosic residues

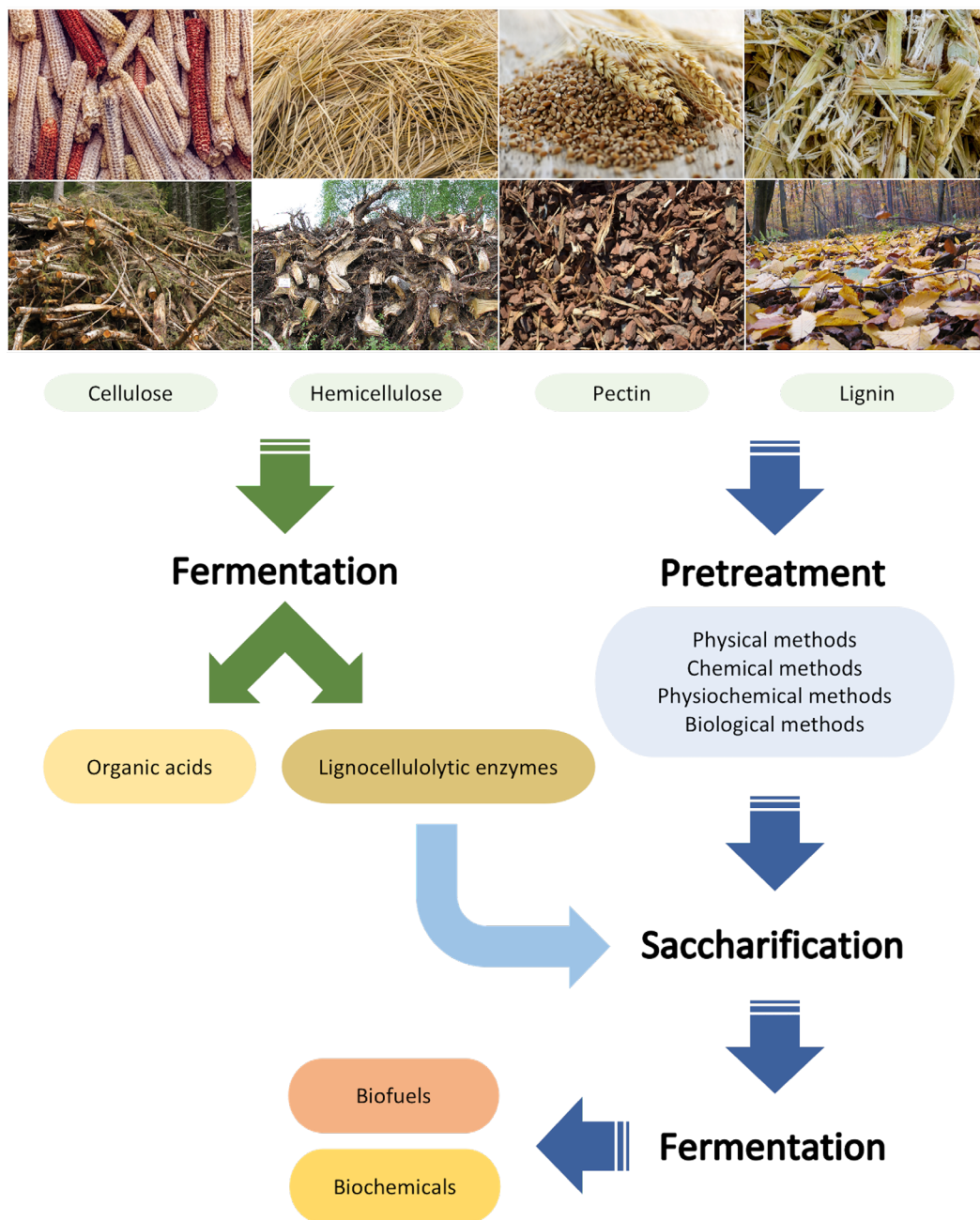


Figure 1. The utilization of lignocellulosic biomass for production of biofuels and biochemicals.

sinapyl alcohol precursors, which significantly hampers the enzymatic degradation of plant cell wall polysaccharides [17,18]. The composition of plant biomass differs considerably and depends on, e.g., plant species, tissue, age and growth environment [19,20]. Therefore, efficient plant biomass degradation requires appropriate and specific enzyme mixtures to compensate for the complex

and heterogenic composition of plant biomass. A large number of reviews have summarized our understanding of fungal degradation mechanisms of plant biomass polysaccharides in detail, which has provided us with insights and knowledge for further research and practical industrial applications [8,21–26].

2.2. Pretreatment of biomass to improve enzymatic depolymerization

Pretreatment is a crucial step to utilize lignocellulosic biomass effectively for bioproduction of valuable compounds. The main purpose of pretreatment is to overcome the recalcitrance of the lignocellulose matrix and separate its major components [27]. In the last decades, different pretreatment approaches have been published, including physical, chemical, physiochemical and biological methods as well as combinations thereof. Every method has its advantages and disadvantages that can affect the subsequent production processes. The choice of the most appropriate and economical pretreatment method depends on the raw material, economic assessment and environmental influences [28,29].

Pretreatment make the biomass more accessible to chemicals and/or enzymes, but the deconstruction of lignocellulose differs for every method. Physical pretreatment methods, such as milling, grinding, extrusion, microwave and sonication, are able to increase the surface area by reducing the size of the biomass particles and decrease crystallinity of the lignocellulosic material. However, these methods require high energy, which results in increasing costs of the whole process [30,31].

Various chemical pretreatment approaches have been reported for enhancing the accessibility of cellulose to hydrolytic enzymes by removing lignin and hemicellulose. Chemical pretreatment methods are the best studied techniques and have been extensively used in various industrial sectors. The most commonly used chemical methods include acid, alkali and ionic liquid treatment. However, these methods are likely to generate by-products and compounds that are toxic for the microorganisms that ferment the resulting sugars to the desired products. They therefore reduce the productivity and yield of target products and require subsequent detoxification treatment [1,32]. Physicochemical methods include liquid hot water, steam explosion, ammonia fiber explosion (AFEX), CO₂ explosion and microwave-chemical pretreatment, which can all hydrolyze hemicellulose. These methods result in low formation of co-products and inhibitors, but generally require high energy and high pressure with high cost and are not effective for biomass with high lignin content [1,33].

Biological pretreatment approaches, which use microorganisms or enzymes to alter the composition and structure of lignocellulosic biomass, have much less impact on our environment compared to chemical and physiochemical treatments [1]. Many wood degrading microorganisms have been reported for biological pretreatment, such as white rot fungi, brown rot fungi and soft rot fungi, as well as a few bacteria [34–40]. While brown and soft rot fungi mainly modify or convert lignin to some extent [41,42], white rot fungi are able to degrade high molecular weight native lignin polymers by producing extracellular oxidative enzymes, such as heme peroxidases and laccases, and are therefore considered as the most promising microorganism for biological pretreatment [43–45]. Besides, various ligninolytic enzymes (heme peroxidases and laccases) and ligninolytic accessory enzymes (feruloyl esterases, glyoxal oxidases and aryl-alcohol oxidase) have been applied in enzymatic treatment for lignin degradation [46–48]. Researchers have paid increasing attention to biological methods, because of their many advantages, such as no addition of chemicals, reduced energy requirement and mild production conditions. However, these processes require cautious control of growth conditions and long incubation times, while most microorganisms also consume cellulose and hemicellulose to support their growth [3]. These drawbacks prevent the industrial use of biological methods, although they are used in fields of biotechnology not covered in this review, such as bioremediation approaches [49–51].

2.3. Conversion of lignocellulosic biomass by filamentous fungi

Filamentous fungi need to recognize low molecular weight compounds defined as inducers released

from complex polymers, such as monosaccharides or disaccharides, before initiating the production of plant polysaccharide-degrading enzymes and activating the metabolic pathways that convert the resulting monosaccharides. Once the inducers are sensed by filamentous fungi, the activation of transcriptional activators is initiated through signaling pathways, and subsequently the expression of their target genes, encoding Carbohydrate-Active enZymes (CAZymes), is triggered [7]. CAZymes are divided into six main groups based on amino acid sequences: glycoside hydrolases (GH), glycosyltransferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), auxiliary activities (AA) and carbohydrate-binding modules (CBM) [52]. These polysaccharide-degrading enzyme sets differ between fungal species and their production corresponds to the specific composition of polysaccharides. As mentioned above, cellulose, hemicelluloses and pectins are the main components of plant cell wall polysaccharides, and their degradation has been described in detail in several reviews (e.g., [8,21,22,53]). The pentoses and hexoses released from these polysaccharides are taken up by the fungal cell and are converted by different metabolic pathways, such as the pentose catabolic pathway, pentose phosphate pathway and other sugar-specific metabolic pathways [4]. In addition, aromatic compounds derived from lignin, such as ferulic acid and vanillin, can be converted to seven central ring-fission intermediates through complicated aromatic metabolic pathways and enter the tricarboxylic acid cycle [5,6]. The production of plant biomass degrading enzymes and metabolic enzymes are both controlled by a network of regulators in fungi. Several of these transcription factors (TFs) have been identified, the majority of which belong to the zinc binuclear cluster (Zn(2)Cys(6)) family of regulators, but they are not conserved across the fungal kingdom. Many studies have aimed to characterize TFs in specific species, as the related regulation of the expression of their target genes directly affects the resulting enzyme cocktails for industrial applications [7].

3. Strategies to optimize the production of plant biomass degrading enzymes in filamentous fungi

Traditional strain improvement, such as by mutagenesis and screening, has been used to increase the production of commercially relevant enzymes. With the availability of whole genome sequences, many novel molecular technologies have been employed for strain improvement, such as CRISPR/Cas9 and RNA interference [11]. Protein engineering strategies contribute to solving the limitations related to enzyme production and functionality [54,55]. In addition, manipulation of TFs, which allow sensing and recognition of free sugars resulting in the activation or repression of enzyme-encoding and metabolic genes, has recently gained increasing attention for improving the production of enzymes and desirable biochemicals. Another strategy used in biotechnological studies is metabolic engineering, which improves the yield and titer of enzymes and other valuable biochemicals produced by filamentous fungi [56].

3.1. Engineering of transcriptional regulators involved in plant biomass conversion

In filamentous fungi the expression of the plant polysaccharide-degrading enzyme-encoding genes is tightly controlled by several TFs, which are activated by specific inducers released from plant polysaccharides. The TFs not only control the expression of the genes encoding plant biomass degrading enzymes, but also regulate the expression of the genes encoding sugar transporters and metabolic enzymes needed for the conversion of the sugars [57].

This regulatory system therefore has a direct effect on the production of extracellular enzyme sets and is closely related to industrial applications of hydrolytic enzymes. It is of industrial interest to manipulate TFs in several fungal species, such as *Aspergillus* spp. [58–63], *Trichoderma reesei* [64–68], *Neurospora crassa* [69–71], and *Penicillium oxalicum* [72–76]. The methods to modulate TFs involve constitutive expression, altering promoters and overexpression/deletion in order to rationally

improve strains towards constitutive or increased production of enzymes and biochemicals (Table 1).

3.1.1. Constitutively active regulators

As mentioned above, the activation of transcriptional regulators is dependent on specific inducers released from lignocellulosic biomass and subsequently affect the production of enzyme mixtures. Therefore, the production of enzyme cocktails for industrial applications requires specific feedstocks. However, the utilization of lignocellulosic biomass results in batch-to-batch variation in the yield and composition of enzymes due to the significantly different composition of biomass, which leads to the limitation of the use of cheap feedstocks for fixed enzyme cocktails [57]. Constructing strains constitutively expressing TFs is a promising strategy for the production of plant biomass degrading enzymes, independent of the carbon source. In the past years, constitutively active TFs have been generated through amino acid substitutions and domain fusion/removal.

The transcriptional activator XlnR from *A. niger* is a zinc binuclear cluster TF, which regulates the expression of genes encoding cellulolytic and hemicellulolytic enzymes. A single amino acid mutation in XlnR (XlnR^{V756F}) was obtained by a forward genetic screen, resulting in constitutive expression of xylanases under repressing conditions [58]. In *T. reesei*, a single point mutation generated in the ortholog of XlnR (Xyr1^{A824V}) by UV mutagenesis led to strong downregulation of xylanase gene expression and highly increased cellulase gene expression, regardless of the used carbon source [64]. By overexpressing the Xyr1^{A824V} version in *T. reesei*, the resulting strain produced increased cellulase activity, including carboxymethyl cellulase, cellobiohydrolase and β -D-glucosidase on Avicel, to a higher level than overexpression of the native *xyl1* [65]. These XlnR/Xyr1 constitutive mutations are located in the C-terminal sequences of XlnR and Xyr1, which have been suggested to be the putative activator domain of these TFs [58,64,65]. Similarly, an amino acid change of the transcriptional activator GaaR (GaaR^{W361R}) and a point mutation in AraR (AraR^{N806I}) both resulted in a constitutively active regulator, leading to constitutive pectinase production and inducer-independent expression of the genes encoding arabinolytic enzymes in *A. niger*, respectively [77,78].

In addition, based on the sequence similarity between XlnR and AraR, a mutation of AraR (AraR^{A731V}) was constructed and expressed under control of the *Aspergillus nidulans* promoter *gpdA* in *P. oxalicum*. The overexpressing strain CX^C-gAraR^{A731V} constitutively produced α -L-arabinofuranosidase in a medium without a carbon source, the level of which was similar to that of the wild type strain cultivated in wheat bran medium [79]. Two heterologous XlnR homologs from *T. reesei* (Xyr1) and *N. crassa* (XLR-1), which have clear regulatory functions on xylanase gene expression, as well as their constitutively active mutants (Xyr1^{A824V} and XLR-1^{A828V}) were also introduced into *P. oxalicum*. Elevated lignocellulolytic enzyme activities were observed in the mutants with overexpression of Xyr1^{A824V} and XLR-1^{A828V}, instead of native Xyr1 and XLR-1 when cultivated in complex carbon medium [80].

In order to produce cellulases under non-inducing conditions, the DNA-binding domain of the cellulase transcriptional activator ClrB was combined with the C-terminal sequence of the hemicellulolytic transcriptional activator XlnR with a constitutively active mutation (XlnR^{A871V}) forming an artificially designed chimeric TF. Considerably improved production of cellulase was observed in *P. oxalicum* strains overexpressing this chimeric TF, compared to overexpressing the native ClrB. It is worth noting that one of these mutants, OE-CX^C-S-1, exhibited 7.3-fold higher cellulase activity compared to the wild type strain on wheat bran [72].

These results support that the modification of transcriptional regulators is a very efficient strategy to engineer filamentous fungi for the increased production of plant biomass degrading enzymes. The constitutively active TFs in engineered strains enable bypassing the requirement of a specific inducing substrate and the utilization of cheap feedstocks for production of the enzymes.

Table 1. The different methods to modulate transcription factors related to plant biomass conversion involving constitutive expression, altering promoters and overexpression/deletion.

| Transcription Factors | Species | Approaches | Improvements | References |
|--|-----------------------------|--|---|------------|
| Constitutively active regulators | | | | |
| XlnR | <i>Aspergillus niger</i> | A single amino acid mutation in XlnR (XlnR ^(756F)) by a forward genetic screen | Constitutive expression of xylanases under repressing conditions | [58] |
| Xyr1 | <i>Trichoderma reesei</i> | A single point mutation in Xyr1 (Xyr1 ^{A824V}) by UV mutagenesis | Strong constitutive expression of xylanase and cellulase encoding genes | [64] |
| GaaR | <i>Aspergillus niger</i> | An amino acid mutation in GaaR (GaaR ^{N361R}) isolated through a forward genetic screen | Constitutive production of pectinases | [77] |
| AraR | <i>Aspergillus niger</i> | The point mutation in AraR (AraR ^{N806S}) by UV mutagenesis | Inducer-independent expression of the genes of arabinolytic enzymes | [78] |
| AraR | <i>Penicillium oxalicum</i> | A mutation of AraR (AraR ^{A731V}) expressed under control of the promoter <i>gpd4</i> | Constitutive production of α -L-arabinofuranosidase | [79] |
| Xyr1 and XLR-1 | <i>Penicillium oxalicum</i> | Two constitutively active mutants (Xyr1 ^{A824V} from <i>T. reesei</i> and XLR-1 ^{A828V} from <i>N. crassa</i>) heterologously expressed | 2.8-fold increase in cellulase production | [80] |
| ClrB | <i>Penicillium oxalicum</i> | The DNA binding domain of ClrB was combined with the C-terminal sequence of XlnR ^{A871V} forming an artificially designed chimeric TF | 7.3-fold increase in cellulase production | [72] |
| Overexpression of transcriptional regulators | | | | |
| AmyR | <i>Aspergillus niger</i> | Overexpression of <i>amyR</i> gene (multicopy) | 2- to 15-fold increase in amylolytic activities | [59] |
| GaaR | <i>Aspergillus niger</i> | Overexpression of <i>gaaR</i> gene under the control of the strong constitutive <i>gpd4</i> promoter | Strongly elevated production of pectinases | [60] |
| ClrB | <i>Aspergillus nidulans</i> | Overexpression of <i>clrB</i> under the control of the constitutive promoter <i>gpd4</i> | Increased expression of cellulase genes | [69] |
| XlnR | <i>Aspergillus nidulans</i> | Overexpression of <i>xlnR</i> under the control of the constitutive promoter <i>gpd4</i> | Enhanced expression of xylanolytic genes | [84] |

| Transcription Factors | Species | Approaches | Improvements | References |
|--|-----------------------------|--|--|------------|
| ManR | <i>Aspergillus oryzae</i> | Overexpression of <i>manR</i> using the constitutive <i>tefI</i> promoter | Increased mannanolytic (1.5-5.5-fold) and cellulolytic (1.5-fold) activities | [61, 62] |
| XlnR | <i>Aspergillus oryzae</i> | Overexpression of <i>xlnR</i> using the constitutive <i>tefI</i> promoter | Improved expression of xylanolytic and cellulolytic genes | [85, 86] |
| Clr-2 | <i>Neurospora crassa</i> | Overexpression of <i>clr-2</i> by a constitutive promoter <i>Pccg-1</i> | Enhanced production of cellulases under inducing conditions and constitutive production under noninducing conditions | [69] |
| PDR-1 | <i>Neurospora crassa</i> | Overexpression of <i>pdr-1</i> under the control of the strong <i>gpd</i> promoter of <i>Myceliophthora thermophila</i> | >2-fold increased pectinase production | [70] |
| Xyr1 | <i>Trichoderma reesei</i> | Overexpression of <i>xyr1</i> via the strong constitutive <i>pdC</i> promoter | 107% increased cellulase activity and 64% increased protein production | [87] |
| Xyr1-Cre1 _b | <i>Trichoderma reesei</i> | Constitutive expression of <i>xyr1-cre1_b</i> using the <i>pdC</i> promoter | Constitutive and increased production of cellulases (12.75-fold) and xylanases (approx. 86-fold) | [66] |
| KojR | <i>Aspergillus oryzae</i> | Overexpression of <i>kojR</i> using the <i>sodM</i> promoter from <i>A. oryzae</i> | 3.1-fold increased kojic acid production | [63] |
| Manipulation of negatively acting regulators | | | | |
| Cre1 | <i>Trichoderma reesei</i> | Deletion of <i>cre1</i> or substitution by a truncated variant (<i>cre1-l</i>) | Derepression of hemicellulase and cellulase production, >20-fold higher cellulase and 10-fold higher xylanase activity | [94] |
| Cre-1 | <i>Neurospora crassa</i> | Deletion of <i>cre-1</i> | Increased amylase and β -galactosidase secretion as well as cellulolytic activity and expression level of cellulolytic genes | [71] |
| | | Deletion of <i>cre4</i> | 8.0-fold increased cellulase and 4.4-fold increased xylanase activities | [95] |
| CreA | <i>Penicillium oxalicum</i> | Deletion of <i>creA</i> and <i>bg/2</i> together with overexpression of <i>clrB</i> | >20-fold improved cellulolytic activity | [74] |
| | | Deletion of <i>cre4</i> , overexpression of <i>clrB</i> and XlnR ^{ASTIV} via a constitutive promoter <i>PDE_02864</i> | 8.9- and 51.5-fold increased production of cellulase and xylanase | [73] |

| Transcription Factors | Species | Approaches | Improvements | References |
|-----------------------|-----------------------------------|---|--|------------|
| CreA | <i>Aspergillus niger</i> | Deletion of <i>creA</i> | A higher expression of some hemicellulolytic genes and a higher specific enzyme production | [96] |
| CreA | <i>Penicillium decumbens</i> | A frameshift mutation at the C-terminus of <i>creA</i> | More than 2- and 3-fold increased production of hemicellulases and cellulases | [97] |
| CreA | <i>Acremonium cellulolyticus</i> | Disruption of <i>creA</i> | Elevated production of cellulase and xylanase with higher activities and increased transcription levels | [98] |
| Cre-1 | <i>Myceliophthora thermophila</i> | Multiple disruptions of four genes of the cellulase production pathway, including <i>cre-1</i> , <i>res-1</i> , <i>gh1-1</i> , and <i>alp-1</i> | Up to 13-fold increased lignocellulase activities | [99] |
| AtfI | <i>Penicillium oxalicum</i> | Deletion of <i>AtfI</i> | 46.1 to 183.2% more cellulase and xylanase production | [75] |
| HCR-1 | <i>Neurospora crassa</i> | Deletion of <i>hcr-1</i> | Increased secreted protein concentration and xylanase activity | [100] |
| MHR1 | <i>Myceliophthora thermophila</i> | Silence of <i>mhr1</i> by RNA interference | 9.56- to 56.14-fold enhanced expression of the main cellulase genes and <i>xyl1</i> ; 1.33- to 1.65-fold higher activity of cellulase and xylanase | [101] |
| Ace1 | <i>Trichoderma reesei</i> | Deletion of <i>ace1</i> | Elevated expression of the main cellulolytic genes as well as several xylanolytic genes | [102] |
| Xpp1 | <i>Trichoderma reesei</i> | Deletion of <i>xpp1</i> | Increased xylanolytic activities | [67] |
| SxIR | <i>Trichoderma reesei</i> | Deletion of <i>sxlR</i> | 0.7- to 14.2-fold higher xylanase activity | [68] |
| Rce1 | <i>Trichoderma reesei</i> | Deletion of <i>rce1</i> | Significant increase of cellulase activities | [103] |

3.1.2. Overexpression of transcriptional regulators

The expression of genes is mainly controlled at the level of transcription initiation, which involves interaction of RNA polymerase II, the promoter of the gene that is expressed and TFs. The promoter is a DNA sequence located near the transcription start site, to which the TFs bind, thus initiating the transcription of the downstream DNA sequence [81]. Increasing the copy number of TF encoding genes leads to an increased amount of the TFs, which in combination with endogenous or strong constitutive or inducible promoters subsequently improves the expression of target genes [57]. Some examples of this approach are discussed below.

An *amyR* multicopy strain of *A. niger* showed increased activities of amylolytic enzymes, including α -glucosidase (AGD), β -glucosidase (BGL), α -galactosidase (AGL) and β -galactosidase (LAC), cultivated in a medium containing lactose, maltose, starch or a low concentration of D-glucose [59]. The *gpdA* promoter is the most well-known constitutive promoter from *A. nidulans* and is extensively used for constitutive expression of target genes [82]. For example, the transcriptional activator GaaR controls the expression of genes encoding pectinases, a D-galacturonic acid (GA) transporter and GA catabolic pathway enzymes in *A. niger* [60]. Overexpression of the TF encoding gene *gaaR* under the control of the strong constitutive *gpdA* promoter of *A. nidulans* resulted in constitutive transcription and significantly elevated production of pectinases in *A. niger* even when grown on fructose [60,83]. Similarly, overexpression of two transcriptional activator encoding genes *clrB* and *xlnR* under the control of the constitutive promoter *gpdA* increased the expression of cellulase genes and enhanced the production of xylanolytic enzymes in *A. nidulans* under inducing conditions, respectively [69,84].

ManR/ClrB not only acts as a transcriptional activator of mannanolytic genes, but also controls the expression of cellulolytic genes in *Aspergillus oryzae*. A *manR*-overexpressing strain of *A. oryzae* using the constitutive *tefl* promoter exhibited highly increased mannanolytic and cellulolytic activities on mannan and Avicel, respectively [61,62]. Overexpressing *xlnR* in *A. oryzae* using the constitutive *tefl* promoter improved the expression of xylanolytic and cellulolytic genes when grown on xylose/xylan and cellobiose/Avicel, respectively [85,86]. In *N. crassa*, overexpression of *clr-2* under the control of a constitutive promoter *Pccg-1* resulted in enhanced production of cellulases under inducing conditions and constitutive production under non-inducing conditions [69]. The pectin degradation regulator-1 (PDR-1) is a multi-functional regulator and plays a major role in pectin deconstruction and catabolism in *N. crassa*. The gene *pdr-1* was overexpressed under the control of the strong *gpd* promoter of *Myceliophthora thermophila*, resulting in substantially increased pectinase production when grown on xylan supplemented with L-rhamnose [70]. Similar examples were reported for *T. reesei*. The industrial strain *T. reesei* RUT C30 obtained by random mutagenesis already has an excellent ability of cellulolytic and xylanolytic enzymes production. In order to further enhance enzyme production, *xyl1* was overexpressed via the strong constitutive *pdC* promoter, leading to highly increased cellulase activity and protein production when the fungus was grown on cellulose [87]. An artificial transcription activator was generated containing the DNA-binding domain of Cre1 and the DNA-binding domain and effector domain of Xyl1. This artificial gene was constitutively expressed using the *pdC* promoter in *T. reesei* RUT C30, resulting in constitutive and significantly increased production of cellulases and xylanases on glucose as a sole carbon source [66].

In addition, several inducible promoters were studied for high gene expression, such as the thiamine-regulatable *thiA* promoter and the *alcA* promoter [88,89]. The *kojR* gene encodes a fungal-specific $\text{Zn(II)}_2\text{Cy}_6$ TF regulating the kojic acid production in *A. oryzae*. Overexpressing *kojR* using the *sodM* (superoxide dismutase) promoter from *A. oryzae* resulted in higher kojic acid production than the wild type strain from glucose and co-expressing of the *kojR* and three cellulase genes enabled the production of kojic acid directly from phosphoric acid swollen cellulose [63].

In conclusion, these results demonstrate that overexpression of TFs is an efficient method to increase

expression of target genes under inducing conditions and even non-inducing conditions, leading to inducer-independent production of lignocellulolytic enzymes and biochemicals.

3.1.3. Manipulation of negatively acting regulators

Carbon catabolite repression (CCR) has a universal function in the regulatory system, which ensures efficient utilization of preferred carbon sources and prevents the activation of unnecessary metabolic pathways to save energy [90]. With respect to the production of extracellular enzymes, when sufficient monomeric sugars are present in the environment, the regulators involved in CCR would repress the expression of genes encoding plant biomass degrading enzymes, because they are not required for releasing monosaccharides from plant biomass [91]. CreA/Cre1 is the best-described negative regulator involved in CCR [7]. In addition, all monosaccharides can induce CreA-mediated CCR with different influences [92,93]. Therefore, the corresponding gene is also a target to be modified by genetic engineering for improving the production of plant biomass degrading enzymes and valuable biochemicals, which has been already applied in many filamentous fungi.

For example, two *T. reesei* mutants were constructed with enhanced enzyme production, in which the *cre1* was completely deleted or substituted by a truncated variant (*cre1-I*). These mutants showed derepression of cellulase and hemicellulase production on glucose as a sole carbon source and produced significantly elevated hydrolytic enzyme levels under inducing conditions. It was suggested that Cre1 regulates the expression of cellulase and hemicellulase genes under both inducing and non-inducing conditions in *T. reesei* [94]. In *N. crassa*, deletion of *cre-1* was not only shown to increase amylase and β -galactosidase secretion, but also resulted in increased cellulolytic activity and expression level of cellulolytic genes when cultivated on Avicel, suggesting that Cre1 may have a direct effect on the production of many lignocellulolytic enzymes [71]. Similarly, the deletion of *creA* or combined with the overexpression of transcriptional activators strongly increased production or activity of the enzymes and accessory proteins relative to lignocellulose degradation in *P. oxalicum* [73,74,95]. The manipulation of *creA/cre1* was also used in other fungi, such as *A. niger*, *Penicillium decumbens*, *Acremonium cellulolyticus* and *Myceliophthora thermophila*, with similar effects on lignocellulolytic activities and production [96–99].

There are also some other negative regulators that influence the expression of enzyme-encoding genes. For example, a novel TF, multiprotein bridging factor 1 (MBF1), has been identified, which regulates the expression of many cellulase and xylanase genes in *P. oxalicum* and is related to stress resistance [76]. Another novel TF Atf1 also regulates the expression of cellulolytic and xylanolytic genes in this fungus, as a deletion mutant of *Atf1* showed higher cellulase and xylanase production than the parental strain [75].

In *N. crassa*, the hemicellulase regulator-1 (HCR-1) is associated with the expression of hemicellulolytic genes on L-arabinose and xylan. Deletion of *hcr-1* increased the secreted protein concentration and xylanase activity, but not endoglucanase activity, suggesting hemicellulase-specific regulation by HCR-1 [100]. Recently, a new identified transcriptional regulator MHR1 of *M. thermophila* has a negative effect on cellulase and xylanase activities and silencing of *mhr1* by RNA interference resulted in strongly enhanced expression of the main cellulase genes and *xyl1* under inducing conditions, indicating that MHR1 is related to carbon catabolite repression as a repressor [101].

Several transcriptional repressors have been manipulated in *T. reesei*. Deletion of the cellulase regulator *acel* caused elevated expression of the main cellulolytic genes as well as several xylanolytic genes on sophorose or cellulose [102]. In addition, deletion of the basic helix-loop-helix TF Xpp1 (Xylanase promoter-binding protein 1) resulted in increased xylanolytic activities at later cultivation stages on xylan, but not the main cellulolytic enzymes, indicating that Xpp1 only regulates the expression of xylanolytic genes [67]. A novel zinc binuclear cluster TF SxIR (specialized xylanase regulator) was identified as a repressor specifically regulating GH11 xylanase genes in *T. reesei*

through binding to the promoter of its target genes. The deletion of *sxIR* resulted in elevated xylanase activity in *T. reesei* cultivated on wheat bran plus Avicel or xylan (inducing conditions) and no significant difference in cellulase activity, as well as improved hydrolytic activity on pretreated rice straw by increasing the xylanase-cellulase ratio [68]. These results showed that appropriate ratio of cellulase and hemicellulase can be conveniently optimized by genetic manipulation of these two TFs, Xpp1 and SxIR, in *T. reesei*. Finally, another novel transcription repressor of *T. reesei*, Rce1, negatively affects the cellulolytic activities but not the xylanolytic activities, and was not involved in Cre1-mediated CCR. Competitive binding assays strongly indicated that Rce1 directly regulates cellulase gene expression by competing with Xyr1 for binding to the *cbh1* (cellobiohydrolase 1-encoding gene) promoter [103].

In summary, the TF Cre1/CreA has an important effect on carbon catabolite repression, a key component for plant biomass degradation. Other transcriptional repressors also negatively regulate the expression of lignocellulolytic enzymes and are therefore interesting targets for genetic manipulation. Through elimination of the negative effect of these TFs, many studies have improved the levels of hydrolytic enzymes, thus showing promise for production of industrial enzymes.

3.2. Promoter engineering to alter the production of specific enzymes

TFs induce or repress the expression of target genes through binding to the promoters of genes. Increasing the expression of TFs using a strong promoter can improve the expression of target genes and thus optimize the production of enzymes and biochemicals. However, the promoters of target genes, such as those encoding lignocellulolytic enzymes, have also been manipulated to achieve the improvement of value-added products, by replacing them with constitutive, inducible/tunable and synthetic promoters [104].

Several promoters have been used for the expression of enzymes in *T. reesei*, among which the best-studied promoter is *CBHI*. For example, this promoter has been used for expression in *T. reesei* of an endoxylanase encoding gene from thermophilic fungus *Chaetomium thermophilum* (*Ct xyn11A*) [105]. Two feruloyl esterase (FAE)-encoding genes *AnfaeA* and *AnfaeB* from *A. niger* were overexpressed under the control of *GPDA* promoter from *A. nidulans* and *CBHI* promoter from *T. reesei* in *T. reesei* RUT-C30 for the production of ferulic acid from wheat bran. A high level of feruloyl esterase was produced in the resulting mutants under solid-state fermentation and released significantly increased ferulic acids from de-starched wheat bran than those released by the parental strain [106].

In addition to *CBHI*, there are some alternative inducible/tunable promoters and constitutive promoters as well as synthetic expression systems for controlling gene expression in *T. reesei*. Constitutive promoters cause gene expression independently of the prevalent carbon source in the environment [107]. For instance, two strong constitutive promoters, *PDC* and *ENO*, were successfully used to obtain high activity levels of a homologous xylanase II in *T. reesei*, cultivated in a medium with high glucose concentration [108]. However, other constitutive promoters have also been commonly used in *T. reesei*, such as *cDNA1*, *PKII*, *TEF1* and *RP2* [104]. Although constitutive promoters are applied conveniently and independent of the used carbon source, the resulting expression level is relatively lower than for the inducible promoters [109].

Gene expression mediated by inducible promoters in the absence of an inducer should be very low and significantly enhanced after the addition of the inducers [110]. As a producer for cellulases and xylanases, the promoters of genes encoding the main hydrolytic enzymes are promising options for overexpression of target genes in *T. reesei* [111]. For example, by using the *CBH2* promoter the *cdh* gene from the basidiomycete fungus *Phanerochaete chrysosporium* was heterologously expressed in *T. reesei*, resulting in significantly increased cellulase production and hydrolysis efficiency of the *cdh*-expressing mutant grown on lactose or cellulose [112]. High production of a thermophilic xylanase enzyme (XynB) from *Dictyoglomus thermophilum* was achieved by expressing *xynB*

gene under the control of *EGL2* (*CEL5a*) promoter in *T. reesei* [113]. There are also several *T. reesei* xylanase promoters that have been used, such as the *XYN3* (xylanase 3) promoter [114,115]. However, the application of those promoters is limited by the specific substrate needed for induction and the repression from carbon catabolite repression.

A new synthetic expression system (SES) was designed for *T. reesei*, consisting of two expression cassettes, the first part containing a constitutive synthetic transcription factor (sTF), and the second part containing a sTF-dependent promoter, enabling strong and tunable expression of target genes [116]. Using SES to express a *T. reesei* native cellobiohydrolase enzyme CBHI, the SES strain secreted more protein compared to the parental strain when cultivated in the glucose containing medium. In addition, the protein produced by SES strain mainly consisted of CBHI, and the amount of CBHI of SES strain on glucose was comparable to the amount produced by the parental strain in cellulase-inducing medium [116].

To facilitate higher gene expression in members of the industrially important fungal genus *Aspergillus*, several studies compared different promoters for the expression of specific target genes [117–119]. In *A. niger*, they were analyzed for their potential for metabolic engineering, through heterologous expression of the *cis*-aconitate decarboxylase (*cad1*) gene of *Aspergillus terreus* to produce the building block chemical itaconic acid in *A. niger* [118]. In *A. vadensis*, the aim was to produce higher levels of extracellular enzymes, and nearly all tested promoters resulted in higher enzyme activity than the commonly used *A. nidulans gdpA*-promoter [119]. Furthermore, a low-pH-inducible promoter, *Pgas*, works efficiently at low pH, but provides basic gene expression at higher pH such as pH 5.0, which has the ability to control the gene expression dynamically for the production of organic acid by metabolic engineering in *A. niger* [120]. In *A. oryzae*, a novel promoter *HLYA* (from a gene encoding a hemolysin-like protein) was identified as a stronger promoter than promoters *PamyA* and *PsodM* for overproduction of plant cell wall degrading enzymes [117]. The production of fatty acids and triglycerides as well as L-malate were significantly increased by overexpression of the fatty acid synthesis-related genes or related degrading enzyme-encoding genes using the constitutive promoter *TEF1* or the engineered *GLAA* promoter in *A. oryzae*, respectively [121,122]. The constitutive promoter *PDE_02864* and the inducible *GLA1* promoter are also used for optimizing enzymes in *P. oxalicum* and *Penicillium verruculosum* [123,124].

In conclusion, promoter engineering has become a promising strategy to manipulate the expression of lignocellulolytic enzyme-encoding genes and biosynthesis-related genes for the production of industrially relevant enzymes and biochemicals.

3.3. Protein engineering to improve enzyme properties

Protein engineering has been applied to improve the characteristics of lignocellulolytic enzymes. Different protein engineering methods have been used, such as rational design, directed evolution and semi-rational design. These methods and their benefits will be discussed in more detail below.

3.3.1. Rational design

The rational design approach mainly includes two methods, site-directed mutagenesis and domain fusion, based on a deep understanding of structures and functions of the proteins of interest [125,126]. Site-directed mutagenesis is a method to create specific mutations in double stranded DNA, and thus changes the enzyme protein to improve its catalytic properties [127]. One category of domain fusion is end-to-end fusion, where the C-terminus of a protein is fused with the N-terminus of another protein by a linkage. Another category of domain fusion is domain insertion by inserting a protein/domain into another protein/domain [128].

Cellulase properties have been improved by protein engineering for many years, such as increasing catalytic activity and improving thermostability [129]. Cellobiohydrolases (CBHs) are important hydrolytic enzymes in cellulose degradation and have been the subject of many structural and

biochemical studies [130]. The function of N-linked glycans on the surface of the catalytic domain of CBHs (Cel7A) from *T. reesei* and *Penicillium funiculosum* was tested using site-directed mutagenesis by adding or deleting N-linked glycosylation motifs. The removal of the N384 glycosylation site in *T. reesei* Cel7A and the addition of a new glycosylation motif at N194 in *P. funiculosum* Cel7A both resulted in 70% higher activities of the modified enzymes compared to the respective native enzymes [131].

Thermostable cellulases with a higher level of stability and activity have many advantages in the bioproduction process at elevated temperatures, which can increase the reaction rates and eliminate contamination [132]. Endoglucanases (EGLs) have been used in the production of many chemicals from lignocellulosic biomass as an important part of enzyme cocktails for industrial applications. However, their commercial applications are restricted because of their low thermostability and catalytic activity at industrially required temperatures. A stable EGL of *Hypocrea jecorina* (anamorph *T. reesei*), Cel5A, engineered by combining thermostabilizing mutations, which were verified by consensus design, chimera experiments and structure-based computational approaches, exhibited a higher optimal temperature than native Cel5A. It also performed more efficiently in hydrolyzing crystalline cellulose at its optimum temperature compared to the native Cel5A [132]. The thermostability of EGLII (Cel5A) from *P. verruculosum* was also enhanced by a structure-based disulfide bond (DSB) engineering [133]. Endoglucanase I (EGI, Cel7B) is the major endoglucanases of the cellulolytic enzyme complex produced by *T. reesei*, and its thermostability was successfully improved by site-directed mutagenesis [134,135].

β -glucosidases can hydrolyze both cellobiose and cellodextrins to fermentable glucose in fungi. For production of biochemicals from lignocellulosic biomass, efficient hydrolysis of cellobiose to glucose by β -glucosidases can increase the levels of free fermentable glucose and eliminate product inhibition of cellulases by cellobioses during saccharification [136]. Due to the essential function of β -glucosidases, improvement of their activity and thermostability is critical for industrial biotechnology. Through amino acid mutations, mutants of *T. reesei* β -glucosidase 2 (TrBgl2) and a *Trichoderma harzianum* β -glucosidase (ThBgl) exhibited improved catalytic efficiency and thermostability as well as increased tolerance to high glucose, making them more suitable for industrial applications [136,137].

Furthermore, site-directed mutagenesis has also been applied for improving the activity and stability of xylanases. A xylanase from *A. niger* was modified by replacing several amino acid residues on the Ser/Thr surface with four or five arginines, effectively increasing the catalytic activity of the modified enzyme on xylan and the thermostability by 18- to 20-fold compared to the wild type enzyme [138]. In addition, xylanase mutants from *T. reesei*, *Penicillium canescens* and *Thermoascus aurantiacus* created by site-directed mutagenesis also showed increased thermostability with longer half-life time and higher residual activity than wild type enzymes at specific temperatures or pH, thus better meeting the extreme conditions of industrial applications [139–142].

There are several studies that have used domain fusion methods to characterize and improve enzymes. Xyloglucan-specific endo- β -1,4-glucanases (XEGs) can specifically hydrolyze xyloglucan (XyG), the most primary hemicellulosic polysaccharide in the primary cell walls of dicots. A GH12 XEG from *Aspergillus niveus* (XegA) was fused with xyloglucan-specific carbohydrate-binding module (CBM) 44 and the chimeric enzyme had improved catalytic efficiency and thermostability over the native XegA [143]. Endo-1,4- β -D-mannanases are the major enzymes related to the hydrolysis of structurally different mannans. Similarly, to improve catalytic properties of a mannanase from *Aspergillus aculeatus* on lignocellulosic biomass, the CBM1 of *A. niger* CBH B was fused at the C-terminal end of this mannanase using a natural linker, and subsequently expressed in *A. niger* under the control of GPDA promoter. The fused enzyme was more thermostable and showed an improved glucose yield on softwood in combination with a *T. reesei* enzyme cocktail compared to the native mannanase [144].

Rational design is an effective method to obtain modified enzymes possessing desired properties,

but it requires a full understanding on the relationship of structures and functions of enzymes, which limits its applications.

3.3.2. Directed evolution

Directed evolution is a powerful method based on natural selection to create genetic diversity and accumulate the desired mutations under specific selective pressure, which does not require knowledge about the protein structure [129]. For this, the first step is to generate a large library of genetically modified mutant enzymes, and then employ an efficient screening or selection approaches to recognize the mutants with improved properties [126]. There are many methods to obtain genetic diversity, including random mutagenesis and genetic recombination.

The most commonly used method of random mutagenesis is error-prone PCR. This is performed by using a DNA polymerase with low fidelity and changing the reaction conditions in standard PCR experiments to generate random mutations [145]. Several lignocellulolytic enzymes have been modified for desired properties by using error-prone PCR. For example, due to the need of an alkali-tolerant cellulase for industrial applications, endoglucanase III (EG III) from *T. reesei* was improved through error-prone PCR. A mutant (N321T) was obtained with a shift of optimal pH level from pH 4.8 to pH 5.4 as well as increased specific activity under alkaline conditions (pH 7.6) than that of wild type [146]. Most mesophilic xylanases are not suitable for industrial processes because they will lose most catalytic activity at elevated temperatures. Therefore, thermostability improvement of *endo*-1,4- β -xylanase (afxnG1) from *Aspergillus fumigatus* was attempted using error-prone PCR. The mutants had higher catalytic activity and increased stability compared to the native xylanase [147]. Directed evolution using error-prone PCR was also used for increasing the catalytic activity and production yields of inulinases [148]. An endoinulinase from *Talaromyces purpureogenus* was modified by directed enzyme evolution to increase its activity and yield for IOS production. The resulting enzyme variants showed significantly improved soluble enzyme yields and increased specific activities [148].

In addition to the random mutagenesis, genetic recombination methods has also been used for improving protein properties, such as random homologous and non-homologous recombination. Homologous recombination is used for combining several mutations from specific variants generated by rounds of mutagenesis. Random non-homologous recombination can generate multi-functional chimeric enzymes by combining several domains [149,150]. For instance, Cel7A from *T. reesei* has a low thermostability limiting its application at high temperatures. To enhance the thermostability of Cel7A by directed evolution, many mutated sites with desired properties were identified and selected by screening *T. reesei* Cel7A mutants. The mutated sites were combined and a Cel7A variant containing 18 mutated sites showed substantially increased T_m and much longer half-life than wild type enzyme, and maintained high activity under industrial process conditions even at 75°C [151]. Random mutagenesis and random recombination of seven potentially beneficial mutations were also used to improve the thermostability of a chimeric *T. reesei* cellobiohydrolase Cel6A (HjPlus). A highly thermostable variant 3C6P had significantly higher catalytic activity on Avicel at high temperatures [152].

Directed evolution has been widely used and does not need the information of structures and functions of enzymes. However, it is necessary to screen a large number of mutants in the library though a high-throughput screening method, which is the main limiting factor of its practical applications.

3.3.3. Semi-rational design

The semi-rational design combines the benefits of random mutagenesis and directed evolution, and identifies the “hotspots” residues/regions of the proteins based on the information of structural and functional data for obtaining desired enzymes [153]. Enzyme modification studies show that most mutations, which have a positive effect on certain enzyme properties, including substrate specificity

and novel catalytic activity, are located near or in the active sites of the proteins, especially the residues related to binding or catalysis. Other properties, such as activity and stability, can be modified by introducing mutations located either near or far away from active sites. Semi-rational approaches use this kind of knowledge to create “small and smart” libraries containing the most likely desired protein variants [153].

A relatively small library of mutants was generated by using biased clique shuffling (BCS) of 11 Cel7A genes from different filamentous fungi, in which most chimeras (85%) were active. 51 chimeras were identified with increased thermostability at 65°C and the majority of them contained mutations in the loop regions over the active site. This BCS library is superior as a source of active and stable chimeras with low mutational loadings, which samples sets of consensus mutations at a probability proportional to their frequency and bias in the parental templates, to the one generated by classic equimolar DNA shuffling [154]. Similarly, to stabilize *Talaromyces emersonii* Cel7A in aqueous-IL (ionic liquid) solutions, Cel7A variants were generated using BCS, resulting in some mutants that were more active and stable in aqueous-IL solutions than the wild type enzyme [155]. Structure-guided protein recombination (SCHEMA) requires three-dimensional structures and a computational algorithm for recombination classified as a semi-rational approach [156]. Non-contiguous recombination (NCR) is a method for researching elements of structure (“blocks”), which are not necessarily contiguous polypeptide sequences, and new chimeric proteins can be generated by swapping these blocks among homologous proteins [157]. A library was generated using non-contiguous structured-guided SCHEMA recombination through swapping structure blocks among CBHI from *T. reesei* (*H. jecorina*) and its two thermostable homologous from *T. emersonii* and *Chaetomium thermophilum*. By analyzing this library, several blocks contributing positively to the stability of a chimeric protein were found and six single amino acid substitutions occurred within two significantly stabilizing blocks, each of which can improve the stability of *T. reesei* CBHI by 1–3°C demonstrating that NCR is a powerful method for identifying stabilizing mutations [157]. In order to improve the thermostability of CBHII (PcCBH2) from the mesophilic fungus *P. chrysosporium*, PcCBH2 variants with consensus mutations were generated based on the amino acid sequences of the catalytic domains of CBHII proteins, and desired mutations were found in this small library [158]. The most thermostable variant Mal4 containing 15 mutations was obtained by accumulating advantageous mutations in the wild type protein. The specific activity of this variant was the same as for the wild type, but its thermostability was improved at 50°C [158]. In addition, the thermostability of EGL Cel7B from *Hypocrea pseudokoningii* (HpCel7B) was improved for the industrial requirements. Random mutants, produced by error-prone PCR of the EGL encoding gene, with elevated activity at higher temperature were screened and sequenced [159]. Comparing the structures of these enzyme mutants with homologous, but more thermostable EGLs indicated several potential hotspots seemed to be important for thermostability. The most potential beneficial mutations based on rational and non-rational methods were randomly recombined using gene synthesis and the resulting variants were analyzed for thermostability. This library contained several improved EGL variants of which the best variant showed 10°C higher melting temperature than the native enzyme, as determined by a thermal unfolding assay. Mutation M63I seemed to have a large effect on the thermostability of enzymes, indicating that hydrophobic interactions are crucial for protein folding and stability [159].

4. Conclusions

Filamentous fungi have the native ability to produce lignocellulolytic enzymes and are able to convert the diverse sugars that are present in plant biomass. Therefore, they provide promising option for the direct utilization of lignocellulosic feedstocks to produce valuable products. However, the improvement of this process is necessary for industrial applications and many efforts have been made for decades. In this review, we summarized the molecular engineering methods that

have been employed for improving the fungal production of lignocellulolytic enzymes and value-added biochemicals from lignocellulosic biomass. Many studies demonstrated that the strategies of transcription factor engineering require the deep understanding of the regulatory network in a specific fungus. Both transcriptional activators and repressors can be manipulated to improve gene expression. Besides, promoter engineering and protein engineering are of great importance for the direct modification of the enzyme production levels or the activity of enzymes. The findings presented in the review demonstrate that only a small part of the potential of employing filamentous fungi in industrial applications has been tapped so far. With increasingly efficient engineering methods, the next generation of fungal cell factories is likely to significantly outperform the current strains, especially with our growing understanding of fungal metabolism [4,5] and the regulatory systems underlying the production of lignocellulose converting enzymes [7].

Aim and outline of this thesis

Aspergillus species play an important role in the biomedicine, agriculture and food industries for the production of enzymes and metabolites, due to their secretion capacity and metabolic versatility. The great advances in *Aspergillus* genomics and modern molecular technologies deepen the understanding of this extremely diverse genus and subsequently provide many promising strategies to the construction of more versatile industrial fungal cell factories, as outlined in **Chapter 1**. Many efforts have been made for the thorough and comprehensive investigation of carbon utilization and regulation mechanisms in *Aspergillus* species, especially *A. niger* and *A. nidulans*. In my thesis, I have addressed several aspects of understanding and engineering sugar metabolism in these fungi.

Chapter 2 describes the construction of four metabolic mutants of *A. niger* for the accumulation of xylitol from D-xylose and lignocellulosic biomass, based on the recent detailed study of the *A. niger* pentose catabolism pathway (PCP). The results showed that all mutants had strongly increased xylitol production from pure D-xylose and several feedstocks compared to the reference strain, but not from all the tested feedstocks. This indicates that metabolic engineering is an effective strategy for the improvement of xylitol production by *A. niger*. It also emphasizes that a careful selection and composition analysis of the substrate is crucial for xylitol production.

The first step of D-xylose utilization is the uptake of D-xylose into the microbial cells. Therefore, increasing D-xylose uptake efficiency in fungal cell factories by manipulation of D-xylose transporters is likely to be of major importance. In **Chapter 3**, a novel D-xylose transporter in *A. niger*, XltD (NRRL3_02351), was identified, and studied together with two previously described D-xylose transporters XltA and XltB. The results demonstrated that XltA and XltD play an important role in D-xylose transport, while XltB does not or only minimally contribute to it. Manipulation of XltA and XltD may present a promising approach to promote D-xylose utilization and improve the production of desired products from lignocellulosic biomass.

L-arabitol occurs intracellularly as an intermediate in the fungal PCP, but is rarely found in high amounts in nature. All metabolic mutants constructed in **Chapter 2** accumulated and secreted xylitol and L-arabitol simultaneously from plant biomass, demonstrating that the capacity of *A. niger* to take up and secrete L-arabitol and the existence of L-arabitol transporters in this fungus. In **Chapter 4**, a L-arabitol transporter, LatA, was identified with high specificity for L-arabitol and it was suggested to be the main L-arabitol transporter in *A. niger*. The results also showed that the deletion of *latA* positively affected L-arabitol production from wheat bran and sugar beet pulp in *A. niger*, indicating that this gene could be a target for strain engineering to generate polyol cell factories. It also showed that LatA homologs are widely present in fungi, providing many candidate polyol transporters for further characterization in other fungi.

A. nidulans is a well-known model species for fundamental fungal research with also a high industrial potential. D-Galactose catabolism and its regulation have been studied previously in *A. nidulans*, but several aspects of its utilization remained unclear, such as the alternative D-galactose

pathways and additional transcription factors involved in the regulation. Two transcription factors, GalR and GalX, control D-galactose catabolism in *A. nidulans*, while the arabinanolytic regulator AraR regulates L-arabinose catabolism. As D-galactose and L-arabinose commonly occur together in plant biomass polysaccharides, the catabolic pathways of D-galactose and L-arabinose are also likely to be active simultaneously. In **Chapter 5**, the interaction between the transcription factors GalX, GalR and AraR in D-galactose and L-arabinose catabolism was investigated, indicating that these regulators all control D-galactose catabolism in *A. nidulans*. The results revealed that the regulation of D-galactose catabolism is highly complex and at least four regulators, GalR, GalX, XlnR, AraR, are involved in it. The compensation phenomenon between different regulators was also confirmed, enabling the fungus to quickly respond to changes in the availability of carbon sources. These results deepen the understanding of the complex regulatory network of filamentous fungi for plant biomass utilization and sugar catabolism and promote the development of efficient microbial cell factories for industrial applications.

The results of the different chapters are summarized and discussed in **Chapter 6**.

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CHAPTER 1

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Chapter 2

Xylitol production from plant biomass by *Aspergillus niger* through metabolic engineering

This chapter was published in *Bioresource Technology*

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Volume 344, 2022, 126199

<https://doi.org/10.1016/j.biortech.2021.126199>

Abstract

Xylitol is widely used in the food and pharmaceutical industries as a valuable commodity product. Biotechnological production of xylitol from lignocellulosic biomass by microorganisms is a promising alternative option to chemical synthesis or bioconversion from D-xylose. In this study, four metabolic mutants of *Aspergillus niger* were constructed and evaluated for xylitol accumulation from D-xylose and lignocellulosic biomass. All mutants had strongly increased xylitol production from pure D-xylose, beechwood xylan, wheat bran and cotton seed hulls compared to the reference strain, but not from several other feed stocks. The triple mutant $\Delta ladA \Delta xdhA \Delta sdhA$ showed the best performance in xylitol production from wheat bran and cotton seed hulls. This study demonstrated the large potential of *A. niger* for xylitol production directly from lignocellulosic biomass by metabolic engineering.

1. Introduction

Xylitol is a five-carbon sugar alcohol and an alternative of sucrose with equivalent sweetness, lower calories and insulin-independent metabolism, which makes it an appropriate sweetener for diabetic patients. Xylitol has also been widely used in toothpastes, chewing gums, confectionery and cosmetics because of its inhibitory effect of dental caries and humectant properties. Besides, it has important application potential in the pharmaceutical industry for treating or preventing acute otitis media, osteoporosis, respiratory infections and inflammatory procedures [1,2]. Industrially, xylitol is produced by catalytic hydrogenation of purified D-xylose from hemicellulose in the presence of Raney nickel, but this process is expensive and environmentally harmful [2]. The biotechnological xylitol production from lignocelluloses in an environmentally friendly way is a promising alternative option to the chemical route and has been studied for decades [3].

To date, some bacteria and fungi have been reported that can convert D-xylose to xylitol. Yeasts are the best strains for xylitol fermentation because of their high pentose assimilation rates and xylitol productivity [4]. *Candida* sp., such as *Candida tropicalis*, *Candida intermedia* and *Candida guilliermondii*, have been extensively studied for xylitol production with high conversion efficiency, as they are native pentose consumers and can maintain equilibrium of oxidation-reduction during xylitol accumulation [5–7]. Other yeasts that have been explored are *Pichia stipitis* [8,9], *Kluyveromyces marxianus* [10] and *Saccharomyces cerevisiae* [11,12]. Several bacteria have the natural ability to synthesize xylitol, but there are only a few efforts on using bacteria for xylitol production, e.g. with *Escherichia coli* [13]. Filamentous fungi, such as *Aspergillus oryzae* and *Trichoderma reesei*, are also studied for xylitol production using D-xylose or xylan as substrates, but low yields have been obtained [1,14].

Currently, researchers mainly focus on lignocellulosic hydrolysates as raw materials for biotechnological xylitol production in yeasts [2]. However, lignocellulosic hydrolysates are obtained through a process involving pretreatment, hydrolysis and detoxification, which significantly increases the production costs. While most yeasts do not produce xylanolytic enzymes, the interest in filamentous fungi as xylitol producers results from their production of a xylanolytic enzyme complex, which releases D-xylose from xylan-rich lignocellulosic biomass, with subsequent xylitol production performed by the same fungi [15,16].

Aspergillus niger is the most commonly used fungal species in industrial applications for producing various extracellular enzymes (e.g., amylases and pectinases) and organic acids, including the well-known citric acid. It is also considered generally recognized as safe (GRAS) by the United States Food and Drug Administration (USFDA), and has been a cell factory for expressing homologous or heterologous proteins for decades [17]. The sugar-specific and central metabolic pathways in *A. niger* have been described, in which the released monosaccharides from biomass are converted to energy or biomolecules [18]. Besides, a network of regulators has been identified in this species, that not only controls the extracellular enzymes involved in biomass degradation, but also the metabolic pathways that convert the resulting sugars [19]. *A. niger* has a gold-standard genome sequence and the CRISPR/Cas9 system has been successfully applied in it for gene manipulation, which both have a great effect on genetic engineering for strain improvement [20,21]. Therefore, *A. niger* is a highly promising candidate to construct strains for direct xylitol production from lignocellulosic biomass. In *A. niger*, D-xylose and L-arabinose are both converted to xylitol through the pentose catabolic pathway (PCP). D-xylose and L-arabinose are converted to xylitol and L-arabitol, respectively, by D-xylose reductase (XyrA, XyrB) and L-arabinose reductase (LarA) (Fig. 1). L-arabitol is subsequently converted in three steps to xylitol. Subsequently, xylitol is converted to D-xylulose by xylitol dehydrogenase (XdhA) and D-xylulose is phosphorylated to D-xylulose-5-phosphate by D-xylulose kinase (XkiA), which then enters the pentose phosphate pathway (PPP) [18]. Recent studies demonstrated that the PCP is more complex than was previously assumed, with several enzymes involved in each metabolic step ([22]; Fig. 1). Two transcription factors, XlnR and AraR,

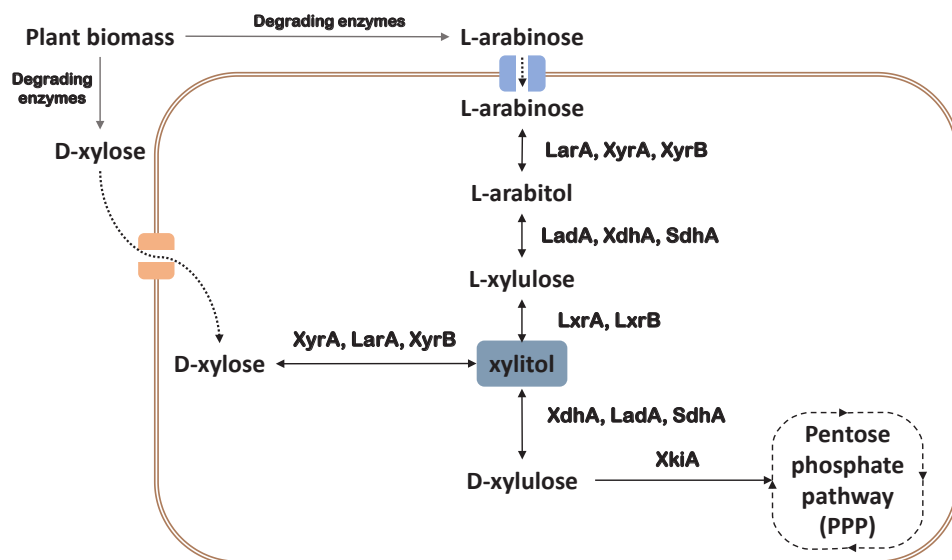


Figure 1. Utilization of D-xylose and L-arabinose by *A. niger* [22]. LarA = L-arabinose reductase, LadA = L-arabitol dehydrogenase, LxrA/LxrB = L-xylulose reductases, XyrA/XyrB = D-xylose reductases, SdhA = sorbitol dehydrogenase, XdhA = xylitol dehydrogenase, XkiA = D-xylulose kinase.

control the PCP and the production of lignocellulose degrading enzymes, such as xylanolytic enzymes [23].

Metabolic engineering is a promising strategy for improving xylitol production from raw materials to fulfill the industrial demand, such as increasing expression of D-xylose reductase and reducing xylitol dehydrogenase expression/activity [24–28]. The activity of D-xylose reductase can be improved by enhancing the expression level of the native D-xylose reductase or constitutively expressing heterologous D-xylose reductase encoding genes for improving the conversion of D-xylose to xylitol and subsequent xylitol productivity [2,29]. The function of xylitol dehydrogenase can be restricted partially or completely for blocking the synthesis of D-xylulose from xylitol and thus improving the accumulation of xylitol [2,30,31]. In *A. niger*, XdhA, LadA and SdhA (sorbitol dehydrogenase) are all involved in the conversion of xylitol to D-xylulose, while XkiA converts D-xylulose to xylulose-5-phosphate ([22]; Fig. 1). Deletion of *xkiA* and *ladA/xdhA/sdhA* in *A. niger* N593 $\Delta ku70$ to generate the $\Delta xkiA$ and $\Delta ladA\Delta xdhA\Delta sdhA$ mutants resulted in blocking the conversion of D-xylulose to xylulose-5-phosphate ([22]; Fig. 1). Deletion of *xkiA* and *ladA/xdhA/sdhA* in *A. niger* N593 $\Delta ku70$ to generate the $\Delta xkiA$ and $\Delta ladA\Delta xdhA\Delta sdhA$ mutants resulted in blocking the conversion of D-xylulose to xylulose-5-phosphate ([22]; Fig. 1). When grown on D-xylose/L-arabinose, $\Delta xkiA$ accumulated intracellularly similar levels of xylitol and L-arabitol (both around 5 mM). Accumulation of xylitol on D-xylose reached a similar level as accumulation of L-arabitol on L-arabinose (both around 20 mM) in $\Delta ladA\Delta xdhA\Delta sdhA$ and these levels were both higher than those observed in single or double deletion mutants of *ladA/xdhA/sdhA* [22]. L-xylulose reductases LxrA and LxrB hydrolyze the conversion of L-xylulose to xylitol in L-arabinose catabolism. The single deletion of *lxrA* or *lxrB* resulted in accumulation of xylitol or L-arabitol on D-xylose or L-arabinose, respectively, and the level of L-arabitol on L-arabinose was 2-fold higher in the double deletion mutant than in single mutants [22]. These results demonstrated that blocking specific steps in the PCP can accumulate upstream intermediates.

In this study, *A. niger* strains were constructed to produce xylitol from D-xylose and lignocellulosic biomass through metabolic engineering. In addition, xylitol production from different feed stocks was evaluated and related this to their sugar composition.

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

| Strains | CBS number | Genotype | Reference |
|---|------------|--|------------|
| N593 $\Delta ku70$ | CBS 138852 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] | [50] |
| $\Delta xkiA$ | CBS 144042 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] , <i>xkiA</i> [−] | [22] |
| $\Delta ladA\Delta xdhA\Delta sdhA$ | CBS 144672 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] , <i>ladA</i> [−] , <i>xdhA</i> [−] , <i>sdhA</i> [−] | [22] |
| $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$ | CBS 145865 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] , <i>lxrA</i> [−] , <i>lxrB</i> [−] , <i>ladA</i> [−] , <i>xdhA</i> [−] , <i>sdhA</i> [−] | [22] |
| $\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$ | CBS 147731 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] , <i>ladA</i> [−] , <i>xdhA</i> [−] , <i>xkiA</i> [−] , <i>sdhA</i> [−] | This study |

2. Materials and methods

2.1. Strains, media, and growth conditions

A. niger strains used in this study are shown in Table 1. The uridine auxotrophic and non-homologous end-joining (NHEJ) deficient *A. niger* strain N593 $\Delta ku70$ was used as the reference strain. *A. niger* mutants were generated using CRISPR/Cas9 genome editing [32]. The primers and plasmids used for the creation of the mutants are listed in a previous study [22]. *A. niger* protoplasting and transformation were performed as described previously [33]. All *A. niger* strains were grown at 30°C on Complete Medium (CM) or Minimal Medium (MM) supplemented with required carbon source [34]. For plate cultivations, 1.5% (w/v) agar was added and 1.22 g/L uridine was supplemented for auxotrophic strains. 0.5 gram lignocellulosic substrates were added into 50 ml MM in each flask and autoclaved for transfer experiments.

2.2. Transfer experiments

All *A. niger* strains were grown on CM plates at 30°C for 5 days. Spores were harvested in ACES buffer and were counted using a haemocytometer. 10⁶ spores/mL were inoculated to 250 mL CM with 2% D-fructose in 1 L Erlenmeyer flasks for precultures and incubated in rotary shakers at 30°C and 250 rpm for 16–18 hours. The mycelia were harvested by filtration on Miracloth under sterile conditions and washed with MM. Equal amounts of mycelia were transferred to 50 mL MM in 250 mL Erlenmeyer flasks containing 25 mM D-xylose, 1% D-xylose, 2% D-xylose, 1% beechwood xylan or 1% wheat bran, and were incubated in rotary shakers at 30°C, 250 rpm. The transfer experiments were performed in triplicate. 2 mL cultures were harvested after 0, 4, 8, 24, 32, 48, 56, 72 and 80 hours, and supernatants were stored after centrifugation for measurement of extracellular monosaccharides. For the comparison of the xylitol production on different biomass, the same approach was used, but samples were only harvested after 0, 4, 8, 24 and 48 hours.

2.3. Monosaccharide and polyol determination

The supernatants were heated at 95°C for 15 min and centrifuged for 5 min at 14000 rpm. The supernatants were 10-fold diluted with MilliQ water prior to analysis. Xylitol, L-arabitol and D-xylose were analyzed by HPLC (Dionex ICS-5000 + system; Thermo Scientific) equipped with CarboPac PA1 column (2 × 250 mm with 2 × 50 mm guard column; Thermo Scientific) as described

previously [35]. 5-250 μM xylitol, L-arabitol and D-xylose were used as standards for identification and quantitation.

2.4. Starch determination in plant biomass substrates

Starch content in the plant biomass substrates was determined with the total starch assay kit (Megazyme, Bray, Ireland) according to the suppliers' instructions.

2.5. Statistical analysis

Statistical significance was determined using a two-sample equal variance t-test with a two-tailed distribution by Microsoft Excel. The maximums of xylitol titer between two strains were compared based on biological triplicates and significant difference was identified with p -value < 0.05 .

3. Results and discussion

3.1. Improvement of xylitol accumulation by *A. niger* from D-xylose through metabolic engineering

A. niger is able to utilize D-xylose and L-arabinose through the PCP and xylitol is an intermediate product in this pathway (Fig. 1). In order to improve xylitol accumulation, *A. niger* metabolic mutant strains were generated by CRISRP/Cas9 genome engineering. In our previous study, three mutants, i.e. $\Delta xkiA$, $\Delta ladA\Delta xdhA\Delta sdhA$ and $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$, were generated and showed that combination of multiple deletions is able to effectively block pentose catabolism and subsequently accumulate pathway intermediates [22]. In this study, $\Delta xkiA$ and $\Delta ladA\Delta xdhA\Delta sdhA$ were combined resulting in the mutant $\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$, in which a higher level of xylitol or L-arabitol production was expected compared to $\Delta ladA\Delta xdhA\Delta sdhA$. Extracellular xylitol production of these four metabolic mutants from D-xylose and lignocellulosic biomass beechwood xylan and wheat bran was evaluated, and compared to the reference strain.

Firstly, the ability of *A. niger* to produce xylitol was evaluated from different concentrations of pure D-xylose, which is the most commonly used substrate for xylitol bioproduction (Fig. 2). The $\Delta xkiA$ mutant produced a maximum extracellular xylitol titer of 1.84 mM, 2.74 mM and 3.03 mM on 25 mM, 1% and 2% D-xylose, respectively (Fig. 2 d-f). These titers were 43, 30 and 19 times higher compared to the *A. niger* reference strain N593 $\Delta ku70$ that produced maximum xylitol titer of 0.04 mM, 0.09 mM and 0.16 mM during growth on 25 mM, 1% and 2% D-xylose, respectively (Fig. 2 a-c). The $\Delta ladA\Delta xdhA\Delta sdhA$ mutant accumulated maximum xylitol titer of 2.45 mM, 2.23 mM and 2.26 mM in the cultures with 25 mM, 1% and 2% D-xylose, respectively (Fig. 2 g-i), and showed the highest yield at 25 mM D-xylose (see Supplementary material). The intracellular xylitol accumulation in $\Delta ladA\Delta xdhA\Delta sdhA$ from 25 mM D-xylose was more than 2-fold higher than in $\Delta xkiA$ in the previous study [22], which was consistent with the lower extracellular xylitol accumulation by $\Delta xkiA$ from 25 mM D-xylose in this study. Maximum xylitol production by $\Delta xkiA$ increased with increasing D-xylose concentrations and was slightly higher than that observed for $\Delta ladA\Delta xdhA\Delta sdhA$ on 1% and 2% D-xylose. The combined mutants $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$ and $\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$ produced a similar amount of xylitol from D-xylose as the triple mutant $\Delta ladA\Delta xdhA\Delta sdhA$ (Fig. 2 j-o). Other studies have supplemented D-xylose cultures with D-glucose or glycerol as co-substrates for cell growth and fed-batch fermentation which could also result in higher xylitol production in *A. niger* [14,36], providing insights into further optimizing the level of xylitol. These results indicated the delicate balance that needs to be considered during metabolic engineering of the PCP.

The $\Delta xkiA$ strain produced a maximum extracellular L-arabitol titer of 1.19, 2.11 and 3.40 mM on

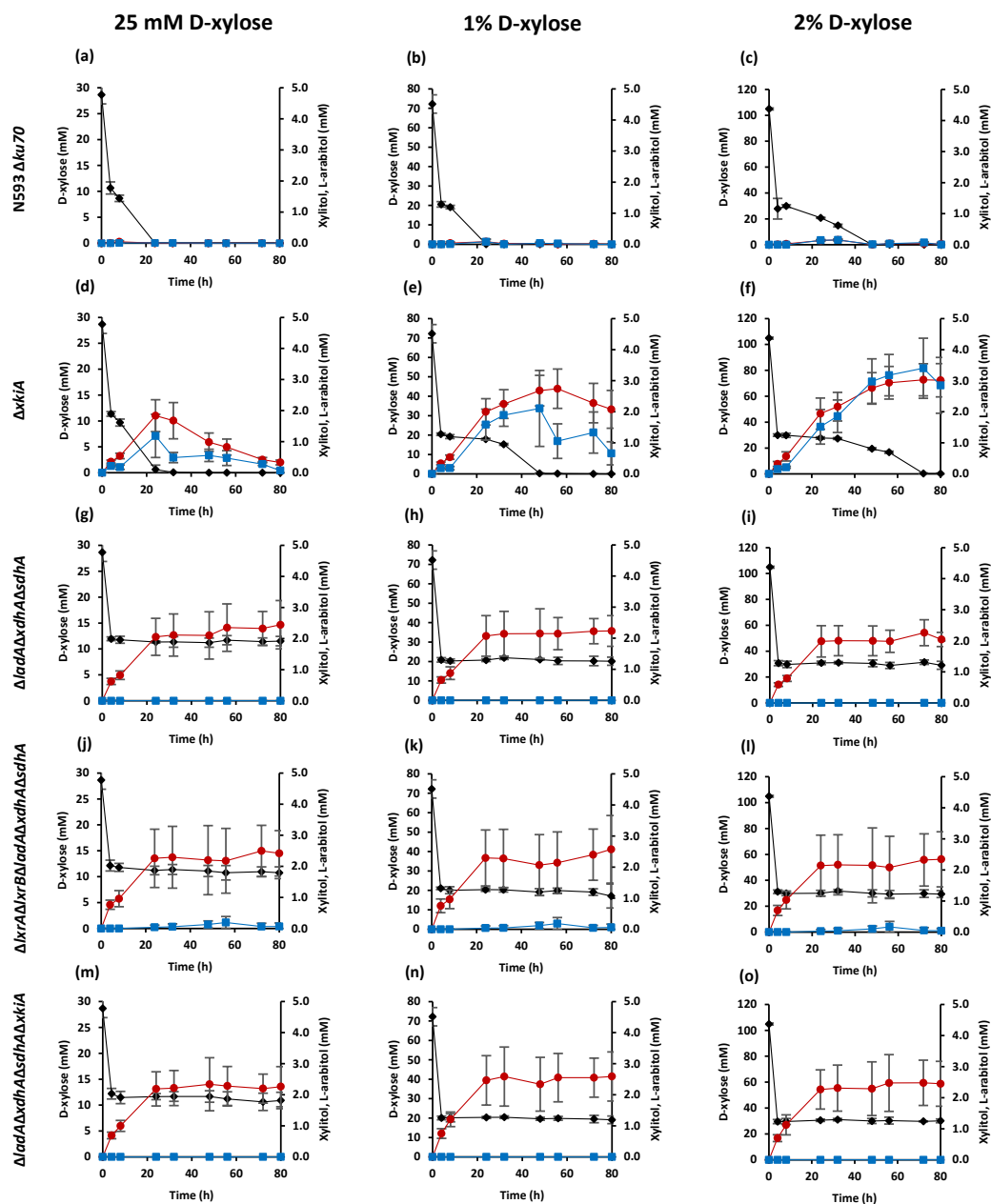


Figure 2. Xylitol production from D-xylose by *A. niger* reference strain (N593 $\Delta ku70$) and metabolic mutants. Strains and substrates are indicated in the figure. Concentration of xylitol (red circle), L-arabitol (blue square) and residual D-xylose (black diamond). The error bars indicate the standard deviation between biological triplicates.

25 mM, 1% and 2% D-xylose (Fig. 2 d-f), which was 132, 25 and 22 times higher, respectively, than detected in the cultures of the reference strain (Fig. 2 a-c). The reversible conversions of xylitol to L-xylulose and L-xylulose to L-arabitol were not blocked in the reference strain and $\Delta xkiA$, which contributed to the accumulation of L-arabitol. Interestingly, a small amount of L-arabitol was

detected in the culture liquids of the $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$ strain after 24 hours, which was higher than the level observed in $\Delta ladA\Delta xdhA\Delta sdhA$ and $\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$ (Fig. 2 g-o). In the previous study, $\Delta lxrA\Delta lxrB$ resulted in reduced growth on L-arabinose and L-arabitol, instead of abolished growth, and still possessed residual LXR activity, indicating that additional enzymes are involved in this reversible metabolic step [22]. This may be an explanation for the conversion of xylitol to L-xylulose in $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$. Growth of $\Delta ladA\Delta xdhA\Delta sdhA$ was completely abolished on both pentoses and intermediates [22], suggesting that there are no other enzymes involved in the conversion of L-xylulose to L-arabitol under these growth conditions. Another possible reason for the accumulation of L-arabitol is that xylitol is converted to L-arabitol by unknown enzymes or a different pathway.

Both $\Delta xkiA$ and the reference strain consumed almost all D-xylose during the cultivation (Fig. 2 a-f), while there was a significant level of residual D-xylose in the cultures of $\Delta ladA\Delta xdhA\Delta sdhA$, $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$ and $\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$, indicating that they cannot take up and consume more D-xylose after 4 hours of cultivation (Fig. 2 g-o). A possible reason is that increased intracellular D-xylose or xylitol levels result in feedback inhibition of the PCP enzymes or the expression of their corresponding genes, which subsequently inactivated D-xylose uptake. Therefore, relieving feedback inhibition by substantial efflux could be a potential way for the further improvement of xylitol bioproduction [37]. Alternatively, high intracellular D-xylose levels could be due to an insufficient conversion of D-xylose to xylitol and therefore negatively affect D-xylose import directly.

As xylitol production of the four mutants was significantly higher than that of the reference strain, these results demonstrated that metabolic engineering is an effective strategy for the improvement of xylitol production by *A. niger*.

3.2. Xylitol accumulation from beechwood xylan and wheat bran

D-xylose is not an ideal substrate for biotechnological production of xylitol because of its high costs. Therefore, bioproduction of xylitol directly from two lignocellulosic biomass substrates, beechwood xylan and wheat bran, was evaluated to confirm the capacity of *A. niger* mutants on more complex substrates (Fig. 3). The maximum xylitol titers from beechwood xylan of four metabolic mutants were 1.97, 2.20, 2.19 and 2.24 mM (Fig. 3 c, e, g, i, respectively), which were significantly higher than that of the reference strain (0.058 mM, Fig. 3 a). The maximum xylitol titer from beechwood xylan produced by metabolic mutants was around 0.34 g/L, which was lower than xylitol production achieved by yeasts [38,39]. However, the *A. niger* strain used in our study is the common laboratory strain, and further optimization could be achieved by improving its ability to release xylose from wheat bran or by selecting another *A. niger* strain with higher basal production levels. It is worth noting that the xylitol concentration of $\Delta xkiA$ reduced after 48 hours of cultivation, suggesting that this strain eventually is able to take up and metabolize xylitol, although which enzymes are involved in this conversion or to which compounds it is converted is currently unknown. L-arabitol was also produced by all strains during growth on beechwood xylan, and the highest production of 1.52 mM was detected by $\Delta xkiA$ after 32 hours. The low content of L-arabinose in beechwood xylan likely explains the lower accumulation of L-arabitol than xylitol from beechwood xylan, by the mutant strains. The reference strain and $\Delta xkiA$ consumed or absorbed almost all the released D-xylose, while the other three metabolic mutants accumulated D-xylose in the media possibly because of feedback inhibition of intracellular D-xylose or xylitol, as mentioned previously.

When wheat bran was used as the substrate, the reference strain did not produce any xylitol in the cultures (Fig. 3 b), suggesting that all released pentose was fully metabolized. Three of the metabolic mutants, except $\Delta xkiA$, produced approximately 2 mM xylitol (Fig. 3 d, f, h, j). Surprisingly, xylitol levels of $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$ and $\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$ reduced after 56 hours of cultivation (Fig. 3 h, j). This effect was even stronger in $\Delta xkiA$, where xylitol levels in the medium

1% beechwood xylan

1% wheat bran

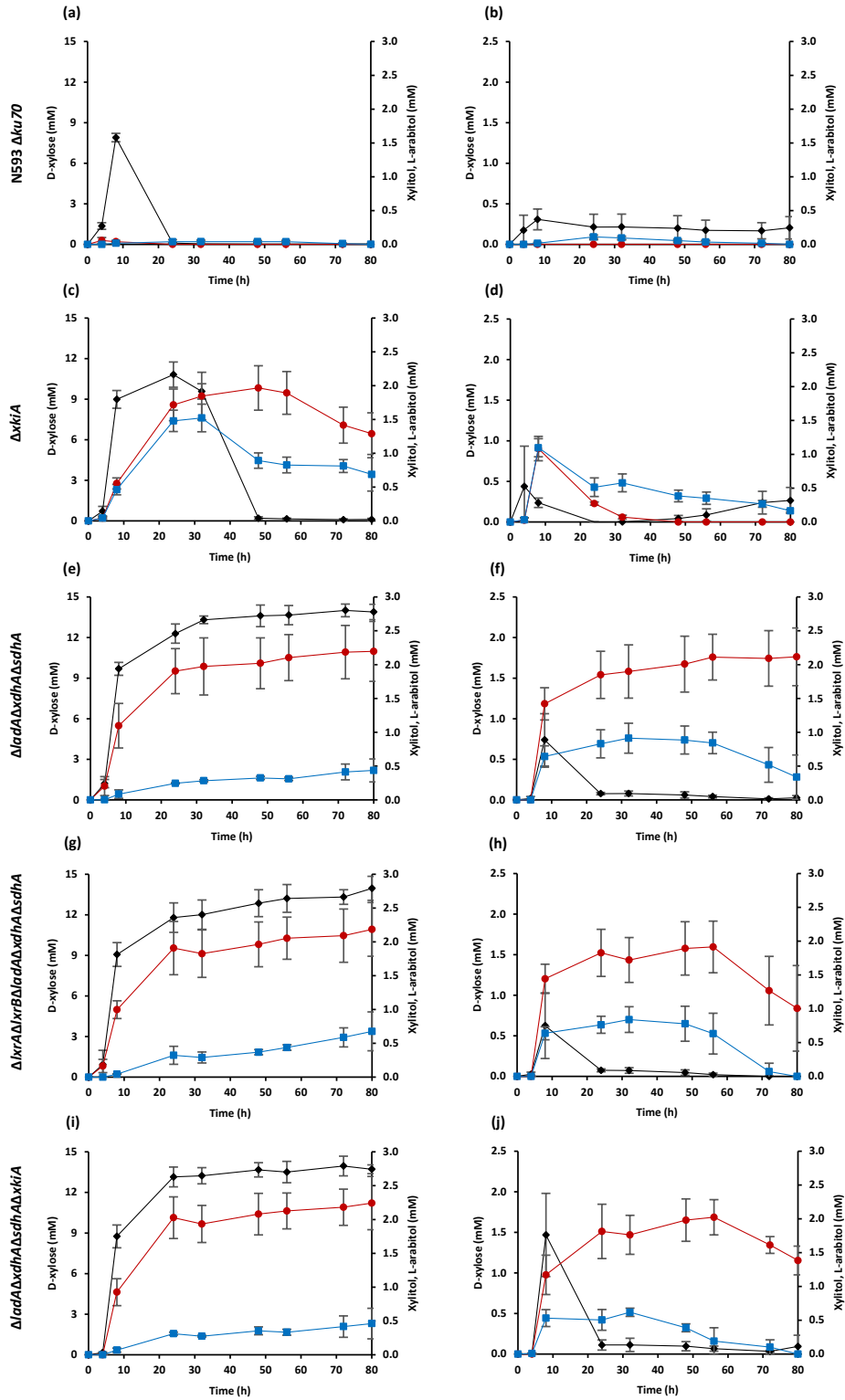


Figure 3. Xylitol production from beechwood xylan and wheat bran by *A. niger* reference strain (N593 $\Delta ku70$) and metabolic mutants. Strains and substrates are indicated in the figure. Concentration of xylitol (red circle), L-arabitol (blue square) and residual D-xylose (black diamond). The error bars indicate the standard deviation between biological triplicates.

reduced to zero after 48 hours of cultivation (Fig. 3 d), in contrast to the good performance of this strain on pure D-xylose (Fig. 2 d, e, f). Moreover, the xylitol titer of $\Delta ladA\Delta xdhA\Delta sdhA$ had no significant difference after 48 hours of cultivation (Fig. 3 f), so 48 hours could be the true fermentation time for industrial production. The xylitol titer from wheat bran by these mutants was lower than in other published *A. niger* strains, but those studies used a hydrolysis pre-treatment of wheat bran, which simplifies xylitol production for industrial application [40]. There was a certain amount of L-arabitol that accumulated in the media by all strains, but it was much lower than the xylitol concentration, except for the reference strain and $\Delta xkiA$ (Fig. 3 b, d). Nearly all released D-xylose was converted by $\Delta ladA\Delta xdhA\Delta sdhA$, $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$ and $\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$ (Fig. 3 f, h, j), but there was a small amount of D-xylose (around 0.2 mM) that accumulated in the medium of the reference strain and $\Delta xkiA$ (Fig. 3 b, d).

These results show that all *A. niger* metabolic mutants can accumulate xylitol from beechwood xylan and wheat bran, although this is a transient situation for some mutants, and that $\Delta ladA\Delta xdhA\Delta sdhA$ strain was the best xylitol producing strain under these conditions. Interestingly, the yield of xylitol on wheat bran was similar to that on 25 mM D-xylose for $\Delta ladA\Delta xdhA\Delta sdhA$ (see Supplementary material), suggesting that a gradual release of a lower level of D-xylose is most efficient for xylitol production.

3.3. Xylitol accumulation by *A. niger* from diverse lignocellulosic biomass

As the composition of different lignocellulosic biomass vary significantly, the effects of these differences on xylitol production by our *A. niger* mutants were evaluated. Sugar beet pulp, corn stover, wheat straw, rice bran, sugarcane bagasse and cotton seed hulls were selected for the comparison, based on the diversity of their composition (Table 2). Xylitol production by the *A. niger* reference strain, $\Delta xkiA$ and $\Delta ladA\Delta xdhA\Delta sdhA$ was analyzed, as the other two mutants did not seem to differ significantly from $\Delta ladA\Delta xdhA\Delta sdhA$. In *A. niger*, D-xylose is an inducer of (hemi-)cellulolytic transcriptional activator XlnR that controls the expression of genes encoding xylanases and enzymes of D-xylose catabolism, and a strong induction is already observed at low concentrations [41,42]. Therefore, 2 mM D-xylose was also added into the media with 1% wheat bran to see if this would accelerate the induction of XlnR and improve degradation of wheat bran.

The highest xylitol titers (1.49, 1.69 and 1.70 mM) were detected in $\Delta ladA\Delta xdhA\Delta sdhA$ after 48 hours of cultivation from 1% wheat bran, 1% wheat bran + 2 mM D-xylose and 1% cotton seed hulls, respectively (Fig 4. (c), (f), (x)). Xylitol could be detected in the culture with 2 mM D-xylose after 4 hours of cultivation, which was earlier than from the sole wheat bran (24 hours), showing that the addition of a low concentration of D-xylose is able to initiate the accumulation of xylitol at earlier time point. Xylitol was only detected in the culture liquids of $\Delta xkiA$ from 1% wheat bran, 1% wheat bran + 2 mM D-xylose, 1% sugar beet pulp and 1% cotton seed hulls, with the maximum xylitol titers of 0.20, 0.48, 0.37 and 0.10 mM, respectively (Fig 4. (b), (e), (h), (w)). However, xylitol titers were reduced to almost zero after 48 hours of cultivation. The reference strain did not produce any xylitol from the tested biomass (Fig 4. (a), (d), (g), (j), (m), (p), (s), (v)). Therefore, wheat bran and cotton seed hulls are the most promising biomass substrates for xylitol bioproduction under our conditions. A large amount of L-arabitol was observed in the culture of $\Delta ladA\Delta xdhA\Delta sdhA$ from 1% sugar beet pulp (2.08 mM, Fig 4. (i)), which is consistent with the high content of L-arabinose in sugar beet pulp (Table 2). L-arabitol was also accumulated by $\Delta ladA\Delta xdhA\Delta sdhA$ from 1% wheat bran, 1% wheat bran + 2 mM D-xylose and 1% cotton seed hulls, (Fig 4. (c), (f), (x)), but

Table 2. Composition of lignocellulosic biomass (in g/100 g substrate) used in this study.

| Biomass | D-xylose | L-arabinose | D-glucose | L-rhamnose | D-mannose | D-galactose | Uronic acid | Starch |
|-------------------|----------|-------------|-----------|------------|-----------|-------------|-------------|--------|
| Wheat bran | 21.4 | 14.4 | 28.5 | 0.0 | 0.1 | 0.7 | 0.0 | 5.9 |
| Sugar beet pulp | 1.1 | 13.8 | 18.6 | 0.8 | 1.2 | 3.8 | 16.7 | 0.1 |
| Corn stover | 19.0 | 2.5 | 35.6 | 0.2 | 0.5 | 1.1 | 3.0 | 0.1 |
| Wheat straw | 18.6 | 2.5 | 31.5 | 0.0 | 0.6 | 1.0 | 2.8 | 0.3 |
| Rice bran | 3.8 | 3.4 | 27.3 | 0.0 | 1.0 | 1.0 | 1.5 | 43.0 |
| Sugarcane bagasse | 21.4 | 1.7 | 39.1 | 0.3 | 0.4 | 0.6 | 2.6 | 0.1 |
| Cotton seed hulls | 21.8 | 0.9 | 19.0 | 0.3 | 0.0 | 0.6 | 4.4 | 0.2 |

at significantly lower levels than the levels of xylitol from the same cultures. In contrast, only low levels of L-arabitol were observed in $\Delta xkiA$, suggesting that this mutant can adapt over time to bypass the block at the end of the PCP.

Initially, D-xylose accumulated in the wheat bran and cotton seed hulls cultures (Fig 4. (a-f), (v-x)), which led to the high accumulation of xylitol by $\Delta ladA\Delta xdhA\Delta sdhA$ from these biomass substrates at later time points. A small amount of L-arabinose was released from wheat bran and sugar beet pulp (Fig 4. (a-i)), which resulted from the higher content of L-arabinose of these substrates (Table 2). In addition, a significant level of D-glucose accumulated from rice bran at early time points (Fig 4. (p-r)), which is in line with the fact that rice bran contains the highest level of D-glucose among all biomass substrates that were selected (Table 2). Accumulation of D-glucose was also detected from other biomass, especially wheat bran (Fig 4. (a-f)). The lower levels of D-glucose in wheat bran and cotton seed hulls (Table 2) resulted in higher xylitol titers. As D-glucose is a preferred carbon source for *A. niger* that consumes sugars in a sequential manner [43], D-glucose could inhibit the use of D-xylose and subsequently affect the xylitol production. D-glucose can originate from either cellulose or starch in the biomass substrates. It was shown previously that cellulose is the least preferred substrate for *A. niger* [44], so it could be expected that the D-glucose in our cultures originates from starch rather than cellulose. Therefore, the amount of starch (normal and resistant) was determined in these substrates (Table 2), which revealed that rice bran resulting in the highest extracellular D-glucose levels was also highest in starch. As no xylitol production was observed on these substrates, this suggests that in the presence of sufficient amount of starch, *A. niger* does not degrade hemicellulose to a level that xylitol accumulates. Whether this is due to repression by the free D-glucose levels, mediated by CreA [45], or whether the starch-related regulator AmyR [46] suppresses hemicellulose degradation remains to be studied.

These results showed that the composition of the biomass substrates strongly affects the production of xylitol in *A. niger* and that the D-xylose amount in the substrate alone does not guarantee xylitol production. Therefore, the choice of lignocellulosic biomass together with the optimally engineered strain is a primary determinant for xylitol bioproduction.

Further optimization of the xylitol levels is possible using a number of approaches. Two transcription factors, XlnR and AraR, control the expression of the main hemicellulolytic enzymes and PCP pathway [47,48]. Constitutively active XlnR leads to increased production of plant biomass degrading enzymes, and D-xylose and L-arabinose release from wheat bran [44]. The constitutively active AraR has also been shown to result in constitutive production of arabinanases under derepressing conditions [49]. Therefore, these two regulators could be manipulated to increase the production of plant biomass degrading enzymes, and subsequently the release of D-xylose and L-arabinose from

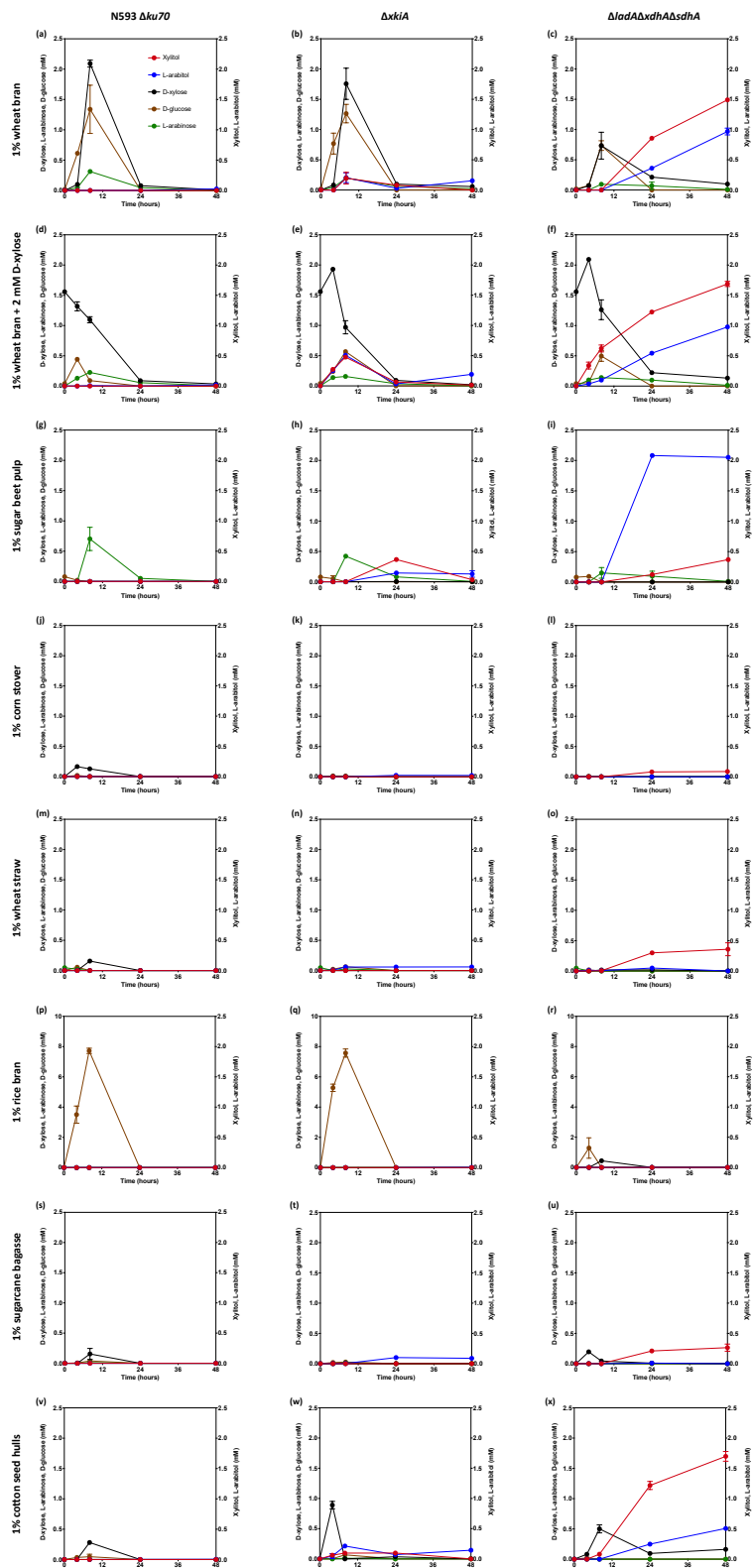


Figure 4. Xylitol production from diverse lignocellulosic biomass by *A. niger* reference strain (N593 $\Delta ku70$), $\Delta xkiA$ and $\Delta ladA\Delta xdhA\Delta sdhA$. Strains and substrates are indicated in the figure. The error bars indicate the standard deviation between biological triplicates.

wheat bran to improve xylitol production. In addition, improvement of D-xylose reductase levels and D-xylose uptake may further stimulate xylitol production. Finally, as this study was performed in the commonly used *A. niger* laboratory strain, screening of other *A. niger* isolates for xylitol accumulation will likely identify other strain lineages with a higher industrial potential.

4. Conclusion

Direct bioproduction of xylitol from lignocellulosic biomass is a highly attractive alternative to chemical synthesis or conversion from pure D-xylose, which was obtained by hydrolysis of lignocellulose. In this study, metabolic engineering of *A. niger* resulted in xylitol production from biomass substrates. It was also demonstrated that the composition of the biomass substrate strongly affects xylitol levels. While the amount of D-xylose in the substrate has a positive effect on xylitol production, large amounts of starch reduce D-xylose release and conversion, indicating that a careful selection and composition analysis of the substrate is crucial for this process.

CRedit authorship contribution statement

Jiali Meng: Funding acquisition, Investigation, Data curation, Writing-original draft. **Tania Chroumpi:** Investigation, Supervision. **Miia R. Mäkelä:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision. **Ronald P. de Vries:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

Dr. Adiphol Dilokpimol is acknowledged for help in the measurement of monosaccharides by HPLC and Kimberly de Vries for the starch determination in biomass substrates.

Funding

This work was supported by the China Scholarship Council (CSC student number: CSC201907720027 to JM) and the Academy of Finland (grant no. 308284 to MRM).

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Supporting information

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2021.126199>.

Table S1. Conversion of D-xylose into xylitol in the engineered *A. niger* strains.

Chapter 3

Identification of a fourth D-xylose transporter XltD in *Aspergillus niger*

This chapter is in preparation for publication

Jiali Meng, Vivien Bíró, Alexandra Márton, Erzsébet Fekete, Levente Karaffa, Miia
R. Mäkelä, Ronald P. de Vries

Abstract

The production of biofuels and chemicals from D-xylose is a promising option as D-xylose is the second most abundant sugar after D-glucose in lignocellulosic biomass. In microbes, efficient D-xylose uptake is a prerequisite for its utilization. Therefore, increasing D-xylose uptake efficiency in fungal cell factories by manipulation of D-xylose transporters would be an attractive strategy to improve fungal cell factories that use D-xylose as a substrate. In this study, we identified a fourth D-xylose transporter, XltD (NRRL3_02351), in *Aspergillus niger*.

The influence of deletion of three D-xylose transporters, XltA, XltB and XltD, on overall D-xylose uptake was investigated. The results demonstrated that XltA and XltD play an important role in D-xylose transport, while XltB does not or only minimally contribute to it. Improving the expression of *xltD* and *xltA* is therefore suggested as a promising target for strain engineering in *A. niger*.

1. Introduction

D-xylose is the second most abundant sugar after D-glucose in nature and a main component of hemicelluloses in lignocellulosic biomass, especially in xylan. Therefore, D-xylose is an attractive substrate for biofuel and biochemical production in industry [1]. The overall process of biofuel and biochemical production from lignocellulosic biomass by microorganisms includes the release of monomeric sugars (mostly D-glucose and D-xylose) from pretreated biomass and the microbial fermentation of sugars to desired end products. Sugar uptake by microbial transporters is likely to be a key rate determining step in the utilization of the released sugars. Increasing D-xylose uptake efficiency is therefore crucial to improve microbial fermentation and may be a promising strategy for developing robust microbial cell factories for D-xylose conversion [2]. High-affinity sugar transporters function at low sugar concentrations, while low-affinity sugar transporters work at high sugar concentrations. Therefore, selection of sugar transporters for manipulation depends on the affinities of the transporters and sugar concentrations in the microbial fermentation.

Only a few native D-xylose transporters have been identified and characterized in bacteria and yeasts with highly diverse affinity for D-xylose (Table 1). *Escherichia coli* has been shown to possess two different D-xylose-specific transport systems, including the major facilitator superfamily (MFS) protein XylE and the ATP binding cassette (ABC) transporter XylFGH [3,4]. XylE is a relatively low-affinity D-xylose/proton symporter, while the proteins XylF, XylG, and XylH comprise a high-affinity D-xylose transporter [5]. The highly reduced growth of the *xylG* deletion mutant compared to the *xylE* deletion mutant on D-xylose has showed that the XylFGH system is dominant for D-xylose uptake in *E. coli* [6]. The expression of XylE or XylFGH improved the rate of D-xylose utilization in the bacterium *Zymomonas mobilis* compared to the control strain [7,8]. In addition, two D-glucose/D-xylose transporters from the yeast *Candida intermedia* (Gfx1, GXS1) have been characterized [9] (Table 1), while three D-glucose transporters (Sut1, Sut2, Sut3) of the yeast *Pichia stipitis* are also able to transport D-xylose, but with a considerably lower affinity than that observed for D-glucose [10]. Heterologous expression of Gfx1 and Sut1 in *Saccharomyces cerevisiae* led to significantly improved D-xylose utilization and ethanol production, respectively [11,12]. In addition, a D-xylose specific facilitator (Xyp29) has been identified from *P. stipitis* [13].

Several transporters with D-xylose transport capacity from filamentous fungi have also been described (Table 1). In *Neurospora crassa*, three D-xylose transporters have been reported, An25 [13], and XAT-1 and XYT-1 [14]. XAT-1 can transport both D-xylose and L-arabinose, while XYT-1 can only transport D-xylose. XLT1 from *Trichoderma reesei* [15] is a high-affinity L-arabinose symporter, with low affinity for D-xylose [16]. Another transporter, Str1, is involved in the utilization of diverse carbon sources in *T. reesei*, and is essential for pentose and pentitol utilization [17], despite having a higher affinity for D-glucose (0.01 ± 0.00 mM) [18]. The MFS transporter XtrD from *Aspergillus nidulans* uses multiple sugars as a substrate, such as D-xylose, D-glucose, D-galactose and D-mannose [19], but has high affinity for D-xylose. A low-affinity D-glucose transporter, HxtB, has also been shown to play a major role in D-xylose transport in *A. nidulans* [20,21].

Aspergillus niger is a major fungal cell factory for the industrial production of organic acids, particularly citric acid, and industrially relevant enzymes [22,23]. Recently, three candidate D-xylose transporters from *A. niger* (Table 1) were functionally validated and biochemically characterized in *S. cerevisiae* [18]. XltA can transport various sugars and showed a very high affinity for D-xylose, while XltB was suggested to be a specific low affinity D-xylose transporter. The affinity of XltC towards D-glucose was approx. 50 times higher than towards D-xylose, suggesting that mainly XltA and XltB have a biological role in D-xylose uptake in *A. niger*.

Our previous study for xylitol production from lignocellulosic biomass showed that the concentration of released D-xylose from biomass was low in the *A. niger* cultures (0 - 2 mM) [24], suggesting that high-affinity D-xylose transporters would play a leading role in taking up D-xylose into *A. niger* cells. In this study, three candidate high-affinity D-xylose transporters were selected, namely XltA

Table 1. Biochemically characterized sugar transporters involved in D-xylose utilization in bacteria and fungi. MFS and ABC transporters represent the major facilitator superfamily and the ATP binding cassette transporters, respectively. The K_m for D-xylose of all transporters except for XylE and XylFGH is determined in *S. cerevisiae*.

| Transporter | Species | Type of transporter | K_m for D-xylose | Reference |
|-------------|-----------------------------|---------------------|---------------------|-----------|
| XylE | <i>Escherichia coli</i> | MFS | 63 - 169 μ M | [3,4,5] |
| XylFGH | <i>E. coli</i> | ABC | 0.2 - 4 μ M | [3,5] |
| Gxf1 | <i>Candida intermedia</i> | MFS | Approx. 50 mM | [9] |
| Gxs1 | <i>C. intermedia</i> | MFS | Approx. 0.4 mM | [9] |
| Sut1 | <i>Pichia stipitis</i> | MFS | 145.0 \pm 1.0 mM | [10] |
| Sut2 | <i>P. stipitis</i> | MFS | 49.0 \pm 1.0 mM | [10] |
| Sut3 | <i>P. stipitis</i> | MFS | 103.0 \pm 3.0 mM | [10] |
| Xyp29 | <i>P. stipitis</i> | MFS | 56 mM | [13] |
| An25 | <i>Neurospora crassa</i> | MFS | 175.7 \pm 21.4 mM | [13] |
| XAT-1 | <i>N. crassa</i> | MFS | 18.17 \pm 3.23 mM | [14] |
| XYT-1 | <i>N. crassa</i> | MFS | 7.58 \pm 0.60 mM | [14] |
| XLT1 | <i>Trichoderma reesei</i> | MFS | Approx. 9 mM | [15,16] |
| Str1 | <i>T. reesei</i> | MFS | 5.70 \pm 0.19 mM | [17,18] |
| XtrD | <i>Aspergillus nidulans</i> | MFS | nd ¹ | [19] |
| HxtB | <i>A. nidulans</i> | MFS | nd ¹ | [20,21] |
| XltA | <i>Aspergillus niger</i> | MFS | 0.09 \pm 0.03 mM | [18] |
| XltB | <i>A. niger</i> | MFS | 15.0 \pm 4.50 mM | [18] |
| XltC | <i>A. niger</i> | MFS | 4.71 \pm 1.04 mM | [18] |

¹nd = not determined

and XltB and the *A. niger* ortholog (XltD, NRLL3_02351) of XAT-1 from *N. crassa*. The role of these three transporters in D-xylose uptake in *A. niger* was investigated by growth profiling and sugar uptake experiments.

2. Materials and methods

2.1. Strains, media and growth conditions

A. niger strains used in this study are shown in Table S1 and were deposited at the CBS culture collection of Westerdijk Fungal Biodiversity Institute. The uridine auxotrophic and non-homologous end-joining (NHEJ) deficient *A. niger* strain N593 $\Delta ku70$ was used as the reference strain. CRISPR/

Cas9 technology was used to create *A. niger* deletion mutants [25]. The primers used for the creation of all deletion mutants are shown in Table S2. *A. niger* protoplasting and transformation were carried out as described previously [26]. All *A. niger* strains were grown at 30°C on Complete Medium (CM) or Minimal Medium (MM) supplemented with required carbon source [27]. For plate cultivations, 1.5% (w/v) agar was added, and 1.22 g/L uridine was supplemented for auxotrophic strains. 1.3 mg/mL 5-fluoroorotic acid (5-FOA) was required in the solid plates for counter selecting colonies containing the *pyrG* marker gene on ANEp8-Cas9 plasmids.

A. niger strains were grown on CM plates with 1% D-glucose at 30°C for 5 days. Spores were harvested in ACES buffer and were counted using a haemocytometer. Solid MM was used for growth profiles supplemented with required carbon sources, including 25 mM D-glucose, 2 mM D-xylose, 25 mM D-xylose, 1% (66.6 mM) D-xylose, 2 mM L-arabinose, 25 mM L-arabinose and 1% (66.6 mM) L-arabinose. 200 spores in 5 µL ACES buffer were inoculated on the plates and incubated at 30°C up to 9 days.

2.2. Sugar utilization rates

Pre-cultures were inoculated with 10⁶ spores/mL and were grown for 16 hours with CM containing 2% D-fructose and 1.22 g/L uridine. Cultures were incubated at 30°C in 500 mL Erlenmeyer flasks containing 100 mL aliquots in a rotary shaker at 250 rpm. Mycelia were then harvested by filtration on a sintered glass funnel without suction, washed with MM and transferred into fresh MM with 5 mM or 25 mM D-xylose, supplemented with 1.22 g/L uridine. The cultures were incubated in rotary shakers at 30°C, 250 rpm and samples were taken at several time points.

The concentration of D-xylose in the culture medium was determined by HPLC analysis, using an H⁺ exchange column (Bio-Rad Aminex HPX-H⁺; Hercules, CA, USA), employing 10 mM H₂SO₄ at 55°C as mobile phase. Compounds were detected by means of a refractive index detector [28].

3. Results and discussion

3.1. At least three D-xylose transporters are involved in D-xylose utilization in *A. niger*

To compare their role in D-xylose uptake, three candidate D-xylose transporters were investigated: XltA, XltB and the ortholog of XAT-1 from *N. crassa* (NRRL3_02351, named XltD) [14,18]. Single and triple deletion mutants of three transporters were generated using *A. niger* N593Δ*ku70* as the reference strain. The contribution of three transporters to D-xylose uptake was analyzed by growth profiling (Fig. 1). The single deletion of *xltA* showed a small reduction in growth compared to the reference strain when 25 mM D-xylose was the sole carbon source, suggesting that XltA could be involved in D-xylose uptake to a small degree at relatively low D-xylose concentrations. Single deletion mutants of *xltB* and *xltD* grew similarly as the reference strain on D-xylose at all selected concentrations, but we cannot exclude their function on D-xylose uptake in *A. niger* yet. No further growth reduction in triple mutant was observed, indicating that *A. niger* likely has more transporters contributing to D-xylose uptake. Presence of several D-xylose transporters in *A. niger* could be explained by the abundant present of D-xylose in the natural biotope of this fungus. The growth of the reference strain and mutants was similar on a second plant-derived pentose sugar, L-arabinose, at all selected concentrations, indicating that three transporters may have no function in L-arabinose uptake or may be not the primary transporters for this sugar.

3.2. XltA and XltD have similar D-xylose transport efficiencies

No obvious difference was showed on agar plates between all strains, but dynamic changes of D-xylose uptake by all strains could be observed in liquid cultures. The absence of a phenotype on agar plates likely indicates that the residual D-xylose transport ability is sufficient to promote normal

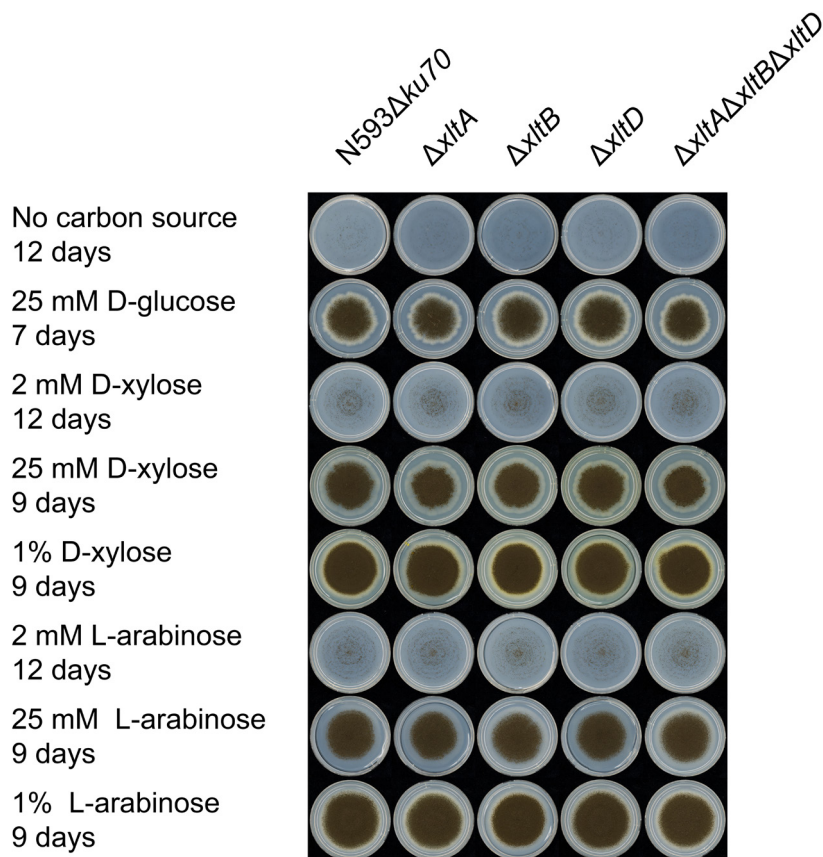


Figure 1. Growth profiling of the *A. niger* reference strain $\Delta ku70$ and deletion mutants of three D-xylose transporters, XltA, XltB and XltD, on pentoses.

growth of *A. niger*. Therefore, the utilization of D-xylose by *A. niger* in the liquid cultures was also analyzed to further study the *xltA*, *xltB* and *xltD* deletion strains (Fig. 2). Two concentrations of D-xylose, 5 mM and 25 mM, were used in this analysis. The single deletion strains of *xltA* and *xltD* decreased the D-xylose utilization rate similarly (~ 2.7 -fold and ~ 2.1 -fold, respectively), compared to the reference strain at both concentrations. This confirmed the function of the previously identified D-xylose transporter XltA [18] and showed that XltD contributes similarly to D-xylose transport as XltA. In a previous study [29], *xltA* was highly expressed on D-xylose and is regulated by the xylanolytic transcriptional activator XlnR on D-xylose, which further supported its function as a D-xylose transporter. The *xltD* gene was not specifically expressed on D-xylose, but was expressed on almost all tested carbon sources (e.g. D-rhamnose, D-galactose, D-fructose, D-glucose, D-mannose, guar gum, apple pectin, arabinan and galactan) [29]. Whether this implies that XltD has a wide sugar specificity needs to be further investigated. Conversely, the single deletion of *xltB* barely affected D-xylose utilization (Fig. 2), suggesting that XltB is not of major importance for D-xylose uptake in *A. niger*, at least under the tested conditions. A previous study showed that *xltB* is lowly expressed on almost all tested carbon sources [29], suggesting that XltB may be involved in the transport of these sugars, including D-xylose. This correlates with the lack of effect of deletion of *xltB* on D-xylose uptake, but contradicts a previous study, in which XltB was suggested to be a specific D-xylose transporter with a slightly lower affinity than XltA [18]. The explanation could be

that XltB has a very minor contribution to D-xylose uptake compared to XltA and XltD, and that therefore the deletion of *xltB* had a little or no effect on the D-xylose utilization rate in this study. In addition, the D-xylose utilization rate of the triple deletion mutant decreased further than those of two single deletion mutants of *xltA* and *xltD* by ~ 1.3 -fold and ~ 7.8 -fold, respectively, at both D-xylose concentrations (Fig. 2). However, the uptake rate did not go down to zero, indicating that additional transporters are involved in D-xylose uptake under these conditions. This correlates with the high expression of many other putative transporter-encoding genes on D-xylose in *A. niger* [29].

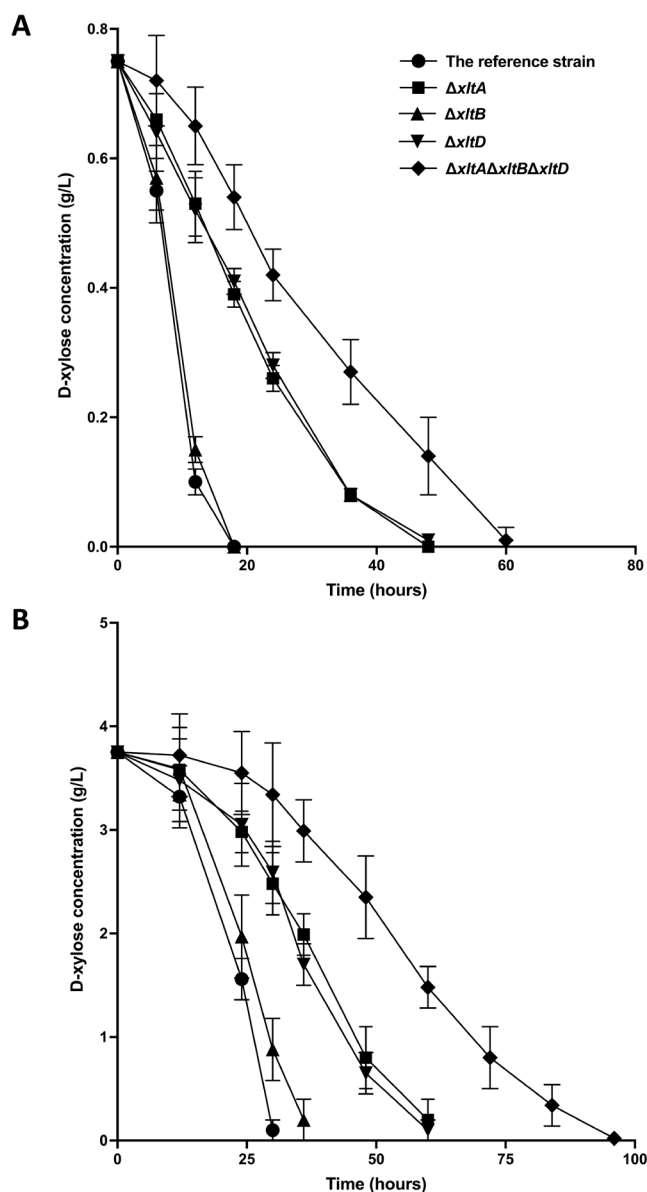


Figure 2. D-xylose utilization rates of the *A. niger* reference strain $\Delta ku70$ and deletion mutants of three D-xylose transporters, XltA, XltB and XltD. The initial D-xylose concentrations are 5 mM (A) and 25 mM (B). The error bars represent standard deviations of biological triplicates.

4. Conclusions

In this study, we identified a new xylose transporter, XltD, of *A. niger* and compared its role in D-xylose uptake to that of two previously identified D-xylose transporters. XltA plays an important role in D-xylose transport in *A. niger in vivo*, while no impact of XltB could be observed. XltD is also involved in D-xylose uptake with similar efficiency as XltA. Biochemical characterization in *S. cerevisiae* is still required for XltD to study its sugar specificity and kinetic properties. Our results suggest that overexpression of *xltA* and *xltD* may promote D-xylose uptake in *A. niger* and therefore improve the efficiency of cell factories that use D-xylose as a substrate.

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Supporting information

Table S1. *Aspergillus niger* strains used in this study.

| Strain | CBS number | Genotype | Reference |
|-------------------------------------|------------|--|----------------------------|
| N593 $\Delta ku70$ | CBS 138852 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] | Meyer <i>et al.</i> , 2007 |
| $\Delta xltA$ | CBS 147732 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] , <i>xltA</i> [−] | This study |
| $\Delta xltB$ | CBS 147733 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] , <i>xltB</i> [−] | This study |
| $\Delta xltD$ | CBS 147734 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] , <i>xltD</i> [−] | This study |
| $\Delta xltA\Delta xltB\Delta xltD$ | CBS 147741 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> [−] , <i>xltA</i> [−] , <i>xltB</i> [−] , <i>xltD</i> [−] | This study |

Table S2. Primers used in this study. The guide RNAs (gRNA) for gene deletion are marked in red and the linkers are shown in lowercase. *Available upon request from the author*

Chapter 4

A specific L-arabitol transporter in *Aspergillus niger*

This chapter was submitted for publication in *FEBS Letters*
Jiali Meng, Miia R. Mäkelä, Ronald P. de Vries

Abstract

L-arabitol is an intermediate of the pentose catabolic pathway in fungi but can also be used as a carbon source by many fungi, suggesting the presence of transporters for this polyol. In this study, an L-arabitol transporter, LatA, was identified in *Aspergillus niger*. Growth and expression profiles indicated that LatA is specific for L-arabitol and is regulated by the arabinanolytic transcriptional activator AraR. Moreover, L-arabitol production from wheat bran and sugar beet pulp was increased in a metabolically engineered *A. niger* mutant by the deletion of *latA*, indicating its potential for improving L-arabitol producing cell factories. Phylogenetic analysis showed that homologs of LatA are widely conserved in fungi.

1. Introduction

Polyols (sugar alcohols) have diverse functions in fungi, such as (1) in storage of the reducing power and coenzyme regulation, (2) to adjust the osmotic pressure by acting as compatible solutes, (3) act as endogenous carbohydrate reserves, (4) act as translocatory compounds and (5) as intermediates of major metabolic pathways [1]. Mannitol is the most common polyol found in fungi, while others include L- and D-arabitol, erythritol, D-threitol, xylitol, galactitol, sorbitol and volemitol [2]. Polyols are widely used in food and pharmaceutical industries with several health-related advantages, such as low-calorie, low-glycemic, low-insulinemic, anticarcinogenic and prebiotic properties [3].

L-arabitol occurs intracellularly as an intermediate in the fungal pentose catabolic pathway (PCP), but is rarely found in high amounts in nature [4]. However, it can be industrially produced from plant biomass derived sugars, and it is therefore widely used in the food and pharmaceutical industries as one of 12 building block chemicals (C3-C6 compounds) derived directly from biomass. Improving L-arabitol production is a major target in industrial biotechnology, focusing in particular on fermentation optimization and strain screening and development [5].

In the fungal PCP, L-arabinose is reduced to L-arabitol and then converted in two steps to xylitol [6,7]. Studies in *A. niger* showed that xylitol and L-arabitol can both support growth as a sole carbon source similar to D-xylose and L-arabinose [6,8–11]. Several mutants of *A. niger* constructed by metabolic engineering have been shown to accumulate and secrete xylitol and L-arabitol from D-xylose or L-arabinose [11,12]. These results demonstrate that *A. niger* has the capacity to both take up and secrete these two polyols, indicating the existence of polyol transporters in this fungus. Reports concerning polyol transporters are limited, but there has been a growing interest in them in recent years. Several polyol transporters have been studied in plants, such as the H⁺/mannitol transporter in celery and the polyol transporter AtPLT5 from *Arabidopsis* [13,14], in red algae, such as transporters from *Galdieria sulphuraria* [15], and in bacteria, such as D-arabinitol and ribitol transporters from *Klebsiella pneumonia* [16]. Some characterized fungal transporters are also able to transport polyols, such as hexose transporters Hxt11, Hxt13, Hxt15, Hxt16 and Hxt17 from *Saccharomyces cerevisiae* [17], two L-arabinose transporters Lat1 and Lat2 from *Ambrosiozyma monospora* [18,19], and five polyol/H⁺ symporters Sgl1, Stl1, Syi1, Syl1 and Syl2 from *Debaryomyces hansenii* [20].

Recently, 86 putative sugar transporter genes were identified in a genome-wide study of the sugar transportome of *A. niger* [21]. These predicted and 61 characterized fungal sugar transporters were phylogenetically classified to nine clades with diverse functional motifs and possible sugar specificity. Clade I contains pentose and glycerol transporters, such as XAT1 [22], Lat2 [19], Xyp29 [23] and Stl1 [24]. Lat2 has transport activities of L-arabinose, L-arabitol and ribitol in *A. monospora* grown on L-arabinose instead of D-glucose [19]. In this study, putative L-arabitol transporter encoding genes from *A. niger* were identified by combining transcriptome and phylogeny analysis. Construction of deletion strains for these genes and subsequent growth profiling revealed that one of them (NRRL3_04757, named *latA*) encodes an L-arabitol transporter. The application of *latA* deletion for L-arabitol production from wheat bran and sugar beet pulp by *A. niger* was also studied.

2. Materials and methods

2.1. Strains, media and growth conditions

Escherichia coli DH5α was used for plasmid construction and was grown on Luria-Bertani (LB) medium supplemented with 50 µg/mL ampicillin. *A. niger* strains used in this study were deposited at the CBS culture collection of Westerdijk Fungal Biodiversity Institute with numbers shown in Table 1. The uridine auxotrophic and non-homologous end-joining (NHEJ) deficient *A. niger* strain N593 Δ*ku70* was used as the reference strain. The mutants were generated using CRISPR/

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

| Strain | CBS number | Genotype | Reference |
|--|------------|---|------------|
| N593 $\Delta ku70$ | CBS 138852 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> ⁻ | [28] |
| $\Delta latA$ | CBS 147737 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> ⁻ , <i>latA</i> ⁻ | This study |
| $\Delta 05659$ | CBS 147735 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> ⁻ , <i>05659</i> ⁻ | This study |
| $\Delta ladA\Delta xdhA\Delta sdhA$ | CBS 144672 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> ⁻ , <i>ladA</i> ⁻ , <i>xdhA</i> ⁻ , <i>sdhA</i> ⁻ | [11] |
| $\Delta ladA\Delta xdhA\Delta sdhA\Delta latA$ | CBS 149003 | <i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> ⁻ , <i>ladA</i> ⁻ , <i>xdhA</i> ⁻ , <i>sdhA</i> ⁻ , <i>latA</i> ⁻ | This study |

Cas9 genome editing [25]. The primers used for creating two deletion mutations are listed in Table S1. *A. niger* protoplasting and transformation were performed as described previously [26]. All *A. niger* strains were grown at 30°C on Complete Medium (CM) or Minimal Medium (MM) [27] supplemented with required carbon source. For plate cultivations, 1.5% (w/v) agar was added, and 1.22 g/L uridine was supplemented for auxotrophic strains. 1.3 mg/mL 5-fluoroorotic acid (5-FOA) was added in the solid medium for counter selecting colonies containing the *pyrG* marker gene on ANEp8-Cas9 plasmids.

A. niger strains were grown on CM plates with 1% D-glucose at 30°C for 5 days. Spores were harvested in ACES buffer and were counted using a haemocytometer. Solid MM was used for growth profiles supplemented with 25 mM D-glucose, 25 mM D-xylose, 25 mM L-arabinose, 25 mM xylitol, 25 mM L-arabitol, 25 mM D-arabitol, 25 mM glycerol, 25 mM galactitol, 25 mM D-sorbitol, 25 mM D-mannitol, 25 mM *myo*-Inositol, 25 mM adonitol (ribitol) or 25 mM DL-threitol. 200 spores in 5 μ L ACES buffer were inoculated on the plates and incubated at 30°C for up to 12 days.

2.2. Transfer experiments

10⁶ spores/mL were inoculated to 250 mL CM with 2% D-fructose in 1 L Erlenmeyer flasks for precultures and incubated in rotary shakers at 30°C and 250 rpm for 16-18 hours. The mycelia were harvested by filtration on Miracloth under sterile conditions and washed with MM. Equal amounts of mycelia were transferred to 50 mL MM in 250 mL Erlenmeyer flasks containing 1% wheat bran or 1% sugar beet pulp, and were incubated in rotary shakers at 30°C, 250 rpm. The transfer experiments were performed in triplicate. 2 mL culture liquid was harvested after 0, 4, 8, 24, 32, 48, 56, 72 and 80 hours, and supernatants were stored at -20°C after centrifugation for measurement of extracellular xylitol and L-arabitol concentrations.

2.3. Quantification of monosaccharides and polyols

The culture liquid samples were heated at 95°C for 15 min and centrifuged for 5 min at 14000 rpm. The supernatants were 10-fold diluted with MilliQ water prior to analysis of xylitol and L-arabitol by HPLC (Dionex ICS-5000 + system; Thermo Scientific) equipped with CarboPac PA1 column (2 \times 250 mm with 2 \times 50 mm guard column; Thermo Scientific) as described previously [29]. 5-250 μ M xylitol and L-arabitol were used as standards for identification and quantitation.

2.4. Phylogenetic analysis

Homologs of LatA from other fungi were obtained using BLASTP based on the amino acid sequence of *A. niger* LatA (NRRL3_04757) on the MycoCosm database (<https://mycocosm.jgi.doe.gov/mycocosm/home>). In this phylogenetic analysis, eight Eurotiomycetes (*A. niger* NRRL3, *Aspergillus nidulans* FGSC A4, *Aspergillus oryzae* RIB40, *Penicillium rubens* Wisconsin 54-1255,

Aspergillus tubingensis v1.0, *Aspergillus carbonarius* ITEM 5010 v3, *Aspergillus fumigatus* Af293 and *Penicillium subrubescens* FBCC1632/CBS 132785), three Sordariomycetes (*Neurospora crassa* OR74A v2.0, *Trichoderma reesei* QM6a and *Fusarium oxysporum* f. sp. lycopersici 4287 v2), one Leotiomycete (*Botrytis cinerea* v1.0), two Dothideomycetes (*Phaeosphaeria nodorum* SN15 v2.0 and *Pseudocercospora* (*Mycosphaerella*) *fijiensis* v2.0) and two Saccharomycetes (*Candida albicans* SC5314 and *Saccharomyces cerevisiae* S288C) species were selected and the best hits were used for construction of phylogenetic tree. The specific L-arabinose transporter Lat2 from *A. monospora* was also included in the phylogenetic analysis [18]. All collected sequences were aligned using MAFFT v7.0 (<https://www.ebi.ac.uk/Tools/msa/mafft/>). Phylogenetic analysis was computed using Neighbor joining method with 500 bootstraps of the Molecular Evolutionary Genetics Analysis (MEGA v7.0) program [30].

3. Results and discussion

3.1. Identification of putative L-arabitol transporters

In a previous study [21], many putative sugar transporters were proposed in *A. niger* CBS 513.88 and 30 predicted transporters grouped to Clade A and Clade I mainly containing inositol/fructose and pentose/glycerol transporters, respectively. The gene NRRL3_04757 (An07g06880) is a homolog of Lat2 of *A. monospora*, which is a characterized L-arabinose transporter that is also able to transport L-arabitol. The gene NRRL3_05659 (An02g07610) is the homolog of two polyol/H⁺ symporters Syl1 and Syl2 from *D. hansenii*. The expression profiles of sugar transporter genes on different carbon sources (Fig. 1) showed that NRRL3_04757 and NRRL3_05659 were highly expressed on L-arabinose. The expression level of NRRL3_04757 was significantly reduced in single deletion mutants of two transcriptional activators AraR and RhaR, respectively, showing that it is regulated by AraR on L-arabinose and by RhaR on L-rhamnose [21]. Therefore, the transporters NRRL3_04757 and NRRL3_05659 were selected as putative L-arabitol transporters.

3.2. Deletion of *latA* resulted in impaired growth on L-arabitol

To confirm the function of NRRL3_04757 and NRRL3_05659, single deletion mutants were generated in the *A. niger* N593 $\Delta ku70$ reference strain and their growth on L-arabitol was compared to the reference strain (Fig. 2 A). The deletion mutant of NRRL3_04757 ($\Delta latA$) showed impaired growth on L-arabitol compared to the reference strain, indicating that NRRL3_04757 is a main L-arabitol transporter under this condition, and the gene was therefore named *latA*. The strong phenotype suggests that there is no redundancy in L-arabitol transport in *A. niger*, which is a clear contrast with the higher number of D-glucose and D-xylose transporters [31–34]. A possible explanation for this is that D-glucose and D-xylose are abundantly present in natural carbon sources of *A. niger*, while L-arabitol is an intermediate of the PCP and therefore much less abundant in the natural biotope of this fungus [4]. In contrast, the NRRL3_05659 deletion strain grew identical to the reference strain on L-arabitol (Fig. 2A), indicating that NRRL3_05659 is not involved in L-arabitol transport.

The effect of LatA on the growth of *A. niger* on several pentoses and polyols was also investigated (Fig. 2 B). No phenotype was observed on any of the other tested compounds, suggesting that LatA is highly specific for L-arabitol. However, we cannot exclude that transport of (some of) these other polyols could be mediated by multiple transporters, affecting a possible phenotype of $\Delta latA$ on these substrates. However, this would mean that LatA has a minor contribution to their transport as at least reduced growth would otherwise be expected. Production of LatA in a heterologous host (e.g. *S. cerevisiae*) could shed more light on the range of compounds it is able to transport.

In a previous study [21], LatA (NRRL3_04757, An07g06880) was assigned to Sugar Transporter

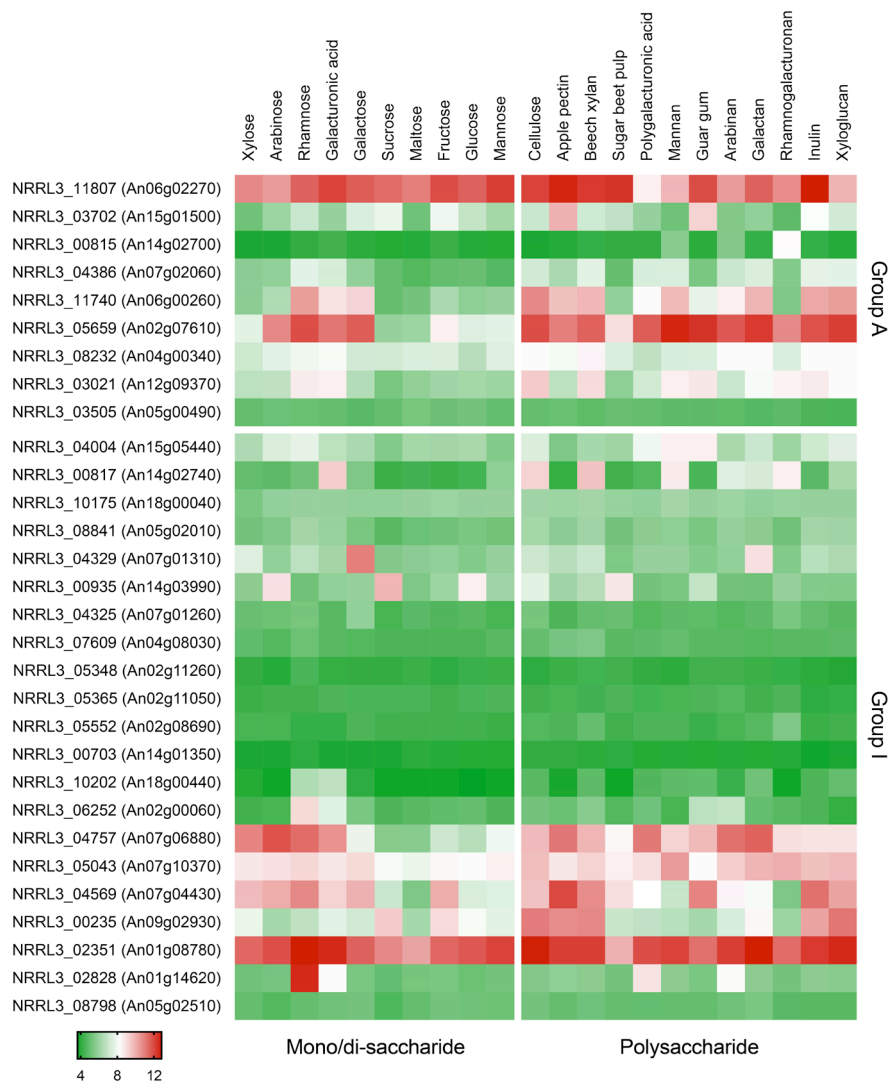


Figure 1. Expression profiles of putative sugar transporters in *A. niger* on diverse carbon sources [21]. Protein IDs of *A. niger* NRRL3 are shown in the figure and numbers in brackets reflect protein IDs from *A. niger* CBS 513.88. The color code represents averaged and logged expression values (FPKM + 1) of replicates. The heat map was drawn using GraphPad Prism (<https://www.graphpad.com/>).

Group I of *A. niger*, containing pentose or glycerol transporters. LatA is the closest homolog of Lat2 of *A. monospora* in the phylogenetic tree (Fig. 3). Lat2 is capable of transporting L-arabinose [19], but no phenotype on this sugar was observed for the *latA* deletion strain in our study (Fig. 2B). As there may be additional L-arabinose transporters in *A. niger*, we cannot exclude at this time that LatA may also contribute to L-arabinose transport. The expression data (Fig. 1) showed that *latA* was induced by D-xylose and L-arabinose, and regulated by AraR on L-arabinose [21], which could support a role for LatA as a pentose transporter. However, since L-arabitol catabolism is also stimulated by L-arabinose and L-arabitol is an intermediate of this pathway, the expression profile also supports the role as a specific L-arabitol transporter.

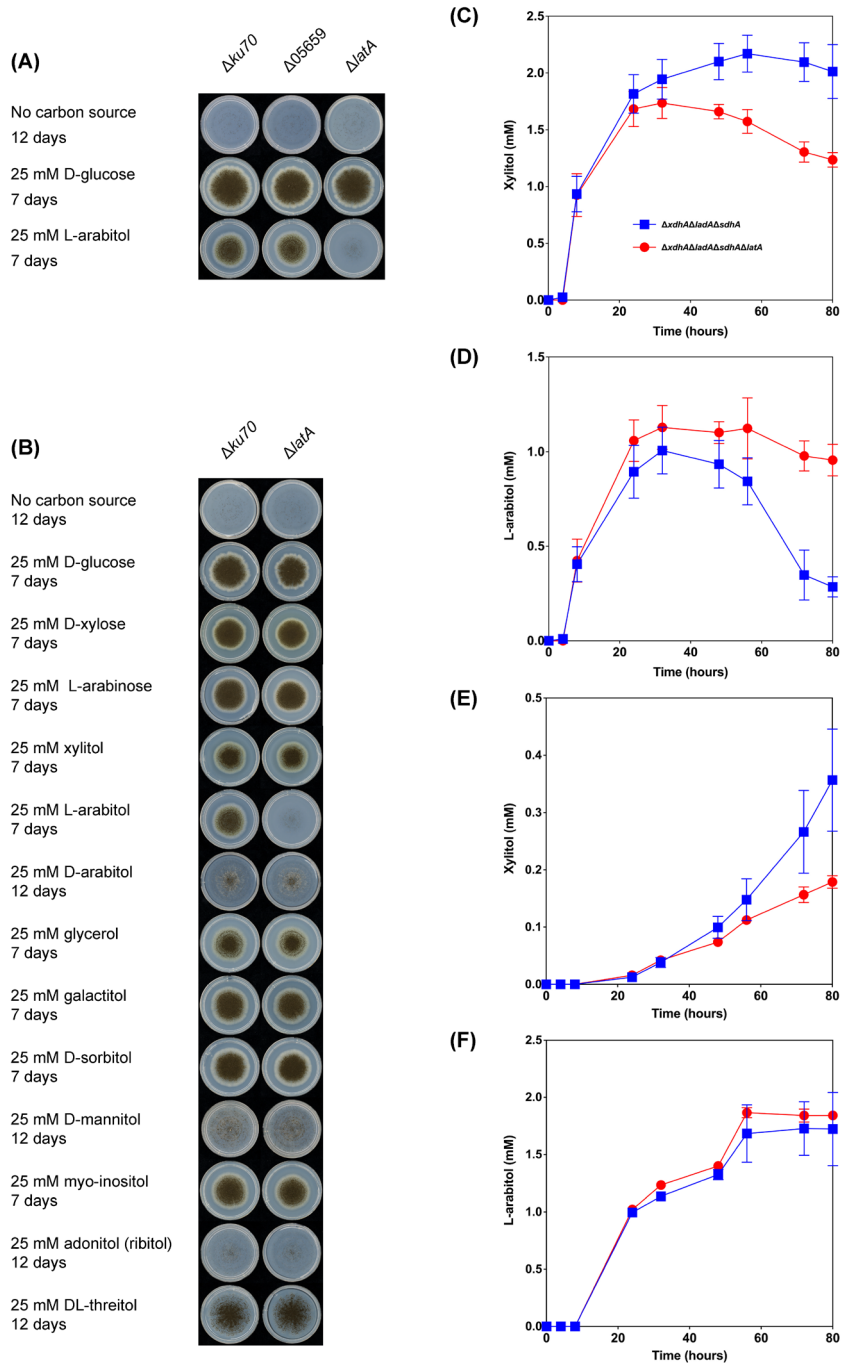


Figure 2. (A) Growth profiling of the *A. niger* reference strain $\Delta ku70$ and two single deletion mutants $\Delta 05659$ and $\Delta latA$ on L-arabitol. (B) Growth profiling of the *A. niger* reference strain $\Delta ku70$ and $\Delta latA$ on different sugars and polyols. (C-F) Extracellular xylitol and L-arabitol titers from wheat bran (C and D) and sugar beet pulp (E and F) by $\Delta latA\Delta xdhA\Delta sdhA$ and $\Delta latA\Delta xdhA\Delta sdhA\Delta latA$. The error bars indicate the standard deviation between biological triplicates.

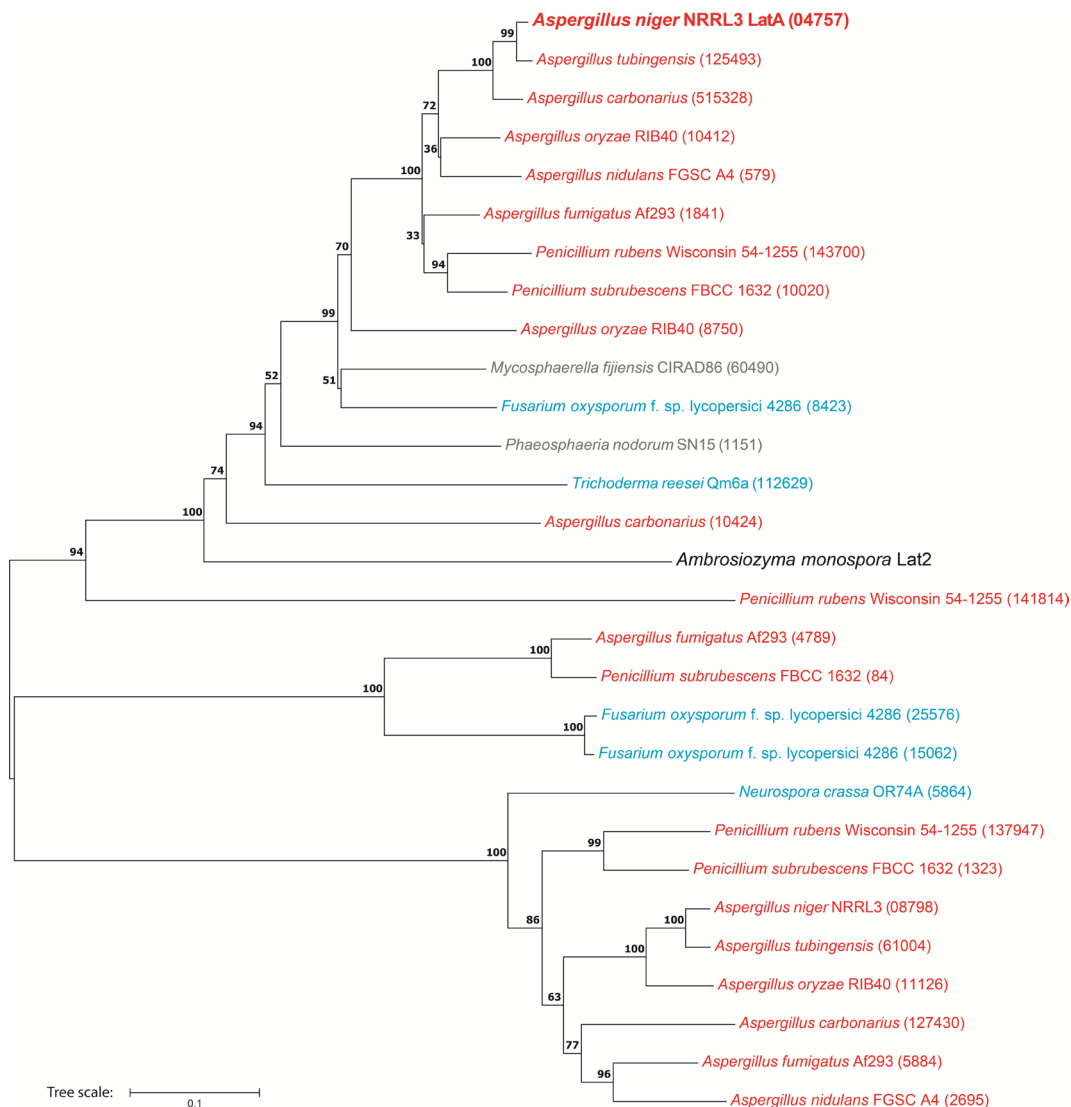


Figure 3. Unrooted phylogenetic tree of homologs of LatA from *A. niger*. The tree includes a specific L-arabinose transporter Lat2 from *A. monospora* (shown in larger font and black), the characterized L-arabitol importer LatA in *A. niger* (shown in larger font and boldface) and homologs of LatA in selected fungi. Bootstrap values are indicated on the nodes which is supported by Neighbor joining method. Colors represent the fungal taxonomic groups. Red = Eurotiomycetes, blue = Sordariomycetes, grey = Dothideomycetes. Numbers in brackets reflect the protein ID from JGI MycoCosm [36].

3.3. The deletion of *latA* positively affected L-arabitol production from wheat bran and sugar beet pulp

Deleting *latA* would potentially prevent the re-consumption of L-arabitol by *A. niger* stimulating L-arabitol production in metabolically engineered strains. To test this, we deleted *latA* in a previously

generated strain ($\Delta ladA\Delta xdhA\Delta sdhA$) [11] where the conversion of L-arabinose to L-arabitol in the PCP was blocked. This triple mutant produces xylitol (approximately 2.0 mM) from wheat bran as well as a small amount of L-arabitol (approximately 1.0 mM) [11,12]. The results showed that the deletion of *latA* in the triple mutant increased the maximum titer of L-arabitol from wheat bran by 12% (Fig. 2 D). The L-arabitol level decreased in the triple mutant after 32 h of cultivation, most likely by re-consumption, while this was delayed until 56 h when *latA* was also deleted. It confirmed that deletion of *latA* can reduce/delay re-consumption L-arabitol when other carbon sources are depleted.

In the previous study, L-arabitol production from sugar beet pulp in the triple mutant was 2-fold higher than from wheat bran and other studied crude biomass [12]. The proportion of L-arabinose in sugar beet pulp is higher than in wheat bran and conversely the content of D-xylose in wheat bran is significantly higher than in sugar beet pulp (Table S2). This difference in composition of these two substrates can explain why the maximum titer of L-arabitol (2.0 mM) from sugar beet pulp was much higher than from wheat bran (1.0 mM) in $\Delta ladA\Delta xdhA\Delta sdhA$ strain. A small increase in L-arabitol titer from sugar beet pulp was observed when *latA* was also deleted (Fig. 2 F).

Conversely, the extracellular xylitol titer was decreased by almost 50% after 80 hours of cultivation when *latA* was deleted (Fig. 2 C and E), most likely due to increased re-consumption, possibly because L-arabitol cannot be re-consumed.

Our results demonstrate that the deletion of *latA* in a xylitol and L-arabitol producing strain has a moderate increase on the L-arabitol concentration, indicating that this gene is a relevant component of a strain engineering approach to generate polyol cell factories.

3.4. LatA is widely present in ascomycete fungi

Homologs of LatA are present in most species selected for our analysis, except *B. cinerea*, *C. albicans* and *S. cerevisiae* (Fig. 3), indicating that homologs of LatA are widely present across the phylum Ascomycota. The phylogenetic analysis provided many candidate polyol transporters for further characterization in other fungi. Some species contain multiple candidate L-arabitol transporters, perhaps for enhancing the activity to transport polyols [35]. However, care should be taken in assigning function to these homologs, as already the functional characterization of Lat2 from *A. monospora* [18] suggests differences in substrate specificity between these transporters.

In conclusion, we identified a highly specific L-arabitol transporter in *A. niger* that contains homologs across Ascomycota and has potential as a target for metabolic engineering of L-arabitol producing fungal cell factories.

Author contributions

JM performed experiments, analyzed data, and wrote the original manuscript. MRM and RPDV designed the experiments, supervised the overall research, and reviewed and edited the manuscript.

Acknowledgements

We would like to thank Chinese Scholarship Council (CSC) for the financial support to J.M. (CSC student number: CSC201907720027). The Novo Nordisk Foundation grant number NNF21OC0067087 “The dual role of sugar transporters in plant biomass conversion by fungi to improve microbial cell factories” (MYCOFACT) to M.R.M. is acknowledged.

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Supporting information

Table S1. Primers used in this study. The guide RNAs (gRNA) for gene deletion are marked in red and the linkers are shown in lowercase. *Available upon request from the author*

Table S2. Composition of wheat bran and sugar beet pulp (mol%). *Available upon request from the author*

Chapter 5

GalR, GalX and AraR co-regulate D-galactose and L-arabinose utilization in *Aspergillus nidulans*

This chapter was published in *Microbial Biotechnology*

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Volume 15, 2022, 1839-1851

<https://doi.org/10.1111/1751-7915.14025>

Summary

Filamentous fungi produce a wide variety of enzymes in order to efficiently degrade plant cell wall polysaccharides. The production of these enzymes is controlled by transcriptional regulators, which also control the catabolic pathways that convert the released monosaccharides. Two transcriptional regulators, GalX and GalR, control D-galactose utilization in the model filamentous fungus *Aspergillus nidulans*, while the arabinolytic regulator AraR regulates L-arabinose catabolism. D-galactose and L-arabinose are commonly found together in polysaccharides, such as arabinogalactan, xylan and rhamnogalacturonan-I. Therefore, the catabolic pathways that convert D-galactose and L-arabinose are often also likely to be active simultaneously. In this study, we investigated the interaction between GalX, GalR and AraR in D-galactose and L-arabinose catabolism. For this, we generated single, double and triple mutants of the three regulators, and analyzed their growth, and enzyme and gene expression profiles. Our results clearly demonstrated that GalX, GalR and AraR co-regulate D-galactose catabolism in *A. nidulans*. GalX has a prominent role on regulation of genes of D-galactose oxido-reductive pathway, while AraR can compensate for the absence of GalR and/or GalX.

1. Introduction

D-galactose and L-arabinose are commonly found together in plant cell wall polysaccharides, such as xylan, xyloglucan, arabinogalactan, and rhamnogalacturonan I and II [1–5]. In filamentous fungi, the carbon catabolic pathways of these monosaccharides can be active at the same time, enabling simultaneous use of these monosaccharides [6].

The key metabolic genes, enzymes and carbon catabolic pathways of *Aspergillus* and other fungi that convert monomers present in plant polysaccharides have been studied for decades [7–9]. The pentoses L-arabinose and D-xylose are converted via the pentose catabolic pathway (PCP) through a number of reductase and dehydrogenase catalyzed reactions, which was recently updated for *Aspergillus niger* ([8], Fig. 1).

There are several D-galactose catabolic pathways in filamentous fungi. The best-known pathway is the Leloir pathway, which exists in both prokaryotic and eukaryotic microorganisms [10]. All the enzymes of the Leloir pathway in *A. nidulans* have been described previously [11]. D-galactose is phosphorylated to D-galactose-1-phosphate by galactokinase (GalE), and then further converted to UDP-galactose and D-glucose-1-phosphate by D-galactose-1-phosphate uridylyltransferase (GalD). UDP-galactose can be converted back to UDP-glucose by UDP-galactose 4-epimerase (GalG) and the conversion of D-glucose-1-phosphate to D-glucose-6-phosphate is catalyzed by phosphoglucomutase (PgmB), which eventually enters glycolysis or the PPP [12–14].

An alternative D-galactose oxido-reductive pathway, with similarity to the PCP, has been described in *A. nidulans* [15], and has also been identified in *Trichoderma reesei* [16,17] and *A. niger* [18–20]. In this pathway, D-galactose is converted to D-fructose-6-phosphate in five enzymatic steps, which then enters glycolysis [15]. The enzymes in this pathway differ between the three species. Only

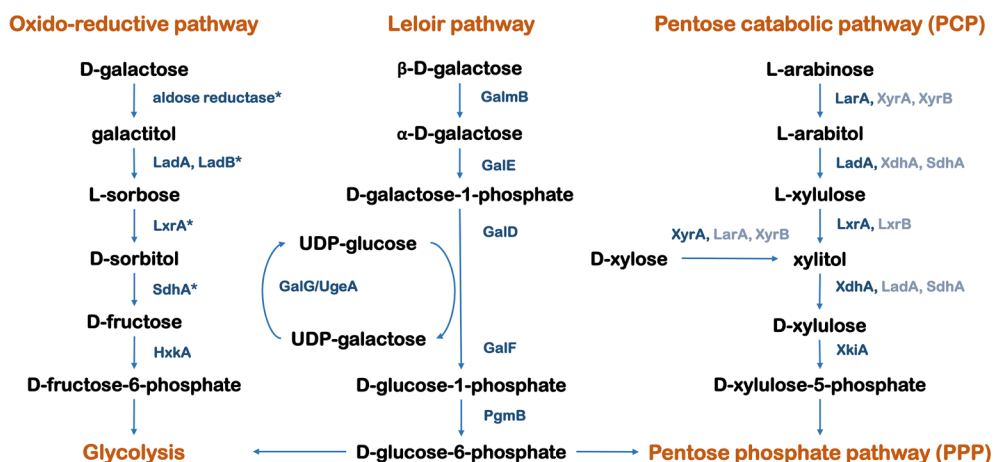


Figure 1. The D-galactose oxido-reductive pathway, Leloir pathway and pentose catabolic pathway in *A. nidulans*. Unidentified or unconfirmed enzymes are marked with a star. Gene function and numbers are: LadA = L-arabitol dehydrogenase (AN0942); LadB = galactitol dehydrogenase (AN4336); LxrA, LxrB = L-xylulose reductase (AN10169, AN8819); SdhA = sorbitol dehydrogenase (AN2666); HxkA = hexokinase (AN7459); GalmB = galactose-1-epimerase (AN3432); GalE = galactokinase (AN4957); GalD = D-galactose-1-phosphate-uridylyltransferase (AN6182); GalG/UgeA = UTP-glucose-1-phosphate uridylyl transferase (AN9148); GalG/UgeA = UDP-galactose-4-epimerase (AN4727); PgmB = phosphoglucomutase (AN2867); LarA = L-arabinose reductase (AN7193); XyrA, XyrB = D-xylose reductase (AN0423, AN1274); XdhA = xylitol dehydrogenase (AN9064); XkiA = D-xylulose kinase (AN8790).

some enzymes involved in the specific steps have been identified in *A. nidulans*, but there are some similarities to *T. reesei* [18]. Three enzymes of the PCP, Xyl1, Lad1 and Xdh1, are involved in D-galactose oxido-reductive pathway in *T. reesei*, and aldose reductase (Xyl1) is a main enzyme in the reduction of D-galactose to galactitol [16]. D-xylose reductase (XyrA) has also been suggested to convert D-galactose to galactitol in *A. niger* [19], but a recent study disproved that claim (Chroumpi *et al.*, unpublished results). Which aldose reductase catalyzes this conversion in *A. nidulans* remains unknown. L-arabitol dehydrogenase (Lad1) from the PCP catalyzes the oxidization of galactitol to L-xylo-3-hexulose in *T. reesei*, but this reaction is catalyzed by a specific D-galactitol dehydrogenase (LadB) instead of LadA in *A. niger*, which is not related to the PCP [17,19]. The *ladB* ortholog also exists in *A. nidulans* and is likely responsible for this reaction. However, the product of galactitol oxidation was identified as L-sorbose in *A. nidulans* [15]. The conversion of L-sorbose to D-sorbitol is suggested to be catalyzed by L-xylulose reductase or a similar enzyme [21]. Conversely, the reduction of L-xylo-hexulose to D-sorbitol is catalyzed by L-xylo-3-hexulose reductase, Lxr4 in *T. reesei* and XhrA in *A. niger* [18]. D-sorbitol is suggested to be converted to D-fructose by sorbitol dehydrogenase (SdhA) in *A. niger*, but a recent study indicated that an alternative sorbitol dehydrogenase may in fact be responsible for this conversion (Chroumpi *et al.*, unpublished data). This reaction was described to be catalyzed by xylitol dehydrogenase (Xdh1) from the PCP in *T. reesei* [18,20].

Transcriptional regulators control the production of polysaccharide degrading enzymes and enzymes of the carbon catabolic pathways for the released monosaccharides. Two transcriptional regulators, XlnR and AraR, together control the PCP in *Aspergillus* [22,23]. XlnR is induced by D-xylose, while AraR is induced by L-arabinose/L-arabitol [23,24]. AraR is the main regulator of the L-arabinose specific PCP genes (*LarA*, *LadA*, *LxrA*), and XlnR regulates *xyrA*. The last two PCP genes (*xdhA*, *xkiA*) are regulated by both regulators [23,25,26].

Two other transcriptional regulators, GalX and GalR, control D-galactose release and catabolism in *A. nidulans* [27]. GalX is conserved in most *Aspergilli*, while GalR is unique to section *Nidulantes*, such as *A. nidulans*, *Aspergillus sydowii* and *Aspergillus versicolor* [28]. In a previous study, the interaction between three transcription factors (XlnR, AraR and GalR) in *A. nidulans* was investigated [28], which demonstrated that to a small extent XlnR, but more prominently AraR not only regulate the PCP, but also the oxido-reductive D-galactose catabolic pathway. In contrast, GalR only controls the genes of the oxido-reductive D-galactose catabolic pathway [28]. It also suggested that three regulators are not the only regulators involved in the D-galactose oxido-reductive pathway and GalX is likely to regulate some enzymes of D-galactose catabolism directly.

In this study, the possible interaction between GalX, GalR and AraR in D-galactose and/or L-arabinose catabolism in *A. nidulans* was investigated in detail. Single, double and triple mutants of the three regulators were generated using CRISPR/Cas9 technology, and their growth, specific enzyme activities, sugar utilization rates and gene expression profiles were analyzed.

2. Results and discussion

2.1. Extreme low concentrations of L-arabinose can induce D-galactose catabolism mediated by AraR

Single, double and triple deletion mutants of *galR*, *galX* and *araR* were generated to investigate possible interaction between GalX, GalR and AraR in D-galactose and/or L-arabinose catabolism in *A. nidulans*. Growth on D-galactose was almost abolished in all strains in which *galR* and/or *galX* was deleted, while the single deletion of *araR* resulted in significant reduction of growth on D-galactose (Fig. 2). This indicates that GalR and GalX are the main regulators of D-galactose catabolism, while AraR has a smaller role. As GalX controls the expression of *galR* [27], this puts

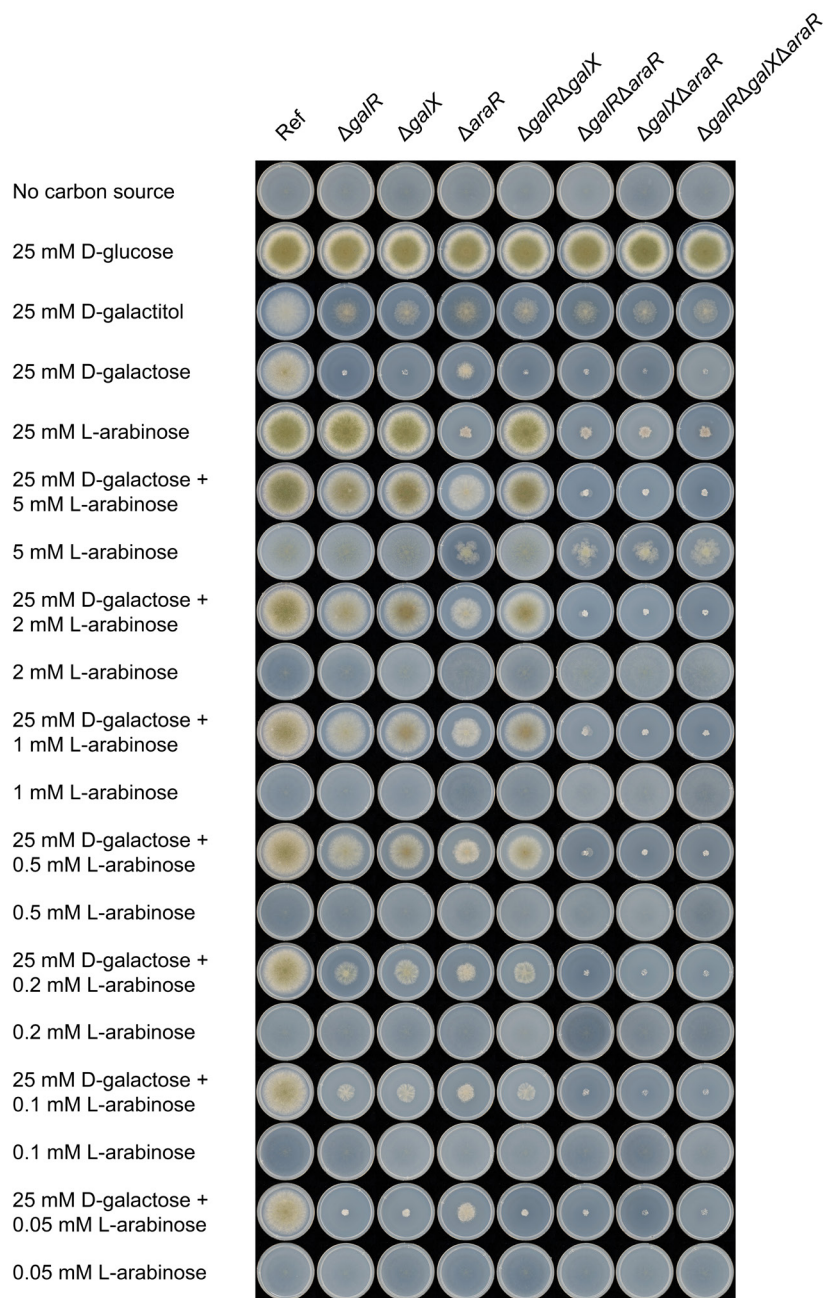


Figure 2. Growth profiling of the *A. nidulans* reference strain and deletion mutants on different carbon sources.

GalX highest in the hierarchy of regulatory control of D-galactose catabolism.

Growth of all single mutants on galactitol was highly reduced, while the growth of double and triple mutants did not reduce further (Fig. 2). It indicates GalX, GalR and AraR all regulate galactitol utilization. galactitol is an intermediate in the D-galactose oxido-reductive pathway. In a previous study, growth on this compound was not reduced when *xlnR* was disrupted, so *XlnR* does not seem

to be a predominant regulator of galactitol or D-galactose utilization [28]. According to these results, there are supposed to be more regulators (except XlnR, AraR, GalR and GalX) involved in the regulation of galactitol or D-galactose utilization.

Growth on L-arabinose was highly reduced when *araR* was deleted (Fig. 2), confirming that AraR regulates L-arabinose utilization. The residual growth on this sugar can be attributed to the influence of XlnR as it also activates the expression of some genes of the PCP [28].

To further study the interaction between GalX, GalR and AraR, we also used the mixtures of D-galactose and L-arabinose as carbon sources. Increasing concentrations of L-arabinose resulted in a gradual growth increase in the single and double mutants of *galR* and *galX* on the mixed carbon sources compared to the sole carbon source. Their growth was comparable to that of the reference strain, especially on the mixture of 25 mM D-galactose and 5 mM L-arabinose. These results showed that already at very low concentrations L-arabinose can induce D-galactose utilization mediated by AraR.

2.2. D-galactose utilization is induced by L-arabinose mediated by AraR

A. nidulans can consume D-galactose and L-arabinose simultaneously, and the utilization rate of L-arabinose is faster in the presence than in the absence of D-galactose [29]. In this study, we also determined sugar utilization rates of the reference strain and mutants in liquid culture. First, we assessed the respective utilization rates of D-glucose, D-galactose and L-arabinose in the reference strain, single ($\Delta galR$, $\Delta galX$ and $\Delta araR$) and triple ($\Delta galR\Delta galX\Delta araR$) deletion mutants (Fig. 3 and Table 1). The utilization rates of D-glucose in all strains were similar as expected. When *galR* and/or *galX* were deleted, the utilization of D-galactose was completely abolished. The transcriptomic data (Fig. S1) showed that the genes AN4590 and AN9173, encoding two putative major facilitator superfamily (MFS) proteins, were highly expressed on L-arabinose and D-galactose compared to D-glucose, and also significantly down regulated on L-arabinose when AraR was absent or on D-galactose GalR and/or GalX were absent. This could suggest that these genes encode L-arabinose transporters and may have partial specificity to D-galactose. *Gsx1* (AN9295) is a predicted Glucose/xylose: H⁺ symporter and the expression level of its encoding gene was significantly down regulated on D-galactose when *galR* and/or *galX* were deleted as well [30]. The significantly reduced expression levels of these three genes could be part of the reason why three mutants $\Delta galR$, $\Delta galX$ and $\Delta galR\Delta galX\Delta araR$ lost the ability to utilization D-galactose. The gene AN2665 encoding another MFS protein was highly expressed on D-galactose compared to D-glucose and also down regulated on D-galactose when *galX* was deleted, suggesting it could be a specific D-galactose transporter. The identical results for these three strains likely indicate that GalR and GalX control D-galactose transport directly. The deletion of *araR* slightly affected the utilization of D-galactose compared to the reference strain. The reason could be that expression levels of transporter genes AN4590, AN9173 and *gsx1* were significantly down regulated when *araR* was deleted on D-galactose. The utilization of L-arabinose was completely abolished when *araR* was absent, possibly in part due to the significantly down regulated expression of predicted MFS transporter encoding genes AN1276, AN9173, AN4590, AN8400, AN8467 and *gsx1* on L-arabinose (Fig. S1). However, the deletion of *galR* or *galX* had no influence on the utilization of L-arabinose. These results indicate the L-arabinose transport is fully under control of AraR.

Moreover, we determined whether the addition of L-arabinose at different concentrations (0.5, 1, 2 and 5 mM) affected the utilization of D-galactose (Fig. 4 and Table 2). The utilization rate of D-galactose in the reference strain was slightly slower when L-arabinose was present. The presence of L-arabinose activated the utilization of D-galactose in $\Delta galR$ and $\Delta galX$, but did not result in D-galactose utilization when *araR* was deleted ($\Delta galR\Delta galX\Delta araR$). The expression levels of MFS transporter encoding genes AN4590, AN8467 and AN9173 were highly reduced in $\Delta galR$, $\Delta galX$ and $\Delta galR\Delta galX\Delta araR$ on D-galactose, but the addition of 5 mM L-arabinose restored their

REGULATION OF D-GALACTOSE AND L-ARABINOSE UTILIZATION

Table 1. D-glucose, D-galactose and L-arabinose utilization rates of the *Aspergillus nidulans* reference strain and mutants.

| Strains | D-glucose (g/L/h) | D-galactose (g/L/h) | L-arabinose (g/L/h) |
|-------------------------------------|-------------------|---------------------|---------------------|
| Reference strain | 0.3 | 0.19 | 0.158 |
| $\Delta galR$ | 0.27 | 0 | 0.147 |
| $\Delta galX$ | 0.277 | 0 | 0.152 |
| $\Delta araR$ | 0.29 | 0.182 | 0 |
| $\Delta galR\Delta galX\Delta araR$ | 0.24 | 0 | 0 |

expression in $\Delta galR$ and $\Delta galX$ to the levels in the reference strain, which could contribute to the restoration of D-galactose utilization in these two mutants (Fig. S1). However, their expression in $\Delta galR\Delta galX\Delta araR$ remained at very low levels when adding 5 mM L-arabinose to D-galactose, which could cause the complete stop of D-galactose utilization in this mutant even after addition of 5 mM L-arabinose. These results clearly showed that L-arabinose induction of D-galactose utilization is mediated by AraR. It is worth noting that the utilization rates of D-galactose in $\Delta galR$ and $\Delta galX$ did not totally restore to the level of the reference strain when L-arabinose was present. This is consistent with their growth phenotypes on the corresponding carbon sources, showing that in the presence of L-arabinose, AraR can largely, but not fully compensate for the lack of GalR or GalX in regulating D-galactose catabolism. Addition of 2 mM D-galactose to L-arabinose resulted in L-arabinose utilization in $\Delta araR$, but not in $\Delta galR\Delta galX\Delta araR$, indicating that GalR and/or GalX can partially restore L-arabinose utilization in the presence of D-galactose (Fig. 5 and Table 2).

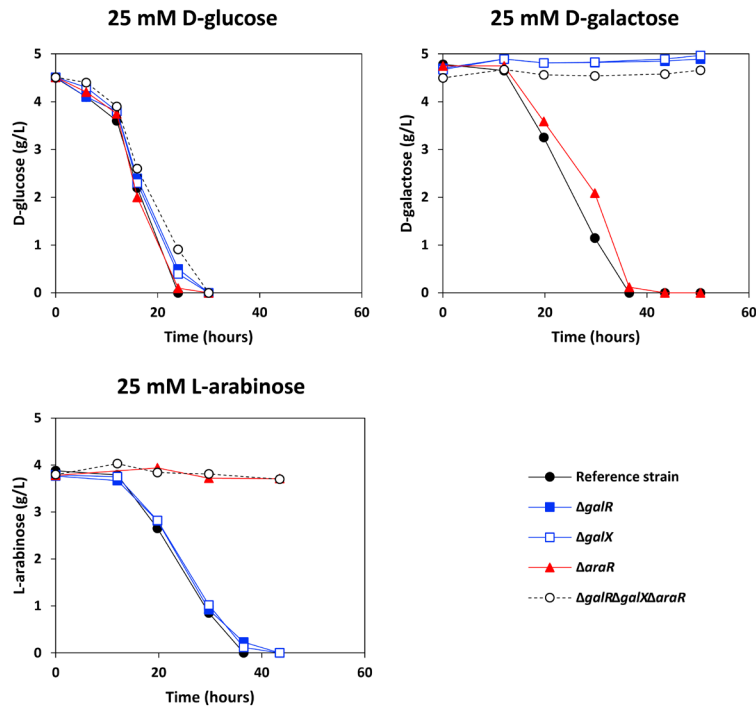


Figure 3. D-glucose, D-galactose and L-arabinose utilization of the *A. nidulans* reference strain and mutants.

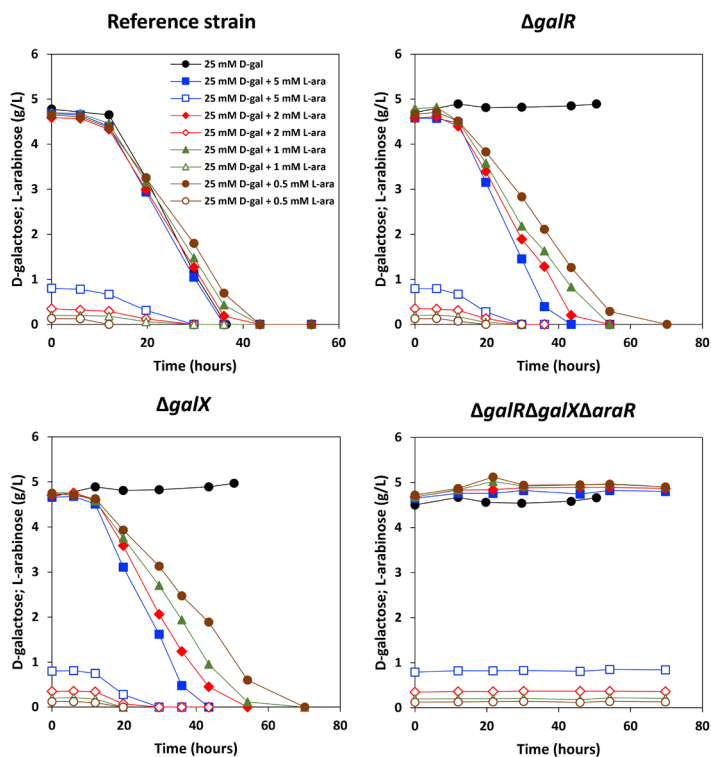


Figure 4. D-galactose and L-arabinose utilization of the *A. nidulans* reference strain and mutants in the mixtures of these two carbon sources. Open markers: L-arabinose concentration; Closed markers: D-galactose concentration. D-gal: D-galactose; L-ara: L-arabinose.

Table 2. D-galactose and L-arabinose utilization rate of the *Aspergillus nidulans* reference strain and mutants in the mixtures of D-galactose and L-arabinose.

| D-galactose utilization rate | | | | |
|--|------------------|---------------|-------------------------------------|-------------------------------------|
| Carbon sources | Reference strain | $\Delta galR$ | $\Delta galX$ | $\Delta galR\Delta galX\Delta araR$ |
| 25 mM D-galactose | 0.19 | 0 | 0 | 0 |
| 25 mM D-galactose + 5 mM L-arabinose | 0.184 | 0.17 | 0.165 | 0 |
| 25 mM D-galactose + 2 mM L-arabinose | 0.172 | 0.133 | 0.135 | 0 |
| 25 mM D-galactose + 1 mM L-arabinose | 0.168 | 0.118 | 0.109 | 0 |
| 25 mM D-galactose + 0.5 mM L-arabinose | 0.151 | 0.102 | 0.093 | 0 |
| L-arabinose utilization rate | | | | |
| Carbon sources | Reference strain | $\Delta araR$ | $\Delta galR\Delta galX\Delta araR$ | |
| 25 mM L-arabinose | 0.158 | 0 | 0 | |
| 25 mM L-arabinose + 2 mM D-galactose | 0.136 | 0.06 | 0 | |

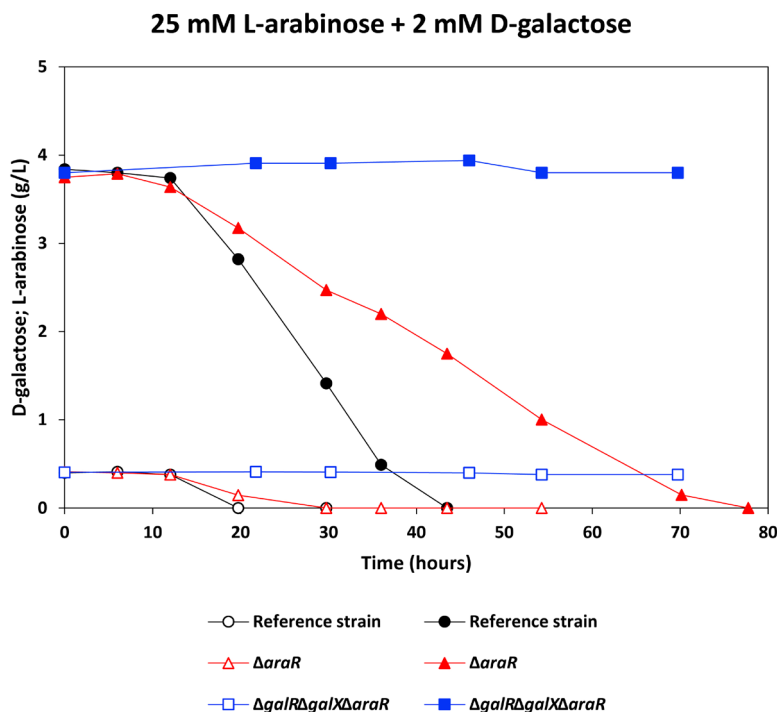


Figure 5. D-galactose and L-arabinose utilization of the *A. nidulans* reference strain and mutants in the mixture of 25 mM L-arabinose + 2 mM D-galactose. Open markers: D-galactose concentration; Closed markers: L-arabinose concentration.

2.3. The single deletions of *galR*, *galX* and *araR* have a greater effect on enzymes of the D-galactose oxido-reductive pathway than on those of the Leloir pathway

Galactokinase (GalE) catalyzes the conversion of D-galactose to D-galactose-1-phosphate in the Leloir pathway and galactitol dehydrogenase (LadB), instead of L-arabitol dehydrogenase (LadA), catalyzes the conversion of galactitol to L-sorbose in the oxido-reductive pathway of *A. nidulans*. In order to study the induction of D-galactose catabolism by L-arabinose in *A. nidulans* in detail, we assayed activities of these two catabolic enzymes from preculture (2% D-fructose) and main culture (25 mM D-galactose and 5 mM L-arabinose) in the reference strain and single deletion mutants ($\Delta galR$, $\Delta galX$ and $\Delta araR$). In the previous study, the expression of *galE* was reduced to a basal level in $\Delta galR$ and no expression was observed in $\Delta galX$ on D-galactose [27]. However, there was a basal level of galactokinase activity in the preculture of all *A. nidulans* strains, while this was not the case for L-arabitol dehydrogenase in this study (Table S1 upper table panel).

In the main culture (Table S1 lower table panel), the galactokinase activity in $\Delta araR$ was almost the same to the reference strain, while its activity slightly decreased in $\Delta galR$ and $\Delta galX$. However, these decreased values were still higher than the basal levels in the pre-culture. These results were consistent with the decreased expression level of *galE* in the three single deletion mutants ($\Delta galX$, $\Delta galR$ and $\Delta araR$) compared to the reference strain on 25 mM D-galactose and 5 mM L-arabinose in this study (Fig. S2). We tested *in vitro* enzyme activity of L-arabitol dehydrogenase on L-arabitol and galactitol, respectively. When using L-arabitol as the substrate, enzyme activity of L-arabitol dehydrogenase decreased in $\Delta galR$ and conversely increased in $\Delta galX$ compared to the reference strain. The possible reason of increased enzyme activity on L-arabitol in $\Delta galX$ is that the deletion

of *galX* slightly increased expression level of *ladA* induced by 5 mM L-arabinose according to the transcriptome data (Fig. S2). The decreased enzyme activity on L-arabitol in $\Delta galR$ could be explained by the slightly reduced expression level of *xdhA*, rather than *ladA*, compared to the reference strain. However, the enzyme activity decreased in $\Delta galR$ and $\Delta galX$ on galactitol, especially in $\Delta galX$, which is consistent with the significantly reduced expression level of *ladB* in both mutants compared to the reference strain (Fig. S2). The deletion of *araR* decreased both specific activities by 28–44% compared to the reference strain, which can be the reason of the reduced growth of $\Delta araR$ on galactitol. The decreased expression level of *ladA* in $\Delta araR$ can explain the reduced enzyme activity of L-arabitol dehydrogenase on L-arabitol. However, the expression level of *ladB* increased slightly in $\Delta araR$ compared to the reference strain, which contradicts with the reduced enzyme activity of L-arabitol dehydrogenase on galactitol (Fig. S2). In the previous study, LadB and LadA from *A. niger* have similar *in vitro* activity with galactitol and LadA induced on D-xylose could substitute for LadB [19]. Therefore, the decreased expression level of *ladA* in $\Delta araR$ could be a possible reason for the reduced enzyme activity on galactitol. These results could also explain the slightly lower D-galactose utilization rates (Table 2) and poorer growth of $\Delta galR$ and $\Delta galX$ (Fig. 2) compared to the reference strain on 25 mM D-galactose and 5 mM L-arabinose.

2.4. GalX, GalR and AraR all regulate D-galactose catabolism, but GalX has the biggest impact

According to growth phenotypes and sugar utilization rates of the reference strain and mutants, GalX, GalR and AraR are all involved in D-galactose catabolism. The analysis of transcriptome data was performed to study transcriptional changes between the reference strain and mutants. The number of differentially expressed genes (DEGs, Fig. 6) showed that the single deletion of *galX* or *araR* has a wider influence on gene expression than the deletion of *galR*, indicating broader regulatory functions of *galX* and *araR* under these conditions. Surprisingly, there was a large number of DEGs due to *galX* deletion on D-glucose, even more than on D-galactose, suggesting that GalX may have an additional role in D-glucose catabolism through glycolysis. These include some GalX-regulated glycolytic genes on D-glucose, such as *pgkA*, *pgmA*, *pfkA* and *glkA*. The absence of *araR* affected the expression of many genes on L-arabinose with and without D-galactose, confirming its clear role in L-arabinose utilization.

The significantly decreased expression levels of the three regulators in the corresponding mutants confirmed their deletion (Fig. S2). The expression level of *galR* also reduced significantly in $\Delta galX$, confirming that GalX controls GalR [27]. It is worth noting that the expression level of *xlnR* was highly increased compared to the reference strain when *araR* was absent on L-arabinose, suggesting that XlnR might be able to partially compensate for the absence of AraR.

PCP genes *larA*, *ladA*, *lxA*, *xdhA* and *xkiA* were highly expressed in the reference strain on L-arabinose, and were expressed at very low levels when *araR* is deleted on L-arabinose (Fig. S2). The decreased expression levels of the first three genes in PCP (*larA*, *ladA* and *lxA*) explains the reduced growth of $\Delta araR$ on L-arabinose. The PCP gene *xyrA* was highly expressed on L-arabinose, but not down regulated when *araR* was deleted, indicating that this gene is mainly controlled by XlnR as mentioned in the previous study [28]. Most of the PCP genes seem to be induced on D-galactose compared to D-glucose, but show lower expression levels on D-galactose than on L-arabinose. As the deletion of *galR* and/or *galX* did highly reduce the expression of *lxA* and *xkiA* on a mixture of D-galactose and L-arabinose, we cannot exclude the involvement of GalR and/or GalX in regulation of PCP genes under these conditions.

All genes involved in Leloir pathway were expressed on D-galactose and their expression levels were not affected by the deletion of the three regulators (Fig. S2). Not all the genes of D-galactose oxido-reductive pathway have been identified in *A. nidulans*. The first enzyme, aldose reductase, involved in the conversion of D-galactose to galactitol remains unknown. The *ladB* (AN4336) gene,

which encodes a specific galactitol dehydrogenase (LadB) in *A. niger*, also exists in *A. nidulans* and is likely responsible for this conversion of galactitol to L-sorbose [17,19]. The highly reduced growth of $\Delta galX$, double and triple mutants on galactitol, but not abolished, indicated that other enzymes could be involved in this conversion, as the expression of *ladB* was highly decreased to around zero. The expression of *ladB* was almost lost when *galX* was deleted confirming that GalX regulates this gene [27]. In this study, the expression of *ladA* was induced on D-galactose, but much lower than on L-arabinose, and was not affected when three regulators were deleted on D-galactose. Therefore, our results confirmed the existence of other enzymes of this reaction. The slightly decreased expression of *ladB* can explain the decreased growth of $\Delta galR$ on galactitol.

The previous study determined that a putative reductase encoding gene *red1* (AN7914) was co-regulated by XlnR, GalR and AraR in *A. nidulans* and was a strong candidate as the unconfirmed L-sorbose reductase [28]. However, the expression of *red1* was not highly induced in the reference strain and did not reduce in any of the mutants on D-galactose in our study, so our results could not support its function in this conversion (Fig. S2). The expression of *lxaA* (AN10169) was induced on D-galactose, but much lower than on L-arabinose. The expression level of *lxaA* was decreased in all mutants by up to 86% compared to the reference strain on D-galactose, indicating that the expression of *lxaA* is regulated by GalX, GalR and AraR. In *T. reesei*, the L-xylulose reductase (LXR1) has activity with L-xylulose, D-xylulose, D-fructose, and L-sorbose [31]. Therefore, LxrA may be responsible for the conversion of L-sorbose to D-sorbitol, at least partially. Its function and the involvement of other enzymes in D-galactose oxido-reductive pathway require further studies. The decreased expression level of *lxaA* can be a reason of the reduced growth of all mutants on D-galactose and galactitol.

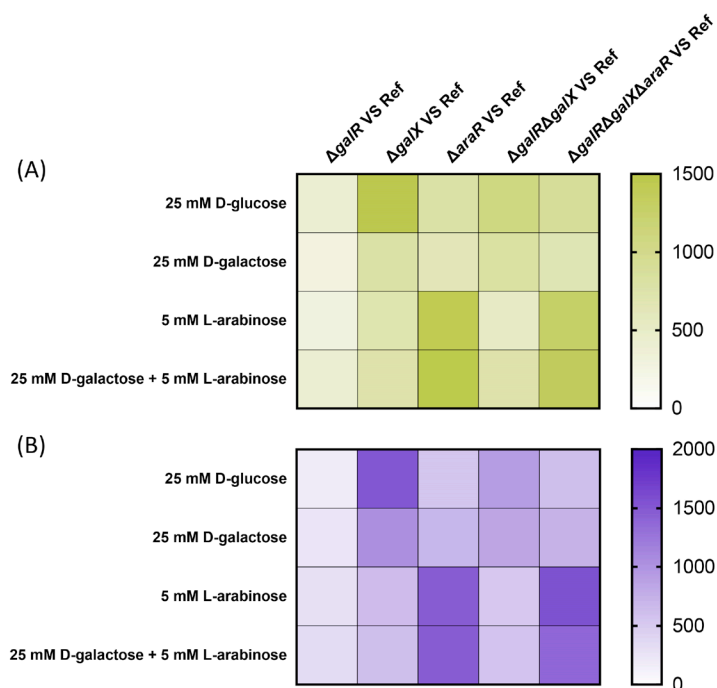


Figure 6. The number of differentially expressed genes in *A. nidulans* mutants compared to the reference strain on different carbon sources. (A) Highly up-regulated genes in mutants (B) Highly down-regulated genes in mutants. Transcripts were considered as differentially expressed if the DESeq2 fold change was > 2 and $P_{adj} < 0.01$.

D-sorbitol is converted to D-fructose by sorbitol dehydrogenase (SdhA) in *A. niger* [20]. The expression of gene AN2666, the ortholog of *sdhA* in *A. nidulans*, was highly induced in the reference strain and its expression level slightly reduced in all mutants on D-galactose, but significantly reduced in $\Delta galX$, indicating the expression of *sdhA* is regulated by GalX, GalR and AraR (Fig. S2). The single deletion of *galX* had the strongest effect on expression of *sdhA*, but the expression of this gene was not abolished in the triple deletion mutant on D-galactose. The residual expression of *sdhA* could be caused by the regulation of XlnR, because the previous study demonstrated that this gene is also regulated by XlnR [28]. These results confirmed that both *ladB* and *sdhA* were highly induced by D-galactose and regulated by different regulators, so they are involved in D-galactose oxido-reductive pathway. Our results also confirmed that GalX directly regulates these two enzymes of D-galactose catabolism in *A. nidulans*. The last step of D-galactose oxido-reductive pathway is catalyzed by hexokinase. The expression of *hxkA* (AN7459) was not significantly affected in all mutants, showing a constitutive level of expression. Growth was not abolished on D-galactose when *galR* and/or *galX* were absent (Fig. 2). The unaffected Leloir pathway can explain the residual growth of these mutants on D-galactose and D-galactose oxido-reductive pathway can be the preferred pathway in *A. nidulans* as previously proposed [28].

3. Conclusions

In summary, the results showed that the regulation of D-galactose catabolism is highly complex and that at least four regulators are involved in its regulation (GalR, GalX, XlnR, AraR). The growth phenotypes and sugar utilization on the mixture of D-galactose and L-arabinose demonstrated a clear role for AraR in D-galactose utilization. The transcriptome data indicated that GalX has a wider effect on regulation of genes involved in D-galactose and D-glucose catabolism than GalR and AraR, while the regulatory function of GalR is not notable on the expression of catabolic genes. It may therefore indicate that GalR mainly regulates D-galactose transport based on sugar utilization study. Several enzymes in D-galactose oxido-reductive pathway remain to be confirmed, and studying these enzymes may be able to provide more detailed evidences about the regulatory role of GalR. The compensation phenomenon between different regulators was also confirmed in the present study as previously described, which can ensure the fungus quickly adapt constantly changing environment.

4. Experimental Procedures

4.1. Strains, media and culture conditions

Escherichia coli DH5 α was used for plasmid construction and was grown on Luria-Bertani (LB) medium supplemented with 50 μ g/mL ampicillin. *A. nidulans* strains used in this study were deposited at the CBS culture collection of Westerdijk Fungal Biodiversity Institute with numbers shown in Table S2. The reference strain *A. nidulans* FGSC A1149 is a uracil auxotrophic and *nkuA* deletion strain used as a parental strain for transformation and efficient gene knockouts. *A. nidulans* strains were grown at 37°C on Complete Medium (CM) or Minimal Medium (MM) supplemented with required carbon source [32]. Solid media were amended with 1.5 % (w/v) agar. 1.22 g/L uridine and 1 mg/L pyridoxine were supplemented for all auxotrophic strains, and 1.3 mg/mL 5-fluoroorotic acid (5-FOA) was added in the solid medium for counter selecting colonies containing the *pyrG* marker gene on ANEp8-Cas9 plasmids.

A. nidulans strains were grown on CM plates with 1% D-glucose at 37°C for 5 days. Spores were harvested in ACES buffer and were counted using a haemocytometer. Solid MM was used for growth profiles supplemented with different monosaccharides, including 25 mM D-glucose, 25 mM galactitol, 25 mM D-galactose, 25 mM L-arabinose, 5 mM L-arabinose, 2 mM L-arabinose,

1 mM L-arabinose, 0.5 mM L-arabinose, 0.2 mM L-arabinose, 0.1 mM L-arabinose and 0.05 mM L-arabinose as well as mixtures of L-arabinose with these concentrations and 25 mM D-galactose. 200 spores in 5 μ L ACES buffer were inoculated on the plates and incubated at 37°C for 5 days.

4.2. Construction of deletion mutants

The CRISPR/Cas9 system used for the construction of deletion mutants was described previously [33]. The gRNA sequences of all ANEp8-Cas9 plasmids were identified using Geneious R11 software (<https://www.geneious.com>) based on *A. niger* NRRL3 genome. The gene deletion cassettes were constructed by fusion of upstream and downstream DNA fragments in a PCR reaction using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Nieuwegein, The Netherlands). Upstream and downstream DNA fragments were amplified using genomic DNA of *A. nidulans* FGSC A1149 as template. A barcode sequence was used as overlapping region for the fusion of two fragments. The primers used in this study are listed in Table S3.

A. nidulans protoplasting and transformation were performed as previously described [34]. Each transformation required 1 μ g ANEp8-Cas9 plasmid and 5 μ g corresponding gene deletion cassette. Several colonies from transformation plates were selected and purified by single colony streaking on MM plates twice. Colonies were subsequently re-cultivated twice on MM plates with uridine to remove the self-replicating ANEp8-Cas9 plasmid. Genomic DNA of putative mutants was isolated and used as a template of colony PCR. Correct mutants were confirmed by amplifying the target region flanking the Cas9 cut site using primers listed in Table S3. All mutants lacking ANEp8-Cas9 plasmid were screened by growth on MM plates containing 5-fluoroorotic acid (5-FOA) before deposit to the CBS culture collection.

4.3. Transfer experiment, RNA isolation and transcriptome analysis

The transfer experiment was performed in biological triplicate. 10⁶ spores/mL were inoculated to 250 mL CM with 2% D-fructose in 1 L Erlenmeyer flasks for precultures and incubated in rotary shakers at 37°C, 250 rpm, for 16-18 hours. The mycelia were harvested by filtration on cheesecloth under sterile conditions and washed with MM. Equal amount of mycelia was transferred to 50 mL MM in 250 mL Erlenmeyer flasks supplemented with 25 mM D-glucose, 25 mM D-galactose, 5 mM L-arabinose and a mixture of 25 mM D-galactose and 5 mM L-arabinose, and were incubated in rotary shakers at 37°C, 250 rpm. After 2 hours incubation, mycelia were harvested by vacuum filtration, dried between tissue paper and frozen in liquid nitrogen. All samples were stored at -80°C for RNA isolation.

The transcriptomes of all strains were analyzed by RNA-seq. Total RNA was extracted from ground mycelia using TRIzol reagent (Invitrogen, Merelbeke, Belgium) and NucleoSpin RNA Clean-up Kit (Macherey-Nagel, Düren, Germany). The quality and quantity of RNA samples were analyzed by a RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies, Middelburg, The Netherlands). Purification of mRNA, synthesis of cDNA library and sequencing were conducted at DOE Joint Genome Institute (JGI) as described previously [35]. Briefly, RNA sample preparation was performed using the Illumina TruSeq Stranded preparation kit and following Illumina poly-A selection protocol. The prepared libraries were quantified using qPCR and then sequenced on the Illumina NovaSeq sequencer following a 2×150 indexed run recipe.

Using BBDDuk (<https://sourceforge.net/projects/bbmap>), raw reads were evaluated for artifact sequence by kmer matching (kmer=25), allowing one mismatch and detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method set at Q6. Finally, following trimming, reads under the length threshold were removed (minimum length 25 bases or one third of the original read length – whichever was longer). Filtered reads from each library were aligned to the *A. niger* NRRL3 (http://genome.jgi.doe.gov/Aspni_NRRL3_1) genome using

HISAT2 version 2.1.0 [36]. FeatureCounts [37] was used to generate the raw gene counts using gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts (-s 2 -p -primary options). The reads from all RNAseq samples were deposited at the Sequence Read Archive NCBI with sample accession numbers SRP296258-SRP296269, SRP296271-SRP296281, SRP296282-SRP296292, SRP307787-SRP307798, SRP307809-SRP307820, and SRP307825-SRP307836.

Statistical analysis was performed using DESeq2 [38]. Transcripts were considered as differentially expressed if the DESeq2 fold change was > 2 and $\text{Padj} < 0.01$. Two heat maps were drawn using GraphPad Prism (<https://www.graphpad.com/>).

4.4. Enzyme activity assays

Pre-cultures were inoculated with 10^6 spores/mL and were grown for 16 hours with CM containing 2% D-fructose, 1.22 g/L uridine and 1 mg/L pyridoxine. Cultures were incubated at 37°C in 500 mL Erlenmeyer flasks containing 100 mL aliquots in a rotary shaker at 200 rpm. Samples were taken right before mycelial transfer. Mycelia were then harvested by filtration on a sintered glass funnel without suction, washed with MM without carbon source and transferred into fresh MM with 25 mM D-galactose + 5 mM L-arabinose, supplemented with 1.22 g/L uridine and 1 mg/L pyridoxine (referred to as main culture). Samples were taken after 4 hours of incubation to assess induction ability. Preliminary trails had established that 4 hours of contact is the time lapse in which maximal induced enzyme activity levels were achieved, with a minimal variation in the biomass concentration. By that time, both L-arabinose and D-galactose have been started to be taken up simultaneously.

To obtain a cell-free extract, 10 mL of culture broth was withdrawn and suction-filtered, and then the harvested mycelia was thoroughly washed with the corresponding buffer used for the respective enzyme activity measurements. The biomass was resuspended in 5 mL of the same buffer, and homogenized in a pre-cooled Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 20,000 x g (20 min, 4°C), and the supernatant immediately used to assay the respective enzyme activities.

Galactokinase activity assay was based on the detection of galactose-1-phosphate in 1 mL of a reaction mixture containing 10 mM ATP, 20 mM D-galactose, 10 mM MgSO_4 , and 0.7 mL crude extract in a 0.1 M phosphate buffer, pH 7.6. The assay was performed at 37°C. The reaction was initiated by the addition of D-galactose, allowed to proceed for 30 min, and then terminated by chilling the mixture on ice. Sulphate was removed by precipitation with an equimolar amount of $\text{Ba}(\text{OH})_2$, followed by centrifugation (room temperature, 20,000 x g, 20 min). The supernatant was assayed for the presence of galactose-1-phosphate by HPLC using an H^+ exchange column (Bio-Rad) at 30°C with 25 mM H_2SO_4 as mobile phase with isocratic elution and a refractive index detection. Within the time and conditions of the assay, the formation of galactose-1-phosphate was linear with respect to time.

L-arabitol dehydrogenase activity assay was performed as described previously [39]. The respective L-arabitol and galactitol concentrations in the assay were 100 mM. The reaction was allowed to proceed for 1 hour at 37°C, and was then terminated by boiling the mixture for 5 min. The reaction mixture was centrifuged (room temperature, 20,000 x g, 20 min) and the supernatant used for HPLC analysis.

Specific enzyme activity values are related to mg protein, which was determined by means of a modified Lowry method [40], using BSA for calibration.

4.5. Sugar utilization rates

The concentration of D-glucose, D-galactose and L-arabinose in the culture broth was determined by HPLC analysis, using an H^+ exchange column (Bio-Rad Aminex HPX- H^+ ; Hercules, CA,

USA), employing 10 mM H₂SO₄ at 55°C as mobile phase. Compounds were detected by means of a refractive index detector [41]. Each point is the result of two averaged measurements, which deviated by not more than 5%.

Acknowledgements

We would like to thank Chinese Scholarship Council (CSC) for the financial support to J.M. (CSC student number: CSC201907720027). S.G. was supported by a grant of the Applied Science Division (TTW) of NWO and the Biotechnology and Safety Program of the Ministry of Infrastructure and Water Management 15807 to R.P.dV. The Academy of Finland grant number 308284 to M.R.M. is acknowledged. This research was supported by the Hungarian National Research, Development and Innovation Fund (grants NN128867 to LK and K138489 to EF).

Author contributions

J.M. performed experiments, analyzed data, and wrote the original manuscript. Z.N. performed experiments and analyzed data. M.P., A.L., V.N., E.S., Y.Z. and I.V.G. performed transcriptomic analysis. S.G. supervised part of the research. E.F. and L.K. designed experiments and supervised part of the research. M.R.M. and R.P.dV. designed the experiments, supervised the overall research, and reviewed and edited the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare no competing interests.

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Supporting information

Supplementary data to this article can be found online at <https://doi.org/10.1111/1751-7915.14025>.

Figure S1. Expression profiles of genes encoding putative MFS transporters in the reference strain and mutants on different carbon sources. The color code represents averaged and logged expression values (FPKM + 1) of triplicates. glc = 25 mM D-glucose, gal = 25 mM D-galactose, ara = 5 mM L-arabinose, gal+ara = 25 mM D-galactose + 5 mM L-arabinose.

Figure S2. Expression profiles of genes encoding related transcription factors and enzymes involved in the PCP and D-galactose catabolic pathway in the reference strain and mutants on different carbon sources. The color code represents averaged and logged expression values (FPKM + 1) of triplicates. glc = 25 mM D-glucose, gal = 25 mM D-galactose, ara = 5 mM L-arabinose, gal+ara = 25 mM D-galactose + 5 mM L-arabinose.

Table S1. Specific enzyme activities ($\text{U mg}_{\text{protein}}^{-1}$) detected from mycelial extracts of *A. nidulans* strains. Upper table panel: pre-culture. Lower table panel: main culture.

Table S2. *Aspergillus nidulans* strains used in this study.

Table S3. Primers used in this study. The guide RNAs (gRNA) for gene deletion are marked in red and the linkers are shown in lowercase.

Chapter 6

Summary & General Discussion

Aspergillus species are among the most common and important filamentous fungi with crucial roles in natural ecosystems and the human economy, although some of them are plant and/or animal pathogens [1,2]. They can produce numerous extracellular enzymes of industrial interest, as well as organic acids and secondary metabolites of major significance in biotechnology [3–6]. The significant development in *Aspergillus* omics deepens the understanding of this extremely diverse genus, and CRISPR/Cas9 technology also remarkably contributes to genetic research and industrial applications of *Aspergillus* strains [2,7–9].

To engineer a more robust and versatile filamentous fungal cell factory, a thorough and comprehensive understanding of carbon utilization and regulatory mechanisms in *Aspergillus* species is crucial. Advances of the omics technologies (i.e. genomics, transcriptomics, proteomics and metabolomics) allows the important development of systems biology in *Aspergilli* and improvement of metabolic engineering strategies [2,10]. Mathematical modeling is the core of systems biology in order to integrate detailed information and quantitatively analyze complex biological processes, thereby predicting physiological changes in the organism or elucidating mechanisms underlying experimental observations [11]. Genome-scale metabolic models are available for several *Aspergillus* species, such as *A. niger* and *A. nidulans* [12,13], providing key aspects of metabolism and its regulatory network. Besides, the key enzymes and reactions of primary carbon metabolism have been identified to validate the recently manually curated model of *A. niger*, thereby improving our understanding of the *A. niger* metabolic network and offering more detailed information for metabolic engineering [14–19].

The main aim of this thesis was to improve our understanding of metabolic and regulatory mechanisms in *A. niger* and *A. nidulans*, and subsequently apply the fundamental knowledge to biotechnological applications, such as the production of intermediates of the Pentose Catabolic Pathway (PCP). Additionally, sugar transporters in *A. niger*, especially those involved in pentose and pentitol utilization, were investigated to gain a better understanding of the complexity and redundancy of sugar transporters in fungi, which may be a promising target for fungal metabolic engineering to improve the production of desired biochemicals from plant biomass.

Metabolic engineering of *A. niger* for efficient xylitol production from plant biomass

Xylitol is one of the top value-added chemicals derived directly from biomass, and is produced industrially by catalytic hydrogenation of D-xylose, a major component of hemicellulose [20]. Although chemical production is currently the main route, microbial processes are more sustainable alternatives due to less harsh production conditions and the possibility to use the abundant and renewable lignocellulosic biomass. Filamentous fungi, such as *Aspergillus oryzae* and *Trichoderma reesei*, have been studied for xylitol production using D-xylose or xylan as substrates, but low yields have been obtained [21,22]. Increasing the expression of D-xylose reductase and reducing xylitol dehydrogenase expression/activity are the most commonly used metabolic engineering strategies for improving xylitol production from raw materials [23]. In *A. oryzae*, the disruption of *xdhA* increased the xylitol production from D-xylose and oat spelt xylan [21], but no further increase in xylitol production from D-xylose was observed in the double deletion mutant of *xdhA* and *ladA* compared to the single deletion mutants of those two genes [24]. The overexpression of the D-xylose reductase gene (*xylI*) and antisense inhibition of D-xylulokinase gene (*xyiH*) in *T. reesei* Δxdh and wild-type (QM9414) strains, respectively, both increased xylitol production from D-xylose [22].

The identification of all enzymes and reactions of *A. niger* PCP facilitates the application of metabolic engineering in this fungus for xylitol production [16]. The intracellular accumulation of xylitol and/or L-arabitol from D-xylose and L-arabinose, respectively, was observed in all deletion mutants of metabolic genes involved in two conversions of xylitol to D-xylulose and D-xylulose to D-xylulose-5-phosphate (*xdhA/ladA/sdhA/xkiA*). Contrary to the observation in *A. oryzae*, the levels of xylitol

on D-xylose and L-arabitol on L-arabinose in the triple mutant $\Delta ladA\Delta xdhA\Delta sdhA$ were higher than those observed in the single or double deletion mutants of *ladA*, *xdhA*, and *sdhA* [16]. The single deletion of *lxrA* or *lxrB* encoding two L-xylulose reductases resulted in accumulation of xylitol or L-arabitol on D-xylose and L-arabinose, respectively, and a 2-fold higher level of L-arabitol on L-arabinose was observed in the double deletion mutant of these two genes. These results demonstrated that blocking a specific step completely requires the deletion of all genes involved in it and thereby results in the accumulation of upstream intermediates to desired levels. In **Chapter 2**, all metabolic mutants showed increased accumulation of extracellular xylitol from 25 mM D-xylose compared to the reference strain, but these levels of xylitol in $\Delta xkiA$ and $\Delta ladA\Delta xdhA\Delta sdhA$ were much lower than intracellular levels in the previous study [16]. Therefore, the improvement of xylitol secretion may be able to relieve product feedback inhibition of PCP enzymes in the fungal cells and subsequently promote the synthesis of xylitol from D-xylose, which also contributes to the recovery of xylitol from fermentation broth with high purity.

The feedback inhibition of D-xylulose after the deletion of *xkiA* may result in the decreased dehydrogenase activities (XdhA/LadA/SdhA) or expression of their corresponding genes in $\Delta xkiA$, followed by the increased level of xylitol in $\Delta xkiA$. Higher levels of xylitol (2.74 and 3.03 mM) were produced by $\Delta xkiA$ at higher D-xylose concentrations (1% and 2% D-xylose), and they were also slightly higher than those of $\Delta ladA\Delta xdhA\Delta sdhA$ (2.23 and 2.26 mM) under the same conditions (**Chapter 2**). The deletion of *xkiA* seems to be more effective on xylitol accumulation at higher D-xylose concentrations than the deletion of three dehydrogenase encoding genes in *A. niger*. Conversely, xylitol production of the *T. reesei* Δxdh (22.8 mM) was 2.6-fold higher than that of strain S6-2-2 (8.6 mM) containing the partially silenced *xyiH* from 25 g/L D-xylose and 10 g/L D-glucose [22]. That could be explained by the partially silenced *xyiH* gene leading to much lower expression of *xyiH* in strain S6-2-2 than in the wild-type strain QM9414. However, expression of *xyiH* was not abolished, which may mean that xylitol conversion still occurs to some degree, which may reduce the accumulation of xylitol. It indicates that the complete block of xylitol assimilation is crucial for the accumulation of xylitol as mentioned above. Multi-omics and enzyme activity analysis could provide more information about the different effects of deletions of *xdhA/ladA/sdhA* and *xkiA* on xylitol accumulation in *A. niger* at higher D-xylose concentrations (1% and 2% D-xylose). The combined mutants $\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$ and $\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$ produced similar amounts of xylitol from D-xylose as the triple mutant $\Delta ladA\Delta xdhA\Delta sdhA$, demonstrating that already fully blocking one specific step of xylitol conversion is very effective for xylitol accumulation.

The ability of *A. niger* to produce xylitol from lignocellulosic biomass was confirmed in **Chapter 2**. Four metabolic mutants all produced xylitol from beechwood xylan and wheat bran, although this is a transient situation for some mutants, especially $\Delta xkiA$. The xylitol levels in $\Delta xkiA$ even reduced to zero after 48 h of cultivation on wheat bran, in contrast to its good performance on pure D-xylose. The expression levels of the other PCP genes (*larA*, *xylA*, *xylB*, *ladA*, *xdhA*, *sdhA*, *lxrA* and *lxrB*) were significantly reduced in $\Delta xkiA$ after 24 h [18], which could be a reason of the highly reduced xylitol production from wheat bran in $\Delta xkiA$ after 24 h of cultivation. The triple mutant $\Delta ladA\Delta xdhA\Delta sdhA$ was the best xylitol producing strain under these conditions. The reduced expression levels of *larA*, *xylA* and *xylB* in $\Delta ladA\Delta xdhA\Delta sdhA$ after 24 h [18], most likely because of the feedback inhibition of intracellularly accumulated xylitol, did not result in obviously enhanced xylitol levels after 48 h of cultivation. Moreover, the huge differences of xylitol production from tested biomass substrates showed that the composition of diverse lignocellulosic biomass strongly affects xylitol production in *A. niger* and that the D-xylose amount in the certain substrate alone does not guarantee xylitol production. Therefore, the choice of lignocellulosic biomass plays an important role in xylitol bioproduction next to strain engineering. The xylitol production from wheat bran and cotton seed hulls by this triple mutant is lower than in other published fungal strains, especially yeasts, but those studies used pretreated biomass resources, such as biomass hydrolysates [25]. The direct use of lignocellulosic biomass by *A. niger* highly simplifies the xylitol bioproduction process

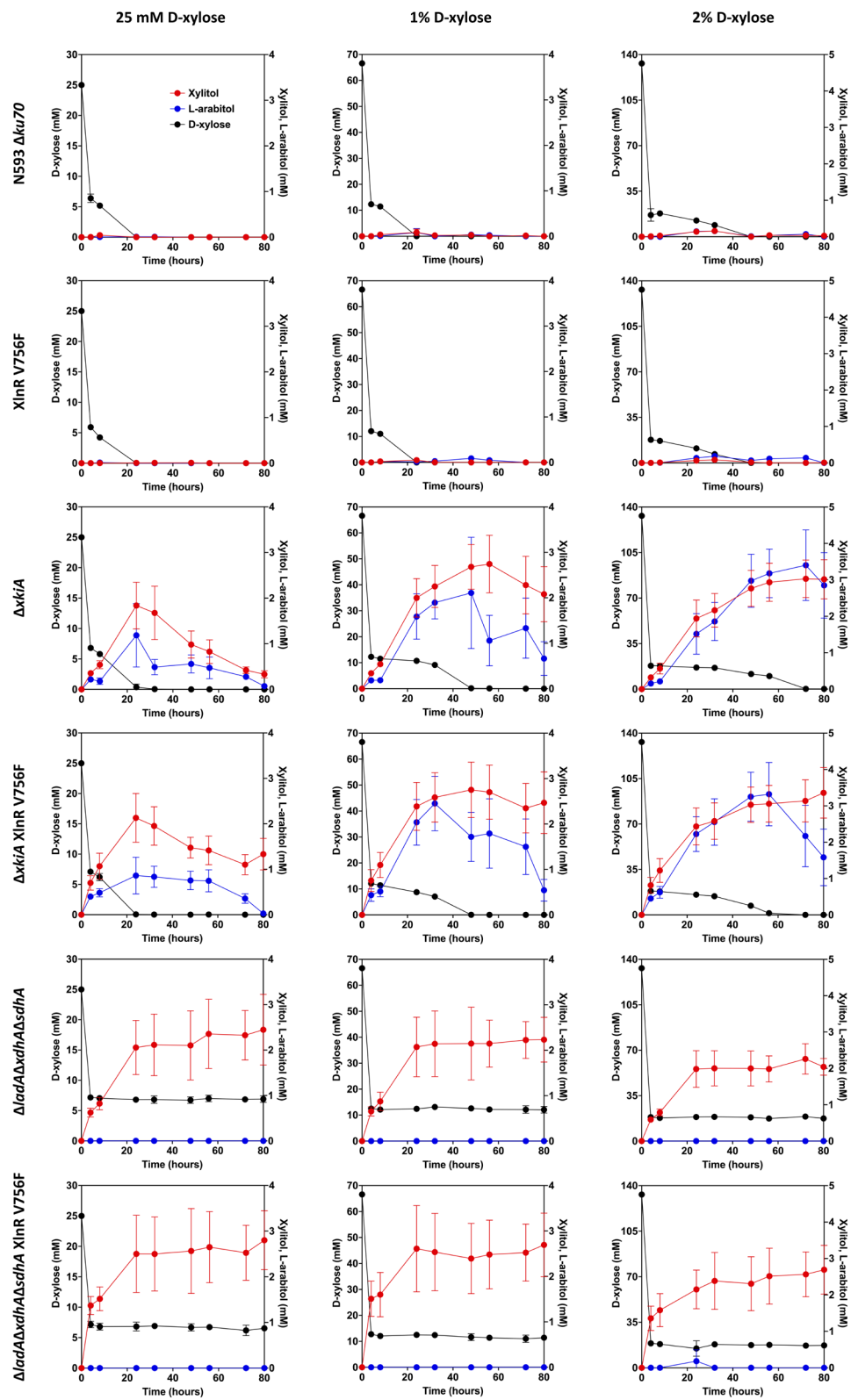


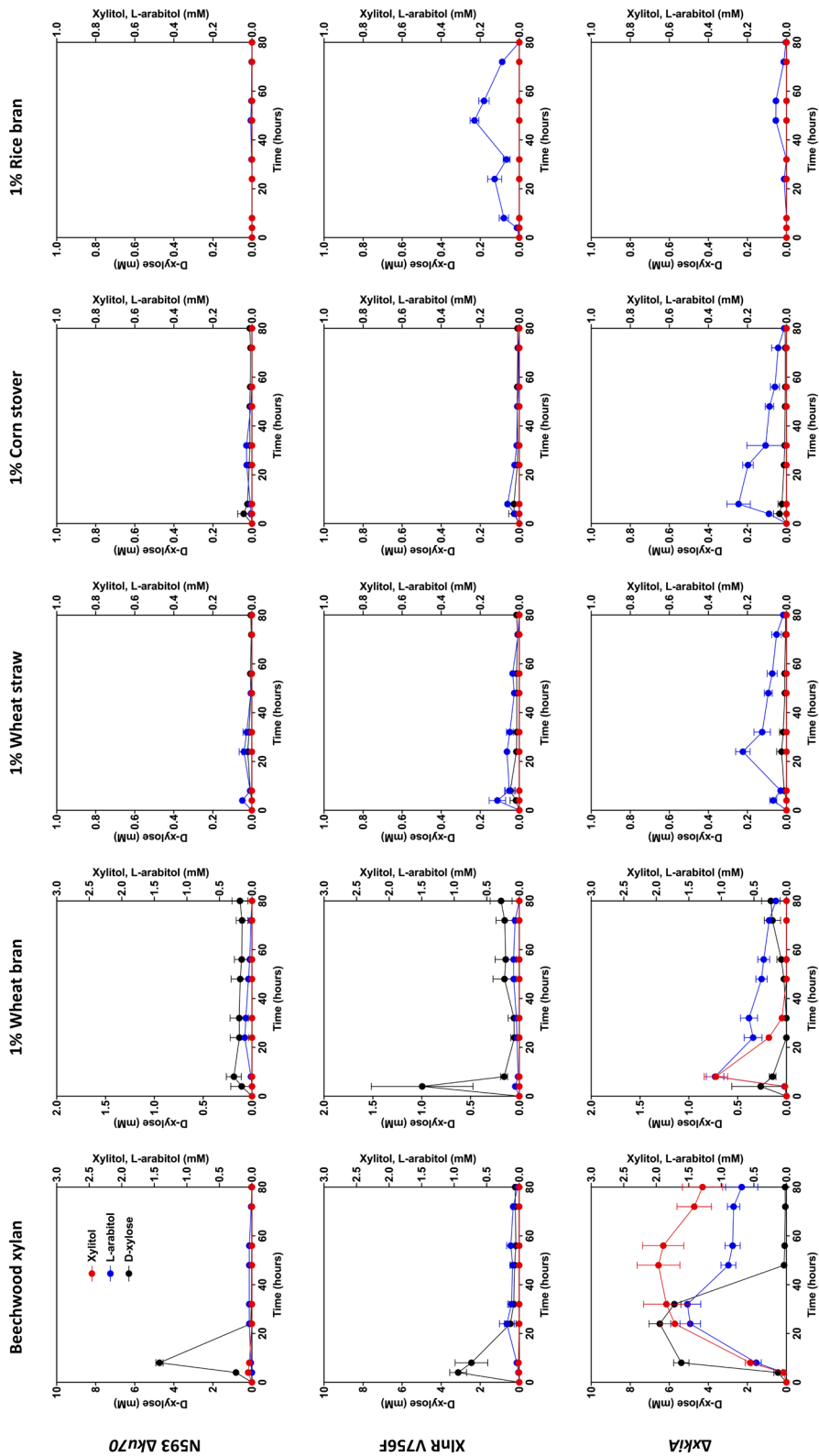
Figure 1. Xylitol production from D-xylose by *A. niger* reference strain (N593 $\Delta ku70$) and metabolic mutants. Strains and substrates are indicated in the figure. The error bars indicate the standard deviation between biological triplicates.

for industrial application. This would become an attractive alternative if the xylitol levels can be improved sufficiently.

Metabolic engineering is an effective strategy for the improvement of xylitol production by *A. niger*, as confirmed in other xylitol producing strains. Further optimization of xylitol titers may be achieved using a number of approaches. Two transcription factors, XlnR and AraR, control the expression of the main hemicellulolytic enzymes and PCP pathway encoding genes [26,27]. The constitutively active AraR has been shown to result in constitutive production of arabinanases under derepressing conditions [28]. Constitutively active XlnR resulted in increased production of plant biomass degrading enzymes, and subsequently enhanced pentose release from wheat bran [9]. These two regulators could be manipulated to increase the production of plant biomass degrading enzymes, and subsequently the release of D-xylose and L-arabinose from plant biomass to improve xylitol production. Therefore, the constitutively active version of *xlnR* (XlnR V756F) was also introduced into two mutants $\Delta xkiA$ and $\Delta ladA\Delta xdhA\Delta sdhA$, respectively. The mutation XlnR V756F increased xylitol titers from all tested carbon sources in $\Delta ladA\Delta xdhA\Delta sdhA$, while it only enhanced xylitol titers from 25 mM and 2% D-xylose in $\Delta xkiA$ (Fig. 1 and 2). Increased D-xylose uptake rates were also observed in two combined mutants containing the constitutive XlnR compared to $\Delta xkiA$ and $\Delta ladA\Delta xdhA\Delta sdhA$, respectively, at higher D-xylose concentrations (1% and 2% D-xylose), indicating that the mutation XlnR V756F may also affect D-xylose transport of *A. niger*. Higher and/or earlier release of D-xylose was due to the mutation XlnR V756F from beechwood xylan, wheat bran and wheat straw (Fig. 2), confirming the function of this mutation as mentioned previously [9]. These results showed that the manipulation of XlnR is a promising strategy for improvement of xylitol production from lignocellulosic biomass, and also confirmed the wide regulatory function of XlnR in the whole process of xylitol production in *A. niger*. In addition, the improvement of pentose reductases, pentose import and pentitol export should also be considered to further stimulate xylitol production.

The complexity and redundancy of sugar transport in *A. niger*

The overall process of biofuel and biochemical production from lignocellulosic biomass by filamentous fungi includes the release of monosaccharides (mostly D-glucose and D-xylose) from (pretreated) biomass substrates by plant biomass degrading enzymes, sugar uptake of the released sugars by diverse sugar transporters and the fermentation of released sugars to desired end products. As sugar uptake is a key step in this process, increasing sugar uptake efficiency may contribute to improved biofuel and biochemical production, such as xylitol accumulation from D-xylose as mentioned above. Several transporters involved in D-xylose uptake have been characterized from fungi (**Chapter 3**), and transport optimization for improved D-xylose uptake have also been used for increasing xylitol production [25]. The previous study for xylitol production from lignocellulosic biomass showed that the amount of released D-xylose from biomass was low in the *A. niger* cultures (0 - 2 mM), suggesting that high affinity D-xylose transporters could play a dominant role in taking up D-xylose to *A. niger* cells (**Chapter 2**). In *A. niger*, only three D-xylose transporters, XltA, XltB and XltC, have been functionally validated and biochemically characterized in *S. cerevisiae* [29]. The results showed that XltA and XltB may be responsible for D-xylose uptake in *A. niger*. The gene NRRL3_02351 from *A. niger* is the homolog of the known pentose transporter XAT-1 from *Neurospora crassa*, with high affinity for D-xylose. Therefore, XltA, XltB and NRRL3_02351 (XltD) were investigated in *A. niger* by growth profiling and sugar uptake experiments (**Chapter 3**). The results demonstrated that XltA plays an important role in D-xylose transport in *A. niger in vivo*,



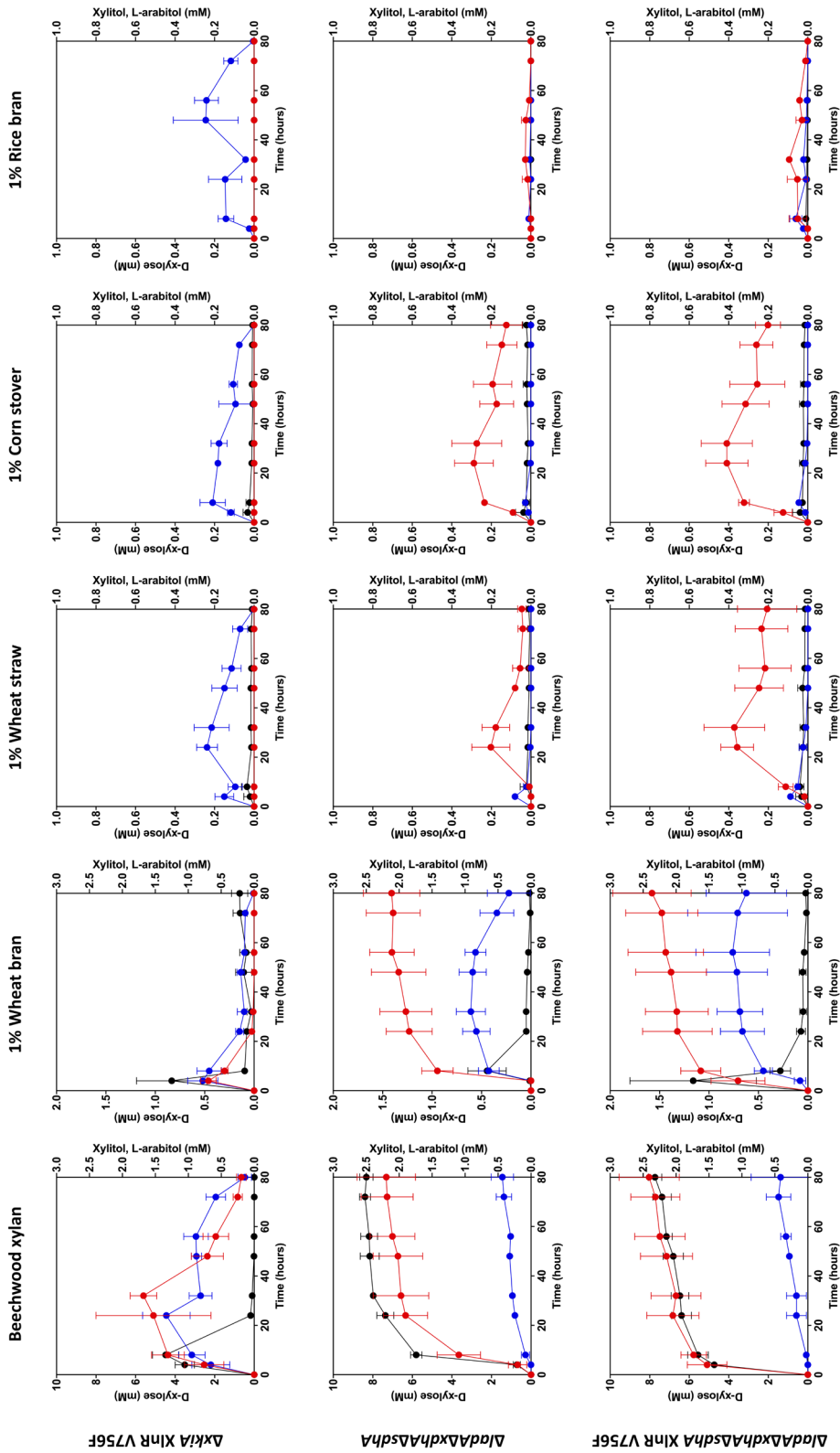


Figure 2. Xylitol production from beechwood xylan and diverse lignocellulosic biomass by *A. niger* reference strain (N593 $\Delta ku70$) and metabolic mutants. Strains and substrates are indicated in the figure. The error bars indicate the standard deviation between biological triplicates.

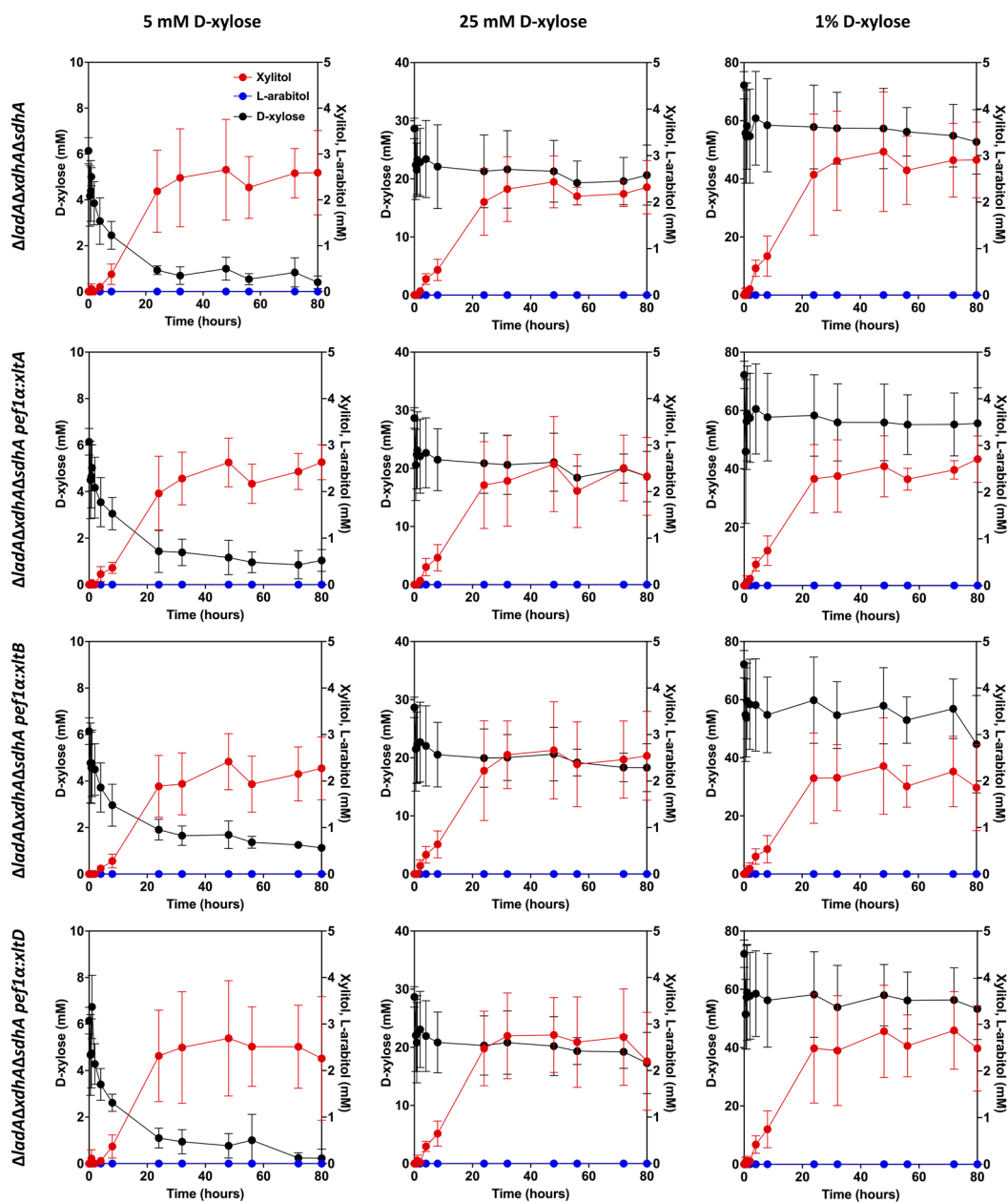


Figure 3. Xylitol production from D-xylose by the triple mutant $\Delta ladA\Delta xd hA\Delta sd hA$ and its overexpression mutants of three D-xylose transporter genes, *xtlA*, *xtlB* and *xtlD*. Strains and substrates are indicated in the figure. The error bars indicate the standard deviation between biological triplicates.

but XltB may not be a major D-xylose transporter. XltD is also involved in D-xylose uptake with similar efficiency as XltA. These results also indicated that there are more transporters involved in D-xylose utilization, such as MstA from *A. niger*, which is a high-affinity sugar/H⁺ symporter and able to transport D-glucose, D-fructose, D-xylose and D-mannose, which could compensate the loss of a certain D-xylose transporter [30]. The existence of more D-xylose transporters correlates with

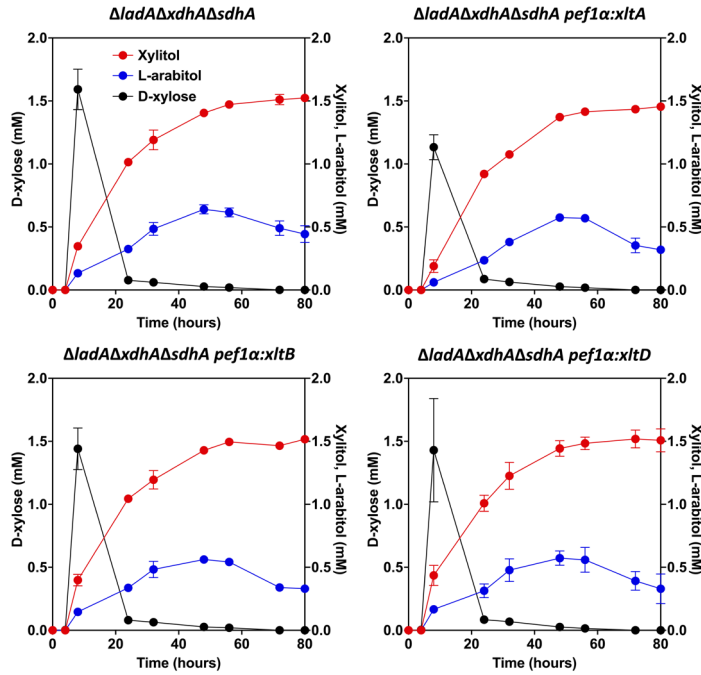


Figure 4. Xylitol production from wheat bran by the triple mutant $\Delta ladA\Delta xdhA\Delta sdhA$ and its overexpression mutants of three D-xylose transporter genes, *xltA*, *xltB* and *xltD*. The error bars indicate the standard deviation between biological triplicates.

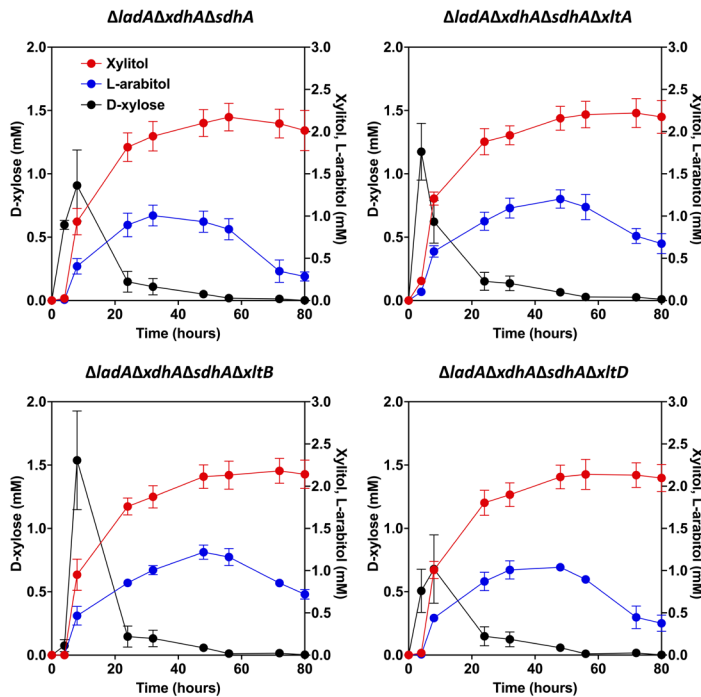


Figure 5. Xylitol production from wheat bran by the triple mutant $\Delta ladA\Delta xdhA\Delta sdhA$ and its deletion mutants of three D-xylose transporter genes, *xltA*, *xltB* and *xltD*. The error bars indicate the standard deviation between biological triplicates.

the high expression of many other putative transporter-encoding genes on D-xylose in *A. niger* [31]. D-xylose is the second most abundant sugar after D-glucose in the natural environment of *A. niger*, so the presence of several D-xylose transporters lets *A. niger* use this sugar efficiently. The same phenomenon is also observed in other fungi, such as *Candida intermedia*, *Pichia stipitis*, *N. crassa* and *T. reesei* [32–37].

The effect of the respective deletion and overexpression of these three D-xylose transporters on xylitol production was also evaluated in the triple mutant $\Delta ladA\Delta xdhA\Delta sdhA$. Unfortunately, the modification of one D-xylose transporter in *A. niger* alone did not have an obvious effect on xylitol production from pure D-xylose and wheat bran (Fig. 3-5). This confirmed that more D-xylose transporters are involved in *A. niger* as mentioned above, and other transporters can compensate for the loss of a specific transporter. It also showed the complexity and redundancy of sugar transport system similar to sugar metabolism in *A. niger* [16], and modification of several D-xylose transporters together may be necessary for the improvement of xylitol production.

Increased L-arabitol accumulation from pure D-xylose and lignocellulosic biomass was observed together with xylitol accumulation in *A. niger* metabolic mutants compared to the reference strain (**Chapter 2**). For example, the $\Delta xkiA$ strain produced a maximum extracellular L-arabitol titer of 1.19, 2.11 and 3.40 mM on 25 mM, 1% and 2% D-xylose, which was 132, 25 and 22 times higher, respectively, than detected in the cultures of the reference strain. A large amount of L-arabitol was observed in the culture of $\Delta ladA\Delta xdhA\Delta sdhA$ from 1% sugar beet pulp (2.08 mM). It indicated the great potential of *A. niger* and efficient metabolic engineering strategies for L-arabitol production. L-arabitol is an intermediate of the PCP in fungi, but it can also be used as a carbon source by many fungi, suggesting the occurrence of polyol transporters. In **Chapter 4**, a specific L-arabitol transporter, LatA, was identified in *A. niger*, and it was shown that the homologs of LatA are widely present in Ascomycete fungi. The impaired growth of $\Delta latA$ on L-arabitol, compared to the reference strain, indicated that LatA is the main L-arabitol transporter in *A. niger*. It also suggested that there is no redundancy of L-arabitol transport in *A. niger* during growth on L-arabitol, which is a clear contrast with the high number of D-glucose and D-xylose transporters, most likely due to the much more abundant presence of D-glucose and D-xylose than L-arabitol in the natural environment of *A. niger*. Moreover, L-arabitol production from wheat bran and sugar beet pulp was increased in $\Delta ladA\Delta xdhA\Delta sdhA$ by the deletion of *latA*, indicating its potential for improving L-arabitol producing cell factories.

Nearly 100 sugar transporter genes are predicted in the *A. niger* genome [38], but only very few transporters have been biochemically characterized in *A. niger*, including four D-glucose transporters [30,39–41], one D-galacturonic acid transporter [42], one L-rhamnose transporter [43] and three D-xylose transporters as mentioned above [29]. The biochemical characterization in *S. cerevisiae* remains to be performed for the novel D-xylose transporter (XltD) and L-arabitol transporter (LatA) to study their sugar specificity and kinetic properties (**Chapters 3 and 4**). The identification of these two transporters deepens the understanding of the complex sugar transport system in *A. niger*, but it is still far away from complete compared to the well-studied *S. cerevisiae* [44,45]. Recently, the comprehensive *in silico* characterization of the sugar transportome of *A. niger* not only provided new insights on the physiological role of sugar transporters on fungal growth, but also provided new target genes for rational engineering of *A. niger* [31].

The simultaneous utilization of diverse sugars and complex regulatory network of sugar utilization

Fungi are confronted with a heterogeneous mixture of carbon sources in natural biotopes, and therefore a complex transcriptional regulatory network controls the expression of the genes required for extracellular polysaccharide degradation, sugar transport into the fungal cells and subsequent intracellular catabolism of sugar residues, which enables the fungus to respond to changes in the

substrate composition [46]. The high level of complexity and redundancy of *A. niger* central carbon metabolism has been described [16–18,47], and similar characteristics of sugar transporter systems of *A. niger* has also been revealed, even with less detailed studies (**Chapters 3 and 4**). With regard to regulatory network, many transcription factors have been reported in filamentous fungi, and the crosstalk and co-regulation between different transcription factors have also been described [48]. Moreover, regulatory systems are poorly conserved in fungi, most likely because of the need of different fungi to adapt to their specific biotope.

D-galactose is a hexose sugar commonly found in plant cell wall polysaccharides, such as xylan, xyloglucan, arabinogalactan, and rhamnogalacturonan I and II, together with L-arabinose and D-xylose, suggesting that the metabolism of these sugars can be active simultaneously [49–52]. The pentoses L-arabinose and D-xylose are converted via the PCP, and D-galactose is converted by two main pathways in filamentous fungi, including the well-known Leloir pathway and the alternative D-galactose oxido-reductive pathway [53]. Some enzymes and intermediates of these pathways differ among fungal species, especially in the oxido-reductive pathway, which involves some enzymes from the PCP in some fungi [54–58]. For example, galactitol is converted to L-sorbose in the oxido-reductive pathway of *A. nidulans* rather than L-xylo-3-hexulose in *A. niger* [54]. Besides, not all enzymes of the oxido-reductive pathway in two species have been identified [47,59].

A previous study showed that D-galactose and L-arabinose can be used simultaneously in *A. nidulans*, and genes of D-galactose catabolism also can be induced by L-arabinose [59,60]. It revealed that two transcriptional regulators XlnR and AraR not only control PCP genes, but also genes of the oxido-reductive D-galactose catabolic pathway, suggesting an interaction between three transcriptional regulators (XlnR, AraR and GalR) in D-galactose catabolism [59]. However, another transcriptional regulator GalX was also reported to control D-galactose release and catabolism with GalR in *A. nidulans* [61]. In **Chapter 5**, the interaction between GalX, GalR and AraR in D-galactose and L-arabinose catabolism was investigated, and our results demonstrated that these three regulators, and possibly others, co-regulate D-galactose catabolism in *A. nidulans*. Among them, GalX plays a prominent role in regulation of genes of the D-galactose oxido-reductive pathway and may have an additional role in D-glucose catabolism, while AraR can largely, but not fully compensate for the absence of GalR and/or GalX. The involvement of GalR and/or GalX in regulation of L-arabinose catabolism cannot be excluded yet by the results of this study. It also suggested that AraR, GalR and GalX control D-galactose transport, while L-arabinose transport is fully under control of AraR. The connection between *A. niger* pentose and D-galactose catabolism was also reported, and AraR, XlnR and GalX were all shown to regulate D-galactose catabolism, but with a different relative contribution and role [47]. This study also indicated that GalX affects L-arabinose catabolism in *A. niger*, while most likely GalR and/or GalX partially regulate L-arabinose transport in the presence of D-galactose in *A. nidulans*. In these two species, the deletion of *galR* and/or *galX* did not affect the expression of genes involved in Leloir pathway. However, the Leloir pathway is controlled by AraR and induced by L-arabinose in *A. niger*, which is not the case in *A. nidulans* (**Chapter 5**). Moreover, GalX does not control D-galactose release from oligo- and polysaccharides in *A. niger*, while the deletion of *galR* did not affect the growth of *A. nidulans* on all tested polysaccharides, indicating that it may not control sugar release from polysaccharides. The contribution of GalR and GalX in *A. nidulans* to polysaccharide degradation still needs to be investigated. The differences of the functions of these regulators in two related species reveals the complexity and diversity of the plant biomass conversion regulatory network in filamentous fungi, even between closely related species. To conclude, it is very important to gain the full knowledge of metabolic and regulatory networks, as well as sugar transport systems, of a specific fungal cell factory before engineering it for new products and increased product levels. In this thesis, metabolic engineering strategies have been successfully employed in *A. niger* for xylitol production from lignocellulosic biomass (**Chapter 2**), while other aspects of xylitol production also have been considered, including transcriptional regulator network and sugar transport systems in *A. niger* (**Chapter 3**). The identification of two

novel sugar transporters (**Chapters 3 and 4**) also deepens the understanding of complex transport systems of *A. niger*. Additionally, the utilization of two different monosaccharides (D-galactose and L-arabinose) in *A. nidulans* was studied, regarding of connections between their related metabolic pathways and co-regulation between several regulators (**Chapter 5**). Therefore, our results strongly improve the knowledge of different aspects of the physiology of *Aspergillus* species and identified several genes and enzymes that are relevant for biotechnological applications. However, additional studies are still required for the new questions that arose in this thesis and other unexplored areas.

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Appendix

English summary

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English summary

Lignocellulosic biomass is an abundant and renewable resource, and has a promising potential as an alternative to fossil resources for industrial bioproduction of second-generation biofuels and value-added biochemicals. Filamentous fungi are the most important and efficient plant biomass degrading microorganisms and are widely used as cell factories in many industries. Fungal strain engineering has been applied for the development of more robust and versatile filamentous fungal cell factories, and considerable progress have been made in recent years, as described in **Chapter 1**. A thorough and comprehensive understanding of fungal physiological processes involved in plant biomass utilization is an essential prerequisite of rational and feasible strain engineering. Advances in omics technologies allow the important development of systems biology, and new genetic tools have been developed for improving the efficiency of genetic engineering of filamentous fungi, such as CRISPR/Cas9 technology. *Aspergillus* species have been used for the production of enzymes, organic acids and secondary metabolites for decades. *Aspergillus niger* and *Aspergillus nidulans* are two of the most important model fungi for basic research, and many efforts have been made to study their metabolism and regulatory networks.

The key enzymes and reactions of primary carbon metabolism have been identified in *A. niger*, such as the Pentose Catabolic Pathway (PCP), facilitating the application of this fungus for xylitol production. **Chapter 2** showed that *A. niger* has the capacity to accumulate xylitol from lignocellulosic biomass and metabolic engineering is highly effective for the improvement of xylitol production in *A. niger*. This provides the industrial production of xylitol with an attractive alternative, as the direct use of lignocellulosic biomass by *A. niger* highly simplifies the xylitol bioproduction process. Besides, there are other crucial aspects in the process of xylitol production, which could be alternative targets for strain engineering, including the release of pentoses from lignocellulosic biomass and the transport of pentoses and polyols. The subsequent study showed that the manipulation of the xylanolytic transcriptional activator XlnR also effectively increased xylitol production from lignocellulosic biomass in *A. niger*. The transport of D-xylose was also considered to further stimulate xylitol production in **Chapter 3**. In addition to three characterized D-xylose transporters (XltA, XltB and XltC), a fourth D-xylose transporter (XltD) was identified in *A. niger*. XltD has similar efficiency as XltA, while XltB may be not a major D-xylose transporter under the tested conditions. The results also showed the existence of more D-xylose transporters in *A. niger*, which contributes to the efficient utilization of this sugar by *A. niger*. Unfortunately, the modification of one D-xylose transporter in *A. niger* alone did not affect xylitol production, showing the complexity and redundancy of sugar transport system similar to sugar metabolism in *A. niger*. Therefore, the modification of several D-xylose transporters together for the improvement of xylitol production may be necessary. In **Chapter 2**, the accumulation of L-arabitol from lignocellulosic biomass was observed together with xylitol accumulation in *A. niger*, indicating the potential of *A. niger* for L-arabitol production and the existence of L-arabitol transporters in this fungus. In **Chapter 4**, an L-arabitol transporter, LatA, was identified with high specificity for L-arabitol in *A. niger* and its homologs are widely present in Ascomycete fungi. Moreover, the deletion of *latA* positively affected L-arabitol production from wheat bran and sugar beet pulp, suggesting that this gene could be a target for the improvement of microbial cell factories. In short, the identification of these two novel transporters (XltD and LatA) deepens the understanding of the complex sugar transport system of *A. niger*, but more remain to be further characterized for rational strain engineering.

In *A. nidulans*, D-galactose catabolism and its regulation have been investigated previously, but the alternative D-galactose pathway and additional related transcriptional regulators remained partially unclear. In **Chapter 5**, the interaction between three transcription factors GalX, GalR and AraR in D-galactose and L-arabinose catabolism was investigated in *A. nidulans*, revealing the involvement of all these regulators in D-galactose catabolism and the compensation phenomenon between different regulators. Comparison to a related study of *A. niger* showed that the functions of

these regulators are different in these two closely related species, highlighting the complexity and diversity of regulatory networks in filamentous fungi.

To summarize, the results of this thesis described different aspects of the physiology of *Aspergillus* species from metabolic and regulatory networks to sugar transport systems, which improve the understanding of these model fungi and facilitate the biotechnological applications of fungal cell factories for the production of valuable biochemicals. Our results also raise many new questions for further research and emphasize the importance of the full understanding of fungal metabolism to facilitate strain engineering for better cell factories.

Nederlandse Samenvatting

Lignocellulose biomassa is een veel voorkomende en hernieuwbare hulpbron en heeft een veelbelovend potentieel als alternatief voor fossiele bronnen voor industriële bioproductie van tweede generatie biobrandstoffen en waardevolle biochemicalïen. Filamenteuze schimmels zijn de belangrijkste en meest efficiënte plantenbiomassa-afbrekende micro-organismen die op grote schaal worden gebruikt als cel-fabrieken in veel industrieën. Schimmel stam verbetering is toegepast voor de ontwikkeling van robuustere en veelzijdigere filamenteuze schimmel cel-fabrieken, en er is de afgelopen jaren aanzienlijke vooruitgang geboekt, zoals beschreven in hoofdstuk 1. Een grondig en uitgebreid begrip van fysiologische schimmelprocessen die betrokken zijn bij het gebruik van plantaardige biomassa is een essentiële voorwaarde voor rationele en haalbare stam verbetering. Vooruitgang in omics-technologieën maakt de belangrijke ontwikkeling van systeembiologie mogelijk, en er zijn nieuwe genetische hulpmiddelen ontwikkeld voor het verbeteren van de efficiëntie van genetische manipulatie van filamenteuze schimmels, zoals CRISPR/Cas9-technologie. *Aspergillus* soorten worden al tientallen jaren gebruikt voor de productie van enzymen, organische zuren en secundaire metabolieten. *Aspergillus niger* en *Aspergillus nidulans* zijn twee van de belangrijkste modelschimmels voor fundamenteel onderzoek en er zijn veel inspanningen geleverd om hun metabolisme en regulerende netwerken te bestuderen.

De belangrijkste enzymen en reacties van het primaire koolstofmetabolisme zijn geïdentificeerd in *A. niger*, zoals de Pentose Catabolic Pathway (pentose katabole route, PCP), waardoor de toepassing van deze schimmel voor de productie van xylitol wordt vergemakkelijkt. Hoofdstuk 2 liet zien dat *A. niger* het vermogen heeft om xylitol op te hopen uit lignocellulose biomassa en dat metabolische engineering zeer effectief is voor de verbetering van de xylitol productie in *A. niger*. Dit biedt de industriële productie van xylitol een aantrekkelijk alternatief, aangezien het directe gebruik van lignocellulose biomassa door *A. niger* het bioproductie-proces van xylitol sterk vereenvoudigt. Daarnaast zijn er nog andere cruciale aspecten in het proces van xylitol productie, die alternatieve doelen zouden kunnen zijn voor stam-engineering, waaronder de afgifte van pentosen uit lignocellulose biomassa en het transport van pentosen en polyolen. De daaropvolgende studie toonde aan dat de manipulatie van de xylanolytische transcriptionele activator XlnR ook effectief de xylitol productie uit lignocellulose biomassa in *A. niger* verhoogde. In Hoofdstuk 3 werd ook bestudeert of het transport van D-xylose de xylitolproductie verder stimuleert. Naast drie gekarakteriseerde D-xylose transporters (XltA, XltB en XltC), werd een vierde D-xylose transporter (XltD) geïdentificeerd in *A. niger*. XltD heeft een vergelijkbare efficiëntie als XltA, terwijl XltB mogelijk geen belangrijke D-xylose-transporter is onder de gebruikte condities. De resultaten toonden ook het bestaan van meer D-xylose transporters in *A. niger*, wat bijdraagt aan een efficiënt gebruik van deze suiker door *A. niger*. Helaas had de modificatie van één D-xylose transporter in *A. niger* geen invloed op de xylitol productie, wat de complexiteit en redundantie van het suiker transportsysteem aantoont, vergelijkbaar met het suiker metabolisme in *A. niger*. Daarom kan het nodig zijn om verschillende D-xylose transporters samen te modificeren om de xylitol productie te verbeteren. In Hoofdstuk 2 werd de ophoping van L-arabitol uit lignocellulose biomassa waargenomen samen met de ophoping van xylitol in *A. niger*, wat wijst op het potentieel van *A. niger* voor de productie van L-arabitol en het bestaan van L-arabitol transporters in deze schimmel. In Hoofdstuk 4 werd een L-arabitol transporter, LatA, geïdentificeerd met een hoge specificiteit voor L-arabitol in *A. niger* en zijn homologen zijn wijdverbreid aanwezig in Ascomycete schimmels. Bovendien had de deletie van *latA* een positieve invloed op de productie van L-arabitol uit tarwezemelen en suikerbietenpulp, wat suggereert dat dit gen een doelwit zou kunnen zijn voor de verbetering van microbiële celfabrieken. Kortom, de identificatie van deze twee nieuwe transporters (XltD en LatA) verdiept het begrip van het complexe suikertransportsysteem van *A. niger*, maar er moeten er nog meer worden gekarakteriseerd voor rationele stam verbetering.

In *A. nidulans* zijn het D-galactose katabolisme en de regulatie ervan eerder onderzocht, maar de

alternatieve D-galactose-route en aanvullende gerelateerde transcriptionele regulatoren bleven deels onduidelijk. In Hoofdstuk 5 werd de interactie tussen drie transcriptiefactoren GalX, GalR en AraR in D-galactose en L-arabinose katabolisme onderzocht in *A. nidulans*, waarbij de betrokkenheid van al deze regulatoren bij D-galactose katabolisme en het compensatiefenomeen tussen verschillende regulatoren werd onthuld. Vergelijking met een verwante studie van *A. niger* liet zien dat de functies van deze regulatoren verschillend zijn in deze twee nauw verwante soorten, wat de complexiteit en diversiteit van regulerende netwerken in filamenteuze schimmels benadrukt.

Samenvattend beschreven de resultaten van dit proefschrift verschillende aspecten van de fysiologie van *Aspergillus* soorten, van metabole en regulerende netwerken tot suiker transportsystemen, die het begrip van deze modelschimmels verbeteren en de biotechnologische toepassingen van schimmel cel-fabrieken voor de productie van waardevolle biochemicalïën vergemakkelijken. Onze resultaten roepen ook veel nieuwe vragen op voor verder onderzoek en benadrukken het belang van de volledige kennis van het schimmel metabolisme voor het maken van betere cel-fabrieken.

Curriculum vitae

Jiali Meng was born on May 23rd, 1992 in Zhejiang, China. She enrolled in the Biological Engineering bachelor program of Zhejiang Gongshang University in 2011 and graduated in 2015. In September 2015, she enrolled in the Biochemistry and Molecular Biology program at Shanghai Normal University. She did her MSc project in the Key Laboratory of Synthetic Biology at the Chinese Academy of Sciences under the supervision of Dr. Yinhua Lu and worked on pristinamycin I biosynthesis in *Streptomyces pristinaespiralis*. She received her master degree in June 2018. In October 2018, she started her PhD at the Fungal Physiology group at the Westerdijk Fungal Biodiversity Institute under the supervision of Prof. dr. ir. Ronald P. de Vries and Dr. Miia Mäkelä.

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Acknowledgements

Yes. Time to say goodbye, even I really don't want to say goodbye. Time flies, and I realize that I have experienced a lot when I look back. It's been a wonderful four years and I've had a lot of moments and a lot of memories here that I'll never forget. Of course, it's because of you who have helped me and supported me. I've really grown up so much and learned so much with you. Here, I'd like to express my warm thanks to all of you.

First, I want to thank my supervisor and promoter Ronald de Vries who gave me the opportunity to join this warm group. Back to 2018, it was the interview when we first met online. When I knew I passed the interview, I was so excited. You helped me to prepare documents for applying CSC, but unfortunately I didn't get it for the first time. Uneasy and eager, I still decided to start my PhD in 2018. Luckily, I got it next year and could continue my PhD. Without your helps, it would never happen. You are always positive and optimistic, even when things went bad, and you have given me confidence to face many difficulties. Your passion for research is also very impressive for me and deeply inspires me. You've always given advice and guided me very patiently. I would like to thank you for your time and support during my PhD.

I also want to thank my co-promoter Miia Mäkelä. We've often met online, but you've provided so many comments and suggestions on my work. I really appreciate your help, especially with my manuscripts, as you always revised my manuscripts with great patience.

My daily supervisors, Tania, Sandra and Roland, you all helped me a lot, not only with my project, but also with many petty things. Tania, thank you for your suggestions and helps. You kindly helped me to adapt to this new group when I just came. You always answered my questions very warmly when I was stuck on a problem. We also had small talk sometimes and, most interestingly, you always called me "Cookie monster" when I ate something sweet. Honestly, I like this lovely nickname because I really love all the sweets. Sandra, thank you for your time and patience. I learned a lot of experimental and computing skills from you, which have been really useful during my PhD. I greatly appreciated your supervision and contribution on Chapter 5. Roland, thank you for patiently answering my questions and helping me with many things. You are such a nice person and I am very happy to meet you. I will remember the first Hungarian word you taught me forever (I believe you know the word. Laugh!).

Thank also to our cooperative partners from University of Debrecen: Levente, Erzsébet, Vivien, Alexandra and Zoltán. Your contribution on Chapter 3 and 5 is of great worth. It is a great pleasure for me to work with you. I am grateful to our partners in JGI. Thank you for all the efforts that you put into Chapter 5.

There are so many people in our group I have met and I would like to thank during these four years. Adi, thank you for bringing so much sweets to feed us and making our lives sweeter. You are a very kind person and it is always interesting to talk with you about delicious food. I also appreciated your help to measure hundreds and thousands of samples for me. Mao, you are always very kind and warm. It is really nice to have a talk with you. Thank you for your help with the complex transcriptome data of Chapter 5 and many helpful suggestions. Astrid, thank you for helping me with the Dionex after Adi. Melva, you worked very hard and did very well during your internship. Thank you for your kindness and contribution. I am also very happy to spend time with many other members of our group: Sonia, Ronnie, Ad, Paul, Sandra L., Natalia, Mar, Aleksandra, Alessia, Sumitha, Agata, Raquel and many more. You all make our group such a warm family.

Thanks to all Chinese people I've met in the Netherlands: Xinxin, Jiajia, Li, Dujuan, Lin, Yuanyuan, Xin, Yanfang and so on. Very pleased to meet you and we really had a great time together. Hope we will see each other in China again.

My family, thank you so much for your love, support and understanding.

感谢老爸老妈无条件支持，没有你们的支持我也不能一直做自己想做的事情。现在我马上就要完成了自己最初的目标，相信你们一定会为我感到骄傲。还有外公外婆和未能看到此

刻的我的爷爷奶奶，相信你们也会为我感到高兴。我的老弟，一直很庆幸有你陪伴我，感谢你陪我走过这些岁月。

I am extremely grateful to China Scholarship Council for the scholarship. The funding support has made my dream come true.

Finally, I would like to thank myself for working hard and playing so hard for these four years.

