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

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HIGHLIGHTS

- *A. niger* metabolic engineering improved xylitol production from lignocellulose.
- The triple mutant $\Delta ladA\Delta xdhA\Delta sdhA$ showed the best performance in xylitol production.
- Wheat bran and cotton seed hulls were the best substrates for xylitol bioproduction.
- Choice of lignocellulosic biomass plays an important role in xylitol bioproduction.

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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 (Mahmud et al., 2013a; Felipe Hernández-Pérez et al., 2019). 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 (Felipe

Hernández-Pérez et al., 2019). 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 (Dasgupta et al., 2017).

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 (Mohamad et al., 2015). *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 (Wang et al., 2016; Wu et al., 2018; Hernández-Pérez et al., 2016). Other yeasts that have been explored are *Pichia stipitis* (Neeru et al., 2013; Phaiboonsilpa et al.,

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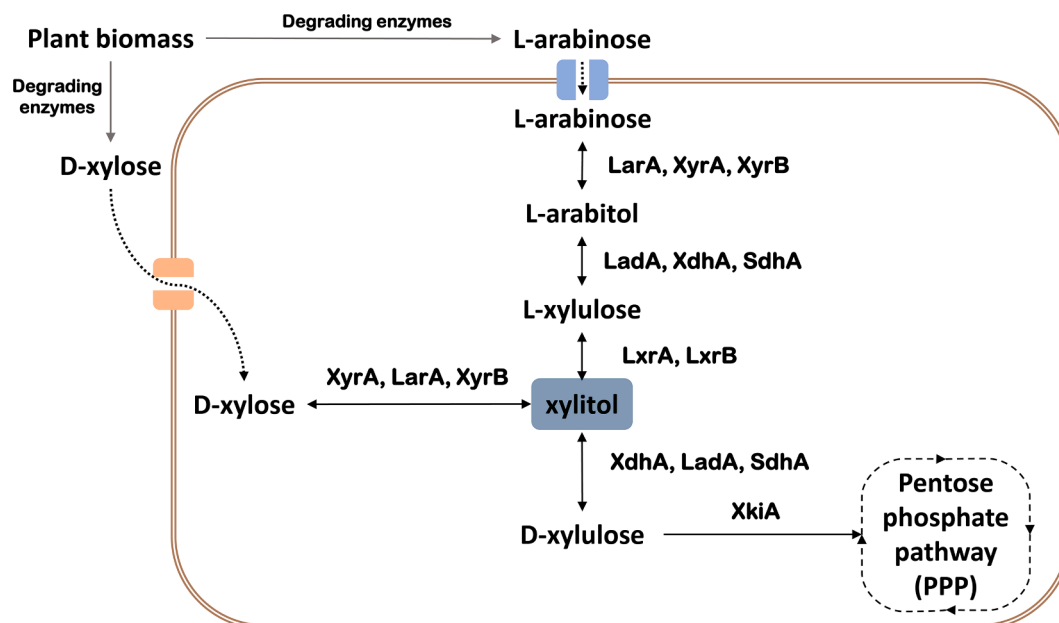


Fig. 1. Utilization of D-xylose and L-arabinose by *A. niger* (Chroumpi et al., 2021). LarA = L-arabinose reductase, LadA = L-arabitol dehydrogenase, LxrA/LxrB = L-xylulose reductases, XyrA/XyrB = D-xylose reductases, SdhA = sorbitol dehydrogenase, XdhA = xylitol dehydrogenase, XkiA = D-xylulose kinase.

2020), *Kluyveromyces marxianus* (de Albuquerque et al., 2015) and *Saccharomyces cerevisiae* (Kogje and Ghoshalkar 2016; Zha et al., 2013). 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* (Jin et al., 2018). 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 (Mahmud et al., 2013a; Hong et al., 2014).

Currently, researchers mainly focus on lignocellulosic hydrolysates as raw materials for biotechnological xylitol production in yeasts (Felipe Hernández-Pérez et al., 2019). 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 (Mäkelä et al., 2014; van den Brink and de Vries 2011).

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 (Park et al., 2017). 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 (Khosravi et al., 2015). 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 (Benocci et al., 2017). *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 (Nødvig et al., 2018; Aguilar-Pontes et al., 2018). 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) (Khosravi et al., 2015). Recent studies demonstrated that the PCP is more complex than was previously assumed, with several enzymes involved in each metabolic step (Chroumpi et al., 2021; Fig. 1). Two transcription factors, XlnR and AraR, control the PCP and the production of lignocellulose degrading enzymes, such as xylanolytic enzymes (Battaglia et al., 2011b).

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 (Zhang et al., 2014; Yang et al., 2020; Zhang et al., 2015; Dashtban et al., 2013; Pal et al., 2013). 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 (Felipe Hernández-Pérez et al., 2019, Oh et al., 2013). 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 (Felipe Hernández-Pérez et al., 2019, Ko et al., 2011, Mahmud et al., 2013b). 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 (Chroumpi et al., 2021; 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 and xylitol to D-xylulose, respectively. 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* (Chroumpi et al., 2021). 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

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> ⁻	Meyer et al., 2007
$\Delta xkiA$	CBS 144042	<i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> ⁻ , <i>xkiA</i> ⁻	Chroumpi et al., 2021
$\Delta ladA\Delta xdhA\Delta sdhA$	CBS 144672	<i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> ⁻ , <i>ladA</i> ⁻ , <i>xdhA</i> ⁻ , <i>sdhA</i> ⁻	Chroumpi et al., 2021
$\Delta lxrA\Delta lxrB\Delta ladA\Delta xdhA\Delta sdhA$	CBS 145865	<i>cspA1</i> , <i>kusA::amdS</i> , <i>pyrG</i> ⁻ , <i>lcrA</i> ⁻ , <i>lcrB</i> ⁻ , <i>ladA</i> ⁻ , <i>xdhA</i> ⁻ , <i>sdhA</i> ⁻	Chroumpi et al., 2021
$\Delta ladA\Delta xdhA\Delta sdhA\Delta xkiA$	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

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 (Chroumpi et al., 2021). 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.

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 (Song et al., 2018). The primers and plasmids used for the creation of the mutants are listed in Chroumpi et al., 2021. *A. niger* protoplasting and transformation were performed as described previously (Kowalczyk et al., 2017). All *A. niger* strains were grown at 30 °C on Complete Medium (CM) or Minimal Medium (MM) supplemented with required carbon source (de Vries et al., 2004). For plate cultivations, 1.5% (w/v) agar was added and 1.22 g/L uridine was supplemented for auxotrophic strains. 0.5 g 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 h. 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 h, 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 h.

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 (Mäkelä et al., 2017). 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 CRISPR/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 (Chroumpi et al., 2021). 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 (Chroumpi et al., 2021), 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

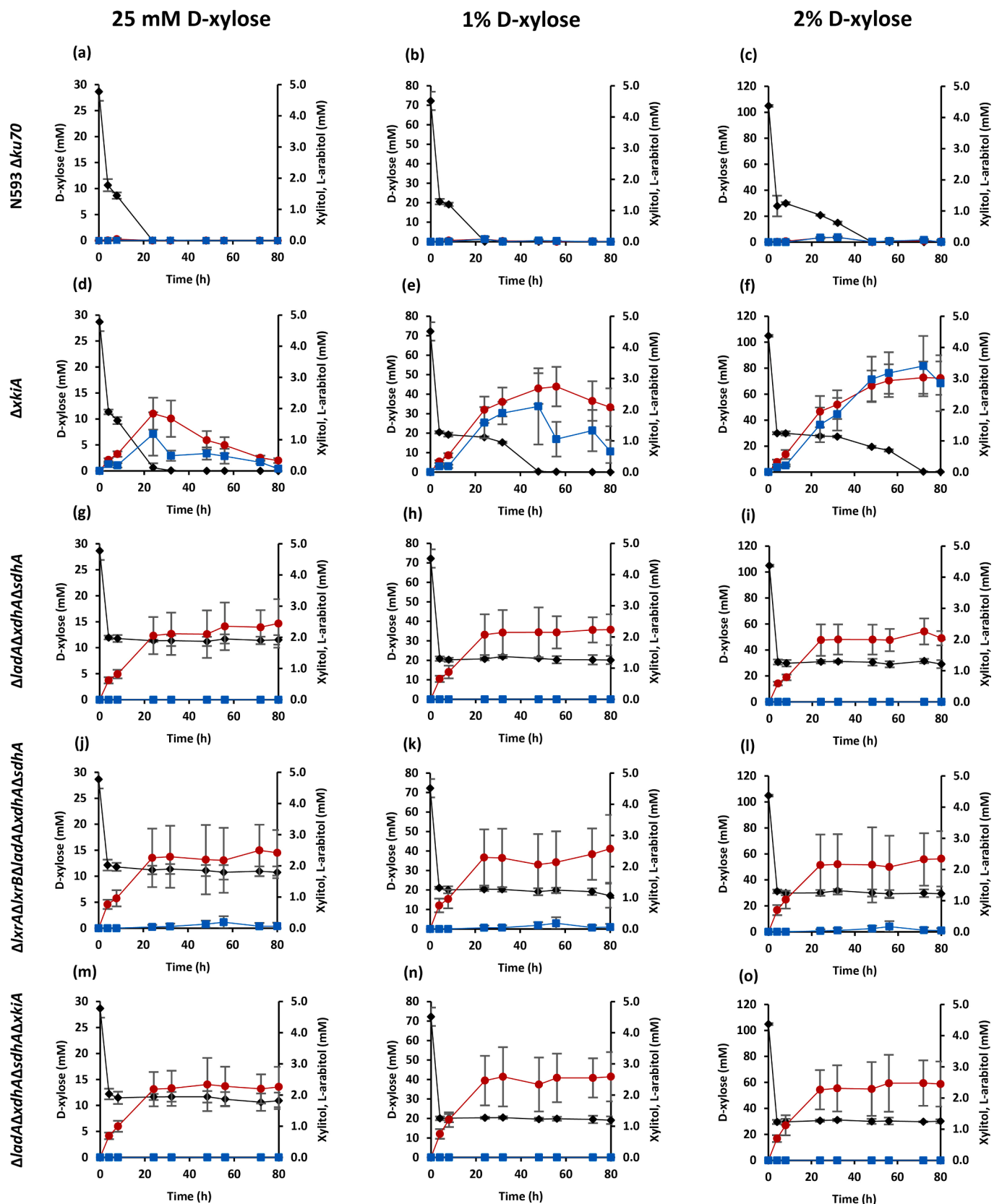


Fig. 2. Xylitol production from D-xylose by *A. niger* reference strain (N593 $\Delta ku70$) and metabolic mutants. Strains and substrates are indicated in the figure. Concentration of xylitol (red circle), L-arabitol (blue square) and residual D-xylose (black diamond). The error bars indicate the standard deviation between biological triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

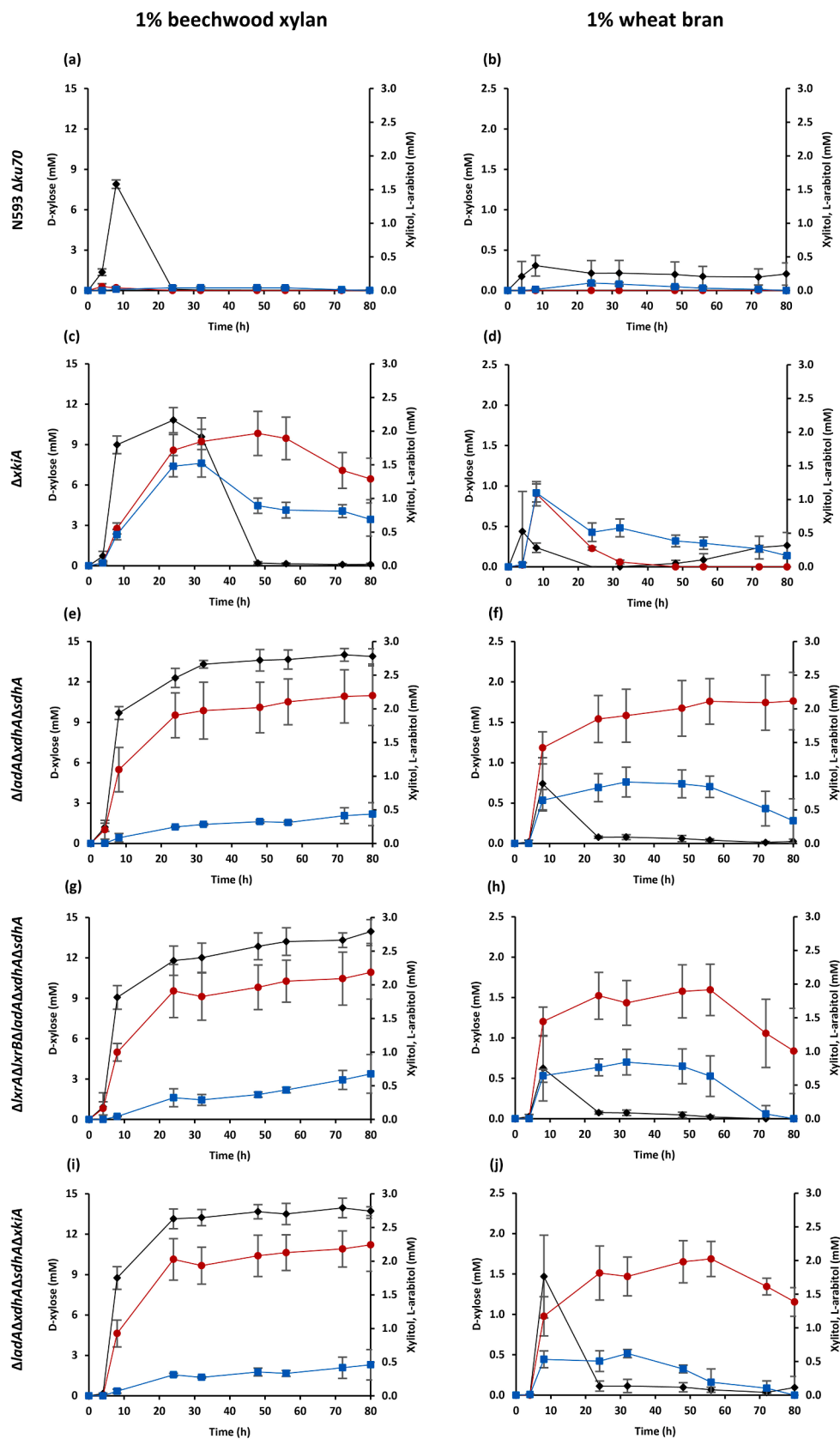


Fig. 3. Xylitol production from beechwood xylan and wheat bran by *A. niger* reference strain (N593 $\Delta ku70$) and metabolic mutants. Strains and substrates are indicated in the figure. Concentration of xylitol (red circle), L-arabitol (blue square) and residual D-xylose (black diamond). The error bars indicate the standard deviation between biological triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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

xylitol production in *A. niger* (Hong et al., 2014; Jeon et al., 2011), 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 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 h, 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 (Chroumpi et al., 2021). 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 (Chroumpi et al., 2021), 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 h 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 (Su et al., 2013). 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 (Li et al., 2013; Guo et al., 2013). 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 h 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 h. 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 h of cultivation (Fig. 3 h, j). This effect was even stronger in $\Delta xkiA$, where xylitol levels in the medium reduced to zero after 48 h 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 h of cultivation (Fig. 3 f), so 48 h 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 (Bedó et al., 2019). 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

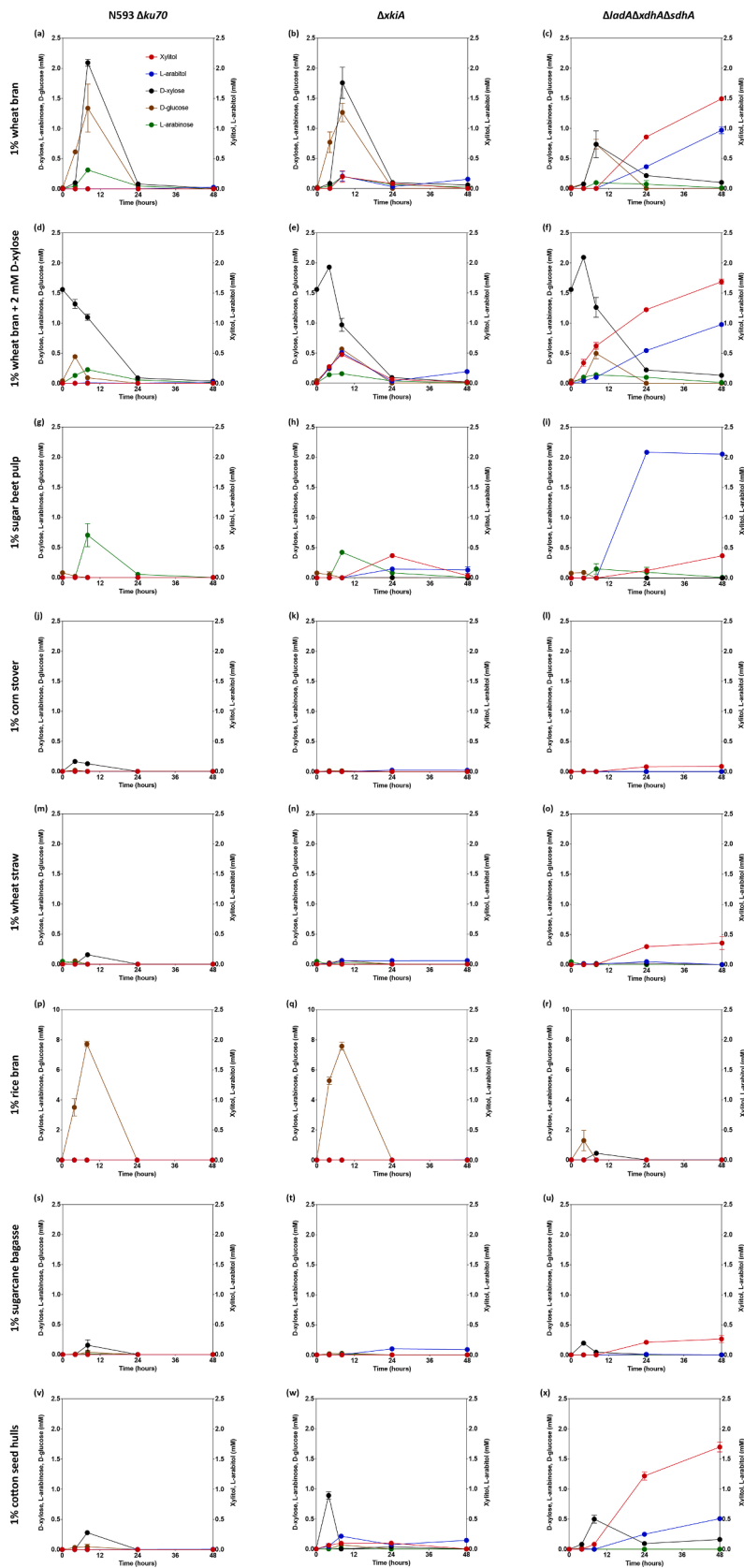


Fig. 4. Xylitol production from diverse lignocellulosic biomass by *A. niger* reference strain (N593 $\Delta ku70$), $\Delta xkiA$ and $\Delta ladA\Delta xhdA\Delta sdhA$. Strains and substrates are indicated in the figure. The error bars indicate the standard deviation between biological triplicates.

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 (de Vries et al., 1999; Mach-Aigner et al., 2012). 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 h 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 h of cultivation, which was earlier than from the sole wheat bran (24 h), 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 h 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 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 (Mäkelä et al., 2018), 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* (Kun et al., 2020), 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 (Peng et al., 2021), or whether the starch-related regulator AmyR (Vankuyk et al., 2012) 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 (Battaglia et al., 2011a; Battaglia et al., 2014). Constitutively active XlnR leads to increased production of plant biomass degrading enzymes, and D-xylose and L-arabinose release from wheat bran (Kun et al., 2020). The constitutively active AraR has also been shown to result in constitutive production of arabinanases under derepressing conditions (Reijngoud et al., 2019). 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 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.

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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 known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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