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Selection and characterisation of a xylitol-derepressed *Aspergillus niger* mutant that is apparently impaired in xylitol transport

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Abstract *Aspergillus niger* is known for its biotechnological applications, such as the use of xylanase enzyme for the degradation of hemicellulose. Depending on culture conditions, several polyols may also be accumulated, such as xylitol during D-xylose oxidation. Also during industrial fermentation of xylose for the production of fuel ethanol by recombinant yeast, xylitol is a by-product. We studied xylitol metabolism by isolating mutants that have impaired xylitol-mediated repression. Genetic and biochemical characterisation revealed that one of these mutants was affected not only in xylitol-mediated carbon repression, but also had impaired xylitol transport.

Keywords Mutant selection · Xylitol transport · Gene regulation · Pentose repression · Xylose fermentation

The first two authors have contributed equally to this work.

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Introduction

Aspergillus is a genus of mainly saprophytic fungi known for their biotechnological applications. Many of these applications are related to hemicellulose degradation, such as the use of xylanases. For the food industry, *Aspergillus niger* enzymes are particularly attractive because they are generally regarded as safe (Archer 2000). Another interest in this research field is related to the production of fuel ethanol from lignocellulose by recombinant yeast strains, expressing fungal hemicellulose-degrading enzymes (Den Haan and Van Zyl 2003; La Grange et al. 2001; Richard et al. 2003). Wild-type *Saccharomyces cerevisiae* does not metabolise D-xylose, but the introduction of pentose-catabolising activities from *Pichia stipitis* amended this problem (Kötter and Ciriacy 1993; Tantirungkij et al. 1993). However, xylitol production during D-xylose fermentation was a problem, which remained despite several strategies (reviewed by Jeffries and Jin 2004). In these fermentations, xylitol is formed as a result of cofactor imbalance, because in yeast D-xylose reductase can use both NADPH and NADH, whereas for the oxidation of xylitol only NAD^+ is used.

A comparable situation exists in *A. niger*; however, only NADP-dependent xylose reductase is found in *A. niger* (de Groot et al. 2005; Witteveen et al. 1989). In *A. niger*, xylitol

is an intermediate of both the D-xylose and L-arabinose catabolism, and it is produced in low amounts during growth on these pentoses (de Groot et al. 2005; Prathumpai et al. 2003; Witteveen et al. 1989). Another pentitol, L-arabitol, is found upon growth on L-arabinose, whilst D-arabitol is produced by several *Aspergilli* during growth on hexoses (Dijkema et al. 1985; Kelavkar and Chhatpar 1993; Ramos et al. 1999; Ruijter et al. 2004; Witteveen and Visser 1995).

Despite the importance of xylitol, only little information is available with regard to transport of xylitol or regulation of metabolism by xylitol. In this study, we investigated whether the mutant selection system used by de Groot et al. (2003) could be applied to identify functions involved in xylitol metabolism. We show that this selection system was capable of selecting a new mutant affected in xylitol-mediated repression, and detailed investigation of a xylitol-derepressed mutant shows it to be severely hampered in xylitol transport.

Materials and methods

Strains and growth conditions

The *A. niger* strains used for this study were derived from *A. niger* N400 (CBS 120.49) and are described in Table 1. Strains N402, N572, NW315, 689.1 and 740.1 have been deposited at the public fungal collection of the Centraalbureau voor Schimmelcultures in The Netherlands (http://www.cbs.knaw.nl/). NW315 was used for mutagenesis and N402 was used as a reference strain. Mycelium cultures were grown at pH 6 in minimal medium (MM) containing per litre 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 10 mg EDTA, 4.4 mg $ZnSO_4{\cdot}7H_2O$, 1.0 mg $MnCl_2{\cdot}4H_2O$, 0.32 mg $CoCl_2{\cdot}6H2O$, 0.32 mg $CuSO_4{\cdot}5H_2O$, 0.22 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1.47 mg $CaCl_2{\cdot}2H_2O$ and 1.0 mg $FeSO_4{\cdot}7H_2O$ and carbon sources as indicated in the text. Culturing was done in a rotary shaker at 250 rpm and 30°C or in a 2.5-l jacketed Applicon bioreactor with pH controlled at 5.0 and dissolved oxygen tension controlled at a minimum of 30%. For growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.

Selection and genetic analysis of mutants

The selection of mutants was done similar to the method described by de Groot et al. (2003); instead of the use of L-arabinose as the repressing pentose, we used xylitol. Conidiospores were irradiated with UV light and 10^5 spores were spread on solid media plates containing 50 mM xylitol in combination with 50 mM sodium D-gluconate. The plates were incubated for 3 days at 30°C and colonies were purified on the same selective medium. The mutants were tested by replica plating for growth on D-xylose and combinations of xylitol, D-xylose or L-arabinose with either D-gluconate or L-alanine. Mutants that remained unable to grow on D-xylose but showed growth on the xylitol + D-gluconate and xylitol + L-alanine combinations were analysed in more detail. Genetic localisation of the pentose-derepressed mutations was determined by mitotic recombination using master strain NW148 (Bos et al. 1988). Complementation of the xylitol-derepressed mutations was tested using heterokaryons of the original mutants with strain 740.1 containing the *xtlA36* mutation.

Consumption and excretion experiments

The experiments were performed as biological duplicates. To analyse consumption of xylitol, strains 740.1 and N402 were pre-cultured on MM containing 2% xylose. After 16 h, mycelium was harvested and washed using 30°C MM by suction over a filter. Aliquots of 33–44 g wet weight were transferred to 2.2 l MM containing xylitol. Samples used for dry weight and polyol analysis were not washed. To investigate excretion of xylitol, strains N572 and 689.1 were pre-grown using MM containing 2% fructose. After 16 h, mycelium was harvested, washed and transferred to MM containing 2% xylose. Samples were taken as described above.

Polyol extraction and determination

Extraction of intracellular polyols was carried out as described previously (Witteveen and Visser 1995). Polyol

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

Strain	Genotype	Reference
N402	*cspA1*	Bos et al. (1988)
N572	*cspA1*; *xkiA1*; *nicA1*	Witteveen et al. (1989)
NW148	*hisD4*; *lysA7 bioA1 cspA1*; *leuA1*; *metB10 argB15*; *pabA1*; *cnxC5*; *trpB2*	de Groot et al. (2003)
NW315	*fwnA1*; *pyrA6 cspA1*; *xkiA1*; *nicA1*	Witteveen et al. (1989)
689.1	*fwnA1*; *pyrA6 cspA1*; *xkiA1 xtlA36*; *nicA1*	This study
689.2	*fwnA1*; *pyrA6 cspA1*; *xkiA1 xtlA46*; *nicA1*	This study
689.3	*fwnA1*; *pyrA6 cspA1*; *xkiA1 xtlA55*; *nicA1*	This study
740.1	*lysA7 bioA1 cspA1*; *leuA1 xtlA36*; *nicA1*	This study, Prathumpai et al. (2003)

and xylose concentrations were measured by high pH anion exchange chromatography (Dionex) with a Carbopac MA1 column using isocratic elution with 0.48 M NaOH.

Results

Isolation of *A. niger* mutants

We adapted the selection method described by de Groot et al. (2003) to obtain *A.niger* mutants altered in xylitol-mediated repression. The selection system uses a parental strain containing the *xkiA1* mutation, which lacks D-xylulose kinase activity. This strain is able to take up L-arabinose, L-arabitol, xylitol and D-xylose, but it is unable to metabolise them beyond D-xylulose, leading to accumulation of catabolites (Witteveen et al. 1989). Xylitol is able to repress the use of poorer carbon sources such as D-gluconate and L-alanine preventing an *xkiA1* strain from growing on a combination of this pentose and D-gluconate. The first selection applied was for mutants capable of growth on D-gluconate in the presence of xylitol. To avoid selection of mutants affected only in D-gluconate catabolism, we considered only those mutants derepressed for the utilisation of both D-gluconate and L-alanine. Xylitol-derepressed mutants were selected in two petri dishes containing MM xylitol + D-gluconate, one containing 10^5 and the other containing 10^6 irradiated spores of NW315. From the plate with 10^5 spores, we isolated three xylitol-derepressed mutants. All three mutants were derepressed for both D-gluconate and L-alanine utilisation on combinations with xylitol, but not with D-xylose or L-arabinose.

Strain 689.1 was subjected to mitotic recombination with tester strain NW148 for three purposes: (1) to obtain information on the genetic localisation of the *xtlA36* mutation; (2) to remove the *xkiA1* background from the *xtlA36* recombinants to determine the phenotype of the *xtlA36* mutation; and (3) to isolate an *xtlA36* recombinant with a different auxotrophic marker, which could then be used in complementation tests with the other *xtl* mutants.

Some of the 53 haploid recombinants we isolated from diploid 689.1//NW148 were unable to grow on 50 mM xylitol, whereas growth on 50 mM L-arabinose, L-arabitol and D-xylose was not affected. This phenotype, apparently resulting from the *xtlA36* mutation, enabled us to determine the presence of the *xtlA36* mutation in all 53 progeny, not just in the *xkiA1*-carrying ones. Recombination frequencies of the genetic markers on chromosome IV with *xtlA36* were 33% for *leuA1* and 17% for *xkiA1*. Recombination frequencies with markers on other chromosomes were approximately 50%, which localised *xtlA36* on chromosome IV. The heterozygous diploid strain, derived from 689.1 and NW148, grew well on xylitol, demonstrating the *xtlA36* mutation to be recessive. This allowed us to perform complementation tests with the other two *xtl* mutations. Recombinant strain 740.1 (Table 1) was forced to form a heterokaryon with 689.2 (*xtlA46*) and 689.3 (*xtlA55*). Both heterokaryons grew on glucose, but not on xylitol, indicating that they are allelic. Because they were isolated from the same petri dish, it is not certain that these *xtlA* mutations are truly independent.

Biochemical analysis of the *xtlA36* mutation

D-xylose and L-arabinose are catabolised via xylitol (Witteveen et al. 1989). Because *xtlA36* strain 740.1 grows like wild type on D-xylose and L-arabinose, it is unlikely that the growth defect on xylitol is caused by a dramatic change in the intracellular pentose metabolism. Therefore, we conducted assays to test whether xylitol was taken up (Fig. 1). During the experiment, the biomass of the wild-type N402 at 60 mM xylitol increased from 0.065±0.003 to

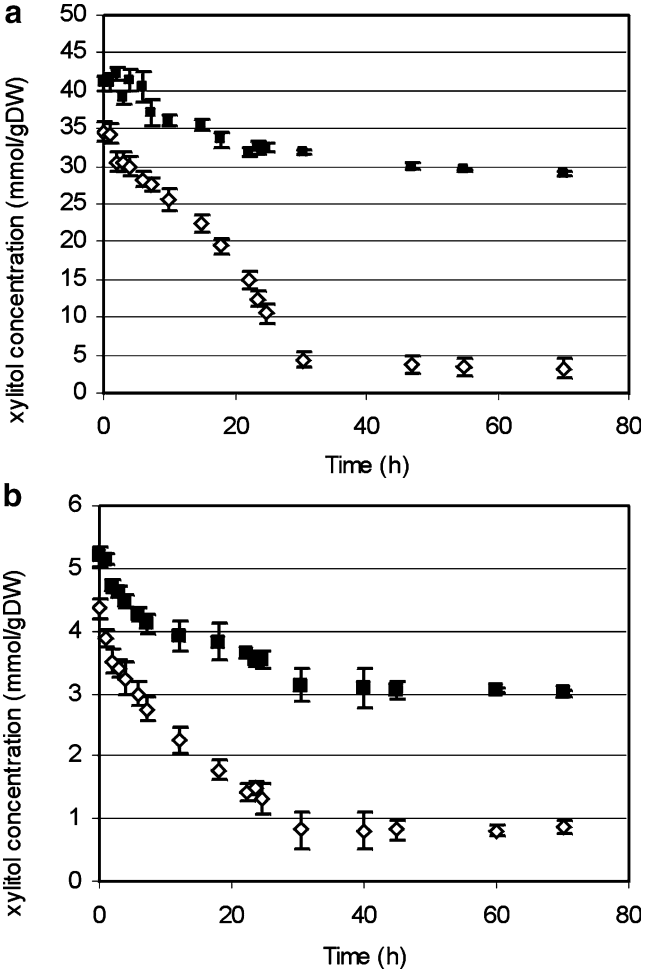

Fig. 1 Xylitol consumption in strains N402 (*diamond*, reference strain) and 740.1 (*black square*, *xtlA36*) at initial concentrations of 60 mM (**a**) and 6 mM (**b**)

0.075±0.004 g/l, but at 6 mM xylitol the biomass remained constant at 0.071±0.005 g/l. The biomass of the *xtlA36* strain remained constant at 0.070±0.005 and 0.060± 0.003 g/l in the 60- and 6-mM xylitol cultures, respectively. In the first 30 h, there was an almost constant decrease of the xylitol concentration in all four cultures, after which the xylitol consumption levelled off. Inspection of the xylitol consumption rate over these 30 h reveals a clear difference between the reference strain and the *xtlA36* mutant. With an initial concentration of 60 mM xylitol, the xylitol consumption rate of the reference strain was 0.92 mmol g^{-1} DW h^{-1}. The *xtlA36* mutant consumed xylitol with a rate of 0.37 mmol g^{-1} DW h^{-1} over the first 30 h. At a lower initial concentration of 6 mM xylitol, there was also a clear difference between the two strains. The xylitol consumption rate during the first 30 h of the reference strain was 0.10 mmol xylitol g^{-1} DW h^{-1}, whereas that of the *xtlA36* mutant was 0.06 mmol xylitol g^{-1} DW h^{-1}.

To investigate whether the *xtlA36* mutation would also affect the excretion of xylitol, we used the *xkiA1* background. With D-xylose as the carbon source, the lack of xylulose kinase in this background causes accumulation of xylitol and arabitol (Witteveen et al. 1989 and Fig. 2). The biomass remained constant at 0.66±0.02 g/l for 689.1 and 0.62±0.02 g/l for N572. We observed excretion of up to 2 mM of xylitol in the *xkiA1* mutant N572, but no significant excretion in the *xkiA1 xtlA36* strain 689.1 (Fig. 3). In the last two samples, the arabitol excretion was higher in the *xkiA1 xtlA36* strain than in the *xkiA1* strain. The polyol accumulation (Fig. 2) in this experiment was comparable between the two strains.

Discussion

The selection method described in this report proves useful for identifying new genes involved in xylitol metabolism and xylitol-mediated repression. We identified a new mutant affected in repression of gluconate and alanine utilisation exerted by xylitol and in the import and export of xylitol. The *xtlA36* mutation

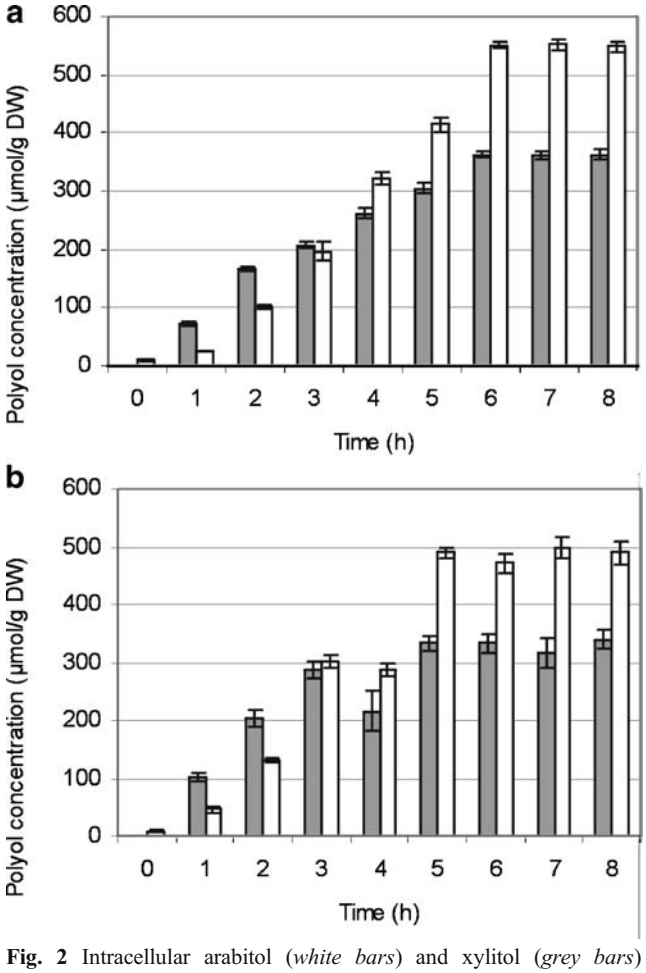

Fig. 2 Intracellular arabitol (*white bars*) and xylitol (*grey bars*) accumulation in strains **a** N572 (*xkiA1*) and **b** 689.1 (*xkiA1 xtlA36*) on 2% D-xylose

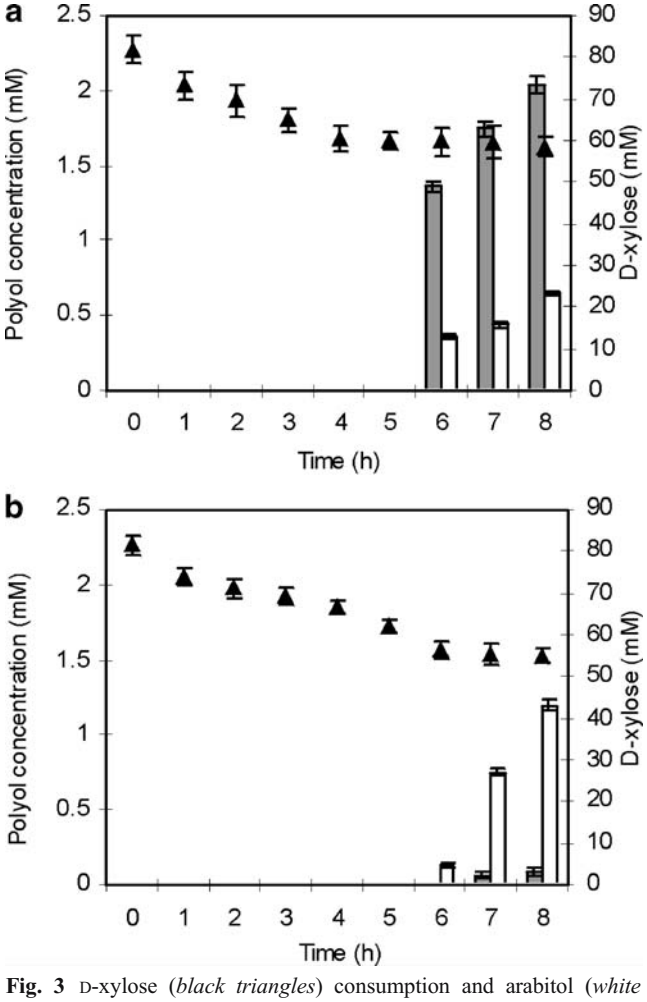

Fig. 3 D-xylose (*black triangles*) consumption and arabitol (*white bars*) and xylitol (*grey bars*) excretion by strains **a** N572 (*xkiA1*) and **b** 689.1 (*xkiA1 xtlA36*) using a initial concentration of 2% D-xylose

causes a partial xylitol non-utilising phenotype. The difference in consumption rate between the *xtlA36* mutant and the reference strain clearly shows that the transport of xylitol into the cell is impaired. This can be explained either by another xylitol importer present, which is not affected by the *xtlA36* mutation, or by a partial effect on a single xylitol transporter.

The polyol excretion experiment shows a marked difference between the excretion of xylitol by the *xkiA1* reference strain and the *xkiA1 xtlA36* double mutant. Because of the *xkiA1* mutation, these strains cannot metabolise any of the substrates from the L-arabinose/D-xylose metabolism beyond D-xylulose. However, the metabolites can be taken up and converted, causing an extreme accumulation of the polyols arabitol and xylitol (Witteveen et al. 1989). It appears that the *xtlA36* mutation prevents the fungus from excreting xylitol produced, but does not reduce the excretion of arabitol compared to the reference strain. A similar intracellular level of both polyols is maintained in the *xtlA* strain and the reference strain. Altogether, these data suggest that the *xtlA36* mutation leads to a severe reduction in xylitol transport and, therefore, could probably be located in a gene encoding the major xylitol transporter. This putative transporter would be responsible for both the import and export of xylitol as by the single *xtlA36* mutation both consumption and excretion are impaired. Also in yeast, bi-directional transporters are found, for example, in the case of glucose (Jansen et al. 2002) and glycerol transport (Karlgren et al. 2004).

It is not difficult to understand why an uptake mutant is isolated with our selection system. In the case of glucose repression, mutants no longer capable of phosphorylating glucose have reduced carbon catabolite repression (Flipphi et al. 2003), indicating that a glucose metabolite is involved in this pathway. In our study, we looked at xylitol-mediated repression, but we do not know which compound or metabolite is the key signal to xylitol-mediated repression. Alternatively, accumulation of xylitol and L-arabitol in the *xkiA1* strain might cause such changes in the anabolic (NADPH/NADP+NADPH) and catabolic (NADH/NAD+NADH) reduction charge that this interferes with the consumption of gluconate and alanine, which requires these reduced cofactors. The types of mutants one can expect to find using our selection method affect xylitol uptake and catabolism to the final metabolite(s) that influence the signalling pathway to pentose-mediated repression. We indeed identified a mutation, *xtlA36*, affecting xylitol transport. Previously, in a screen using L-arabinose instead of xylitol to isolate mutants able to grow both on gluconate and alanine, this selection system resulted in the isolation of the signalling mutants *araA4* and *araB*3, which are surprisingly diverse in their phenotype (de Groot et al. 2003). They are involved in the regulation of the arabinose catabolic pathway as well as the extracellular enzymes involved in arabinan degradation and have reduced growth on L-arabinose and L-arabitol in a *xkiA* + background. We did not find these types of mutations, nor did we find mutations affecting enzymes in the catabolic pathways leading from xylitol. An explanation for this could be redundancy in xylitol catabolism (Witteveen et al. 1989; de Groot et al. 2005).

In conclusion, we demonstrated this mutant selection technique to allow the identification of a new function in xylitol catabolism, and it is likely that the *xtlA* gene encodes a transporter involved in xylitol uptake and excretion. In *A. niger*, this mutation can prevent xylitol excretion, a finding particularly of interest in ethanol production in yeast.

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