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The 'true' L-xylulose reductase of filamentous fungi identified in *Aspergillus niger*

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ABSTRACT

L-Xylulose reductase is part of the eukaryotic pathway for L-arabinose catabolism. A previously identified L-xylulose reductase in *Hypocrea jecorina* turned out to be not the 'true' one since it was not upregulated during growth on L-arabinose and the deletion strain showed no reduced L-xylulose reductase activity but instead lost the D-mannitol dehydrogenase activity [17]. In this communication we identified the 'true' L-xylulose reductase in *Aspergillus niger*. The gene, *lxrA* (JGI177736), is upregulated on L-arabinose and the deletion results in a strain lacking the NADPH-specific L-xylulose reductase activity and having reduced growth on L-arabinose. The purified enzyme had a K_m for L-xylulose of 25 mM and a v_{max} of 650 U/mg.

1. Introduction

L-Arabinose is the second most abundant pentose sugar after D-xylose and is used as a carbon source by a variety of microorganisms living on decaying plant material. Microorganisms use at least four different pathways for the catabolism of L-arabinose. Bacteria use either the isomerase pathway or an oxidative pathway. The isomerase pathway is used by e.g. *Escherichia coli* or *Bacillus subtilis*. The intermediates are L-ribulose and L-ribulose-5-phosphate and the end product is D-xylulose-5-phosphate, which is an intermediate of the pentose phosphate pathway. The enzymes are L-arabinose isomerase, ribulokinase and L-ribulose phosphate-4-epimerase and they are encoded by the ara operon [1]. Other bacteria such as *Pseudomonas fragi* [2] or *Herbaspirillum seropedicae* [3] use an oxidative pathway where the intermediates are L-arabinolactone, L-arabonate, L-2-keto-3-deoxy-arabonate and 2,5-dioxovalerate. The enzymes of this pathway are L-arabinose dehydrogenase, L-arabinolactonase, L-arabonate dehydratase, L-2-keto-3-deoxy-arabonate dehydratase and 2,5-dioxovalerate dehydrogenase [4,5]. The end product of this pathway is α-ketoglutarate which is a metabolite in the TCA cycle. Also a modification of this oxidative pathway exists where L-2-keto-3-deoxy-arabonate is split by an aldolase to pyruvate and glycoaldehyde [6].

Fungal microorganisms catabolise L-arabinose through an oxyreductive pathway. The intermediates in this pathway are L-arabitol, L-xylulose, xylitol and D-xylulose. The end product is D-xylulose-5-phosphate as in the bacterial isomerase pathway. The enzymes are L-arabinose reductase, L-arabitol-4-dehydrogenase, L-xylulose reductase, xylitol dehydrogenase and xylulokinase (Fig. 1). The fungal pathway was first described in filamentous fungi by Chiang and Knight for *Penicillium chrysogenum* [7] and by Witteveen et al. for *Aspergillus niger* [8]. In filamentous fungi the reductases use NADPH as a cofactor while the dehydrogenases use NAD as cofactor. This results in an imbalance of redox cofactors while the overall process is redox neutral. Yeast species use the same pathway as filamentous fungi, however some yeast have an L-xylulose reductase that uses NADH as a cofactor [9,10]. For the filamentous fungus *Hypocrea jecorina* the genes coding for the enzymes L-arabinose reductase, L-arabitol-4-dehydrogenase, L-xylulose reductase and xylitol dehydrogenase have been described [11–14]. Also for *A. niger* the enzymes of this pathway and the corresponding genes with the exception of the L-xylulose reductase gene have been characterized [15,16].

While there is little doubt about L-arabinose reductase, L-arabinitol-4-dehydrogenase, xylitol dehydrogenase and xylulokinase, it has been questioned whether the L-xylulose reductase is the 'true' L-xylulose reductase.

A gene coding for an enzyme with L-xylulose reductase activity had been described in *H. jecorina*. This gene was identified in a screen, where a *H. jecorina* cDNA library in an yeast expression vector was screened for growth on L-arabinose in an *Saccharomyces cerevisiae* strain that contained all the other genes of the pathway [13]. The only gene that appeared in this screen was the *lxr1*, coding for an enzyme with L-xylulose reductase activity. However this

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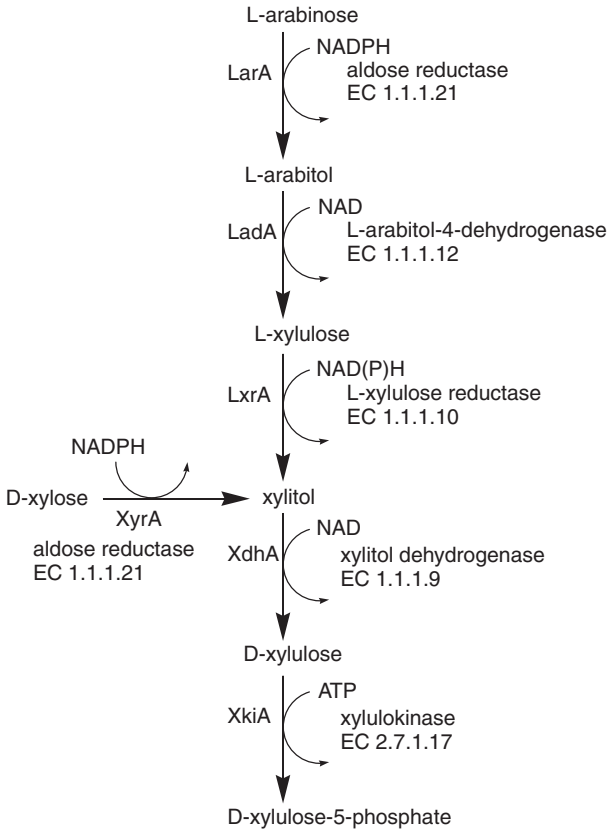

Fig. 1. The *A. niger* pathway for L-arabinose and D-xylose catabolism. The first three steps of the L-arabinose pathway are catalysed by L-arabinose-specific enzymes, whereas the last two steps are shared with the D-xylose utilization pathway. D-Xylulose-5-phosphate enters the central metabolism via the pentose phosphate pathway.

enzyme turned out to be not the 'true' L-xylulose reductase. The *lxr1* gene is transcribed on L-arabinose but even more on other carbon sources and the deletion of this gene does neither affect the growth on L-arabinose nor the L-xylulose reductase activity of the cell extract [17]. The mannitol dehydrogenase activity on the other hand was drastically reduced in the strain with the *lxr1* deletion which led to the conclusion that the *lxr1* actually codes for a mannitol dehydrogenase and the 'true' L-xylulose reductase is still to be discovered [17].

2. Materials and methods

2.1. Strains and chemicals

The *A. niger* strain ATCC1015 was obtained from CBS (CBS 113.46). L-Xylulose was produced from xylitol as described previously [18,19].

The spores of the *A. niger* strains were inoculated and grown in YPG medium containing 10 g/l of yeast extract and 2 g/l of Bacto peptone, and 3% Difco™ gelatine (Beckton, Dickinson and Company; Sparks; USA) to produce mycelium. In order to assess growth on L-arabinose and D-xylose, the spores of the *A. niger* strains were grown on agar plates containing 6.7 g/l of yeast nitrogen base (YNB, Becton, Dickinson and Company; Sparks; USA), synthetic complete amino acid mixture [20], 20 g/l agar, and 20 g/l of L-arabinose or D-xylose, respectively.

For the qPCR analysis, strains were cultivated in YPG medium overnight. The mycelia were transferred to fresh medium containing 10 g/l yeast extract, 2 g/l Bacto peptone, and 20 g/l of D-glucose, D-xylose or L-arabinose. The mycelia were incubated in this medium for up to 10 h at 28 °C and the samples were taken at different time intervals for RNA isolation and transcription analysis.

Table 1
Primers used for PCR.

Primer	Sequence
lxrA-5-F	ATTAGGATCCACGCGTTATACTGCAACATGCCGACCA
lxrA-5-R	ATTAAGATCTTTGGATGCTATGTGGTGCA
lxrA-3-F	ATTAAGATCTTTTTCTGCCTCTCTTTGGCT
lxrA-3-R	ATTAGAATTCACGCGTAGACGAGGGCATTAAGGACA
lxrA-ORF_F	GAAGGCAAATTCGCCATCAT
lxrA-ORF_R	AACCCCCCATTAGCACAAA
pyrG-del-F_n	TATACCCGGGTGATTGAGGTGATTGGCGAT
pyrG-del-R_n	TATACCCGGGTTATCACGCGACGGACAT
AnLXRHisEcoRI	GAATTCATGCATCACCATCACCATCACGGGTCCAGATCCTTGGAAGGTA
AnLXRXhoI	CTCGAGTTAACGCGTGAACT

For L-xylulose reductase activity measurements in *A. niger*, the wild type ATCC1015, and Δ*lxrA* strains were cultivated in YPG medium overnight. The mycelia were transferred to fresh medium containing 10 g/l yeast extract, 2 g/l Bacto peptone, and 20 g/l of L-arabinose, and cultivated for 4 h. The mycelia were collected by filtration, washed with 50 mM Tris–Cl buffer (pH 7.0) and lysed for the enzyme activity measurement with the crude extract as described below.

2.2. Transcription analysis

To quantify the transcription of *ladA* and *lxrA* after induction with D-glucose, D-xylose or L-arabinose, the mycelia were isolated by filtration after different time intervals for up to 10 h. The total RNA was extracted and purified from the mycelia with RNeasy®Plant Mini Kit (QIAGEN) according to manufacturer's manual. The RNA extraction step was modified; the frozen mycelia were disintegrated in RTL buffer (QIAGEN) with VWR™ Pellet Mixer (VWR International). Six micrograms of purified total RNA was used in the reverse transcription reaction (SuperScript; Invitrogen). cDNA produced in the reverse transcription reaction was diluted 1:20 with water and 5 μl of diluted solution was used in qPCR (LightCycler 480 SYBR Green I Master; Roche, Switzerland). The primers are listed in Table 2. The reactions were carried out in a LightCycler 480 Instrument II (Roche, Switzerland) and the analysis was performed with the accompanying software (Advance Relative Quantification tool). The signal was normalised to that of actin.

2.3. Deletion of the L-xylulose reductase gene, lxrA, in A. niger ATCC 1015 ΔpyrG

The construction of the ATCC 1015 Δ*pyrG* that has a deletion in the orotidine-5′-phosphate decarboxylase, *pyrG*, was described previously [21]. The cassette for deletion of the *lxrA* gene (JGI177736) contains 1596 bp from the *A. niger lxrA* promoter region, 1583 bp from the *A. niger lxrA* terminator region, and a 1923 bp fragment containing the *pyrG* gene flanked with its native

Table 2
Primers used for qPCR.

Primer	Sequence
lxrA-qPCR_F	GTTGATTAGGGAGAAGATGGG
lxrA-qPCR_R	CATTAGCACAAACCACACTC
ladA_qPCR_F	ATTTGGAGTGTCAAGTTCGG
ladA_qPCR_R	TGTCACAAGCTTCTTCAAGTC
act_qPCR_F	CAACATTGTCATGTCTGGTGG
act_qPCR_R	GGAGGAGCAATGATCTTGAC

promoter and terminator. These fragments were obtained by PCR using primers lxrA-5-F, lxrA-5-R, lxrA-3-F, lxrA-3-R, pyrG-del-F_n, and pyrG-del-R_n (Table 1) and *A. niger* ATCC1015 genomic DNA as template. The *lxrA* terminator fragment (lxrA-3) digested with *Eco*RI and *Bgl*II (both NEB) was inserted into the plasmid pRSET-A (Invitrogen), which was digested with *Eco*RI and *Bgl*II. This intermediary construct was digested with *Bam*HI (NEB) and *Ecl*136II (Fermentas) and ligated with the *Bam*HI digested promoter fragment (lxrA-5). The resulting vector was digested with *Bgl*II (NEB) and treated with Klenow polymerase and phosphatase. The *pyrG* DNA fragment, after digestion with *Sma*I, was inserted between the two *lxrA* flanking regions. The resulting plasmid was verified by restriction analysis and sequencing. The deletion cassette, 5087 bp, containing the *lxrA* flanking regions and the *pyrG* gene, was released by *Mlu*I digestion and transformed to *A. niger* ATCC1015 Δ*pyrG*. Transformants were selected for the ability to grow in the absence of uracil. Strains with successful deletions were verified by PCR. The primers for testing the presence/absence of the ORF are listed in Table 1.

2.4. Heterologous expression of lxrA

A gene that was codon optimised for *S. cerevisiae* coding for the same amino acid sequence as the *lxrA* including restriction sites to facilitate the ligation to an expression vector was custom made (Geneart, Regensburg Germany). For the expression of the *lxrA* in *S. cerevisiae* and to obtain the protein with a C-terminal HA-tag the codon optimised gene was released as an *Eco*RI/*Mlu*I fragment and ligated to the corresponding sites of the pYX212 vector (R&D Systems). To obtain a non-tagged version of the protein it was released as an *Eco*RI/*Xho*I fragment and ligated to the corresponding sites of the pYX212. In order to obtain an N-terminally histidine-tagged protein the codon optimised gene was amplified by PCR and thereby the N-terminal amino acid sequence MHHHHHHG was introduced that replaced the initial methionine. Also restriction sites were introduced and the *Eco*RI/*Xho*I fragment ligated to the corresponding sites of the pYX212. The pYX212 is a yeast expression vector with TPI1 promotor and URA3 selection. The plasmids were transformed to the *S. cerevisiae* strain CEN.PK2-1D and the transformants selected for their ability to grow in the absence of uracil.

2.5. Protein extraction and enzyme activity measurements

To measure the L-xylulose reductase activities in *A. niger*, the mycelium was collected after 4 h induction with L-arabinose as described above. It was mixed with 800 μl of 50 mM Tris (pH 7.0) buffer with protease inhibitors (Complete, Roche) and 800 μl of acid-washed glass beads (Sigma). The cells were disrupted in two 40 s sessions in the Fast Prep (Bio 101). The cell extracts were clarified by centrifugation and the diluted supernatants were used in the enzyme assay. Protein concentration was analysed using the Protein Assay Kit (BioRad). In the tests performed with the *A. niger* protein extracts L-xylulose was 4.5 mM and NADPH or NADH of 200 μM. The enzymatic activity was measured at room temperature by monitoring the NADH/NADPH disappearance at 340 nm in microtiter plates (NUNC) using the Varioskan (Thermo Electron Corporation) spectrophotometer.

S. cerevisiae strains were grown overnight in 6.7 g/l of yeast nitrogen base (YNB, Becton, Dickinson and Company), synthetic complete amino acid mixture without uracil [20] and 20 g/l glucose. The yeast cells were lysed by vigorous vortexing with glass beads in a buffer containing 100 mM Tris–Cl pH 7.0 and Complete (Roche, Basel, Switzerland) protease inhibitor. The lysed cells were then centrifuged (4 °C, 25 000×g, 30 min) and the supernatants analysed. The histidine-tagged LxrA protein was purified from the cell extract using Ni-NTA resin (Qiagen). The activity was measured at 30 °C in 50 mM Tris–Cl pH 7.0 supplemented with 200 μM NADPH and 10 mM L-xylulose if not otherwise specified. The reaction was started by the addition of L-xylulose the rate of the disappearance of NADPH followed by measuring the absorbance at 340 nm. In reverse direction we used 100 mM Tris–Cl pH 8.0 and 1 mM NADP. The reaction was started by the addition of a sugar alcohol at a final concentration of 200 mM. A unit, U, of enzyme activity converts of 1 μmol of substrate per min. The enzyme assays were performed in an Arena 20XT (Thermo, Vantaa, Finland) automated analyser.

3. Results

Andersen et al. [22] identified in three different *Aspergilli* species genes that were upregulated on D-xylose. Among these genes was also the L-arabinitol-4-dehydrogenase, *ladA*, which is in the L-arabinose pathway and has no function in D-xylose catabolism. This suggested that the genes of the L-arabinose pathway might be upregulated on D-xylose. Among the genes upregulated on D-xylose was the *A. niger* gene JGI177736 coding for a short chain dehydrogenase of unknown function. To test if it had a role in L-arabinose metabolism we tested the transcription of this gene after a shift to medium with L-arabinose.

In a transcription analysis we monitored the *ladA* after a shift to D-glucose, D-xylose or L-arabinose. While there was no transcription on D-glucose, we saw an increased transcription on D-xylose during the first 2 h which then dropped to basal level transcription after 4 h. However after a shift to L-arabinose we observed upregulated transcription during the whole time period of 10 h (Fig. 2A). The same pattern, no transcription on D-glucose, transcription on D-xylose only during the first 2 h and continuing transcription on L-arabinose, we observed with the gene JGI177736 (Fig. 2B).

The gene JGI177736 which we will call in the following *lxrA* was deleted in *A. niger* and the growth on L-arabinose tested. The deletion strain was able to grow on agar plates containing L-arabinose as a sole carbon source, but the growth rate was slightly reduced (based on visual assessment), while there was no detectable difference in growth between mutant and wild type strain on D-xylose (not shown). We tested the L-xylulose reductase activity with NADH or NADPH as a cofactor. In the crude extract of the wild type strain we found L-xylulose reductase activities with NADH and with NADPH while in the deletion strain the activity with NADPH had more or less disappeared (Fig. 3).

After we had established that the *lxrA* gene is upregulated on L-arabinose and essential for NADPH L-xylulose reductase activity we expressed it in a heterologous host for further characterization. We chose *S. cerevisiae* because it does not have an endogenous L-arabinose pathway that would interfere with the analysis. The synthetic gene was codon optimised for the expression in *S. cerevisiae*. It coded for an enzyme consisting of 299 amino acids and a molecular mass of 32.044 g/mol. It was expressed from a multicopy plasmid with a strong constitutive promoter. In the crude *S. cerevisiae* cell extract we found L-xylulose reductase activity that was not present in the control strain. For a more detailed enzyme kinetic analysis we purified the enzyme. To facilitate the purification we tagged the enzyme. Tagging the protein at the C-terminus with an HA-tag resulted in an inactive protein. The addition of an N-terminal histidine tag on the other hand did not affect the activity. In the crude cell extract we obtained the same activity as with the non-tagged protein indicating that the N-terminal tag does not interfere with the catalytic reaction.

The histidine-tagged protein was then purified and used for a kinetic characterization. The purified enzyme had a v_{max} of 650 U/mg and a K_m for L-xylulose of 25 mM (Fig. 4). The enzyme

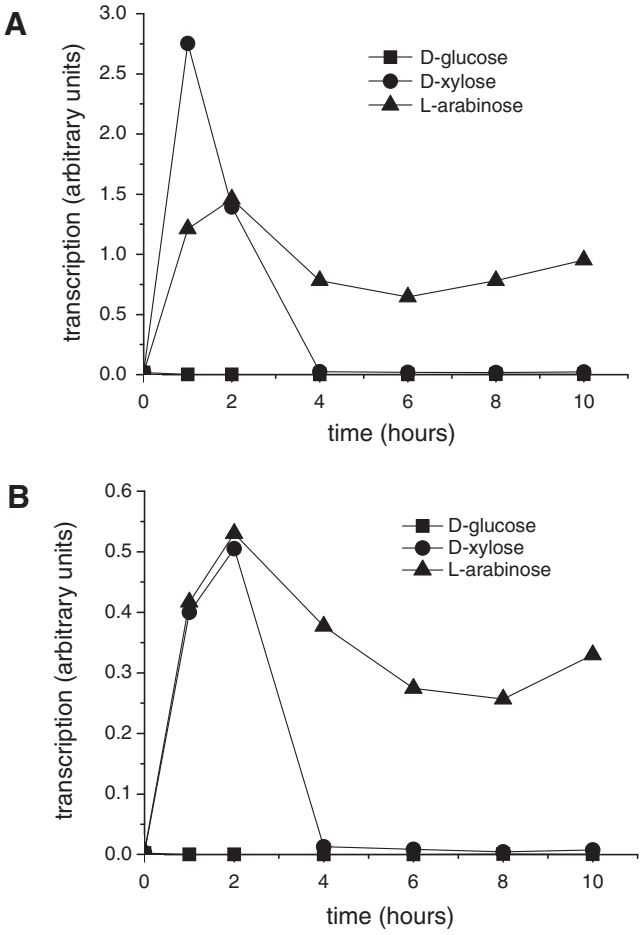

Fig. 2. Transcription analysis of the *ladA* and *lxrA* in *A. niger*. The transcription of *ladA* (part A) and *lxrA* (part B) was analysed after the transfer of the mycelia to medium containing D-glucose, D-xylose or L-arabinose. The mRNA was quantified by using qPCR and normalised to actin mRNA. In the arbitrary units the actin signal equals 1.

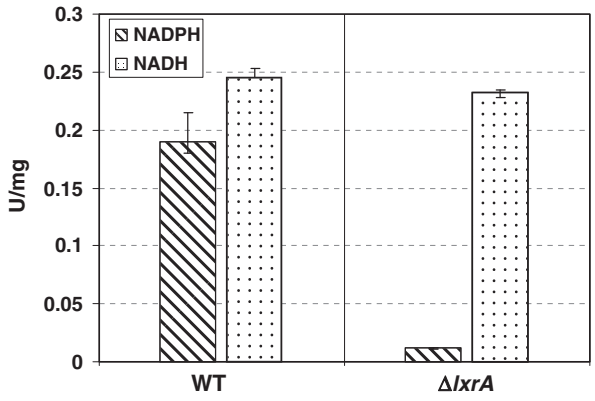

Fig. 3. NAD(P)H L-xylulose reductase activity in *A. niger*. NADPH- and NADH-specific L-xylulose reductase activities were tested in the wild type and the Δ*lxrA* strains. The activities were measured in the presence of 4.5 mM L-xylulose and 200 μM NADH or 200 μM NADPH at pH 7.0. The activity is given in units (U) per mg of total protein in the crude cell extracts.

showed no activity when NADH was used as a cofactor. We also tested if the enzyme would exhibit activity with other sugars. We tested D-ribulose, D-fructose, D-sorbose, L-sorbose and D-xylulose at a concentration of 20 mM. With D-ribulose and D-xylulose we observed activities of 1.4 U/mg and 1.8 U/mg, respectively.

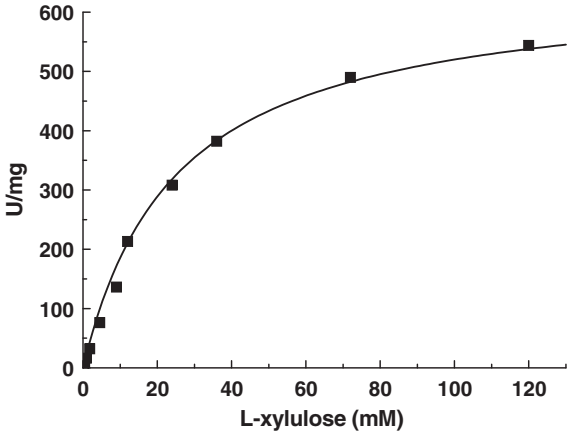

Fig. 4. Kinetic properties of the purified L-xylulose reductase: The drawn line is a fit of the data points to the Michaelis–Menten equation. The K_m for L-xylulose was 25 mM and the v_{max} 650 U/mg of protein. This was measured at pH 7.0, 30 °C and an NADPH concentration of 200 μM.

With the other sugars no activity was observed. In the reverse direction we tested the sugar acids xylitol, D-arabitol, L-arabitol, adonitol (ribitol), D-sorbitol and galactitol. With xylitol we observed an activity of about 1 U/mg. With the other sugar acids no activity was detected.

4. Discussion

The pathways for L-arabinose catabolism and for D-xylose catabolism in eukaryotic microorganisms have a few enzymes in common. The first enzyme, an aldose reductase, converts D-xylose and L-arabinose to xylitol and L-arabitol, respectively. While in some organisms such as *H. jecorina* only one gene (*xylA*) and corresponding enzyme seems to be responsible for both reactions [11], in others such as *A. niger* there is D-xylose-specific (*XyrA*) and L-arabinose-specific reductase (LarA) [8,15,16]. The last two enzymes, the xylitol dehydrogenase and xylulokinase, are also shared. While the D-xylose pathway is complete with these three enzymes, the L-arabinose pathway has additionally an L-arabitol-4-dehydrogenase and an L-xylulose reductase. Obviously the genes that are needed in both pathways are also induced on both carbon sources. For the genes that are only needed for L-arabinose the situation is more complex. In *H. jecorina* the transcription of *lad1* coding for the L-arabinitol-4-dehydrogenase is not induced on D-xylose [23]. In *A. niger* on the other hand the corresponding gene, the *ladA*, is upregulated on D-xylose [22,24]. The fact that genes of the L-arabinose pathway are upregulated on D-xylose might be an artefact since according to Andersen et al. [22] D-xylose can contain small amounts of L-arabinose but it also might be that D-xylose triggers a response to degrade complex carbohydrates such as hemicellulose that contains also L-arabinose [22].

In view of the fact that the 'true' L-xylulose reductase is still not identified, we considered it promising to search among the genes that are up-regulated on D-xylose in *A. niger*. Even more promising would have been a search among genes up-regulated on L-arabinose but such a dataset is not publically available.

Among the genes that were upregulated on D-xylose, the JGI177736 turned out to be an L-xylulose reductase that we named *lxrA*. We demonstrated that the transcription of the *lxrA* is induced on L-arabinose and we expressed the gene in a heterologous host resulting in and active enzyme. The purified protein confers high affinity and specific activity with L-xylulose. We also deleted the enzyme and observed that the resulting strain has a slightly reduced growth rate on L-arabinose as a sole carbon source and that

it lacked NADPH L-xylulose reductase activity. These are indicators that we identified the 'true' L-xylulose reductase.

The strain with the deletion in the *lxrA* was still able to grow on L-arabinose which is probably due to the NADH L-xylulose reductase activity. In *A. niger* the both L-xylulose reductase activities had been described previously. NADPH and NADH L-xylulose reductase activities were induced on L-arabinose and to a lesser extent also on D-xylose [8]. In crude cell extract with NADPH and with NADH as a cofactor activities of 0.29 and 0.24 U/mg protein were observed [8] which is in good agreement with our data (Fig. 3). The NADPH dependent L-xylulose reductase activity had been purified from *A. niger*. The enzyme showed a single band in SDS–PAGE after purification and had a molecular mass of 32 kDa. The K_m was 17 mM and the v_{max} 200 U/mg. [25]. This is again in quite good agreement with the enzyme described in this communication. The LxrA has a K_m of 25 mM and a v_{max} of 650 U/mg. The higher v_{max} we described here might be due to the different assay conditions. Witteveen et al. [25] used 100 mM phosphate buffer, whereas we used Tris–Cl buffer. The phosphate had an inhibitory effect of LXR1 from *H. jecorina* [13] and it might also affect the *A. niger* LxrA. The LxrA of *A. niger* had also a much higher activity than the LXR1 of *H. jecorina*. At an L-xylulose concentration of 63 mM the activity of the purified LXR1 was 4.8 U/mg [13] compared to 450 U/mg of the LxrA under similar conditions.

The *A. niger* LxrA is quite specific for the substrate. The LXR1 of *H. jecorina* was active with the ketose sugars D-xylulose, D-fructose and L-sorbose [13] and the *Ambrosiozyma monospora* ALX1 also catalysed the conversion of D-ribulose to D-arabinitol [9]. These side activities are nearly absent in the LxrA further supporting the suggestion that this is the 'true' L-xylulose reductase.

We have also performed database search for close homologues of *A. niger lxrA* in other fungal species. There seems to be a single *lxrA* gene present in a range of fungi often annotated as a 3-oxoacyl-(acyl-carrier-protein) reductase. Some of the highly homologous hits include *H. jecorina* JGI22771, *Aspergillus nidulans* AN10169, and *P. chrysogenum* XP_002560861.

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