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Xyr1 (Xylanase regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*

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Running title: Xyr1 is the major regulator of the hydrolytic enzyme system of *H. jecorina*

Xyr1 (xylanase regulator 1) of the ascomycete *Hypocrea jecorina* (*Trichoderma reesei*) was recently demonstrated to play an essential role in transcriptional regulation of *xyn1* (xylanase 1-encoding) gene expression. Consequently, this study reports on the deletion of the *xyr1* gene from the *H. jecorina* genome. Comparative studies on the growth behavior of the different mutant strains (deleted and retransformed *xyr1*) on various carbon sources pointed at the strongly reduced ability of the *xyr1* deletion strain to utilize D-xylose and xylan. Transcriptional analysis of *xyl1* (D-xylose reductase 1-encoding) as well as measurements of corresponding enzymatic activities gave evidence that Xyr1 takes part in control of the fungal D-xylose pathway, in particular in the regulation of the D-xylose reductase. It could be demonstrated that the uptake of D-xylose into the fungal cell is uninfluenced in the Δ*xyr1*-strain. Furthermore, transcriptional regulation of the major hydrolytic enzymes encoding genes *xyn1*, *xyn2* (xylanases 1 and 2-encoding) *cbh1*, *cbh2* (cellobiohydrolases 1 and 2-encoding) and *egl1* (endoglucanase 1-encoding) is strictly dependent on Xyr1. Regulation of the respective genes via Xyr1 is not affected by the substances mediating induction (xylose, xylobiose, sophorose) and is indispensable for all modes of gene expression (basal, de-repressed, induced). Xyr1 was moreover revealed to activate transcriptional regulation of inducer-providing enzymes such as β-xylosidase BXLI and β-glucosidase BGLI, but not to be involved in the regulation of BGLII.

Introduction

Hypocrea jecorina (anamorph *Trichoderma reesei*) is a fungus of noteworthy industrial importance, mainly because of its employment both in fermentative production of native extracellular enzymes as well as in heterologous protein production. Hydrolases secreted by this fungus achieved a broad area of application covering e.g. pulp and paper (4, 35, 50), food and feed (9, 27, 49) as well as textile industries (23, 26, 36). The set of hydrolytic enzymes produced by *H. jecorina* comprises of two main cellobiohydrolases, CBHI and CBHII (EC 3.2.1.91) (e.g. (43)), endo-β-1,4-glucanases, EGI to EGV (EC 3.2.1.4) (e.g. (37)), 1,4-β-glucosidases, BGLI and BGLII (EC 3.2.1.21) (e.g. (8, 40)), two major specific endo-β-1,4-xylanases, XYNI and XYNII (EC 3.2.1.8) (45), and one β-xylosidase BXLI (EC 3.2.1.37) (17) – to mention only the best characterized. This set of hydrolases is synergistically working together to attain a complete degradation of bio-polymeric substrates of which cellulose and xylan are predominant. In this particular brake-down process these enzymes cause hydrolysis to smaller, soluble oligo- and monosacharides which are finally either acting directly as low-molecular weight inducer substances (e.g. xylobiose, xylose) (29, 53) or are converted to respective inducers (e.g. sophorose) via the transglycosylation activity of some of these enzymes (46).

Whereas in *Aspergillus* the xylanolytic and cellulolytic system is strictly co-regulated via the inducer xylose (e.g. (10, 15)), enzymes participating in the respective *T. reesei* hydrolytic systems are not. Their differential expression has been reported in several studies: Briefly summarizing, all discussed hydrolytic genes are inducible by respective degradation and/or transglycosylation products of xylan and/or cellulose, e.g. *xyn1* (xylanase 1-encoding) by xylose (30), *xyn2* (xylanase 2-encoding) by xylobiose and sophorose (53) and *bxl1* (β-xylosidase 1-encoding) by xylobiose (33), cellulases such as *cbh1* (cellobiohydrolase 1-encoding), *cbh2* (cellobiohydrolase 2-encoding), and *egl1* (endoglucanase 1-encoding) (20) as

well as *bgl1* (β-glucosidase 1-encoding) (8) and *bgl2* (β-glucosidase 2-encoding) (40) by sophorose.

We recently reported that during xylose-mediated induction of *xyn1*, Xyr1 (xylanase regulator 1) plays a main role in *H. jecorina* (38). Xyr1 is a zinc binuclear cluster protein binding to a GGCTAA-motif arranged as an inverted repeat in the *xyn1* promoter (38), closely resembling the consensus sequence for binding of the *Aspergillus niger* XlnR transactivator (48). XlnR is not only a central regulator protein responsible for activation of more than 10 genes involved in degradation of xylan and cellulose but also contributes to the regulation of the D-xylose metabolism (10, 15, 47).

Ancillary to the Xyr1/XlnR-mediated induction, the carbon catabolite repressor Cre1/A has for both organisms been described as a wide domain repressor of particular hydrolase-encoding genes (e.g. (6, 7, 21, 30)). In *T. reesei* only some of the major hydrolases, namely *cbh1* and *xyn1* are under direct Cre1 control (21, 30), whereas other hydrolytic genes such as *cbh2*, *xyn2* and *bgl1* are not regulated Cre1-dependent (31, 33).

In addition, the isolation of the two transcription factors Ace1 and Ace2, potentially involved in the regulation of hydrolase formation in *H. jecorina,* has been reported (2, 39). While the previously described repressor Ace1 (1) was proven to directly antagonize Xyr1 function by competing for one of its binding sites in the *xyn1* promoter (38) deletion of *ace2* was demonstrated to clearly reduce expression levels of main cellulase genes and of *xyn2* on cellulose but did not effect induction on sophorose (2). A more detailed study revealed that Ace2 contacts the xylanase activating element XAE (essential for *xyn2* expression) in the *xyn2* promoter (52) but is not involved in *xyn1* transcription (2, 38). Up to now no mechanisms involving respective orthologous regulators have been identified in the expression of *Aspergillus* hydrolases.

In this study, we report deletion of *xyr1* from the *H. jecorina* genome. Strikingly reduced growth on D-xylose and restricted utilization of xylan by the *xyr1* deletion strain could be

observed when compared to the wild type. Consequently, we identified Xyr1 as a general and essential transcriptional activator of not only *xyn1*, but also *xyn2*, *cbh1*, *cbh2* and *egl1* gene transcription. Furthermore, Xyr1 was demonstrated to strictly control xylanolytic as well as cellulolytic enzyme formation under inducing and non-inducing conditions in *H. jecorina*. Moreover, Xyr1 could be shown to regulate gene expression of at least some inducer-providing enzymes, e. g. BGLI and BXLI. Finally, we have proven the involvement of Xyr1 in D-xylose metabolism, namely its strong impact on the expression of D-xylose reductase activity. Summarizing, we revealed Xyr1 to govern the expression of the xylanolytic and cellulolytic enzyme system as well as D-xylose metabolism in *H. jecorina*.

Experimental procedures

Strains and growth conditions

H. jecorina (*T. reesei*) QM9414 (ATCC 26921) was used as parental strain throughout this study and as recipient strain for *amdS*-mediated transformation to delete *xyr1*. It was maintained on malt agar.

Analysis of growth on different carbon sources was performed on plates with Mandels-Andreotti (MA) medium (32) supplemented with 1 % (w/v) of the corresponding carbon source and 2 % (w/v) Agar-Agar at 30 °C.

For replacement experiments mycelia were pre-cultured in 1-l-Erlenmeyer flasks on a rotary shaker (250 rpm) at 30 °C for 18 h in 250 ml of MA medium applying 1 % (w/v) glycerol as a carbon source. 10^8 conidia per liter (final concentration) were used as inoculum. Pre-grown mycelia were washed and thereafter equal amounts were resuspended in MA media containing 1 % (w/v) oat spelt xylan (Sigma, Steinheim, Germany), carboxymethylcellulose (Calbiochem, San Diego, CA), glucose or xylose as carbon source. Mycelia were also

transferred to MA media without carbon source (control) or on media without carbon source but supplemented with 2 mM sophorose or xylobiose as respective inducer molecules. Incubation was continued for 8 and 24 h if cultivated on xylan or carboxymethylcellulose, for 3, 5 and 8 h if cultivated on xylose, xylobiose or on medium without carbon source, for 5 and 8 h if cultivated on sophorose and for 3 and 5 h if cultivated on glucose.

Direct cultivations were performed in 500-ml-Erlenmeyer flasks with 100 ml MA medium containing 1 % (w/v) xylan or carboxymethylcellulose and inoculated with 10^8 conidia per liter (final concentration). Incubation was performed for 24, 48 and 72 h at 30 °C and 250 rpm.

Deletion and retransformation of xyr1 *from the* H. jecorina *genome*

The plasmid containing the *xyr1* deletion cassette was constructed as follows: To generate *Eco*RI and *Acc65*I terminal sites of a *xyr1* gene fragment, pXR51.1, bearing the 4.3 kb *xyr1* gene (38), served as template in a four-primer PCR mutagenesis strategy (22) by using overlapping primer pairs Xyr1.1.f and Xyr1.2.r (yielding a 1021 bp fragment) as well as Xyr2.1.f. and Xyr2.2.r (yielding a 1450 bp fragment); a full-length 2.5 kb fragment of *xyr1* was re-amplified using the flanking primers Xyr1.1f and Xyr2.2.r. An iCycler (Biorad, Herkules, US) was used to run 30 cycles of 1 min 95 °C, 1 min 57 °C (59 °C for the full-length fragment) and 1 min 30 s (2 min 30 s) 72 °C applying a Taq2000 Polymerase (Stratagene, La Jolla, US). Primers sequences are given in Table 1. The 2.5 kb fragment was cloned into the pGEM-T vector (Promega, Wisconsin, US) according to the manufacturer's instructions to create pD0xlr1. In order to remove a *Sal*I restriction site from pD0xlr1, the plasmid was cleaved *Not*I/*Nde*I, treated with Sequenase version 2.0 (Amersham Biosciences, UK) and religated to obtain pD1xlr1. A *Sal*I/*Eco*RI *amdS* gene fragment from the plasmid pamdS (pUC 19 containing *amdS*-gene) from our department stock and the *Sal*I/*Eco*RI

fragment of pD1xlr1 were ligated to yield pD2xlr1. For linear transformation, the deletion cassette (6.4 kb) was released from pD2xlr1 by *Acc65*I digestion. Transformation of *H. jecorina* QM9414 was carried out according to the optimized transformation protocol described by (13) using the *amdS* gene of *A. nidulans* (19) as dominant selection marker. Retransformation of the obtained *xyr1* deletion strain was performed according to an optimized protocol for particle bombardment (16), applying co-transformation of pXR51.1, bearing the 4.3 kb *xyr1* gene (38), and pRLMex$_{30}$ (28), bearing the *hph* gene (encoding hygromycin B-phosphotransferase) of *E. coli*.

Southern blot analysis

Fungal genomic DNA was isolated as described previously (12). Southern hybridization was carried out as described by (41). Chromosomal DNA of the *xyr1* deletion strain was digested with *Nde*I, and the obtained blot was hybridized with the [α-^{32}P]dCTP-labeled 6.4 kb deletion cassette whereas chromosomal DNA of the *xyr1*-retransformants was digested with *Eco*RV, and the obtained blot was hybridized with the [α-^{32}P]dCTP-labeled 1.5 kb *Sac*II-fragment of *xyr1*.

Parallel DNA- and RNA-extraction, reverse transcription, real-time PCR

Harvested mycelia were homogenized in 1 ml peqGOLD TriFast DNA/RNA/protein purification system (PEQLAB Biotechnologie, Erlangen, Germany) using a FastPrep FP120 BIO101 ThermoSavant cell disrupter (Qbiogene, Carlsbad, US). DNA and RNA were simultaneously isolated in a two-step-process according to the manufacturer's instructions.

Synthesis of cDNA from mRNA was carried out applying RevertAid^{TM} H Minus First Strand cDNA synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions.

All PCRs were performed in an iCycler iQ, Real-Time Detection System (Biorad). The software of the iCycler (iCycler iQ, Optical System Software, Version 3.0a, Biorad) was used to compile PCR protocols and define plate set-ups. All reactions were accomplished three-fold in 25-µl-mixtures including 1 x iQ Supermix (Biorad), 0.1 µM corresponding taqman probe (MWG, Ebersberg, Germany), 0.1 µM forward primer, 0.1 µM reverse primer, 1 mM $MgCl_2$ (only for *xyn1*- and *cbh1*-real-time PCR), and as template DNA or cDNA (100-fold diluted), respectively. Primers and probes are given in Table 1. Each run included a blank (sterile bi-distilled water instead of sample) and a no-amplification control (0.01 % SDS added to the reaction mixture). The following optimized (to an efficiency of at least 1.9) PCR protocols were followed: 3 min initial denaturation at 95 °C, followed by 45 cycles of 15 s at 95 °C, 15 s at 60 °C (for real-time PCR *of xyr1*) or 59.8 °C (for real-time PCR *of cbh1*-, *cbh2*- and *egl1*) or 59 °C (for real-time PCR of *xyn1*- and *xyn2*) and 15 s at 72 °C. For all reactions performed the efficiency was calculated essentially following (44). Because of the high similarity of the respective PCR efficiencies no correction factors had to be applied. The threshold level was set automatically to noise-to-signal ratio conditions by the Optical System Software. Calculation of relative transcription levels was performed as follows: After choosing a reference sample (in all cases the sample from replacement of QM9414 mycelium to MA medium without carbon source incubated for 3 h) all threshold cycle (C_t)-values of DNA-samples were referred to the C_t-values of the DNA of the reference sample to obtain a correction factor f_{DNA} for each DNA-sample. Then C_t-values of cDNAs of all samples were corrected by dividing through f_{DNA}. That corrected cDNA-C_t-values (C_t-$value_{corr.}$) were subtracted from C_t-value of cDNA of the reference sample to obtain ΔC_t-$value_{corr}$. After establishing a relationship between cDNA-C_t-values and DNA-C_t-value of the reference

sample, differences between initial amounts of cDNA were calculated. According to the equation $y = 10^{-1/k}$, and with y to be the PCR-efficiency ($y_{100\ \%\ efficiency} = 2$) and k to be the slope ($k_{100\ \%\ efficiency} = -3.32$), the ΔC_t-$value_{corr}$ was divided by 3.32 to obtain S. 10 was raised to the power of S, because initial target amounts differing in one decimal power should differ in their C_t-values about 3.32. This procedure allows describing differences in gene transcription not in C_t-values, but in amounts of DNA-corrected cDNA-targets. Thus, results of transcription analysis are given in relative amounts of mRNA (cDNA) per gene dose.

Enzyme assays

D-xylose reductase activity and L-arabinose reductase activity were measured as described previously (5). One unit of activity is defined as the amount of enzyme responsible for consumption of one micromole NADPH per minute due to its oxidation in presence of D-xylose or L-arabinose under the defined assay conditions. Cell-free extract for the above mentioned enzyme assay was prepared as described previously (42).

Xylanase activity was measured applying Xylazyme AX Tablets (Megazyme, Wicklow, Ireland) according to the manufacturer's instructions. One unit of activity is defined as the amount of enzyme required to release one micromole of xylose reducing-sugar-equivalents per minute under the defined assay conditions.

Cellulase activity was measured using the DNS method according to Miller (34). One unit of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar-equivalents per minute under the defined assay conditions.

Activity of β-glucosidase was assayed as described by Kubicek (25) using p-nitrophenyl-β-D-glucoside as substrate. One unit of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar-equivalents per minute under the defined assay conditions.

Activity of β-xylosidase was assayed as described by (24) using p-nitrophenyl-β-D-xylopyranoside as substrate. One unit of activity is defined as the amount of enzyme required to release one micromole of xylose reducing-sugar-equivalents per minute under the defined assay conditions.

Determination of fungal growth on xylan

Harvested mycelia were suspended in 1 ml 0.1 N NaOH in a reaction tube with screw cap (2 ml, Brand GmbH+Co KG, Wertheim, Germany). Glass beads (0.37 g, diameter: 0.1 – 0.01 mm, 0.25 g, diameter: 1 mm, 1 glass bead, diameter: 3 mm) (Braun Biotech International GmbH, Melsungen, Germany) were added to each mixture. This suspension was solubilized in a Teflon-Homogenizer (FastPrep 120, BIO 101 Savant Instruments, Hobrook, NY, US). After an incubation of the samples at room temperature for three hours, they were centrifuged at 14000 rpm and 4 °C for 10 min. Protein concentration was determined via Biorad Protein Assay (Bradford) reagent according to the manufacturer's guidelines.

Determination of D-xylose uptake

QM9414- and Δ*xyr1*-mycelia pre-grown as described above and were transferred to 5 ml phosphate buffer (20 mM, pH 5) containing 2 mM D-xylose pulsed with $2 \cdot 10^{-6}$ mM [^{14}C]D-xylose (1,85 MBq/250 µl) (Amersham Biosciences, UK). After an incubation time of 20 min at 30 °C, 0.1 M D-xylose was added to a final concentration of 50 mM. Mycelia were harvested, washed thoroughly and measured in a scintillation counter LKB Wallac 1219 Rack-beta (PerkinElmer, Boston, US).

Results

Deletion of the xyr1 *gene of* H. jecorina

To delete *xyr1* (gene bank accession no.: AF479644) from the *H. jecorina* genome, transformation with a deletion cassette containing the *xyr1* up- and downstream regulatory regions interrupted by the *amdS* gene of *A. nidulans* (gene bank accession no.: M16371) thereby replacing the *xyr1* encoding region, was performed and yielded two mitotically stable transformants. Retransformation of *xyr1* was carried out via co-transformation using the full-length *xyr1*gene (38) and the vector pRLMex$_{30}$ conferring hygromycin B resistance yielded seven strains. Southern blot analysis revealed deletion of *xyr1* and insertion of one to three copies of *xyr1* on ectopic loci, respectively (data not shown). Transcriptional analysis via real-time PCR indicated absence or regain of *xyr1* transcript in all deletion or retransformation strains, respectively. It shall be noted that transcript levels of *xyr1* did not importantly vary in the retransformant strains when compared to the wild type (data not shown). The Δ*xyr1*-strain showed similar growth rates on malt extract and synthetic media supplemented with different low-molecular weight carbon sources with exception of D-xylose (Table 2) when compared to the parental strain QM9414. In all cases tested neither conidio-spore formation on agar plates nor germination times differed comparing the mutant with the parental strain.

Xyr1 controls D-xylose reductase activity

To examine the influence of Xyr1 on the utilization of different carbon sources we conducted a series of growth experiments on agar plates. In detail, growth of the parental strain and of respective deletion and retransformation strains on plates containing glucose, glycerol, L-arabinose, L-arabitol, D-xylose, xylitol, xylan and cellulose was observed for six days (Table 2). While the parental strain as well as the *xyr1* retransformation strains (data are shown for

one strain, Re*xyr1*A) were able to utilize all carbon sources investigated, the Δ*xyr1*-strain showed strongly reduced growth on D-xylose as sole carbon source (Fig. 1A). As known from the D-xylose pathway, in the first step of D-xylose metabolism D-xylose reductase converts D-xylose into xylitol. Xylitol is reduced to D-xylulose and then goes into the pentose phosphate pathway (Fig. 1B). In contrast to D-xylose, when applying xylitol (next intermediate of the D-xylose utilization pathway) as sole C-source no growth differences between the parental and the Δ*xyr1*-strain could be observed (Fig. 1A). As fungi are not able to reduce D-xylose by means of a xylose isomerase (51), D-xylose is converted into xylitol via D-xylose reductase in *H. jecorina*. Consequently, the regulatory influence of Xyr1 on this enzyme system was further examined.

Transcriptional analysis via real-time PCR of the parental, Δ*xyr1* and *xyr1* retransformation strains replaced on D-xylose as carbon source showed *xyl1* (D-xylose reductase-encoding) transcript to be absent in the Δ*xyr1*-strain under all conditions tested. Regain of D-xylose-dependent *xyl1* transcript formation was observed for the retransformation strains for all time-points (5 or 10 hours of cultivation), (data not shown). Measuring D-xylose reductase activities in cell-free extracts also provided evidence for a regulatory impact of Xyr1 on D-xylose reductase since strongly reduced activities could be detected from the deletion strain (Fig. 2A).

To investigate if D-xylose reductase could at least partially be replaced by L-arabinose reductase, both reductase activities were analyzed in cell-free extracts of mycelia replaced either to D-xylose or L-arabinose. The measuring of L-arabinose reductase activity in cell-free extracts of mycelia of both strains grown on L-arabinose showed similar activities (Fig. 2B). We therefore conclude that L-arabinose reductase is not or only weakly affected by Xyr1. D-xylose reductase activities could also be detected in the parental strain and to a lesser extent in the Δ*xyr1*-strain when replaced to L-arabinose (Fig. 2C) and *vice versa* (Fig. 2D). These

findings indicate that those two enzymes are at least partially able to substitute one another in *H. jecorina* in their activities, thereby explaining the weak residual growth of the deletion strain on D-xylose plates (Table 2).

Transport of D-xylose into the cell is not influenced by Xyr1

Results described s*upra vide* demonstrate a main regulatory influence of Xyr1 on D-xylose reductase, whereas only negligible to no effects on the downstream enzymes of the D-xylose pathway can be deduced. Summarizing this set of data, it still remains unclear, whether deletion of *xyr1* additionally causes an inhibition of the transport of D-xylose into the cell. Therefore, the parental and the deletion strain were pre-cultivated on glycerol and thereafter transferred into a D-xylose-containing medium spiked with [^{14}C]D-xylose. Measuring the mycelia after 20 minutes incubation in a liquid scintillation counter gave 13400 cpm/g dry weight for the Δ*xyr1*-strain and 12700 cpm/g dry weight (both values are means of three independent experiments) for the wild-type indicating that the uptake of D-xylose into the cell is uninfluenced by Xyr1.

Xyr1 regulates transcription of xyn1*,* xyn2*,* cbh1*,* cbh2 *and* egl1

The fact that a GGCTAA-palindrome within the *xyn1* promoter is bound by Xyr1 under inducing and non-inducing conditions as recently shown (38), prompted us to examine *xyn1* transcript formation in the respective mutant strains. After pre-cultivation the parental and the Δ*xyr1*-strain were transferred to media without carbon source or media containing either glucose, D-xylose or xylan as sole carbon source and incubated 3 and 5 hours or 3, 5 and 8 hours or 8 and 24 hours, respectively. After parallel DNA- and RNA-extraction followed by cDNA synthesis, transcription levels were analyzed via real-time PCR. Whereas the parental

strain showed transcript formation on xylan and on D-xylose, both carbon sources already known to activate *xyn1* expression (30), no *xyn1* transcript could be detected from the Δ*xyr1*-strain under those conditions (Table 3A). No transcript formation occurred from any strain on glucose a carbon source known to completely repress *xyn1* transcription (30).

Consequently, *xyn2* transcription in both strains replaced to medium either without carbon source or with xylan or to a medium containing 2 mM sophorose or xylobiose as inducers was examined and similar results were found. On the activating carbon source xylan and the inducing compounds sophorose and xylobiose, *xyn2* transcription in the parental strain QM9414 was strongly induced as previously published (53), but from the Δ*xyr1*-strain no *xyn2* transcript arose (Table 3B). In contrast to the parental strain, from which small amounts of transcript could also be detected when replaced on medium without carbon source (Table 3B), no *xyn2* transcript was formed by the Δ*xyr1*-strain (Table 3B), strongly indicating that Xyr1 is also involved in the regulation of the previously described carbon source independent (basal) transcriptional level of *xyn2* (52, 53).

To ascertain if Xyr1 also influences transcription of cellulase-encoding genes we accordingly determined *cbh1*- (Fig. 3A), *cbh2*- (Fig. 3B) and *egl1*-transcription levels (Fig. 3C) - all giving similar results. Under activating conditions applying either 2 mM sophorose as inducer or cellulose as carbon source, no transcripts of any of those three genes were observed for the Δ*xyr1*-strain, although the parental strain showed high amounts of transcript formation which is in strict accordance with previous publications (e.g. (20)) (Fig. 3).

Role of Xyr1 in regulation of the expression of xylanolytic and cellulolytic enzyme system

To investigate whether the transcriptional regulation of the above mentioned genes via Xyr1 can be extended to a general influence on xylanolytic and cellulolytic enzyme activity formation in *H. jecorina*, xylanase activity was measured in supernatants of cultivations after

transferring equal amounts of mycelia to media with corresponding carbon sources. Xylanase activity from the parental strain could be detected when either applying the low-molecular weight inducers sophorose or xylobiose or during growth on D-xylose. Very high activities were formed if the strain was grown on xylan or cellulose which is in accordance to former results (e.g. (18)) (Fig. 4). Only marginal but still detectable activity could be obtained from the parental strain on medium without carbon source after 8 h, most probably due to de-repression of *xyn1* expression (30). In contrast no xylanase activity was yielded from the Δ*xyr1*-strain under any conditions tested (Fig. 4). This findings perfectly fit the observations that cultivation of the Δ*xyr1*-strain on xylan plates led to no distinct clearing zone formation due to xylanolytic activity (data not shown), indicating that the strain is not any more able to degrade the D-xylose-backbone of xylan. The remaining residual growth on xylan plates (Table 2) is most likely due to the utilization of the carbon sources comprised in the side groups linked to the D-xylose-backbone of xylan. The oat spelt xylan used during this study (Sigma, Steinheim, Germany) contains approximately 10 % arabinose- and 15 % glucose-residues according to the manufacturer.

Obtained results strongly point at Xyr1 as the general regulator of the xylanolytic and cellulolytic enzyme system of *H. jecorina*. This assumption is further affirmed by measuring enzyme activities in supernatants of direct cultivations of longer time periods. To this end both strains were directly cultivated on xylan and cellulose in a time course experiment (24, 48 and 72 h). As expected, the parental strain showed formation of xylanase as well as cellulase activity after 48 and 72 hours on the respective carbon source whereas the Δ*xyr1*-strain never formed those enzyme activities (Table 4).

In order to exclude that deletion of *xyr1* leads to formation of inactive enzymes, Western blot analysis of supernatants of the replacement experiment and the direct cultivation of both strains was performed applying monoclonal antibodies against CBHI, CBHII, XYNI and XYNII. Distinct bands from supernatants of strain QM9414 applying 2 mM sophorose or

xylobiose as respective inducers for 8 h and from cellulose or xylan cultures grown for 48 h as well as 72 h could be obtained, but no bands appeared from supernatants of the Δ*xyr1*-strain (data not shown).

Summarizing, we conclude that Xyr1 is the general regulator of the xylanolytic and cellulolytic enzyme activity formation in *H. jecorina* independent of whatever inducer or inducing carbon source is used.

Involvement of Xyr1 in regulation of bgl1, bgl2 *and* bxl1

As already mentioned, hydrolytic enzyme-encoding genes are inducible by their respective degradation/transglycosylation products, e.g. xylose, xylobiose, sophorose. Since activation of expression of those genes is dependent on Xyr1, we wondered if enzymes partaking in providing those inducers, such as BGLI, BGLII and BXLI (11, 17, 40, 46) are also affected by Xyr1. By examination of transcriptional levels of *bgl1*, *bgl2* and *bxl1* from glycerol pre-grown mycelia replaced to media containing 2 mM sophorose or xylobiose as inducers and incubated for 8 or 5 hours respectively (Table 5) as well as by determination of corresponding enzyme activities (Fig. 5A, B), we found that Xyr1 strictly regulates *bgl1* and *bxl1* expression (Table 5, Fig. 5A, B), but is not involved in activation of *bgl2* expression (Table 5)..

Discussion

In absence of easily utilizable carbon sources saprophytes as *H. jecorina* are able to metabolize heterogeneous polysaccharides. The naturally high secretory capacity of this fungus was further improved and recently led to *H. jecorina* strains secreting up to 100 g/L extracellular protein (M. Ward, personal communication). A set of its native enzymes allows degradation of xylan to smaller saccharides and finally to monomeric D-xylose (3) which can

enter the fungal cells and acts as an inducer (30). In this study we report effects of the deletion of the *xyr1* gene from the *H. jecorina* genome on D-xylose utilization. The observation that a Δ*xyr1*-strain was almost unable to grow on D-xylose plates (Table 2, Fig. 1A) prompted us to examine transcription levels of *xyl1* as well as activity of D-xylose reductase and L-arabinose reductase. As can be inferred from Fig. 2A, D-xylose reductase is tightly regulated by Xyr1 in contrast to L-arabinose reductase which is not affected in the Δ*xyr1*-strain (Fig. 2B). Therefore, we presume that the weak residual growth on D-xylose plates of the Δ*xyr1*-strain is resulting from a more general aldose reductase activity of the L-arabinose reductase. It shall be noted that a similar mechanism has previously been proposed for *A. niger* (15).

It is a well established fact that D-xylose is an inducer of *xyn1* gene expression in *H. jecorina* (30, 38, 53). Since Xyr1 affects the metabolism of that inducer, it stood to reason whether and how Xyr1 influences the expression of *xyn1*. Recently, we could demonstrate the basic necessity of Xyr1 binding to the *xyn1* promoter for both derepressed (release from Cre1 mediated glucose repression (30)) as well as induced *xyn1* gene expression (38). In this study we could demonstrate that transcript formation of *xyn1* is completely abolished on D-xylose and xylan in a Δ*xyr1*-strain (Table 3A). While in the parental strain both a basal *xyn2* transcription level as well as clear induction on sophorose, xylobiose and xylan could be detected, the Δ*xyr1*-strain did not form any corresponding transcript (Table 3B). Hence, we conclude that there is an indispensable Xyr1 dependence both on induction as well as on low basal *xyn2* transcription. As transcription analysis of *cbh1*, *cbh2* and *egl1* of both strains portrayed the same picture (Fig. 3A, B, C), consequently formation of expression products of the above mentioned genes was checked by determination of activities of hydrolytic enzymes. Measuring xylanase activities in supernatants of mycelia replaced to various carbon sources confirms the inability of the Δ*xyr1*-strain to form xylanolytic enzymes (Fig. 4) and thus asserted findings of analysis on the transcriptional level. Deletion of *xyr1* does not only lead

to the loss of induction of xylanase formation but also to the absence of low activity on medium without any carbon source that could be detected in the parental strain (Fig. 4). That low activity could on the one hand result from derepression of *xyn1* expression being regulated in a Cre1-dependent manner (30). Such an assumption is also in accordance with the fact that *xyn1* expression is strictly dependent on a Xyr1 binding motif even in the background of inactivated Cre1 sites in the *xyn1* promoter (38) hence strongly indicating the involvement of Xyr1 in the derepression mechanism. On the other hand, the low xylanase activity on medium without carbon source could be due to a basal level of *xyn2* transcription (52, 53). However, induction of xylanase enzyme activity formation is strictly depending on Xyr1 and it suggests itself that Xyr1 is additionally involved in both derepression and basal expression mechanisms.

Determination of cellulase activities again revealed a complete loss of enzyme formation in the Δ*xyr1*-strain (Table 4) thereby strongly pointing at Xyr1 as the general regulator of the *H. jecorina* xylanolytic and cellulolytic enzyme system. A similar regulatory function has previously been described for XlnR, the Xyr1 orthologue of *A. niger* (10, 15).

In addition, Xyr1 was shown to regulate the inducer-providing enzymes BGLI and BXLI (Fig. 5A, B, Table 5). Transcriptional levels of *bgl2* (40) are uninfluenced in contrast to those of *bgl1* that are absent in the Δ*xyr1*-strain (Table 5). Measuring of β-glucosidase activity in the supernatants confirmed regulation via Xyr1 since we could not detect any activity in the Δ*xyr1*-strain (Fig. 5A). Previously, it has been published that the *bgl1* gene product is mainly secreted into the medium (29) whereas antibodies raised against BGLII showed the presence of this enzyme in *H. jecorina* cell lysates but not in the culture supernatant (40). The fact that we measured reduced total β-glucosidase activities (mycelia plus supernatant) from the Δ*xyr1*-strain together with the findings that transcription of *bgl1* but not *bgl2* is activated in a Xyr1-dependent manner (Table 5, Fig. 5A), leads us to the conclusion that only the β-

glucosidase activity present in the supernatant is regulated by Xyr1 and is exclusively due to the *bgl1* gene product.

Thus Xyr1 regulates on the one hand the expression of the main xylanolytic and cellulolytic genes regardless which inducer substances they respond to and on the other hand some genes whose products are responsible for making these inducers available (*bxl1* and *bgl1*). A respective model outlining the extensive impact and involvement of Xyr1 on mechanisms leading to xylan and cellulose degradation can be found in Fig. S1 in the supplemental material.

Xyr1 being the central activator of expression of main xylanolytic and cellulolytic enzymes in *H. jecorina* relates this factor to *A. niger* XlnR which is known to be the main transcriptional activator of cellobiohydrolase- and xylanase-encoding genes (10, 47). Although both are regarded to be the main regulatory factor in the corresponding hydrolytic enzyme system and the D-xylose metabolism of the respective fungus, the molecular mechanisms of transcriptional activation involving Xyr1 must significantly differ from those concerning XlnR. Whereas XlnR-mediated induction of important hydrolase-encoding genes as *cbhA*, *cbhB, xlnB* and *xlnD* in *A. niger* require D-xylose (10, 15), the induction pattern of the best characterized hydrolytic enzymes in *H. jecorina* is more differentiated.

In recent years, low-molecular weight inducer substances as xylobiose and D-xylose as well as cellobiose and sophorose were shown to act as inducers of the formation of main hydrolases or transcription of the encoding genes of XYNI, XYNII, CBHI and CBHII (e.g. (18, 20, 30, 33, 53)).

Activation of the corresponding genes was previously proven to be caused by different inducers, e.g. the different inducibility of *xyn1* and *xyn2* by D-xylose and xylobiose respectively (53) or the inducibility of *xyn2* by xylobiose and sophorose in contrast to *cbh1* only by sophorose (33). Nevertheless, in transcriptional regulation of all of them one common regulator plays the essential role. Therefore our current working hypothesis includes the

existing of a general, substrate-unspecific activator - Xyr1 - next to inducer-specific transcriptional regulators responsible for the fine-tuning of regulation of the corresponding gene, such as recently exemplarily shown for the antagonistic relationship of Xyr1 and Ace1 concerning *xyn1* regulation (38). This working model concurs with the indication that Xyr1 binds to an inverted repeat within the *xyn1* promoter either as a homo- or hetero-dimer, respectively, thereby providing the opportunity for specific regulatory proteins to interact with the accordant promoter and/or Xyr1. Moreover, preliminary studies suggest Ace2 to pose a *xyn2*-specific transcriptional factor modulating activation caused by Xyr1 (A. R. Stricker, P. Trefflinger, and R. L. Mach, unpublished data). For *A. niger* XlnR a possible participation of Ace1 in transcriptional regulation of hydrolase-encoding genes has not yet been shown and an Ace2 homologue could not even be found in the genome. In addition, it was suggested that XlnR binds as a monomer since it was shown to bind to a non-palindromic consensus in the promoter (14, 48). In contrary to Xyr1, XlnR seems to act as an unspecific transactivator of a wide variety of genes encoding hydrolytic enzymes responding mainly to D-xylose induction, largely independent of other substrate-specific regulatory proteins.

It remains to be resolved if and how Ace2 interacts with Xyr1 and which role further proteins involved in regulation of *xyn2* under repressing conditions are playing (e.g. the postulated AGAA–binding repressor protein of the *xyn2* promoter; (52)). Above all, a fundamental understanding of the regulation of Xyr1 itself would enlighten important mechanisms within the xylanolytic transcriptosome and pose a powerful tool for strain design and protein production improvement.

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1 TABLE 1. Primers and probes used throughout the study.

2

Name	Sequence (5' - 3')	Employment[a]
Actf	TGAGAGCGGTGGTATCCACG	actin real-time PCR
Actr	GGTACCACCAGACATGACAATGTTG	actin real-time PCR
bgl1f	CAGACAGTCACTCAACATCGGG	*bgl1* real-time PCR
bgl1r	ACCTTATCTTGGAGATTGAGCTTTGCC	*bgl1* real-time PCR
bgl2f	ACGGCTGCCTACCAGATCG	*bgl2* real-time PCR
bgl2r	AGCCGTCGGCGATCTTGC	*bgl2* real-time PCR
bxl1f	GCCAACTTCGCCACCAAGG	*bxl1* real-time PCR
bxl1r	CGGCAATCTGGTGGATCAATGTG	*bxl1* real-time PCR
Taqman cbh1 FAM	CTGGACTCCACCTACCCGACAAACGAGACC	*cbh1* real-time PCR
chb1f	GATGATGACTACGCCAACATGCTG	*cbh1* real-time PCR
cbh 1Df2	TGTTACAGTACTACGCCAACATGCTG	*cbh1* real-time PCR
cbh 1r	ACGGCACCGGGTGTGG	*cbh1* real-time PCR
Taqman cbh2 TR	GACTTGCCGGATCGCGATTGCGCTGCCC	*cbh2* real-time PCR
cbh2f	CTATGCCGGACAGTTTGTGGTG	*cbh2* real-time PCR
cbh2Dr	GTCAGGCTCTGGAAGAAGG	*cbh2* real-time PCR
cbh2r	GTCAGGCTCAATAACCAGGAGG	*cbh2* real-time PCR
Taqman egl1 FAM	CAACTCGAGGGCGAATGCCTTGACCCCTCACTC	*egl1* real-time PCR
egl1f	CTGCAACGAGATGGATATCCTGG	*egl1* real-time PCR
egl1Dr	GAGAGCGCCAGGAAAGGG	*egl1* real-time PCR
egl1r	GTAGTAGCTTTTGTAGCCGCTGC	*egl1* real-time PCR
Xorf	CTGTGACTATGGCAACGAAAAGGAG	*xyl1* real-time PCR
Xorr	CACAGCTTGGACACGATGAAGAG	*xyl1* real-time PCR
Taqman xyn1 FAM	CGTCCAACCAACGCCCACAACAA	*xyn1* real-time PCR
Taqxyn1f	CAGCTATTCGCCTTCCAACAC	*xyn1* real-time PCR
Taqxyn1Dr	GAGGAGTCCTCCTACGCAGAA	*xyn1* real-time PCR
Taqxyn1r	CCAAAGTTGATGGGAGCAGAA	*xyn1* real-time PCR
Taqman xyn2 FAM	CTGCCATCCCTTGCCGCC	*xyn2* real-time PCR
Taqxyn2f	GGTCCAACTCGGGCAACTTT	*xyn2* real-time PCR

Taqxyn2Dr	GGTAAGGGTAGGTAGTCTTACTTGTTC	*xyn2* real-time PCR
Taqxyn2r	CCGAGAAGTTGATGACCTTGTTC	*xyn2* real-time PCR
Taqman xyr1 FAM	CGCGCTTGTGACCAGTGCAACCAGCTTCGTACC	*xyr1* real-time PCR
xyr1f	CCCATTCGGCGGAGGATCAG	*xyr1* real-time PCR
xyr1Dr	GATCAGTACATGTGCTTGAGCGC	*xyr1* real-time PCR
xyr1r	CGAATTCTATACAATGGGCACATGGG	*xyr1* real-time PCR
Xyr1.1.f	GGTACCAATTGTGAGCGCATCAC	Construction of deletion cassette
Xyr1.2.r	CTGTCGACGATGGGAATTCGGGTCAAATGAC	Construction of deletion cassette
Xyr2.1.f.	CCGAATTCCCATCGTCGACAGGCACCTGG	Construction of deletion cassette
Xyr2.2.r	GGTACCGAACATAGCCCAACG	Construction of deletion cassette

[a] Employment of the oligonucleotides during the study is given.

TABLE 2**.** Growth of the parental strain and the Δ*xyr1*-strain on plates containing MA medium supplemented with different carbon sources (1 % w/v) at 30 °C.

Carbon source	avg diam [cm] after 3 days			avg diam [cm] after 6 days		
	QM9414	Δ*xyr1*	Re*xyr1*A	QM9414	Δ*xyr1*	Re*xyr1*A
Glucose	3.7 ± 0.29[a]	3.9 ± 0.17	3.5 ± 0.19	8.0 ± 0.00	8.0 ± 0.00	8.1 ± 0.04
Glycerol	3.0 ± 0.15	3.4 ± 0.00	3.2 ± 0.12	7.2 ± 0.35	7.1 ± 0.10	7.2 ± 0.12
L-(+)-Arabinose	3.3 ± 0.10	3.5 ± 0.21	3.5 ± 0.17	7.4 ± 0.12	7.5 ± 0.40	7.3 ± 0.15
L-(-)-Arabitol	3.1 ± 0.17	3.7 ± 0.06	3.3 ± 0.08	7.7 ± 0.15	7.7 ± 0.12	7.6 ± 0.22
D-(+)-Xylose	4.1 ± 0.23	1.1 ± 0.10	4.2 ± 0.21	8.0 ± 0.00	2.4 ± 0.06	8.2 ± 0.16
Xylitol	3.3 ± 0.12	3.5 ± 0.06	3.5 ± 0.12	7.5 ± 0.06	7.5 ± 0.00	7.6 ± 0.17
Xylan	2.0 ± 0.17	2.5 ± 0.12	2.2 ± 0.19	5.5 ± 0.38	4.6 ± 0.12	5.6 ± 0.16
Cellulose	2.8 ± 0.17	3.3 ± 0.17	3.0 ± 0.14	5.7 ± 0.10	6.0 ± 0.00	5.9 ± 0.11

[a] Values are derived from three independent experiments; ± indicates standard deviation

TABLE 3A. Relative *xyn1* transcription levels of the wild-type and the Δ*xyr1*-strain.

Carbon source, cultivation time [h]	amt of mRNA (cDNA) per gene dose	
	QM9414	Δ*xyr1*
No carbon source 3	1.0 ± 0.5[a]	nd[b]
No carbon source 5	0.5 ± 0.1	nd
No carbon source 8	0.4 ± 0.2	nd
Glucose 3	nd	nd
Glucose 5	nd	nd
Xylose 3	1.9 ± 0.4	nd
Xylose 5	169.3 ± 16.5	nd
Xylose 8	0.0 ± 0.0	nd
Xylan 8	16.1 ± 2.6	nd
Xylan 24	9.8 ± 0.0	nd

[a] values are derived from three independent experiments; ± indicates standard deviation

[b] nd means no detection

TABLE 3B. Relative *xyn2* transcription levels of the wild-type and the Δ*xyr1*-strain.

Carbon source, cultivation time [h]	amt of mRNA (cDNA) per gene dose	
	QM9414	Δ*xyr1*
No carbon source 3	1.0 ± 0.1	nd
No carbon source 5	3.8 ± 0.5	nd
No carbon source 8	nd	nd
Sophorose 5	97.9 ± 9.6	nd
Sophorose 8	6701.7 ± 531.1	nd
Xylobiose 3	6085.9 ± 637.1	nd
Xylobiose 5	265.6 ± 11.2	nd
Xylobiose 8	37.5 ± 3.2	nd
Xylan 8	4305.9 ± 415.3	nd
Xylan 24	3819.4 ± 277.6	nd

TABLE 4. Xylanase- and cellulase activities in supernatants of direct cultivations of the parental strain and the Δ*xyr1*-strain on xylan or cellulose as carbon source, respectively.

Cultivation time [h]	Xylanase activity [a] [U/µg protein] in xylan supernatants		Cellulase activity [b] [U/µg protein] in cellulose supernatants	
	QM9414	Δ*xyr1*	QM9414	Δ*xyr1*
24	nd [c]	nd	0.003 ± 0.000 [d]	nd
48	0.206 ± 0.015	nd	0.655 ± 0.012	nd
72	0.142 ± 0.009	nd	0.885 ± 0.022	nd

[a] One Unit of activity is defined as the amount of enzyme required to release one micromole of xylose reducing-sugar-equivalents per minute under the defined assay conditions.

[b] One Unit of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar-equivalents per minute under the defined assay conditions.

[c] nd means no detection

[d] Values are derived from three independent experiments; ± indicates standard deviation

TABLE 5. Relative transcription levels of *bgl1*, *bgl2* and *bxl1* of the wild-type and the Δ*xyr1*-strain.

Analyzed gene, carbon source, incubation time [h]	Transcript ratios [a]	
	QM9414	Δ*xyr1*
bgl1, Sophorose 8	1.0 ± 0.1 [b]	nd [c]
bgl2, Sophorose 8	1.3 ± 0.2	1.1 ± 0.0
bxl1, Xylobiose 5	58.2 ± 0.4	nd

[a] values were calculated with reference to sample QM9414, *bgl1*, Sophorose 8

[b] values are derived from three independent experiments; ± indicates standard deviation

[c] nd means no detection

Legends to figures

FIG. 1: (A) Growth of parental strain QM9414, the *xyr1* retransformation strain (Re*xyr1*A) and the Δ*xyr1*-strain on plates containing MA media supplemented with 1 % (w/v) L-arabinose (AN), L-arabitol (AL), xylitol (XL) and D-xylose (XO) and 2 % (w/v) Agar-Agar at 30 °C after four days. (B) Schematic presentation of the D-xylose catabolic pathway. In *H. jecorina* D-xylose cannot be directly converted into D-xylulose because fungi do not have xylulose isomerase at their disposal (51).

FIG. 2: Enzyme activities of D-xylose reductase (A, C) and L-arabinose reductase (B, D) given in U/g measured in cell free extracts of QM9414 (QM) and the Δ*xyr1*-strain. All strains were pre-cultured in MA medium containing 1 % (w/v) glycerol. For analysis of both reductase activities, mycelia were transferred to MA media comprising 1 % (w/v) D-xylose (XO) (A, D) and L-arabinose (AN) (B, C) and grown for 5 and 10 h. One Unit of activity is defined as the amount of enzyme responsible for consummation of one micromole NADPH per minute due to its oxidation in the presence of D-xylose or L-arabinose under the defined assay conditions (5). Values are means of three independent experiments. Error bars indicate standard deviations.

FIG. 3: Relative transcription levels of *cbh1* (A), *cbh2* (B) and *egl1* (C). The parental strain QM9414 (QM) and the Δ*xyr1*-strain were pre-cultured on glycerol and thereafter transferred to MA media containing 2 mM sophorose (SO) as inducer or 1 % (w/v) cellulose (CL) as sole carbon source and incubated 5 and 8 hours or 8 and 24 hours, respectively. After parallel extraction of RNA and DNA and reverse transcription of RNA, real-time PCR analysis of the

respective genes was performed. Values are means of three independent experiments. Error bars indicate standard deviations.

FIG. 4: Xylanase activities in culture supernatants of the parental strain QM9414and the Δ*xyr1*-strain. Both strains were pre-cultured in MA medium containing 1 % (w/v) glycerol. Detection of xylanase activity formation was accomplished after transfer of equal amounts of wet weight of mycelia to MA media without carbon source or comprising 2 mM sophorose or xylobiose as inducer incubated for 5 and 8 h or 1 % (w/v) D-xylose grown for 5 and 8 h or to MA media containing 1 % (w/v) oat spelt xylan or carboxymethylcellulose, grown for 8 and 24 h. One Unit of activity is defined as the amount of enzyme required to release one micromole of xylose reducing-sugar-equivalents per minute at 40 °C. Data are means of results from three independent experiments. Error bars indicate standard deviations.

FIG. 5: (A) Total (extracellular and mycelial-bound) β-glucosidase activity (+) and activity in the culture supernatant (-) of the parental and the Δ*xyr1*-strain after replacement of glycerol pre-grown mycelia into media containing 2 mM sophorose (SO) and incubation for 5 and 8 h. (B) Activity of β-xylosidase in supernatants of the parental and the Δ*xyr1*-strain after replacement of glycerol pre-grown mycelia into media containing 2 mM xylobiose (XB) and incubation for 5 h. All data are means of results from three independent experiments. Error bars indicate standard deviations.

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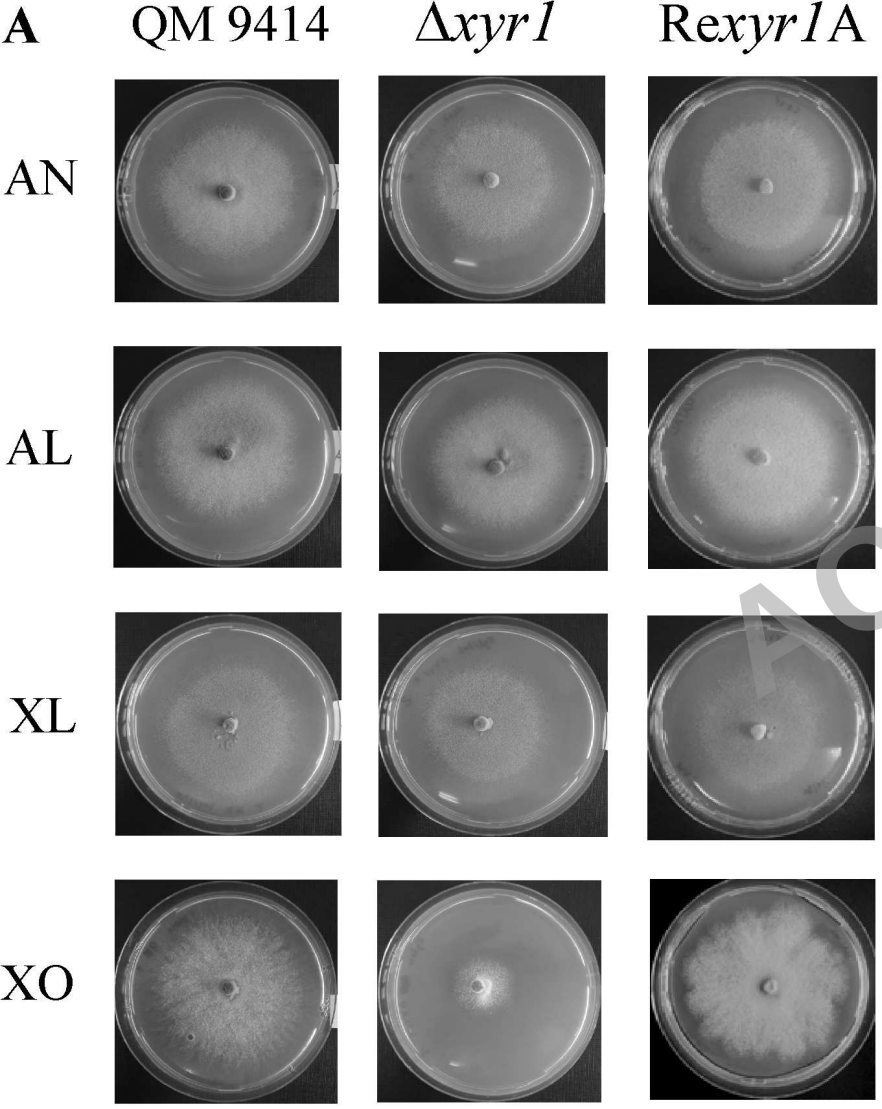
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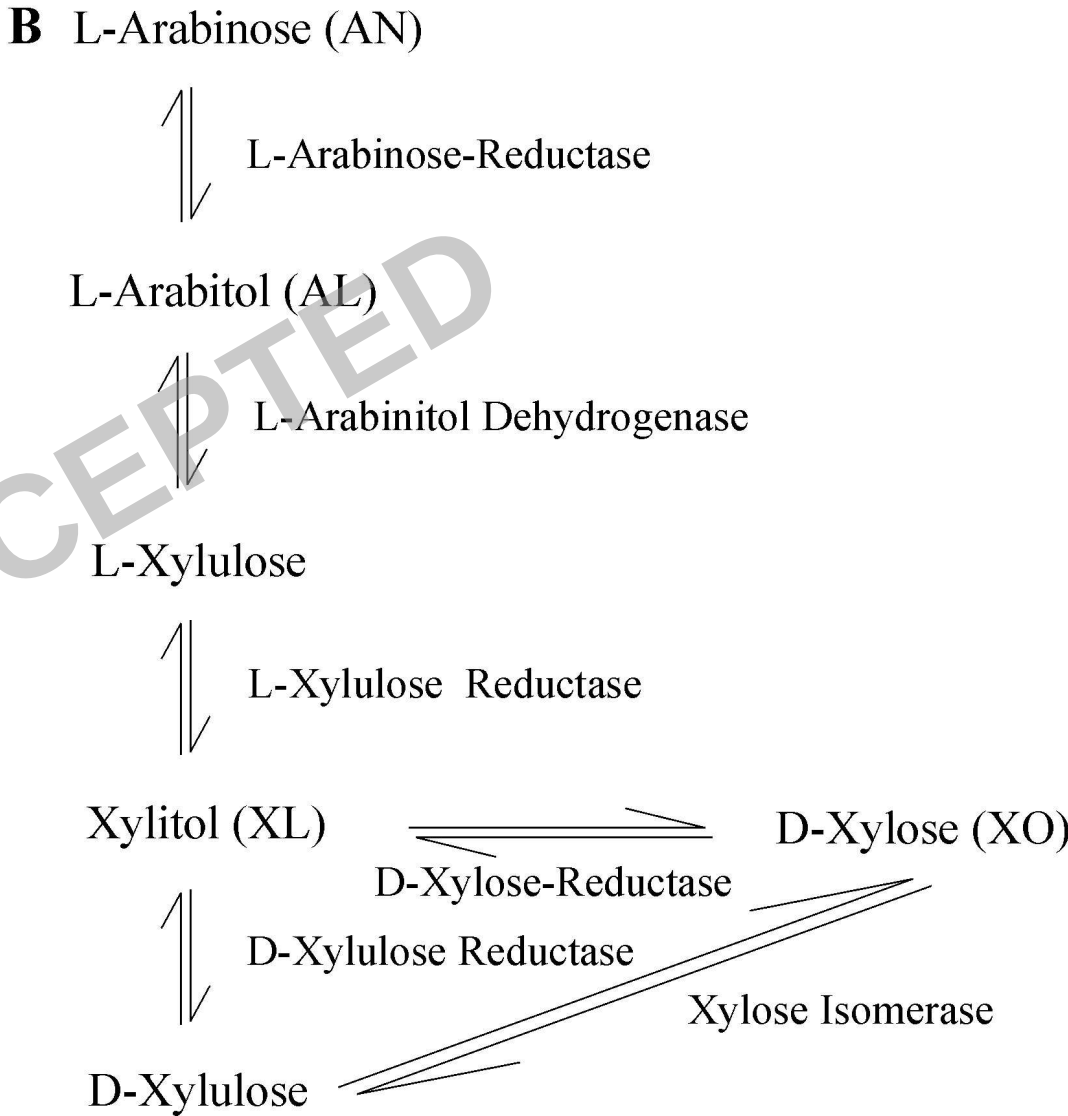
XL
XO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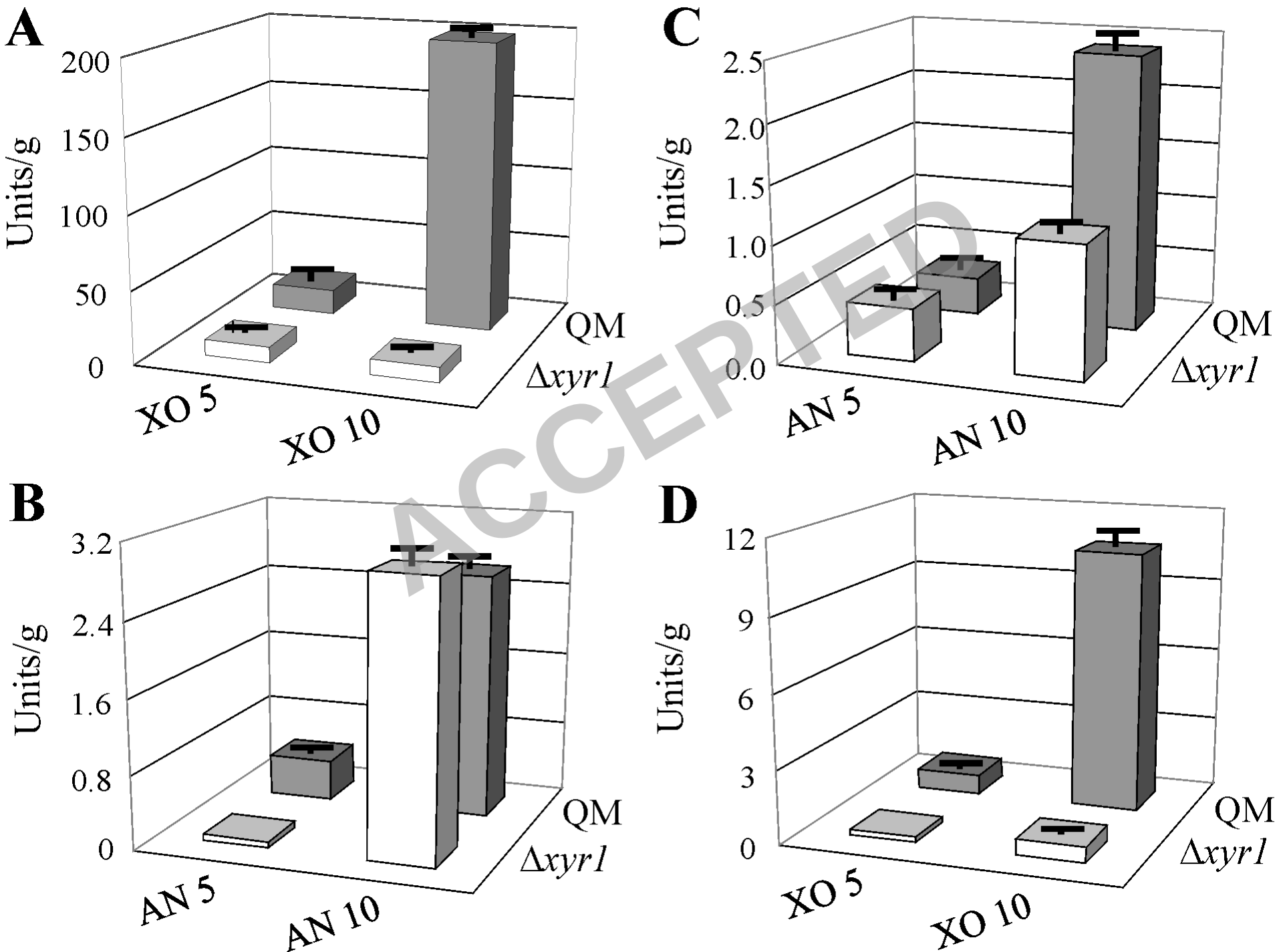
D-Xylulose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XO 10
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0
Units/g
AN 5
AN 10
QM
Δxyr1
C
2.5
2.0
1.5
1.0
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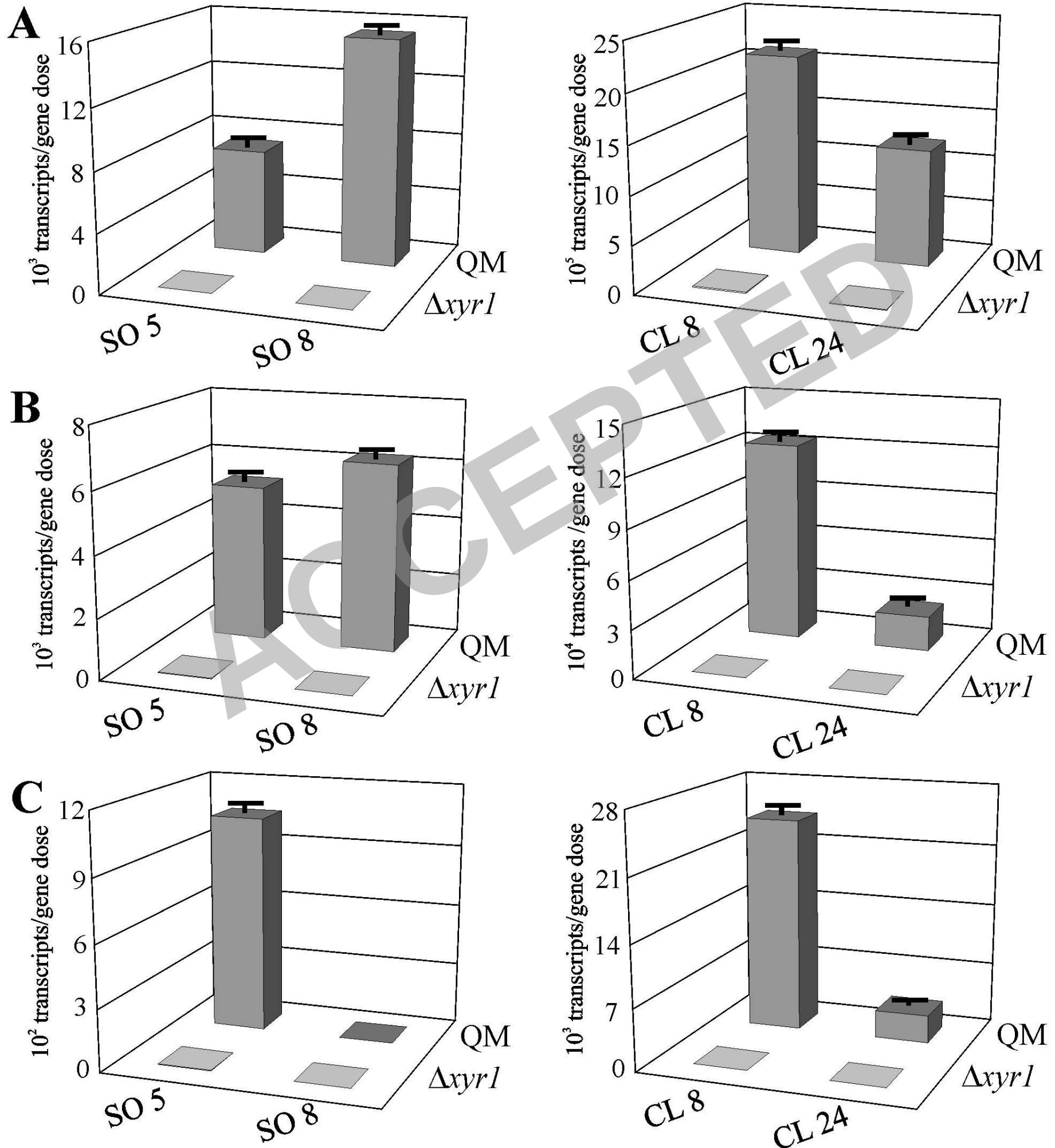
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10
5
0
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CL 24
QM
Δxyr1
B
10^3 transcripts/gene dose
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6
4
2
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SO 8
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10^4 transcripts /gene dose
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12
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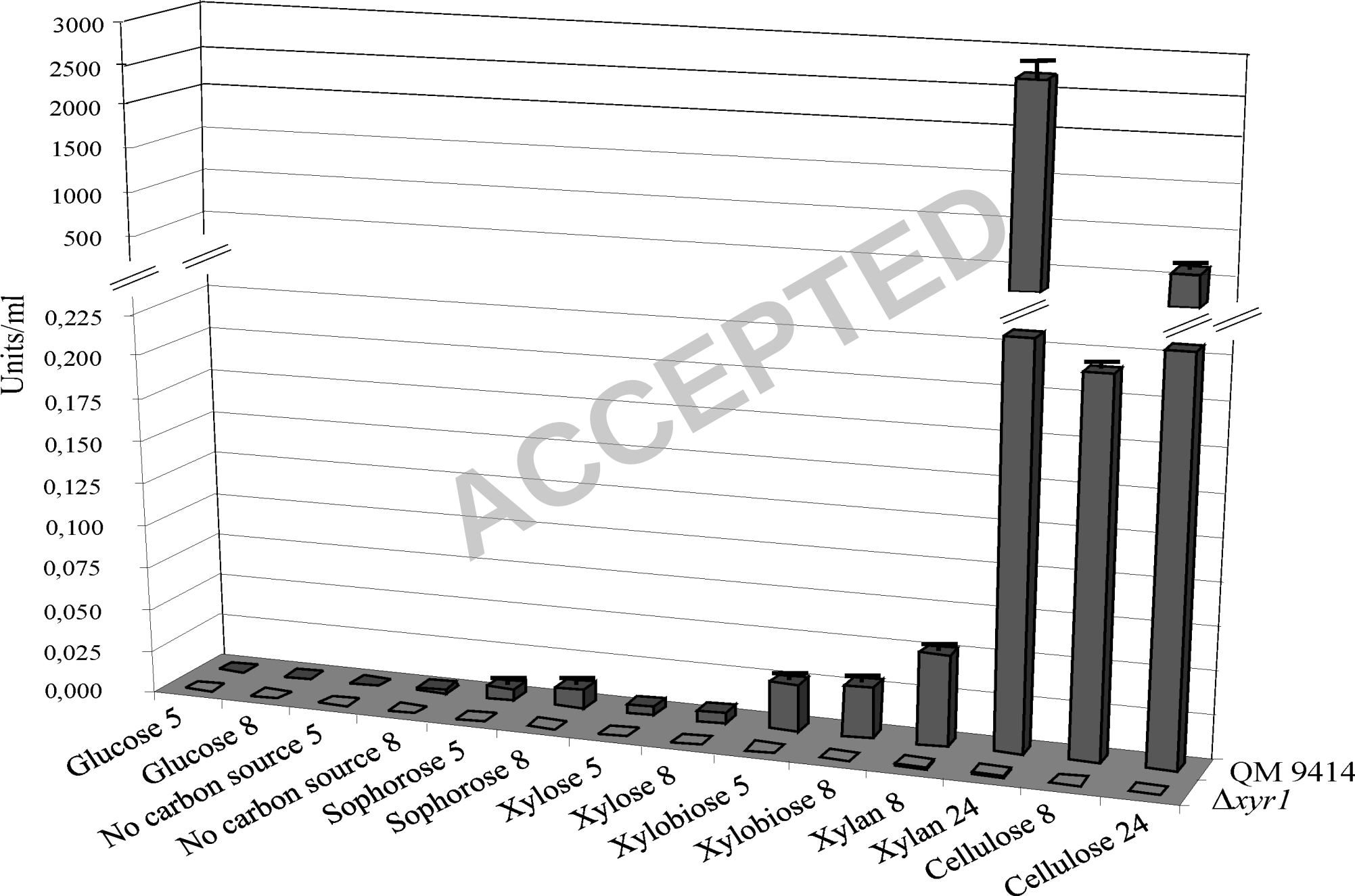
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Glucose 8
No carbon source 5
No carbon source 8
Sophorose 5
Sophorose 8
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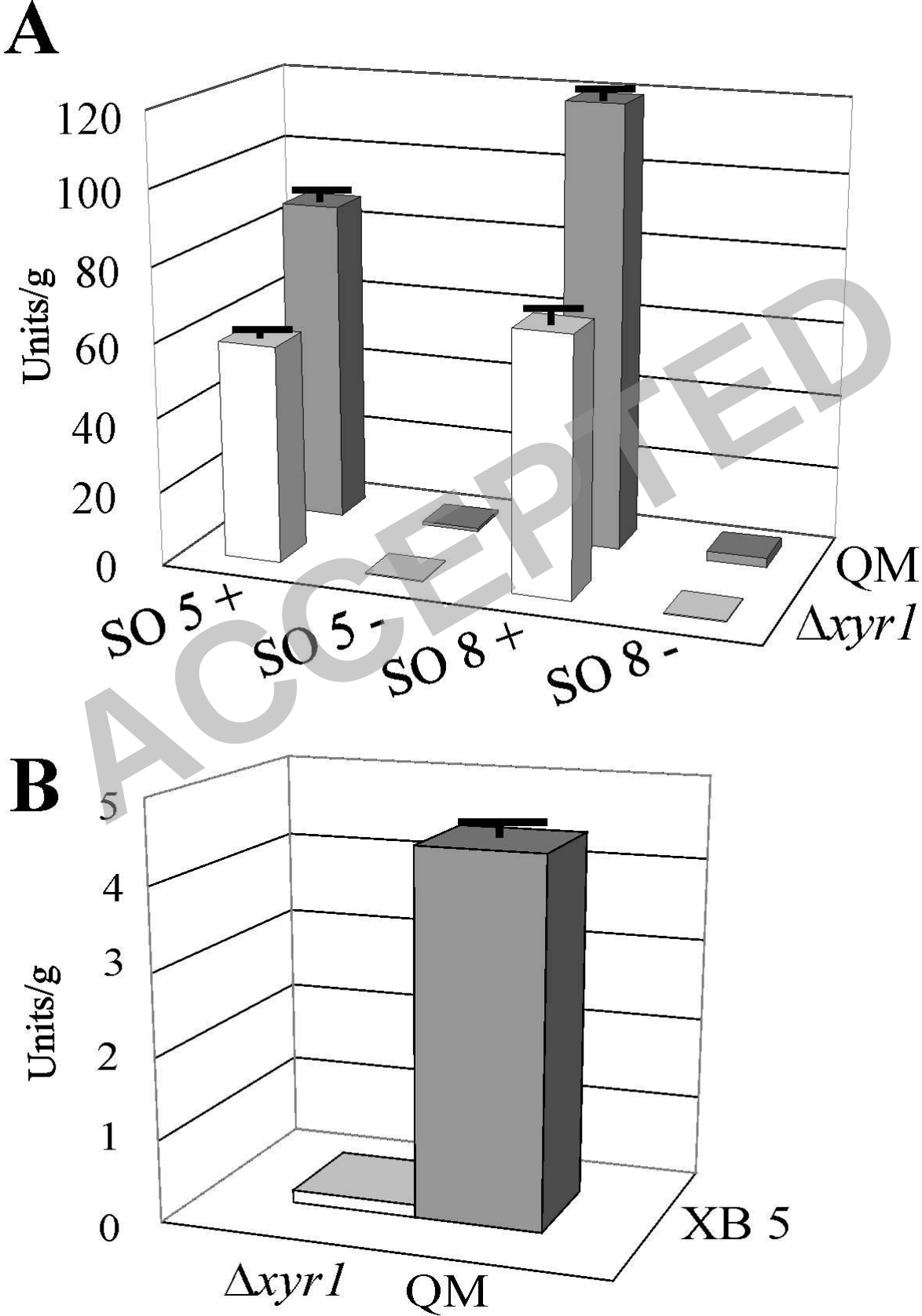
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SO 8 -
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Units/g
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QM
XB 5