Electronic Supplementary Information

Substrate promiscuities of a bacterial galactokinase and a glucose-1-phosphate uridyltransferase enable xylose salvaging

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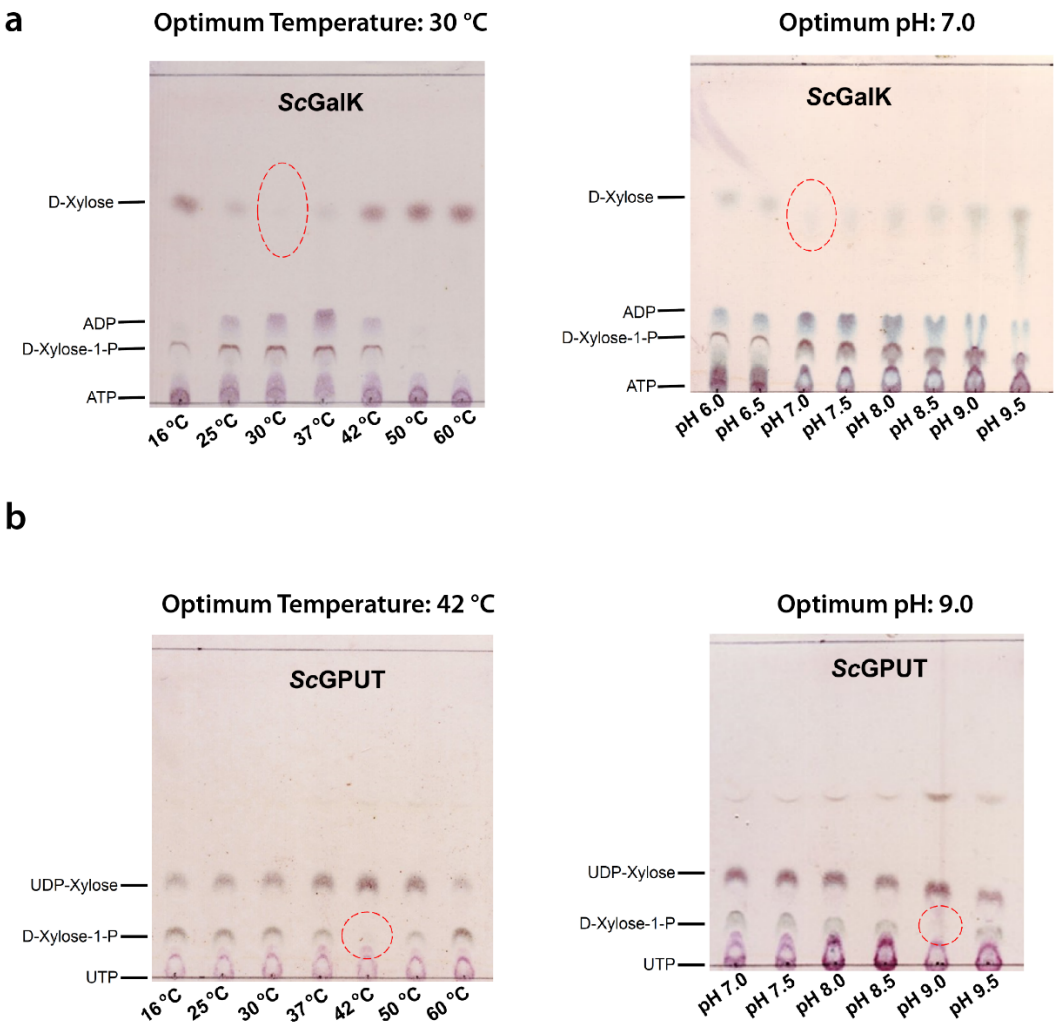
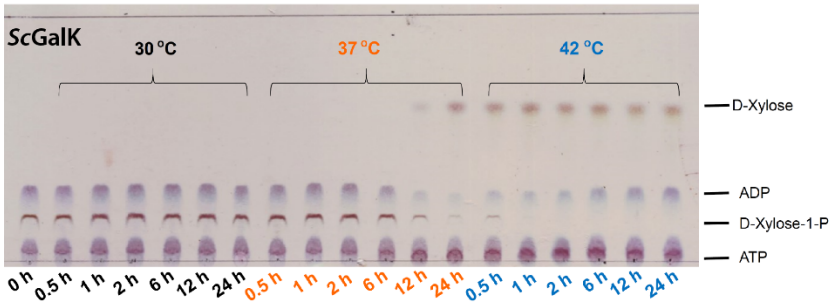


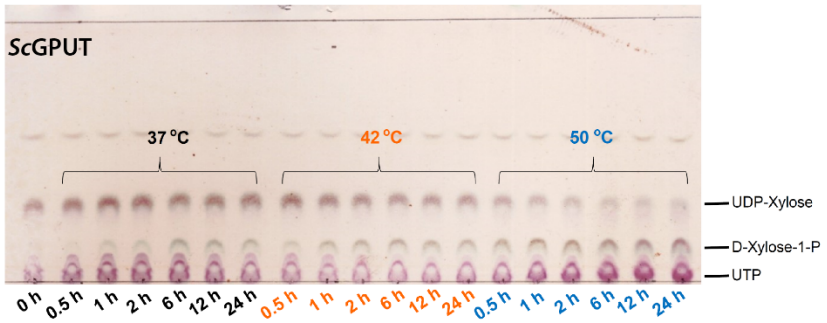
Fig. S1: Thin-layer chromatography (TLC) to determine the temperature and pH optima of (a) *Sc*GalK and (b) *Sc*GPUT. The red circles show where the highest depletion of the respective donor substrate occurred. The temperature optimum of *Sc*GalK was determined as follows: Reaction mixtures (final volume 50 µL) containing D-xylose (1 mM), ATP (2 mM), $MgCl_2$ (2 mM) Tris/HCl (50 mM, pH 8.0), and *Sc*GalK (210 µg) were incubated for 18 h at the respective temperatures. The pH optimum of *Sc*GalK was determined as follows: Reaction mixtures (final volume 50 µL) containing D-xylose (1 mM), ATP (2 mM), $MgCl_2$ (2 mM), *Sc*GalK (145 µg), and 200 mM of the respective pH buffer (sodium phosphate buffer for pH 6.0/6.5, and Tris/HCl buffer for pH values 7.0-9.5). The temperature optimum of *Sc*GPUT was determined as follows: Reaction mixtures (final volume 50 µL) containing D-xylose-1-phosphate (1 mM), UTP (3 mM), $MgCl_2$ (2 mM), Tris/HCl (50 mM, pH 7.5), and *Sc*GPUT (185 µg) were incubated for 18 h at the respective temperatures. The pH optimum of *Sc*GPUT was determined as follows: Reaction mixtures (final volume 50 µL) containing D-xylose-1-phosphate (1 mM), UTP (3 mM), $MgCl_2$ (2 mM), *Sc*GPUT (150 µg), and 200 mM of the respective Tris/HCl buffer adjusted to pH values 7.0-9.5. After centrifugation (20,000 × *g*, 5 min), 1 µL of the supernatant was spotted onto silica gel 60 F_{254} TLC sheets (on aluminium, Supelco), and sample spots were separated using an *n*-butanol/ethanol/water (5:3:2, v:v:v) solvent system. The analytes were visualised using an orcinol stain (consisting of 40 mg orcinol monohydrate dissolved in 20 mL of aqueous H_2SO_4 (3.6 M)) was used for visualisation.

a



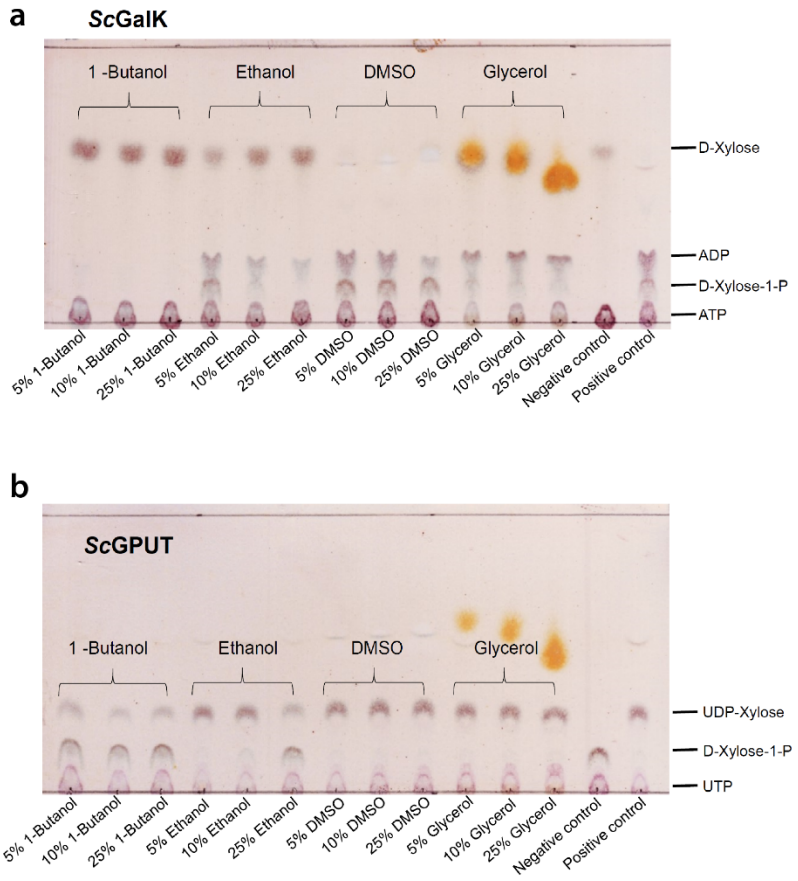
	0.5 h	1 h	2 h	6 h	12 h	24 h
30 °C	+++++	+++++	+++++	+++++	+++++	+++++
37 °C	+++++	+++++	+++++	+++++	+++	+
42 °C	+	-	-	-	-	-

b



	0.5 h	1 h	2 h	6 h	12 h	24 h
37 °C	+++++	++++	++++	+++	+++	+++
42 °C	++++	+++	+++	+++	+++	+++
50 °C	+++	++	++	+	+	+

Fig. S2: Thin-layer chromatography (TLC) to determine stability (a) *Sc*GalK and (b) *Sc*GPUT at various temperatures. The temperature stability of *Sc*GalK was determined as follows: Purified *Sc*GalK enzyme (250 µg in 20 µL) were incubated for 0.5-24 h at the respective temperatures, and then used as enzyme source for 50 µL reaction mixtures which contained D-xylose (1 mM), ATP (2 mM), $MgCl_2$ (2 mM), and Tris/HCl (50 mM, pH 8.0). These samples were incubated for 12 h at 30 °C. The temperature stability of *Sc*GPUT was determined as follows: Purified *Sc*GPUT enzyme (240 µg in 20 µL) were incubated for 0.5-24 h at the respective temperatures, and then used as enzyme source for 50 µL reaction mixtures which contained D-xylose-1-phosphate (1 mM), UTP (3 mM), $MgCl_2$ (2 mM), and Tris/HCl (50 mM, pH 7.5). These samples were incubated for 8 h at 42 °C. After centrifugation (20,000 × *g*, 5 min), 1 µL of the supernatant was spotted onto silica gel 60 F_{254} TLC sheets (on aluminium, Supelco), and sample spots were separated using an *n*-butanol/ethanol/water (5:3:2, v:v:v) solvent system. The analytes were visualised using an orcinol stain (consisting of 40 mg orcinol monohydrate dissolved in 20 mL of aqueous H_2SO_4 (3.6 M)) was used for visualisation.



c

	5% (v/v) 1-Butanol	10% (v/v) 1-Butanol	25% (v/v) 1-Butanol	5% (v/v) Ethanol	10% (v/v) Ethanol	25% (v/v) Ethanol	5% (v/v) DMSO	10% (v/v) DMSO	25% (v/v) DMSO	5% (v/v) Glycerol	10% (v/v) Glycerol	25% (v/v) Glycerol
*Sc*GalK	-	-	-	++	+	-	+++++	+++++	+++++	+	+	+
ScGPUT	++	++	++	+++++	++++	+++	+++++	+++++	+++++	+++++	+++++	+++++

Fig. S3: Thin-layer chromatography (TLC) to determine activities of (a) *Sc*GalK and (b) *Sc*GPUT in the presence of various organic solvents. For *Sc*GalK, the enzymatic activity in the presence of various organic solvents was determined as follows: Reaction mixtures (final volume 50 µL) containing D-xylose (1 mM), ATP (2 mM), $MgCl_2$ (2 mM) Tris/HCl (50 mM, pH 8.0), and *Sc*GalK (330 µg) were incubated in the presence of organic solvents (concentration between 5% and 25% (v/v)) for 12 h at 37 °C. For *Sc*GPUT, the enzymatic activity in the presence of various organic solvents was determined as follows: Reaction mixtures (final volume 50 µL) containing D-xylose-1-phosphate (1 mM), UTP (3 mM), $MgCl_2$ (2 mM), and Tris/HCl (50 mM, pH 7.5), and *Sc*GPUT (275 µg) were incubated in the presence of organic solvents (concentration between 5% and 25% (v/v)) for 12 h at 37 °C. After centrifugation (20,000 × *g*, 5 min), 1 µL of the supernatant was spotted onto silica gel 60 F_{254} TLC sheets (on aluminium, Supelco), and sample spots were separated using an *n*-butanol/ethanol/water (5:3:2, v:v:v) solvent system. The analytes were visualised using an orcinol stain (consisting of 40 mg orcinol monohydrate dissolved in 20 mL of aqueous H_2SO_4 (3.6 M)) was used for visualisation.

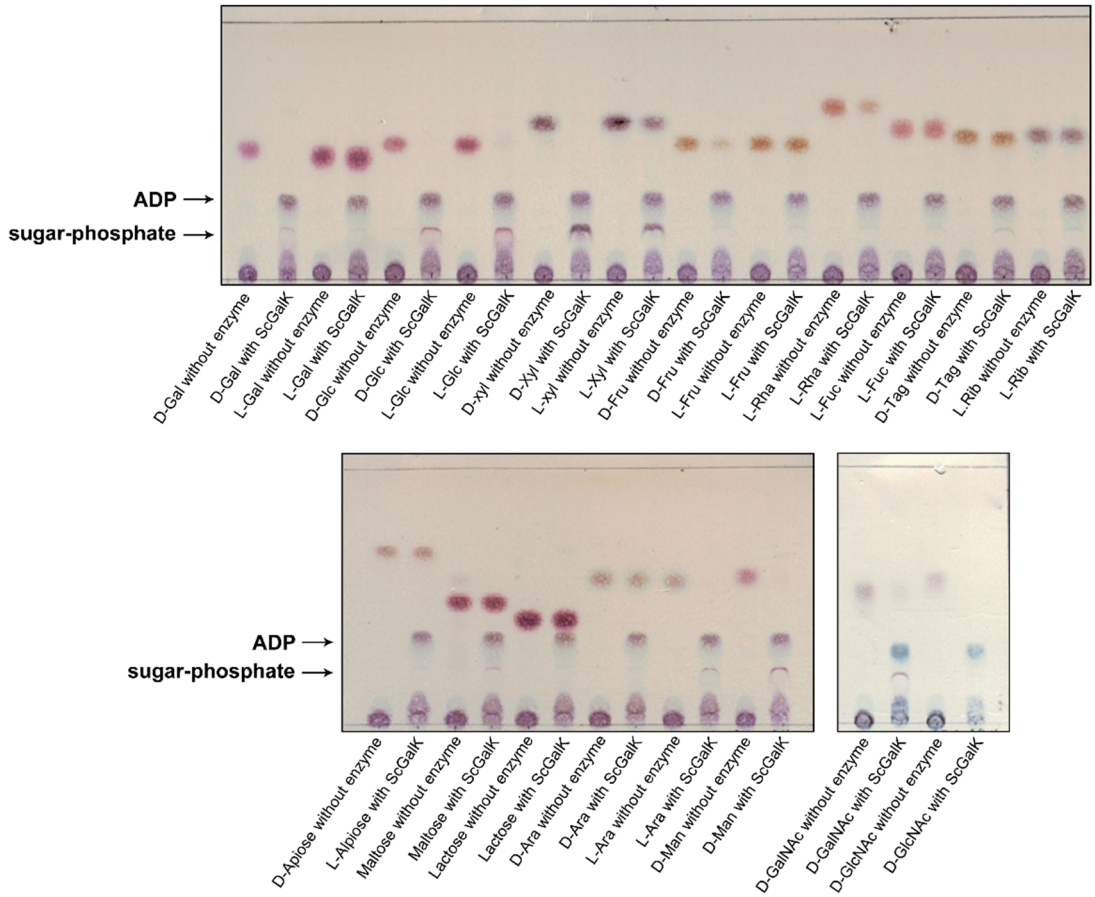


Fig. S4: Thin layer chromatography (TLC) of *Sc*GalK reaction mixtures with various acceptor sugar substrates. The applied reaction mixtures (50 µL) contained the acceptor sugar solution (7.5 µL, 20 mM), phosphate buffer (5 µL, pH 8.0, 500 mM), $MgCl_2$ solution (1 µL, 100 mM), ATP solution (3 µL, 100 mM), and recombinant, purified and desalted *Sc*GalK enzyme solution (33.5 µL, protein concentration 5.1 mg/mL). The reaction mixtures were incubated at 37 °C for 18 h and the reaction stopped by heating at 95 °C for 5 min. Control samples replaced the *Sc*GalK enzyme solution with 33.5 µL of water. After centrifugation (20,000 × *g*, 5 min), 1 µL of the supernatant was spotted onto silica gel 60 F_{254} TLC sheets (on aluminium, Supelco), and sample spots were separated using an *n*-butanol/ethanol/water (5:3:2, v:v:v) solvent system. The analytes were visualised using a DPA stain (consisting of aniline (2% v/v), diphenylamine (2% w/v) and phosphoric acid (10% v/v) in acetone) for GalNAc and GlcNAc. For all other sugar substrates an orcinol stain (consisting of 40 mg orcinol monohydrate dissolved in 20 mL of aqueous H_2SO_4 (3.6 M)) was used for visualisation.

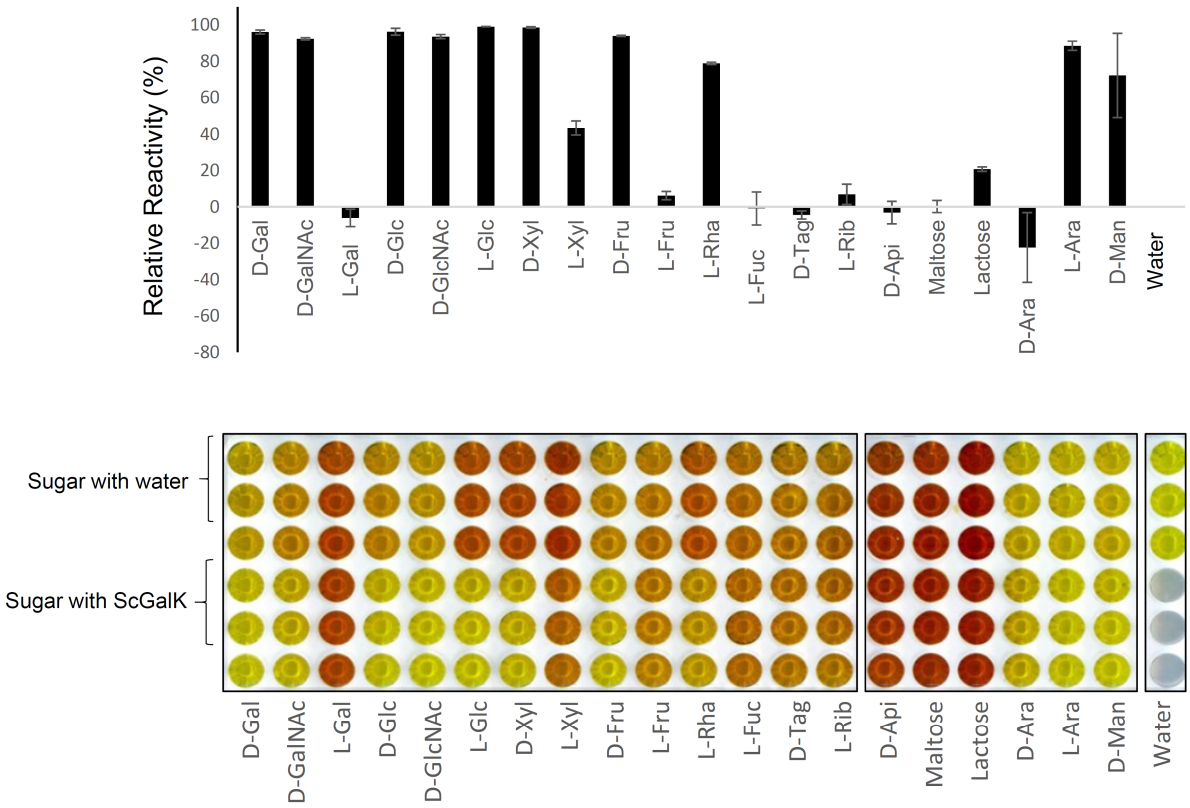


Fig. S5: Colorimetric determination of the *Sc*GalK activity towards various acceptor sugar substrates. The applied reaction mixtures (50 µL) contained the acceptor sugar solution (7.5 µL, 20 mM), phosphate buffer (5 µL, pH 8.0, 500 mM), $MgCl_2$ solution (1 µL, 100 mM), ATP solution (3 µL, 100 mM), and recombinant, purified and desalted *Sc*GalK enzyme solution (33.5 µL, protein concentration 5.1 mg/mL). The reaction mixtures were incubated at 37 °C for 18 h and the reaction stopped by heating at 95 °C for 5 min. Control samples replaced the ScGalK enzyme solution with 33.5 µL of water. After centrifugation (20,000 × *g*, 5 min), 30 µL of the clear supernatant were mixed with 45 µL of a DNS reagent (a 200 mL solution consists of 0.63 g 3,5-dinitrosalicylic acid, 4 g NaOH, 18.2 g potassium sodium tartrate tetrahydrate, 0.5 g phenol, and 0.5 g of sodium sulfite in water) and heated at 100 °C for 5 min. From each sample, 20 µL aliquots were then transferred into individual wells of a 384-microtitre plates and the absorbance measured at 550 nm. The mean values and standard error was calculated from the pairwise comparison of Absorbance values of the *Sc*GalK samples and control samples (reaction mixtures without *Sc*GalK) using the same sugar substrate with the Absorbance value of the blank sample (only 'Water' Sample) using the formula: $Relative\ Reactivity\ (\%) = (Abs_{'Water'\ Sample} - Abs_{ScGalK\ Sample}) / (Abs_{'Water'\ Sample} - Abs_{Control\ Sample})$.

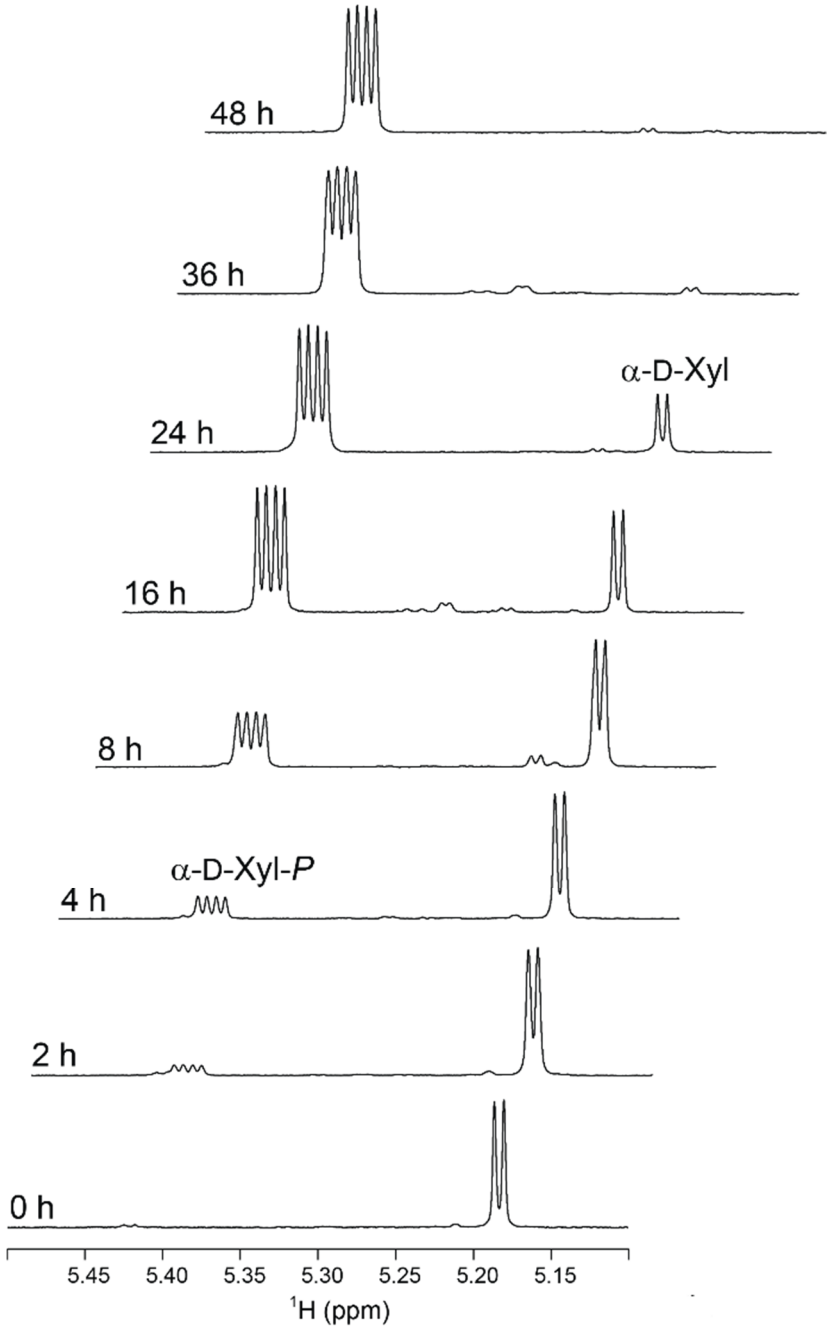


Fig. S6: Time course analysed by ^{1}H NMR spectoscopy for the kinase reaction carried out by *Sc*GalK transforming D-xylose into α-D-xylose 1-phosphate. The progress was monitored by sampling the reaction at different time points (isolated sample) as shown for the appearance of the resonance from the anomeric proton of the product α-D-xylopyranose 1-phosphate (left) and the disappearance of the resonance from the anomeric proton of the substrate α-D-xylopyranose (right).

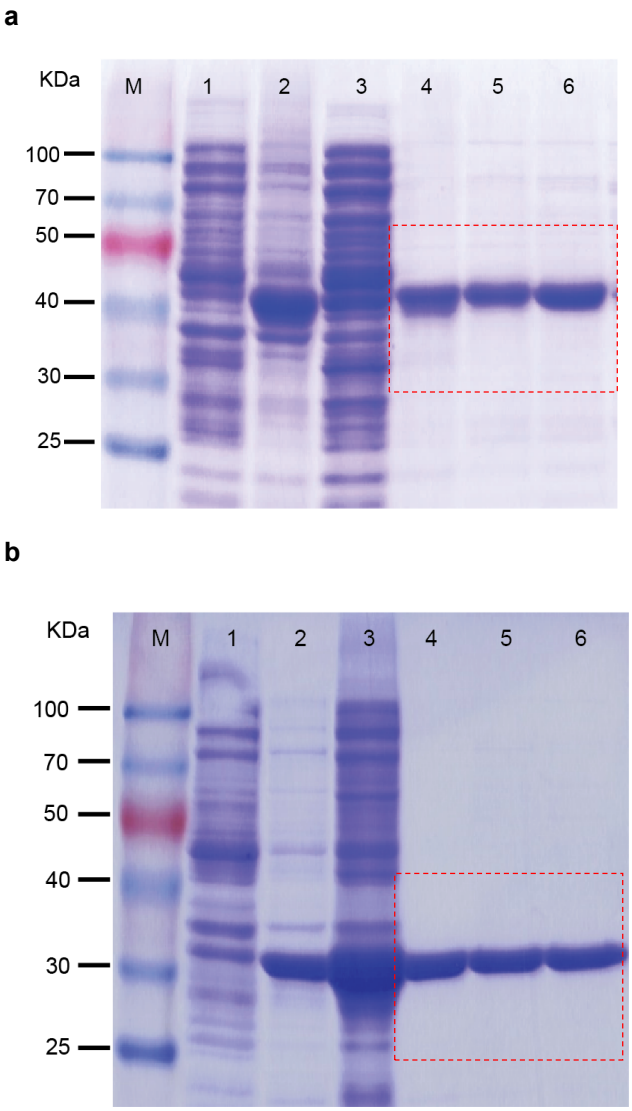


Fig. S7: SDS-PAGE analysis of *Sc*GalK and *Sc*GPUT. The dotted red area shows the clipped images used in figure panels 1e and 2b, respectively. **(a)** M: Protein marker; 1: Crude protein preparation of BL21 cells transformed with *Sc*GalK wild-type variant before IPTG induction; 2: Crude protein preparation of BL21 cells transformed with *Sc*GalK wild-type variant after 20 h IPTG induction; 3: supernatant of lysed BL21 cells transformed with the *Sc*GalK wild-type variant; 4: Ni-NTA purified *Sc*GalK wild-type variant;5: Ni-NTA purified *Sc*GalK Asp169Asp mutant variant; 6: Ni-NTA purified *Sc*GalK Asp169Ala mutant variant; **(b)** M: Protein marker; 1: Crude protein preparation of BL21 cells transformed with *Sc*GPUT wild-type variant before IPTG induction; 2: Crude protein preparation of BL21 cells transformed with *Sc*GPUT wild-type variant after 20 h IPTG induction; 3: supernatant of lysed BL21 cells transformed with the *Sc*GPUT wild-type variant; 4: Ni-NTA purified *Sc*GPUT wild-type variant;5: Ni-NTA purified *Sc*GPUT Asp107Asp mutant variant; 6: Ni-NTA purified *Sc*GPUT Asp107Ala mutant variant.

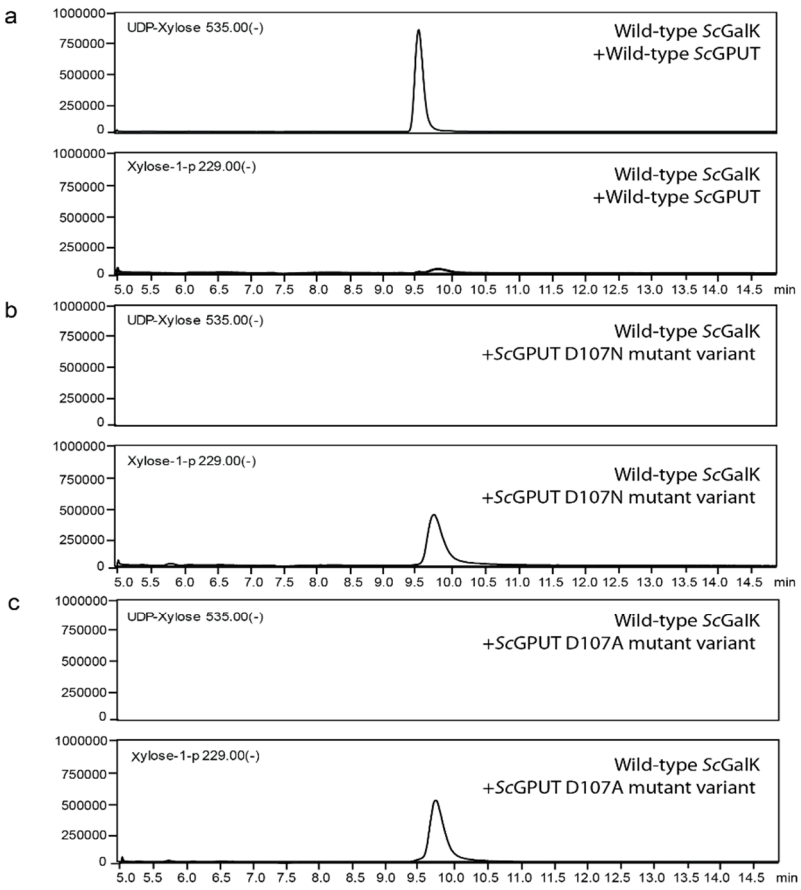


Fig. S8: LC-ESI-MS monitoring of the two-enzyme transformation of xylose to UDP-xylose by *Sc*GalK and *Sc*GPUT. Each sample contained D-xylose (2.5 µL, 20 mM), Tris/HCl buffer (5 µL, pH 7.5, 500 mM), $MgCl_2$ solution (5 µL, 10 mM), ATP solution (2 µL, 25 mM), UTP solution (2.5 µL, 20 mM), *Sc*GalK wild-type (23 µL, 2.1 mg/mL), and *Sc*GPUT wild-type or mutant variants (10 µL, 3.2 mg/mL). The reaction mixtures were incubated at 37 °C for 18 h and the reaction stopped by heating at 95 °C for 5 min. After centrifugation (20,000 × *g*, 5 min), 5 µL of the clear supernatant were mixed with 10 µL of water and 35 µL of acetonitrile, and 10 µL of this mixture was then subject to LC-ESI-MS analysis. The employed Shimadzu LCMS 8040 system consisted of a LC-30AD pump equipped with a low-pressure gradient mixing unit and a SIL-30AC autosampler coupled to the ESI mass spectrometer. The separation of the analytes was achieved by HILIC separation (Hydrophobic interaction liquid chromatography) using an ethylene-bridged hybrid (BEH) UPLC column (Waters Acquity glycan column, 1.7 µm, 2.1 × 150 mm). Samples were analyzed at a column temperature of 60 °C. The flow rate was 0.5 mL/min, and aqueous NH_4COOH (pH 4.5, 50 mM, solvent A) and acetonitrile (solvent B) were used as mobile phases. The separation of the D-xylose-1-*P* and UDP-D-xylose was achieved using a linear gradient from 95 – 20% of solvent B within the 14.5 min. MS signals correlating with D-xylose-1-*P* $[M]^-$=229.1 and UDP-D-xylose $[M]^-$=535.0 were monitored for reaction mixtures containing combinations of (a) wild-type *Sc*GalK and wild-type *Sc*GPUT (showing UDP-Xylose formation and depletion of the D-xylose-1-*P* intermediate) and (b,c) wild-type *Sc*GalK and the respective *Sc*GPUT mutant variants (showing no UDP-Xylose formation but an accumulation of the D-xylose-1-*P* intermediate).

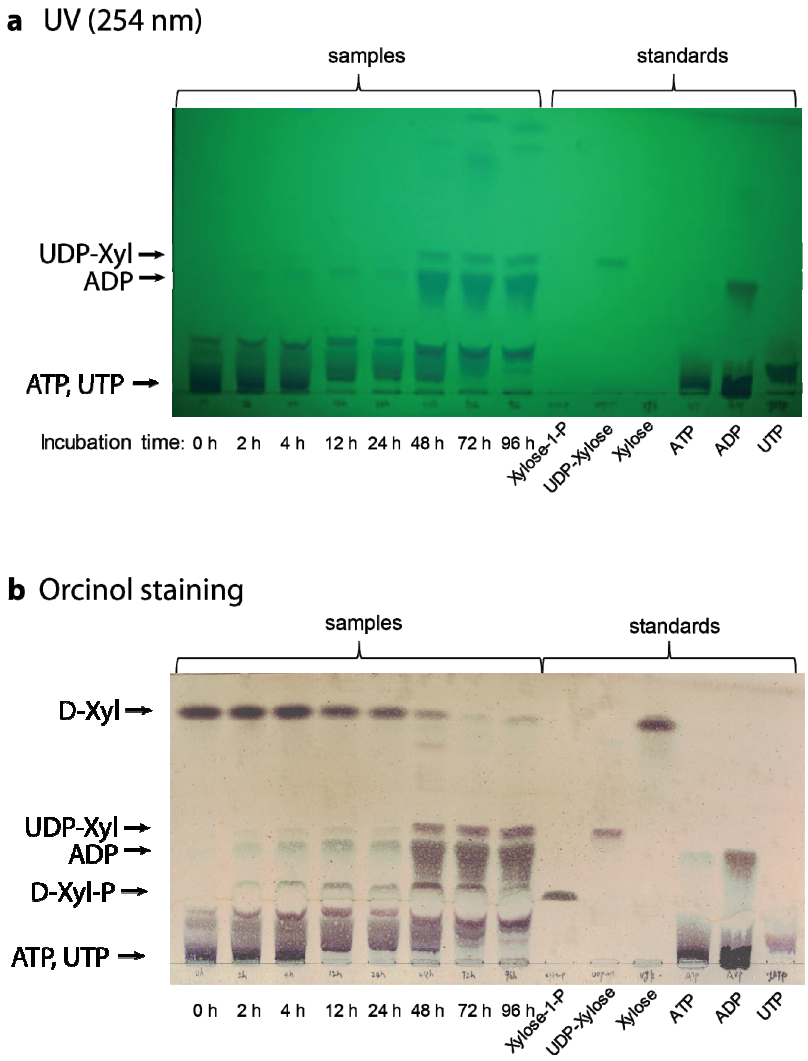


Fig. S9: Thin-layer chromatography (TLC) of the two-enzyme cascade conversion of xylose to UDP-xylose by *Sc*GalK and *Sc*GPUT in mg scale. Each sample (final volume 2 mL) contained D-xylose (50 µL, 200 mM), phosphate buffer (200 µL, pH 7.5, 500 mM), $MgCl_2$ solution (40 µL, 100 mM), ATP solution (100 µL, 200 mM), UTP solution (100 µL, 200 mM), *Sc*GalK (810 µL, 4.0 mg/mL), *Sc*GPUT (700 µL, 6.3 mg/mL). The buffer conditions (pH 7.5) and reaction temperature of 37 °C were chosen as a compromise between the pH and temperature optima of *Sc*GalK and *Sc*GPUT (see also Fig. S1). The reaction mixtures were incubated between 0 and 96 h, whereafter the enzymes were heat-inactivated at 95 °C for 5 min. After centrifugation (20,000 × *g*, 5 min), 4 µL of the supernatant was spotted onto silica gel 60 F_{254} TLC sheets (on aluminium, Supelco), and sample spots were separated using an *n*-butanol/ethanol/water mixture (5:3:2, v:v:v) as the solvent system. After drying the TLC plate this separation was repeated twice (for a better separation of ADP and UDP-xylose). Compounds were revealed by UV light (254 nm) or orcinol staining (consisting of 40 mg of orcinol monohydrate dissolved in 20 mL of aqueous H_2SO_4 (3.6 M)).

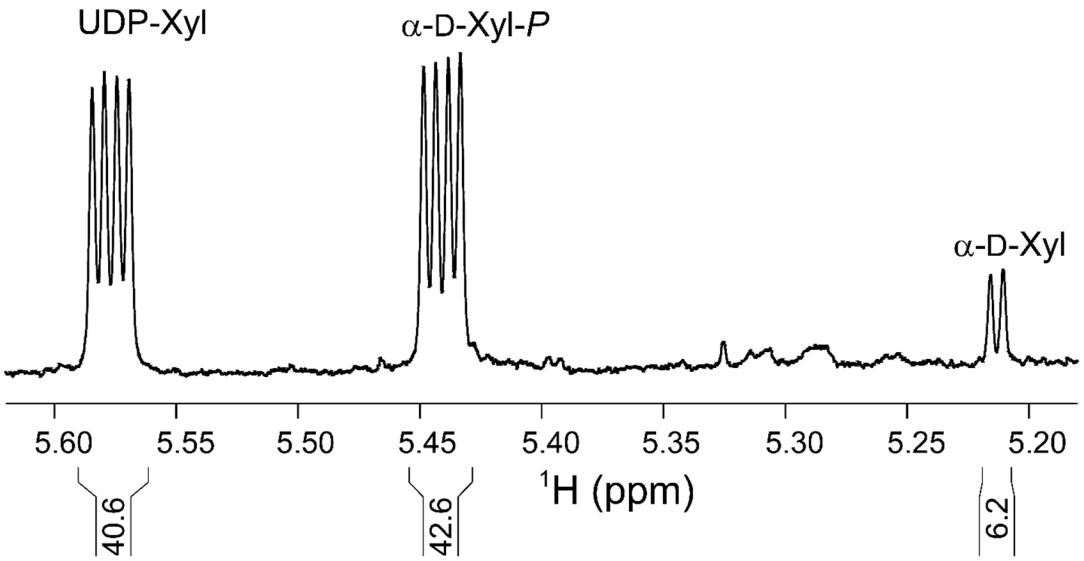


Fig. S10: Selected anomeric region of the ^{1}H NMR spectrum for the cascade reaction after 96 h using *Sc*GalK and *Sc*GPUT. Anomeric proton resonances of sugars can be identified, viz., from the product UDP-xylose, the intermediate α-D-xylose 1-phosphate and D-xylose, where the latter is present as an anomeric mixture with δ_{H1} 5.21 for α-D-xylose (the anomeric proton of β-D-xylose resonates at 4.56 ppm). The progress of the reaction after 96 h was analysed and normalised for the above components to 100, in which the relative ratio α-D-xylose:β-D-xylose is 37:63 at 310 K as determined from a separate ^{1}H NMR spectrum of D-xylose.

Table S1. High-resolution mass spectrometry data for α-D-Xyl*p*-1-*P* and UDP-Xyl.

α-D-xylopyranose 1-phosphate product from *Sc*GalK reaction. ESI-MS: *m/z* $[M-H]^-$ calc. for $C_5H_{10}O_8P$ 229.0119, found 229.0114.
UPD-Xyl product from *Sc*GPUT reaction. 96 h: ESI-MS: *m/z* $[M-H]^-$ calc. for $C_{14}H_{21}N_2O_{16}P_2$ 535.0371, found 535.0372. 72 h: ESI-MS: *m/z* $[M-H]^-$ calc. for $C_{14}H_{21}N_2O_{16}P_2$ 535.0371, found 535.0377.

Table S2. Oligonucleotide primers used in this study. Underlined sequence parts show the restriction sites NdeI and XhoI. Bold nucleotides show the position of the mutated Asp codon. N/A: not applicable.

Designation	UniProt ID	Putative Annotation	Primer Sequence
*Sc*GalK	H8KL58	Galactokinase	Sense primer: 5'-CATATGATGGAGAACAATCTATCCTTGG-3' Anti-sense primer: 5'-CTCGAGATTAGCTATTCTAACCCCATCTC-3'
*Sc*GalK Variant Asp169Asn	N/A	N/A	Sense primer: 5'-ggtgtaaactgtggaataatg**AAC**cagtttgcggtggctttcgg-3' Anti-sense primer: 5'-ccgaaagccaccgcaaactg**GTT**cattattccacagtttacacc-3'
*Sc*GalK Variant Asp169Ala	N/A	N/A	Sense primer: 5'-ggtgtaaactgtggaataatg**GCG**cagtttgcggtggctttcgg-3' Anti-sense primer: 5'-ccgaaagccaccgcaaactg**CGC**cattattccacagtttacacc-3'
*Sc*GPUT	H8KMA8	Glucose-1-phosphate thymidylyltransferase	Sense primer: 5'-CATATGATGAAAGGTATTATTCTCGCAG-3' Anti-sense primer: 5'-CTCGAGTAAGTACGCTTCTTCCTCTTGAT -3'
*Sc*GPUT Variant Asp107Asn	N/A	N/A	Sense primer: 5'-gtgtagcattagtcttagga**AAC**aatattttcttctcaaacgg-3' Anti-sense primer: 5'-ccgtttgagaagaaaatatt**GTT**tcctaagactaatgctacac-3'
*Sc*GPUT Variant Asp107Ala	N/A	N/A	Sense primer: 5'-gtgtagcattagtcttagga**GCG**aatattttcttctcaaacgg-3' Anti-sense primer: 5'-ccgtttgagaagaaaatatt**CGC**tcctaagactaatgctacac-3'