Supporting information

Synthesis of the Rare Disaccharide Nigerose by structure-based design of a phosphorylase mutant with altered Regioselectivity

M. Kraus,[a] J. Görl[a], M. Timm[a] and Jürgen Seibel*[a]

[a] Institute for Organic Chemistry, Julius-Maximilians University Würzburg, Am Hubland C1, Würzburg, Germany.
Email: seibel@chemie.uni-wuerzburg.de;
Fax: +49 (0) 931 31 84625; Tel: +49 (0) 931 31 85326

Experimental Section

Cloning of wt-BaSP:

Freeze-dried cultures of *Bifidobacterium adolescentis* (DSM 20083) were obtained from DSMZ (Deutsche Sammlung von Mikroorgansimen und Zellkulturen GmbH), and grown under anaerobic conditions in DSMZ medium Nr.58 without resazurin. Cells were harvested and the genomic DNA isolated, using a GenJet Genomic DNA purification Kit (Thermo Fisher). The BaSP gene was amplified from genomic DNA using the primers 5'-ATAACCATGGCTATGAAAAACAAGGTGCAGCTCATCAC-3' and 5'-CAATCCGCCTGTCGTCGCCCTCGAGTAAT-3'. The amplicon was inserted into pET-28b(+) using the NcoI and XhoI restriction sites yielding plasmid pET-28b(+)-BaSP-wt.

Cloning, expression and purification of the enzymes

E. coli Bl21 star™ cells were heat shock transformed with plasmid pET-28b(+)-BaSP-wt. Overnight cultures of the transformed host in LB-medium containing 50 mg/l kanamycin sulfate were grown and 1.8 ml were used to inoculate 250 ml of LB-Medium (50 mg/l kanamycin sulfate). The cultures were incubated at 37 °C and 180 rpm until they reached an OD_{600} of 0.6, at which point the temperature was adjusted to 19 °C and IPTG was added to a final concentration of 0.5 mM. The cells were grown for additional 18 h after which they were harvested by centrifugation (4000 g for 10 min). The sediment was resuspended in lysis buffer (60 mM phosphate, 250 mM NaCl, 11 mM imidazole, 5 mM β-mercaptoethanol, pH=8). Cells were lysed using a sonifier and centrifuged at 17000 g for 10 min at 4 °C. The lysate was loaded onto 0.5 ml Ni-NTA columns equilibrated with lysis buffer and incubated at 4 °C and slow rotation for a minimum of 2 hours. The column was washed with 2.5 ml of lysis buffer and the protein was eluted with 1.5 ml of elution buffer (60 mM phosphate, 250 mM NaCl, 230 mM imidazole, 5 mM β-mercaptoethanol, pH=8). The buffer was exchanged to 20 mM MOPS-buffer (pH=7) using 5 ml Hi-Trap columns from GE Healthcare.

Construction and purification of the variants

The mutations were constructed applying the Megaprimer method. 5'-CAATCCGCCTGTCGTCGCCCTCGAGTAAT-3' was used as reverse primer for the creation of the megaprimer and 5'-ATAACCATGGCTATGAAAAACAAGGTGCAGCTCATCAC-3' was used as the forward primer in the second PCR. For the construction of the D316C_N340C and D312C_L341C, the variants N340C and L341C, respectively, were created first. The mutations D316C and D312C, respectively, were introduced in a second megaprimer PCR.

variant	forward mutagenic primer in first PCR
Q345Y	5'-CCAATCTCGACCTCTACTTCGTCAACAGCACCTAC-3'
N340C	5'-CCGCCGCATCCTGTCTCGACCTC-3'
L341C	5'-CCGCATCCAATTGCGACCTCTACC-3'
D316C	5'-GGATGAGGACGTGTGCAACCTCGTCAAC-3'
D312C	5'-GGTCTCGTGCCGTGTGAGGACGTGG-3'

Cloning and purification of the variants followed the procedure described for the wildtype. The desired mutations were confirmed by DNA sequencing (GATC biotech AG, Konstanz, Germany).

Enzyme assays (final concentrations are given).

If not stated otherwise enzyme assays were carried out in MOPS buffer ((3-(*N*-morpholino) propane sulfonic acid, 50 mM, pH 7) supplemented with sucrose (100 mM) in a total volume of 100 or 200 µl. Reactions were incubated at 37 °C. The variants D316C_N340C and D312C_L341C were pretreated with 15 mM TCEP (tris(2-carboxyethyl)phosphine) for 1 h at 30 °C to ensure reduction of disulfide bonds.

Unit definition

1 U was defined as the enzyme activity that hydrolases 1 µmol of sucrose in one minute (200 mM sucrose, 50 mM MOPS buffer pH 7, total reaction volume 200 µL, 37 °C).

Immobilization of baker's yeast

1.25 g sodium alginate (purchased from VWR) was dissolved at 60 °C in 80 ml water. 10.0 g baker's yeast (Dr. Oetker, "frische Backhefe") was suspended in the solution and the resulting slurry was loaded into a syringe and added dropwise (6.0 ml min^{-1}, via syringe pump) to 500 ml of a stirred $CaCl_2$-solution (150 mM).[1]

Production of nigerose

In a total volume of 10 ml, sucrose (400 mM) was supplemented with glucose (200 mM) in MOPS buffer (20 mM, pH 7) and 30% DMSO. 1.0 mL of BaSP Q345F (5 mg/mL, activity: 62 U/g) was added and the reaction was incubated at 37 °C under slow agitation. After 4 d (90% sucrose consumption) the reaction was stopped by the addition of 20 ml MeOH. The occurring precipitate was removed by centrifugation (10 min, 6000 g). After evaporation of the solvent and freeze-drying, the residual syrup was supplemented with 50 ml water and baker's yeast (immobilized on calcium alginate beads, 20 beads). The consumption of sugars at 20 °C was monitored by HPAEC (figure S1).

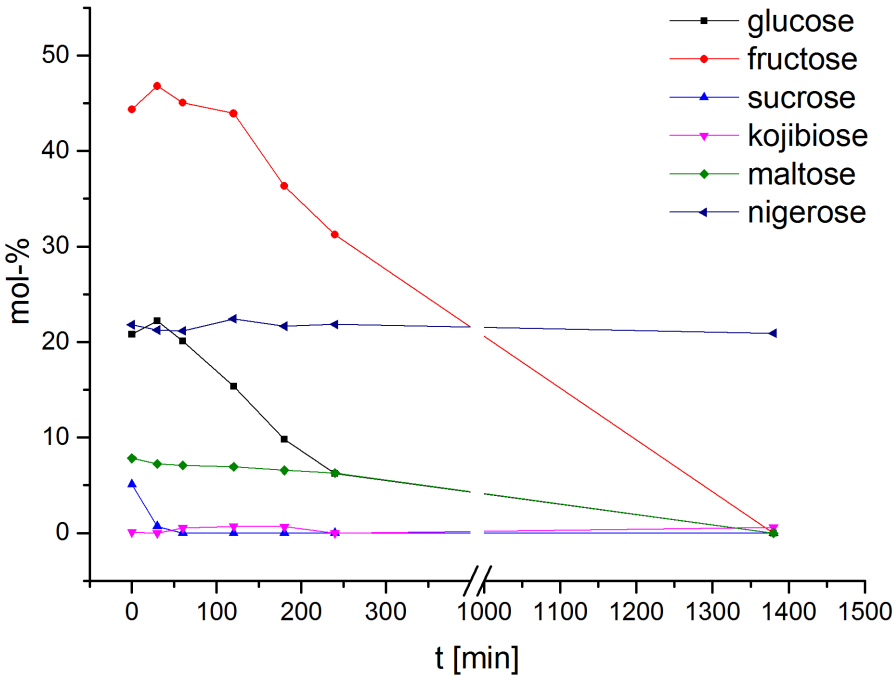
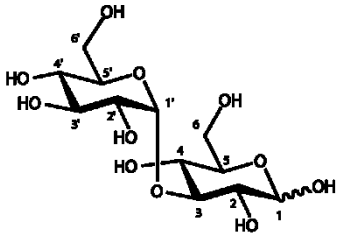


Figure S1 Consumption of sugars of the reaction mixture by baker's yeast (mol-% is based on the total sugar concentration at time 0).

After reaction completion, the baker's yeast was removed by filtration and the solvent was removed by freeze-drying. Silica gel chromatography (0.063-0.200 mm, MeCN/MeOH = 4:1) yielded pure nigerose (430 mg, 24%).

NMR

^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) were recorded on a Bruker Avance III HD 600 instrument using D_2O as solvent and acetone as internal ^{1}H (2.225 ppm) and ^{13}C (31.45 ppm) standard. The obtained NMR data for nigerose is in agreement with data previously published.[2]



α-anomer (40%):

^{1}H NMR (600 MHz, D_2O) δ 5.37 (d, *J* = 3.9 Hz, 1H, H-1'), 5.23 (d, *J* = 3.8 Hz, 1H, H-1), 4.02 (m, 1H, H-5'), 3.87-3.81 (m, 4H, H-3, H-5, H-6a, H-6'a), 3.80-3.73 (m, 2H, H-6'b, H-6b), 3.75 (dd, *J* = 9.5, 9.5 Hz, 1H, H-3'), 3.64 (dd, *J* = 9.5, 7.0 Hz, 1H, H-4), 3.62 (dd, *J* = 9.8, 3.9 Hz, 1H, H-2), 3.57 (dd, *J* = 9.9, 5.1 Hz, 1H, H-2'), 3.46 (dd, *J* = 9.2, 10.2 Hz, 1H, H-4') ppm.

^{13}C NMR (150 MHz, D_2O) δ 100.25 (C-1'), 93.43 (C-1), 80.73 (C-3), 74.05 (C-3'), 72.93 (C-5'), 72.89 (C-2'), 72.38 (C-5), 71.29, 71.22 (C-2, C-4), 70.60 (C-4'), 61.56, 61.53 (C-6, C-6') ppm.

ß-anomer (60%):

^{1}H NMR (600 MHz, D_2O) δ 5.36 (d, *J* = 3.9 Hz, 1H, H-1'), 4.66 (d, *J* = 8.0 Hz, 1H, H-1), 4.02 (m, 1H, H-5'), 3.89 (dd, *J* = 2.2, 12.3 Hz, 1H, H-6a), 3.84 (dd, *J* = 12.7, 2.2 Hz, 1H, H-6'a), 3.78 (ddd, *J* = 12.7, 8.4, 4.0 Hz, 1H, H-6'b), 3.74 (dd, *J* = 9.6, 9.6 Hz, 1H, H-3'), 3.72 (dd, *J* = 12.4, 5.9 Hz, 1H, H-6b), 3.64 (dd, *J* = 9.5, 7.0 Hz, 1H, H-3), 3.63 (m, 1H, H-4), 3.56 (dd, *J* = 9.8, 4.1 Hz, 1H, H-2'), 3.47 (ddd, 9.7, 5.4, 2.8, 1H, H-5), 3.44 (dd, *J* = 10.6, 9.5 Hz, 1H, H-4'), 3.33 (dd, *J* = 9.3, 8.0 Hz, 1H, H-2) ppm.

^{13}C NMR (150 MHz, D_2O) δ 100.21 (C-1'), 97.16 (C-1), 83.25 (C-3), 76.84 (C-5), 74.08 (C-3'), 74.03 (C-2), 72.90 (C-5'), 72.82 (C-2'), 71.25 (C-4), 70.47 (C-4'), 61.74 (C-6), 61.37 (C-6') ppm.

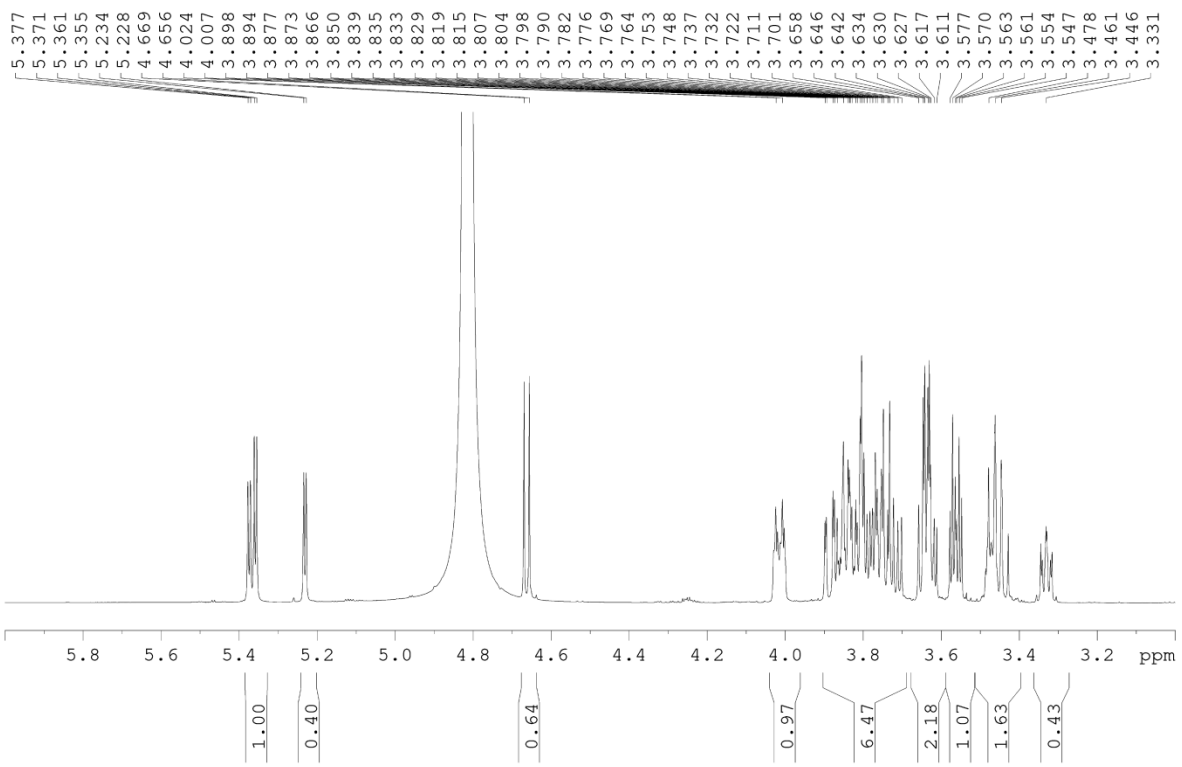


Figure S2 ^{1}H-NMR spectrum of nigerose.

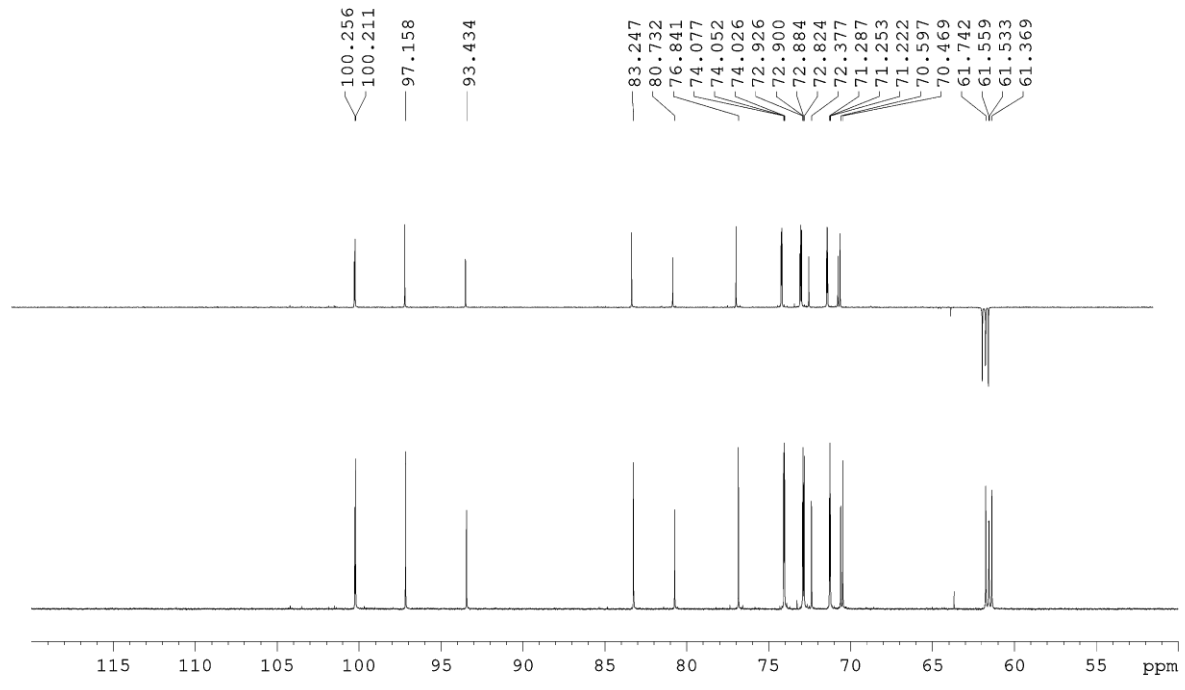


Figure S3 ^{13}C- and DEPT-135 spectra of nigerose.

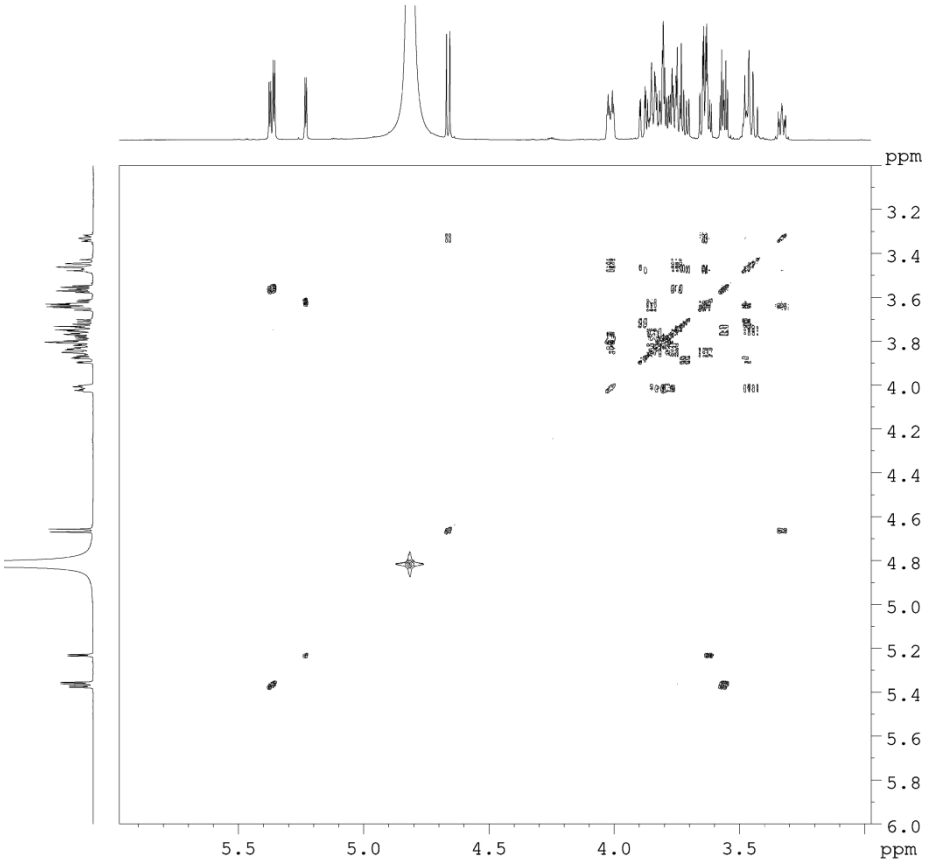


Figure S4 ^{1}H-^{1}H-COSY spectrum of nigerose.

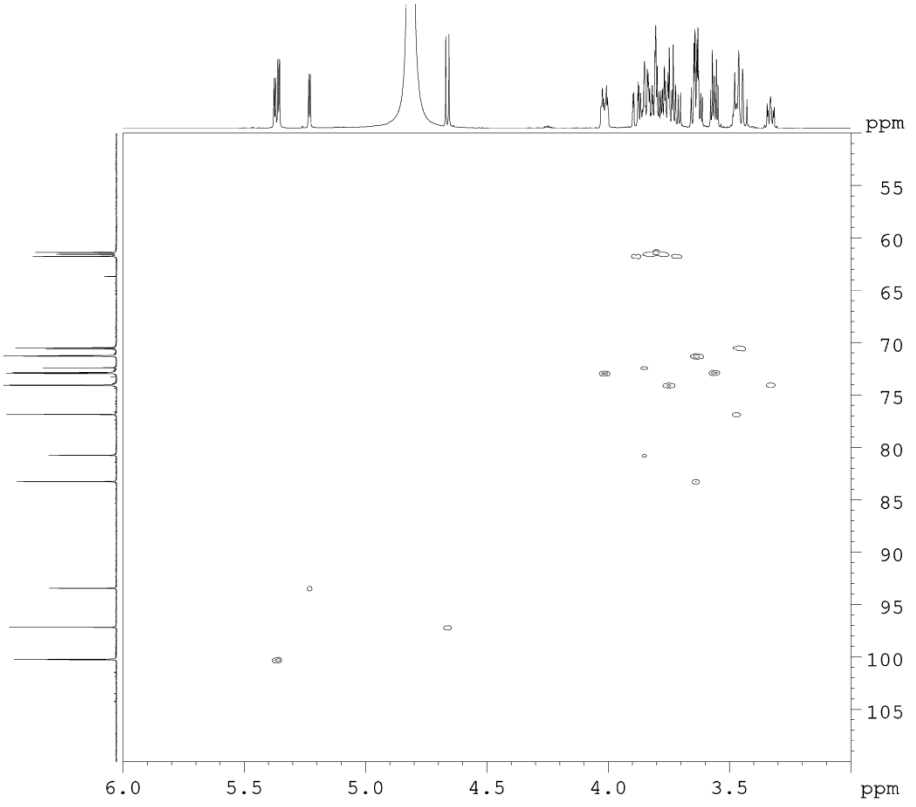


Figure S5 ^{1}H-^{13}C HSCQ spectrum of nigerose.

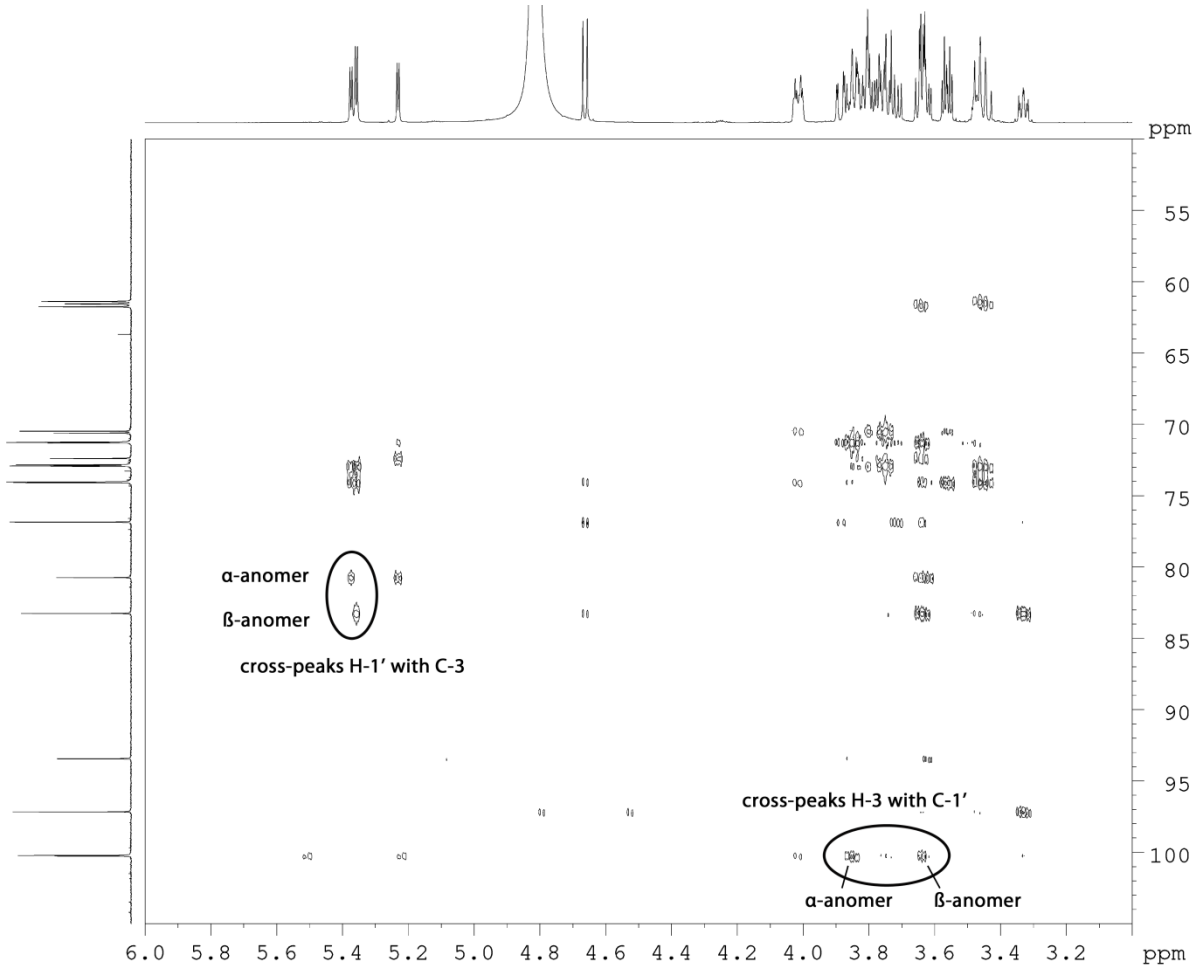


Figure S6 ^{1}H-^{13}C HMCQ spectrum of nigerose; the cross-peaks defining the α-(1,3)-linkage are highlighted.

HPAEC-sample preparation

Collected samples were diluted (1:50) with water and boiled at 95 °C for 5 min to stop the catalytic reaction. After centrifugation at 17.000 g for 5 min the samples were furthermore diluted (final total sugar concentration: 100-200 µM).

HPAEC-PAD analysis

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) was performed with a Dionex ICS-5000+ SP system utilizing a Carbopac PA10 (2x250 mm) column. The saccharides were resolved using an isocratic program (100 mM NaOH and 30 mM NaOAc at 250 µL/min). Yields were determined by peak area using external standards (fructose, glucose, sucrose, kojibiose, nigerose and maltose) of known concentrations (10 µM, 25 µM, 50 µM, 75 µM and 100 µM).

Determination of Glucose and solvent influence on Nigerose Production

Glucose. In a total volume of 100 µl, sucrose (100 mM) was supplemented with glucose (0-100 mM, in 25 mM intervals), in MOPS-buffer (50 mM, pH 7) and 30% DMSO. 95 U/l BaSP Q345F was added and the reaction mixture was incubated at 37 °C. After 52 h, samples of 10 µl were taken and yields were determined using HPAEC-PAD.

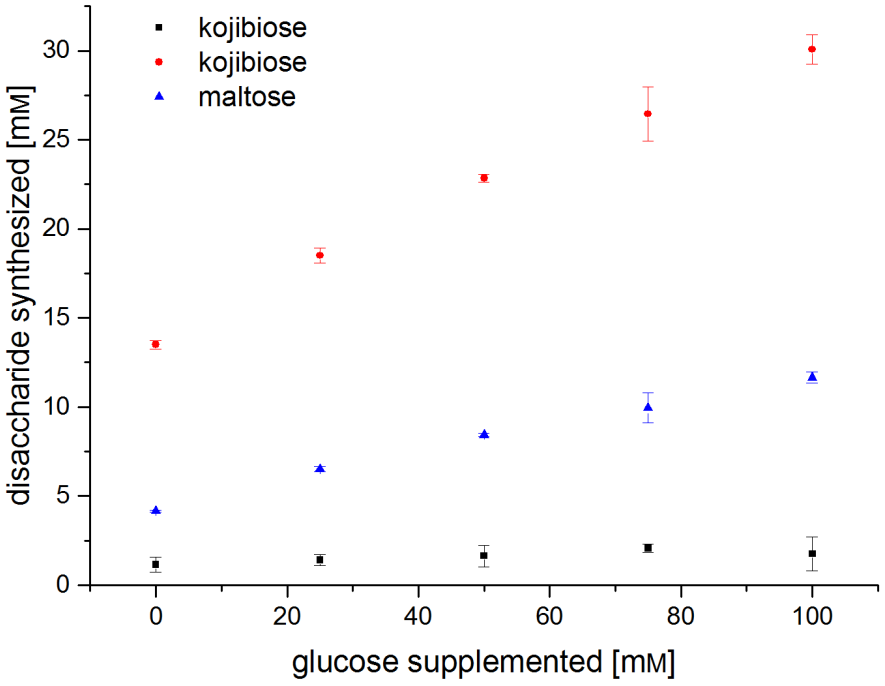


Figure S7 Effect of glucose supplementation on the disaccharide formation by BaSP Q345F; product yields after complete (>90%) conversion of sucrose.

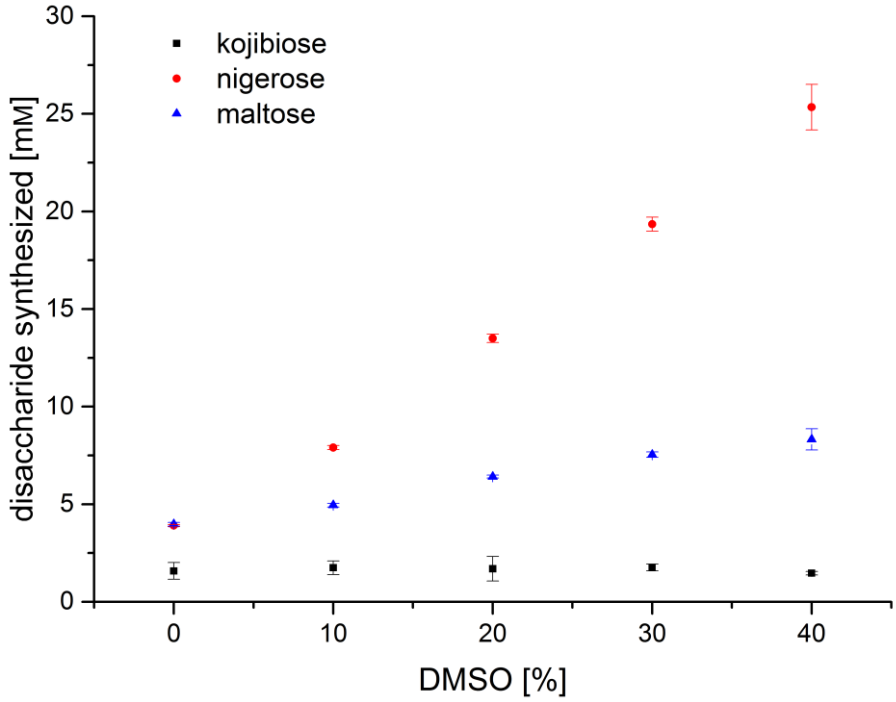


Figure S8 Effect of DMSO concentration on the disaccharide synthesis of BaSP Q345F; Product yields after complete (>90%) conversion of sucrose.

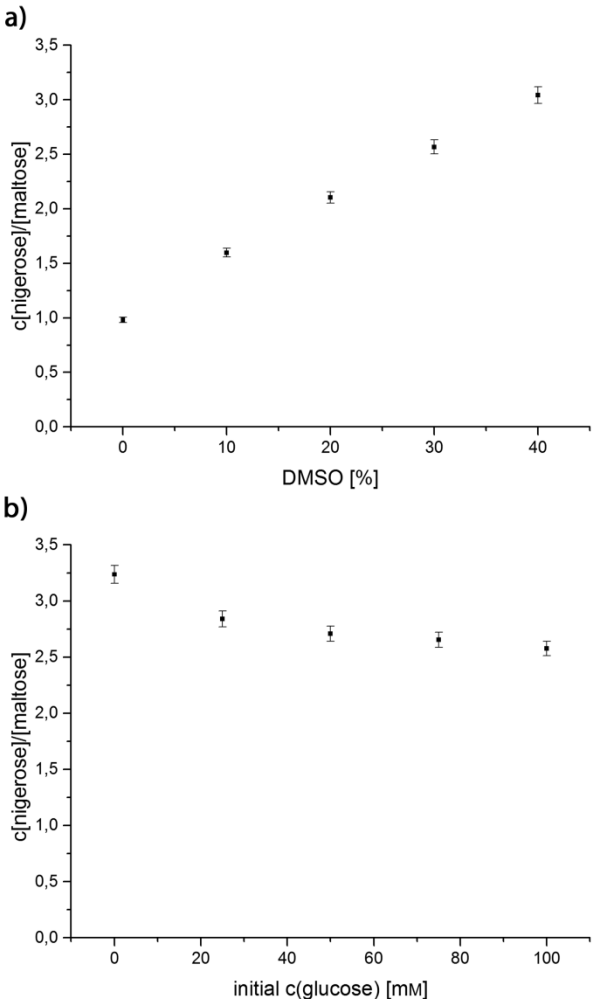


Figure S9 a) shift of nigerose/maltose ratio with increaseing DMSO concentration (100 mM sucrose, 50 mM glucose, 50 mM MOPS, 37 °C); b) shift of nigerose/maltose ratio with increasing glucose concentration (100 mM sucrose, 30% DMSO, 50 mM MOPS, 37 °C).

DMSO. In a total volume of 100 µl, sucrose (100 mM) was supplemented with glucose (50 mM) in MOPS-buffer (50 mM, pH 7) and DMSO (0-40% in 10% intervals). 95 U/l BaSP Q345F was added and the reaction mixture was incubated at 37 °C. After 52 h, samples of 10 µl were taken and yields were determined using HPAEC-PAD.

Other solvents. In a total volume of 100 µl, sucrose (100 mM) was supplemented with glucose (50 mM) in MOPS-buffer (50 mM, pH 7). Different solvents (EtOH, *i*-PrOH, acetone, *n*-BuOH, *t*-BuOH, 0-30% in 10% intervals) were added and the reactions were started by the addition of 95 U/l BaSP Q345F. Samples were taken after incubation at 37 °C for 17, 48 and 72 h and yields were determined using HPAEC-PAD.

Molecular Docking

Protein and ligand setup

The crystal structure of the BaSP E232Q mutant (pdb code 2gdu, chain A) was retrieved from PDB as "receptor" for the docking calculations. All water molecules and ligand entries were removed, non-polar hydrogens were added using AutoDockTools 1.5.6r.[3] Grid box center and grid dimensions (16x16x16 Å, grid spacing: 1.0 Å) were determined via AutoDockTools and transferred to the AutoDock Vina configuration file.

Ligands were built and geometries were optimized using ChemBioOffice 13 (MM2 energy minimization with default settings). Both α- and ß-anomers of kojibiose and nigerose were prepared. Gasteiger charges were added and rotatable bonds were assigned using AutoDockTools.

AutoDock Vina

AutoDock Vina[4] was used for docking calculations. The docking parameters "exhaustiveness" and "energy_range" were set to "25" and "4", respectively. Sucrose was used a test ligand for the docking procedure resulting in an excellent agreement with the binding mode of the co-crystallized sucrose of the 2gdu structure. Conformations in which the non-reducing glucose-moiety was in agreement with the one of the 2gdu structure were considered productive binding modes and within these the best-scored modes were chosen for closer analysis. From the analyzed binding modes no significant difference was observed between the α- and ß-anomers of the ligands.

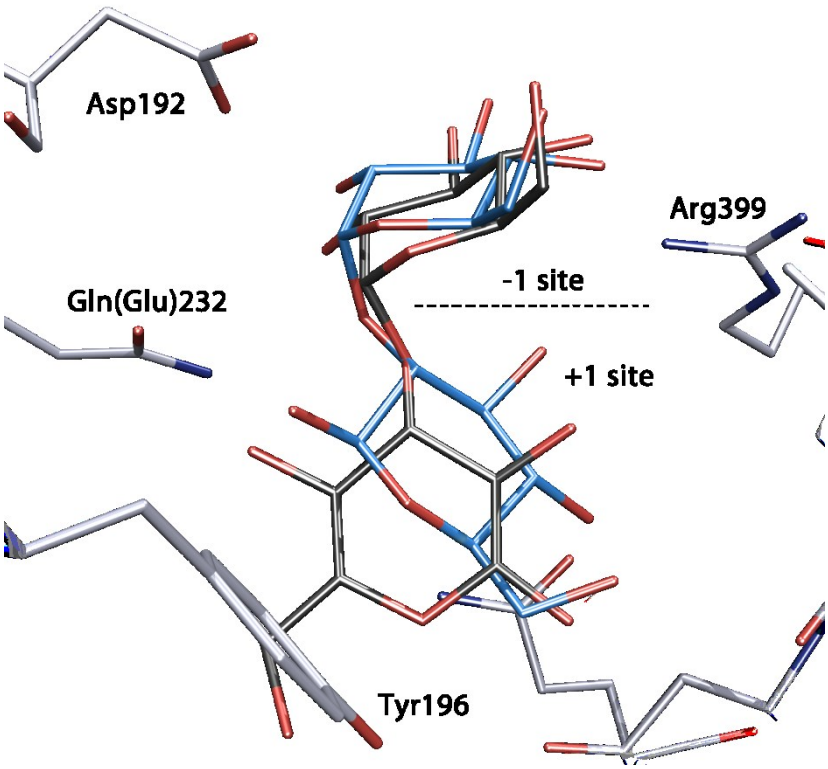


Figure S10 Best docking modes for nigerose (black) and kojibiose (blue): while the non-reducing glucose-moiety of kojibiose (-1 site) is in agreement with the sucrose (not shown for reasons of clarity) co-crystallized in the 2gdu structure, the best docking mode of nigerose is significantly shifted.

Structure and sequence alignments. Structure alignments of BaSP (2gdu and 2gdv, chain A and B) with LmSP were performed using I-TASSER[5] (data not shown). The section of the sequence alignment (ClustalW2) neighboring the ^{134}PRP^{136} motif is given below.

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BaSP 123 ATEEDLAGIYRPRPGLPFTHYKFAG 147
LmSP 126 PTQADVDLIYKRKDKAPTQEITFDD 150
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References:

[1] N. Qureshi, D. V. Tamhane, *Appl Microbiol Biot* **1985**, *21*, 280-281.

[2] D. Goffin, P. Bystricky, A. S. Shashkov, M. Lynch, E. Hanon, M. Paquot, A. V. Savage, *B Korean Chem Soc* **2009**, *30*, 2535-2541.

[3] M. F. Sanner, *J Mol Graph Model* **1999**, *17*, 57-61.

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[5] Y. Zhang, *Bmc Bioinformatics* **2008**, *9*.