Recombinant *Aspergillus* β-galactosidases as a robust glycomic and biotechnological tool

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Supplemental Tables

Supplemental Table S1. Influence of galactose and glucose on enzymatic activity.

Activity of recombinant *Aspergillus* galactosidases and non-recombinant *A. oryzae* galactosidase (*) in the presence of different concentrations of galactose and glucose. For each enzyme, the activities at various conditions are shown in relation to the maximal activity measured for that enzyme. Average values (% activity) +/- standard error are shown.

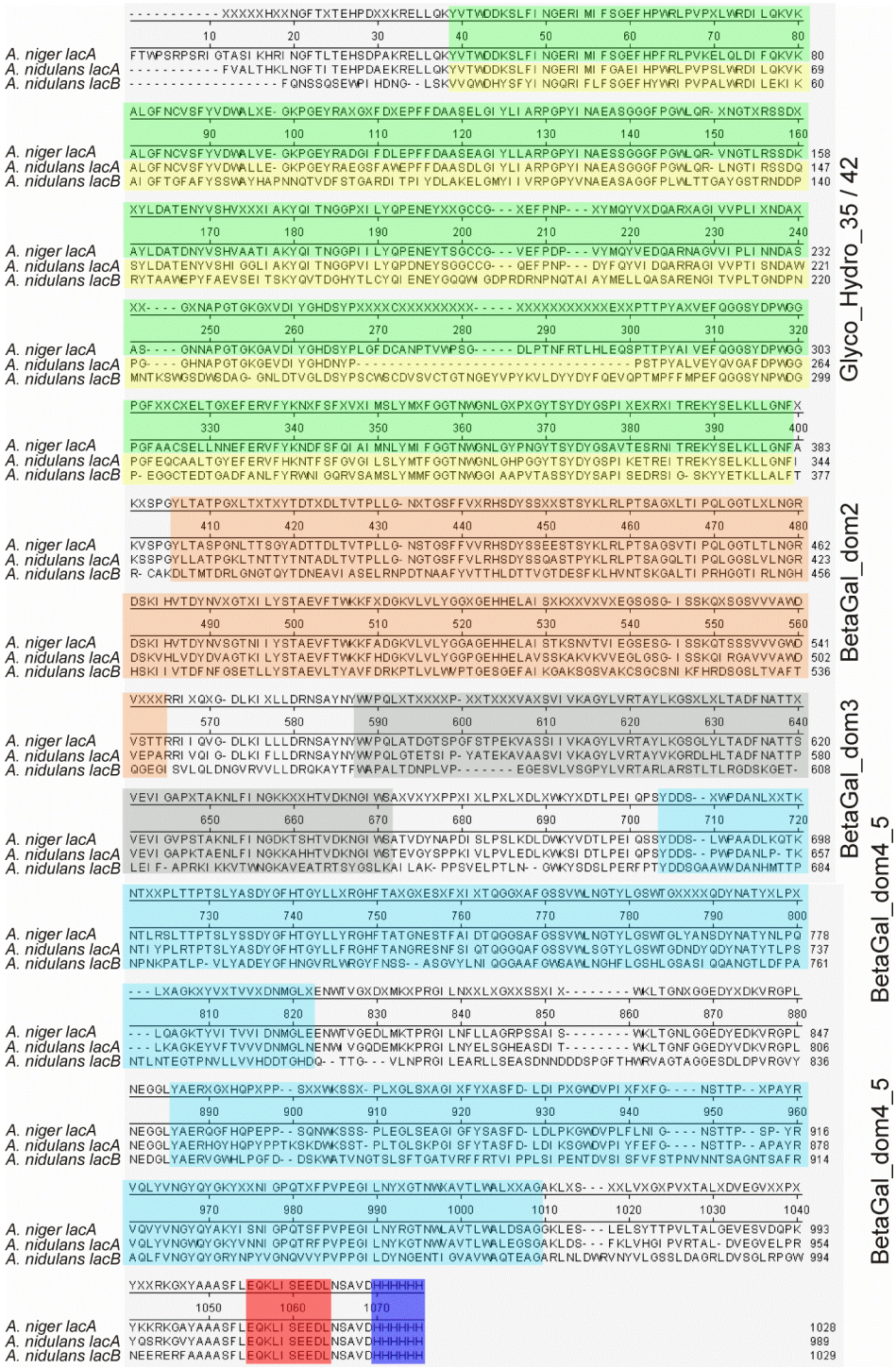
galactose [mM]	*A. niger* rlacA	*A. nidulans* rlacA	*A. nidulans* rlacB	*A.oryzae**
0	**100 +/- 0.0**	58.9 +/- 0.1	**100 +/- 0.5**	**100 +/- 2.6**
0.95	90.3 +/- 1.5	**100 +/- 0.1**	71.9 +/- 4.2	30.4 +/- 5.4
4.75	65.8 +/- 1.7	92.2 +/- 3.4	33.7 +/- 2.7	23.8 +/- 1.2
9.5	47.9 +/- 0.3	39.7 +/- 4.8	24.8 +/- 2.7	23.4 +/- 4.6
47.5	15.9 +/- 0.2	34.6 +/- 5.9	4.6 +/-0.7	12.5 +/- 3.0
95	5.1 +/- 0.2	16.6 +/- 0.2	2.6 +/-0.3	8.9 +/- 1.7
475	2.5 +/- 0.1	4.14 +/- 0.0	0.2 +/- 0.1	2.5 +/- 1.4
950	1.0 +/- 0.3	1.72 +/- 0.3	0.2 +/- 0.5	0.3 +/- 0.0
glucose [mM]	***A. niger* rlacA**	***A. nidulans* rlacA**	***A. nidulans* rlacB**	***A.oryzae****
0	82.2 +/- 7.3	45.3 +/- 1.7	79.6 +/- 5.9	62.6 +/- 5.3
9.5	90.2 +/- 5.2	51.7 +/- 1.0	80.1 +/- 0.3	67.5 +/- 2.9
23.75	96.5 +/-5.3	51.2 +/- 3.4	97.1 +/- 10.4	70.0 +/- 5.5
47.5	91.6 +/- 3.1	57.3 +/- 1.8	97.7 +/- 3.1	77.7 +/- 1.5
95	99.2 +/- 0.5	71.4 +/- 3.4	99.1 +/- 13.3	86.9 +/- 5.1
190	**100.0 +/- 1.3**	93.0 +/- 2.8	**100.0 +/- 8.5**	**100.0 +/- 10.1**
950	90.8 +/- 3.2	**100 +/- 6.6**	82.2 +/- 8.2	88.1 +/- 13.0
1900	61.8 +/- 0.9	76.0 +/- 3.1	61.8 +/- 1.6	72.2 +/- 1.9

Supplemental Table S2. β-1,3 activity using lacto-*N*-tetraose as substrate.

Ratio of β-1,3 activity and β-1,4 activity after 2, 24 and 48h is shown. For *A. niger* and *A. nidulans* rlacA, values after 24 and 48h (marked with an asterisk) overestimate the activity as almost complete conversion of lacto-*N*-neotetraose (β-1,4 substrate) already occurred after 2h (see Figure 4 in the main manuscript).

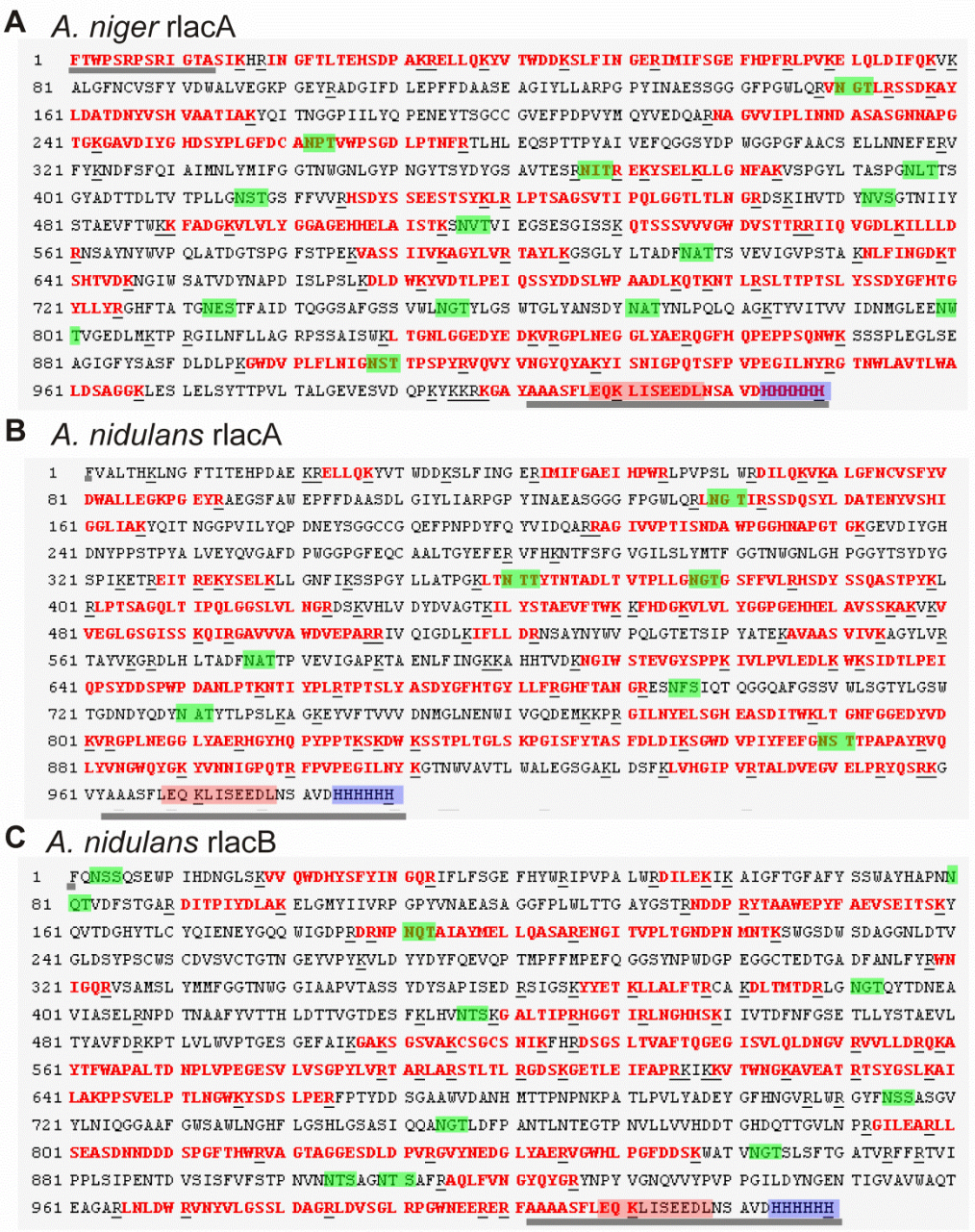
Ratio of β-1,3 activity vs β-1,4 activity after [h] of incubation	**2**	**24**	**48**
***A. niger* rlacA**	0.027	0.242*	0.616*
***A. nidulans* rlacA**	0.043	0.429*	0.979*
***A. nidulans* rlacB**	0.005	0.195	0.280
***A. oryzae* galactosidase**	0.019	0.081	0.429

Supplemental Figures



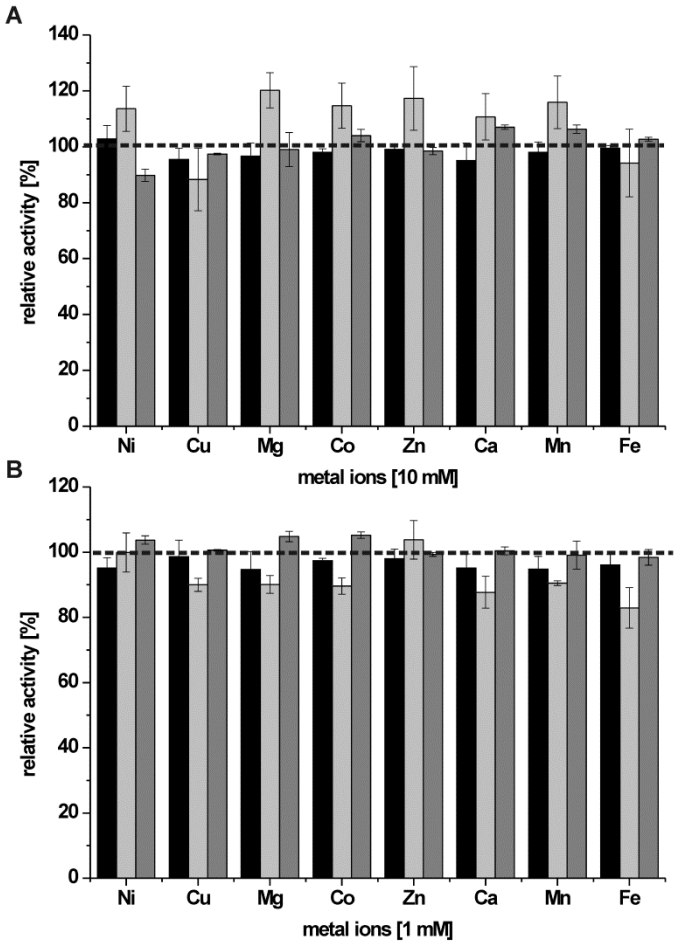
Supplemental Fig. S1 Sequence alignment of the three recombinant galactosidases

The sequences used in the current study are depicted. Conserved sequence motifs (yellow, green, orange, grey, light blue) and C-terminal myc (red) and HIS tags (dark blue) are highlighted.



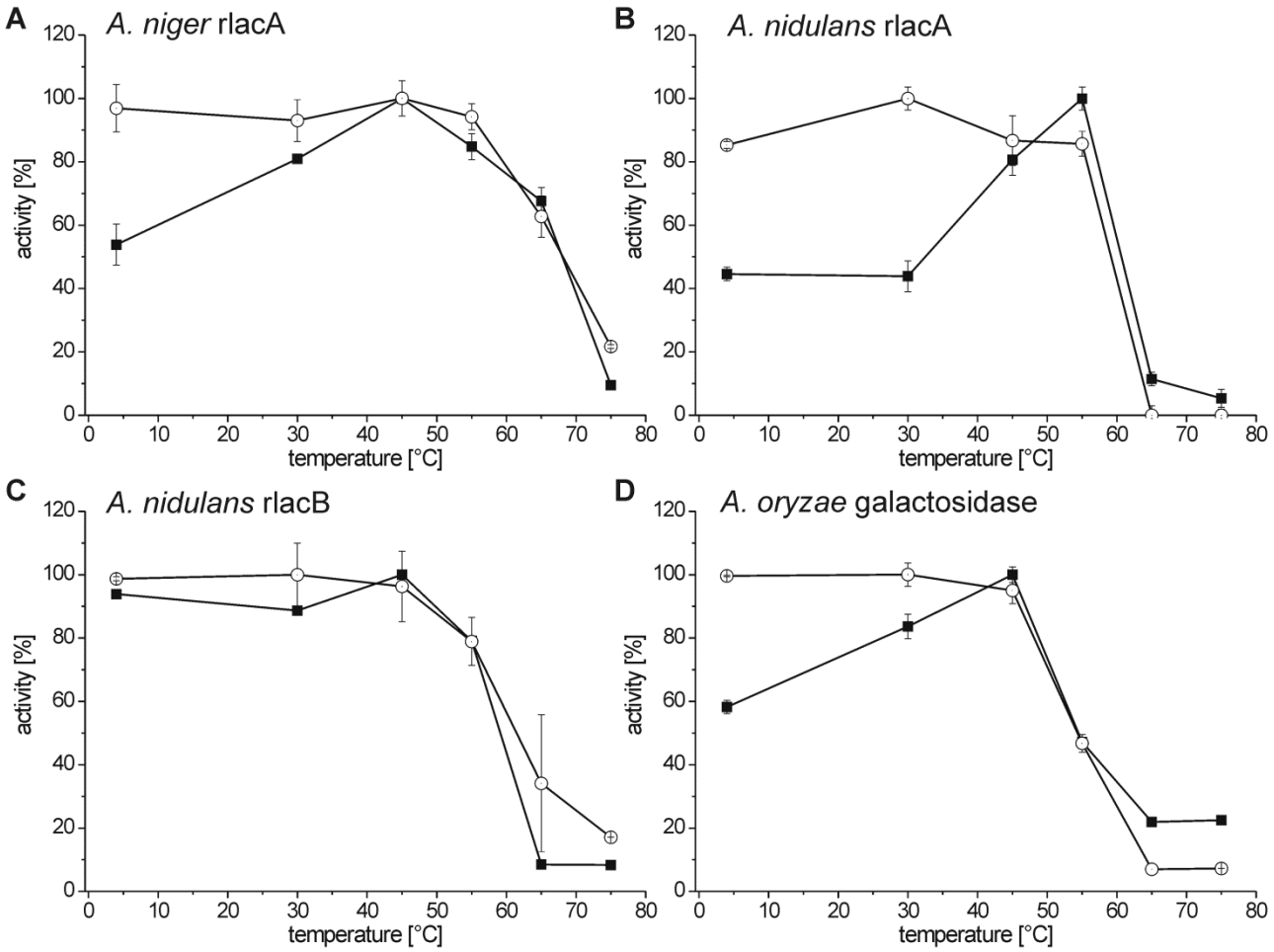
Supplemental Fig. S2 Protein identity verification by mass fingerprint analysis

Purified (A) *A. niger* rlacA (B) *A. nidulans* rlacA and (C) *A. nidulans* rlacB were subjected to digestion with trypsin and subsequently analyzed by MALDI-TOF/TOF MS. Amino acid sequences in red font represent protein sequence covered by tryptic peptides whereas underlined positions indicate trypsin cleavage sites. Sequences underlined in grey represent amino acid sequences that were attached to the native protein during cloning. Sequences highlighted in red and blue indicate recombinant c-myc and His-tag and amino acid triplets highlighted in green indicate potential N-glycosylation sites.



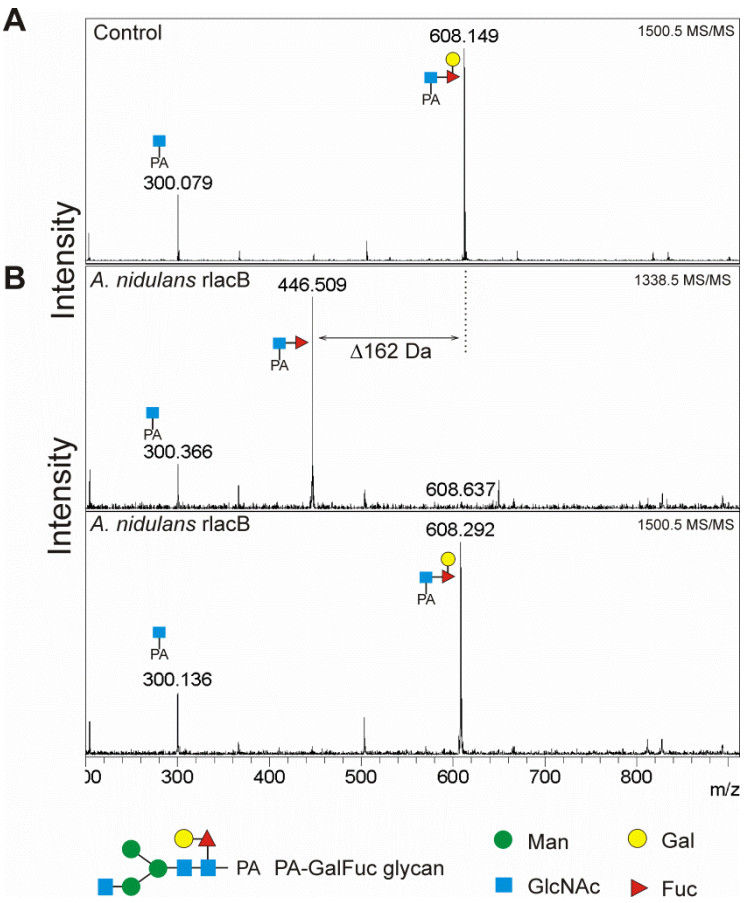
Supplemental Fig. S3 Divalent metal ion susceptibility of recombinant galactosidases.

PNPG Reactions were supplemented with (A) 10 mM and (B) 1 mM of the respective metal salts. Black bars - *A. niger* rlacA, light grey bars - *A. nidulans* rlacA and grey bars - *A. nidulans* rlacB. Values represent averages +/- standard error. % relative activity compared with standard reactions without ion supplementation (= 100 %) is shown.



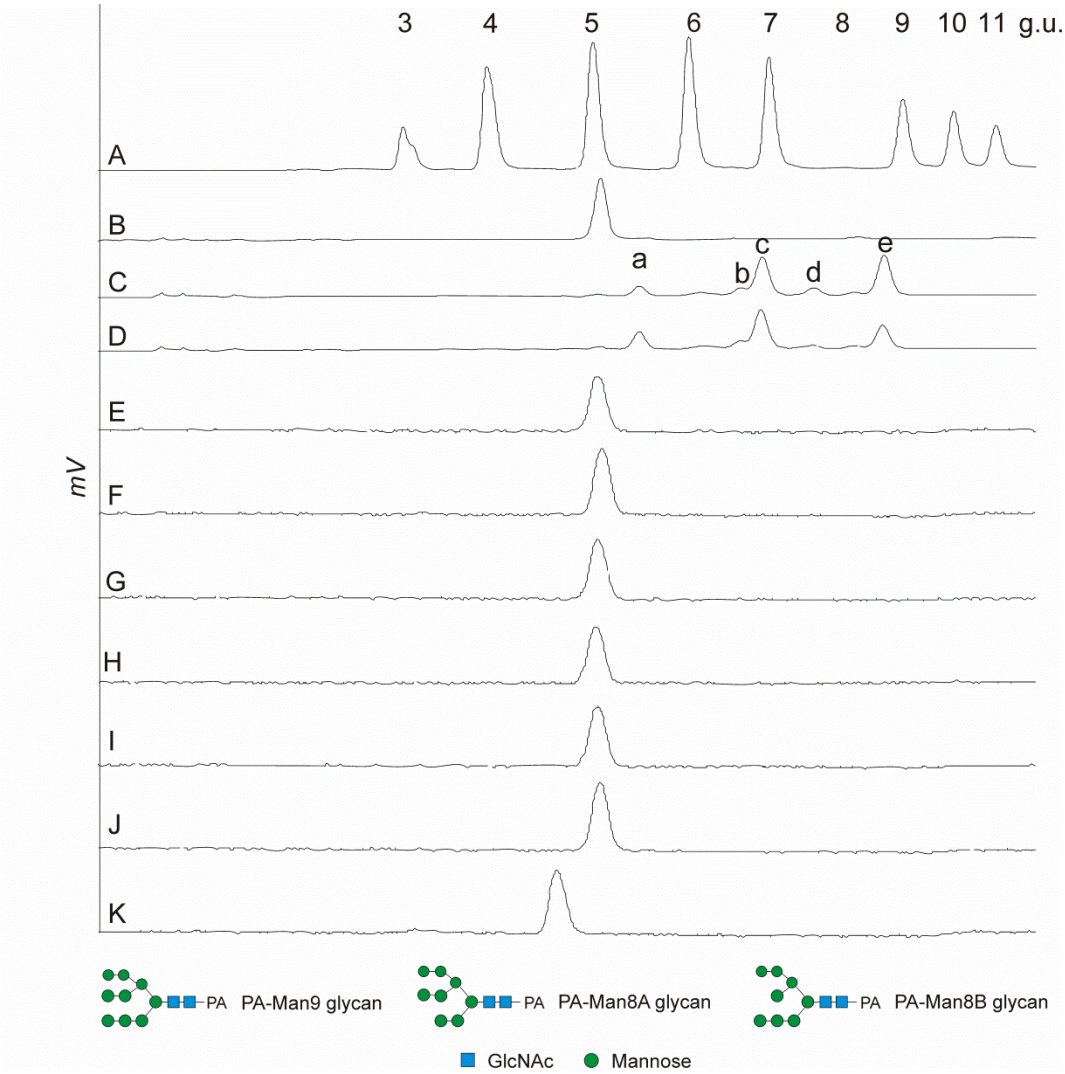
Supplemental Fig. S4 Temperature stability of *Aspergillus* β-galactosidases.

The β-galactosidases were incubated at indicated temperatures for 2 hours and remaining activity was determined by standard PNPG assay. Black squares represent incubations without BSA and open circles represent incubations supplemented with BSA. % activity +/- standard error is shown.



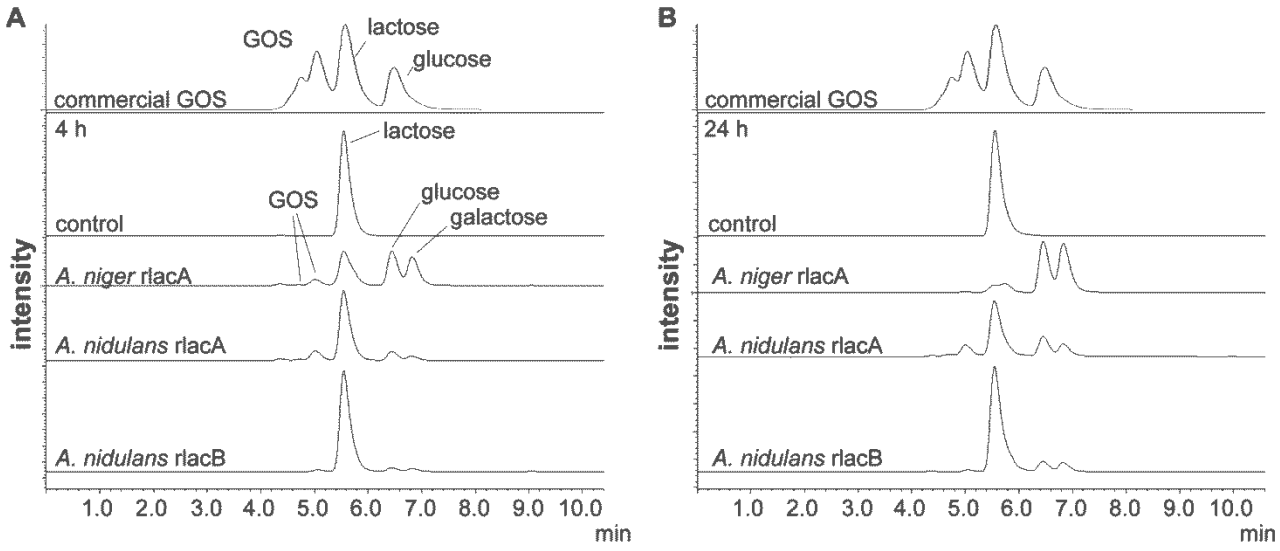
Supplemental Fig. S5 MS/MS data of PA labeled N-glycan structure containing Galβ1,4Fuc used a substrate for recombinant β-galactosidases.

The parent ions of the major structures observed in the experiments described in Figure 5 were subject to MS/MS analyses to verify the removal of the galactose residue. (A) no enzyme control (parent ion *m/z* equals 1500.5 is substrate (hydrogen adduct)) (B) *A. nidulans* rlacB (parent ion *m/z* equals 1338.5 for processed and 1500.5 for unprocessed substrate (both as hydrogen adducts)). The data show that the 162 Da loss from the substrate is due to galactose removed from the core fucose (*m/z* 446 rather than 608) and not due to mannose cleavage. Comparable results were obtained for *A. niger* rlacA and *A. nidulans* rlacA (data not shown). The glycans are depicted following the nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org).



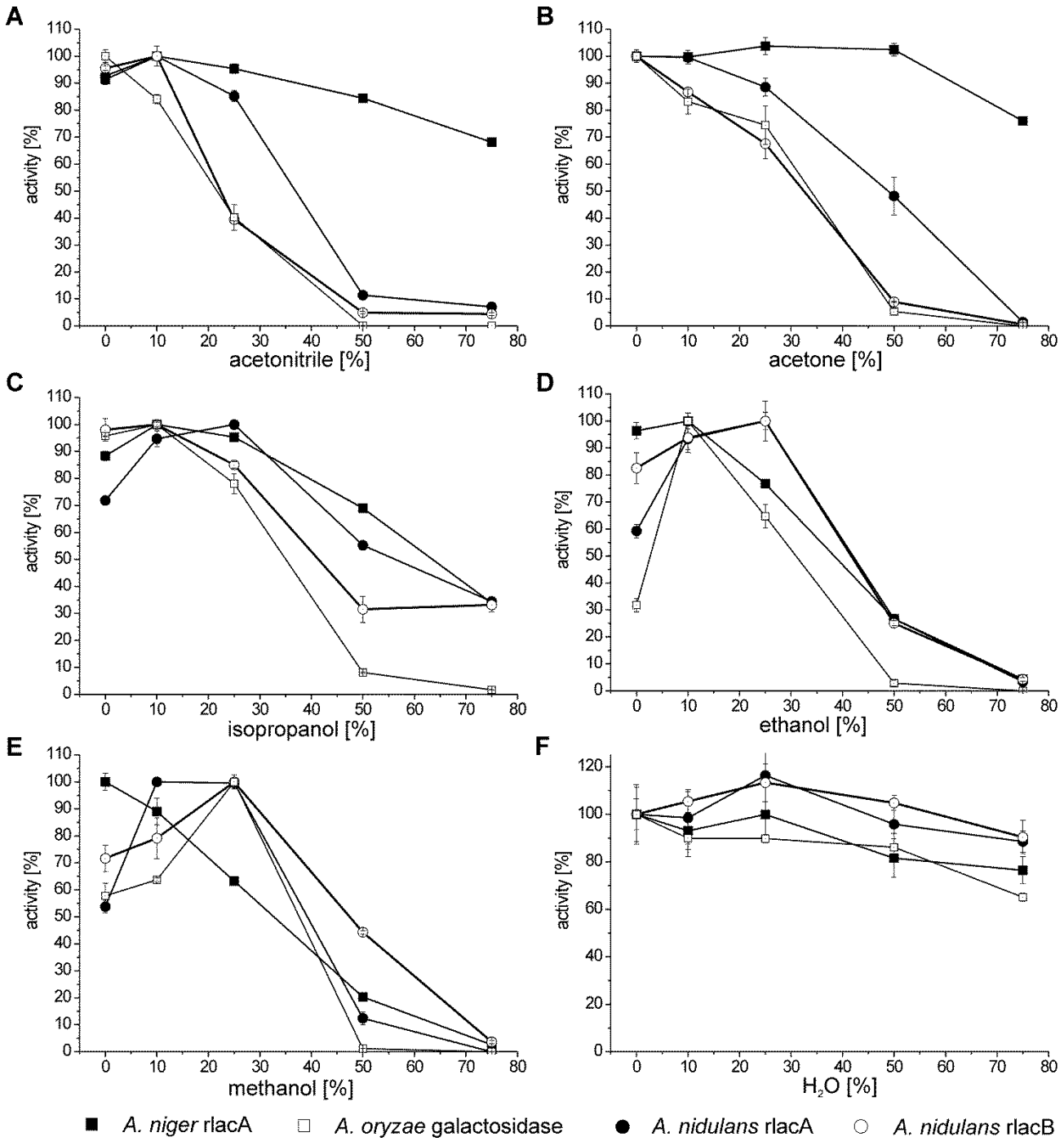
Supplemental Fig. S6 Analysis of potential mannosidase activity of the enzyme preparations.

2-aminopyridine-labeled Man9 glycan was incubated with both native and recombinant enzymes at optimal pH for fungal galactosidases (pH 4.5 or pH 5.0) and optimal pH for typical α1,2-mannosidases (pH 6.9). (A) dextran standard (B) Man9-PA control (C) *A. oryzae* galactosidase pH 4 (D) *A. oryzae* galactosidase pH 6.9 (E) *A. niger* rlacA at pH 4.5 (F) *A. niger* rlacA at pH 6.9 (G) *A. nidulans* rlacA at pH 5.0 (H) *A. nidulans* rlacA at pH 6.9 (I) *A. nidulans* rlacB at pH 5.0 (J) *A. nidulans* rlacB at pH 6.9 and (K) positive control reaction at pH 6.9 using a recombinant mannosidase I derived from *P. pastoris* producing Man8B. Lower case letters above peaks in panel C represent (a) Man8A (b,c) Man 6 and 7 isomers (d) Man5 and (e) Man6. The glycans are depicted following the glycan nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org).



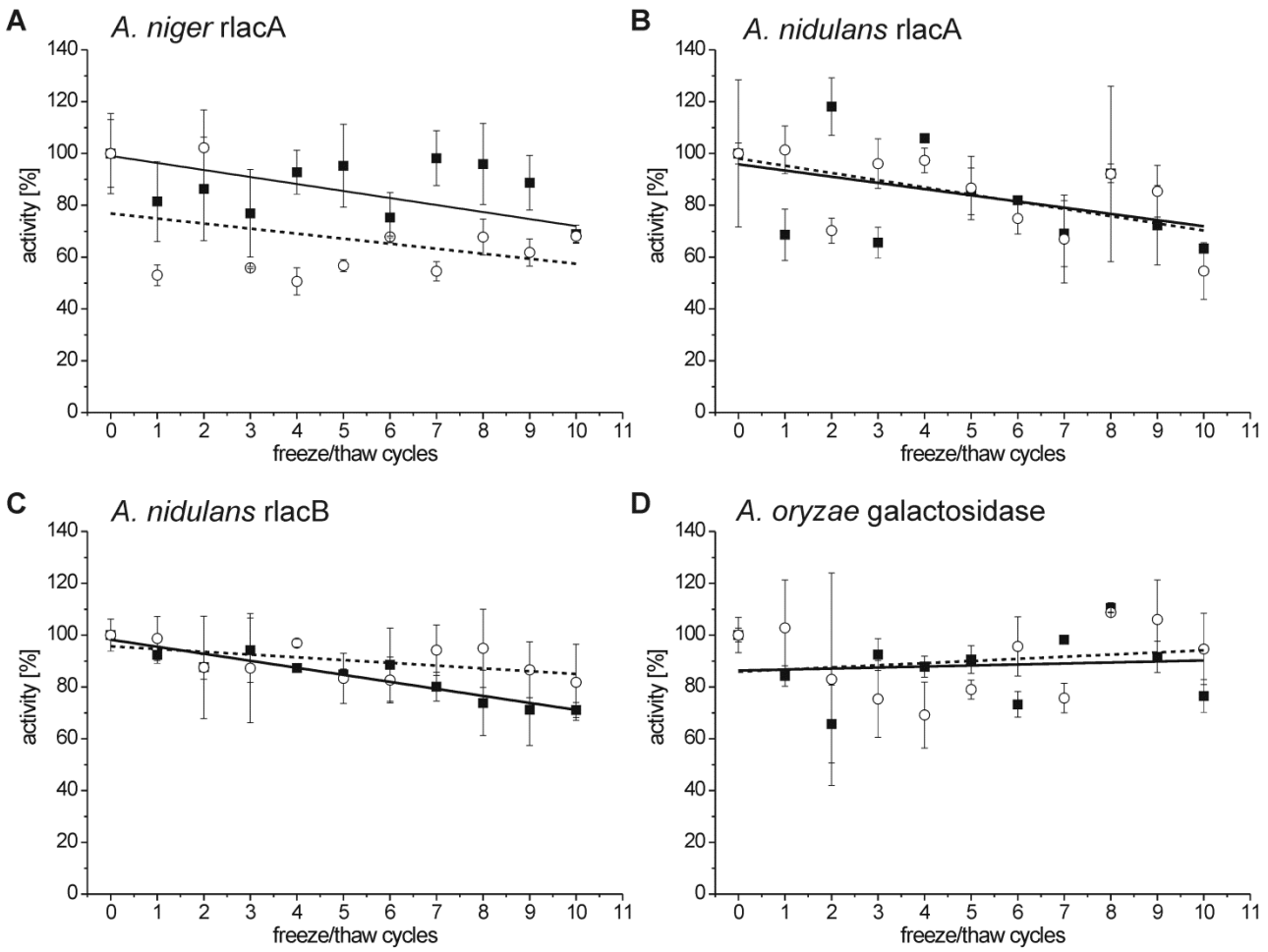
Supplemental Fig. S7 Recombinant *Aspergillus* galactosidases process lactose.

4 µg of either heat inactivated or functional *A. niger* rlacA, *A. nidulans* rlacA and *A. nidulans* rlacB were used per reaction in a total volume of 50 µL containing 20% w/v lactose. Incubations were analyzed after 4 h (A) and 24 h (B). The high efficiency of *A. niger* rlacA leads to production of GOS in the first phase of incubation. GOS and lactose are mostly digested into glucose and galactose after 24 hours. The low conversion of lactose when using *A. nidulans* rlacB stems from the low efficiency of this enzyme. Retention times of lactose, galactose, glucose and galacto-oligosaccharides (GOS) are indicated in (A).



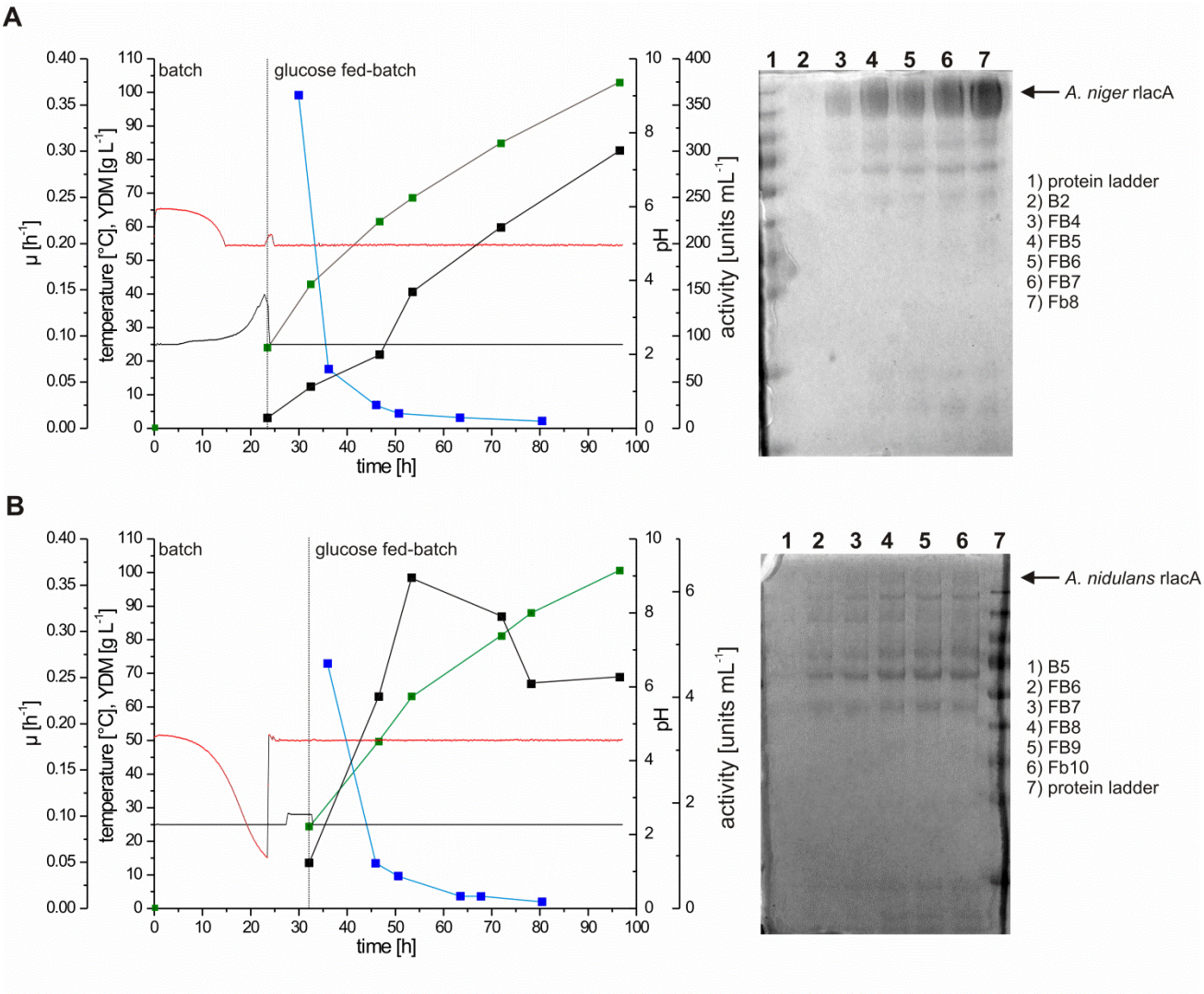
Supplemental Fig. S8 Influence of organic solvents on activity of analyzed β-galactosidases.

Reactions were performed with PNPG as substrate at 37°C with different concentrations of various solvents: (A) acetonitrile, (B) acetone, (C) isopropanol, (D) ethanol, (E) methanol and (F) water at different concentrations. Values represent averages +/- standard error.



Supplemental Fig. S9 Stability of analyzed β-galactosidases subjected to freeze/thaw cycles.

β-galactosidase activity after several freeze/thaw cycles of the respective enzymes at a concentration of 100 µg mL^{-1}. Data without BSA (black squares, solid trendline) and with BSA (1 mg mL^{-1}) supplementation (open circles, dashed trendline) are shown. Values represent averages +/- standard error.



Supplemental Fig. S10 Summary of bioreactor cultivation of *P. pastoris* X-33 expressing *A. niger* and *A. nidulans* rlacA.

Cultivations were performed essentially as described previously (Gasser*, et al.*, 2010). Figures show the time-course for the production of *A. niger* rlacA (A) and *A. nidulans* rlacA (B). Red lines indicate pH, whereas black lines indicate the temperature throughout the cultivation. Yeast dry mass (YDM) and growth rate (μ) are indicated by green and blue squares, respectively. Black squares indicate β-galactosidase activity in culture supernatant. Total protein samples from culture supernatants were analyzed by SDS-PAGE (on the right in A and B) at the time points indicated in the diagrams. B - batch samples, FB - fed-batch samples. Gels were stained with Coomassie Brilliant Blue.