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Research article

Biochemical characterization of three *Aspergillus niger* β-galactosidases

Dandan Niu [a,b,*], Xiaojing Tian [b], Nokuthula Peace Mchunu [c], Chao Jia [b], Suren Singh [c], Xiaoguang Liu [d], Bernard A. Prior [e], Fuping Lu [b]

[a] Fujian Provincial Key Laboratory of Marine Enzyme Engineering, College of Biological Science and Engineering, Fuzhou University, Fuzhou 350116, China
[b] College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, China
[c] Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa
[d] Department of Biological Chemical Engineering, College of Chemical Engineering and Material Sciences, Tianjin University of Science and Technology, Tianjin 300457, China
[e] Department of Microbiology, Stellenbosch University, Matieland, South Africa

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ABSTRACT

Background: β-Galactosidases catalyze both hydrolytic and transgalactosylation reactions and therefore have many applications in food, medical, and biotechnological fields. *Aspergillus niger* has been a main source of β-galactosidase, but the properties of this enzyme are incompletely studied.
Results: Three new β-galactosidases belonging to glycosyl hydrolase family 35 from *A. niger* F0215 were cloned, expressed, and biochemically characterized. In addition to the known activity of LacA encoded by *lacA*, three putative β-galactosidases, designated as LacB, LacC, and LacE encoded by the genes *lacB*, *lacC*, and *lacE*, respectively, were successfully cloned, sequenced, and expressed and secreted by *Pichia pastoris*. These three proteins and LacA have N-terminal signal sequences and are therefore predicted to be extracellular enzymes. They have the typical structure of fungal β-galactosidases with defined hydrolytic and transgalactosylation activities on lactose. However, their activity properties differed. In particular, LacB and lacE displayed maximum hydrolytic activity at pH 4–5 and 50°C, while LacC exhibited maximum activity at pH 3.5 and 60°C. All β-galactosidases performed transgalactosylation activity optimally in an acidic environment.
Conclusions: Three new β-galactosidases belonging to glycosyl hydrolase family 35 from *A. niger* F0215 were cloned and biochemically characterized. In addition to the known LacA, *A. niger* has at least three β-galactosidase family members with remarkably different biochemical properties.

1. Introduction

β-Galactosidase (β-D-galactohydrolases, EC 3.2.1.23) is an abundant glycoside hydrolase enzyme that catalyzes the hydrolysis of terminal β-D-galactosyl moieties to monosaccharides by breaking the glycosidic bond of substrates such as disaccharides, diverse glycoconjugates, and polysaccharides. Under defined reaction conditions, many β-galactosidases can catalyze transglycosylation reactions using various acceptor molecules. Depending on its biochemical activities, β-galactosidase activity has been used for (1) hydrolysis of lactose (thereby rendering dairy products consumable for lactose-intolerant individuals), analytical studies, glycan remodeling, and various processes of biotechnological and medical importance [1,2,3] and (2) synthesis of galactooligosaccharides (GOS) from lactose by transglycosylation [4].

β-Galactosidases can be derived from microbial sources including bacteria, yeasts, and filamentous fungi [4,5,6,7]. *Aspergillus*-sourced β-galactosidases, especially those from *Aspergillus oryzae*, are most widely used, and detailed data on their characteristics and many applications have been previously reported [8,9,10].

The *A. niger* β-galactosidase was identified, purified, and characterized more than four decades ago [11,12]. The *lacA* gene encoding *A. niger* β-galactosidase was first cloned and expressed in the yeast *Saccharomyces cerevisiae* in 1992 [13,14] and later overexpressed in yeast [7] and *Eremothecium gossypii* (formerly *Ashbya gossypii*) [15]. Experimental evidence, especially from transcriptomic data, indicates that the β-galactosidase activity of *A. niger* is contributed by different isoenzymes [12,16,17,18]. However, gene cloning, overexpression, structural analysis [19], and applications [20] have mainly been focused on *A. niger* LacA (or lactase A encoded by *lacA*). Advances in

* Corresponding author at:
E-mail address: ddniu0529@fzu.edu.cn (D. Niu).
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the genome sequencing of *A. niger* [21] opened possibilities to further exploit this fungus to identify additional β-galactosidase-like enzymes, and a total of five putative β-galactosidases have been identified at genome-level [16,17,21]. In this study, in addition to the known LacA, genes encoding four new lactose-hydrolyzing enzymes were cloned, sequenced, and heterologously expressed, and their enzyme properties were established.

2. Materials and methods

2.1. Strains and culture conditions

A. niger CICIM F0215 used in this study was isolated from a natural sample, identified, and stored at the Culture and Information Center for Industrial Microorganisms of China Univerisities (CICIM-CU, Jiangnan University, China). The strain was grown in minimal medium (MM) containing 7 mM KCl, 11 mM KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO_3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 6.2 nM Na_2MoO_4, and 174 nM EDTA (pH 5.5). Erlenmeyer flasks (250 mL) containing 50 mL MM supplemented with 0.1% (w/v) casamino acids and 2% (w/v) lactose (Sigma) were inoculated with 1×10^6 spores mL^{-1} and incubated at 32°C on a rotary shaker at 240 rpm for 24 h. The mycelium was recovered on a nylon membrane by suction, washed with MM, and stored at -80°C prior to the isolation of total RNA. The bacterial strain used for transformation and amplification of recombinant DNA was *Escherichia coli* XL-1 Blue (Stratagene). Minimal dextrose medium (MD; 13.4 g/L YNB w/o, 0.4 mg/L biotin, and 20 g/L glucose), yeast extract peptone dextrose medium (YPD; 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose), buffered glycerol complex medium (BMGY, 10 g/L yeast extract, 20 g/L peptone, 13.4 g/L YNB w/o, 0.4 mg/L biotin, 1% glycerol, and 100 mM potassium phosphate, pH 6.0) and buffered minimal methanol medium, (BMMY, 10 g/L yeast extract, 20 g/L peptone, 13.4 g/L YNB w/o, 0.4 mg/L biotin, 0.5% methanol, and 100 mM potassium phosphate, pH 6.0) were used to cultivate *Pichia pastoris* GS 115 or its recombinants.

2.2. Database mining of A. niger genome

A. niger LacA amino acid sequence (GenBank ID: AGS42424) was used for a reciprocal BLAST (website: http://www.aspergillusgenome.org/cgi-bin/compute/blast_clade.pl) against the genome sequence of *A. niger* CBS 513.88, and the corresponding protein sequences were extracted. Sequences were aligned using the CLUSTAL W program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Multiple sequence alignments of known fungal lactases, based on full-length predicted protein sequences, were performed using the CLUSTAL W interface in MEGA version 5.0 (www.megasoftware.com). Signal peptide prediction was made using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/).

2.3. Cloning of sequences encoding A. niger β-galactosidases

Open reading frames (ORFs) from *A. niger* CICIM F0215 were amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (TaKaRa Biotechnology-Dalian Co. Ltd., China) and 50 ng cDNA templates using the primers listed in Table 1. RNA sample was extracted using High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol from *A. niger* cultures as described above. cDNA was produced from 200 ng total RNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's guidelines. The PCR products including the region encoding the original signal peptide were amplified using the primers lacA and lacA_2, lacB and lacB_2, lacC and lacC_2, lacD and lacD_2, or lacE and lacE_2 and were sequenced by Sanger sequencing [22].

The genes encoding the mature peptides were prepared using the primers lacA_1 (lacB_1, lacC_1, lacD_1, or lacE_1) and lacA_2 (lacB_2, lacC_2, lacD_2, or lacE_2). The products were digested with *Xba*I, ligated with T_4 ligase into *Avr*II- and *Sna*BI-digested pPIC9k (Invitrogen), and used to transform *E. coli* XL-1 Blue. Positive clones were selected on LB plates containing 100 $\mu g \cdot mL^{-1}$ ampicillin, and the *Pst*I-digested restriction pattern and Sanger sequencing verified the inserted sequence. Correct recombinant plasmid DNAs were extracted using Qiagen plasmid mini kit (Beijing, China).

Table 1
Primer sequence.

Primer	Nucleotide sequence (5′ → 3′)[a]
lacA	ATGAAGCTTTCCTCCGCTTGTG
lacA_1	*GTA*GCGTCCATTAAGCATCGAATCA
lacA_2	TGCTCTAGACTAGTATGCACCCTTCCGCTTCTT
lacB	ATGAAGCTGCAGTCCATACTTTCATG
lacB_1	*GTA*ACTACAGATGGTCTGACAGACCTGGT
lacB_2	TGCTCTAGATCAGGTGTACTTTGCCCGAGCT
lacC	ATGACGCGGATCACCAAGTTATG
lacC_1	*GTA*GCCCAGAACCAGACGGAGACT
lacC_2	TGCTCTAGATCAAGCAAACTTCAACCTCTCCG
lacD	ATGCAAGCCTTTTCAGAGTATATTGA
lacD_1	*GTA*ATGCAAGCCTTTTCAGAGTATATTGA
lacD_2	TGCTCTAGATTACGCGTACACGCTCCTACC
lacE	ATGAAGACCTCATTTTTGCTTGCTATAG
lacE_1	*GTA*CCGAATTATGTTCGTCAGATTAATGC
lacE_2	TGCTCTAGATCAATAGCTATCAACCCGTTCCTTG

[a] Underlined sequences represent the added *Xba*I site; italic letters represent the half *Sna*BI site added. The primers lacA (lacB, lacC, lacD, or lacE) and lacA_2 (lacB_2, lacC_2, lacD_2, or lacE_2) were used to amplify the gene including the region encoding the original signal peptide. The primers lacA_1 (lacB_1, lacC_1, lacD_1, or lacE_1) and lacA_2 (lacB_2, lacC_2, lacD_2, or lacE_2) were used to amplify the region encoding the mature peptide.

2.4. Expression in P. pastoris

The recombinant plasmids carrying the ORF of the galactosidase were linearized with restriction enzymes (*Stu*I, *Sac*I, or *Bgl*II) and transformed into *P. pastoris* GS115. Positive clones were selected on MD plates containing histidine and YPD plates containing 2 mg/mL G418 (Invitrogen) according to the manufacturer's instructions. A single colony from the YPD plate was inoculated into BMGY for growth and BMMY for expression and induction of the recombinant protein. All enzyme expression studies were performed in baffled shake flasks at 30°C and 200 rpm for 7 d with 0.5% (v/v) methanol added daily, and samples were obtained every 24 h. The culture medium was centrifuged at 4000 rpm for 20 min, and the supernatant was collected and dialyzed against 10 mM phosphate buffer (pH 6.0) overnight and lyophilized for further tests.

2.5. β-Galactosidase activity assays

β-Galactosidase activity was determined according to a method described previously [23]: the ortho-nitrophenol release from ortho-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) as substrate under pH 5.0 and 37°C was measured at 420 nm. The activity was further confirmed using lactose as substrate under pH 5.0 but at 50°C. One enzyme unit is defined as the quantity of enzyme that catalyzes the liberation of 1 μmol of ortho-nitrophenol from ONPG per minute or 1 μmol of glucose from lactose under assay conditions. Protein concentrations were determined by the method of Bradford [24] with crystalline bovine serum albumin fraction V (Serva Feinbiochemica, Germany) as standard.

2.6. Determination of the pH optimum, temperature optimum, and metal ion susceptibility

Assays were performed as described above using 5 mM ONPG as substrate. For optimum temperature determination, the activity of the recombinant enzyme was measured at temperatures of 30°C, 40°C,

45°C, 50°C, 55°C, 60°C, 70°C, and 80°C. To calculate the optimum pH, the enzymatic activity was measured at 37°C under different pH conditions: pH 3.0–5.5 with acetate buffer, pH 6.0–7.5 with phosphate buffer, and pH 8.0–9.0 with Tris–HCl buffer. For the determination of the effect of cations and EDTA on the enzymatic activity, the enzyme was incubated with 5 mM cation or chemical in pH 5.0 buffer, and the activity was measured as previously described.

2.7. Kinetic studies

The β-galactosidase activity was tested with both ONPG and lactose as substrates. The determination of the β-galactosidase activity was performed at pH 5.0 and 37°C, as described above, but in the presence of different concentrations of substrates. Michaelis constant (K_m) and V_{max} were calculated by the Lineweaver–Burk method using the nonlinear curve fitting function available in the OriginPro 8 software package.

2.8. Substrate specificity and galactooligosaccharide formation

The substrate specificity was tested with substances including lactose (Sigma), xylan (Sigma), xyloglucan (Sigma), pectin (Sigma), arabinoxylan (Sigma), ONPG (Sigma), maltose (Shanghai Biotech, China), and sucrose (Shanghai Biotech, China). They were used as substrates at a concentration of 30% (w/v) in acetate buffer at 50°C and pH 5.0. Reactions commenced with the addition of 10 units of the enzyme per gram of substrate to a total volume of 1 mL. Samples were taken at 4 and 24 h for analysis. For GOS formation, the condition was the same as above; however, 30% lactose was used as the substrate, and the reaction was carried out at pH 3.0, 4.0, 5.0, and 6.0.

Products were determined by HPLC (Agilent 1200 Series HPLC System) with an evaporative light-scattering detector (Alltech ELSD detector 2000s, Grace co. Ltd.) using a TSKgel Amide-80 column (4.6 mm ID × 250 mm, Tosoh, Japan) with acetonitrile:water (65:35, v/v) as solvent at a flow rate of 1 mL·min^{-1} at 30°C. Monosaccharides and disaccharides used as HPLC standards were purchased from Sigma. The GOS standard was obtained from Jiangsu Ruiyang Biotech Co. Ltd., China.

2.9. Homology modeling

The models of the *A. niger* β-galactosidases were prepared using the fully automated protein structure homology-modeling server Swiss-Model (http://www.swissmodel.expasy.org). The protein structures were visualized using PyMOL (http://bibdesk.sourceforge.net/).

The nucleotide sequence and their predicted protein sequences have been submitted to the GenBank database under accession numbers KU847417 (*lacB*), KU847418 (*lacC*), and KU847419 (*lacE*).

3. Results

3.1. Identification of β-galactosidase isoenzymes in the A. niger genome

The amino acid sequence of *A. niger* β-galactosidase A (LacA) was used to blast the whole genome of *A. niger* CBS 513.88. Five significant sequences were revealed, including the previously described LacA (β-galactosidase A or β-galactosidase E), β-galactosidase B, β-galactosidase B, β-galactosidase B, and β-galactosidase E in the genome (Table 2). This is in agreement with the five sequences originally reported by de Vries et al. [16,17]. The genes encoding five β-galactosidases were cloned and sequenced by the methods described above. The amino acid sequences were deduced based on the nucleotide sequences. The main results are summarized in Table 2.

Of the five cloned genes from *A. niger* F0215, *lacA*, *lacB*, *lacC*, and *lacE* possessed a complete ORF. The nucleotide sequence of *lacA* cDNA was

Table 2
β-Galactosidases from *A. niger* CBS 513.88 and F0215.

Genes[a]	Nomination in *A. niger* CBS 513.88	*A. niger* CBS 513.88				No. of amino acids: CBS 513.88/F0215	Signal peptide	Deduced molecular weight of matured peptides (Da) in F0215	Differences in F0215 from CBS 513.88
		Gene ID	mRNA ID	Protein ID	No. of introns				
lacA	β-Galactosidase E	An01g12150 ANI_1_1636014	XM_001389585.2	XP_001389622.1	8	1007/1007	1–18	108,019.7	Same sequence
lacB	β-Galactosidase B	An06g00290 ANI_1_366054	XM_001390820.2	XP_001390857.2	6	991/994	1–19	106,502.1	Three more amino acids and 6 other amino acids different
lacC	β-Galactosidase B	An01g10350 ANI_1_1398014	XM_001389416.2	XP_001389453.2	5	1016/1014	1–20	109,461.9	Two less amino acids and 6 other amino acids different
lacD	β-Galactosidase B	An07g04420 ANI_1_1714064	XM_001391487.2	XP_001391524.2	11	1044/–	/	/	Pseudogene
lacE	β-Galactosidase E	An14g05820 ANI_1_1530124	XM_001401168.1	XP_001401205.1	5	1015/1030	1–18	111,841.1	First predicted intron had coding meaning

[a] Renamed in this study.

the same as that of *lacA* in *A. niger* CBS 513.88. The nucleotide sequence of *lacB* cDNA in *A. niger* F0215 contained three additional codons and differed by 40 nucleotides from that of *lacA*. The resulting protein comprised of three additional amino acid residues with six amino acid residues variation in different positions compared to LacB of *A. niger* CBS 513.88. The nucleotide sequence of *lacC* cDNA in *A. niger* F0215 differed by 39 nucleotides, with two additional codons missing. The resulting proteins comprised of six amino acid residues variation, with two amino acid residues missing in different positions compared to LacC of *A. niger* CBS 513.88. The nucleotide sequence of *lacE* cDNA in *A. niger* F0215 included the 45 base pairs of the first intron predicted in *A. niger* CBS 513.88. However, *lacD* cDNA in *A. niger* F0215 failed to be amplified. A further attempt to amplify and sequence by redesigning the sense primer and using chromosomal DNA as template also failed

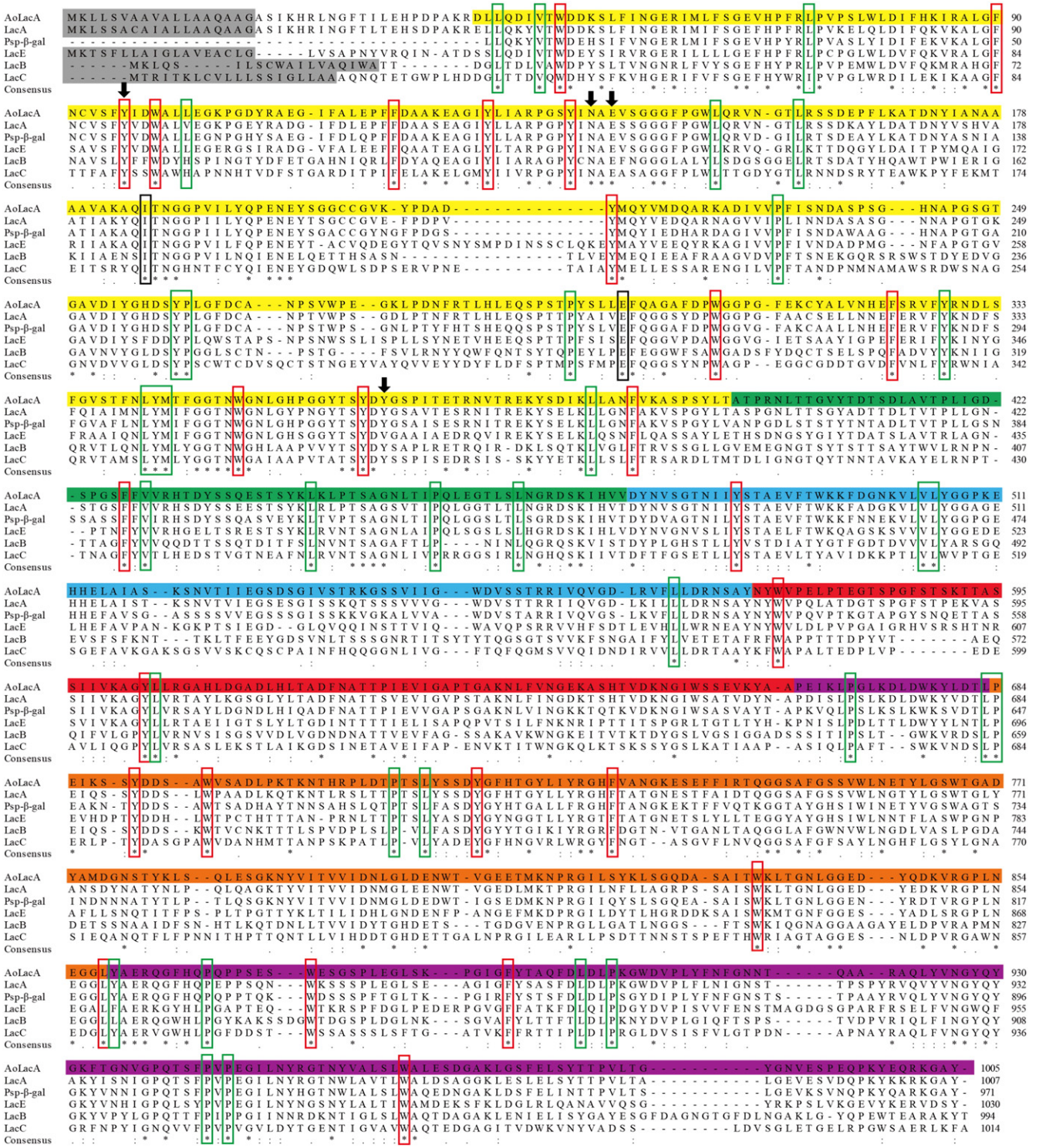

Fig. 1. Multiple sequence alignment of β-galactosidases from *Penicillium* sp. (Psp-β-gal), *A. oryzae* (AoLacA), and *A. niger* F0215 (LacA, LacB, LacC, and LacE). The sequences of signal peptides are colored in gray. The conserved amino acids, conserved replacement, and semi-conserved replacement are marked with asterisks, colon, and point, respectively. The two conserved glutamic acid residues (E) and some conserved hydrophobic and aromatic amino acid residues are shown in black, green, and red rectangles, respectively. Domains 1 to 6 in AoLacA are colored by yellow, green, blue, red, orange, and purple, respectively. Amino acid residues known to bind galactose are marked with arrows above the sequence.

(data not shown). Therefore, *lacD* in *A. niger* F0215 is probably disrupted or a pseudogene.

3.2. Amino acid sequence comparison of *A. niger* β-galactosidases

Database searches with *A. niger* LacA sequence as queries showed homology to other known β-galactosidase sequences. The sequences were aligned and subsequently used to generate a phylogenetic tree using the program MEGA 4.0 with the neighbor-joining algorithm [25]. Interestingly, the LacA, LacB, LacC, and LacE proteins identified in this study were significantly different in their amino acid sequences and belonged to different subgroups. LacA amino acid sequence had 39%, 36%, and 51% similarity with LacB, LacC, and LacE, respectively, while LacB showed 41% and 37% similarity with LacC and LacE, and LacC revealed 35% similarity with LacE.

Four functional domains were well-conserved in the tertiary structure of fungal β-galactosidases. They contained many highly conserved aromatic and hydrophobic residues that are observed in members of glycosyl hydrolase family 35. These highly conserved functional domains, as well as other conserved domains, were also present in most of the fungal β-galactosidases (Fig. 1). The amino acid sequence known to bind galactose in β-galactosidases was conserved in LacA, LacB, LacC, and LacE, implying that these four enzymes contained galactose-binding domains [26]. The two catalytic glutamates (Glu200 and Glu298), an acid/base catalyst, and a nucleophile in β-galactosidases from *A. oryzae* [26], were also highly conserved in these four *A. niger* β-galactosidases. Moreover, some conservative hydrophobic amino acid residues were present in β-galactosidases both from *A. niger* and *A. oryzae*.

To improve this protein sequence alignment, 3D protein models were predicted for the catalytic domains of these four β-galactosidases and structurally aligned with crystal structures of AoLacA (β-galactosidase from *A. oryzae*, PDB ID: 4IUG) [26]. The sequence similarities between AoLacA and LacA, LacB, LacC, and LacE from *A. niger* F0215 were 54%, 39%, 38%, and 45%, respectively. The model was evaluated with QMEAN (the QMEAN4 score is a composite score composed of a linear combination of four statistical potential terms (estimated model reliability between 0 and 1)) [27]. The QMEAN4 scores of the constructed 3D structures were 0.725, 0.657, 0.692, and 0.722, respectively, and QMEAN Z-scores were -0.32, -2.86, -3.38, and -1.75, respectively. From the QMEAN4 score, these modeled structures were considered valid for use. Despite having a valid QMEAN4 score, the LacC modeled structure was less valid because the QMEAN Z-score was less than -3.0. Despite the low sequence similarities, the conservative domains of the four 3D putative structures were similar to those of the template (Fig. 2).

3.3. Enzyme kinetics and biochemical characteristics of recombinant galactosidases

The enzymes were expressed and secreted by *P. pastoris*. The hybrid expression plasmids containing *lacA*, *lacB*, *lacC*, and *lacE* lacked the region encoding the original signal peptide. The plasmids pPIC-*lacA*, pPIC-*lacB*, pPIC-*lacC*, and pPIC-*lac*E were linearized and genetically transformed into *P. pastoris* GS115. The resulting recombinants, named GS115-*lacA*, GS115-*lacB*, GS115-*lacC*, and GS115-*lacE* and GS115-9k (*P. pastoris* GS115 integrated with pPIC9k) as control were selected and cultured in shake flask experiments, and the supernatants were collected. The enzyme activities in the supernatants using ONPG as substrate were 33.9, 1.3, 1.1, and 1.4 units per mL, respectively. The activities in the supernatants using lactose as substrate were 169.8, 8.1, 6.9, and 9.6 units per mL, respectively. No activity was detectable in the supernatant of GS115-9k. The basic enzyme kinetic parameters were determined using ONPG or lactose as substrate. The K_m values of *A. niger* LacA, LacC, and LacE were similar, whereas LacB revealed a slightly higher affinity toward ONPG. LacC showed the highest V_{max} value on ONPG, and LacA revealed the highest V_{max} value on lactose (Table 3).

The temperature profiles of the enzymes differed considerably (Table 3). LacA and LacC showed a temperature optimum of about 60°C, with a very broad temperature range of activity; LacA showed 60% activity at 55°C or 70°C and LacC at 50°C or 70°C. LacB and LacE showed a temperature optimum of about 50°C. At temperatures between 40°C and 60°C, LacB maintained more than 60% activity, while LacE held more than 70% activity.

The pH optimum of LacA was between 3.5 and 4.0, and it retained 60% of its maximal activity at pH 3.0 or pH 4.5. The maximum activity of LacC occurred at pH 3.5, and the enzyme retained more than 80% of its maximum activity at pH 3.0 or 4.5. The pH optima of LacB and LacE were between pH 4.0 and 5.0, with both retaining 60% of their maximum activity at pH 3.5 or 5.5. All except LacC revealed less than 10% of their maximum activity at pH 6.5, while LacC retained 40% activity at pH 7.0 (Table 3). Because of its larger pH spectrum and higher pH flexibility, LacC will potentially perform better than the other β-galactosidases from *A. niger* under higher pH values or in processes with fluctuating pH conditions.

The effect of cations and EDTA on the activity is shown in Table 4. The activity of LacA was significantly enhanced with 5 mM Ca^{2+} or

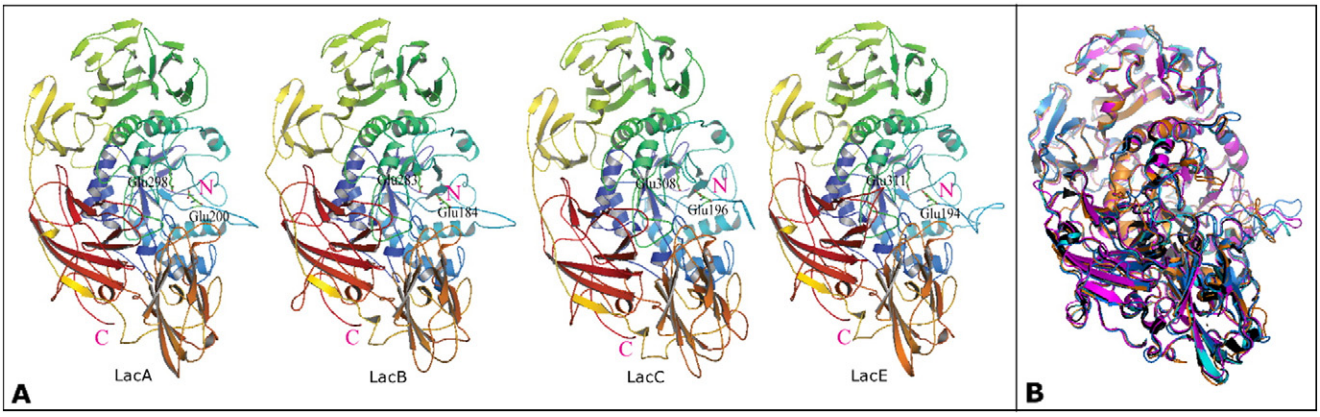

Fig. 2. Putative 3D structures of the *A. niger* β-galactosidases aligned with 4IUG by PyMOL. Box A presents the 3D protein models of the *A. niger* F0215 β-galactosidases LacA, LacB, LacC, and LacE predicted for the catalytic domains and structurally aligned with crystal structures of AoLacA, a β-galactosidase from *A. oryzae*; PDB ID: 4IUG. Box B presents the overlapped four 3D models.

Table 3
Properties of *A. niger* β-galactosidases.

β-Galactosidase	ONPG as substrate[a]		Lactose as substrate[a]		T_{opt} (°C)	pH_{opt}
	K_m (mM)	V_{max}	K_m (mM)	V_{max}		
LacA	0.43 ± 0.09	0.026 ± 0.003	0.71 ± 0.04	0.067 ± 0.003	60	3.5–4.0
LacB	0.22 ± 0.03	0.022 ± 0.002	0.57 ± 0.05	0.039 ± 0.001	50	4.0–5.0
LacC	0.37 ± 0.04	0.065 ± 0.001	0.74 ± 0.03	0.030 ± 0.005	60	3.5
LacE	0.46 ± 0.08	0.011 ± 0.002	0.65 ± 0.08	0.039 ± 0.002	50	4.0–5.0

[a] Mean ± standard deviation of triplicate determinations.

Mn^{2+}, whereas LacB and LacC were more sensitive to Mn^{2+}, Mg^{2+}, Ca^{2+}, or Na^{+}. The activity of LacE was enhanced with Mg^{2+}, Ca^{2+}, Mn^{2+}, or Na^{+}. Except for LacA, EDTA had minimal effect on the remaining enzymes.

3.4. Substrate specificity and galactose oligosaccharide formation

The substrate specificity of the four β-galactosidases was examined in the enzyme assays with lactose, xylan, xyloglucan, pectin, arabinoxylan, maltose, and sucrose as natural substrates or with chemically synthesized ONPG. All the β-galactosidases predominantly hydrolyzed ONPG and lactose. However, no activity on xylan, xyloglucan, pectin, arabinoxylan, maltose, or sucrose was detected. GOS formation from lactose by transgalactosylation of the enzymes was further examined. At a lactose concentration of 20% and after 24 h of incubation, GOS formation was confirmed by HPLC analysis. All four β-galactosidases could synthesize GOS. The observed ratio between oligosaccharides and monosaccharides suggested that LacE and LacC were superior to LacB and LacA for GOS formation, which made these two newly characterized enzyme interesting candidates for biotechnological applications for transgalactosylation/GOS production. The transgalactosylation was optimal in acidic conditions (pH 3.0) but no transgalactosylation activity, except for LacC, was found at pH 6.0 or above (Fig. 3). Therefore, LacC might influence the GOS production process.

4. Discussion

In this study, we identified five ORFs from *A. niger* F0215 representing putative β-galactosidases. The ORFs represented a previously described β-galactosidase (LacA) [7,13,15] and three other β-galactosidases (LacB, LacC, and LacE) that had been identified [16] but not previously biochemically characterized. In the present study, we cloned and expressed the four genes and characterized the biochemical properties and structures of the gene products. A sequence similar to the β-galactosidase ORF (XP_001391524.2) previously found in the genome of *A. niger* CBS 513.88 and designated as LacD was failed to be obtained and expressed. This ORF appears to represent a pseudogene. These results suggest that the β-galactosidase family of *A. niger* F0215 consists of four functional members (LacA, LacB, LacC, and LacE) belonging to family 35 of the glycoside hydrolases (www.cazy.org).

Evidence reported by Widmer and Leuba [12] pointed to the possibility of the *A. niger* β-galactosidase family consisting of more than one member. They purified three β-galactosidases and identified them as glycoproteins with molecular weights of 124,000, 150,000, and 173,000; isoelectric points of about 4.6; pH optima between 2.5 and 4.0; and heat stability up to 60°C. These properties are similar to those established in our detailed investigations. However, the variation in the properties of the three glycoproteins was proposed to be mainly due to dissimilar carbohydrate contents and related to culture conditions [12]. Subsequently, a β-D-galactosidase from *A. niger* was purified and characterized [28]. Gel-filtration chromatography and SDS-PAGE analysis showed that its molecular weight was 300,000 and 130,000, respectively, suggesting that it was heavily glycosylated. This β-galactosidase was designated *lacA*, and the gene was cloned and expressed in *S. cerevisiae* [13,14], yeast [7], and *E. gossypii* [15]. The transcription of *lacA* was induced by arabinose, xylose, xylan, and pectin [29].

The advent of the genomic era has allowed greater detailed investigation of enzymatic properties and has shown that specific enzyme activity is linked to the substrate utilization profiles of the isoenzymes [30,31]. Not surprisingly, the functional four β-galactosidases from *A. niger* F0215 shared similar structures (Fig. 1 and Fig. 2), similar molecular weights between 106,500 and 111,840 (Table 2), yet there was substantial variation in the kinetic profiles (temperature and pH optima, substrate affinity). This might explain the multiple form patterns of the *A. niger* β-galactosidase reported in the initial study of Widmer and Leuba [12].

Table 4
Effect of cations and EDTA on the enzymatic activity of *A. niger* β-galactosidases.

Cation and chemical	Relative activity (%)[a]			
	LacA	LacB	LacC	LacE
Control	100 ± 2.0	100 ± 2.0	100 ± 2.0	100 ± 2.0
Mg^{2+}	112.7 ± 2.0	147.9 ± 2.0	126.7 ± 2.0	150.6 ± 2.0
Cu^{2+}	68.8 ± 1.0	85.4 ± 1.2	76.6 ± 1.1	71.1 ± 1.5
Fe^{2+}	66.6 ± 1.1	69.9 ± 1.0	58.0 ± 1.0	49.0 ± 1.0
Ca^{2+}	127.8 ± 2.1	136.0 ± 2.0	130.1 ± 2.0	134.2 ± 2.0
Na^{+}	102.7 ± 1.0	133.3 ± 2.0	125.1 ± 1.8	117.9 ± 2.0
Ni^{2+}	82.2 ± 1.5	86.3 ± 1.0	72.1 ± 1.0	77.0 ± 1.0
Zn^{2+}	61.7 ± 1.0	61.3 ± 0.8	70.5 ± 1.2	52.5 ± 1.2
Mn^{2+}	130.9 ± 2.7	172.9 ± 2.0	164.0 ± 2.3	123.0 ± 2.0
Li^{+}	82.0 ± 1.3	81.2 ± 1.0	86.7 ± 1.0	89.4 ± 1.0
EDTA	84.6 ± 1.0	99.4 ± 1.2	98.4 ± 1.5	95.4 ± 1.5

[a] Mean ± standard deviation of triplicate determinations.

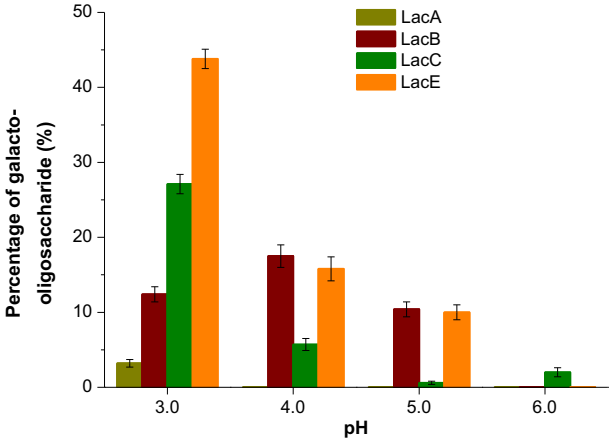

Fig. 3. Percentage of galactooligosaccharide (GOS) formed from lactose at different pH with the enzymes LacA, LacB, LacC, and LacE. GOS formation from lactose by transgalactosylation of the enzymes was further examined. At a lactose concentration of 20% and 24 h incubation, the amount of GOS formed was determined by HPLC analysis. All four β-galactosidases were pH-dependently able to synthesize GOS with different transformation rates.

Many potential applications of these enzymes have been identified. Two are particularly attractive: (1) hydrolysis of lactose in dairy products or administration as a digestive supplement to prevent or treat lactose intolerance [32] and (2) transglycosylation of lactose to produce the valuable prebiotic GOS. All four β-galactosidases hydrolyzed and transgalactosylated lactose but with unique kinetic properties. pH is an important factor affecting the kinetics of lactose hydrolysis and GOS synthesis. In many cases, similar pH optima are reported for GOS synthesis and galactoside hydrolysis [18,33]. In contrast, the *A. niger* β-galactosidases optimally synthesized GOS in acidic pH values (Fig. 3), although lactose hydrolysis occurred optimally at higher pH values (Table 3). These enzymes have been reported to be useful in the modification of the glycosyl side chains on xylan, pectin, xyloglucan, and arabinoxylan amongst other polymers [29]. The potential of *A. niger* β-galactosidases to modify polymers requires further investigation.

5. Conclusion

In addition to the known LacA in *A. niger*, three new β-galactosidases belonging glycosyl hydrolase family 35 from *A. niger* F0215 were cloned, expressed, and biochemically characterized. *A. niger* has at least four β-galactosidase family members with remarkably different biochemical properties.

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Conflict of interest

The authors declare that they have no competing interests.

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