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Molecular cloning and high-level expression of a β-galactosidase gene from *Paecilomyces aerugineus* in *Pichia pastoris*

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ABSTRACT

A β-galactosidase gene (designated *PaGalA*) was cloned for the first time from *Paecilomyces aerugineus* and expressed in *Pichia pastoris* under the control of the *AOX*1 promoter. The coding region of 3036 bp encoded a protein of 1011 amino acids with a deduced molecular mass of 108.7 kDa. The *PaGalA* without the signal peptide was cloned into a vector pPIC9K and was expressed successfully in *P. pastoris* as active extracellular β-galactosidase. The recombinant β-galactosidase (PaGalA) was secreted into the medium at an extremely high levels of 22 mg ml^{-1} having an activity of 9500 U ml^{-1} from high density fermentation culture, which is by far the highest yield obtained for a β-galactosidase. The purified enzyme with a high specific activity of 820 U mg^{-1} had a molecular mass of 120 kDa on SDS-PAGE. PaGalA was optimally active at pH 4.5 and a temperature of 60 °C. The recombinant β-galactosidase was able to hydrolyze lactose efficiently at pH 5.0 and 50 °C. It also possessed transglycosylation activities at high concentrations of lactose. PaGalA exhibited better lactose hydrolysis efficiency in whey than two other widely used commercial lactases. The extremely high expression levels coupled with favorable biochemical properties make this enzyme highly suitable for commercial purposes in the hydrolysis of lactose in milk or whey.

1. Introduction

β-Galactosidases (EC 3.2.1.23) belong to the class of hydrolytic enzymes which catalyze the conversion of lactose to glucose and galactose. Due to their hydrolytic property, these enzymes have long been used in the dairy industry to improve the digestibility, solubility and sweetness of lactose. They also hydrolyze the β (1 → 4) linkage of lactose [galactosyl β (1 → 4) glucose] to glucose and galactose & transfer the galactose formed from lactose cleavage onto the galactose moiety of another lactose to yield galactooligosaccharides (GOSs) which are galactose-containing oligosaccharides of the form Glu β 1–4[β Gal 1–6]*n* in which *n* = 2–5 [1]. GOSs are regarded as functional foods and are being increasingly used as prebiotics. They are known to have several health benefits as they are non-digestible and cause selective increase in the beneficial microflora of the intestine [2].

A large number of β-galactosidases have been isolated from different microbial sources such as bacteria, yeast and fungi [3–5]. However, isolation and extraction of enzyme from natural sources suffer drawbacks such as intracellular localization of the enzyme and low expression levels. Fungi are regarded as a good source for isolation of these enzymes due to their extracellular secretion and properties such as acidic pH optima and broad pH stability. Acid-stable β-galactosidases from filamentous fungi are suitable for processing acid whey, acid whey permeate or fermented dairy products. Concentrated hydrolyzed whey or whey permeates can be used as a sweetener in products such as canned fruit syrups and soft drinks. Moreover, it can help to solve environmental pollution problems caused by large amount of whey disposal from cheese manufacturing factories. Hence, β-galactosidases from several mesophilic fungi such as *Penicillium notatum* and *Aspergillus* sp. as well as from thermophilic fungi have been isolated, purified and characterized extensively [6–9]. The fungi, *Aspergillus niger* and *Aspergillus oryzae* are considered to be safe and have been widely used in the commercial production of this enzyme. However, the industrial application of β-galactosidase has been hampered by the difficulty and expense of producing the enzyme in good yield. For the commercial success, it is necessary that the cost of production should be minimal which depends on the expression level of the

Abbreviations: AOX, alcohol oxidase; CAPS, (cyclohexylamino)-1-propane sulfonic acid; CHES, 2-(cyclohexylamino)ethane sulfonic acid; DTT, dithiothreitol; GH, glycosyl hydrolase; GOS, galactooligosaccharide; *o*NP, *o*-nitrophenol; *o*NPG, *o*NP-β-D-galactopyranoside; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; *p*NP, *p*-nitrophenol; TLC, thin-layer chromatography.

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enzyme as well as the purification costs. Due to this, there is an increased interest in producing the enzyme by recombinant methods. Although many β-galactosidase genes from different micro-organisms have been cloned, there are few reports on the cloning and expression of recombinant β-galactosidase genes from fungi in heterologous hosts [10–12]. The methylotrophic yeast, *Pichia pastoris* has been successfully used for expressing several different proteins. *P. pastoris*, apart from being easy to manipulate as *Escherichia coli*, combines several advantages of eukaryotic system such as protein processing, folding and post-translational modifications. It can be used for commercial production of recombinant proteins owing to its high expression levels, efficient secretion mechanism and the potential to grow to a high cell density [13].

A new fungal strain of *Paecilomyces aerugineus* was isolated from soil samples and found to produce organic acid in our lab (unpublished data). This fungal strain has not been explored before for the production of enzymes. This is the first time, a β-galactosidase gene from *P. aerugineus* has been identified, cloned and expressed in *P. pastoris*. The recombinant β-galactosidase was purified and the biochemical and functional properties of the enzyme were studied. The recombinant β-galactosidase was found to possess attractive properties that are suitable for industrial use for lactose hydrolysis in milk or whey permeate.

2. Experimental

2.1. Strains and plasmid

P. aerugineus GY701 identified by the China General Microbiological Culture Collection (CGMCC, Beijing, China) is preserved under the registration CGMCC NO. 2733. The host, *E. coli* JM109, the plasmid vector *p*MD-18T, T4DNA ligase and restriction enzymes were purchased from TaKaRa Corporation (Japan). DNA polymerase *Pfu* was obtained from Promega (Madison, WI). *P. pastoris* strain GS115 (Invitrogen, USA) was used for heterologous expression with pPIC9K as the expression vector. Trizol reagent was purchased from Invitrogen Corporation whereas Oligotex mRNA Mini Kit was from Qiagen (Germany).

2.2. Reagents

All chemicals and reagents were of analytical grade unless otherwise stated. The substrates, *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG) and *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) were from Sigma Chemical Company (St. Louis, MO, USA). Sephacryl S-200 gel filtration matrix and SP sepharose fast flow resin used for purification of recombinant β-galactosidase were purchased from GE Life sciences (USA). The enzyme was concentrated using a 10 kDa MW cut-off ultrafiltration membrane in an amicon stirred cell (Millipore, USA). Whole milk was purchased locally. The commercial lactase from *Kluyveromyces lactis* (Lactozym 3000L) was kindly provided by Novozymes, Denmark. The β-galactosidase from *A. oryzae* was bought from Sigma Chemicals Co. High Performance Liquid Chromatography (Shimadzu, Japan) was performed on amino column (Sugar-D, 4.6 mm × 250 mm) from Waters, America.

2.3. Cloning and sequencing of a β-galactosidase gene

The genomic DNA was isolated from mycelia of *P. aerugineus* after growth in a medium induced with lactose for 3 days. Fungal mycelia were collected by centrifugation (5000 × *g*, 10 min), washed with water twice at 4 °C and ground to a powder in liquid nitrogen. The total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, USA) and mRNAs were purified using the Oligotex mRNA Midi kit.

Table 1
Primers used in this study.

Primer	Primer sequence (5′–3′)[a]
GalDF	GGAGGATTCCCAGGAtggytncarmg
GalDR	TTTCTGGTTGGTATAGTATTATTGGTCCnccrttngtdat
Gal5′GSP	CCCGTTGGTGATCTGCGCCTTTGC
Gal5′NGSP	GTGCCATTTACCCTTTGAAGCCATCCAG
Gal3′GSP	GGTGAGCGATTGATGATGTTCAGTGGTG
Gal3′NGSP	CTACTTGCTAGCCCGTCCCGGTCCTT
GalF[b]	GAATTCGCTGCTATCAGCCACAAGCTTGACG
GalR[b]	GCGGCCGCCTAGTACGCCCCCTTTCGAGACTTGTAC

[a] D = A/G/T, M = A/C, N = A/T/C/G, R = A/G, Y = C/T.
[b] Underlined sequences refer to the restriction sites incorporated into the primers.

Based on the conserved amino acid sequences (GGFPGWLQR and ITNGGPIILYQPEN) of known fungal β-galactosidases, degenerate primers, GalDF and GalDR (Table 1) were designed using the CODEHOP algorithm [14]. The genomic DNA extracted from the fungus was used as the template for PCR amplification using the degenerate primers, GalDF and GalDR. The conditions used for PCR were: a hot start at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 65–55 °C for 45 s and 72 °C for 1 min. The amplified PCR product was purified, ligated to pMD18-T vector and sequenced. The results of sequencing were deposited in the GenBank and subjected to BLAST analysis.

In order to obtain the full length cDNA sequence of the β-galactosidase gene (designated *PaGalA*), 5′ and 3′ RACE were carried out using a BD SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA). 5′ end of the cDNA was amplified using the primers Gal5′GSP and adapter primer UPM, followed by a nested PCR using nested gene specific primer Gal5′NGSP and adapter primer, NUP. Similarly, 3′ RACE was performed using the primers Gal3′GSP and UPM, followed by a nested PCR using the nested gene specific primer, Gal3′NGSP and NUP. Nucleotide and deduced amino acid sequences were analyzed with the ExPASy Proteomics tools (http://www.expasy.ch/tools/). Database homology searches of nucleotide sequences obtained were carried out using BLAST in GenBank at the NCBI. Signal peptide was analyzed by Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignalP). Search analysis of conserved domain and signature sequences was carried out using ScanProsite (http://www.expasy.ch/tools/ScanProsite). The active site was predicted using the online software Motifscan (http://muhits.isb-sib.ch/cgi-bin/motif-scan/). *N*- and *O*-glycosylation sites were predicted using NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively.

2.4. Expression of the β-galactosidase gene in P. pastoris

The *PaGalA* encoding β-galactosidase mature protein (without signal sequence) was amplified from the *P. aerugineus* cDNA using the primers, GalF and GalR to which *EcoR*1 and *Not*1 restriction sites (underlined, Table 1) were added at both ends. PCR amplification was carried out using the DNA polymerase *Pfu*. The amplified PCR product was cloned in-frame at the downstream site of the α-factor (signal peptide) in pPIC9K vector. The resultant recombinant plasmid designated as pPIC9K-PaGalA was used to transform *P. pastoris* as described below.

The recombinant plasmid, pPIC9K-PaGalA was linearized by *Sal*1 in order to integrate the β-galactosidase gene into the chromosomal DNA of *P. pastoris* at the *AOX*1 locus. Transformation into *P. pastoris* GS115 strain was done by electroporation according to the manufacturer's instructions (Invitrogen). The transformant colonies were grown on minimal dextrose plates (1.34% yeast

nitrogen base, 4×10^{-5}% biotin, 2% dextrose, and 1.5% agar) for 2–3 days until colonies appeared. The colonies with multiple copies of the integrated plasmid were selected based on their growth on high concentration (4–8 mg ml^{-1}) of G418. Positive colonies from YPD-geneticin plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar containing 4 mg ml^{-1} or 8 mg ml^{-1} G418) were selected and grown in 5 ml BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol) in a rotary shaker at 30 °C at 230 rpm till the OD_{600} of the culture was between 2 and 6. The *P. pastoris* cells were then harvested by centrifugation at 1500 × *g*, 5 min and resuspended in appropriate volume of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol) to obtain a final OD_{600} of 1.0. The cells were further grown for 5 days during which, methanol was added to the medium at a final concentration of 0.5% every 24 h to maintain the induction. The cell-free supernatant was collected by centrifugation (12,000 × *g*, 20 min) and β-galactosidase activity was assayed as described.

2.5. High cell-density fermentation of β-galactosidase

The transformed strain showing the highest β-galactosidase activity in shake-flask culture was cultivated in high cell density fermentor. Methods used are described in the *Pichia* Fermentation Guidelines (Version B, 053002, Invitrogen Inc.). All cultivations were carried out in a 5.0 l fermentor with a 1.5 l working volume at 30 °C. The fermentor was inoculated with 150 ml of a shake flask culture grown at 30 °C and 250 rpm overnight (18 h) to an optical density (OD_{600}) of more than 10 in BMGY medium. The fermentor medium was composed of FBS (Fermentation basal salt) medium with 4.35 ml of PTM_1 trace salt solution (per liter). The cultivation was maintained at constant pH with the aid of ammonium hydroxide which was provided during the cultivation if needed. The agitation rate was maintained at 950 rpm. Initially, the dissolved oxygen levels were maintained at >20% air-saturation. Upon depletion of initial glycerol, fed-batch fermentation was initiated with a fed-batch medium at a rate of 18.4 ml h^{-1} l^{-1} of initial volume. After a 6 h glycerol fed-batch phase, the methanol induction phase was initiated with a feed containing 12 ml PTM_1 trace salts (per liter) at pH 6.0. The concentration of methanol was kept stable by monitoring the dissolved oxygen (DO) content and maintaining it at greater than 20%. Samples were withdrawn at various time-intervals during the methanol induction phase and the OD_{600}, wet weight of the cells, β-galactosidase activity and the protein content were determined.

2.6. Purification of the recombinant β-galactosidase

The cell-free crude supernatant was obtained by centrifuging the fermentation culture of *P. pastoris* at 12,000 × *g* at 4 °C for 20 min. The supernatant (50 ml) was dialyzed against buffer A (50 mM acetate buffer, pH 5.0) for 16 h. The crude extract was then subjected to cation exchange chromatography by loading onto a SP sepharose column pre-equilibrated with 3 column volumes (CV) of buffer A. Following a wash with buffer A till the OD_{280} reached baseline, the protein was eluted at a flow-rate of 1 ml min^{-1} with a linear gradient from 0 to 100% buffer B (50 mM acetate buffer, pH 5.0 containing 0.5 M NaCl). The fractions obtained from the above column were pooled, concentrated by ultra filtration (10 kDa MW cut-off membrane, Millipore) and further fractionated on a Sephacryl S200 gel filtration column. The protein was eluted in 50 mM acetate buffer (pH 5.0) at a flow-rate of 0.3 ml min^{-1}. The fractions from gel filtration were pooled and analyzed as described below. All the studies were performed using the purified enzyme.

2.7. SDS-PAGE analysis and determination of protein content and molecular mass

Protein concentration was determined by the method of Lowry et al. [15] using bovine serum albumin as the standard. The purified enzyme was analyzed on a 10% SDS-PAGE [16] and the bands were stained with Coomassie brilliant blue R-250. The molecular weight was estimated by comparison with molecular mass standard proteins (Beijing Biomed Co. Ltd., Beijing, China). The sub-unit composition of the enzyme and its native molecular mass were determined on a Sephacryl S200 gel filtration column by comparison with proteins of known molecular weights (Sigma–Aldrich, USA) such as alcohol dehydrogenase (150 kDa), fetuin (68.0 kDa), albumin from chicken egg white (45.0 kDa), α-chymotrypsinogen (25.7 kDa).

2.8. Deglycosylation of recombinant β-galactosidase

10 μg of the purified recombinant β-galactosidase was treated with 250 U of endo-β-N-acetylglucosaminidase H (Endo H) for 2 h at 37 °C according to the supplier's instructions (New England Biolabs). The untreated (control) and above treated samples were analyzed by SDS-PAGE. The β-galactosidase activity of the control and deglycosylated samples was also determined as given below.

2.9. Enzyme assays

The β-galactosidase activity was determined using *o*NPG as the substrate. 25 μl of purified enzyme (diluted with 50 mM citrate buffer, pH 5.0) was added to 225 μl of reaction mixture consisting of 5 mM *o*NPG in 50 mM citrate buffer (pH 5.0). After incubation at 55 °C for 10 min, the reaction was stopped by adding 750 μl of 2 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was measured colorimetrically by measuring the absorbance at 410 nm. One unit of enzyme activity was defined as 1 μmol of *o*NP released per minute under the conditions described.

2.10. Biochemical properties of the recombinant β-galactosidase

The pH optimum of the recombinant enzyme was determined at 55 °C with 5 mM of the substrate, *o*NPG in the pH range of 3.0–11.0. The buffers used were McIlvane (pH 3.0–7.5), citrate (pH 3.0–6.0), phosphate (pH 6.0–8.0), Tris–HCl (pH 7.0–9.0), glycine–NaOH (pH 9.0–10.5), CHES (pH 8.0–10.0) and CAPS (pH 10.0–11.0). For pH stability determination, the enzyme was incubated at 55 °C for 30 min in the same buffers and the residual activity was measured under the standard conditions.

The optimum temperature for β-galactosidase activity was determined by performing the enzyme assay at different temperatures in 50 mM citrate buffer (pH 4.5). Thermo-stability studies were performed by incubating suitably diluted enzyme sample at various temperatures (30–85 °C) in 50 mM citrate (pH 4.5) for 30 min followed by cooling on ice. The residual β-galactosidase activity was determined at 55 °C in 50 mM citrate buffer (pH 4.5).

The influence of different modulators such as metal ions and other reducing and chelating agents on the enzyme activity was also tested. The purified recombinant β-galactosidase was incubated with various metal ions and other chemicals (DTT, SDS, EDTA) at a final concentration of 2 mM in citrate buffer (pH 5.0) at 55 °C for 30 min. The residual enzyme activity of the above treated samples was measured at 55 °C in citrate buffer (pH 4.5) as described earlier. All the experiments were done in triplicates.

2.11. Specificity and kinetic parameters of the recombinant β-galactosidase

The specificity of the enzyme for various nitrophenyl substrates such as *p*NPG, *p*NP-α-D-galactopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside was tested as mentioned above.

The Michaelis–Menten constant (K_m) and the maximum reaction velocity (V_{max}) for the synthetic substrates (*o*NPG and *p*NPG) and for its natural substrate (lactose) were determined by incubating the enzyme with six different concentrations of the substrate in 50 mM citrate buffer (pH 4.5) at 55 °C for 5 min. The enzyme activity was determined as given above. The non-linear regression analysis of the data was performed using the software program 'Grafit' to calculate the K_m and K_{cat} values. When lactose was used as the substrate, the reaction was carried out in the same way as described above. However, after 10 min of incubation at 55 °C, the reaction was stopped by boiling the mixture for 10 min. The reaction mixture was cooled and the release of D-glucose was determined using a glucose-oxidase kit (Beijing BHKT clinical Reagent Co. Ltd.). Glucose standards were used to make a standard plot for estimating the enzyme activity. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of D-glucose per minute under the given conditions.

2.12. Lactose hydrolysis by the recombinant β-galactosidase

The ability of β-galactosidase to hydrolyze lactose was monitored by incubating 15 U ml^{-1} of pure enzyme with 5% lactose or 30% lactose in 50 mM sodium citrate buffer (pH 5.0) at 50 °C for 6 h. Samples were withdrawn at various time intervals, boiled for 15 min to stop the reaction and analyzed by thin layer chromatography (TLC). The reaction mixtures were deionized and spotted onto a silica gel plate (Merck Silica Gel 60F 254, Germany), and developed twice in a solvent system containing butan-1-ol–ethanol–water (5:3:2, v/v). Saccharides were detected by heating in an oven after spraying the plates with a mixture of methanol: sulfuric acid (95:5, v/v).

The hydrolysis of lactose in whey by recombinant β-galactosidase was further investigated. For preparation of whey, whole milk was centrifuged at 12,000 × *g* for 30 min. After separating the fat, the skimmed milk was acidified with HCl until the pH reached 4.8 [17]. The whey was centrifuged at 12,000 × *g* for 30 min and the supernatant was used for the hydrolysis reaction. The pH of a small portion of the whey supernatant was adjusted to 6.6 [18]. The same amount (1.5 U) of different β-galactosidases (lactose units determined using glucose-estimation kit, as described before) was added to 1 ml whey and incubated at 40 °C for 8 h. Hydrolysis was performed with acid whey (pH 4.8) as well as sweet whey (pH 6.6). Aliquots were removed at various time-intervals, boiled for 5 min to deactivate the enzyme and centrifuged. The amount of residual lactose was analyzed on a Sugar-D Waters column (4.6 mm × 250 mm), operated by an HPLC system equipped with a Refractive Index Detector (RID). Elution was done at 40 °C with mobile phase of 75:25 (v/v) acetonitrile–water at a flow rate of 1.0 ml min^{-1}. The hydrolysis of lactose was evaluated by the following equations: lactose hydrolysis (%) = 100-residual lactose concentration (g l^{-1}) × 100/initial lactose concentration (g l^{-1}).

The generation of galactooligosaccharides (GOSs) by the purified recombinant β-galactosidase was studied. The time-course of GOS synthesis was monitored by incubating 20 U ml^{-1} of the purified enzyme with 30% (w/v) lactose at 50 °C in 50 mM citrate buffer (pH 5.0) for 6 h. Samples were withdrawn at various time-intervals and the amount of lactose, monosaccharides (galactose and glucose) and GOS (tri-, tetra- and penta-saccharides) was analyzed on a Sugar-D Waters column (4.6 mm × 250 mm) by High Performance Liquid Chromatography equipped (HPLC) with a Refractive Index Detector (RID).

3. Results and discussion

3.1. Cloning of a β-galactosidase gene from P. aerugineus

Using the degenerate primers, GalDF and GalDR (Table 1), a fragment of about 231-bp was generated from *P. aerugineus* genomic DNA by PCR amplification. Using the GenBank database, a blast analysis was performed which showed that the nucleotide sequence of the generated fragment shares 100% identity with that of β-galactosidase gene from *Penicillium expansum* (accession number EU543998.1) [19]. Fragments of 550 bp and 2.8 kbp were obtained by 5′ and 3′ RACE by splicing the gene sequence of 3.4 kbp which contains 3036 bp of the full ORF of β-galactosidase gene (designated *PaGalA*). ATG at the nucleotide position, 84–86 was identified as the initiation codon whereas TAG at 3116–3118 was identified as the stop codon at the 5′ terminal region. A poly (A+) tail was found at the 3′ end. The mature β-galactosidase is predicted to consist of 1011 amino acids with a signal peptide of 18 amino acids. The mature protein has a molecular weight of 108,667.6 Da and a theoretical pI of 6.6. Five potential *N*-glycosylation sites were identified in the amino acid sequence of the mature protein. The nucleotide sequence has been submitted to GenBank database and has been allocated the accession number GU721031. The deduced amino acid sequence of *PaGalA* from *P. aerugineus* was aligned with other fungal β-galactosidases. PaGalA showed significant identity to the reported sequences of β-galactosidase from *Penicillium canescens* (83%, CAA49852.2), *A. niger* (71%, CAQ53697.1), and *Neosartorya fischeri* NRRL 181 (70%, EAW22442.1), *Hypocrea jecorina* (57%, CAD70669.1), and *Bispora* sp. MEY-1 (55%, ACR78153.1).

Recombinant production of commercially important enzymes such as β-galactosidases has several advantages such as high expression levels, ease of purification and possible modifications by site-directed mutagenesis. Fungal β-galactosidases have received relatively less attention, only a few β-galactosidase genes from fungi have been cloned [10–12]. Actually, fungal β-galactosidases are useful in the dairy industry for hydrolysis of whey and whey permeate since they are fairly stable at acidic pH. Cloning of novel β-galactosidase genes from fungi and their expression in heterologous hosts are thus important from commercial point of view. In this report, we describe for the first time, cloning of a β-galactosidase gene from the acidophilic fungus, *P. aerugineus*.

The phylogenetic dendrogram based on full-length amino acid sequences of fungal β-galactosidases shows the position of β-galactosidase from *P. aerugineus* related to other fungal β-galactosidases. The result shows that PaGalA shares high homology with other GH family 35 β-galactosidases. On the basis of amino acid sequence similarity with other known fungal β-galactosidases, PaGalA can be classified into GH family 35.

β-Galactosidases have been classified into glycosyl hydrolase (GH) family 1, 2, 35 and 42 on the basis of amino acid sequence similarity [20]. Most of the β-galactosidases including the widely studied β-galactosidase from *E. coli* belong to family 2 whereas those from thermophilic, psychrophilic and halophilic organisms are included in family 42. Fungal β-galactosidases such as those from *A. oryzae*, *Bispora* sp., *Penicillium* sp., *A. niger*, *H. jecorina* have been included into GH family 35 [12,21–23]. Most of the β-galactosidases from GH family 35 share conserved amino acids "GGPXILYQPENEY" at residues 189–201 where X is V or I. The catalytic residues (E200 and E299) which act as proton donor and

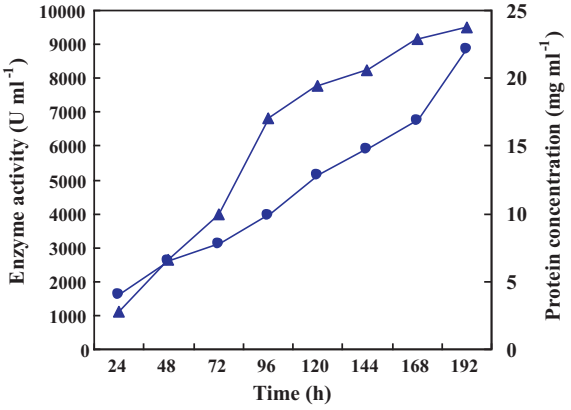

Fig. 1. Time-course of recombinant β-galactosidase expression in high cell density fermentation. Protein concentration (●) and enzyme activity (▲) were monitored at various time intervals during the fermentation. The values correspond from three different experiments.

proton acceptor during enzymatic hydrolysis of sugar molecules, respectively were found to be conserved in the β-galactosidase from *P. aerugineus* [22].

3.2. Expression of PaGalA in P. pastoris

The *PaGalA* without the native signal sequence was inserted into pPIC9K to generate an in-frame fusion between the yeast α-mating factor and the mature form of the β-galactosidase. The resultant recombinant plasmid was transformed into *P. pastoris*, and the transformants that contained multiple copies of the integrated gene were selected based on their growth on high concentrations of geneticin (G418). The geneticin resistant colonies were screened for expression of β-galactosidase. The highest β-galactosidase activity obtained after optimization of the growth conditions was 218 U ml^{-1} after growing for 5 days in the shake-flask culture. The colony showing highest β-galactosidase activity was grown in a 5-l high cell density cultivation. As shown in Fig. 1, the β-galactosidase activity showed a continuous rise with time, reaching to a maximum of 9500 ± 391 U ml^{-1} after 192 h. The OD_{600} reached to a maximum of 663 ± 18 with a wet cell mass of 411 ± 9 g l^{-1}. A very high amount of protein (22.1 mg ml^{-1}) which comprises majorly of β-galactosidase was secreted into the medium (Fig. 1).

We have made use of the *P. pastoris* expression vector that allows increasing the number of copies of the gene of interest in *P. pastoris* leading to higher expression level. The expression of the foreign gene is driven by the strong promoter, *AOX*1 and is tightly regulated and induced by methanol to very high levels. Since the yeast *P. pastoris* is easily adaptable for large scale fermentation, we have carried out high cell density fermentation of the *P. pastoris* transformant showing the highest expression level in a 5-l fermentor. A very high amount of protein (22.1 mg ml^{-1}) was secreted into the fermentation media. The β-galactosidase activity reached to a maximum of 9500 U ml^{-1} which is by-far the highest expression level reported. The expression levels of β-galactosidases in *P. pastoris* were reported to be much lower than those obtained in this study. For instance, a β-galactosidase gene *bgalA* from the meso-acidophilic fungus, *Bispora* sp. MEY-1 expressed in *P. pastoris* yielded only 0.08 U ml^{-1} of β-galactosidase after 48 h of induction [12]. Similarly, a β-galactosidase from *Alicylcobacillus acidocaldarius* yielded 90 mg ml^{-1} enzyme having an activity of 20 U ml^{-1} [24]. Various bacterial and fungal β-galactosidases expressed in other systems too, showed relatively lower expression levels. The β-galactosidase gene from *Pyrococcus woesei* was cloned and expressed in *E. coli* at a level of 0.02 mg ml^{-1} culture [25] whereas β-galactosidases from *Lactobacillus* were over-expressed in the same host strain at an expression level of 0.1 mg ml^{-1} amounting to 55% of the total host cell proteins and having an enzyme activity of 23 U ml^{-1} [26]. Cloning and expression of high copy number of LacA in *A. oryzae* resulted in an enzyme activity of ~1000 U ml^{-1} when induced with maltose and cultured for 200 h [21].

Table 2
A summary of purification of β-galactosidase from the cell-free fermentation culture of *Pichia pastoris*.

Purification step	Total activity (U)[a]	Total protein (mg)[b]	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude lysate	235,000	520	452	100	1.0
Cation-exchange	199,000	336	593	84.8	1.3
Sephacryl S-200	128,000	156	820	54.4	1.8

[a] Activity was measured in 50 mM citrate buffer (pH 5.0) at 55 °C for 10 min using *o*NPG as substrate.
[b] The protein was measured by the method of Lowry at al. [15], using BSA as the standard.

3.3. Purification of the recombinant β-galactosidase

A major advantage of expressing heterologous proteins as secreted proteins in *P. pastoris* is that it secretes very low levels of host proteins thereby facilitating ease of purification. Our results also indicate that a major proportion of the protein present in the culture supernatant comprised of the recombinant β-galactosidase with very few other host proteins which simplified the purification process. The recombinant β-galactosidase (PaGalA) was purified to electrophoretic homogeneity from 50 ml of the cell-free fermentation culture (Table 2). Following ion exchange and gel filtration chromatography, the specific activity of the β-galactosidase increased from 452 U mg^{-1} to 820 U mg^{-1} resulting in a 1.8 fold of purification. An overall yield of 54.4% was achieved. Considering the high expression levels and the amount of purified enzyme obtained, we believe it is a highly cost-effective process for production of β-galactosidase for commercial applications.

PaGalA migrated on SDS-PAGE (Fig. 2A) as a single band of around 120 kDa which is slightly higher than the predicted molecular mass of 109 kDa. Many β-galactosidases from family 35 are known to possess multiple *N*-glycosylation sites [12,22,27]. The recombinant PaGalA contains five potential *N*-glycosylation sites. Deglycosylation of the purified enzyme yielded a band on SDS-PAGE at around 110 kDa (Fig. 2B), indicating that the enzyme is indeed glycosylated. In order to check whether the high β-galactosidase activity is due to glycosylation, the enzyme activity of the glycosylated and deglycosylated enzymes was compared. The deglycosylated enzyme showed an activity of 555 ± 8.3 U ml^{-1} as compared to glycosylated enzyme which showed an activity of 679 ± 4.6 U ml^{-1}. The deglycosylated enzyme was thus shown to retain most of its activity (81.7%) indicating that its activity is significantly affected by glycosylation. The native molecular mass of PaGalA as determined on gel filtration column was found to be 118 kDa (data not shown). The recombinant β-galactosidase was thus found to be a monomer which is consistent with the finding that most of the fungal β-galactosidases belonging to family 35 are high molecular weight monomeric enzymes [23–28].

3.4. Biochemical properties of recombinant β-galactosidase

The biochemical properties of the purified PaGalA were analyzed. The enzyme showed maximum activity at pH 4.5 and displayed stability in the pH range of 4.5–8.5 at 55 °C for 30 min (Fig. 3A and B). The recombinant β-galactosidase showed an optimum activity at 60 °C (Fig. 4A). More than 90% of the activity was

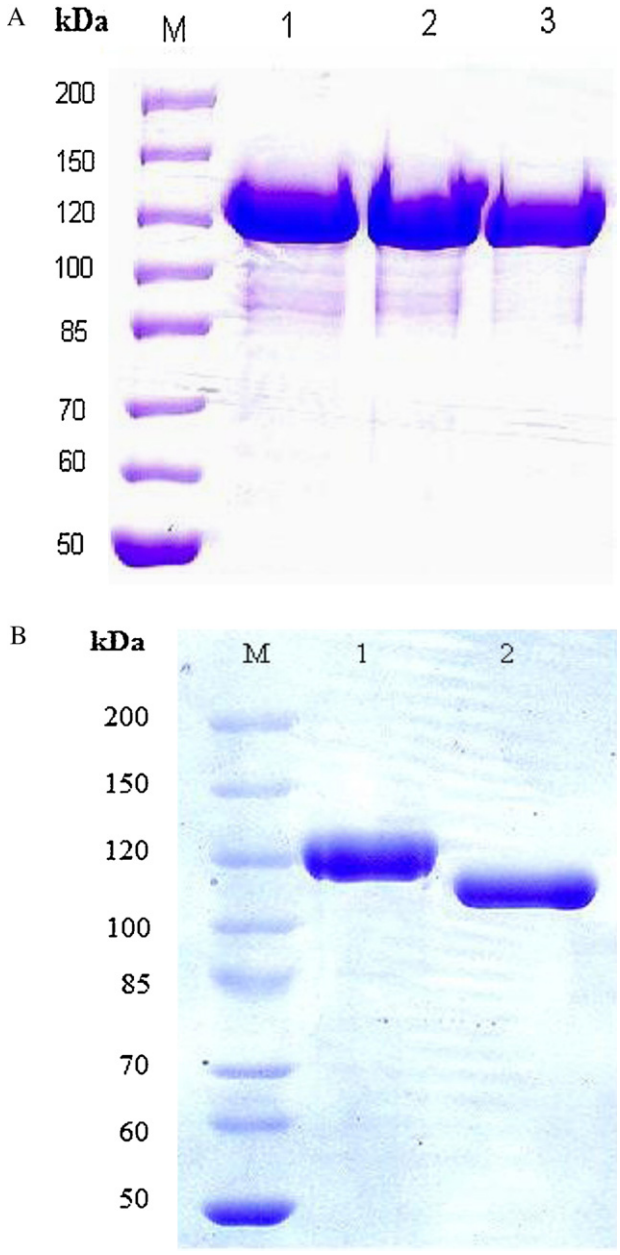

Fig. 2. Analysis of (A) purity of the recombinant β-galactosidase (PaGalA) from *P. aerugineus* expressed in *Pichia pastoris* and (B) deglycosylation by EndoH on 10% SDS-PAGE. (A) Lane M, molecular weight standard; lane 1, crude secreted protein; lane 2, purified by cation-exchange chromatography; lane 3, purified by Sephacryl S-200 gel filtration chromatography. (B) Lane M, molecular weight standard; lane 1, purified β-galactosidase; lane 2, deglycosylated β-galactosidase. The gel was stained with Coomassie brilliant blue R-250.

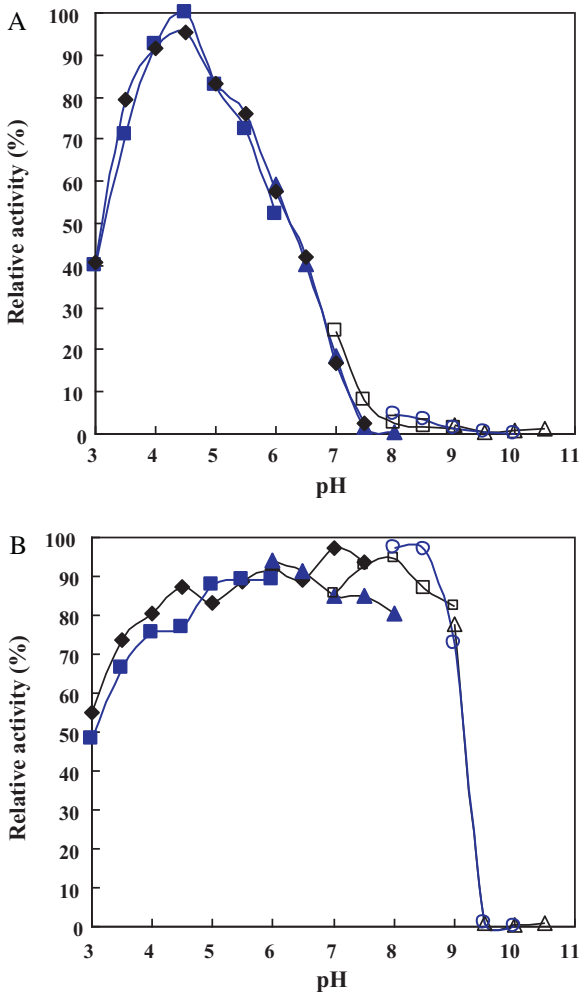

Fig. 3. Determination of optimal pH (A) and pH stability (B) of PaGalA. The β-galactosidase activity assay was performed with 5 mM *o*NPG at 55 °C in 50 mM of different buffers (McIlvane (♦), pH 3.0–7.5; citrate (■), pH 3.0–6.0; phosphate (▲), pH 6.0–8.0; Tris–HCl (□), pH 7.0–9.0; glycine–NaOH (△), pH 9.0–10.5; CHES (○), pH 8.0–10.0). To determine pH stability, the sample was incubated for 30 min at 55 °C over various pH ranges and the remaining activity was measured (in citrate buffer pH 4.5 at 55 °C).

retained up to a temperature of 55 °C for 30 min (Fig. 4B). The enzyme activity was not affected by most of the metal ions tested as well as the metal chelator, EDTA. The enzyme showed marginal activation in presence of Fe^{3+} (121%). The metal ions, Hg^{2+} and Ag^{+}, on the other hand completely inhibited the enzyme activity (1.1% and 17.6%, respectively). SDS inhibited the enzyme activity significantly (70.7%). The reducing agent, DTT, however did not have any effect on the enzyme activity (101%) indicating that disulfide bonds are not crucial to the catalytic mechanism of the enzyme.

The substrate specificity of β-galactosidase for various artificial substrates was determined. The enzyme showed 1.5 fold higher activity for the substrate, *o*NPG (820 U mg^{-1}) as compared to *p*NPG (554 U mg^{-1}). PaGalA did not act on other substrates tested such as *p*NP-α-D-galactopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside. The Michaelis–Menten constants of β-galactosidase for *p*NPG, *o*NPG and lactose were determined. The kinetic constants, K_m and V_{max} for the artificial substrates *p*NPG and *o*NPG were 0.51 ± 0.01 mM and 0.65 ± 0.02 mM; and 704 ± 8.1 μmol min^{-1} mg^{-1} and 944 ± 20.1 μmol min^{-1} mg^{-1},

Table 3
Determination of kinetic parameters of the recombinant β-galactosidase.[a]

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
*p*NPG	704 ± 8.1	0.51 ± 0.01	11.7	23.0
*o*NPG	944 ± 20.1	0.65 ± 0.02	15.7	24.2
Lactose	315 ± 7.2	32.6 ± 1.3	5.3	0.2

[a] Enzymatic reactions were carried out for 5 min at 55 °C in 50 mM citrate buffer (pH 4.5).

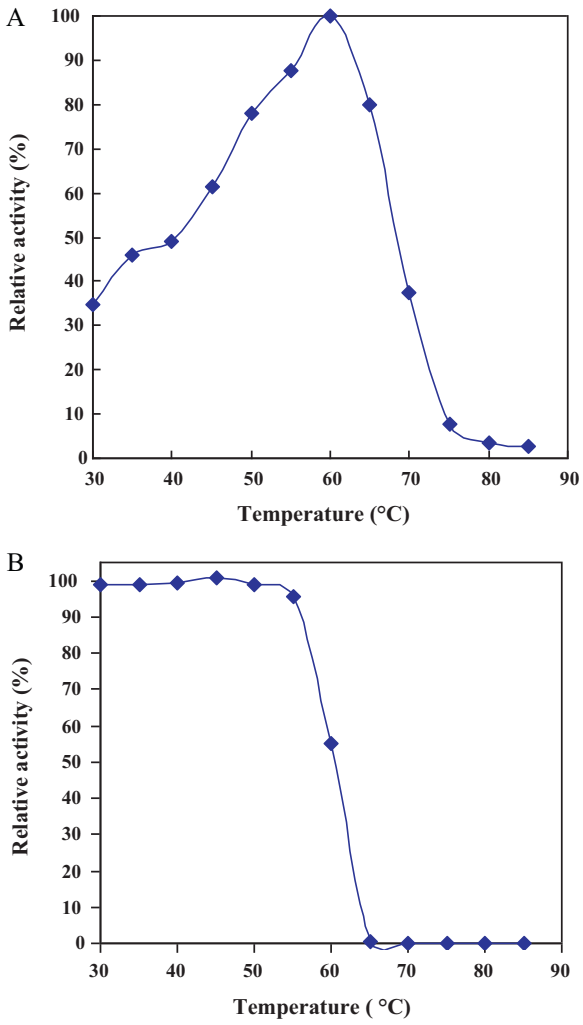

Fig. 4. Determination of optimal temperature (A) and thermal stability (B) of PaGalA. The enzymatic reaction was carried out at different temperatures in 50 mM citrate buffer (pH 4.5). For determination of thermo-stability, the residual activity of the treated enzyme was measured in 50 mM citrate buffer, pH 4.5 at 55 °C after 30 min incubation at different temperatures in the same buffer.

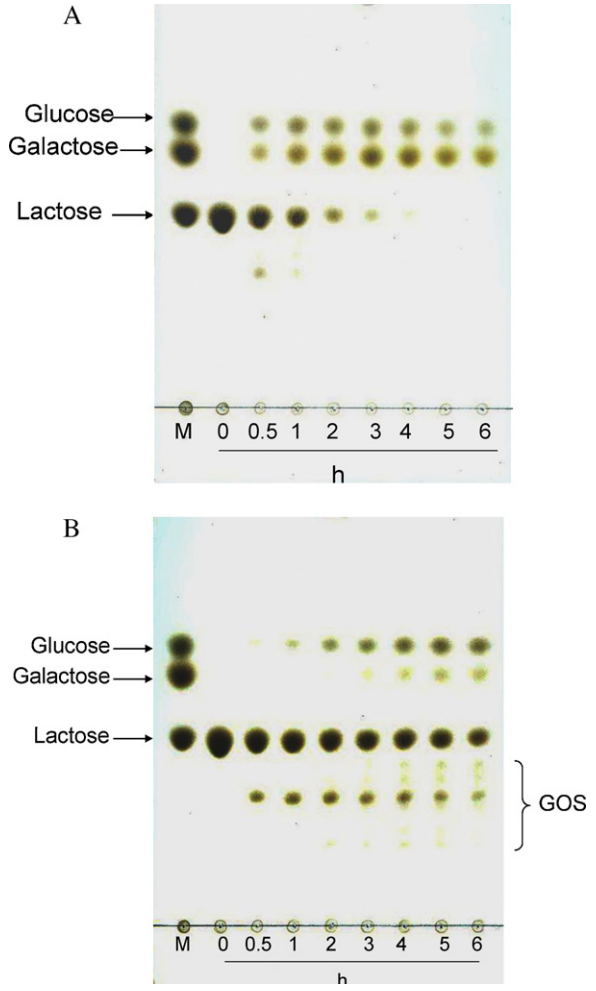

Fig. 5. Analysis of hydrolytic property of PaGalA by TLC. Hydrolysis of 5% lactose (A) and 30% lactose (B) was performed using 15 U ml^{-1} of the purified enzyme at pH 5.0 and 50 °C for 6 h. Lane M: a mixture of galactose, lactose and glucose; samples were analyzed after 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 8 h time-intervals.

respectively (Table 3). The K_m and V_{max} values for the natural substrate, lactose were observed to be 32.6 ± 1.3 mM and 315 ± 7.2 μmol min^{-1} mg^{-1}, respectively.

Fungal β-galactosidases are generally found to have pH optima in the acidic range of 2.5–5.4 making them suitable for hydrolysis of acid whey. The pH optimum of PaGalA was found to be at pH 4.5 (Fig. 3A) which is comparable with that reported for other fungal β-galactosidases [9,23,29]. Since the enzyme was stable over a broad pH range of 4.5–8.5 (Fig. 3B), it is also useful in the dairy industry for hydrolysis of lactose. PaGalA displayed a temperature optimum of 60 °C (Fig. 4A) which is higher than that reported from the fungi, *A. oryzae* (46 °C), *Penicillium chrysogenum* (50 °C) and *Beauveria bassiana* (30 °C) [28–30]. PaGalA showed high substrate specificity with a preference for *o*-linked nitrophenyl compound, *o*NPG followed by *p*NPG. The enzyme also displayed higher affinity towards *p*NPG and *o*NPG as compared to lactose (Table 3). This behavior has been observed in general for most of the β-galactosidases including those of fungal origin [6,31]. The K_m values we have obtained for the synthetic substrates are in accordance with those observed for other fungal β-galactosidases. A β-galactosidase from *Aspergillus fonsecaeus* exhibited K_m values of 1.78 mM and 61.3 mM for the substrates, *o*NPG and lactose, respectively [32]. The recombinant enzyme in the present study also showed better affinity towards lactose in comparison to few other fungal β-galactosidases (the K_m value of β-galactosidase from *A. niger* ranged from 85 to 125 mM whereas that of β-galactosidase from *Rhizomucor* sp. was 50 mM) [9,33].

3.5. Lactose hydrolysis by the recombinant β-galactosidase

The recombinant β-galactosidase was able to hydrolyze 5% lactose completely to its products, galactose and glucose at 50 °C and pH 5.0 (Fig. 5A). Complete lactose hydrolysis was achieved by 4 h with 15 U ml^{-1} of purified β-galactosidase. This property along with its stability in a broad pH range makes the enzyme suitable for hydrolysis of lactose in milk or whey permeate.

The practical application of recombinant purified PaGalA in the food industry was further evaluated by studying its efficiency in lactose hydrolysis present in whey. Commercial lactases such as those from *Kluyveromyces fragilis* (Lactozym) and *A. oryzae* are widely used for lactose hydrolysis in milk and whey. The fungal lactases (from *Aspergillus*) have acid pH optima (pH 2.5–4.5) which makes them especially suitable for hydrolysis of lactose in acid whey [34]. The yeast enzymes such as that from *K. fragilis* (Lactozym), on the other hand have neutral pH optima (pH 6–7) making them suitable for the hydrolysis of lactose in milk or sweet whey [35]. Hence, in the present investigation, hydrolysis of lactose in sweet whey (pH

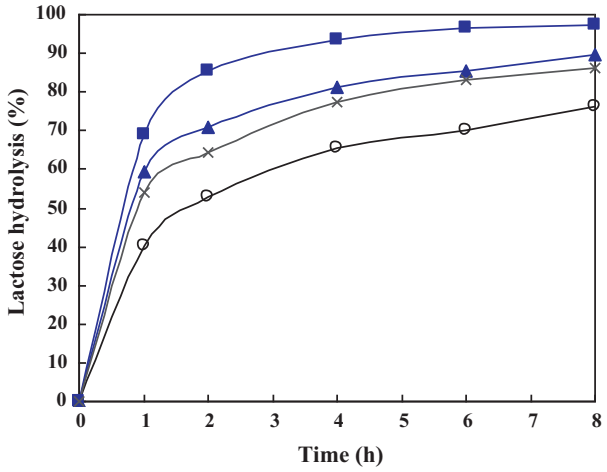

Fig. 6. Hydrolysis of lactose in whey by different β-galactosidases. Comparison of lactose hydrolysis with purified PaGalA (■) and β-galactosidase from *Aspergillus oryzae* (○) in acid whey (pH 4.8) as well as with PaGalA (▲) and Lactozym (×) in sweet whey (pH 6.6) was done at 40 °C. Samples at various time-intervals were analyzed by HPLC for the amount of residual lactose.

6.6) and acid whey (pH 4.8) by PaGalA, Lactozym and *A. oryzae* β-galactosidase was evaluated. As shown in Fig. 6, 1.5 U ml^{-1} of the β-galactosidase from *A. oryzae* was able to hydrolyze 76.2% lactose in acid whey (pH 4.8) at 40 °C. In comparison, PaGalA could almost completely hydrolyze (97.4% hydrolysis) lactose in 8 h under the same conditions. Thus, PaGalA was found to be better than the commercial fungal enzyme in hydrolyzing lactose present in acid whey. PaGalA also exhibited fairly good hydrolysis in sweet whey resulting in 89.6% lactose hydrolysis after 8 h. The lactose hydrolysis efficiency of PaGalA in sweet whey (89.6%) was found to be slightly superior to the commercial yeast enzyme (Lactozym) which could hydrolyze 86.3% lactose after 8 h. Thus, PaGalA displayed better lactose hydrolytic capability over a wide pH range than the commercially used β-galactosidases.

PaGalA also possessed glycosyl transferase activity at higher concentrations of lactose (Fig. 5B). β-Galactosidases isolated from various fungi as well as a few recombinant fungal β-galactosidases have been employed either as free enzymes or as immobilized onto a support for production of galacto-oligosaccharides. We monitored the rate of GOS synthesis up to 6 h at 50 °C in the presence of 30% lactose and our data suggests that a maximum yield of 19.7% GOS was synthesized in 4 h.

4. Conclusions

A β-galactosidase gene (*PaGalA*) from the acidophilic fungus, *P. aerugineus* was cloned and expressed at high levels in *P. pastoris*. The β-galactosidase belongs to GH family 35 and shares high degree of sequence similarity with other fungal β-galactosidases. The purified PaGalA displayed optimum activity at acidic pH and stability over a broad pH range. Moreover, PaGalA was able to hydrolyze lactose present in acid-whey and sweet-whey. Its lactose hydrolysis ability was found to be superior to two commercially available β-galactosidases suggesting its potential use in the hydrolysis of lactose in whey and milk.

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