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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