Cloning of a β-Glucosidase Gene (*BGL1*) from Traditional Starter Yeast *Saccharomycopsis fibuligera* BMQ 908 and Expression in *Pichia pastoris*

Le Thuy Mai and Vu Nguyen Thanh

***Abstract*—**β-Glucosidase is an important enzyme for production of ethanol from lignocellulose. With hydrolytic activity on cellooligosaccharides, especially cellobiose, β-glucosidase removes product inhibitory effect on cellulases and forms fermentable sugars. In this study, β-glucosidase encoding gene (*BGL1*) from traditional starter yeast *Saccharomycosis fibuligera* BMQ908 was cloned and expressed in *Pichia pastoris*. *BGL1* of *S. fibuligera* BMQ 908 shared 98% nucleotide homology with the closest GenBank sequence (M22475) but identity in amino-acid sequences of catalytic domains. Recombinant plasmid pPICZαA/*BGL1* containing the sequence encoding *BGL1* mature protein and α-factor secretion signal was constructed and transformed into methylotrophic yeast *P. pastoris* by electroporation. The recombinant strain produced single extracellular protein with molecular weight of 120 kDa and cellobiase activity of 60 IU/ml. The optimum pH of the recombinant β-glucosidase was 5.0 and the optimum temperature was 50°C.

***Keywords*—**β-Glucosidase, *Pichia pastoris, Saccharomycopsis fibuligera,* recombinant enzyme.

I. Introduction

ENZYME β-glucosidase (EC 3.2.1.21) catalyses the hydrolysis of terminal, non-reducing β-D-glucose residues of β-D-glucosides with the release of β-D-glucose. The enzyme is wide spread in nature and plays various vital functions. In bacteria and fungi, β-glucosidase is mainly associated with cellulose degrading enzyme systems [3]. In insect and plants β-glucosidase was found to play role in the release of cyanides from cyano-glucoside precursors as a part of the host defense mechanisms [5]. The functions of the enzyme in plants also include the hydrolysis of phytohormone precursors, pigment metabolisms and biomass conversion [3].

β-Glucosidase has found wide range of applications in food industry and agriculture. By catalyzing the hydrolysis of naringin to prunin, β-glucosidase is used for the removal of bitterness from citrus fruit juices [12]. Thermostable β-glucosidase from *Paecilomyces thermophila* was used for conversion of isoflavone glycosides in soybean to increase the isoflavone aglycones in soy products [16]. In wine industry, both commercial preparations of β-glucosidase and selected yeasts, malolactic bacteria with glycosidase activities are employed for hydrolysis of glycoconjugated aromatic precursors in order to enhance the organoleptic quality of wine [11]. In diary industry, the enzyme is utilized for processing of lactose-containing products [15]. β-Glucosidase supplementation was found beneficial for single-stomached animals such as pigs and chickens [3].

Recently, production of fuel ethanol from lignocellulose has received much attention as an alternative energy source. Hydrolysis of cellulose by both endo- and exo-glucanases was found to be inhibited by cellobiose, the reaction product. When β-glucosidase is added, the enzyme converts cellobiose into fermentable glucose and allows efficient and complete saccharification of cellulose. It is projected that the demand for β-glucosidase will have a sharp increase when lignocellulosic ethanol technology enters commercialization phase.

Fungi are considered as promising genetic source for commercial production of recombinant β-glucosidase. β-Glucosidase from *Aspergillus niger* could be expressed in both *Saccharomyces cerevisiae* and *Pichia pastoris*. Comparing with *S. cerevisiae*, the level of expression and specific activity of the recombinant enzyme in *P. pastoris* was in an order of magnitude higher [4]. β-Glucosidases from *A. kawachii, Histoplasma capsulatum, Humicola grisea, Phanerochaete chrysosporium, Talaromyces emersonii, Trichoderma* spp. have been cloned and expressed in bacterial (*E. coli*), yeast (*S. cerevisiae, P. pastoris*), and fungal (*A. oryzae, T. reesei*) hosts [3]. Two β-glucosidase encoding gens (*BGL1* and *BGL2*) from *S. fibuligera* have been cloned and expressed in *S. cerevisiae*. The two β-glucosidases showed the same enzymatic characteristics, such as thermodenaturation kinetics and dependencies on pH and temperature, but quite different substrate specificities. BGL1 hydrolyzed cellobiose efficiently, while BGL2 did not [10]. In this study, *BGL1* from a Vietnam traditional starter strain *S. fibuligera* BMQ 908 was cloned and expressed in methylotrophic yeast *P. pastoris*. Our

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objective was to obtain recombinant β-glucosidase for application in fuel ethanol production.

II. Materials and Methods

A. Microbial strains and plasmid

For cloning of *BGL1*, 6 *S. fibuligera* strains isolated from Vietnam traditional alcohol fermentation starters (*banh men*) were utilized. These strains were previously identified based on morphological appearance and stored at the Culture Collection of Food Industry Microorganisms (FIRI, Vietnam) since 2005. *S. fibuligera* was considered as main amylolytic microorganism in *banh men* [14]. Vector pPICZαA (Invitrogen) was used for both cloning of *BGL1* into *E. coli* DH5α and for expression in *P. pastoris* strains (KM71H and X33, Invitrogen).

B. DNA extraction from yeast

For DNA extraction, yeast was grown on YMA (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar) for 3 days at 25°C. One loop-full of cells was transferred to a micro-tube containing 1 ml of 2×SSC and heated at 99°C for 10 min. Cells were collected and washed once with 1 ml of sterile de-ionized water by centrifugation. To the cell pellet, *ca*. 75 µl of glass beads (0.2-0.5 mm in diameter), 75 µl of phenol-chloroform, and 100 µl of de-ionized water were added. The tube was shaken at 1400 rpm for 10 min and then centrifuged at 13000 rpm for 10 min. The upper layer was transferred to a new micro-tube and used directly as template for PCR.

C. Yeast identification

Strains were compared by PCR fingerprinting using micro-satellite primer $(GTG)_5$, which allowed differentiation of strains at subspecies level [9]. The PCR condition was as follows: an initial denaturation step of 95° C for 2 min followed by 35 cycles of 94°C for 40 sec, 55°C for 40 sec and 72°C for 2 min. The final extension step was 72°C for 10 min. Strain BMQ 908 was identified to species level by sequencing D1/D2 domains of the 26S rDNA and comparing with sequences of the type strains deposited at GenBank as described previously [8].

D. PCR amplification of BGL1

Primers S-BGL1-EcoRI-F (5'-GCTGAATTCGTCCCAATTCAAAACTATACC-3') and S-BGL1-XbaI-R (5'-TGTTCTAGAATAGTAAACAGGACAGATGT-3') were designed based on GenBank sequence M22475 for amplification of 2577 bp encoding mature BGL1 (nucleotide 328 to 2904). *Eco*RI and *Xba*I restriction sites were introduced into forward and reverse primer respectively in order to make an advantage of ligation with pPICZαA. At the 5' end of each primer, 3 nucleotide overhangs were added to facilitate digestion with restriction enzymes. The secretion of recombinant protein was insured by *S. cerevisiae* α-mating factor leading sequence included in pPICZαA. Amplification of *BGL1* was carried out with 0.5 µl of genomic DNA, 10 pmol of each primer in 50 µl of DreamTaq™ PCR mixture (Fermentas). PCR was performed in GeneAmp® PCR System 9700 Applied Biosystems (USA) using a thermo-program consisting of preheating step at 94°C for 3 min followed by 35 cycles of 40 sec at 94°C, 40 sec at 52°C, and 2 min at 72°C. PCR product was purified using QIAEX II agarose gel extraction kit (Qiagen).

E. Cloning of BGL1 into pPICZαA

Purified PCR product and plasmid pPICZαA were double digested using restriction enzymes *Eco*RI and *Xba*I from Fermentas. After purification, *ca*. 30 ng of plasmid DNA and 15 ng of the insert (molar ratio 1:1) in a total volume of 10 µl were ligated using T4 DNA ligase (Fermentas) at 22°C for 3 h. Recombinant vector pPICZαA/*BGL1* was transformed into *E. coli* DH5α by heat-shock method [13] and plated on LB low salt agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.6% agar, pH 7.0) containing 25 mg/l zeocin (Invitrogen). The presence of recombinant plasmid in growing colonies was examined by PCR using α-factor and 3'AOX1 primers (Invitrogen).

F. Transformation into P. pastoris

E. coli DH5α carrying pPICZαA/*BGL1* was grown in 50 ml Erlenmeyer flask containing 10 ml of LB low salt-zeocin at 37°C with shaking at 150 rpm. After 24 h, cells were collected and plasmids were extracted using NucleoSpin® Plasmid extraction kit (Macherey-Nagel, Germany). pPICZαA/*BGL1* was digested with *Pme*I and then purified with Wizard® SV (Promega). Approximate 7 µg of linearized plasmid was transformed into *P. pastoris* strains X33 and KM71H using MicroPulser electroporator (Bio-Rad). Cell suspensions were spread on YPDS agar plate (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing 100 mg/l zeocin and incubated at 28°C for 3 days. Formed colonies were purified on YPD-zeocin (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 100 mg/l zeocin) and the integration of *BGL1* was verified by PCR using cloning primers. Trasformants with likelihood of having multiple inserts were screened using YPD media with elevated concentration of zeocin (1000 - 2000 mg/l zeocin).

G. Expression of recombinant β-glucosidase

P. pastoris containing pPICZαA/*BGL1* was cultured at 28°C for 24 h in BMG (100 mM potassium phosphate, pH 6.0, 1.34% YNB (Difco), 40 ppm biotin, 1% glycerol) media. The cells were harvested, washed twice with sterile distilled water by centrifugation at 6000 *g* and re-suspended in BMM (100 mM potassium phosphate, pH 6.0, 1.34% YNB (Difco), 40 ppm biotin, 0.5% methanol). The yeast was incubated at 28°C with shaking at 200 rpm and methanol was added to final concentration of 0.5% every 24 h to maintain induction. The expression was carried out for 7 days and samples were taken each day for analysis.

H.Assay of β-glucosidase

β-Glucosidase activity was evaluated by the action of the enzyme on cellobiose with formation of glucose. Hydrolysis was carried out at 50°C in 0.1 M sodium acetate buffer, pH 5.0 with an initial cellobiose concentration of 15 mM. After 30 min, the reaction was stopped by heating at 100°C in a water bath for 10 min and cooled down with tap water. Glucose released was quantified by enzymatic method based on glucooxidase-peroxidase (POD-GOD) system. Briefly, 0.5 ml of sample diluted in 0.1 M sodium acetate buffer, pH 5.0 was added to 1 ml of POD-GOD mixture containing 0.45 mM 3,3',5'5'-tetramethylbenzidine (MP Biomedicals), 0.75 U/ml glucooxidase (Roche), 0.75 U/ml horse radish peroxidase (MP Biomedicals) in the same buffer. The mixture was incubated at 25°C for 20 min, and then 0.5 ml of 0.8 M HCl was added. Optical density was measured at 450 nm using UV-1650PC spectrophotometer (Shimadzu). Glucose concentration was calculated using a standard curve. One unit of β-glucosidase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of cellobiose substrate per minute.

III. Results and Discussion

A. Species identification and screening for BGL1

In order to create a strain of *P. pastoris* expressing extracellular β-glucosidase, 6 strains previously assigned to *S. fibuligera* based on morphological properties were chosen as potential donors (Fig. 1). PCR fingerprints generated by primer $(GTG)_5$ for all 6 strains were identical (data not shown) and this indicated the conspecificity of the strains. 26S rDNA D1/D2 sequence of the representative strain (BMQ 908) was identical to GenBank sequence U40088 of NRRL Y-2388, the type strain of *S. fibuligera*. PCR screening using cloning primers S-BGL1-EcoRI-F and S-BGL1-XbaI-R designed specifically for *BGL1* yielded in 5 positives. The mobility of the bands fitted well with the calculated size of 2594 bp including additional restriction sites and overhangs (Fig. 2). Thus, phenotypic identification of *S. fibuligera* was supported by DNA fingerprinting and sequencing data. The lack of PCR band in one *S. fibuligera* strain indicates that *BGL1* may be missing or has significant variation in some specific case. In subsequent study, *S. fibuligera* BMQ 908 was chosen as donor strain.

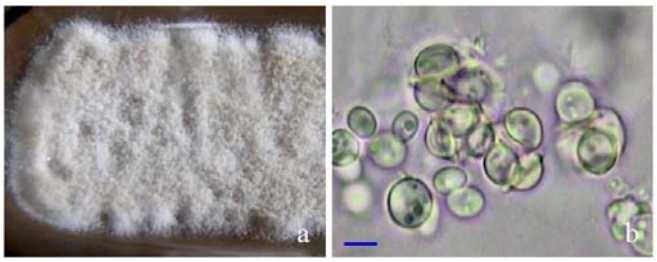

Fig. 1. Phenotypic characteristics of traditional starter yeast *S. fibuligera* BMQ 908. (a) - Colony grown on Malt-Glucose agar; (b) - hat shaped ascospores. Bar represents 4 μm.

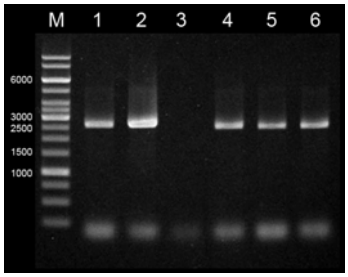

Fig. 2. PCR screening for *BGL1* of *S. fibuligera* strains using cloning primers S-BGL1-EcoRI-F and S-BGL1-XbaI-R. M - GeneRuler™ 1 kb DNA Ladder (Fermentas); 1 - strain BMQ 545.2; 2 - BMQ 908; 3 - BMQ 517; 4 - BMQ 535; 5 - BMQ 544; 6 - BMQ 546.

B. Cloning BGL1 of S. fibuligera BMQ 908 and expression in P. pastoris

β-Glucosidase of *S. fibuligera* is an extracellular enzyme. As in other yeasts and fungi, excretion of the enzyme is responsible by a leading signal peptide which is cleaved off during insertion into the endoplasmic reticulum membrane by signal peptidase. Although some leading sequences may function cross species, the process is rather specific. In order to ensure excretion possibility of the engineered enzyme we adopted the α-factor sequence from pPICZαA. Thus, only the sequence encoding mature BGL1, corresponding 2577 bp from nucleotide 328 to nucleotide 2904 of *S. fibuligera BGL1* was cloned (Fig. 3). To assist purification and detection, c-myc epitope and poly-histidine tag from pPICZαA were also utilized. Under the control of AOX1 promoter, recombinant gene could be expressed efficiently.

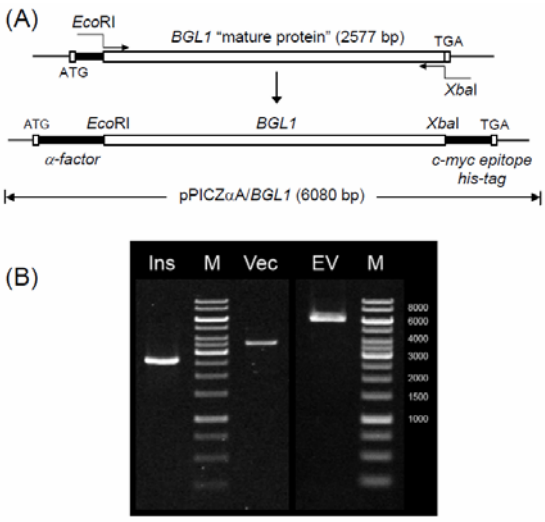

Fig. 3. Cloning scheme (A) and construction of the expression vector pPICZαA/*BGL1* (B). Ins - *BGL1* PCR insert digested with *Eco*RI and *Xba*I; Vec – vector pPICZαA opened with *Eco*RI and *Xba*I; EV - Expression vector pPICZαA/*BGL1* linearized with *Pme*I.

After digestion with *Eco*RI and *Xba*I, purified *BGL1* fragment was ligated to pPICZαA previously opened with the same restriction enzymes and transformed into *E. coli* DH5α.

BGL1 insert and flanking regions were sequenced. *BGL1* from *S. fibuligera* BMQ 908 shared 98.0% similarity with sequences M22475 of *S. fibuligera* HUT7212 and FJ028723 of *S. fibuligera* BCRC 20455. The latter two sequences were identical. Amino acid sequences deduced from DNA sequences were slightly more conserved and shared 98.6% similarity. Amino acid substitutions occurred outside the conserved catalytic domain of *BGL1* which comprised of 7 amino acids (VVSDWGA) [2], [6], [7].

Recombinant vector pPICZαA/*BGL1* of 6080 bp was linearized within 5′ AOX1 region by restriction enzyme *Pme*I and transformed to *P. pastoris* (Fig. 3). The vector is expected to integrate into the host 5′ AOX1 region by gene insertion. Both methanol utilization phenotypes X-33 (Mut+, able to utilize methanol) and KM71H (MutS, slow methanol utilization) were used as hosts for β-glucosidase expression.

Both *P. pastoris* KM71H and X33 could effectively express *BGL1*. In total, 158 clones from *P. pastoris* KM71H and 92 clones from *P. pastoris* X33 were obtained. In average, clones of *P. pastoris* X33 showed 70% higher extracellular β-glucosidase (cellobiase) activity than KM71H. Integration of *BGL1* not always guaranteed expression since we have found several clones containing *BGL1* but displayed no β-glucosidase activity. Extracellular protein fraction of recombinant yeast contained one strong band of 120 kDa according to SDS-PAGE while none was detected with "wild-type" of *P. pastoris* (Fig. 4). Some degrees of glycosylation occurred with the expressed BGL1 since with 876 amino acid residues, unglycosylated BGL1 would have molecular weight of 103 kDa. The deduced amino acid sequence encoded by *BGL1* of *S. fibuligera* BMQ 908 contains 16 potential N-glycosylation sites. BGL1 expressed in *P. pastoris* was significantly less glycosylated than in *S. cerevisiae*. BGL1 of the latter showed molecular weight of 220 kDa [10].

C. Enzymatic properties of recombinant β-glucosidase

The optimal temperature, thermo-stability, optimal pH, and pH stability of recombinant BGLI were determined using cellobiose as substrate. Properties of recombinant β-glucosidase from both *P. pastoris* KM71H and X33 were similar. The enzyme hydrolyzed cellobiose efficiently at the temperature range from 40°C to 60°C with optimum at 50°C (Fig. 5A). For thermo-stability testing, the enzyme was incubated for 1 hour at different temperature and then remaining activity was determined. Recombinant enzyme retained 50% of activity for 1 hour at 60°C. Beyond that, the activity rapidly decreased and completely lost at 70°C (Fig. 5C). The optimum pH for recombinant BGL1 was 5.0 (Fig. 5B). The enzyme retained about 80% activity at the pH range from 4.0 to 6.0 after holding for 1 h at 4°C (Fig. 5D). Thus, BGL1 expressed in *P. pastoris* was slightly more thermo-stable than in *S. cerevisiae*. The latter completely lost activity after incubation at 60°C for 30 min. Temperature and pH optimums of BGL1 expressed in both systems were similar [10].

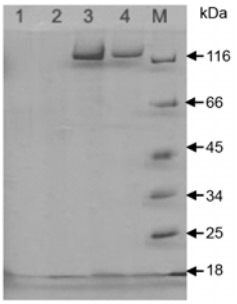

Fig. 4. SDS-PAGE of the crude recombinant β-glucosidase produced by *P. pastoris*. Bands 1, 2 - "wild-type" of *P. pastoris* KM71H and X33 respectively; Band 3, 4 - recombinant *P. pastoris* KM71H and X33 respectively; M - protein size marker.

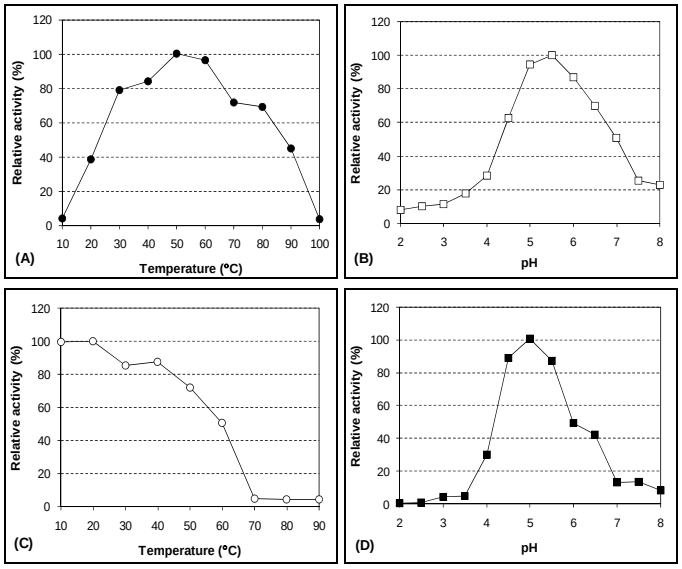

Fig. 5. Effect of temperature and pH on recombinant β-glucosidase. A – relative activity of β-glucosidase at different temperatures; B – relative activity at different pH; C - effect of temperature on stability of the enzyme; D – enzyme stability at different pH. The relative activity of BGL1 was expressed in percentage of the maximum.

D.Selection of β-glucosidase over-expressing clones

After electroporation, linearized pPICZαA/*BGL1* is expected to integrate into the host 5′ AOX1 region by homologous recombination. In *P. pastoris* system, multiple integrations may occur at the rate of 1-10% of the single insertion events. The increase in the number of expression cassette often leads to the increase in expression level and zeocin resistance [1]. In order to obtain β-glucosidase over-expressing clones, transformants were spread on medium with elevated concentration of zeocin. Several clones resisting zeocin concentration of 2000 mg/l were obtained. With these clones, we achieved maximum extracellular β-glucosidase (cellobiase) activity of 60 IU/ml. Given the fact that Novozym 188, a widely distributed β-glucosidase preparation from Novozymes has cellobiase activity of 250 IU/ml, the expression level obtained is promising.

IV. Conclusion

BGL1 from traditional starter yeast *S. fibuligera* BMQ 908 has been cloned and expressed as an active, extracellular β-glucosidase in methylotrophic yeast *P. pastoris* under the control of AOX1 promoter and α-factor signal peptide. The recombinant strain produced single extracellular protein with molecular weight of 120 kDa and cellobiase activity of 60 IU/ml.

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