

Molecular Cloning and Expression of a Synthetic Gene Encoding a β -glucosidase of *Aspergillus Niger* in the Methyilotrophic Yeast *Pichia Pastoris*

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Abstract

A 2526 bp gene encoding *Aspergillus niger* β -glucosidase was chemically synthesized for its heterologous expression in methyilotrophic yeast *Pichia pastoris*, using methanol as inducer. The enzyme was purified from the induction medium to homogeneity by using ammonium sulfate precipitation, DEAE-cellulose chromatography and Sephadex G-200 gel filtration. The recombinant β -glucosidase catalyses the hydrolysis of cellobiose and salicin. The specific activity (51.2 U/mg) for cellobiose hydrolysis was enriched 7.4 fold with a recovery of 13.6%. Optimum activity was observed in pH 5.0 at 50°C. The enzyme was a monomer with an apparent molecular mass of 90 kDa as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Keywords: Chemical synthesis, β -glucosidase, *Pichia pastoris*, Methanol induction, Heterologous expression

1. Introduction

Cellulose, a polymer of glucose with β -1, 4 linkages, is the world's most abundant natural biopolymer and a potential important source for production of industrially useful material such as fuels and chemicals. Ethanol is one of the most important renewable fuels, contributing to the reduction of negative environmental impacts generated by worldwide consumption of fossil fuels. Production of ethanol as renewable fuel from cellulose biomass is actively investigated (Lynd et al., 2002). However, the lack of biocatalysts and cellulase preparation for converting biomass into fuel ethanol has always been cited as roadblocks to bioethanol industry (Dien et al., 2003). Enzymatic hydrolysis of cellulose offers the potential for higher glucose yields and milder process

conditions (Aristidou and Penttilä, 2000). The complete degradation of cellulose involves a synergistic action of three main cellulolytic enzymes. These enzymes include: a) β -1, 4-endoglucanase (EC 3. 2. 1. 4) which randomly cleaves internal β -1,4-glycosidic bonds; b) Cellobiohydrolases (EC 3. 2. 1. 91) sequentially releases molecules of cellobiose from reducing and non-reducing ends of cellulose and c) β -glucosidase (β -D-glucoside glucohydrolase; EC 3. 2. 1. 21) that hydrolyses cellobiose to glucose which can then further be converted to other chemicals such as ethanol (Bhat and Bhat, 1997). The presence of β -glucosidase in cellulase preparations has been reported to stimulate the rate and extent of cellulose hydrolysis (Ryu and Mandel 1980; Wood and McCrae, 1982). This effect has been explained by the concept that it relieves the inhibition by cellulose-derived cellobiose of cellulase activity, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose. Developing cellulase enzymes, and preferably, suitable microorganisms that produce such enzymes which can be used for the efficient depolymerization of a complex sugar and subsequent rapid fermentation of the sugar into alcohol would be of great benefit. Various cellulases and β -glucosidase genes have been expressed in *Saccharomyces cerevisiae* with aim of direct ethanol production from cellulose (Van Rensburg *et al.*, 1998; Jeon *et al.*, 2009; Fujita *et al.*, 2002, Hong *et al.*, 2006). The methylotrophic yeast *Pichia pastoris* is a powerful tool for the heterologous expression of proteins (Cereghino and Cregg, 2000). The increasing popularity of this expression system can be attributed to several factors, such as the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris*, many eukaryotic posttranslational modifications and high-level gene expression (Hasslacher *et al.*, 1997; Hollenberg and Gellissent, 1997; Sreekrishna *et al.*, 1997). Protein expression in *P. pastoris* is based on the use of the alcohol oxidase 1 (AOX1) promoter. This promoter is strongly induced by methanol (Li *et al.*, 2007). Chemical synthesis is an attractive alternative to conventional gene cloning for the reason that codon usage can be optimized for the proposed organism of expression favoring high yields of protein. In this work, we used a synthetic gene encoding a β -glucosidase from *Aspergillus niger* to develop a recombinant yeast *Pichia pastoris* expressing a cellobiase activity. Expression, structural and functional characterizations of the yeast synthesized polypeptide are described.

2. Material and Methods

2.1 Strain, plasmid and medium

Escherichia coli (*E. coli*) DH5 α was used as a host for sub-cloning. *Pichia pastoris* GS115 was used as a host for expression. Plasmid pPIC9K was used for expression. *E. coli* was cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or on LB agar plate. When needed, ampicillin was added at a concentration of 100 μ g/ml. The expression strains were screened in minimal dextrose (MD) medium [1.34% yeast nitrogen base (YNB), 0.00004% biotin, 2% dextrose] and minimal methanol (MM) medium (1.34% YNB, 0.00004% biotin, 0.5% methanol). Yeast was cultured in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar), buffered glycerol-complex (BMGY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.00004% biotin, 1% glycerol) and induced in buffered methanol-complex (BMMY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.00004% biotin, 1% methanol).

2.2 Construction of cloned β -Glucosidase expression vector

A gene encoding a β -glucosidase (β -Gl2) from *Aspergillus niger* was designed according to its nucleic acid sequence published at GenBank (Accession n° EU233788.1). This sequence was synthesized by Shanghai Sangon Bioengineering Company (Shanghai, China) and cloned into pUC19 plasmid named pUC19- β -Gl2. DNA manipulations and subcloning were performed according to standard procedures (Sambrook and Russell 2001). The full-length sequence of β -Gl2 without the signal peptide was amplified from pUC19- β -Gl2. PCR reaction, using *ExTaq* polymerase (TAKARA), was as follows: Denaturation 5 min at 94°C, 30 cycles of denaturing at 94°C for 45 sec, annealing at 59.1°C for 1 min and elongation at 72°C for 3 min. Forward primer 5'- AATACGTAGATGAATTGGCCTACTCC - 3' and reverse 5'- ATAGTTTATCGGCCCGCTTAGTGAACAGTAGGCA - 3' were designed to introduce *Sna*BI and *Not* I restriction sites (underlined) respectively. Amplified PCR product was resolved by 0.8% agarose gel electrophoresis. The gel slice containing the expected size band was excised and extracted with SANGON gel extraction kit. The purified β -Gl2 fragment was first treated with restriction enzymes and then ligated, using DNA ligation kit (Takara), to the purified *Sna*BI-*Not* I double-digested secretory expression vector pPIC9K. *E. coli* strain DH5 α was transformed with the ligation mixture. Bacterial transformants were selected for their ability to grow in LB medium in the presence of 100 μ g/ml ampicillin. Transformants were screened by colony-PCR using the above primers. The recombinant expression vector, designated as pPIC9K- β -Gl2, was then prepared using a plasmid miniprep kit, identified by double enzyme digestion and insert further sequenced by GenScript.

2.3 Construction and screening of *P. pastoris* expression strains.

pPIC9K- β GI2 used for transformation was linearized by *PmeI*. *P. pastoris* GS115 strain was made competent and transformed with *PmeI*-linearized pPIC9K- β GI2 by electroporation following INVITROGEN protocol. About 10 μ g of linearized vector and 80 μ l of electro-competent GS115 cells were used. Immediately after pulsing (1.5kv, 5ms), 1 ml of cold 1M sorbitol was added to the cuvette. After incubation of 30 min at 30°C, cells were plated on MD medium and incubated at 30°C and monitored each day till appearance of colonies. His⁺ transformants in each plate were resuspended in 2-3 ml of sterile water and thereafter pooled. The cells density was determined by reading OD at 600 nm. Then 10⁵ cells were plated on MD plates containing increasing concentrations of G418 from 0.25 up to 4 mg/ml to screen for multi integrant transformants.

2.3.1 PCR analysis of *Pichia* integrants

Yeast genomic DNA was extracted from selected clones grown overnight in 5ml YPD as follows: Cells from 1.5 ml of the cultures were pelleted in a microcentrifuge tube and the cell pellets were resuspended in 200 μ l of lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] (Harju et al., 2004). 200 μ l of phenol-chloroform (1:1) and 0.3g acid washed glass beads were added and the tubes were vortexed for 2 minutes. Thereafter 200 μ l TE buffer pH8 were added to tubes, followed by 5 minutes centrifugation 12,000 \times g at room temperature. The aqueous layer was transferred to a tube containing 1 ml of ice-cold 100% ethanol. The samples were allowed to precipitate 5 minutes at room temperature and then centrifuged 2 minutes at room temperature at 12,000 \times g. Supernatants were poured off and DNA pellets reconstituted in 400 μ l TE buffer pH8 containing 30 μ g/ml RNase A and incubated for 5 minutes at 37°C. Then, 18 μ l of 3M ammonium acetate and 1ml ice-cold 100% ethanol were added and tubes were kept at -20°C for few hours. DNA was spun down 10 minutes at 12,000 \times g resuspended in 25 μ l water. The so-extracted DNA served as a template for Taq DNA polymerase PCR using 5'*AOXI*: 5'-GACTGGTTCCAATTGACAAGC-3' and 3'*AOXI*: 5'-GCAAATGGCATTCTGACATCC-3' primers (Invitrogen)

2.4 *Pichia* expression studies

Single colony of one the positive transformants was grown at 30 °C in 25 ml BMGY medium in 250ml flasks for 16 to 20 h with vigorous shaking (225~250 rpm) until an OD₆₀₀ value of 2-5 had been reached, then harvested by centrifugation at 3000 g at 25 °C for 5 min. Supernatant was decanted and the cell pellet was resuspended to an OD₆₀₀ of 1.0 in induction medium BMMY in 1l flasks and allowed to grow at 30°C. 1% (final concentration) methanol was added to the flask every 24 h in order to maintain induction. Supernatants were taken after induction for 0h, 12 h, 24h, 36h, 48h, 60h, 72h and 84h for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis using 10% acrylamid gel (Laemmali, 1970).

2.4.1 Production and purification of the recombinant β -glucosidase

Recombinant *P. pastoris* was cultured in BMGY and induced in BMMY as previously described. Expression was induced for 60 hours at 30°C under shaking and the cell-free extract obtained after centrifugation 3000xg for 5min at 25°C was used as the “crude extract”. **Ammonium sulfate fractionation:** The crude extract (360 ml) was fractionated by gradual addition of solid ammonium sulfate to give 30% saturation with stirring at 4°C for 3 hours. After, the mixture was centrifuged at 12000xg for 15min. Solid ammonium was added to the supernatant to 80% saturation and the solution was stirred overnight at 4°C and thereafter centrifuged at 12000 g for 20min. The precipitate was resuspended in 50mM citric-phosphate buffer pH6 and dialyzed overnight at 4°C against 10mM phosphate buffer pH7. **Ion exchange chromatography:** The dialysate was loaded onto a DEAE-32 (Pharmacia) column (1.9cm x 30cm) pre equilibrated with 10mM phosphate buffer pH7. The elution was performed with the starting buffer (~2.8 column volume) and then a gradient of 0-0.3M NaCl in 10mM phosphate buffer pH7. The column was washed with 0.5M NaCl in 10mM phosphate buffer pH7. Fractions containing β -glucosidase activity were pooled and concentrated through Amicon Ultra Centrifugal Devices, MW cut off 10,000 (Millipore) at 7,500xg for 10min at 25°C. The sample was dialyzed overnight at 4°C against 10mM phosphate buffer pH7. **Gel filtration:** The filtrate was further chromatographed on a Sephadex G-200 (Pharmacia) column using 10mM phosphate buffer pH7 as elution buffer. Active fractions were pooled and dialyzed against 10mM phosphate buffer pH7 then concentrated by ultrafiltration. The filtrate was the “purified enzyme”. Purity was monitored throughout the fractionation by SDS-PAGE. Detection of β -glucosidase in eluates was carried out using 4-methylumbelliferyl- β -D glucopyranoside (MuGlc) as substrate for enzyme reaction. 10 μ l of each sample was placed on 3% agar plate containing 0.2mM MuGlc in 50mM citric-phosphate buffer pH5. The plate was then incubated at 50°C for 1 hour and thereafter illuminated with UV light. An intense fluorescence was an indicator of β -glucosidase activity.

2.4.2 Enzyme assay

Protein concentration was determined spectrophotometrically by coomassie (BRADFORD) Protein Assay, using the protein assay kit (Nanjing Jiancheng Bioengineering Institute). Three milliliters of diluted reagent were pipetted into 50 μ l of sample solution. The mixture was then incubated at room temperature for 10min. The absorbance was measured at 595nm. β -glucosidase activity assay was performed by monitoring the release of glucose or reducing sugar from cellobiose and salicin. Enzyme preparation, 500 μ l, was incubated in the presence of 500 μ l (1% w/v) substrate in 50mM citric-phosphate buffer pH5 at 50°C for 30min. Amount of glucose or reducing sugar released was measured with a glucose determination kit (Rongsheng Biotech Co) as described in the manufacturer's instructions. One international unit (IU) of enzyme activity was defined as the amount of enzyme that releases one micromole of glucose per minute under assay conditions. Specific activity was expressed as unit per milligram of protein.

2.4.3 Optimum pH and temperature

The pH optima for β -glucosidase activity was determined by performing assay at 50°C, in sodium phosphate buffer (50mM) with pH values of 3.3, 4.5, 5, 6.5, 7.3 and 8. For optimum temperature assay, β -glucosidase activity was assayed at temperature 30, 40, 50, 60, 70, and 80°C in sodium phosphate buffer (50mM, pH 5). All reactions for activity test were carried out as previously described.

3. Results and discussion

3.1 Construction of expression vector and screening of positive transformants

The full length DNA of synthetic β -glucosidase gene was inserted into pPIC9K vector and transformed into *E. coli* DH5 α to give pPIC9K- β GI2 expression plasmid. The plasmid pPIC9K- β GI2 contained an insert with 2526 bp; confirmation of insertion was achieved by PCR (fig 1) and DNA sequencing. Figure 2 shows the nucleotide sequence of the insert DNA. It encodes a polypeptide of 841 amino acids with a predicted pI value of 4.52, having an estimated molecular weight of 91.5kDa. A BLAST search against sequences deposited in the GenBank database revealed that the β GI2 contains conserved domains which belong to glycosyl hydrolysis family 3. Figure 3 presents amino acid alignment of β GI2 with other isolated beta-glucosidases. The target gene was cloned in frame and downstream of the α -factor signal sequence to allow secretion of the recombinant protein into the culture medium. In addition pPIC9K vector contains the AOX1 promoter that allows methanol-inducible high level expression in *Pichia*. Plasmid pPIC9K- β GI2 was prepared, then linearized and transformed into the genome of *P. pastoris* strain GS115 and plated on MD plates. His⁺ transformants were observed after three days. Transformants could only grow on plates containing G418 at 0.25 mg/ml when screened for multi integration of the β GI2 gene. Subsequently, integration of the gene into the *P. pastoris* genome was verified on seven G418 resistant clone by PCR, using 5'AOX1 and 3'AOX1 primers. Figure 4 shows a successful PCR amplicon with the size of ~2500 bp for five of the seven selected clones, meaning integration of β GI2 gene downstream of AOX1 promoter in their genome. In addition, by yielding two products after PCR reaction, selected transformants appeared to be Mut⁺ phenotype (methanol utilization fast).

3.2 Expression of recombinant β -glucosidase in *Pichia pastoris*

Time-course for β -glucosidase production in the culture medium was studied for 80 hours (fig 5). The presence of the recombinant protein was checked via SDS-PAGE analysis on sample prepared as described (material and methods). The enzyme was successfully excreted into the culture medium by *P. pastoris* from pPIC9K- β GI2 vector, using 1% final concentration of methanol as inducer. AOX1 gene in *P. pastoris* is tightly regulated and induced by methanol to high levels (Higgins and Cregg, 1998). A protein band corresponding to the recombinant β -glucosidase (r β GI2) could be detected by coomassie Blue staining 24 hours after induction (fig 5). The production of glucosidase was increasing and reached maximum after 60 hours of induction. Accordingly, the subsequent experiments of beta-glucosidase were carried out with an induction time of 60 hours.

3.3 Enzyme production and purification

The recombinant beta-glucosidase produced by *Pichia pastoris* was purified from the culture supernatant. Summary of purification steps was recorded in table 1. Ammonium sulfate fractionation at range 30-80% saturation produced around 61% of protein from the initial protein amount in the supernatant. This fraction retained much of the enzyme activity (~87%). The precipitated enzyme was then bound to DEAE-cellulose at 10mM phosphate buffer, pH7.0. Elution of the column using a linear sodium chloride gradient could further remove about 80% contaminating protein from the previous step (or ~87% protein from the culture filtrate). In this step, β -glucosidase was purified 3.1 fold with a yield of 42.6%. The final purification was achieved by gel filtration through Sephadex G-200. This resulted in an increase of the purity by about 2 times from

DEAE-cellulose. By these steps, the specific β -glucosidase activity reached 51.2 U.mg^{-1} . A similar protocol using Sephacryl 300 yielded approximative results during purification of a plant β -glucosidase (Rakrudee, 2004). The denatured enzyme (boiled for 10min in the presence of β -mercaptoethanol) showed a single homogenous band, having an apparent molecular mass of approximately 90kDa (fig 6), which is consistent with the predicted value from deduced protein encoded by the cloned gene.

3.4 Effect of pH and temperature on enzyme activity

The recombinant β -glucosidase secreted by *Pichia pastoris* was assayed for cellobiose and salicin hydrolysis. The enzyme was most active towards cellobiose [17.7 U/ml against 3.9 U/ml for salicin at optimum conditions (fig 7)]. The optimum pH was 5.0 for both substrates as shown in figure 7 (A). The activity decreased at pH above 5. Salicinase and cellobiase activities were highest at temperatures of 40 and 50°C respectively. Similar properties were observed for some β -glucosidases (Dhake and Patil, 2005; Pornphimon, 2005; Zhou and Yin, 2009).

Overexpression of beta-glucosidase in *Pichia pastoris* offers an advantage for its large scale production via fermentation for industrial bioconversion processes. In addition, construction of yeast expressing cellulases appear to be very useful for simultaneous saccharification and fermentation

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Table 1. Summary of purification of recombinant β -glucosidase from *Pichia pastoris*

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Fold purification	Yield (% recovery)
Culture filtrate	313.56	45.36	6.9	-	-
Ammonium sulfate	275.6	27.8	9.9	1.4	87.9
DEAE-cellulose	117.34	5.45	21.53	3.1	37.42
Sephadex G-200	42.56	0.83	51.2	7.4	13.6

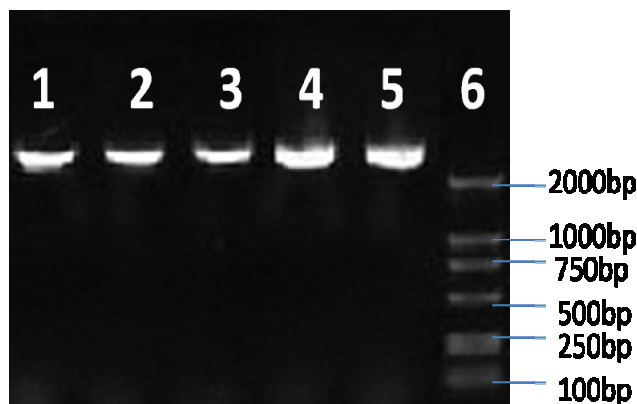


Figure 1. Screening of DH5 α transformants harboring the expression vector pPIC9K- β GL2. Colony PCR was performed using selected colonies from LB plate + ampicillin. An aliquot of PCR product were analyzed by electrophoresis on 0.8% agarose gel, Lane 1 to 4; Lane 5: plasmid pUC19- β GL2. Lane 6: DNA marker

GATGAATTGGCCTACTCCCGCCGTATTACCCATCCCCTTGGGCCAATGGCCAGGGTGACTGGCCGGAAGCATACCAACGCCCTGTTGATATCGTCTCGC
 D E L A Y S P P Y Y P S P W A N G Q G D W A E A Y Q R A V D I V S
 AGATGACATTGGCTGAGAAGGTCAATTTGACTACGGGAAGTGGATGGGAATTGGAATTATGTGTTGGTCAGACTGGAGGTGTTCCCGCGTTGGGAATTCC
 Q M T L A E K V N L T T G T G W E L E L C V G Q T G G V P R L G I P
 GGGAAATGTGTGCACAGGATAGCCCTCTGGGTGTTGCTGACTCCGACTACAACCTCTGCGTTCCTGCGCGGTGTCAACGTGGCCGCAACCTGGGACAAGAAT
 G M C A Q D S P L G V R D S D Y N S A F P A G V N V A A T W D K N
 CTGGCTTACCTTGGTGGCCAGGCTATGGGTGAGGAGTTAGTGACAAGGGTGCTGATATCCAATTGGGTCCAGCTGCCGCCCTCTCGGTGCTAGTCTG
 L A Y L R G Q A M G Q E F S D K G A D I Q L G P A A G P L G R S P
 ACGCGCGTGGTAACTGGGAGGGCTTCTCCCGAGACCGGCCCTCAGTGGTGTGCTCTTTGACAGAGACAATCAAGGGTATTGAGGATGCTGGTGTGTTG
 D G G R N W E G F S P D P A L S G V L F A E T I K G I Q D A G V V A
 AACGGCTAAGCACTACATCGCTACGAGCAGGAGCATTTCGCTCAGCGCGCTGAAGCTCAAGGCTACGGATTCAATATTACGAGAGTGGAGCGCGAAC
 T A K H Y I A Y E Q E H F R Q A P E A Q G Y G F N I T E S R S A N
 CTCGACGATAAGACTATGCATGAGCTGTACCTCTGGCGGTTGCGGATGCCATCGCTGCAGGTGCCGGTGTGTGATGTGCTGTACAACAGATCAACA
 L D D K T M H E L Y L W P F A D A I R A G A G A V M C S Y N Q I N
 ACAGCTATGGCTGCCAAACAGCTACACTCTGAACAAGCTGCTCAAGGCTGAGCTGGGTTTCCAGGGCTTTGTCATGAGTGATTGGCGCGCTCACCATGC
 N S Y G C Q N S Y T L N K L L K A E L G F Q G F V M S D W A A H H A
 CGGTGTGAGTGGTCTTTGGCGGGATTGGACATGTCTATCGCGGGAGAGCTGATTACGACAGTGGCAGCTTACTGCGGTACCAACTTGACCATCAGT
 G V S G A L A G L D M S M P G D V D Y D S G T S Y W G T N L T I S
 GTGCTCAACGGGACGGGCCACAATGGCGGTGTTGATGACATGGCTGTCCGATCATGGCCGCTACTACAAGGTGCGCGGTGACCGTCTGTGGACTCCTC
 V L N G T A P Q W R V D D M A V R I M A A Y Y K V G R D R L W T P
 CTAACCTCAGCTCATGGACCGTGATGAATACGGCTCAAGTACTACTATGTCTCGGAGGGACCGTATGAGAAGGTCAACCAAGTTCGTGAACGTGCAACG
 P N F S S W T R D E Y G F K Y Y Y V S E G P Y E K V N Q F V N V Q R
 CAACCATAGCGAGTTGATCGCGGTATTGGAGCAGACAGCAGGTGCTCCTCAAGAAGCATGGCGCTCTTCCATTGACTGGAAGAGAGCGCTTGGTCGCC
 N H S E L I R R I G A D S T V L L K N D G A L P L T G K E R L V A
 CTTATCGGAGAAGATGGCGGTTCCAATCCTTATGGTGGCAACGGCTGCAGTGACCGTGGGTGCGACAATGGAACATTGGCGATGGGCTGGGGAAGTGCCA
 L I G E D A G S N P Y G A N G C S D R G C D N G T L A M G W G S G
 CTGCCAACCTTTCGTAAGTGGTGACCCCTGAGCAGGCCATCTCGAAGAGGTGCTCAAGAACAAGATGGCGTATTCACTGCGACCGATAACTGGGCTAT
 T A N F P Y L V T P E Q A I S N E V L K N K N G V F T A T D N W A I
 TGATCAGATTGAGGCGCTTGCTAAGACCGCAGTGTCTCTTGTCTTTGTCACGCGGACTCTGGTGAGGGTTATATCAATGTGACGGAACCTGGGT
 D Q I E A L A K T A S V S L V F V N A D S G E G Y I N V D G N L G
 GACCGCCGGAACCTGACCTGTGGCGTAACGGCGACAATGTGATCAAGGCTGCTGCTAGCAACTGCAACAACACGATCGTTATTACTCTGTGCGGC
 D R R N L T L W R N G D N V I K A A A S N C N N T I V I I H S V G
 CAGTCTTGGTTAACGAGTGGTAGACAACCCAAATGTTACCGTATTCTCTGGGGTGGTCTTCCGGGTGAGGAGTCTGGCAACTCCCTCGCGGACGTGCT
 P V L V N E W Y D N P N V T A I L W G G L P G Q E S G N S L A D V L
 CTACGGCGGTGTAACCCCTGGTGCCAAGTCCGATTACCTGCGGCAAGACTCGTGAGGCGCTACCAAGATTACTTGTACACCGAGCGCAACAACGGCAAC
 Y G R V N P G A K S P F T W G K T R E A Y Q D Y L Y T E P N N G N
 GGAGCGCCTCAGGAAGACTTCGTGAGGGCGTCTTATTGACTACCGCGGATTTGACAAGCGCAACGAGACTCCTATCTATGAGTTCGGCTATGGTCTGA
 G A P Q E D F V E G V F I D Y R G F D K R N E T P I Y E F G Y G L
 GCTACACCACCTTCAACTACTCGAACCTTCAGGTGGAGGTTCTGAGCGCCCTCGGTACGAGCCTGCTTGGGCGGAGACTGAGGAGCGCGGACTTTTCGG
 S Y T T F N Y S N L Q V E V L S A P A Y E P A S G E T E A A P T F G
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 E V G N A S D Y L Y P D G L Q R I T K F I Y P W L N S T D L E A S
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 S G D A S Y G Q D A S D Y L P E G A T D G S A Q P I L P A G G G A
 GCGGCAACCCCTCGCTGTACGAGGAGCTCATCCGCTGTGGTGACTATCAAGAACACCGGCAAGGTTGCGGGTGTGAAGTTCCTCAACTGTATGTTTC
 G G N P R L Y D E L I R V S V T I K N T G K V A G D E V P Q L Y V S
 TCTTGGCGGCGCTAACGAACCTAAGATCGTGTGCTGCTCAATTCGAGCGTATCAGCTGCAGCGCTCGAAAGAGACGAGTGGAGCACAACCTCTGACCGCG
 L G G P N E P K I V L R Q F E R I T L Q P S K E T Q W S T T L T R
 CGTGACCTTGGCAACTGGAATGTTGAGACGAGGACTGGGAGATTACGTCGTATCCAAAGATGGTGTGTTGGCGGAAGCTCCTCGCGGAAGCTGCCGCTCC
 R D L A N W N V E T Q D W E I T S Y P K M V F A G S S S R K L P L
 GGGGCTCTGCTACTGTTCACTAA
 R A S L P T V H

Figure 2. Nucleotide and deduced amino acid sequences of β -glucosidase

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BGL2   141 SPDGGRNWEGFSPDPALSGVLFAETIKGIQDAGVVATAKHYIAYEQEHFR---gygfniTeSRSANLDDKT
214
AGCBG1 105 SGLNGRNFECYSEDPALTAACAVAYINGVQSQGVAAATIKHFVANESEIER-----Q-TMSSDVDERT
165
PABGL   164 KAAGGRGWEGHGPDPYLEGVIAYLQTIGIISQGVVSTAKHLIGNEQEHFR-----tSsSLSSEIDDRA
243
ACABGL  152 SPDGGRNWEGFSPDPVNSGLLVAETIKGIQDAGVIACVKHFIGNEQERFR---gygfdiSeSSSSNIDDVT
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AACBGL  151 SPDGGRNWEGFSPDPALTGVLFAETIKGIQDAGVVATAKHYILNEQEHFR---gygfniSdTSSNVDDKT
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SFBGL1  165 KARGGRNFEAFGSDPYLQGTAAATIKGLQENNVMACVKHFIGNEQEKYR---atnqttKeASANIPDRA
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CHBGL1  156 KPRGGRNWEGFSGDPVLQAFGGSLSVEGIQSTGVIAATIKHLIANEQEMYR-----ivKpGYSSNVDDRT
222
CIBGL1  167 KPRGGRNWEGFSGDPSLQAIAAVETIKGVQSKGVIAATIKHLVGNEQEMYR-----ivQrAYSANIDDRT
233
KCBGLN  161 VATGGRNWEGFETNDPYLAGALVYETTKGIQ-ENVIACTKHFIGNEQETNR-----gtYNqSVSANIDDKT
227

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Figure 3. Alignment of β GL2 amino acid with other glycosyl hydrolases family. Conserved residues are highlighted. AGCBG1 represents *Agrobacterium tumefaciens* CBG1 (Accession n° P27034), PABGL1 represents *Pichia anomala* BGL (Accession n° P06835), ACABGL represents *Ajellomyces capsulatus* (Accession n° Q00025), AACBGL represents *Aspergillus aculeatus* BGL1 (Accession n° P48825), SFBGL1 represents *Saccharomycopsis fibuligera* BGL1 (accession n° P22506), CHBGL1 represents *Cochliobolus heterostrophus* BGL1 (Accession n° O13385), CIBGL1 represents *Coccidioides immitis* BGL1 (Accession n° O14424), KCBGLN represents *Kuraishia capsulate* BGLN (Accession n° Q12653), BGL2 represents Beta-glucosidase (this study).

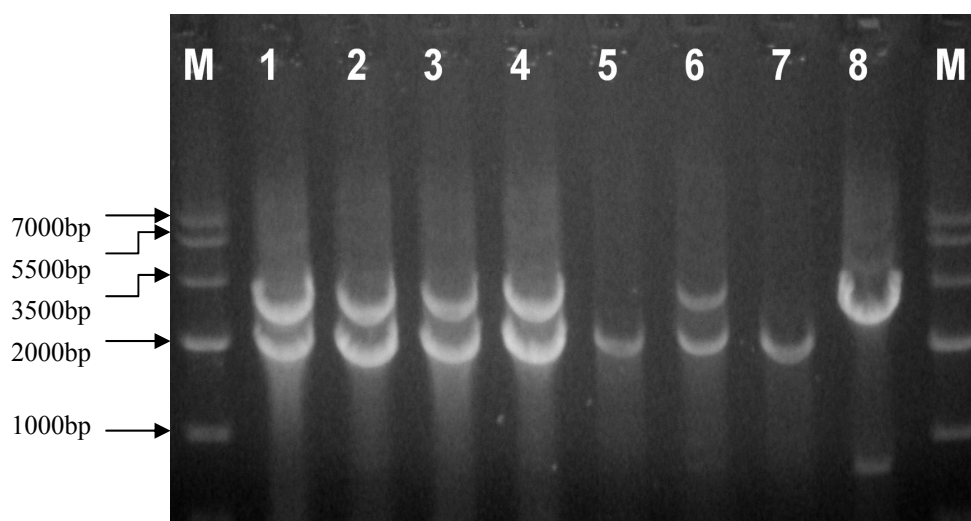


Figure 4. PCR analysis of the β -glucosidase gene (β GL2) integration. Genomic DNA of *Pichia pastoris* clones transformed by pPIC9K- β GL2 was analyzed by PCR using AOX1-specific primers. Integrated sequence yields a PCR product of 2526bp while the wild-type AOX1 gene yields a product of 2200bp. Clones 1 to 4 and 6 had incorporated the β GL2 sequence; Clones 5 and 7 were false positives; Lane 8: Control pPIC9K- β GL2 plasmid. M: molecular marker.

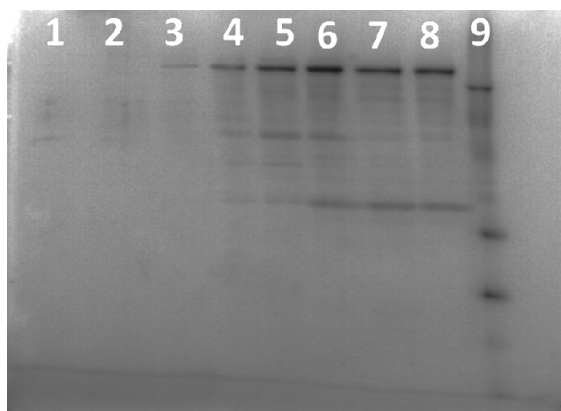


Figure 5. Time course production of β -glucosidase into the induction medium. Samples were taken from the *Pichia pastoris* culture medium at time intervals 0h (lane1), 12h (lane 2), 24h (lane 3), 36h (lane 4), 48 (lane 5), 60h (lane 6), 72h (lane7) and 80h (lane 8). Level of expression was measured on supernatants on 10% SDS-PAGE gel. High level was after 60 hours induction (Lane 6). Marker (Lane 9).

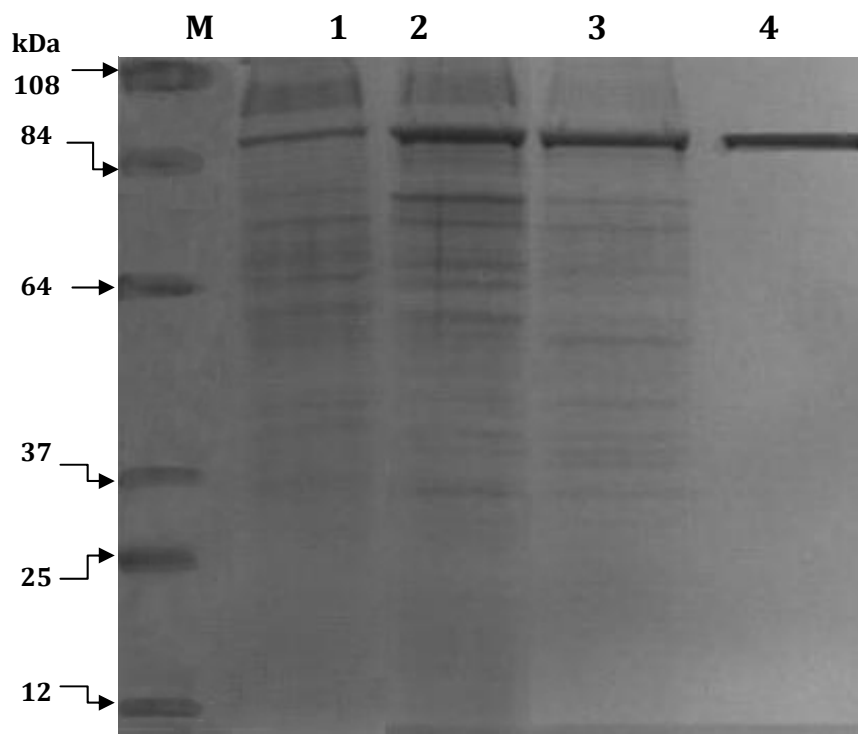


Figure 6. SDS-PAGE profile β -glucosidase purification's steps. Lane 1 Molecular marker, Lane 2: culture supernatant, Lane 3: 30-80% ammonium sulfate fraction, Lane 4: DEAE-cellulose, Lane 5: purified β -glucosidase

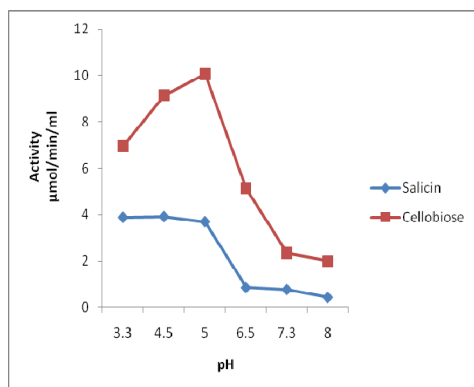


Fig 7 (A)

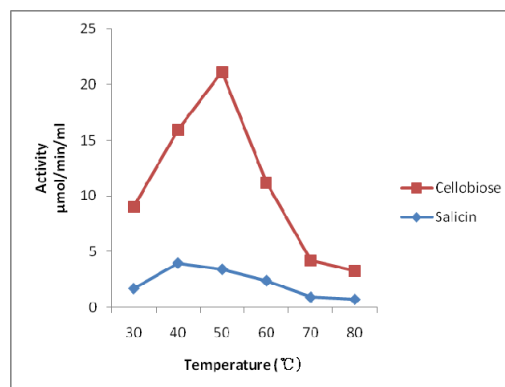


Fig 7 (B)

Figure 7. Determination of the optimum pH and temperature of recombinant β -glucosidase activity. The enzyme activity was tested in presence of cellobiose and salicin at different pH, ranging from 3.3 to 8 (A) and at different temperature from 30 to 80 $^{\circ}\text{C}$ (B)