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RESEARCH ARTICLE

BETA-GLUCOSIDASE FROM THERMOTOLERANT YEAST *PICHIA ETCHELLSII*: GENE SEQUENCING, CLONING AND FUNCTIONAL EXPRESSION IN METHYLOTROPHIC YEAST *PICHIA PASTORIS*

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ABSTRACT

Pichia etchellsii Bgl1 gene coding for BGLI protein was fished out from yeast genomic DNA using PCR based strategies. The primers were designed based on the internal peptide sequences of native BGLI protein. *Bgl1* gene was cloned and expressed in *Pichia pastoris.* The deduced amino acids encoded by *Bgl1* showed high similarity with the sequences of Glycoside hydrolase family 3 members. The predicted isoelectric point (pI) of the protein was 5.2 and A+T% and G+C% were 58.06% and 41.95% respectively. The multiple sequence alignment using ClustalV program of DNASTAR software showed 98.6 % identity with a hypothetical 765 aa protein of *Kluyveromyces lactis* and 73.4% with 845 aa β-glucosidase protein of *Kluyveromyces fragilis*. The recombinant enzyme showed maximal activity at pH 6.0 and was stable between pH 3.5–9. More than 80% of enzyme activity was retained in this pH range on incubating enzyme for 24 h. The enzyme had temperature optimum of 50 °C under optimal pH with pNPG as substrate.

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INTRODUCTION

A consortium of enzymes, endocellulases, exocellulases and β-glucosidases is required for complete saccharification of cellulose into fermentable sugars. The conversion of cellobiose and soluble cellodextrins into glucose by β-glucosidases is the major rate limiting step in the reaction (Sternberg *et al*., 1977; Philippidise *et al*., 1993). β-D Glucoside glucohydrolases (3.2.1.21) or β-glucosidases are well characterized, biologically and industrially important enzymes. These enzymes catalyse the transfer of glucosyl group between oxygen nucleophiles which under physiological conditions results in hydrolysis of β-glucosidic bond linking carbohydrate residues in alkyl-, aryl-, or amino-glucosides, cyanogenic glucosides, disaccharides and short chain oligosaccharides. In addition to cellulosic biomass degradation in bacteria and fungi, these enzymes also catalyse liberation of terpenols from their glucoside precursors in yeasts, hydrolysis of glucosylated flavonoids in plants and breakdown of glycosyl ceramides in mammalian lysosomes. In addition to hydrolytic activity, the use of β-glucosidases in biosynthesis of glycoconjugates (oligosaccharides, glucosides) has been widely reported (Bhatia *et al*., 2002a; Thiem, 1995; Vic and Thomas 1992).

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Glycosidases have been classified into 115 families on the basis of amino acid sequence and folding similarities (Henrissat and Davis, 1996). β-glucosidases belong to family 1 and family 3 except glucosylceramidases which fall in family 30. Family 1 contains enzymes from archea, bacteria, plants and mammals whereas family 3 contains enzymes from archea, bacteria, fungi and yeasts. These enzymes hydrolyze the substrate with net retention of anomeric configuration via double displacement mechanism (Sinnott 1990; McCarter and Withers 1994; Davies *et al*., 1998). The active site of these enzymes contains two carboxylic acid residues which act as nucleophile and acid base catalyst in the catalysis. In the glycosylation step, the nucleophilic residue attack the anomeric center of substrate and acid base catalyst protonates the glycosidic oxygen resulting in departure of aglycone moiety. This leads to formation of covalent α-glucosyl enzyme intermediate. In deglycosylation step, hydrolysis of intermediate occurs by base catalyzed attack of water at the anomeric centre with the release of glycone moiety with net retention configuration. Formation and hydrolysis of intermediate proceed by oxocarbenium transition state. A substantial amount of work has been done on cloning structural genes of β-glucosidase from a variety of microorganisms and plants into high-yielding expression systems such as *Escherichia coli*, *Saccharomyces cerevisiae,* and filamentous fungi to understand the molecular basis of these enzymes. However, β-glucosidases from yeast are least

explored and only few enzymes have been cloned and expressed. Yeast enzymes have been expressed in eukaryotic hosts like *S. cerevisiae* and *Candida* sp., however *Candida wickerhamii* and *P. etchellsii* gene have been expressed in *E. coli* (Skory and Freer, 1995; Pandey and Mishra, 1995; Sethi *et al*., 2002). *K. fragilis* β-glucosidase and *Saccharomycopsis fibuligera* BGL1 and BGL2 were also cloned in *S. cerevisiae* (Raynal *et al*., 1987; Machida *et al*., 1988). The use of methylotropic yeast *P. pastoris* as host for expression of recombinant proteins has gained importance in recent times. The yeast is easy to genetically manipulate, provides soluble, correctly folded recombinant proteins and has potential to grow to a very high cell density (Kim *et al*., 1997; Zhu *et al*., 1995). *P. pastoris* expression system has also been explored for functional expression of bacterial, fungal, plant and human liver cytoplasmic β-glucosidase genes. *P. etchellsii*, a themotolarant yeast produces two inducible, cell wall bound β-glucosidases viz. BGLI and BGLII which have been purified and characterized (Wallecha and Mishra, 2003). In addition two other enzymes, BglI and BglII have been identified by way of cloning genomic DNA and expression in *E. coli* (Pandey and Mishra, 1997; Sethi *et al*., 2002). The present work accounts for cloning *Bgl1* gene, encoding BGLI enzyme from *P. etchellsii* and functional expression in *P. pastoris.*

MATERIALS AND METHODS

Strains and plasmids

P. etchellsii [Deutsche Von Mikroorganismen (DSM), Germany] was used as donor of *Bgl1* gene. The cloning host was *E. coli* DH5α (Clontech, USA) whereas *P. pastoris* GS115 (Invitrogen, USA) was used as expression host. Yeast expression vector pPIC9 (Invitrogen, USA) was taken for expression of *Bgl1* gene. *E. coli* transformants were selected on LB + amp (50 µg/ml) plates, where as selection of His^+ GS115 transformants was done on minimal dextrose (MD) plate.

Media and culture conditions

P. etchellsii was grown in YPD medium (0.5% yeast extract, 1% bacto-peptone, 2% D-glucose) at 40 °C. *E. coli* DH5α was grown in Luria- Bertani (LB) medium at 37 °C. GS115 was grown on YPD medium at 30 °C. The heterologous expression of β-glucosidase in GS115 was carried out in BMGY and BMMY. The ingredients of MD (Minimal Dextrose Medium), MM (Minimal Methanol Medium) BMGY (Buffered Glycerol Complex medium), BMMY (Buffered Methanol Complex medium) and culture conditions of *P. pastoris* were in reference of the Invitrogen Pichia Expression Kit manual (Invitrogen). For RNA isolation, *P. etchellsii* was grown in Phosphate Succinate Medium (0.25% yeast extract, 0.5% peptone, 0.6% succinic acid, 0.03% $CaCl_2$, 0.87% K_2HPO4, 0.40% $(NH_4)_2SO_4$ and 0.05% $MgSO_4$, pH 4.7) for 14 h and then supplemented with 10 mM of inducer, cellobiose. YPM medium (1% yeast extract, 2% peptone, and 0.5 % methanol, pH 6.0) was used as the induction medium for activity screening in plates (Kawai *et al*., 2003).

Isolation of gene coding for β-glucosidase

Chromosomal DNA from *P. etchellsii* was prepared according to Cregg *et al*. (1985) with some modifications. Plasmid DNA was isolated by alkaline lysis method (Sambrook *et al*., 1989). Total RNA was extracted using Nucleospin RNA Isolation Kit (Biolinkk) in guidance of the manufacturer's instructions. Primers were designed to fish out complete *Bgl1* gene from *P. etchellsii* genomic DNA (Table 1). The internal peptide sequences of native BGLI protein (Wallecha and Mishra, 2003) were aligned with GHF3 β-glucosidases using ClustalW program of MegAlign program of DNASTAR software (*DNASTAR,* Madison, WI*)*. The primers were designed from the 5' end gene sequences of *K. fragilis* and *K. lactis* proteins showing homology with BGLI peptide. Degenerate primers were designed from an internal peptide sequence 'FPFGYGI/L' of BGLI protein which was towards C terminus of the protein and was conserved in family 3 members. The gene specific primer BGL5F and Oligo dT-AYY dT was used for 3' RACE. The oligonucleotide primers were synthesized by MGW Biotech. Step down PCR (Hecker et al., 1996), 3' RACE (Frohman, 1990) and Primer walking technique (Studier, 1989) were used to find out complete gene of *Bgl1* in parts. All PCR products were sequenced from MGW sequencing facility and DNA sequencing facility, Department of Biochemistry, University of Delhi, South Campus. Subsequently, the overlapping sequences of PCR products were assembled to get complete *Bgl1* gene sequence and submitted to NCBI database.

Construction of recombinant expression vector

The *Bgl1* gene was amplified from *P. etchellsii* chromosomal DNA by PCR using DNA polymerase (TAKARA, Japan) and cloned in yeast shuttle expression vector, pPIC9 in-frame with the native *Saccharomyces cerevisiae* α-factor secretion signal sequence under the control of Alcohol Oxidase 1 promoter (AOX1) to generate a recombinant plasmid, pPIC9*Bgl1*. The forward primer BGL PIC F and the reverse primer BGL PIC R incorporate enzyme cleavage sites for *Xho*I and *Not*I (underlined sequences, Table 1), respectively. All cloning steps were done as per standard protocols (Sambrook *et al.*, 1989). In-frame fusion and authenticity of the *Bgl1* gene in recombinant plasmid, (pPIC9*Bgl1*) was verified through sequencing of the recombinant construct.

Yeast transformation and screening of His^+ recombinants

Expression vector pPIC9*Bgl1* was linearized with *Stu*I enzyme and transformed in GS115 by electroporation using Gene Pulser X cell (Bio-Rad) and screened on MD plates as per the *Pichia* Expression Kit Instruction Manual (Invitrogen Corporation). After incubation for 3 days at 30 °C on MD plates, colonies were inoculated onto YPM plate to induce the expression of the *Bgl1* gene as described by Kawai *et al*. (2003) using 1 mM 4- methylumbelliferyl β-D-glucoside (MUG) as substrate. Integration of *Bgl1* gene in the *Pichia* genome was checked by PCR using gene specific primers BGL F1 and PEBGL2R (Table 1) and Dot blot analysis. The selected clones were screened for Mut^+ and Mut^s phenotype as per Invitrogen manual protocol.

Expression of *Bgl1* gene in *P. pastoris* and purification of recombinant enzyme

The two transformants, GS115pPIC9*Bgl1*-5 and GS115pPIC9*Bgl1*-41 were cultured at 30 °C in BMGY

medium till OD reached 2-6. The cells were pelleted and resuspended in induction medium BMMY to an OD of 1.0. After 6 days of induction, the supernatant was harvested by centrifugation. Effect of induction temperature on enzyme activity was also studied. The secretory proteins in culture supernatant were precipitated by 85% ammonium sulphate precipitation. The precipitated pellet of protein was solubilized in 50 mM sodium phosphate buffer and loaded on Sephadex G 200 gel filtration matrix (XK26/100). The samples containing β glucosidase activity were pooled and further purified by anion-exchange chromatography using Q Sepharose fast flow resin (XK16/30).

pH and temperature studies

The partially purified recombinant BGLI was characterized in terms of pH optimum, pH stability, temperature optimum, temperature stability using standard protocol as described previously by Wallecha and Mishra, 2003. The effect of metal ions and chemical inhibitors on enzyme activity was also studied.

Protein and Enzyme activity assay

β-glucosidase activity was assayed using p-nitrophenyl-β-D-glucopyranoside as described previously (Wallecha and Mishra, 2003). One unit of β-glucosidase activity corresponds to release of 1 µmol p-nitrophenol/min. Protein concentration was estimated by the method of Bradford using BSA as standard (Bradford, 1976). The specific activity of the enzyme was expressed in units of enzyme activity per milligram of protein.

Electrophoretic procedures

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% polyacrylamide gel with Tris/ Glycine buffer in presence of SDS. Protein was stained with Coomassie Brilliant Blue R-250 (Lammmlli, 1970). PAGE-zymogram analysis of the culture supernatant was also carried using 4-methylumbelliferyl β-D-glucoside (MUG) as substrate as described previously (Wallecha and Mishra, 2003).

Transcriptional studies

Expression was also confirmed by Northern hybridization. For this, total RNA from the transformant GS115pPIC9*Bgl1*-5 and control GS115 was extracted using Nucleospin RNA Isolation Kit (Biolinkk). RNA was separated in 1.5% denaturing formaldehyde agarose gel, blotted and subsequently hybridized to the radiolabelled 340 bp *Bgl1* internal gene fragment under conditions of high stringency (Sambrook *et al.,* 1989).

RESULTS

Complete nucleotide sequence of *Bgl1* gene and sequence analysis

The complete sequence of *Bgl1* was assembled from the sequences of the PCR products obtained from all PCR reactions. The figure 1 represents the position of various primers and PCR products sequenced to get *Bgl1* gene sequence as described previously. The intronless *Bgl1* gene (GenBank Accession No. EU914813) ORF consisted of 2,544 bp nucleotides encoding a protein of 847 amino acids. The sequence homology search using BLAST (Altschul *et al*., 1990) and multiple sequence alignment using ClustalV program of MegAlign program of DNASTAR software revealed sequence identity with several members of GHF3 (Fig.2). The deduced amino acid sequence matched with the internal peptide sequences of the native BGLI determined earlier. The ORF predicted a protein of molecular mass of 93.4 kDa. The sequence analysis by DNASTAR showed the presence of 90 strongly basic amino acids (K, R), 114 strongly acidic amino acids (D, E), 298 hydrophobic amino acids (A, I, L, F, V) and polar 215 amino acids (N, C, Q, S, T, Y). The predicted isoelectric point (pI) of the protein was 5.2 and A+T% and G+C% were 58.06% and 41.95% respectively. The multiple sequence alignment using ClustalV program of DNASTAR software showed 98.6% identity with hypothetical 765 aa protein of *K. lactis* and 73.4% with 845 aa β-glucosidase of *K. fragilis* (Fig.3). The sequence was thus concluded to belong to GHF3.

Cloning and expression of *Bgl1* in *P. pastoris*

Complete *Bgl1* gene was amplified using BGL PIC F and BGL PIC R primers and cloned in pPIC9 vector in-frame with the secretion signal. After transformation, the presence of *Bgl1* in clone DH5α-16 containing pPIC9*Bgl1* was confirmed by restriction digestion, PCR and sequencing. GS115pPIC9*Bgl1*-5 transformant showed strong fluorescence under UV when screened for secretory expression of rBGL1 by agar plate assay (Fig. 4. A1& A2), confirming successful extracellular expression of *P. etchellsii* BGLI. Integration of *Bgl1* gene in *Pichia* genome of GS115pPIC9*Bgl1*-5, GS115pPIC9*Bgl1*-8 and GS115pPIC9*Bgl1*-41 was confirmed by PCR with gene specific primers. Further, hybridization signals in Dot Blot analysis(data not shown) with *Bam*HI digested genomic DNA of above mentioned clones using radiolabelled probe also confirmed successful integration of the *Bgl1* gene in the yeast chromosomal DNA. The selected clones were found to be His^+ Mut^+ as transformants grew well on both MD and MM plates.

Expression of Recombinant *Pichia* Strains in baffled flasks

Based on results of PCR, dot blot and agar plate assay, clone GS115pPIC9*Bgl1*-5 and GS115pPIC9*Bgl1*-41 were selected for expression studies. The recombinant enzyme activity reached a maximum of 560 IU/L on eighth day in case of GS115pPIC9*Bgl1*-5 and 415 IU/L for GS115pPIC9*Bgl1*-41 (Fig.4.B). The effect of temperature during culture growth and induction was also studied and optimized. The maximum enzyme activity of 974 IU/L was achieved (Fig.4.C) in clone GS115pPIC9*Bgl1*-5 on day 6 when the induction temperature was set to 23 °C, which was about 1.74 fold higher than in unoptimized culture. Thus, temperature of cultivation was found to affect expression. Similar results have been reported for other *P. pastoris* expression systems (Shi *et al* 2003; Li *et al* 2001, Bencurova *et al* 2003). Northern analysis confirmed that the increase was at mRNA level and maximum expression occurred on 6^{th} day of induction (Fig.5).

Table 1. Primers used in gene isolation, cloning and sequencing

A. Primers designed based on gene sequences of *K. fragilis* and *K. lactis*
Forward primer: 2F KLU- 5' GCTGCTGTGATTTTGGGTCC 3'
Reverse primer: 2R KLU- 5'CCATCTTGTTGGTCCAGGG 3'
Reverse primer: PEBGL1R-5' TCCAACCCATTCTTGATAGC 3'
Forward primer: F5KL- 5'TCATGTCCAAATTTGATGTCG 3'
Reverse primer: PEBGL2R- 5'CCACGACCACCCAAAGGACC 3'
B. Degenerate primers
Reverse primer : FPF RI
M R W
5' (A/C)CC (A/G)TA (A/T)CC GAA GGG GAA 3'
C. Primers for 3' RACE
Forward primer: BGL5F – 5' TTC CAT GGG TAG AAC AAG CC 3'
Reverse primer: Oligo dT-AYY dT- 5' AYY TTTTTTTTTTTTTT 3'
D. Gene specific primer
Forward primer: BGL F1 – 5' ATGATCATGTCCAAATTTGATG 3'
E. Primers used for primer walking
Forward primer: 2F KLU- 5' GCTGCTGTGATTTTGGGTCC 3'
Forward primer: BGL SEQ F1: 5' ATAGGTCCAAACGCCAAAGCC 3'
F. Primers used for cloning in pPIC9 vector
Forward primer: BGL PIC F: 5'-GTA TCT ***CTC GAG*** AAA AGA GAG GCT GAA GCT ATG ATC ATG TCC AAA TTT GAT G -3'
Reverse primer: BGL PIC R: 5'- CCC AT***G CGG CCG C***GT CGC AGC AAT GTA AAT TTG TAC GAC-3'

Table 2. Purification of recombinant β-glucosidase expressed in GS115pPIC9*Bgl*-5 transformant of *P. pastoris*

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery of units (%)
Culture supernatant (200 ml)	220	182	0.827	100
Ammonium sulphate precipitation	180	155	0.86	85
Sephadex G 200	47	71	1.5	54
Q Sepharose	8	28	3.5	15

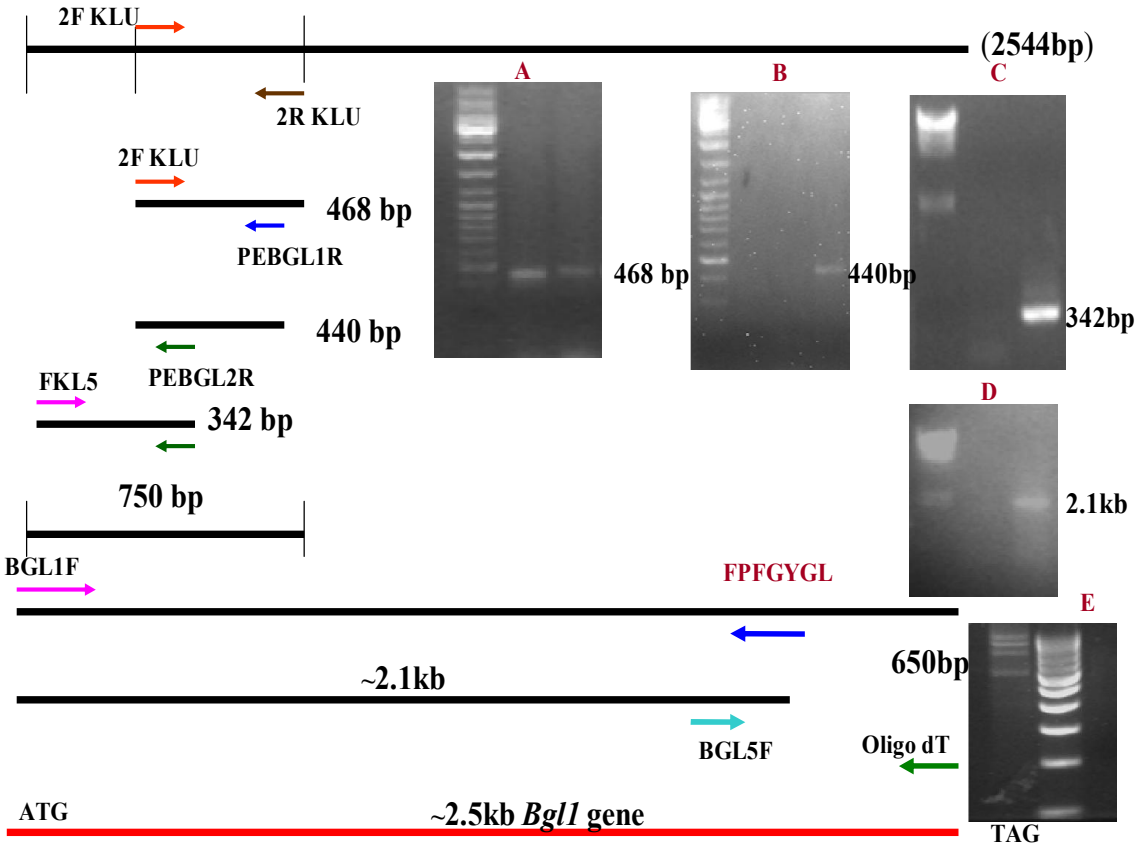
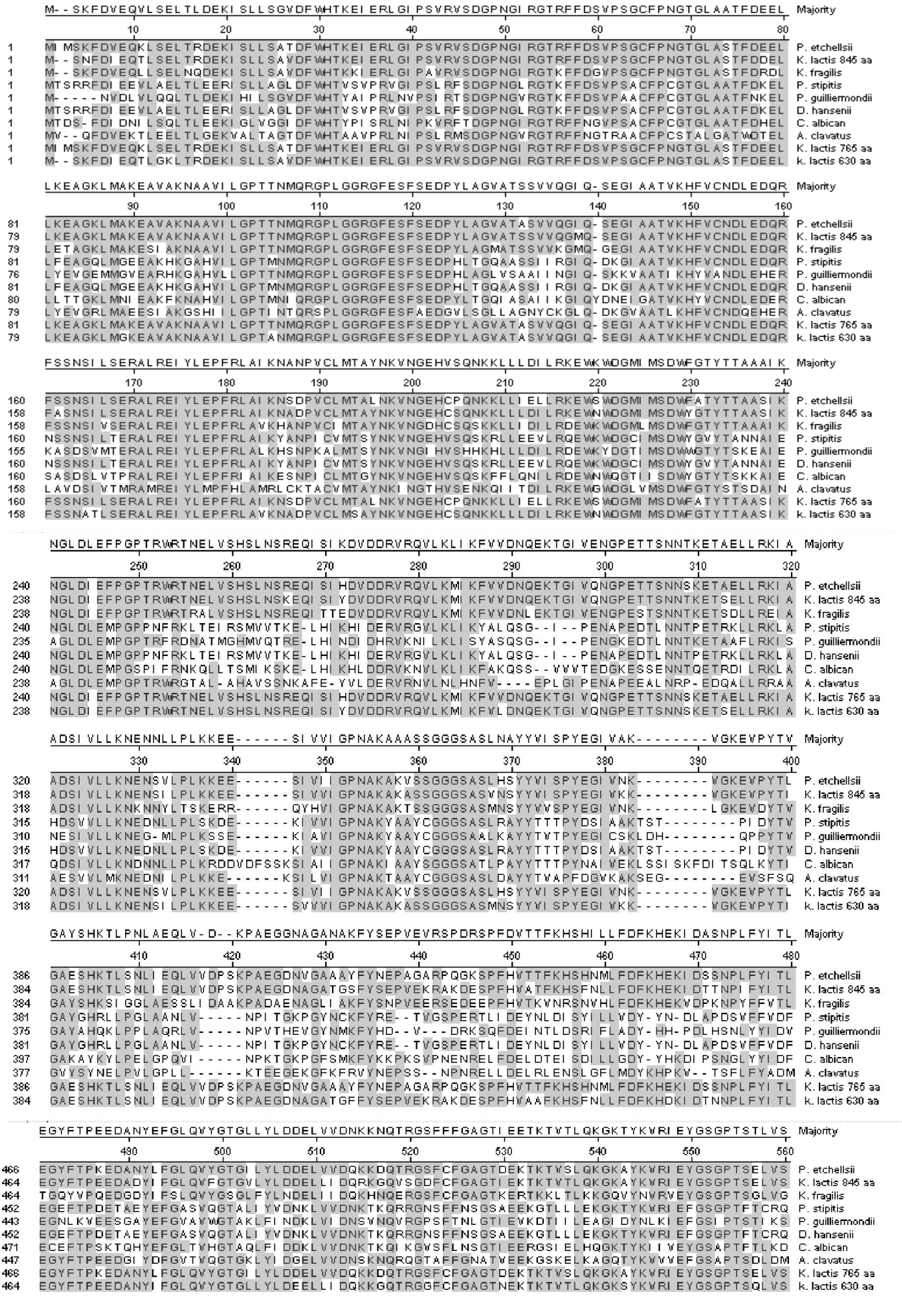


Fig.1. Amplification of *Bgl1* gene from *P. etchellsii* genomic DNA using PCR. Figure shows various primers used for Step down PCR and 3'RACE as described in text to get PCR products which on sequencing provide complete *Bgl1* gene sequence


Majority
P. etchellsii
K. lactis 845 aa
K. fragilis
P. stipitis
P. guilliermondii
D. hansenii
C. albican
A. clavatus
K. lactis 765 aa
k. lactis 630 aa

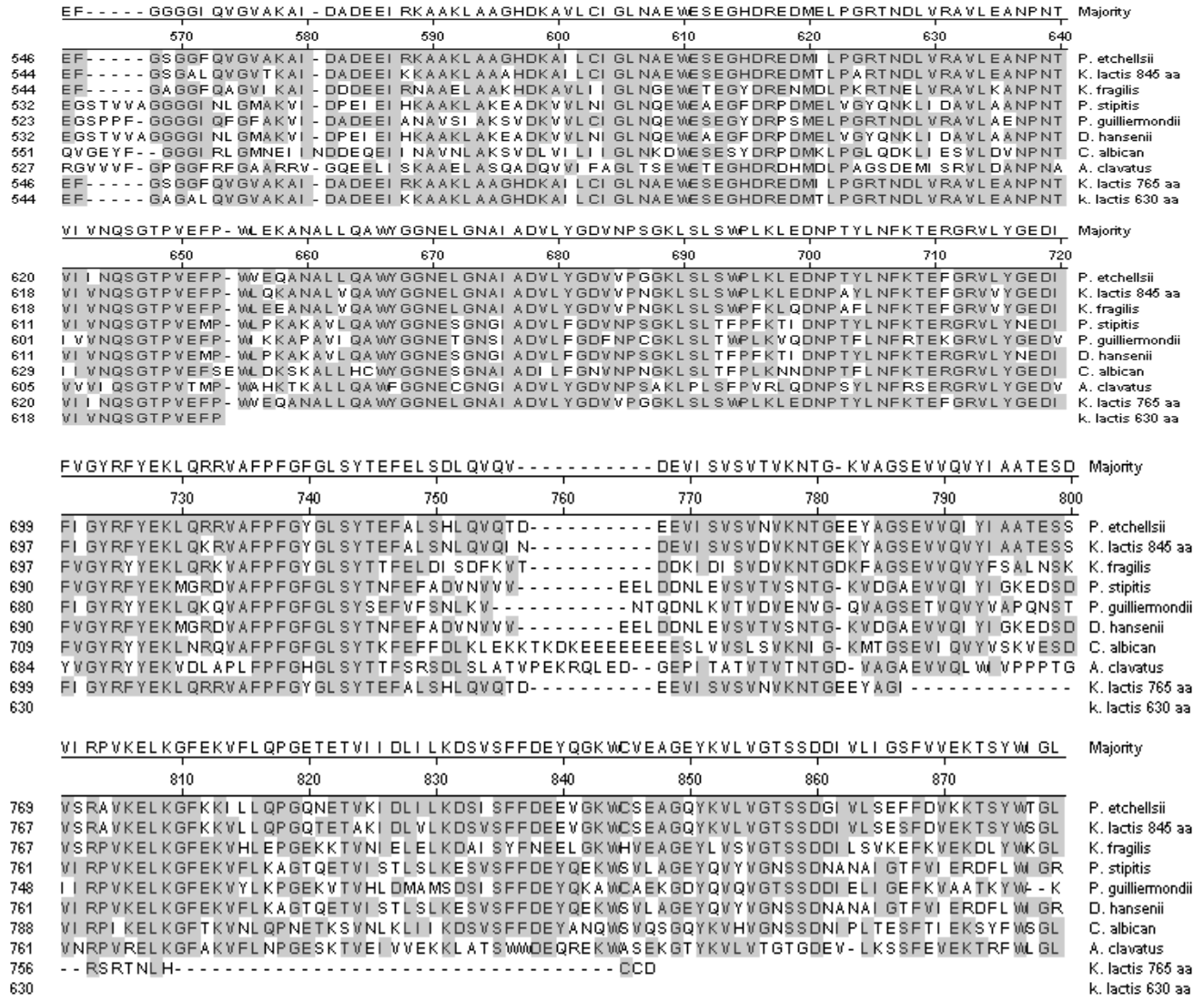


Fig. 2. Multiple sequence alignment of identical amino acids sequences of GHF3 β-glucosidases. Identical amino acids have been shown in gray boxes. P. etchellsii β- glucosidase (this study, ACF93471.1), K. lactis 845 aa protein, K. fragilis (P07337), Pichia stipitis, P. gulliermondii, Debaromyces hansenii, Candida albicans, Aspergillus clavatus, K. lactis 765aa protein and K. lactis 630 aa protein

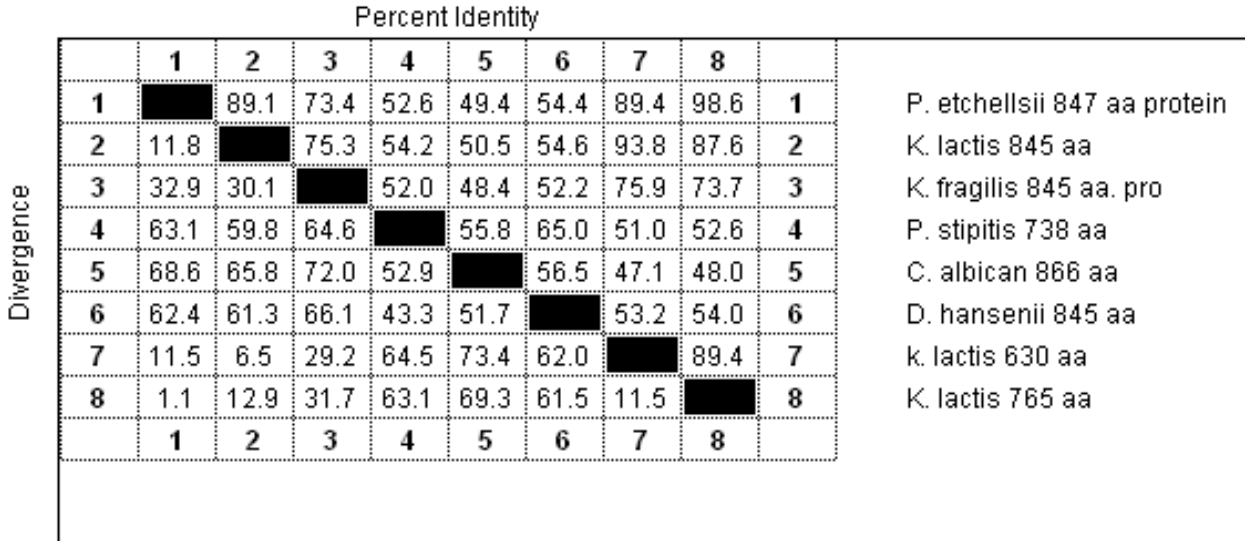


Fig. 3. Sequence homology of *P. etchellsii* BGLI with GHF3 proteins of various yeasts

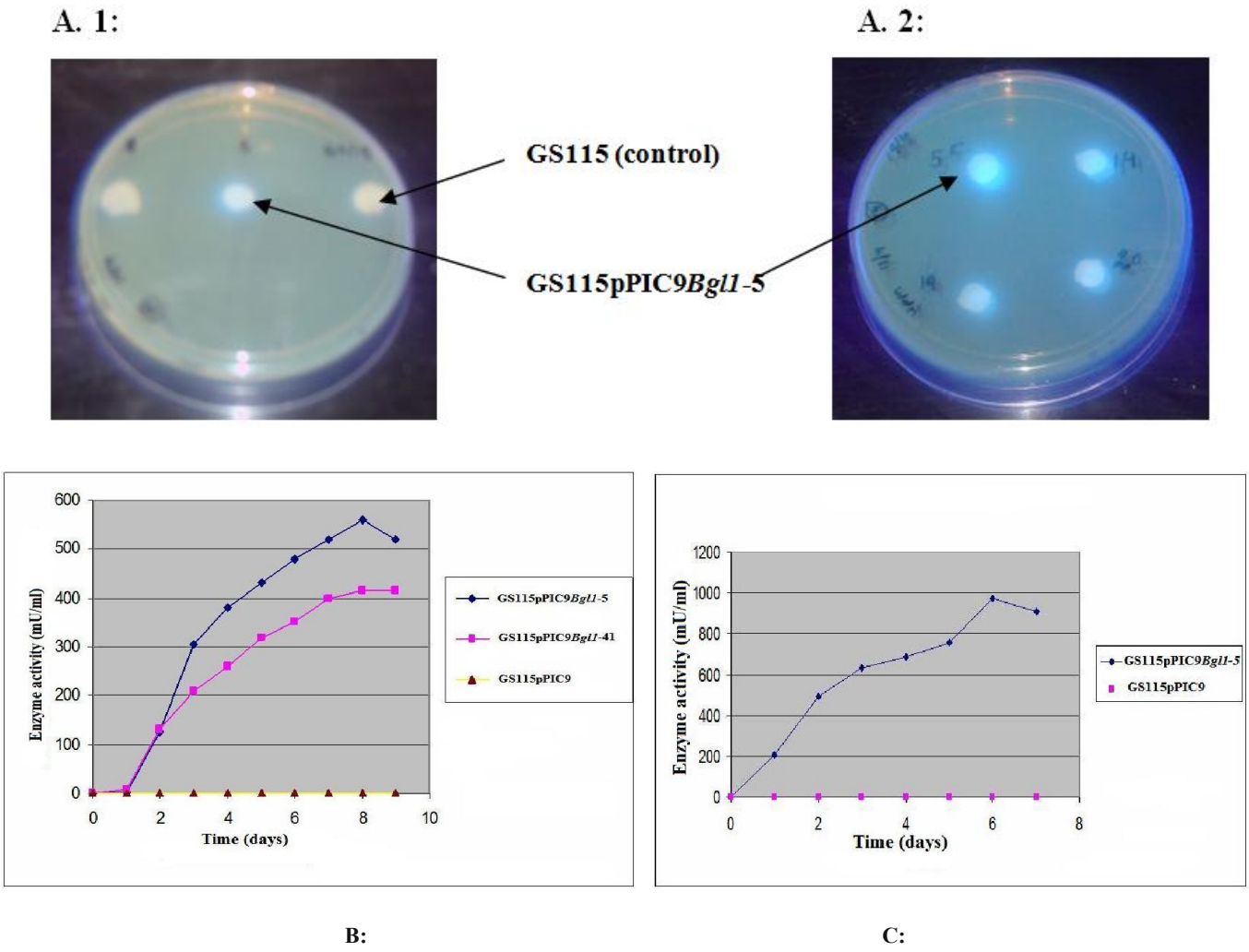


Fig. 4. Expression of *Bgl1* gene in *P. Pastoris* GS115. The enzyme activity was detected on the plate (A) and in the liquid medium (B). β-glucosidase activity was detected as described in materials and methods (C). Effect of induction temperature (23 °C) on enzyme activity of clone GS115pPIC9*Bgl1*-5

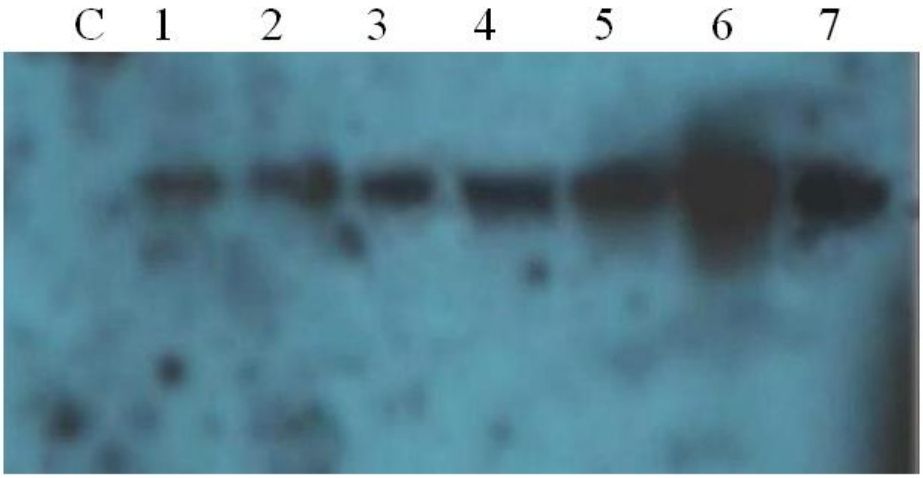


Fig. 5. Northern hybridization of *Bgl1* gene expressed in *P. pastoris* GS115 strain. Lane C- Reference strain GS115 (Negative control); Lane 1-7 *Bgl1* expressing GS115pPIC9*Bgl1*-5 (day 1-day7)

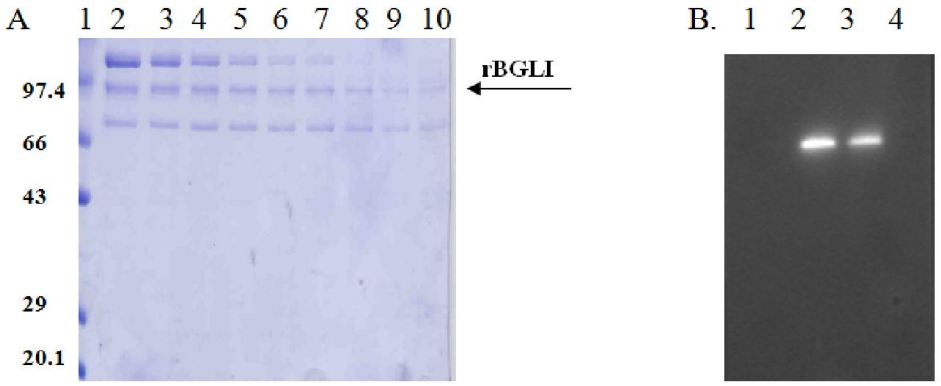


Fig. 6 A: SDS-PAGE profile of partially purified recombinant BGLI (reBGLI). Lane 1 molecular mass marker; lane 2. Partially purified protein after ion exchange chromatography (2-10) B. Native-PAGE of culture supernatant: activity staining was done using MU-Glc as a substrate; lane 1 GS115 (negative control) , lane 2 GS115pPIC9*Bgl1*-5, lane 3 GS115pPIC9*Bgl1*-41 and lane 4 GS115pPIC9 (negative control). Molecular masses of standard proteins are indicated

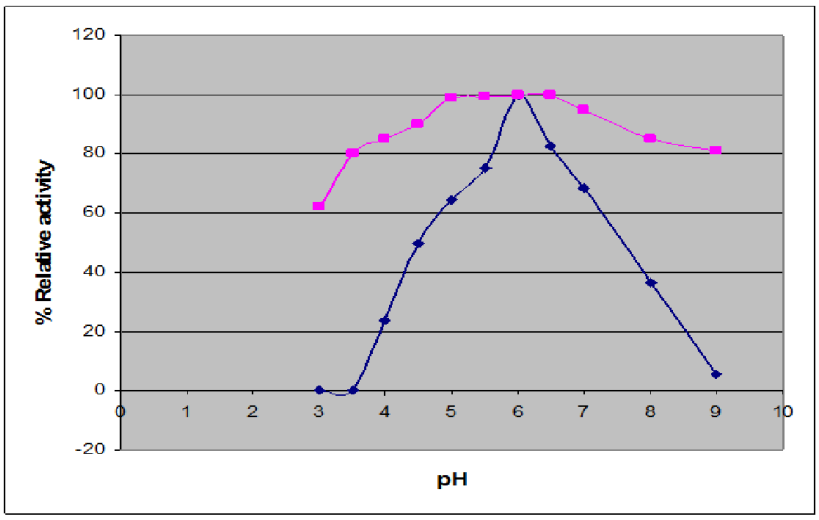


Fig. 7. Effect of pH on activity and stability of recombinant BGL1. For optimum pH, enzyme activity was measured in phosphate citrate buffer, pH 3.0 - 7.0 and pH range of 8.0-9.0 at 50 °C

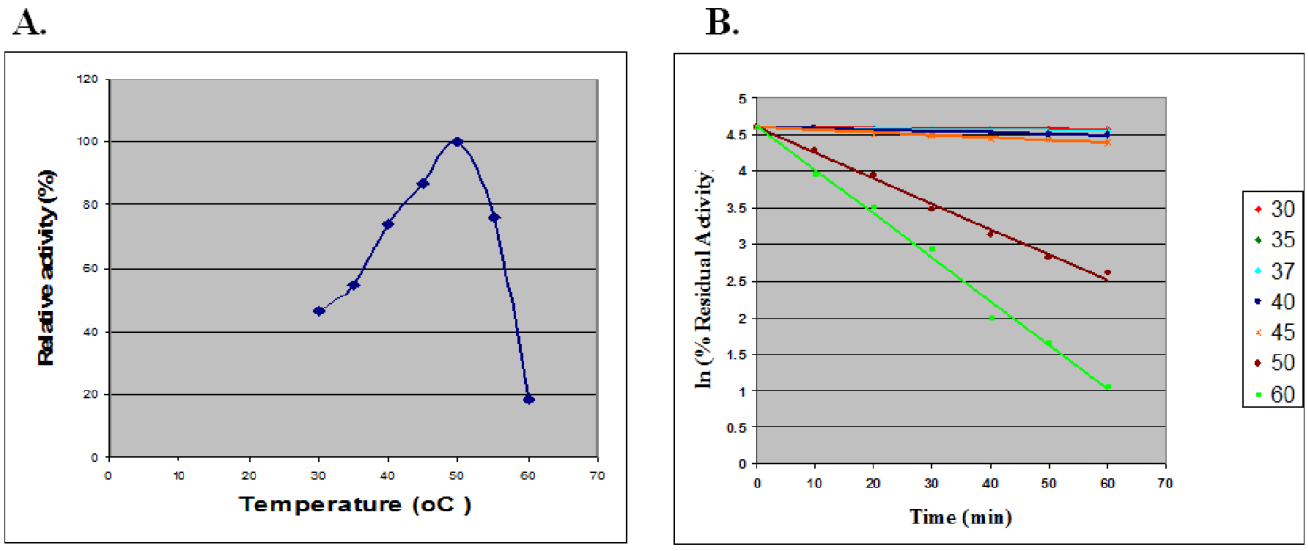


Fig. 8. β-glucosidase activity as a function of temperature. A. For optimal temperature enzyme activity was measured in phosphate citrate buffer, pH 6.0. at various temperatures using pNPG as substrates. B. Thermal stability was studied by incubating the enzyme at different temperatures (30 to 60°C) and assaying for residual activity using pNPG as substrate

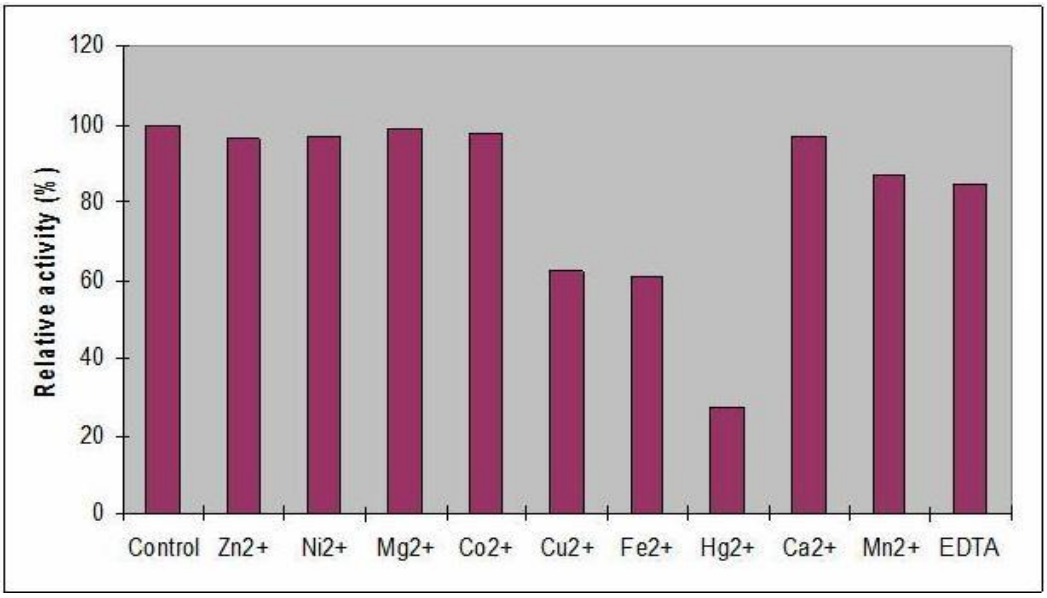


Fig. 9. Effect of metal ions on β-glucosidase activity of rBGLI

Many recombinant proteins have been expressed successfully in *P. pastoris* (Daly and Hearn, 2005; Etemad *et al*., 2008).

Purification and characterization of recombinant protein from culture medium

Native PAGE showed fluorescent high molecular weight band corresponding to rBGLI under UV light in GS115pPIC9*Bgl1*-5 medium supernatant which was not present in untransformed GS115 host (Fig.6.B). This further confirmed the secretory expression of protein in culture medium. The enzyme was purified partially by a combination of ammonium sulfate precipitation, Sephadex G 200 chromatography and ion exchange chromatography to a specific activity of 3.5 IU/mg of protein with 15% yield (Table 2). The dependence of β-glucosidase activity on pH was studied in the pH range of 3.5 to 9. The enzyme showed maximal activity at pH 6.0 (Fig.7). The recombinant enzyme was stable between pH 3.5–9. More than 80% of enzyme activity was retained in this pH range on incubating enzyme for 24 h (Fig.7). However at pH 3.0, 60% of enzyme activity was remained. The enzyme had temperature optimum of 50 °C under optimal pH with pNPG as substrate (Fig.8.A). Half life values of enzyme at 30 °C, 35 °C and 37 °C were found to be 16.5 h, 14.4 h and 12.8 h. However at 40 °C, 45 °C and 50 °C $t_{1/2}$ values of 6.0 h, 3.4 h and 0.5 h were obtained (Fig.8.B) A decline in enzyme activity was observed with Cu^{2+} and Fe^{2+} (~38 and 40% respectively) ions and only 27.34 % of activity was left in presence of 1 mM Hg^{2+} ions (Fig.9). The properties of the enzyme produced in *P. pastoris* were thus found similar to the native enzyme from *P. etchellsii*.

DISCUSSION

In this paper we presented molecular cloning of *Bgl1* gene coding for BGLI enzyme from genomic DNA of yeast *P. etchellsii* and functional expression in *P. pastoris*. Initially studies on cloning of structural genes of β-glucosidase were performed to make engineered strains of *S. cerevisiae* which could efficiently ferment cellobiose to ethanol. The brewing yeast, *S. cerevisiae* posses the structural gene for β-glucosidase, but it is very poorly expressed and thus can't grow on cellobiose as carbon source (Duerksen and Halvorson 1958). Most of the cloned genes failed to confer cellobiose-fermenting ability on *S. cerevisiae*, since their gene products were either not secreted or unable to hydrolyze cellobiose (Kohchi and Toh-e, 1985; Raynal and Guerineau, 1984). However, *S. cerevisiae* strain harbouring *S. fibuligera* BGLI was able to produce ethanol but transformation was low as it was a laboratory strain and not an industrial strain (Machida *et al*., 1988). Currently, the research on β-glucosidases in several laboratories is going on in order to understand the molecular basis of their wide substrate specificity, their assembly into multimodular entities, identifying amino acid residues occurring at the enzyme active site of enzyme for revealing the structure-function relationship as well as designing mutant enzymes with improved characteristics. Therefore, molecular cloning and functional expression of yeast enzymes is necessary. Glycosynthases, the engineered enzymes in which active site nucleophile is mutated by site directed mutagenesis are also gaining importance rapidly because of very high synthetic activity of oligosaccharides (Mackenzie, 1998). From yeasts, only one α-glycosynthase (Family 31 enzyme) D481G from *Schizosaccharomyces pombe* has been reported (Okuyama *et al*, 2002). This has lead to continuous search for novel and more efficient microbial glycosynthases.

Pichia etchellsii Bgl1 gene on sequencing revealed the nucleotide sequence devoid of introns. An Open reading frame of 2,544 bp nucleotides encodes a protein of 847 amino acids with a molecular mass of 93.4 kDa. However, *P. pastoris* secreted enzyme had an apparent molecular weight of 97.3 kDa, similar to the native BGLI in size (97.7kDa). This may be due to glycosylation of recombinant enzyme as at moves through the secretory pathway. The biochemical properties of recombinant enzyme were found similar to native enzyme. The sequence homology search using BLAST revealed sequence

identity with several members of GHF3. The multiple sequence alignment using ClustalV program of DNASTAR showed high degree of homology with members of Genus *Kluveromyces*, 98.6 % identity with a *K. lactis* hypothetical 765 aa protein and 73.4 % with *K. fragilis* β- glucosidase protein of (Raynal *et al*., 1987) rather than members of genus *Pichia*. The consensus sequences of GHF3; SDW and 'FPFGYGI/L', are found in BGL1 sequence**.** The sequence was thus concluded to belong to GHF3. Comparitive studies reveal conserved amino acids in yeast β- glucosidases which may be important to enzyme function. A comparative modeling of the three-dimensional structure of β-glucosidases of GHF3 using the barley enzyme showed that the catalytic nucleophile (Asp-285 of barley enzyme) is conserved across the family. The signature sequence of active site of GHF3 was found at amino acids 267–284 of BGLI (LLKSELDFQGFVMSDWGA) and Asp-281 was identified as a potential nucleophile in the catalytic center (Harvey *et al*. 2000). Multiple sequence alignment and molecular modeling of *P etchellsii* BGLI protein with Barley enzyme enzyme using Program 3Djigsaw (available online at http://www.bmm.icnet.uk/servers/3djigsaw/) showed that the two catalytic amino acids (Asp 227 and Glu 590) are also placed in a position to the ones found in the known structure of *Hordeum vulgare* (Unpublished data). Although they seem to be placed farther apart probably due to the greater number of amino acids between the two catalytic amino acid and unlike in the protein from *Hordeum vulgare*, the latter domain is larger than the Barrel domain.

Hyperglycosylation of the recombinant protein in *S. cerevisiae* has been reported to limit the activity and stability of enzymes as the yeast adds 50 – 150 mannose residues per side chain. We therefore chose to examine expression of *Bgl1* in *P. pastoris* since *Pichia* typically adds only 8–14 mannose residues per side chain. Expression of the *C. wickerhamii bglB* in *P. pastoris* GS115 strain revealed that all constructs accumulate equal amount of transcripts as shown by northern hybridization studies, however none of the isolates produced β-glucosidase activity, either intracellular or extracellular, that was significantly different from controls (Skory *et al*., 1996). Transcriptional studies of *P. etchellsii* expressed protein in *P. pastoris* showed that expression occurs slowly and maximum enzyme production occurred on 6^{th} day of induction (Fig. 5). In addition to yeasts, expression of β-glucosidase proteins in *P. pastoris* from fungal, plants and human liver cytosol have also been reported. *P. pastoris* transformed with *A. niger bgl1* secreted high levels of rBGL1 to the medium (about 0.5 g/liter) appearing as almost pure protein in the culture supernatant (Dan *et al*., 2000). BGLI1 and BGL2 from *Thermoascus auranticus* have also been successfully cloned and secreated in *P. pastoris* (Hong *et al*., 2006 and 2007). Dalcochinin-8'-O-β-glucoside (β-glucosidase (dalcochinase) from the Thai rosewood *(Dalbergia cochinchinensis* Pierr) when expressed in pPIC9K vector of *Pichia*, protein was expressed intracellularly at a low level of 1.6 mg/liter in minimal medium (BMM) or 5.2 mg/liter in rich medium (BMMY pression is low (Cairns *et al*., 2000). A full-length cytosolic β-glucosidase cDNA (cbg-1) was cloned from a human liver cDNA library and expressed in the methylotrophic yeast *P. pastoris* at a secretion yield of 10 mg/L (Berrin *et al*., 2002). We have demonstrated the successful cloning and secretion of *P. etchellsii* rBGLI from in GS115 strain of *P. pastoris* at a level of 120 mg/L. We have shown the successful expression of high molecular weight yeast β-glucosidase in *P. pastoris.* In addition, the temperature of induction played an important role on enzyme secretion and activity. It may be due to the fact that low induction temperature inhibits proteases activity in the culture medium leading to secretion of more stable enzyme with improved activity.

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