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International Journal of Current Research Vol. 8, Issue, 02, pp.26199-26209, February, 2016 INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

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

*Dr. Richa Baranwal

Scientist-III, National Institute of Biologicals (Min. of Health and Family Welfare, Gov. of India)

ARTICLE INFO	ABSTRACT				
Article History: Received 09 th November, 2015 Received in revised form 24 th December, 2015 Accepted 13 th January, 2016 Published online 14 th February, 2016	<i>Pichia etchellsii Bgl1</i> 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. <i>Bgl1</i> gene was cloned and expressed in <i>Pichia pastoris</i> . The deduced amino acids encoded by <i>Bgl1</i> 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				
Key words: β-glucosidase <i>Pichia etchellsii</i> , Heterologous expression, Glycosynthase.	DNASTAR software showed 98.6 % identity with a hypothetical 765 aa protein of <i>Kluyveromyces lactis</i> and 73.4% with 845 aa β -glucosidase protein of <i>Kluyveromyces fragilis</i> . 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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Citation: Richa Baranwal, 2016. "Beta-glucosidase from thermotolerant yeast *Pichia etchellsii*: Gene sequencing, cloning and functional expression in Methylotrophic yeast *Pichia pastoris*", *International Journal of Current Research*, 8, (02), 26199-26209.

INTRODUCTION

A consortium of enzymes, endocellulases, exocellulases and βglucosidases 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 βglucosidic 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).

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

^{*}Corresponding author: Dr. Richa Baranwal,

Scientist-III, National Institute of Biologicals (Min. of Health and Family Welfare, Gov. of India)

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 \beta-glucosidase genes. P. etchellsii, a themotolarant yeast produces two inducible, cell wall bound βglucosidases 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* DH5 α (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 DH5a 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% CaCl₂, 0.87% K₂HPO4, 0.40% (NH₄)₂SO₄ and 0.05% MgSO₄, 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 *XhoI* and *NotI* (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, GS115pPIC9Bgl1-5 and GS115pPIC9Bgl1-41 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 anionexchange 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 Bgll 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 DH5a-16 containing pPIC9Bgl1 was confirmed by restriction digestion, PCR and sequencing. GS115pPIC9Bgl1-5 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 GS115pPIC9Bgl1-5, GS115pPIC9Bgl1-8 and GS115pPIC9Bgl1-41 was confirmed by PCR with gene specific primers. Further, hybridization signals in Dot Blot analysis(data not shown) with BamHI 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 6th 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 TTTTTTTTTTTTT 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 CAC AAA AGA AGA GAG GCT GAA GCT ATG ATC ATG TCC AAA TTT GAT G -3'
Reverse primer: BGL PIC R: 5'- CCC AT GCGC CCG CGC AGC AAT GTA AAT TTG TAC GAC-3'

Table 2. Purification of recombinant β-glucosidase expressed in GS115pPIC9Bgl-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

	MSKEDVEQVLSE	<u>LTLDEKI SLLS</u>	GVDFWHTKEL	ERL GI PSVRV	SDGPNGI RGTR	FFDSVPSGC	FPNGTGLAATF	DEEL	Majority
1 1 1 1 1 1 1 1	10 MI MSKFDVEQKLSE M-SKFDVEQLLSE MTSRREDIEEVLAE MTSRREDIEEVLAE MTSRREDIEEVLAE MTDS-FDIDNILSQ MVQFDVEKTLEE MI MSKFDVEQKLSE M-SKFDIEQTLGK	LTRDEKI SLLS LTRDEKI SLLS LTRDEKI SLLS LTLEERI SLLA LTLEERI SLLA LTLEEKI GLVG LTLEEKI GLVG LTLGEKVALTA LTRDEKI SLLS	30 ATDFWHTKEI AYDFWHTKEI GVDFWHTKKI GUDFWHTVSVI GVDFWHTVSVI GIDFWHTVSVI GIDFWHTYPI GTDFWHTKEI ATDFWHTKEI	40 ERL GI PSVRV ERL GI PSVRV RVGI PSVRV PRLNVPSI RT PRVGI PSLRF SRLNI PSLRF SRLNI PSLRM ERL GI PSVRV ERL GI PSVRV	50 SDGPNGI RGTR SDGPNGI RGTR SDGPNGL RGTK SDGPNGL RGTK SDGPNGL RGTK SDGPNGL RGTR SDGPNGI RGTR SDGPNGI RGTR SDGPNGI RGTR	60 FFDSVPSGC FFDSVPSGC FFDSVPSAC FFDSVPSAC FFDSVPSAC FFNGVPSAC FFNGTRAAC FFNGTRAAC FFDSVPSGC	70 FPNGTGLASTFE FPNGTGLASTFE FPCGTGLAATFE FPCGTGLAATFE FPCGTGLAATFE FPCGTGLAATFE FPCGTGLAATFE FPCSTALGATWE FPNGTGLASTFE	BU DEEL DDEL DRDL DRDL DREL DREL DREL DREL	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
81	LKEAGKLMAKEAVA 90 LKEAGKLMAKEAVA	KNAAVILGPTT 100 KNAAVILGPTT	NMQRGPLGGR(110 NMQRGPLGGR(3FESFSEDPY 120 3FESFSEDPY	LAGVATSSVVQ 130 LAGVATASVVQ	GIQ-SEGIA 140 GIQ-SEGIA	ATVKHEVCNDLE 150 ATVKHEVCNDLE	EDQR 160 EDQR	Majority P. etchellsii
79 79 81 76 81 80 79 81 79	LKEAGKLMAKEAVA LETAGKLMAKESIA LFEAGQLMGEEAKH LYEVGEMMGVEARH LFEAGQLMGEEAKH LLTTGKLMNIEAKF LYEVGRLMAEESIA LKEAGKLMAKEAVA	KNAAVILGPTT KNAAVILGPTT KGAHVLLGPTT KGAHVLLGPTT KGAHVILGPTM KNAHVILGPTM KSSHILGPTT KNAAVILGPTT	NMQRGPLGGR NMQRGPLGGR NMQRGPLGGR NMQRGPLGGR NMQRGPLGGR NIQRGPLGGR NTQRSPLGGR NTQRSPLGGR NMQRGPLGGR	3FESFSEDPY 3FESFSEDPH 3FESFSEDPH 3FESFSEDPH 3FESFSEDPY 3FESFSEDPY 3FESFSEDPY 3FESFSEDPY	LAGVATSSVVQ LAGMATSSVVK LTGQÅASSIIR LAGLVSAAIIN LTGQÅASSIIR LTGQIASAIIK LSGLLAGNYCK LAGVATASVVQ LAGVATASVVQ	GMQ- SEGI A GMQ- GEGI A GI Q- DKGI A GI Q- SKKVA GI Q- DKGI A GI QVDNEI G GL Q- DKGVÅ GI Q- SEGI A GI Q- SEGI A	A T VKHF V CNDLE A T VKHF V CNDLE A T VKHF V CNDLE A T I KHY V A NDLE A T I KHY V A NDLE A T VKHF V CNDLE A T VKHF V CNDLE A T VKHF V CNDLE A T VKHF V CNDLE	DQR DQR DQR HER DQR HER DQR DQR	K. lactis 845 aa K. fragilis P. stipitis D. hansenii C. albican A. clavatus K. lactis 765 aa k. lactis 630 aa
	FSSNSILSERALRE	I YLEPFRLAIK	NANP VCL MTA	<u>YNKVŅGEHVS</u>			MSDWFGTYTTA/		Majority
160 158 158 160 155 160 160 158 160 158	170 FSSNSI LSERALRE FASNSI VSERALRE NSSNSI VSERALRE NSSNSI LTERALRE SASDSVMTERALRE SASDSLVTPRALRE LAVDSI VTMRAMRE FSSNSI LSERALRE	180 I YLEPFRLAIK I YLEPFRLAIK I YLEPFRLAIK I YLEPFRLAIK I YLEPFRLAIK I YLEPFRLAIK I YLEPFRLAIK I YLEPFRLAIK I YLEPFRLAIK	190 NSDPVCLMTAI NADPVCLMTA' HANPVCIMTA' YANPICVMTS' HSNPKALMTS' YANPICVMTS' ESNPICLMTS' LCKTACVMTA' NSDPVCLMTAI NADPVCLMSA'	200 NKYNGEHCP YNKYNGEHCS YNKYNGEHVS YNKYNGEHVS YNKYNGEHVS YNKYNGEHVS YNKYNGEHCP YNKYNGEHCP YNKYNGEHCS	210 QNKKLLI ELLR QNKKLLDI LR QSKKLLEEVLR QSKRLLEEVLR QSKRLLEEVLR QSKFFLQNI LR QSKFFLQNI LR QNKKLLI ELLR QNKKLLI ELLR	220 KEWSWDGMI KEWNWDGMI DEWKWDGCI DEWKYDGTI QEWKYDGTI REWSWDGMI KEWGWDGLY KEWSWDGMI KEWNWDGMI	230 MSDWFATYTTA/ MSDWFGTYTTA/ MSDWFGTYTTA/ MSDWWGTYTSKE MSDWFGTYTANI SDWFGTYSKE MSDWFGTYSKE MSDWFGTYSKE MSDWFGTYSTA/ MSDWFGTYTTA/	240 A SI K A SI K A AI K NAI E EAI E NAI E CAI E DAI N A SI K A SI K	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
	NGLDLEFPGPTRW	RTNELVSHSLNS	REQISIKOVO	DRVRQVLKLI	KEVVONQEKT	GI VENGPETT	ISNNTKETAELL	RKLA	Majority
240 238 240 235 240 240 238 240 238 240	250 NGL DI EFP GPT RWF NGL DI EFP GPT RWF NGL DI EFP GPT RWF A GL DL EMP GPT RFF NGL DL EMP GPT RFF NGL DL EMP GSPT FF A GL DL EMP GPT RWF NGL DI EFP GPT RWF	260 RTNELVSHSLNS RTNELVSHSLNS RTRALVSHSLNS RKLTEI RSMVV1 RNLTEI RSMVV1 RNKQLTSMIKS RTNELVSHSLNS RTNELVSHSLNS	270 SREQI SI HDVD SREQI SI HDVD SREQI TTEDVD TKE-LHI KHI D TKE-LHI KHI D SKE-LHI KHI D SKE-LHI KHI D SKE-LHI KHI D SKEQI SI HDVD SREQI SI YDVD	280 DRVRQVLKM DRVRQVLKM DRVRQVLKM DRVRQVLKLI DRVRQVLKLI DRVRQVLKLI DRVRQVLKM DRVRQVLKM	290 KFVVDNQEKT KFVVDNLEKT KYALQSG1 SYASQSG1 KYALQSG1 KFAKQSSV INFVEPL KFVVDNQEKT KFVLDNQEKT	300 GI VQNGPET GI VQNGPES GI VENGPES - PENAPED - PENAPED - PENAPES GI PENAPES GI PENAPES GI VQNGPET GI VQNGPET	310 ISNNSKETAELL ISNNTKETSELL ISNNTKETSELL ILNNTPETRKLL SENNTQETRKLL SENNTQETROLL ISNNSKETAELL ISNNSKETSELL	32 RKI A RKI A RKI A RKI A RKI A RKI A RKI A	0 P. etchellsii K. Iragilis P. stipitis P. guilliermondii D. hansenii C. albican A. clavatus K. lactis 765 aa K. lactis 630 aa
	ADSI VLLKNENNLI	. PLKKEE	- SI VVI GPNA	KAAASSGGG	SASENAYYVI S	PYEGI VAK	VGKEV	PYTY	Majority
320 318 315 310 315 317 311 320	330 ADSI VLLKNENSVI ADSI VLLKNENSVI HDSVVLLKNEDNLI NESI VLLKNEDNLI QDSI VLLKNEDNLI AESVVLMKNEDNI I	340 PLKKEE PLKKEE PLSKDE PLKSSE PLKKE PLKKE	350 SI VII GPNA QYHVI GPNA QYHVI GPNA KI AVI GPNA KI AVI GPNA SKSI AII GPNA KI VI GPNA SI VII GPNA	360 KAKVSSGGG KAKSSGGG KYAAYCGGG KYAAYCGGG KYAAYCGGG KIAAYSGGG KTAAYCGGG KAKVSSGGG	370 SASLHSYYVIS SASVNSYYVIS SASNNSYYVVS SASLRAYYTTT SASLRAYYTTT SASLRAYYTTT SASLDAYYTVA SASLHSYYVIS	380 PYEGI VNK PYEGI VKK PYEGI VNK PYEGI CSKLI PYEGI CSKLI PYEGI VNK- PFEGI VNK	390 VGKEN LGKEN 3TQF 3TQF 3TQF SISKFDITSQL 5G	40 PYTL DYTV DYTV DYTV PYTV CYTV KYTI SFSQ PYTL	0 P. etchellsii K. fragilis P. stipitis P. guilliermondii D. hansenii C. albican A. clavatus K. lactis 765 aa
318	ADSI VLLKNENSI L	PLKKEE	- SVVVI GPNA	KAKASSGGGS	SASMNSYYVI S	РҮЕСІ VKK	VGKEN	РҮТІ УЛ ТІ	k. lactis 630 aa
386 384 381 375 381 397 377 386 384	GAYSHKTLPNLAE(410 GAESHKTLSNLIE(GAESHKTLSNLIE(GAYSHKSIGGLAES GAYGHRLLPGLAA GAYGHRLLPGLAA GAYGHRLLPGLAA GAKAYKYLPELGP(GYSYNELPVLGPL GAESHKTLSNLIE(420 420 2L V V DP S K P A E (2L V V DP S K P A E (3 S L I D A A K P A D) VL V NPI 1 2L V NPI 1 2V NPI 1 2V NPI 1 2V NPI 1 2U K T E E 2L V V DP S K P A E (2L V V DP S K P A E (430 GDNV GAAAY FY GDNA GATGSFY AENAGLI AKFY GKP GY NCKFY FGKP GY NCKFY FGKP GY NCKFY FGKP GFSMKFY GKP GFSMKFY GKP GFSMKFY GDNV GAAAY FY GDNA GATGFFY	<u>SEPVEVRSP(</u> 440 NEPAGARPQ(SEPVEKRAK(SNPVEERSE(RE- TVGSPE RE- TVGSPE KKPKSVPNE(NEPSS- NP) NEPAGARPQ(SEPVEKRAK(460 3KSPFHVTTFK DESPFHVTKVN ERTLI DEYNLD KSQFDEI NTL KSQFDEI NTLD RTLI DEYNLD RTLI DEYNLD RELEDEL NLE SKSPFHVAAFK	H <u>SHILLEDEH</u> 460 HSHNMLEDEH HSENLLEDEH RSNVHLEDEH SYILLVDY- ISYILLVDY- ISYILLVDY- ISDILLGDY- NSLGELMDY- HSHNMLEDEH HSENLLEDEH	KHEKI DASNPLF 470 (HEKI DSSNPLF (HEKI DTTNP) F (HEKI DTTNP) F (HEKI DTNNPYF YN- DL APDSYF YN- DL APDSYF YHKDI PSNGLY (HPKI DSSNPLF (HDKI DTNNPLF	• YI TL 48 • YI TL • YI TL • FV TL • FV DF • YI DV • YI DF • YI DF • YI DF • YI DF • YI TL • YI TL	_ Majority 0 P. etchellsii K. lactis 845 aa K. fragilis P. suilliermondii D. hansenii C. albican I. A. clavatus K. lactis 766 aa K. lactis 630 aa
	EGYFTPEEDANYEF(<u>SLQVYGTGLLYI</u> 500	<u>DDEL VVDNKK</u> 510	NQTRGSFFF	<u>530</u> 530	<u>TLQKGKTYK</u> 540	VRIEYGSGPTS 550	TLVS 560	Majority
466 464 464 452 443 452 471 447 469	EGYFTPEEDANYLFC EGYFTPEEDANYLFC TGQYVPQEDGDYIFS EGEFTPDETAEYEFC EGETPDETAEYEFC EGEFTPDETAEYEFC EGEFTPSKTQHYEFC EGYFTPEEDGIYDFC	SUPPORT SLQVYGTGILYL SLQVYGSGLFYL SASVQGTALIYY SVAVWGTAKLFI SASVQGTALIYY SLTVHGTAQLFI SVTVQGTQLFI	DELVVDQKK DELLIDQKK NDELIDQKK NDELIDQK NDKLVVDNKT NDKLVVDNKT DDKLVVDNKT DGELVVDNKT	GQVSGDFCFI (GQVSGDFCFI INQERGSFCFI (NQVRGPSFTI (NQVRGPSFTI (NQVRGPSFTI (NQRGGTSFFI (NQRQGTSFFI (NQRQGTSFFI	GAGTDEKTKTV GAGTLEKTKTV GAGTKERTKKL NSGSAEEKGTL NSGSAEEKGTL NSGSAEEKGTL NSGSIEERGSI GNATVEEKGSK GNATVEEKGSK	SLQKGKAYK TLQKGKAYK TLKKGQVYN LLEKGKTYK ILEKGKTYK ELHQGKTYK ELHQGKTYK SLQKQVAY	VRI EYGSGPTSI VRI EYGSGPTSI VRI EFGSGPTSI VRI EFGSGPTFI LKI EFGSGPTFI VRI EFGSGPTFI I VEYGSAPTFI VVVEFGSAPTSI VBI EVGSGPTSI	ELVS ELVS GLVG TCRQ TIKS TCRQ TLKD DLDM	P. etchellsii K. lactis 845 aa K. fragilis P. stipitis P. guilliermondii D. hansenii C. albican A. clavatus K. lacei: 205

444 EGYFTPKEDANYLFGLQVYGTGLLYLDDELVVDQKKDQTRGSFCFGAGTDEKTKTVSLQKGKAYKVRIEYGSGPTSELVS K. lavtis 765 ag
464 EGYFTPEEDANYIFGLQVYGTGLLYLDEELUIDQKKGQTRGGFCFGAGTNEKTKTVTLQKGKSYKVRIEYGSGPTSQLVS k. lavtis 630 aa

	EFGGGGI Q	GVAKAL - DADEE	I RKAAKLAA	GHDKAVLCI	GLNAEWESEG	HOREDMELP	GRTNDL VRAVL	EANPNT	Majority
	570	580	590	600	610	620	630	640	
546 544	EFGSGGFQ	GVAKAL - DADEE	I RKAAKLAA		GLNAEWESEG		GRTNDLVRAVL	EANPNT	P. etchellsii K. Iactic 845 aa
544	EFGAGGEQ/	AGVI KAL - DDDEE	RNAAELAA	KHDKAVLII	GLNGEWETEG	SYDRENMOL P	KRTNEL VRAVL	KANPNT	K. fragilis
532 522	EGSTVVAGGGGI NU	GMAKVI - DPELE	I HKAAKLAK Lanavslak	EADKVVLNI SVDKVVLCI	GLNQEWEAEG	SFORPOMEL V	GYQNKLI DAVL	AANPNT	P. stipitis P. quillierroopdii
532	EGSTVVAGGGGI NL	. GMAKVI - DPELE	I HKAAKLAK	EADKVVLNI	GLNQEWEAEG	SFORPOMEL V	GYQNKLI DAVL	AANPNT	D. hansenii
551 527	QVGEYFGGGI RL BGYYYE-GPGGERE	. GMNELL NDDEQE FGAARRY - GQEEL	I I NAVNLAK I SKAAFLAS	SVDLVILII GADGVVIFA	GLNKDWESES	SYDRPDMKLP SHORDHMDLP	GLQDKLIESVL Agsdemisrvl	DVNPNT	C. albican A. clayatus
546	EFGSGGFQ\	GVAKAI - DADEE	RKAAKLAA	GHDKAI L CI	GLNAEWESEG	SHDREDMILP	GRTNDL VRAVL	EANPNT	K. lactis 765 aa
544	EFGAGAL QI	GVAKAI - DADEE	I KKAAKLAA	GHDKAI L CI	GLNAEWESEG	SHOREDMTLP	GRENDLVRAVL	EANPNT	k. lactis 630 aa
	VI VNQSGTPVEFP-	WLEKANALLQAW	Y GGNEL GNA	I ADVLYGDU	NPSGKLSLSV	MPLKLEDNPT	YLNFKTERGRV	LYGEDI	Majority
	650	660	670	680	690	700	710	720	
620 618	VIINQSGTPVEFP- VIVNQSGTPVEFP-	·WWEQANALLQAW ·WUQKANALVQAW	Y GGNEL GNA Y GGNEL GNA	I ADVLYGD\ I ADVLYGD\	/VPGGKLSLSV /VPNGKLSLSV	MPLKLEDNPT MPLKLEDNPA	YLNFKTEFGRV Ylnfktefgrv	LYGEDI Vygedi	P. etchellsii K. lactis 845 aa
618	VI VNQSGTPVEFP-	WLEEANALVQAW	Y GGNEL GNA	I ADVLYGDV	(VPNGKLSLSV	MPFKLQDNPA	FLNFKTEFGRV	VYGEDI	K. fragilis
611 601	IVVNQSGTPVEMP-	·WIKKAPAVIQAW	YGGNESGNG YGGNETGNS	I ADVLEGDU I ADVLEGDE	NPSGKLSLIF NPCGKLSLIV	MPLKVQDNPT	FLNFRTEKGRV	LYNEDI Lygedv	P. stipitis P. guilliermondii
611 620	VI VNQSGTPVEMP	WEPKAKAVEQAW	YGGNESGNG	I ADVLEGD	(NPSGKLSLTF	PERTI DNPT	YLNEKTERGRV	LYNEDI	D. hansenii C. albiana
605	VVVIQSGTPVEFSE	·WAHKTKALLQAW	FGGNESGNA	I ADVLYGDU	NPNGKESETF NPSAKEPESF	PVRLQDNPS	YLNFRSERGRV	LYGEDI	C. albican A. clavatus
620 649	VIINQSGTPVEFP-	WVEQANALLQAW	Y GGNEL GNA	I ADVLYGDV	/VPGGKLSLSV	MPLKLEDNPT	YLNFKTEFGRV	LYGEDI	K. lactis 765 aa k. lactis 820 aa
010	OF UNQUOIF OFF								K. Iacus 030 aa
	FVGYRFYEKLORR	/AFPFGFGLSYTE	FELSDLQVG	QV	DEVISV:	SVTVKNTG-H	<pre>vagsevvqvyi</pre>	AATESD	Majority
	730	740	750	760	770	780	790	80(N
c00				TD ITD	EEVI OV				
697	FIGYRFYEKLOKR	VAFPFGYGLSYTE	FALSNLQVG	N	DEVI SV:	SVDVKNTGE	(YAGSEVVQVYI	AATESS	K. lactis 845 aa
697	FVGYRYYEKLORK	VAFPFGYGLSYTT	FELDI SDFK	VT	DDKI DI	SVDVKNTGD	<pre><pre><pre><pre><pre><pre><pre><pre></pre></pre></pre></pre></pre></pre></pre></pre>	FSALNSK	K. fragilis
690	FVGYRFYEKMGRD	VAFPFGFGLSYTN	FEFADVNVV	/ V	- EEL DDNL EV	SVTVSNTG-H	KVDGAEVVQI YI	GKEDSD	P. stipitis
680	FI GYRYYEKLQKQ	VAFPFGFGLSYSE	FVFSNLKV-		NTQDNLKV	TVDVENVG-0	QVAGSETVQVY1	VAPQNST	P. guilliermondii
690 709	EVOVEVENT NROY	VAFPFGFGLSTIN Vaepegegievtv	FEFADUNUU FEFEDI VIE		- EEL DUNLEU: EEEEEESI VV	SULUSNIG-1	(VDGAEVVQIY) (Mtgseviovy)	USKEDSD VSKVESD	D. nansenii C. albican
684	YVGYRYYEKVDLAF	PLEPEGHGLSYTT	FSRSDLSLA	TVPEKRQL	EDGEPITA	TVTVTNTGD-	VAGAEVVQL W	VPPPTG	A. clavatus
699	FI GYRFYEKLORRI	VAFPFGYGLSYTE	FALSHLQVG	ΩTD	EEVI SV:	SVNVKNTGE	EYAGI		K. lactis 765 aa
630									k. lactis 630 aa
	VIRPVKELKGEEK	VELOPGETETVII	DLILKDSVS	FEDEYQGK	WCVEAGEYKV		VLIGSEVVEKT:	SYWIGL	Majority
	910	<u>820</u>	920	940		960	970		
700						LVETCODELY		e v wriei	D. stabollaŭ
767	VSRAVKELKGEKKI	(LLQPGQNETAK)	DEVEKDSVS	FFDEEVGK	WESEAGQYKVI	LVGTSSDOLV	VLSESFDVEKT:	SYMSGL	K. lactis 845 aa
767	VSRPVKELKGFEK	HLEPGEKKTVNI	ELELKDAI S	YFNEELGK	WHVEAGEYLV	SVGTSSDDI I	SVKEFKVEKDI	LYWKGL	K. fragilis
761	VI RPVKELKGFEK)	VFLKAGTQETVIS	TLSLKESVS	FFDEYQEK	WSVLAGEYQV	YVGNSSDNA	NAL GTEVLERD	FLWIGR	P. stipitis
748	I RPVKELKGFEK)	VYLKPGEKVTVHL	DMAMSDSIS	FFDEYQKA	WCAEKGDYQV	QVGTSSDDI B	ELI GEFKVAATI	<ΥWΚ	P. guilliermondii
761	VI RPVKELKGFEK)	VFLKAGTQETVIS	TLSLKESVS	FFDEYQEK	WSVLAGEYQV	YVGNSSDNA1	NALGTEVI ERDI		D. hansenii C. albicaa
788	VIRPIKELKGEIK)	VELNEGESKTVEL	KULI KUSVS VVEKKLATS	CHECKANQ CMMDEORE	WOYQSGQYKVI WASEKGTYPYI	HUGNSSUNI H	LIESEILEKS Iksseevevti	REWUGL	u. aibican A clavatus
756	RSRTNLH		N A C C M C A I G		- CCD		EKOOLEVENII	A DE DE	K. lactis 765 aa
630									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

.

	1	2	3	4	5	6	7	8		
1		89.1	73.4	52.6	49.4	54.4	89.4	98.6	1	P. etchellsii 847 aa protein
2	11.8		75.3	54.2	50.5	54.6	93.8	87.6	2	K. lactis 845 aa
3	32.9	30.1		52.0	48.4	52.2	75.9	73.7	3	K. fragilis 845 aa. pro
4	63.1	59.8	64.6		55.8	65.0	51.0	52.6	4	P. stipitis 738 aa
5	68.6	65.8	72.0	52.9		56.5	47.1	48.0	5	C. albican 866 aa
6	62.4	61.3	66.1	43.3	51.7		53.2	54.0	6	D. hansenii 845 aa
7	11.5	6.5	29.2	64.5	73.4	62.0		89.4	7	k. lactis 630 aa
8	1.1	12.9	31.7	63.1	69.3	61.5	11.5		8	K. lactis 765 aa
	1	2	3	4	5	6	7	8		

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



A. 2:



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 GS115pPIC9Bgl1-5 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 Hg²⁺ 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 cellobiosefermenting 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 βglucosidase 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 6th 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 bgll 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.

Acknowledgements

Sincere thanks to Prof. Saroj Mishra, Department of Biochemical Engineering and Biotechnology, IIT Delhi for her valuable guidance during this research work. The institutional Doctoral fellowship awarded to author is sincerely acknowledged.

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