Cloning and Expression of β-Glucosidase Gene from the Yeast *Pichia etchellsii*

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A 4.8-kilobase pairs DNA fragment from thermophilic yeast *Pichia etchellsii* was cloned into the vector plasmid pUC19 to form plasmid pBG55 and the encoded β-glucosidase expressed in *Escherichia coli*. The effect of different carbon sources on growth and enzyme synthesis was studied in the pBG55 transformant and 0.2% (w/v) cellobiose found to be the most suitable carbon source for enzyme biosynthesis. The level of intracellularly produced β-glucosidase was slightly reduced on 0.2% (w/v) glucose and 0.2% (w/v) maltose. The partially purified enzyme from the *β-glu* transformant was active against a wide range of aryl β-glucosides and β-linked disaccharides and the preferred substrates were *p*-nitrophenyl-β-D-glucoside (*p*NPG), cellobiose, gentiobiose, sophorose and sucrose. While maximum enzyme activity of 62 U/l was against *p*NPG at 50°C, the activities in the range of 120–170 U/l were against various β-linked disaccharides at 37°C. The enzyme displayed glucose tolerance and a temperature optima profile slightly different from that exhibited by the native yeast glycosylated enzyme. The β-glucosidase in the crude extract of pBG55 transformant was identified as a stably produced protein of 200 kDa by PAGE-Zymogram analysis.

[**Key words:** cloning, β-glucosidase expression, *Pichia etchellsii* β-glucosidase]

The enzyme β-glucosidase (EC 3.2.1.21) is an ubiquitous enzyme occurring in several microbial, plant and animal cell systems. It catalyzes scission of β-1,4-glucosidic linkage in cellobiose, short-chain cello-oligosaccharides (degree of polymerization, 3–8), aryl β-D-glucosides (such as *p*-nitrophenyl-β-D-glucoside) and alkyl glucosides (such as methyl β-D-glucoside) (1). Under controlled reaction conditions, the enzyme also displays transglycosylase and alkyl transferase activities leading to synthesis of short-chain oligosaccharides and alkyl glucosides (2). In cellulolytic micro-organisms, the enzyme is believed to play an important role in the induction of cellulase enzymes (3, 4) and cellulose hydrolysis (5) during which the breakdown of cellobiose and short-chain dextrins is catalyzed by the enzyme. Thermostable and glucose tolerant enzymes that enable continuous cellulose hydrolysis around 45–50°C are preferred in such bioconversions. In some plants, this enzyme is involved in the catabolism of glycosides of various flavanones and anthocyanins that constitute pigments in flowers, while in some other plants, β-glucosidases are involved in hydrolysis of glycoside arbutin (3-hydroxy phenyl β-D-glucoside) releasing hydroxyphenol which is toxic to the invading organism. The lysosomal β-D-glucosidase in cells of the reticuloendothelial system is essential for normal catabolism of glucosyl ceramides (For review, see 6). The deficiency of this enzyme in endothelial cells results in accumulation of excess glucosyl ceramides which causes organs such as the spleen, liver and lymph nodes to enlarge (Gaucher's disease) (7).

A number of bacterial, yeast, and fungal β-glucosidase genes have been cloned and expressed either in the bacterium *Escherichia coli* or the yeast *Saccharomyces cerevisiae* (For review, see 5) with the intent to produce the enzyme for use in cellulose saccharification. Relatively fewer reports have appeared on the molecular cloning and characterization of β-glucosidase from thermophilic organisms (8–13). In an attempt to identify a β-glucosidase that has a temperature optimum and increased stability around 45–50°C, as well as being glucose resistant over the various glucose concentrations generated during cellulose saccharification, we have screened a number of bacterial and yeast systems and identified the yeast *Pichia etchellsii* as the producer of the desired β-glucosidase. The enzyme in this yeast was found to be cell bound, glycosylated (35%, w/w) and exhibited increased stability at 45°C. The native enzyme retained 40% activity in the presence of up to 2% (w/v) glucose (Pandey, M. *et al.*, Abstr. Natl. Conf. Fungal Biotech., Bhopal, India, p. 28, 1994).

As part of a long term strategy to produce the enzyme on a large scale and to understand the properties of the enzyme at the molecular level, we report on the cloning and expression of the gene *β-glu*, encoding the *P. etchellsii* β-glucosidase, in *E. coli*. The inter-relationship between enzyme biosynthesis and host growth properties was investigated on different carbon sources. The partially purified enzyme from recombinant *E. coli* was characterized in terms of substrate specificity, glucose tolerance and a temperature optima profile.

MATERIALS AND METHODS

Strains, plasmids and culture conditions *P. etchellsii* (Deutsche Sammlung Von Mikroorganismen (DSM), Germany) was used as the β-glucosidase gene donor, *E. coli* C600 and XL1-Blue as cloning hosts and YEp13 (*E. coli*-yeast shuttle vector) and pUC19 as cloning vectors. *P. etchellsii* was grown in YPD (0.5% yeast extract, 1% bacto-peptone, 2% D-glucose) medium. *E. coli* was grown in LB or M9CA synthetic medium at 37°C according to standard protocols (14). For *E. coli* transformants, ampicillin at 50 μg/ml was added to the medium.

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Cloning of β-glucosidase gene Chromosomal DNA from *P. etchellsii* was prepared according to Cregg *et al.* (15) with some modifications. The yeast sphaeroplasts were prepared by incubation of the exponentially grown cells of *P. etchellsii* in YPD with 1.5% Zymolyase 30,000 (Miles Inc., Elkhart, Ind., USA; gift from Prof. A. Dutta, JNU, New-Delhi, India). The DNA was spooled out on a glass rod after alkaline lysis of the sphaeroplasts (15). Chromosomal DNA of *E. coli* C600 and XL1-Blue were prepared according to Maniatis *et al.* (14). Large scale YEp13 (kind gift from Prof. Michels, CUNY, NY, USA) and pUC19 plasmid DNA preparations were made by alkaline lysis method followed by centrifugation to equilibrium in a cesium chloride-ethidium bromide density gradient. Small scale plasmid DNA and competent cells of *E. coli* C600 and XL1-Blue were prepared by the standard procedures (14).

P. etchellsii chromosomal DNA was partially digested with *Sau*3A to yield fragment sizes of 5–20 kbp size. The appropriate 5–15 kbp size fragments were obtained from a 5–20% sucrose density gradient centrifugation as described (14). These were ligated to dephosphorylated YEp13 and pUC19 at the *Bam*HI site. The recombinant plasmids were transformed into competent *E. coli* C600 (for YEp13 derived plasmids) or XL1-Blue (for pUC19 derived plasmids) cells. Test transformations performed with the plasmids gave a transformation efficiency of 2×10^6 transformants/μg of DNA. The size of the insert DNA was between 4–15 kbp in recombinant plasmids.

Transformants were cultivated overnight on LB medium containing ampicillin at 37°C. All white colonies were grid on LB+amp and chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-glu; Sigma Chemical Co., MO, USA), 50 $\mu g \cdot ml^{-1}$ and the plates incubated overnight at 37°C. These were incubated for an additional 2–3 h at 50°C and colonies appearing blue selected. The β-glucosidase positive transformants on X-glu were further plated on LB plus ampicillin plates covered with 1 mM 4-methyl umbelliferyl-β-D-glucopyranoside (MUG; Sigma Chemical Co.) at 50°C. Hydrolysis of MUG by the cloned β-glucosidase resulted in the release of 4-methylumbelliferone, a fluoroscent compound detectable under UV-transilluminator.

Southern hybridizations Chromosomal DNA of *P. etchellsii* and *E. coli* were digested to completion with *Bam*HI, electrophoresed along with *Bam*HI cut pBG5 and pBG55 and separated by horizontal gel electrophoresis. The DNA was blotted on to Hybond N membrane (Schleicher and Schuell). The electroeluted intragenic *Hin*dIII fragments from pBG55 labelled by the nick-translation method in the presence of α-^{32}P dCTP (BARC, Bombay, India) served as the probe. Hybridization of the DNA on the membrane with the probe was according to standard protocols (14).

Influence of carbon source on growth and enzyme activity The influence of carbon source on growth and enzyme biosynthesis was investigated for the transformants of C600 with pBG5 and XL1-Blue with pBG55 in M9CA synthetic medium (14). Five hundred ml flasks containing 200 ml of growth medium supplemented with required sugar concentration (0.2% and 1% w/v) were inoculated to 10% with an overnight grown inoculum in the same medium. Flasks were removed at suitable time intervals, in duplicate, for measurement of growth, by measuring turbidity at O.D. 610 nm, and enzyme activity. The O.D. values were converted to mg cell dry wt from a standard plot. The entire contents of the flasks were centrifuged (6,000 *g*, 10 min, 4°C) and the cells concentrated to about 1/10 of the original volume in 50 mM sodium phosphate buffer, pH 7.0. The cell suspension was sonicated in Soniprep at maximum power output for 10 min and then centrifuged for 30 min (15,000 *g*, 4°C) to remove the cellular debris. The cell free extract was stored at 4°C and used to measure enzyme activity. The β-glucosidase was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside (16). One unit of enzyme activity was defined as the quantity that released one μmol of *p*-nitrophenol/min/ml of reaction mixture. Protein concentration was estimated in the crude extract by Lowry's method using Bovine Serum Albumin as the standard. The β-lactamase activity in the crude extract of the transformants was measured according to standard methods (17).

The overall specific growth rate (μ) for each sugar was computed from the slopes of ln cell dry wt (x) vs. t plot. The instantaneous specific growth rate was determined from $(1/X \cdot dX/dt)$ computed at different time intervals. The specific product formation rate, here called the enzyme activity, was computed from $(1/X \cdot dE/dt)$ at different time intervals.

Substrate specificity of the cloned β-glucosidase The substrate specificity of the cloned β-glucosidase was measured in the partially purified enzyme preparation obtained from the transformant of XL1-Blue with pBG55. The crude-extract (prepared as described above) was treated with 1% streptomycin sulfate to remove the nucleic acids. The sample was centrifuged at 8,000 *g* for 30 min at 4°C and the clear supernatant was given an ammonium sulphate cut off of 30–90%. The protein precipitate obtained therein was resuspended in 50 mM sodium phosphate buffer, pH 7.0. The salts were removed and the sample concentrated by ultrafiltration in an Amicon cell with a PM10 membrane.

The substrate specificity of the partially purified enzyme (as described above) was determined by measuring (i) the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside (aryl-β-glucoside) and *p*-nitrophenyl-β-D-xylopyranoside and (ii) the glucose released from cellobiose, gentiobiose, lactose, sophorose, sucrose, salicin and carboxymethyl cellulose. The listed substrates were from Sigma Chem. Co. The enzyme assays were performed in PC buffer, pH 6.5 according to Ait *et al.* (16). The glucose released was estimated by glucose oxidase-peroxidase kit (Ranbaxy Laboratories, New-Delhi). One unit of enzyme activity is defined as the release of 1 μmol of *p*-nitrophenol (in the 1st case) or 1 μmol of glucose (in the 2nd case)/min/ml of reaction volume. The protein concentration in all assays was held constant at 15 $mg \cdot ml^{-1}$.

The partially purified enzyme from *P. etchellsii* was obtained in the following manner. The yeast cells were grown overnight in phosphate-citrate medium (18) supplemented with 0.075 M cellobiose and harvested. The cells were broken with glass beads in Dynomill (30 min, 4°C). The resulting cell suspension was centrifuged at 10,000 *g* for 30 min at 4°C. To the clear supernatant, streptomycin sulfate was added to 1% (w/v) and mixture stirred for 4 h at 0°C. The nucleic acids were removed by centrifugation and, to the supernatant, an ammonium sulphate cut off of 40–90% given. The precipitated enzyme was dissolved in 50 mM sodium phosphate buffer, pH 7.0, extensively dialysed against the same

buffer and concentrated by ultrafiltration using PM10 membrane in an Amicon cell. The protein was purified about twelve fold.

The enzyme from recombinant *E. coli* (prepared as described above) and *P. etchellsii* were investigated for temperature optima and tolerance to externally added glucose. All assay conditions were the same as described above.

PAGE-zymogram The partially purified enzyme from recombinant *E. coli*, obtained as described above, (in 200 μg total protein), was electrophoresed on 10% native polyacrylamide gel in Tris-Glycine buffer, pH 8.4 according to standard procedures (19). Half of the gel was stained with Coomassie Blue and destained according to standard methods and the other half was incubated with 4 mM PNPG solution prepared in PC buffer at 50°C for 30 min. One molar Na_2CO_3 was then added to stop the reaction. A single clear yellow band defining the location of the β-glucosidase was observed on a clear gel background.

RESULTS

Cloning of β-glucosidase gene The hosts *E. coli* C600 and XL1-Blue do not produce detectable β-glucosidase activity. This was determined by the plate assay method and measurement of enzyme activity in the crude extract of the two strains. Our initial attempts to clone and express the *P. etchellsii* β-glucosidase gene using *E. coli*-yeast shuttle vector, YEp13 in *E. coli* C600 were successful with maximum activity of 20 U·l^{-1} obtained on LB plus ampicillin medium in the β-glucosidase transformant pBG5 : C600. However, the large size of the plasmid (>25 kbp) and low copy number (1–2 plasmids per cell) in the pBG5 : C600 transformant, determined by measuring β-lactamase activity in the cell-free extract (1 U/ml as opposed to 51.6 U/ml for YEp13 : C600), resulted in low plasmid yield. Further cloning was done with pUC19 as the vector and XL1-Blue as the host strain. Out of approximately 1000 *E. coli* transformants selected by antibiotic marker, only one tested positive for β-glucosidase activity using the LB+Amp+X-glu plate assay. This transformant, pBG55 : XL1-Blue, also hydrolyzed 4 methyl umbelliferyl β-D-glucopyranoside (MUG) confirming positive β-glucosidase activity. The plasmid pBG55 was purified and in subsequent transformations, the β-glucosidase phenotype co-transformed with ampicillin resistance confirming the β-glucosidase phenotype to be plasmid borne. The restriction map of pBG55 is shown in Fig. 1 and it indicates the structural gene for β-glucosidase to be encoded on the 4.8 kbp insert. The relative copy number in this transformant was about 8–10 plasmids per cell.

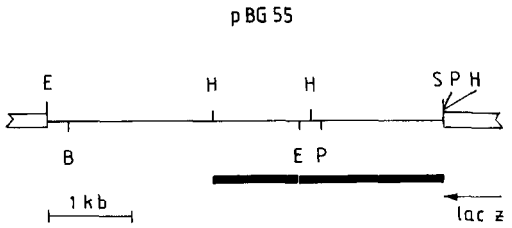

FIG. 1. Restriction map of the yeast insert encoding β-glucosidase activity in pBG55 transformant. The thin line is for *P. etchellsii* DNA and the clear boxed regions are of vector pUC19. B: *Bam*HI; E: *Eco*RI; H: *Hin*dIII; P: *Pst*I; S: *Sal*I. Intragenic *Hin*dIII fragments (both) used as probes for Southern hybridizations are indicated by the shaded bar. The orientation of transcription of *lacZ* in pUC19 is shown as an arrow.

The ^{32}P-labelled *Hin*dIII fragments (shown in Fig. 1) of the plasmid pBG55 were hybridized to the *Bam*HI digested *P. etchellsii* chromosomal DNA (Fig. 2A, lane 2), *Bam*HI treated pBG55 (Fig. 2A, lane 3), and *E. coli* XL1-Blue chromosomal DNA (Fig. 2A, lane 4) and the results shown in Fig. 2B indicate the probe to hybridize to a single yeast DNA fragment of about 5.0 kbp

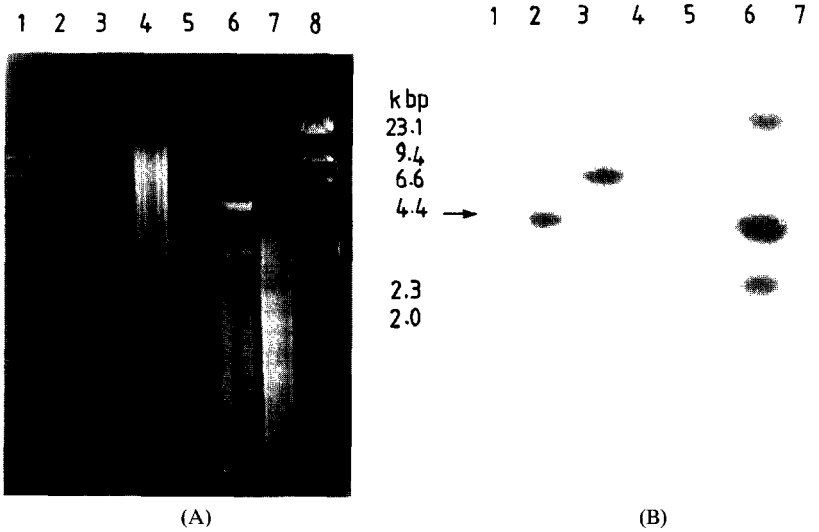

FIG. 2. Southern hybridization showing homology between cloned DNA and *P. etchellsii* chromosomal DNA digested with *Bam*HI. (A) Agarose gel electrophoretogram of different DNAs: lanes 1 and 8, λDNA digested with *Hin*dIII; lane 2, *P. etchellsii* chromosomal DNA digested with *Bam*HI; lane 3, pBG55 linearized with *Bam*HI; lane 4, *E. coli* XL1-Blue chromosomal DNA digested with *Bam*HI; lane 5, Blank; lane 6, pBG5 digested with *Bam*HI; lane 7, *E. coli* C600 chromosomal DNA digested with *Bam*HI. (B) Hybridized pattern from panel A. Arrow indicates position of the *Pichia* DNA inserted into pBG55.

confirming the origin of insert to be yeast DNA (Fig. 2B, lane 2). The same probe also hybridized to *Bam*HI digested pBG5 DNA (YEp13 derivative) and three DNA fragments, one of large molecular weight and two of 4.4 kbp and 2.5 kbp, were detected (Fig. 2B, lane 6). This indicated either insertion of multiple fragments into the vector or rearrangements of the insert DNA in the recombinant plasmid. No hybridization was observed between the yeast β-glucosidase gene and *E. coli* host DNA (Fig. 2B, lanes 4 and 7).

Growth and enzyme biosynthesis on different carbon sources Nearly 15% of the total β-glucosidase activity in the recombinant transformants pBG5 and pBG55 was localized to the *E. coli* cell surface while the remaining 85% of the activity was found intracellularly as a soluble enzyme. No activity was detected in the periplasmic space. The effect of various carbon sources viz. cellobiose, glucose and maltose (at 0.2% and 1% w/v) in M9CA synthetic medium was studied on growth and intracellular enzyme levels in pBG5 : C600 and pBG55 : XL1-Blue transformants and the results obtained on 0.2% carbon source are shown in Fig. 3 (panel A–C). The profile of cell growth and intracellular enzyme activity on 0.2% cellobiose was similar for both the transformants with maximum cell concentration of 1.76 mg/ml and 1.64 mg/ml obtained for pBG5 : C600 and pBG55 : XL1-Blue respectively (Fig. 3A). Maximum enzyme activities were also similar and were obtained at the 8th hour of growth. Matching growth profiles and intracellular β-glucosidase activity profiles were also obtained for the two transformants, bearing different copy numbers, on 0.2% glucose and 0.2% maltose and only the representative data for pBG55 : XL1-Blue is shown in Figs. 3B and 3C respectively. While a higher cell con-

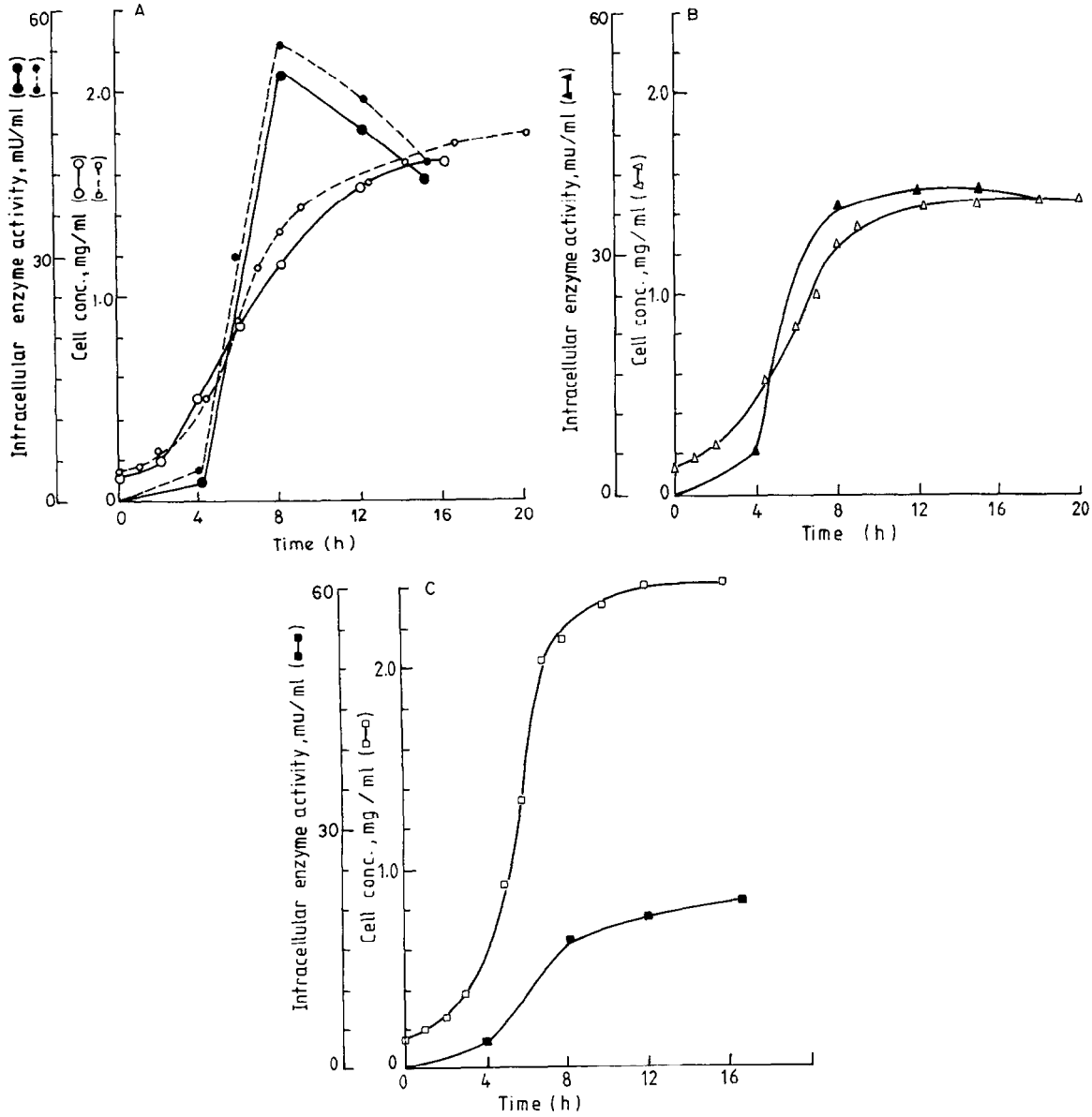

FIG. 3. Growth (open symbols) and enzyme activity (closed symbols) in transformant pBG5 (on 0.2% cellobiose, Panel A, broken lines) and pBG55 (on 0.2% cellobiose, Panel A; 0.2% glucose, Panel B; and 0.2% maltose, Panel C; all solid lines).

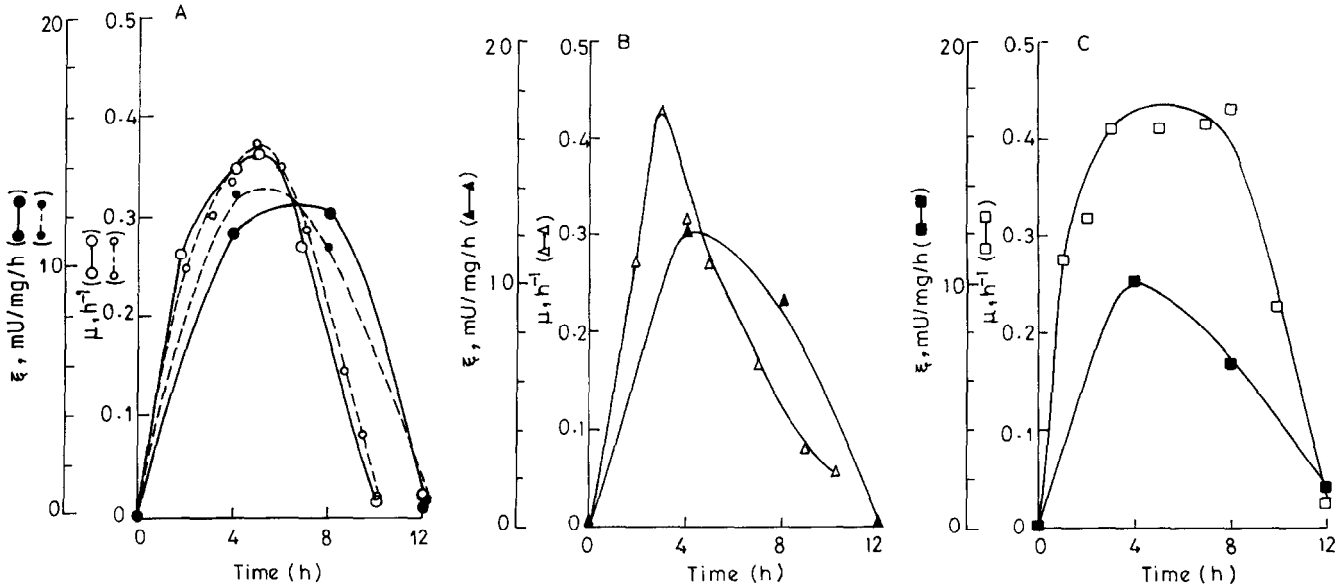

FIG. 4. Specific growth rate (μ) and specific enzyme formation rate (ε) plotted as a function of time for transformant pBG5 (on 0.2% cellobiose. Panel A; broken lines) and pBG55 (on 0.2% cellobiose, Panel A; 0.2% glucose, Panel B; and 0.2% maltose, Panel C; all solid lines).

centration of 2.4 mg/ml was observed on 0.2% maltose, growth was slightly less on 0.2% glucose. Maximum enzyme activity was obtained on 0.2% cellobiose supplemented medium. An increase in sugar concentration to 1% (w/v) did not lead to an increase in enzyme biosynthesis (data not shown).

The exact relationship between growth and enzyme levels produced is a specific property unique to the cells under study and is of importance for large scale enzyme production. This relationship was derived for the two transformants from the data of Fig. 3A. The resulting value for specific growth rate (μ) and enzyme biosynthesis rate (ε) was plotted as a function of time and the plots are shown in Fig. 4A. The results for pBG55 obtained on 0.2% glucose and 0.2% maltose are shown in Figs. 4B and 4C respectively. It is seen that for pBG5, as μ increased during the exponential phase, so did the enzyme biosynthesis rate and a growth associated enzyme profile was observed (Fig. 4A). A slight shift in enzyme biosynthesis rate was observed for pBG55 with maximum enzyme synthesis rate of 12 mU/mg/h obtained at the 8th hour of growth. The specific growth rate at this stage was considerably lower than the maximum specific growth rate of 0.37 h^{-1} (at 4 h). The period of maximum enzyme biosynthesis can be considered constant between 4 and 8 h of growth during which μ undergoes a change from 0.35 h^{-1} to 0.15 h^{-1}. Growth on glucose was rapid (Fig. 4B) with a maximum specific growth rate of 0.42 h^{-1} around 3 h while the maximum enzyme synthesis rate was at about 4 h. The enzyme biosynthesis was supported in a narrow zone of growth rates. A similar pattern was obtained for 0.2% maltose grown cells. In all cases, as cells entered into stationary phase, the enzyme production rate dropped dramatically.

Substrate specificity of the cloned β-glucosidase

The enzyme produced by pBG55 : XL1-Blue was partially purified (14 fold) and substrate specificity determined against a number of substrates. The results are shown in Table 1. The cloned β-glucosidase was more active on *p*-nitrophenyl-β-D-glucopyranoside at 50°C. Low activity was detected against *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-xylopyranoside. The enzyme displayed no activity against carboxymethylcellulose (CMC), lactose and salicin. Interestingly, amongst a number of β-linked disaccharides, activities were detected on cellobiose, gentiobiose, sophorose, and sucrose at 37°C. At the increased temperature of 50°C, no activity was detected on these substrates (Table 1). Thus, aryl-β-glucoside was preferentially hydrolyzed at 50°C, whereas the specific β-linked disaccharides were hydrolyzed at 37°C.

Effect of temperature on β-glucosidase activity

The influence of reaction temperature was investigated on β-glucosidase activity in the range of 30–70°C (Fig. 5). Interestingly, the partially purified enzyme obtained from recombinant pBG55 : XL1-Blue cells had temperature optimum marginally shifted to 50°C as opposed to the optimum of 45°C for the partially purified enzyme from native *P. etchellsii* cells. The rapid inactivation of enzyme from both sources occurred as the temperature increased to 55°C, beyond which the *E. coli* expressed enzyme activity was lost slowly. While 25% of the enzyme activity was retained for this enzyme at 60°C, the native enzyme was totally inactivated.

Influence of glucose on enzyme activity The enzyme activity was monitored in PC buffer, pH 6.5, in the presence of (0–110 mM, or 0–2%) glucose, and the residual activity expressed as a function of glucose concentration is shown in Fig. 6. The activity of the partially purified native yeast enzyme is also shown for comparison. At a glucose concentration up to 30 mM, the native yeast enzyme was inactivated more rapidly and only 48% of the activity remained. Further loss in enzyme activity was slow as glucose concentration increased to 110 mM. The β-glucosidase expressed in pBG55 : XL1-Blue was inactivated more slowly and residual activity of 63% was observed in the presence of 45 mM glucose (Fig. 6). A further increase in glucose concentration to 110 mM, resulted in final levelling off of enzyme activity

TABLE 1. Substrate specificity of the β-glucosidase activity in partially purified enzyme preparation from the clone pBG55 *β-glu*

Substrate	Enzyme activity (mU/ml)	
	37°C	50°C
p-Nitrophenyl-β-D-galactoside	2.8	11.1
p-Nitrophenyl-β-D-glucoside	36.8	62.0
p-Nitrophenyl-β-D-xyloside	7.1	19.3
Salicin [2-(hydroxymethyl)phenyl-β-D-glucoside]	0.3	—
Carboxymethyl cellulose	—	—
Cellobiose (Glcβ1-4Glc)	160.0	—
Gentiobiose (Glcβ1-6Glc)	120.0	—
Lactose (Galβ1-4Glc)	—	—
Sophorose (Glcβ1-2Glc)	160.0	—
Sucrose (Fruβ2-1Glc)	170.0	—

The protein concentration in the assay mixture was 15 mg/ml.
—, No detectable activity.

at 58%.

PAGE-zymogram The partially purified enzyme was subjected to native PAGE and β-glucosidase enzyme localized on the gel by activity staining. A single band at 200 kDa position marked the presence of β-glucosidase in the preparation (data not shown).

DISCUSSION

There are three β-glucosidases classified on the basis of substrate specificity. Aryl β-glucosidase hydrolyzing aryl β-glucoside linkage as found in *p*-nitrophenyl-β-D-glucosides, true cellobiases capable of hydrolyzing a β-1-4-glucosidic linkage as in cellobiose and short chain cellodextrins, and members of the third group classified under broad substrate specificity β-glucosidases and these display significant activity on both of the above categories of substrates. These enzymes from thermophilic microbes would be more versatile from application point of view. In an attempt to study a β-glucosidase with broad substrate specificity, we have initiated studies on a β-glucosidase from a thermophilic yeast *P. etchellsii*. The enzyme in the native yeast was cell bound, 35% w/w glycosylated with a molecular weight of approximately 400 kDa. The purification of the enzyme to homogeneity from the yeast was met with a number of difficulties on account of aggregation of the protein due to heavy glycosylation. Hence, molecular biological investigations were initiated to clone and express the structural gene in *E. coli* to obtain a pure protein for study of its function.

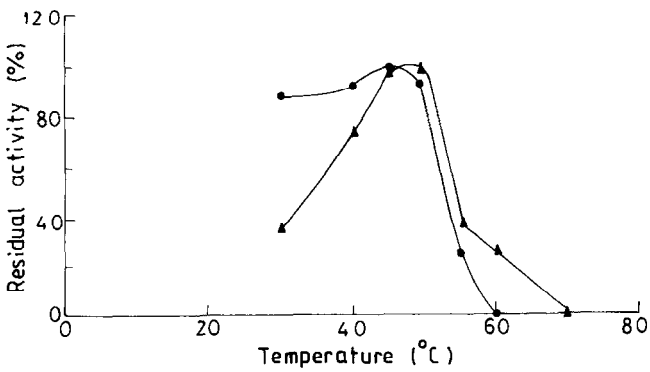

FIG. 5. Influence of temperature on β-glucosidase activity; (●), native *P. etchellsii* enzyme; (▲), β-glucosidase in pBG55 transformant.

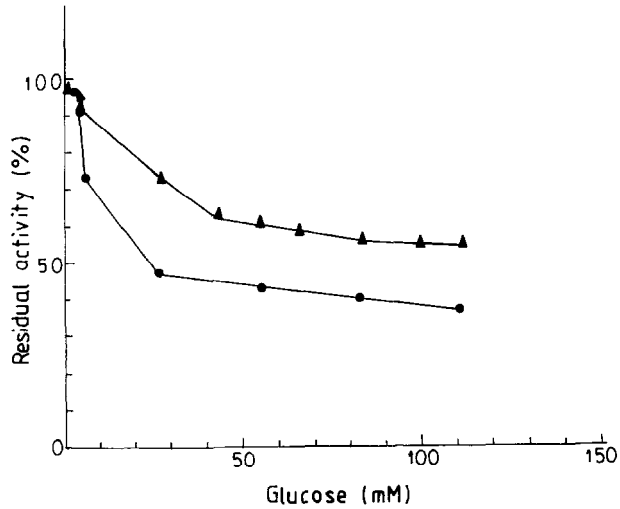

FIG. 6. Influence of glucose (0–110 mM) on β-glucosidase activity; (●), native *P. etchellsii* enzyme; (▲), β-glucosidase in pBG55 transformant.

The recombinant β-glucosidase producing clone pBG55 : XL1-Blue was selected (pUC19 derivative) although work with *E. coli*-yeast shuttle vector YEp13 resulted in the selection of the pBG5 transformant. While the levels of intracellular β-glucosidase were similar for these transformants, the recovery of the plasmid was very poor from the pBG5 transformant. The β-glucosidase activity was encoded on a 4.8 kbp DNA fragment which is large enough to contain information for a large polypeptide chain. Our work suggests the enzyme to be multimeric in nature and this is also reported for other yeast β-glucosidases such as that from *Candida pelliculosa* (20), *S. cerevisiae* (21), *S. fibuligera* (22), *Trichosporon adeninovorans* (23). The only exception is the enzyme from *Kluyveromyces fragilis* (24) where the enzyme is monomeric with a mol wt of 94,000 Da. In fact since many β-glucosidases also transfer glucose residues to a growing saccharide chain or to a suitable glycosyl acceptor (2), these can be considered as glycosyl transferases. Glycosyl transferases have been predicted to be large molecular weight enzymes with a multidomain structure (25).

Our studies on the effect of carbon source on enzyme biosynthesis rates of *P. etchellsii* enzyme expressed in *E. coli* indicate little difference between the various sugars. A maximum enzyme biosynthetic rate of 12 U/g/h was obtained on 0.2% cellobiose medium at the 8 h of growth. However, the period of maximum enzyme biosynthesis was supported over longer time periods and a greater zone of specific growth rates on this sugar. A slow release of glucose arising out of cellobiose metabolism may be favorable for enzyme biosynthesis. No inductive effect of cellobiose on β-glucosidase synthesis was noticed in the recombinant bacterial cell in contrast to the cellobiose induction of the enzyme in the native yeast. This suggests that the regulatory sequences responsible for this effect may be distantly located from the structural gene and were not picked up in the cloned fragment or that these sequences were ineffective in the bacterial host. Such regulation of β-glucosidase by carbon source was reported in *Streptomyces* sp. (26). Since the levels of intracellular enzyme activity obtained for pBG5 : C600 and pBG55 : XL1-Blue were similar, the

final levels of enzyme obtained do not appear to be determined by the plasmid size or plasmid heterogeneity in this system.

The substrate specificity determination of the partially purified enzyme from recombinant clone pBG55 : XL1-Blue indicated that hydrolysis of aryl β-glucosidic linkage in *p*NPG and β,1–4 glucosidic linkage, as in cellobiose, were equally and efficiently attacked by the enzyme although some degree of preference was shown for the latter. In addition to the β,1–4 linkage, the β,1–2 and β,1–6 linkages were also attacked by the enzyme indicating a relatively broad substrate specificity. This property is also exhibited by the cloned β-glucosidases from *Clostridium thermocellum* (27), *Thermotoga maritima* (12) and BGL1 from the yeast *S. fibuligera* (22). In fact, the *Pichia* enzyme shares the property to hydrolyze sucrose with a *S. fibuligera* enzyme (22). The β-glucosidase from the related yeast *Candida wickerhamii*, on the other hand, has been found to be active only against some synthetic substrates (Skory, C. N. and Freer, S. N., Abstr. Gen. Meet. Am. Soc. Microbiol. 94th Meeting, USA, p. 366, 1994).

The *Pichia* enzyme expressed in *E. coli* had a temperature optimum of 37°C for cellobiose, gentiobiose, sophorose and sucrose hydrolysis while hydrolysis of aryl β-glucosides occurred at the optimum temperature of 50°C. Although different temperature optima have been observed for these two substrates by the β-glucosidase B from *C. thermocellum* (27), the *Pichia* enzyme is different in that no hydrolysis of β-linked disaccharides occurred at 50°C. While this may be due to a conformational change in the enzyme at a higher temperature, the property is advantageous in controlling the substrate specificity of the enzyme.

Eventhough the stability of enzyme activity in the presence of glucose is a rare property among β-glucosidases, the cloned enzyme displayed this property. Among the reported β-glucosidases, only the enzyme from thermophilic bacterium *Microbispora bispora* was stabilized in the presence of glucose (11). A slight shift in the temperature optimum to 50°C was also noticed for the cloned enzyme. Lack of glycosylation may lead to this change for the recombinant enzyme. These results show that the *E. coli* expressed enzyme displays broad substrate specificity with a stronger preference for β-linked disaccharides. The enzyme also displayed advantageous temperature and glucose tolerance profiles when compared to the native enzyme. A slightly different profile for the purified enzymes cannot be ruled out but, in the absence of the pure enzyme from the yeast, the recombinant clone reported here is useful for obtaining the pure enzyme and for further evaluation of the enzyme at molecular level.

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