Biotechnol. Bioprocess Eng. **2002**, 7: 43-51

Cloning, Characterization of *Pichia etchellsii* β-Glucosidase II and Effect of Media Composition and Feeding Strategy on its Production in a Bioreactor

Benu Sethi, Monika Jain, Manish Chowdhary, Yogesh Soni, Yukti Bhatia, Vikram Sahai, and Saroj Mishra*

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Delhi, Hauz-Khas, New-Delhi 110016, India

Abstract The cloning and expression of β-glucosidase II, encoded by the gene *βglu2*, from thermo-tolerant yeast *Pichia etchellsii* into *Escherichia coli* is described. Cloning of the 7.3 kb *Bam*HI/*Sal*I yeast insert containing *βglu2* in pUC18, which allowed for reverse orientation of the insert, resulted in better enzyme expression. Transformation of this plasmid into *E. coli* JM109 resulted in accumulation of the enzyme in periplasmic space. At 50°C, the highest hydrolytic activity of 1686 IU/g protein was obtained on sophorose. Batch and fed-batch techniques were employed for enzyme production in a 14 L bioreactor. Exponential feeding rates were determined from mass balance equations and these were employed to control specific growth rate and in turn maximize cell growth and enzyme production. Media optimization coupled with this strategy resulted in increased enzyme units of 1.2 kU/L at a stabilized growth rate of 0.14 h^{-1}. Increased enzyme production in bioreactor was accompanied by formation of inclusion bodies.

Keywords: *Pichia etchellsii*, β-glucosidase, recombinant *Escherichia coli*, fed-batch cultivation

INTRODUCTION

The enzyme β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) catalyses the hydrolysis of glucosides containing residues linked by β, 1-4 linkage. The enzyme is ubiquitous and occurs in microbes, plants and animals. In cellulolytic microorganisms, the enzyme is associated with the breakdown of terminal products of cellulose hydrolysis viz. cellobiose and cellodextrins [1,2]. The role of β-glucosidase in cellulase induction due to transglycosylation properties resulting in formation of low molecular weight inducers has also been proposed [3]. The functions of this enzyme are quite diverse in plants such as release of aroma rich terpenes from flavorless terpene-glucosides during fruit maturation, release of phenol acting as insect repellant from phenolic-glucosides, cell wall development during growth etc. (for review, see [4]). In animals, the deficiency of β-glucosidase leads to accumulation of glucosyl-ceramides which trigger neurological disorders [5]. Apart from the use of this enzyme in natural cellular hydrolytic as well as biosynthetic reactions, it can also be used *in vitro* to synthesize a variety of oligosaccharides and other glycoconjugates using the transferase property of this enzyme. Such reactions occur either by (i) reverse hydrolysis or by (ii) transglycosylation activity of the enzyme [6,7]. Both the approaches have been used for biosynthesis of a variety of compounds.

In the past few years, several β-glucosidases have been cloned from bacteria, yeast, fungi, plant and animal sources with the intent of producing this enzyme on a large scale for various applications. However, the level of enzyme expression in the recombinant hosts has been low. Very few enzymes were expressed in stable form in the host to facilitate their purification. The use of the enzyme for investigation of biosynthetic activity is generally limited due to non-availability on large scale. Non-recombinant enzyme from almonds is commercially provided by several suppliers and is the only enzyme investigated in detail for biosynthesis of some compounds [8]. Our own interest in the area of glycoconjugate biosynthesis led to identification of a thermo-tolerant yeast *Pichia etchellsii*. In our previous work [9] we described the cloning of *βglu* (now renamed as *βglu1*) gene encoding β-glucosidase (now renamed as β-glucosidase I or BglI). The enzyme was purified and used to demonstrate biosynthesis of a limited number of cello-oligosaccharides [10].

In this work, we report the cloning and expression of another β-glucosidase gene, named *βglu2*, from *P. etchellsii* encoding β-glucosidase II or BglII. The unique substrate specificity profile of the enzyme necessitated its production on a larger scale. Among different strategies used for overproduction of proteins from recombinant *Escherichia coli* (re-*E. coli*) considerable emphasis has been laid on supplying the carbon source slowly

* **Corresponding author**
Tel: +91-011-659-1007 Fax: +91-011-686-8521
e-mail: saroj@dbeb.iitd.ernet.in, saroj98@hotmail.com

throughout the fermentation in a fed-batch mode and by optimizing the composition of the media ingredients. The optimal medium composition depends on the *E. coli* strain and a variety of medium formulations have been developed [11,12]. These allow for better control of specific growth rate and accumulation of inhibitory metabolite, acetate. In aerobic cultures of *E. coli* large excess of nutrients, particularly the carbon source, leads to increase in specific growth rate and when that exceeds a certain threshold level, acetate accumulates. We describe adopting nutrient composition and feeding strategy to regulate specific growth rate to levels below those responsible for accumulation of inhibitory metabolites. The strategies used here should be applicable for scaling up of re-*E. coli* for other important products.

MATERIALS AND METHODS

Strains, Plasmids and Growth Conditions

The yeast *Pichia etchellsii* (Deutsche Sammlung Von Mikrorganismen (DSM) Germany) was used as the donor of the β-glucosidase gene. The bacterial strains used in this study were *E. coli* DH5α[13] and JM109 [14]. Plasmids pUC19 and pUC18 were obtained from Bangalore Genei, Bangalore. Plasmid pAH3 [15] was a kind gift from Dr. J. Gowrishankar, CCMB, Hyderabad. It contains an up promoter mutation in the *lac*I promoter and makes more amount of the *lac* repressor. This is routinely used to reduce expression of genes cloned under the *lac* promoter if the gene product is toxic to the cell. It titrates out the *lac* operator/promoter sequences in the multi copy plasmid pUC19/pUC18.

Routine growth and maintenance of the yeast was carried out on yeast extract-peptone-malt extract medium containing 2% cellobiose as described previously [9]. *E. coli* DH5α or JM109 was grown at 37°C in Luria-Bertani (LB) medium (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl per liter). In addition, LB ampicillin (amp) + *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) plates and LB amp + 4-methylumbelliferyl-β-D-glucoside (MUG) plates were used for screening of β-glucosidase positive transformants. LB amp + *p*NPG contained LB with 100 μg/mL amp, 4 mM *p*NPG and 2% agar. The LB amp + MUG plates contained LB with 100 μg/mL amp over layered with 0.5% MUG in 0.6% agar, 0.1% SDS (sodium dodecyl sulphate) and 0.2% Triton X-100. The recombinant cells were grown at 37°C unless otherwise mentioned.

DNA Methods

The chromosomal DNA of *P. etchellsii* was prepared according to Cregg *et al.* [16] from cells grown on YPD (1% yeast extract, 2% peptone, 2% glucose) and harvested at the end of the exponential phase. Zymolyase 30,000 (Miles Inc., Elkhart, Ind., USA) was used to prepare the DNA as described previously [9]. The genomic DNA library was prepared with 5-20 kb size fractionated DNA fragments with pUC19 vector according to standard methods [17] in *E. coli* DH5α host. Restriction mappings were carried out by comparing parallel digestions of plasmids with and without insert DNA. For sub-cloning work, restriction fragments (as indicated by open boxes in Fig. 1) were purified by electro elution from agarose gels in dialysis bags. The fragments were cloned into *Bam*HI-calf-intestinal alkaline phosphatase (CIP) treated or *Hin*dIII-CIP treated pUC19 vectors. The 7.3 kb *Bam*HI/*Sal*I insert, containing *βglu2*, was also cloned into *Bam*HI/*Sal*I digested pUC18 allowing for reverse orientation of the fragment downstream of *lac*Z promoter. The construct pBG22 (Fig. 1) was transformed into *E. coli* DH5α and JM109.

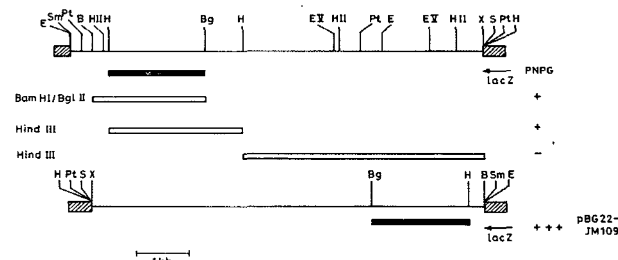

Fig. 1. Restriction map of the yeast insert encoding *βglu2* gene of *Pichia etchellsii*. The thin line represents the yeast DNA and adjacent hatched regions are of vector polylinker DNA.. Fragments sub-cloned into pUC19 vector were 2.1 kb *Bam*HI/*Bgl*II. 2.5 kb *Hin*dIII and 4.5 kb *Hin*dIII. Recombinant *E. coli* containing the construct was analysed for *p*NPG hydrolysing activity. The activity is indicated by + or – next to the relevant fragment. The shaded box indicates the approximate position of the identified coding region. The lowermost figure indicates cloning of the 7.3 kb *Bam*HI/*Sal*I fragment of the original insert in pUC18. The activity due to this construct is indicated by +++. The direction of transcription of *lacz* in indicated by an arrow. The letters denoting restriction sites: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Eco*RV (EV), *Hin*cII (HII), *Hin*dIII (H), *Pst*I (Pt), *Sal*I (S), *Sma*I (Sm), *Xba*I (X).

The 2.1 kb *Bam*HI/*Bgl*II fragment (Fig. 1) was labeled with α-^{32}P-dCTP (BARC, Bombay, India) using the Nick-translation kit (Promega) as per suppliers instructions. Southern hybridizations were performed using *Bam*HI digested chromosomal DNA as described in Sambrook *et al.* [17].

Isolation of plasmid DNA, ligation and transformation into *E. coli* was performed according to standard methods [17]. Restriction enzymes were obtained from MBI Fermentas Inc.

Localization and Characterization of BglII

The β-glucosidase activity in the recombinant clones was detected by screening on (i) LB amp + MUG and (ii) LB amp + *p*NPG plates. β-Glucosidase positive clones appeared fluorescent under UV on MUG plates, due to release of 4-methyl umbelliferone. The positive clone was also tested on LB amp + *p*NPG plates. A yel-

low halo surrounding the clone indicated β-glucosidase positive activity. The construct pBG22, derived from the original isolate pBG52, when transformed into JM109 demonstrated higher production of β–glucosidase and this transformant was investigated for enzyme localisation and subsequent characterization. A small-scale day culture (5 mL) was inoculated in 100 mL LB containing 100 μg/mL amp and grown overnight with shaking at 37°C. Cells were harvested by centrifugation at 5000 × *g* for 10 min at 4°C. Supernatant was collected as extracellular fraction. Harvested cells were given osmotic shock according to Nossal and Heppel [18] with some modifications, to release the periplasmic enzymes. The concentration of EDTA was observed to affect the release of the enzyme and it was optimized at 40 mM level. The *E. coli* cell pellet, after the shock treatment, was suspended in 1/10 of original volume in 50 mM sodium phosphate buffer, pH 7.0 and sonicated, at an amplitude of 10 μ for 10 min with intermittent cooling in the presence of 1 mM PMSF (phenyl methyl sulfonyl fluoride). The cell-free extract obtained after centrifugation at 15,000 × *g* for 20 min was the intracellular fraction. The extracellular and the periplasmic fractions were concentrated 10-fold by ultrafiltration through a 10 kDa (Nalgene) membrane. β-Glucosidase was assayed in each of the three fractions.

The substrate specificity of periplasmically located enzyme was determined by measuring (i) the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenyl β-D-galactopyranoside (*p*NPGal), *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) and *p*-nitrophenyl-β-D-xylopyranoside and (ii) the release of glucose from cellobiose, gentiobiose, sophorose, sucrose, salicin and carboxymethyl cellulose.

PAGE and Zymogram Analysis

The periplasmic fraction obtained from pBG22: JM109 was subjected to ammonium sulphate fractional precipitation (40-80%). The precipitated enzyme was solubilized in 50 mM sodium phosphate buffer (pH 7.0), extensively dialysed against the same buffer and concentrated by ultrafiltration using PM10 membrane. The protein was purified about 7-fold. About 150 μg protein was electrophoresed on 8% native polyacrylamide gel in Tris-Glycine buffer, pH 8.4 and run along with BSA markers [10] according to standard procedures [19]. Half of the gel was stained with Coomassie blue and the other half was incubated in 1 mM MUG solution (prepared in PC buffer). The gel was incubated at 37°C for 30 min and observed for fluorescence, caused by release of 4-methyl umbelliferone, under UV (366 nm).

Media Optimisation and Cultivation in a Bioreactor

The effect of media composition on cell growth and enzyme activity was first studied at shake flask level. A single colony of pBG22:JM109 was inoculated into 5 mL LB + amp and grown overnight at 37°C with shaking at 250 rpm. Two mL of overnight culture was used to inoculate media (50 mL in 250 ml flask) containing different combinations of peptone, yeast extract, malic acid, sodium chloride and glucose. The cultures were grown for 16 h and harvested. The cell dry weight was measured and enzyme activity determined in the periplasmic fraction of these cultures.

The cells were also cultivated in a 14-L capacity bioreactor (Chemap AG interfaced with indigenously procured IBM compatible PC-AT-286). It was equipped with pH, temperature and dissolved oxygen (DO) monitoring probes. The bioreactor was run in both batch and fed-batch mode, the latter being run via the computer with an in-house developed software. Media composition for various runs is tabulated in Table 1. The components were autoclaved in groups to prevent precipitation. The volume of the batch was 6 L after inoculation with 200 mL of overnight grown culture of pBG22:JM109. For fed-batch mode, as the available carbon in the batch medium depleted the DO increased. At this time, the feed was started at a rate controlled by the computer according to the following equation:

$$F(t) = [\mu/Y_{X/S} + m]\frac{X_0 V_0}{S_0}\exp[\mu^*(T_{FB} - T_B)]$$

where,

$F(t)$ = Instantaneous feed flow rate, L/h
μ = set value of specific growth rate, L/h
$Y_{X/S}$ = yield coefficient, biomass per carbon (g/g)
m = maintenance coefficient, carbon per biomass per h (g/g. h)
X_0 = cell mass at the end of batch, g/L
V_0 = culture volume at the end of batch, L
T_B = duration of the batch phase, h
T_{FB} = time elapsed since inoculation, h
S_0 = concentration of carbon per volume in the feed, g/L

The feed pump was calibrated and an equation as V (volts) = f (F, mL/h) was found. This equation was incorporated into the software to achieve predetermined feed. A load cell was used below the feed bottle to monitor the actual amount of media fed compared to the desired value. The feed rate and the fed-batch phase duration were calculated based on the volume in the batch phase, final volume at the end, growth rate to be maintained, desired cell dry weight at the end of the batch phase, cell yield, batch duration and maintenance coefficient. The value of maintenance coefficient was taken as 0.01 h^{-1} from the available literature [20] to calculate the feed rate. The DO was controlled at 25% by automatic change in agitation (270-550 rpm) and inlet air (0.7-1.25 vvm) with the help of a PID controller algorithm in the computer. When air and agitation reached maximum limits oxygen was mixed with air (0-50%). The temperature and pH were maintained at preset levels using computer operated PID controllers. The feed volume for the first three fed-batch runs was 2 L and for the fourth fed batch run, it was 4 L. A booster

Table 1. Media composition of various bioreactor runs

Run # / Component	MTP01	MTP02	MTP03 B[a]	MTP03 F	MTP04 B	MTP04 F	MTP05 B	MTP05 F	MTP06 B	MTP06 F
Yeast extract	5.0	10.0	10.0	100.0	10.0	100.0	10.0	100.0	10.0	100.0
Peptone	10.0	10.0	5.0	100.0	5.0	50.0	5.0	50.0	5.0	50.0
Salts										
NaCl	5.0	5.0	5.0	-	5.0	-	5.0	-	5.0	-
KH_2PO_4	2.0	2.0	2.0	-	2.0	-	2.0	-	3.0	-
K_2HPO_4	2.3	2.3	2.3	-	2.3	-	2.3	-	3.3	-
Citric acid	-	-	-	-	1.7	-	1.7	-	1.7	-
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2	-	0.2	1.0	0.2	1.0	1.0	1.0
Trace Metals[b]										
$CuCl_2 \cdot 2H_2O$	1.0	1.0	1.0	-	1.0	-	1.0	-	1.0	-
$MnCl_2 \cdot 4H_2O$	2.0	2.0	2.0	-	2.0	-	2.0	-	2.0	-
$CoCl_2 \cdot 6H_2O$	1.0	1.0	1.0	-	1.0	-	1.0	-	2.0	-
H_3BO_3	1.0	1.0	1.0	-	1.0	-	1.0	-	1.0	-
$Na_2MoO_4 \cdot 2H_2O$	1.0	1.0	1.0	-	1.0	-	1.0	-	3.0	-
$ZnCl_2$	3.0	3.0	3.0	-	3.0	-	3.0	-	6.0	-
Fe_3citrate	25.0	25.0	25.0	-	25.0	-	25.0	150.0	25.0	217.5
$EDTA \cdot 2H_2O$	10.0	10.0	10.0	-	10.0	-	10.0	-	10.0	
Thiamine	1.0	1.0	1.0	-	1.0	-	1.0	-	1.0	-

[a]B: batch, F: fed-batch, [b]Trace metal solution contents and thiamine were in mg/L. All other components were in g/L.
Ampicillin was added at 100 μg/L concentration in all the runs.

shot of ampicillin containing 50% of the original amount was added at about 24 h in view of the reported 50% hydrolysis of ampicillin in 24 h. The temperature was initially maintained at 37°C. In the batch phase, as the agitation reached its maximum limits, the temperature was slowly lowered to 28°C in steps of 2°C. This led to a slowing of the oxygen demand of the cells and consequent control of the DO without resorting to an increase in air flow rate. In the last two runs, the temperature was maintained at 37°C, which led to a faster completion of the batch phase of the fermentation. The samples were removed periodically and analysed for cell OD_{600nm} and β-glucosidase activity. The data on pH, DO and temperature was monitored on-line.

Analytical Methods

Dry cell weight was estimated using a calibration curve plotted between the optical density at 600 nm and the dry cell weight. The β–glucosidase activity was measured on the indicated substrates (See listing above) in 50 mM phosphate citrate (PC) buffer, pH 6.5, as described previously [9]. Glucose released in the assays was quantified using glucose oxidase peroxidase kit (Ranbaxy Ltd., Delhi, India). Enzyme activity is reported in international units (IU) and one IU corresponds to release of 1 μmol of *p*NP/min/mL enzyme or release of 1 μmol glucose/min/mL enzyme depending on the substrates.

RESULTS

Cloning, Restriction Mapping and Sub-cloning of β-Glucosidase II Gene from *Pichia etchellsii*

From *Sau*3AI partially digested chromosomal DNA of *P. etchellsii*, fragments in the range of 5-20 kb were prepared and ligated into *Bam*HI linearized/ CIP treated vector pUC19. The screening on (i) MUG followed by on (ii) *p*NPG resulted in the identification of clone pBG52. The transformant was subcultured as single colonies and plasmids recovered from fluorescent colonies on MUG selection plates. The plasmids were used again in transformation experiments. Ampicillin transfer and MUG hydrolysis occurred in the same step confirming the plasmid borne nature of β–glucosidase. A 7.7 kb yeast insert containing the second β–glucosidase gene, *βglu2*, was identified and its restriction map is shown in Fig. 1. To localize the region responsible for β–glucosidase activity on the 7.7 kb insert in plasmid pBG52, sub-cloning of the various fragments, as shown in Fig. 1, was performed. The smallest sequence necessary for enzyme activity, indicated by a solid bar in Fig. 1, was localized to a 1.8 kb *Hin*dIII/*Bgl*II fragment. The enzyme activity in the *E. coli* DH5α containing the original insert as well as the 2.1 kb *Bam*HI/*Bgl*II fragment was low. The activity in the cell-free extract was less than 10 mU/mL.

Southern hybridisation to determine the origin of

βglu2 from genomic DNA of *P. etchellsii* was performed. The data showed hybridisation signals with a single 8.5 kb fragment of chromosomal DNA. Dot-blot hybridisation experiments were also performed to examine whether DNA sequences from 2.1 kb *Bam*HI/*Bgl*II fragment, containing the *βglu2* gene, showed homology to previously reported *βglu1*. Analysis revealed no hybridisation between the two DNA fragments.

In an attempt to study if the reverse orientation of the insert and change of host would have any effect on enzyme levels, the 7.3 kb *Bam*HI/*Sal*I fragment, containing *βglu2*, was cloned into *Bam*HI/*Sal*I cut pUC18. The recombinant thus constructed, pBG22, was transformed into DH5α and *E. coli* JM109. While the level of β-glucosidase did not improve in DH5α host, it was substantially increased to 88-100 IU/L in pBG22:JM109 transformant. Further, the enzyme was localized to the periplasmic space of re-*E. coli* JM109. The effect of IPTG was investigated in this construct to study any possible effect by being located downstream of *lacZ* promoter. Accordingly, different concentrations of IPTG (0.5, 1.0 mM) were added at either (i) 1 h after inoculation of seed culture into production flask or (ii) 6 h after inoculation (as described in Material and Methods). While no stimulation in specific enzyme activity per protein (0.87 U/g) was observed in the first case with 1 mM levels, about 20% increase (1.3 U/g) was observed in the latter case over control (1.1 U/g) where no IPTG was added. In view of the multi copy nature of pUC18 in which all *lac* regulatory sites may not be titrated out by the level of repressor in the uninduced culture (and thereby mask the difference between the two experimental conditions, uninduced and induced), an attempt was made to titrate out the repressor by co-transformation with plasmid pAH3 (for description, see Materials and Methods) and selecting the transformants on LB + amp and kanamycin. However, stable transformants were not obtained. This was most likely due to incompatibility of the plasmids.

Localization and Characterization of BglII

The localization of BglII in different sub-cellular fractions of pBG22:JM109 was investigated and the results indicated it to be periplasmically located. The pH, temperature optima and substrate specificity of the periplasmically extracted BglII was investigated. The pH optima for *p*NPG and cellobiose hydrolysis was 6.0-6.2. The temperature optima against the two substrates lied between 45-50°C. Maximum hydrolytic activity in terms of IU per g protein, 1686 IU/g, was obtained on sophorose (β1→2 linked glucose dimer) followed by *p*NPG (230 IU/g) and gentiobiose (β1→6 linked glucose dimer) (198.3 IU/g) (Table 2). The enzyme displayed low activity on cellobiose (β1→4 linked glucose dimer) and salicin (2-hydroxymethyl phenyl β-D-glucoside). The anomeric specificity was indicated by preferential hydrolysis of *p*NPG as compared to *p*NPGal. The activity on *p*NPG , the substrate on which maximum activity has been reported for a large number of β-glucosidases,

Table 2. Substrate Specificity of β-glucosidase II of *Pichia etchellsii*

Substrate	50°C Specific activity (mU/mg of protein)	37°C Specific activity (mU/mg of protein)
Cellobiose (Glc β1-4 Glc)	60.00	33.00
Salicin [2 (hydroxymethyl) phenyl β-D-glucoside)	45.00	31.66
Sophorose (Glc β1-2 Glc)	1686.33	1091.16
Gentiobiose (Glc β1-6 Glc)	198.33	165.33
Lactose (Gal β1-4 Glc) -	–[a]	–
Maltose (Glu α1-4 Glc)	–	–
Sucrose (Fru β2-1 Glc)	–	–
Carboxymethyl cellulose	–	–
pNP-β-D-glucopyranoside	230.00	100.00
pNP-β-D-galactopyranoside	21.66	14.66

[a]not detected

was less by about an order of magnitude when compared to the activity obtained on sophorose. The cell extracts were prepared from parallel control transformants (in which pUC18, without the insert, was transformed into *E. coli* JM109) and assayed for β-glucosidase activity. No activity was observed against the maximally hydrolyzed substrates sophorose, gentiobiose and *p*NPG. The molecular mass of the enzyme was estimated to be about 180 kDa on the basis of the presence of a single MUG hydrolysing activity in the periplasmically extracted proteins following Native PAGE and Zymogram analysis (data not shown). This was further confirmed by gel-permeation chromatography where the molecular mass was determined to be about 176 kDa (manuscript submitted for publication).

Media Optimisation and Cultivation in a Bioreactor

Media optimisation with a view to produce high cell density culture of *E. coli* and eventually high enzyme units was carried out. For this purpose, the recombinant pBG22:JM109 was first cultivated in different combinations of LB with or without the salts. Decline in cell growth and enzyme activity were observed when either component of LB, *i.e.*, peptone or yeast extract, was absent. Similarly substituting glucose with malic acid did not affect enzyme activity. In addition, using a rich carbon and nitrogen source such as tryptone, did not significantly alter the enzyme levels. This data was used for enzyme production in a bioreactor.

The cultivation in a bioreactor was carried out in both batch and fed-batch modes with an objective to control oxygen demand within the oxygen transfer capabilities of the bioreactor and to avoid accumulation of acetate and ethanol. For batch runs, the media contained LB supplemented with salts and trace elements. The final cell dry wt per litre was 4.85 g/L with enzyme levels of 57.6 IU/L (Table 3). A maximum specific growth

Table 3. Summary of batch and fed-batch fermentation processes

Run	Maximum cell density (g dry cell wt/L)	Specific growth rate [a] (h^{-1})	β-Glucosidase activity (IU/L)
Batch			
MTP01	4.8	0.58	58.0
MTP02	5.4	0.63	276.0
Fed-batch			
MTP03	14.7	.05 (0.1)	726.0
MTP04	10.6	.03-.08 (0.07)	724.0
MTP05	8.6	0.1 (0.1)	919.0
MTP06	14.5	0.14 (0.16)	1210.0

[a] The numbers in parentheses indicate the set growth rates in the fed-batch processes.

rate (μ_{max}) of 0.58 h^{-1} was obtained. By increase-ing yeast extract to peptone ratio (1:1) in the second batch run, a significant increase in activity was observed from 57.6 to 276 IU/L. This information was used to design media for fed-batch runs. The third run was started as batch phase, which lasted for 12.5 h, and continued as fed-batch phase that lasted for 31 h. The enzyme production at the end of the batch phase was considerably higher at 368 IU/L. The growth rate monitored in the fed-batch phase was low, at 0.05 h^{-1}, which was significantly lower than the set value of 0.1 h^{-1} suggesting that some trace elements were limiting. In this run, at the end of 42 h, final cell dry wt of 14.7 g/L and enzyme activity of 725.6 IU/L was achieved (Fig. 2). Subsequent fed-batch runs were carried out with improvements in media composition in order to attain set specific growth rate and higher enzyme activities.

The role of magnesium and iron has been emphasized in several studies. Magnesium and iron may have been limiting in the first three runs, thus these were added to the medium in the following runs (run 4 and run 5, Table 1) along with citric acid. Addition of all the three ingredients did not have a significant effect on cell dry wt but the enzyme activity rose to 919 IU/L (run 5). The fourth fed-batch run (run 6) contained yeast extract : peptone ratio of 2:1 in both batch and fed-batch media along with magnesium and added iron. The specific growth rate was set at 0.16 h^{-1}. The detailed temperature, DO, pH, volume fed and agitation profiles as observed on-line are shown in Fig. 3 along with experimentally determined values of biomass and activity. The specific growth rate in the batch phase increased to a maximum of 0.47 h^{-1} and then progress-sively fell to a value close to zero as the substrate was utilised (Fig. 4a).The specific growth rate in the fed-batch phase was more or less stabilised at 0.1 h^{-1} (Fig. 4b) passing through a maxima of 0.14 h^{-1}. Thus the combination of nutrients in which yeast extract:pep-tone ratio was kept at 2:1 along with added Mg^{2+} ions and iron citrate in batch and fed-batch mode resulted in reaching the tar geted specific growth rate. The enzyme activity increased to a high value of 1.2 kU/L. This represented a

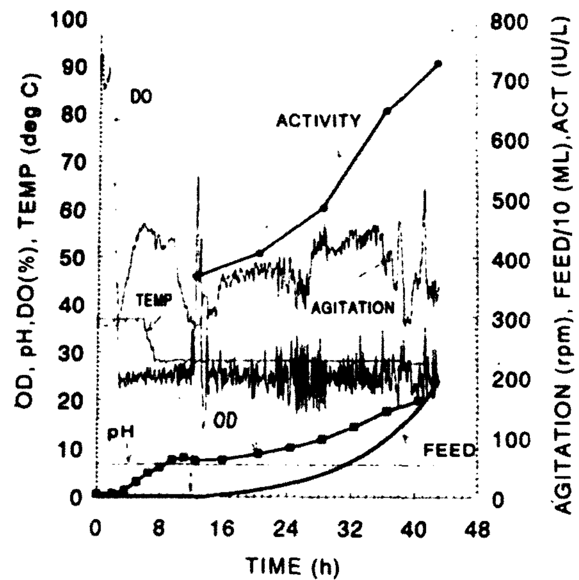

Fig. 2. Cell growth and β–glucosidase production in 14 L bioreactor by re- *E. coli,* containing pBG22 construct, in fed-batch culture (MTP03). The duration of the batch phase was 12.5 h. The specific growth rate in the fed-batch was set at 0.1 h^{-1}. The on-line determined parameters were pH, dissolved oxygen (DO), temperature (TEMP), volume fed (FEED) and agitation (AGITATION). Samples were removed at regular intervals and optical density (OD) and enzyme activity (ACT) determined.

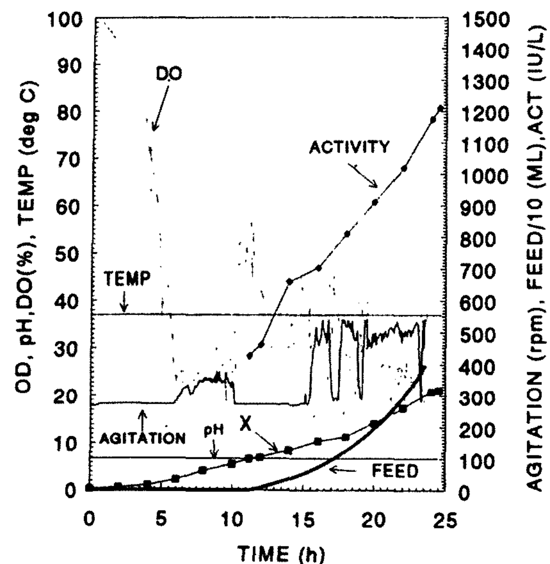

Fig. 3. Cell growth and β–glucosidase production in a 14-L bioreactor by re- *E. coli,* containing pBG22 construct, in fed-batch culture (MTP06) at set specific growth rate of 0.16 h^{-1}. The duration of the batch phase was 11.1 h. The pH, dissolved oxygen (DO), temperature (TEMP), volume fed (FEED) and agitation (AGITATION) as observed on-line are plotted. Samples were removed at regular time intervals for measurement of cell optical density (OD) and enzyme activity (ACT).

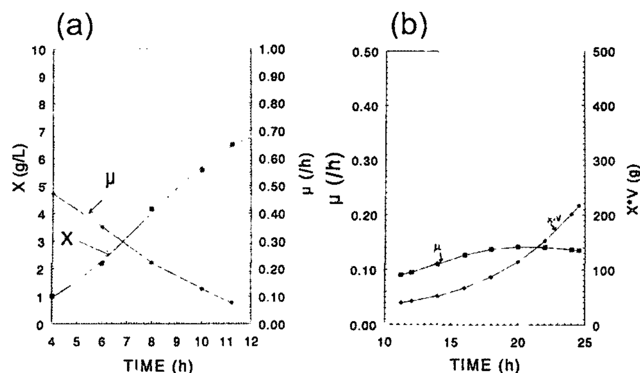

Fig. 4. (a) Profile of cell mass (X) and instantaneous specific growth rate (μ in batch phase of cultivation in MTP06 (For details, see text), (b) Profile of specific growth rate (μ in fed-batch phase of cultivation in MTP 06. The cell mass concentration X (g dry cell weight/L) multiplied with culture volume V (L) at the end of the fed-batch phase is also indicated on the Y-axis.

1,200% increase in enzyme level over the shake-flask data. A microscopic analysis was done on samples drawn at various times. It was observed that as the fermentation in the fed-batch stage progressed the cell size progressively decreased and the cells lost their characteristic rod shaped morphology and became swollen and round. Structures that stained darkly, indicative of inclusion bodies, were observed. The drop in specific enzyme productivity in this batch was associated with inclusion body formation. The cells were also plated on solid medium (LB + amp). Upon plating, the cells regained the rod shaped morphology. Table 3 gives a summary of all bioreactor runs.

DISCUSSION

The cloning and characterisation of another β-glucosidase enzyme (BglII) of yeast *Pichia etchellsii* in *E. coli* is described in this paper. On the basis of restriction mapping of the gene encoding this protein, *βglu2*; lack of hybridisation between *βglu2* and *βglu1*, and different substrate specificity profile of the enzyme, it was considered to be different from the previously reported β-glucosidase I [10]. The effect of several media constituents was investigated to maximise expression of the enzyme. Controlled growth in a bioreactor with predetermined specific growth rate was carried out and its effect on cell growth and enzyme production studied.

A 7.7 kb yeast fragment bearing the gene *βglu2*, encoding BglII, was isolated and the region encoding the enzyme activity was localised to an approximately 1.8 kb *Hin*dIII/*Bgl*II region. The reverse orientation of the 7.3 kb *Bam*HI/*Sal*I fragment, containing *βglu2*, in pUC18 along with a change in the host to *E. coli* JM109 resulted in conditions leading to higher enzyme activities. This strain has been reported [17] to be defective for the synthesis of bacterial cell walls. We believe this to be responsible for increased enzyme secretion in this construct as transformation of pBG22 into *E. coli* DH5α did not show increased enzyme production over the original transformant pBG52:DH5α. In an earlier study, Shima *et al.* [21] have made similar observations with respect to release of cloned cellulase enzyme of *Clostridium cellobioparum* in this host. The construct pBG22 also responded to IPTG addition and a 20% increase in specific enzyme activity was noticed over the uninduced control. The time of addition of IPTG was critical and its addition to cells that had grown for 6 h resulted in higher levels of enzyme than when IPTG was added in the beginning. The stimulation by IPTG has been reported for a number of other cloned β-glucosidases as well such as BglA of *Bacillus polymyxa* [22] and Cel B of *Pyrococcus furiosus* [23] in re-*E.coli*. No satisfactory explanation is available for these observations. In a recent study lactose was substituted for IPTG as an inducer in the production of type 1 ribosome inactivating protein from *Phytolacca insularis* with 55% enzyme levels compared to levels obtained with IPTG [24]. Hence other inducers, less expensive, also need to be investigated for such overproduction purposes.

The enzyme BglII was localised to the periplasmic fraction of the re- *E. coli* unlike BglI which was intracellular. Zymogram analysis of the periplasmic fluid extract showed the presence of a single MUG hydrolysing activity at about 180 kDa position. BglA and BgxA from *Erwinia herbicola* [25] and *E. chrysanthemii* [26] respectively were also localized in periplasmic space of host *E. coli*. Apparently, the sequences involved in the secretion process are being recognized in the host. The pH and the temperature optima of the enzyme were different from the previously reported BglI [10]. The unique feature of BglII was its ability to hydrolyse sophorose most effectively. To our knowledge this is the first report of a yeast β-glucosidase with highest relative activity on sophorose. It is also expected that unique oligosaccharides can be synthesized with this enzyme. Maximum activity on laminaribiose over cellobiose and *p*NPG has been reported for the enzyme from *Thermoanaerobacter brockii* [27].

Although a number of bacterial and yeast β-glucosidases have been cloned, the expression in respective host (*E. coli* or *Saccharomyces cerevisiae*) has been low. While these enzyme levels are sufficient for detection in plate assay methods, the activity in the cell-free extracts of the host has been found to be very low limiting the usefulness of these enzymes for practical applications. One of the major objectives in the present study was to develop a strategy for overproduction with media manipulation. Media composition has been reported to influence production of proteins in re-*E. coli* cells by several workers (For review, see [28]). For batch runs the media contained salts and trace elements in addition to LB and slight improvements in enzyme production were noticed (276 IU/L) over the shake flask data. However, growth in rich medium results in accumulation of acetic acid leading to toxicity of the cell [29].

Acetate formation is reported to increase drastically when growth rate exceeds 0.35 h^{-1} (in defined medium) and 0.2 h^{-1} (in complex medium). In a number of microbial processes therefore the approach of exponential feeding has been used to achieve some of the highest cell concentrations due to constant substrate utilisation and control of specific growth rate μ, especially for *E. coli* producing a recombinant product [30,31]. This is mainly due to the ability to control specific growth rate (μ) at a value low enough to minimise inhibitory by-product formation [32,33]. This strategy was adopted in the present study and growth rate was kept constant by maintaining a pre-determined feed rate. Marked improvements in enzyme production were achieved. By maintaining specific growth rate at 0.1 h^{-1}, final cell dry wt per litre of 14.7 g/L and enzyme activity of 725.6 IU/L was obtained in the first fed-batch experiment. High concentration of nutrients and precursors for macromolecular synthesis were provided by feeding complex nitrogenous medium. Proper nutrient feeding levels minimised the lowering of product yield at low μ value. Iron is an essential co-factor for many enzymes and along with magnesium has been shown to reduce acetic acid accumulation along with significant increase in recombinant protein productivity [34]. Such modifications in the media were introduced and these led to production of high enzyme levels. In the final fed-batch run, enzyme activity of 1.2 kU/L was obtained. An increase in specific growth rate beyond 0.14 h^{-1} may result in decrease in enzyme synthesis. It must be emphasized that synthetic (or defined) as well as semi-synthetic medium containing yeast extract and peptone, in addition to salts, has also been used successfully by several workers [For review, see 28] to achieve high cell densities of *E. coli* and in most cases this was accompanied by high levels of product formation. For instance, high levels (800 mg insulin B-chain) of the product were produced by re-*E. coli* on synthetic medium, with glucose as sole carbon source, in fed-batch mode of cultivation [35]. Semi-defined medium in microfiltration bioreactor was used for overproduction (11,500 U/L) of α–glucosidase of *Sulfolobus solfataricus* in re-*E. coli* [36]. In the present study, strong influence of yeast extract has been shown on product formation and this has been emphasized earlier [28] and also very recently to increase the cell biomass and protein production in *P. insularis* [24]. The increase in β–glucosidase enzyme units was accompanied by increase in specific enzyme activities as well. The last fed-batch run was accompanied by changes in cell morphology with darkly staining bodies distinctly visible in the cell cytoplasm indicating a change in the cellular distribution of enzyme. The decrease in specific enzyme activity in this particular case could be attributed to precipitation of enzymes in the cellular environment.

In conclusion, in this study we have identified a new β–glucosidase from thermo-tolerant yeast *P. etchellsii*. The enzyme has unique substrate hydrolysis profile and should be useful for special applications including synthesis of oligosaccharides. With the choice of a suitable host and manipulation of common media ingredients higher levels of enzyme production were achieved in the host. Some of these strategies should be applicable for overproduction of other proteins using re-*E. coli*.

Acknowledgements The authors wish to thank Department of Biotechnology, Ministry of Science and Technology, Government of India for providing financial assistance. Scholarship awarded to Ms. Yukti Bhatia by Council of Scientific and Industrial Research (New-Delhi) is duly acknowledged.

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[Received October 18, 2001; accepted February 7, 2002]