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CLONING OF STRUCTURAL GENES FOR ß-GLUCOSIDASE FROM *CELLULOMONAS BIAZOTEA* INTO *E.COLI* AND *SACCHAROMYCES CEREVISIAE* USING SHUTTLE VECTOR pBLU-D

Muhammad I. Rajoka*, S. Parvez & K.A. Malik
National Institute for Biotechnology & Genetic Engineering
P.O. Box 577, Jhang Road, Faisalabad, Pakistan.

ABSTRACT

A SmaI genomic library from *Cellulomonas biazotea* NIAB 442 was constructed in *Escherichia coli* HB101 using shuttle vector pBLU- D. Three clones with ability to hydrolyse esculin were isolated. These clones were compared *in vivo* and *in vitro* tests to select for hyper-secretion of ß-glucosidase. The recombinant plasmids were transformed to competent cells of a Cir° yeast. *In vivo* studies indicated that the genes were fully expressed in yeast as well.

INTRODUCTION

Enzymatic conversion of cellulose to metabolizable sugars is an important step in terms of their further conversion to other useful products including ethanol production. The microbial conversion of lignocellulosic biomass into useful products is a complex process and involves synergistic action of three enzymes of the cellulase complex namely endoglucanase (EC 3.2.1.4), exo-cellobiohydrolase (EC 3.2.1.91) and ß-glucosidase (EC. 3.2.1.21) (Marsden and Gray, 1986).

Recently cellulase genes from a wide variety of micro- organisms have been cloned (Knowles et al, 1987; Gilkes et al, 1991; Penttila et al. 1989). Structural genes of different cellulases from *Cellulomonas* spp. have been cloned in *E. coli* and *Saccharomyces cervisiae* (Whittle et al, 1982; Nakamura et al, 1986; Wong et al, 1988; Curry et al, 1988, Silva et al, 1988). In this paper, we report cloning of ß-glucosidase genes from *Cellulomonas biazotea* into *E. coli* and cir° strain of *S. cerevisiae*; the recombinants secrete gene product very efficiently *in vivo* in both *E. coli* and yeast recombinants. This work adds to our previous work reported elsewhere (Rajoka et al, 1992).

MATERIALS AND METHODS

Restriction enzyme SmaI & T4 DNA ligase were from New England Biolab, USA. Lysozyme, esculin, paranitrophenyl-ß-D- glucopyranoside (pNPG), ferric ammonium citrate, ampicillin, cellobiose, Deoxy-D-glucose and agar were from Sigma, USA. All other chemicals were of analytical grade.

Strains and Plasmid: *Cellulomonas biazotea* was isolated from a bagasse heap (Rajoka & Malik, 1986). Plasmid pBLU-D (Ludwig and Bruschi, 1991) was a gift from Prof. Bruschi, ICGEB, Trieste Italy. *E. coli* HB101 was supplied by New England Biolabs. Cir^o strain of *S. cerevisiae* FAS-21 was also a gift from ICGEB.

Culture Media: *Cellulomonas biazotea* was grown in Dubos salts minimal medium consisting $NaNO_3$, 1 g; KCl, 0.5g; K_2HPO_4, 1g; $MgSO_4$, 0.5g; $FeSO_4$, 0.1g per litre of water of pH 7.3. For isolation of chromosomal DNA, the medium was supplemented with cellobiose (0.5gl^{-1}). *E. coli* cells were grown in LB medium or Dubos salts-0.4% yeast extract-cellobiose medium. All above media were supplemented with ampicillin (50 μg ml^{-1}) whereever needed.

Isolation of DNA: *C. biazotea cultures* (100 ml) were grown for 20 hours and harvested by centrifugation. Chromosomal DNA was extracted from the cell pellet using CTAB after Ausubel et al (1990). Recombinant and other plasmids were isolated by the method of Birnboim and Doly (1979). Large scale recombinant plasmids were isolated after Ausubel et al (1990) and purified on cesium chloride-ethidium bromide density gradient.

Cloning Procedures: Two *C. biazotea* DNA samples containing 5 μg each were digested with SmaI at 25°C partially (for 1h) or completely (2 hours). The DNA samples were purified by extraction with phenol-chloroform, phenol-chloroform-isoamyl alcohol solutions and subsequently precipitated with isopropanol and dissolved in TE buffer. Plasmid pBLU-D was digested completely with SmaI and purified. The partially and completely digested chromosomal DNA (5 μg) each was ligated with 5μg of SmaI cut-purified preparation of pBLU-D using 4 units of T4 DNA ligase. The ligation mixture was maintained at 4°C for approximately 2 days after which it was transformed to competent cells of *E. coli* HB101 following protocol described by Ausubel et al (1990). The transformants were selected on Dubos-esculin-Deoxy-D-glucose- ferric ammonium citrate-ampicillin-agar medium. The positive clones converted esculin to esculitin which reacted, in turn, with ferric ions to form blackening zone. The diameter of zone of blackening (in mm) was taken as measure of ß- glucosidase secretion and compared for in-vivo production of ß-glucosidase

Subcloning in Cir^o yeast strain: The recombinant plasmids, prepared as above, were transformed to competent cells of Cir^o yeast after Ausubel et al, 1990; the recombinants were compared as above.

Preparation of Enzyme Extracts: *E. coli* strains were grown at 37°C in Dubos Salt-0.4% yeast extract supplemented with 50 μg Amp/ml using 0.25% cellobiose as carbon source. Yeast recombinants were grown in Del Rosario's medium having 50μg/ml ampicillin. The *E. coli* and yeast cultures were grown to late logarithmic phase using 1% (v/v) inoculum from overnight cultures grown in above media. Extracts were harvested by centrifuging cells in 5mM acetate buffer after sonicating on ice for two 3 minute bursts; cell debris was removed by centrifugation for 5 min in a Beckman centrifuge and the supernatant was preserved for enzyme assay.

Enzyme assays: *In-vivo* secretion of ß-glucosidase was performed by pouring 10µl cells of equal OD on the suerface of esculin-ferric ammonium citrate-amp-medium. The enzyme activity was determined by measuring zone of blackening in mm.

In-vitro assays on cell extracts were performed towards pNPG or cellobiose after Nakamura & Kitamura (1982) and Rajoka & Malik (1986). Enzyme activity was expressed as units/ml hr.

RESULTS

Cloning of ß-glucosidase genes: A total of 300 Amp^r clones were obtained. Three clones showed detectable ß-glucosidase activity. The recombinant plasmids isolated from these clones were named pRPI, pRP2 & pRP3.

Expression of ß-glucosidase genes: Results of in-vivo screening of ß-glucosidase secretion in *E. coli* and yeast recombinants are shown in Table 1 & Fig.1. These assays were performed as described in 'Material and Methods'. The zone of blackening measured in mm was taken as measure of enzyme secretion. The yeast recombinants produced 3-4 fold more ß- glucosidase than that produced by the donor.

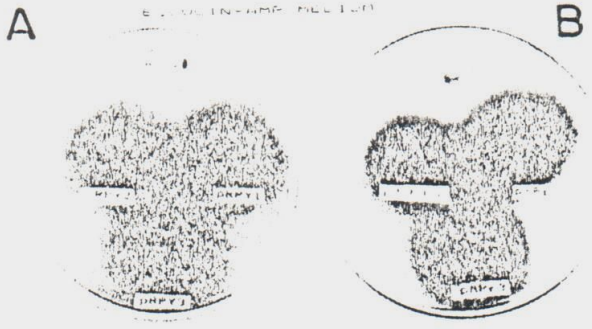

Fig.1: Secretion of ß-glucosidase by yeast (A) and *E. coli* 11(B) recombinants after growth on esculin-feric ammo nium citrate-Amp. medium. Cir^o yeast (A) and *E. coli* HB101 host carrying pBLU-D shuttle vector were used as control.

The rec-plasmids from *E. coli* recombinants were isolated after Birnboim and Doly (1979) and were transformed to competent cells of Cir^o Strain of *S. cerevisiae*. *In vivo* culture screening tests on yeast indicated that the genes were expressed at high level in yeast as well (3-4 fold improvement). When cells were grown in 0.25% cellobiose added to yeast fermentation medium (del Sario et al, 1979); *E. coli* and yeast recombinants grew to an OD of 2-3 measured on 5 fold diluted cultures at 610 nm.

Table-1:Production of ß-glucosidase by *E. coli* and yeast recombinants harboring pBLU-D rec-plasmids in plate tests.

	ß-glucosidase activity (dia of zone of blackening) (mm)	
	E. coli Recombinants	Yeast Recombinants
Control (pDLU-D)	0	0
Control donor	10	8
pRP1	19	32
pRP2	22	24
pRP3	27	30

10 µl of overnight culture of OD=0.6 was poured on the surface of esculin-ferric ammonium citrate solid medium, allowed to grow overnight and zone of blackening measured. All were grown in the presence of ampicillin except the donor which was grown without ampicillin.

Results of *in-vitro* screening after production of enzyme in 0.25% cellobiose are shown in Table 2. Maximum enzyme activity produced by the best recombinant was 2 fold the activity produced by the donor.

DISCUSSION

Molecular cloning of structural genes for ß-glucosidase from *C.uda* have been cloned in *E. coli* (Nakamura et al 1986). These authors reported that the cloned genes expressed very well in the host; the clones produced 1.2 to 4 times the activity produced by the donor respectively. The enzyme activity produced was mainly cell bound. In the present studies, bacterial recombinants produced 2-3 fold more ß-glucosidase in vivo as well as *in vitro* studies while yeast recombinants produced 3 to 4 times the activity produced by the donor in *in vivo* studies. Endo-ß-glucanase genes from *Ruminococcus albus* have also been cloned in *E. coli* and *S. cerevisiae* (Honda et al, 1988). The enzyme was secreted interacellularly in yeast transformants and produced a level of enzyme activity similar to the *E. coli* transformants. ß-glucosidase gene from *Aspergillus niger* has also been cloned in *S. cerevisiae*. Sacco et al (1984) reported the expression of an endo-glucanase gene in yeast from *Clostridium thermocellum*. Similarly endo-glucanase structural gene from *C.fimi* has also been cloned in yeast (Skipper et al, 1985). Cloning of the genes for cellobiohydrolase, CBH1 and CBH2 in *S. cerevisiae* has resulted in greater understanding of the cellulase system (Penttila et al, 1987). In this study, the enzyme was mainly produced cell bound as well and the electrophoretic mobility of ß- glucosidase from the donor as well as from the recombinanats was identical. The meximum activity produced by the yeast recombinanat towards

Table-2: Production of ß-glucosidase by *E. coli* and yeast recombinents harbouring chromosomal genes from *Cellulomonas biazotea* in Sma1 site of pBLU-D after growth on 0.5% cellobiose.

Recombinants harbouring plasmids	OD.610 on 5 fold diluted culture		ß-glucosidase (1U/ml hr)	
	B.R^{+}	Y.R^{++}	B.R^{+}	Y.R^{++}
Control*	0.0	0.0	0.0	0.0
pRP1	2.4	2.0	5.18	2.0
pRP2	2.3	2.2	6.15**	2.25
pRP3	3.2	2.8	7.17	3.1

* *E. coli* or yeast host harbouring shuttle vector pBLU-D was used as control.
** Processed in the presence of PMSF
\+ Bacterial recombinants
\+ + Yeast recombinants

NB. The donor under its optimum conditions of growth produced ß-glucosidase equivalent to 3 IU/ml hr after 20 hours of growth. Both bacterial and yeast recombinanats were grown as described in Materials and Methods.

cellobiose was 2 IU/ml hr. There is possibility that the other enzyme namely cellobiose phosphorylase may also has been cloned along with that for ß-glucosidase. We are trying vectors harbouring strong promoter which may further enhance the enzyme secretion by the recombinants. Our future efforts are directed towards cloning other genes in yeast to convert cellulosic substrates into ethanol.

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