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Cloning and expression of choline dehydrogenase gene (*betA*) of *Escherichia coli* in yeast *Saccharomyces cerevisiae*

Triwibowo Yuwono*[1,2], Jajah Fachiroh[2] and Joedoro Soedarsono[1,2]

1) Laboratory of Microbiology, Faculty of Agriculture, Gadjah Mada University,Yogyakarta, Indonesia
2) Inter University Center for Biotechnology, Gadjah Mada University, Yogyakarta, Indonesia

Abstract

The complete DNA sequence of betA, *encoding choline dehydrogenase of* Escherichia coli *has been cloned in a yeast expression-secretion vector and expressed in* Saccharomyces cerevisiae *DBY 746. The recombinant plasmid (designated as pYYF9) was stably maintained in yeast cells at a level of 24% after 30 generations. Gene expression analysis was carried out at transcription and translation levels. The mRNA analysis by DNA-RNA hybridisation using a specific* betA *probe did not reveal any signal of transcription of the cloned* betA *gene in yeast. Protein analysis by SDS-PAGE did not show any specific protein secreted into the yeast supernatant either. However, analysis of the intracellular proteins of yeast demonstrated differences of protein profile between the untransformed with the transformed cells.*

Keywords : *choline dehydrogenase gene* (betA) – *gene expression* – Saccharomyces cerevisiae

Introduction

Microbial adaptation to osmotic shock may be accomplished through the synthesis or accumulation of osmolytes. In *Escherichia coli*, tolerance to osmotic shock is conferred by the synthesis of glycine betaine using choline as the precursor. Choline dehydrogenase is the first enzyme involved in the synthesis of glycine betaine in *E. coli*. The enzyme oxydises choline into betaine aldehyde which is then followed by second oxidation, by betaine aldehide dehydrogenase, to glycine betaine. Choline dehydrogenase is encoded by *betA* gene, while betaine aldehyde dehydrogenase is encoded by *betB* gene. The DNA sequences of both genes have been completely characterised (Lamark *et al.*, 1991). A study has demonstrated that the expression of *betA* gene alone in tobacco resulted in the enhanced tolerance of transgenic tobacco to NaCl stress (Lilius *et al.*, 1995). This study thus suggested that choline dehydrogenase alone can accomplished the conversion of choline into glycine betaine.

Saccharomyces cerevisiae is a unicellular eukaryot which has been extensively used in industry. One of the limiting factors in the application of yeast in industry is the low

*) Corresponding author. Mailing address : Inter University Center for Biotechnology, Gadjah Mada University, Yogyakarta 55281, Indonesia, Telp. (0274) 902284, Fax. (0274) 520842, e-mail: tribowo@iuc-biotec.ugm.ac.id

tolerance to osmotic stress. The osmotic stress may occur due to the use of high concentration of sugar in the medium. High osmotic stress may result in the decrease of growth rate which consequently will also affect the net yield of fermentation. In *S. cerevisiae*, tolerance to osmotic stress imposed by high sugar and salt content is conferred by glycerol (Norbeck and Blomberg, 1997; Myers *et al.*, 1997). It was of interest to investigate whether *betA* gene of *E. coli* could be expressed in yeast. If the gene could be expressed in yeast, this would open up a novel approach in improving yeast tolerance to osmotic shock. This study describes the cloning and expression of *betA* gene of *E. coli* in the yeast *S. cerevisiae*.

Materials and Methods

Bacterial and yeast strains

1. *Escherichia coli* JM109 [*F' traD36 lacI*^{q} *Δ(lacZ)M15 proAB/recA1 endA1 gyr96 (Nal*^{R}*) thi hsdR17* (r_k-m_k) *supE44 relA1 Δ(lac-proAB)]* was used as the host for cloning.
2. *Saccharomyces cerevisiae* strain DBY 746 (*MATα his3-Δ1 leu2-3,112 ura3-52 trp1-289*) was used to express the choline dehydrogenase gene (*betA*) of *E. coli*.

Plasmid

1. pUMA19 was the recombinant plasmid carrying the complete structural gene of *betA* of *E. coli* (Yuwono *et al.*, 1996) and was used as the DNA source for the cloning of *betA* gene in yeast expression-secretion vector.
2. pYSV9 was the yeast expression-secretion vector used to express *betA* gene (kindly provided by Dr Peter A. Meacock, University of Leicester, UK).

Cloning of *betA* gene in yeast expression-secretion vector

The DNA sequence encoding choline dehydrogenase (*betA* gene) was digested from pUMA19 using *Hind*III and *Bam*HI enzymes followed by electrophoresis on 0.8% agarose gel. The *Hind*III - *Bam*HI fragment was cut from the gel, followed by extraction and purification using Agarose Gel DNA Extraction Kit (Boehringer Mannheim). The purified fragment was then ligated into pYSV9 which was also digested with *Hind*III and *Bam*HI enzymes. The ligation reaction was then used for transformation of *E. coli* JM109. All cloning procedures were carried out using the protocol as described by Sambrook *et al.* (1989).

Transformation of yeast

The recombinant plasmid (pYYF9) was subsequently used for transformation of *S. cerevisiae* DBY 746 using lithium acetate-induced transformation method (Ito *et al.*, 1983).

Plasmid stability analysis

The analysis of recombinant plasmid stability in yeast cells was performed by plating the cells carrying plasmid on a non-selective medium, followed by replica plating the colonies onto selective medium. Plasmid stability was expressed as the percentage of total colonies on the non-selective plates that grew on selective plates (Semi Defined medium: 6.7 g/l Yeast Nitrogen Base [without amino acids but supplemented with ammonium sulphate], 20g/l glucose, 20 µg/ml L-histidine, 20 µg/ml L-tryptophan, 30 µg/ml uracil). The cells carrying plasmid were firstly grown in non-selective broth YEPD medium (10 g/l yeast extract, 20 g/l peptone, glucose 20g/l) until reaching mid-exponential phase. The culture was then maintained at this growth phase by repeated dilution in the same medium.

Samples of cells were drawn from the culture when the cells have reached mid-exponential phase and plated onto YEPD agar medium. Colonies grew on YEPD agar were then replica plated onto selective medium on which only cells carrying the plasmid would be able to grow. The ratio (in percent) of colonies grew on selective plates to colonies grew on non-selective plates was expressed as plasmid stability (Bitter *et al.*, 1989; Yuwono, 1991).

RNA analysis

Total RNA was isolated from the yeast cells by using the method of Schmitt *et al.* (1990). RNA obtained was then electrophoresed on an agarose gel using the method as described by Farrel (1993), followed by northern transfer (Sambrook *et al.*, 1989) onto nylon membrane (Boehringer Mannheim). RNA blotted onto nylon membrane was subsequently hybridised with a probe developed for *betA* gene (Yuwono *et al.*, 1996). The probe used was the full-length DNA sequence of *betA* gene and prepared by random priming method using Non-radioactive DNA Labeling and Detection Kit (Boehringer Mannheim). RNA-DNA hybridisation was detected by ELISA method using anti-digoxigenin alkaline phosphatase conjugate and visualised using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Protein analysis

Protein was prepared both from the extracellular and intracellular preparations. The extracellular protein preparation was carried out by precipitation using cold acetone (Hames and Rickwood, 1990) and by TCA precipitation (Baldari *et al.*, 1987). The intracellular proteins were prepared by breaking-up the cell using sonicator. Cells were sonicated in cell-breaking buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA). The extracellular and intracellular protein preparations were subsequently electrophoresed on SDS-PAGE (Hames and Rickwood, 1990) and stained with Coomassie blue.

Results and Discussion

Cloning of *betA* gene in yeast expression-secretion vector

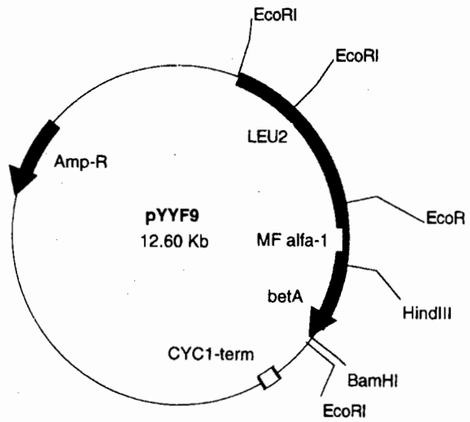

Figure 1. Scheme of recombinant plasmid carrying *betA* gene (pYYF9). *MF alfa-1*: DNA sequence encoding the regulatory and the prepro-sequences of the yeast α-factor gene, *LEU2*: Scerevisiae *LEU2* gene, *CYC1*-term: the *CYC1* terminator sequence, Amp-R: ampicillin resistance gene, *betA*: the complete structural DNA sequence of *betA* gene inserted in-frame between *Hind*III *and Bam* HI sites.

The scheme of recombinant plasmid carrying the complete structural gene of *betA* (pYYF9) is outlined in Figure 1. Restriction analysis of the recombinant plasmid (pYYF9) is presented in Figure 2. The plasmid vector, pYSV9, consists of regulatory region and prepro portion of the *MFα-1* gene of *S. cerevisiae*. The *MFα-1* regulatory region and leader sequence is essential for regulating the expression of the downstream heterologous gene and directing the polypeptide

into the secretion pathway. In addition, the vector also contains the *CYC1* termination sequences. The heterologous gene (*betA*) to be expressed was inserted in-frame at *Hind*III site which is part of the lys-arg residues. The lys-arg residues will be cleaved up by the action of dipeptidyl aminopeptidase (DPAPase A) encoded by *STE13* gene of *S. cerevisiae*. Thus, cloning of heterologous gene in-frame at *Hind*III site will result in the correct transcription and translation of the gene.

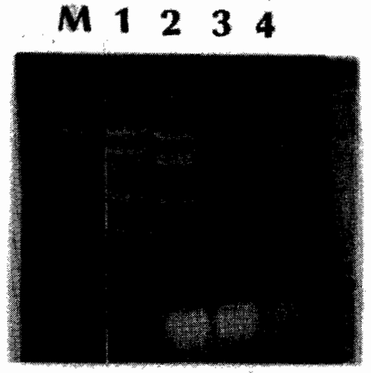

Figure 2. Restriction analysis of recombinant plasmid carrying *betA* gene (pYYF9): pYSV9 digested with *Eco*RI, 2 - 4: pYYF9 digested with *Eco*RI, M: marker, λDNA digested with HindIII + EcoRI.

Figure 2, lane 1 showed the results of digestion of the vector, pYSV9, with *Eco*RI which gave four fragments. The plasmid vector, pYSV9, contains four *Eco*RI sites which, upon cleavage, will result in four fragments of different sizes. One of the *Eco*RI sites is located just upstream of the *MFα-1* regulatory region and leader sequence. Figure 2, lane 2 - 4 showed that the *betA* gene has been inserted into the pYSV9 plasmid. The inserted fragment will increase the size of one of four *Eco*RI fragments.

Transformation of *S. cerevisiae* with recombinant *betA* gene

The recombinant plasmid (pYYF9) carrying the complete structural DNA of *betA* was subsequently used for transformation of *S. cerevisiae* DBY 746. Transformant obtained was then reconfirmed by culturing it in a selective medium. The results (data not shown) showed that the transformants carrying either the recombinant plasmid, pYYF9, or the vector only, pYSV9, grew on the selective media. The untransformed cells, on the other hand, did not grow in the same selective medium.

Recombinant plasmid stability

Recombinant plasmid, pYYF9, present in *S. cerevisiae* DBY 746 was analysed for its stability. The result (Figure 3) demonstrated that the plasmid was maintained in the cell, in a non-selective medium (YEPD), up to the level of 24% after 30 generations. The fact that the plasmid was relatively stably maintained in the cells under non-selective condition suggested that the *betA* gene expression was not toxic to the cells.

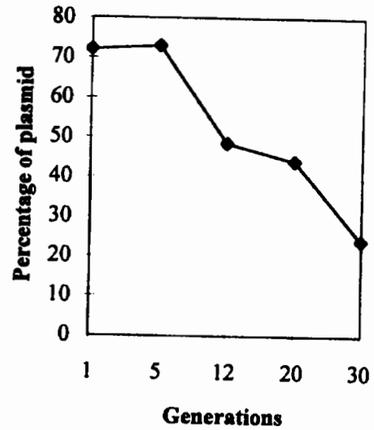

Figure 3. Stability of recombinant plasmid carrying *betA* gene in *S. cerevisiae* DBY 746 grown in YEP supplemented with 2% glucose

Expression of *betA* gene in *S. cerevisiae*

The analysis of *betA* expression in *S. cerevisiae* DBY 746 was carried out by analysis of transcription and translation. Transcription analysis was carried out by isolating

total RNA from yeast grown under non-selective condition in YEPD medium. The RNA was then electrophoresed and blotted onto nylon membrane. The RNA blot was subsequently probed by using *betA* DNA probe (Yuwono *et al.*, 1996). The result of hybridisation, however, did not show any positive signal, even after altering the temperature and hybridisation time. The results of transcription analysis thus suggest that the *betA* gene may not be efficiently transcribed. Therefore, the level of mRNA obtained may not be enough to be detected using the non-radioactive detection kit as used in this study. The possibility of mRNA degradation following isolation can be excluded, as it has been confirmed (data not shown) that after blotting the RNA could be clearly stained by methylene blue (Sambrook *et al.*, 1989) on the membrane.

Translation analysis was carried out by analysing both extracellular and intracellular proteins. It was assumed that if BetA protein was expressed in yeast, it would be secreted out of the cell. Therefore, extracellular protein was extracted from the supernatant culture grown in YEPD to a late exponential phase. The protein was concentrated and electrophoresed on SDS-PAGE. The results did not show any additional protein band. The absence of specific protein in the supernatant may be attributed to the low level of expression or the inefficiency of secretion. The relatively large (65 kDa) size of BetA protein may have imposed difficulty on secretion of the protein into the supernatant. In addition, it may also be possible that the SDS-PAGE may not be sensitive enough to visualise protein present at low level in the supernatant.

To determine whether or not the BetA protein (choline dehydrogenase) was retained in the cell, intracellular protein preparation was carried out after disrupting the cells by sonication. Electrophoresis of the intracellular proteins (Figure 4) demonstrated differences of protein profiles between untransformed cells (lane A1 - 3), cells transformed with pYSV9 (lane B1 - 3), and cells transformed with recombinant plasmid, pYYF9 (lane C1 - 3). When the cells were grown in YEP supplemented with 2% glucose (lane C1) more distinct new protein bands appeared (arrow).

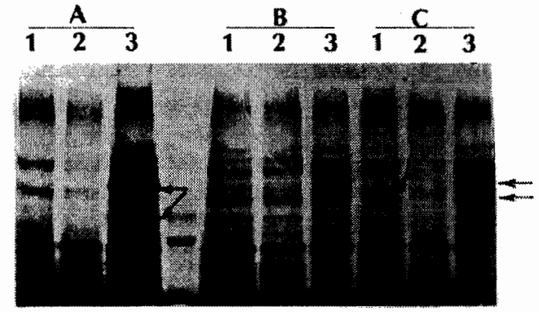

Figure 4. Profiles of intracellular proteins extracted from *S. cerevisiae* DBY 746. A: untransformed yeast cells, B: yeast carrying the vector only (pYSV9), C: yeast carrying recombinant plasmid harboring betA gene (pYYF9). The yeast was grown in:
1. YEPD supplemented with 2 % glucose
2: YEPD supplemented with 20 % glucose
3: YEPD supplemented with 2 % glucose and 5mM choline
Arrows showed new protein bands emerged

The protein band of this size was not observed in the preparation of cells carrying the vector only (pYSV9) grown under the same condition (lane B1). The more significant difference was observed when the cells were grown in YEP supplemented with 20% glucose and 5mM choline (lane C3, see the arrow). The emergence of the two protein bands in this cell suggested specific gene expression. Protein bands of the similar sizes did not appear in the cells transformed with pYSV9 (lane B3). However, new protein of different sizes also appeared when the untransformed cells were grown in YEP supplemented with 20% glucose and 5mM choline (lane A3, see the arrow). The data presented here suggested that the presence

of recombinant *betA* gene in yeast resulted in the changes of protein profiles expressed. The difference of protein profile was observed when the cell was grown in the presence of choline and high sugar content. Interestingly, changes of protein profile was also observed in the untransformed cells, despite the fact that the sizes of the new proteins were different. Similarly, Norbeck and Blomberg (1997) also observed changes of protein profiles of *S. cerevisiae* grown in the presence of 1.4M NaCl. It was not clear at this state, however, whether or not the changes of protein profile in the cells transformed with recombinant plasmid was due to the specific expression of *betA* gene in the plasmid. The fact that the cells transformed with the plasmid vector only (pYSV9) did not show signal of the synthesis of new protein (lane B3) suggested that the presence of recombinant gene (*betA*) might have some effects on the pattern of protein expression.

Acknowledgment

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References

Baldari, C., Murray, J.A.H., Ghiara, P., Cesareni, G., and Galeotti, C.L. (1987) A novel leader peptide which allows efficient secretion of a fragment of human interleukin 1b in *Saccharomyces cerevisiae*. *EMBO J.* 6:229-234.

Bitter, G.A., Egan, K.M., Koski, R.A., Jones, M.O., Elliott, S.G., and Giffin, J.C. (1989) Expression and secretion vectors for yeast. *In*: R. Wu, L. Grossman, K. Moldave (eds.) Recombinant DNA Methodology. Aacademic Press, Inc., San Diego.

Farrel, R.E. (1993) RNA Methodologies: A laboratory Guide for Isolation and Characterization. Acadermic Press, California

Hames, B.D. and Rickwood, D. (1990) Gel Electrophoresis of Proteins. A Practical Approach. Second edition. ORL Press, Oxford.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163-168.

Lamark, T., Kaasen, I., Eshoo, M.W., Falkenberg, P., McDougall, J. and Strom, A.R. (1991) DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-glycine pathway of *Escherichia coli*. *Mol. Microbiol.* 5:1049-1064.

Lilius, G., Nicklas, H., and Leif, B. (1995) Enhanced NaCl stress tolerance in transgenic tobacco expressing bacterial choline dehydrogenase. *Bio/Technol.* 14:177-180.

Myers, D.K., Lawlor, D.T.M., and Attfield, P.V. (1997) Influence of invertase activity and glycerol synthesis retention on fermentation of media with a high sugar concentration by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 63:145-150.

Norbeck, J., and Blomberg, A. (1997) Metabolic and regulatory changes associated with growth of *Saccharomyces cerevisiae* in 1.4M NaCl. *J. Biol. Chem.* 272:5544-5554.

Sambrook, J., Maniatis, T., and Fritsch, E.F. (1989) Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory, New York.

Schmitt, M.E., Brown, T.A., and Trumpower, B.L. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acid. Res.* 18:3091.

Yuwono, T. (1991) A Study of Actinidin Expression in Yeast. PhD Thesis. University of Leicester, UK.

Yuwono, T., Shovitri, M., Mursyanti, E., and Soedarsono, J. (1996) Development of probes for detection of betaine genes in rhizobacteria using PCR-amplified betain-encoding DNA sequences of *Escherichia coli*. *Indon. J. Biotech.* December:75-81.