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Genetic Stability of the Integrated Structural Gene of Guamerin in Recombinant *Pichia pastoris*

LIM, HYUNG-KWON, KYEONG-YEON KIM, KONG-JU LEE, DOO-HONG PARK, SOO-IL CHUNG, AND KYUNG-HWAN JUNG*

Mogam Biotechnology Research Institute, 341 Pojung-Ri, Koosung-Myun, Yongin, Kyonggi-Do 449-910, Korea

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Abstract Genetic characteristics of the structural gene of guamerin (a novel elastase inhibitor from Korean leech), integrated into the *HIS4* locus of chromosomal DNA of *Pichia pastoris* along with the α-factor leader sequence, were investigated. In the selected clone from candidates, two copies of the integration cassette including the structural gene of guamerin were found in the integration site of the chromosomal DNA of *P. pastoris*. It was demonstrated that the integrated structural gene of guamerin was stable up to about 70 generations in the relay flask culture. Then, a high-cell-density culture could be fulfilled easily by DO-stat fed-batch culture, in which the cell growth and the recombinant guamerin production reached about 250 of $OD_{600\,nm}$ and 260 mg/l, respectively. Finally, it was revealed that the DNA sequence of the integrated structural gene of guamerin in *P. pastoris* was maintained correctly in the end of production cells of relay flask culture and high-cell-density culture.

Key words: Guamerin, genetic stability, master cell bank, *Pichia*, integration

The establishment of an industrial process producing a recombinant protein by rDNA technology requires an extensive validation of the genetic stability of host and vector system. Those data should be documented as a file to be submitted to the regulatory authorities [5]. In particular, the genetic characterization of the master cell bank (MCB), which served as a source of seed culture for the lifetime of recombinant protein product, is recognized to be a prerequisite for establishing the commercial production process.

Many heterologous proteins have been successfully expressed and secreted into the culture medium using the *Pichia pastoris* (*P. pastoris*) expression system at commercially interesting concentrations [2, 3, 9, 10]. In a *P. pastoris* system, for the purpose of preventing its genetic instability during the cultivation process, the expression vector is generally integrated into chromosomal DNA of *P. pastoris*. In addition, it is known that the production system using *P. pastoris* has many advantages. One of the benefits is to easily fulfill a high-cell-density culture and to carry out the scale-up process.

Guamerin is a novel elastase-specific inhibitor isolated first by Jung *et al.* (1995) [4] from Korean leech (*Hirudo nipponia*). Recently, recombinant guamerin-producing *P. pastoris* had been constructed (K. J. Lee *et al.* 1998. cDNA and its expression vector for an elastase inhibitor from Korean blood sucking leech. *Korean Patent* 98-27344), in which the structural gene of guamerin was fused with an α-factor leader sequence for the secretion of guamerin, and it was integrated into the host chromosomal DNA.

In this study, to examine the genetic stability of the structural gene of guamerin integrated into the chromosomal DNA of *P. pastoris*, the genetic characterization of recombinant *P. pastoris* was performed using the cassette number estimation, the restriction analysis, and the DNA sequencing. Finally, a high-cell-density culture for the production of recombinant guamerin was accomplished on a lab-scale.

MATERIALS AND METHODS

Construction of Expression Strain

The structural gene for guamerin was obtained from the cDNA library of the entire leech by using PCR. [6]. To construct the expression strain using *P. pastoris*, its cDNA was fused with an α-factor leader sequence containing the KEX2 cleavage site and thereafter transformed into *P. pastoris* GS115, a *his4* strain (K. J. Lee *et al.* 1998. cDNA and its expression vector for an elastase inhibitor from

*Corresponding author
Phone. 82-31-260-9845; Fax: 82-31-260-9808;
E-mail. khjung@greencross.com

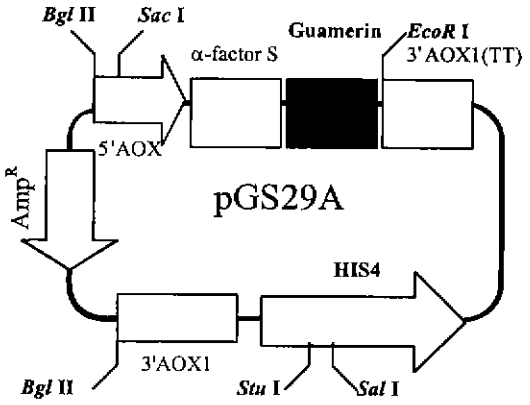

Fig. 1. Plasmid vector for the expression of recombinant guamerin in *P. pastoris*.
pGS29A (8.2 kbps) contains c-DNA for guamerin inserted between the AOX1 promoter (5'AOX) and the AOX1 terminator (3'AOX(TT)) for methanol-inducible expression/secretion.

Korean blood sucking leech. *Korean Patent* 98-27344). Gene insertion events arose from a single crossover between the *his*4 locus in the chromosome and the *HIS*4 marker in the vector. The host and vector system was purchased from Invitrogen Co. (San Diego, U.S.A.). Figure 1 shows the expression vector, pGS29A.

Clone Selection and Preparation of Master Cell Bank (MCB)

For the clone selection, the candidate clone was cultured for 15 h in a minimal medium (glycerol, 10 g/l; yeast nitrogen base, 13.4 g/l; and biotin, 0.4 mg/l) and then it was induced by resuspending the clone culture into the minimal medium containing 0.5% (w/v) methanol instead of glycerol. Methanol was repeatedly supplied every 24 h to maintain the methanol concentration of 0.5% (w/v) for approximately three days.

To prepare MCB, recombinant *P. pastoris* was cultured in the YPD medium (yeast extract, 10 g/l; Bacto-peptone, 20 g/l; dextrose, 20 g/l) for 16 h. An equal mixture of culture broth and 50% (w/v) glycerol were made in a cryo-tube (Corning, Cambridge, U.S.A.) and MCB was stored at - 70°C.

Determination of Elastase Inhibition Activity

Porcine pancreatic elastase (PPE, Sigma, U.S.A.) and *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma, U.S.A.) were used as an enzyme and its substrate, respectively. The reaction was performed in 0.05 M Tris-HCl buffer (pH 8.0). To 1 volume of buffer solution containing PPE (0.1 unit), 1 volume of sample solution was added, mixed thoroughly, and 3 volume of 0.1 mM substrate was added. After 10 min reaction at 25°C, the absorbance at 410 nm was measured. One inhibition unit (iu) was defined as the amount of inhibitor to suppress formation of 1 μmol *p*-nitroanilide from *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide per min under the given condition [4]. The percent of elastase inhibition activity was defined as the ratio of the decrease in the production of *p*-nitroanilide by the sample to the control assayed in 0.05 M Tris-HCl (pH 8.0).

Cassette Number Estimation and Restriction Analysis

To identify the cassette number of integration of the structural gene of guamerin, the genomic DNA analysis was performed by Southern blotting with both restriction enzyme and probe, as previously described [7, 8]. Total DNA isolation from recombinant *P. pastoris* was performed using the Easy-DNA kit (Invitrogen Co., U.S.A.). Isolated DNA was restricted and probed with *Bgl*II and *HIS4*, respectively (Gene Images™, random prime labeling module, Amersham International plc, U.K.).

Restriction analysis was also performed using the various combinations of restriction enzymes and probes such as *Bgl*II/guamerin, *Eco*RI/*HIS4*, and *Eco*RI/guamerin. The interpretation of the results of Southern blotting was based on the principle of homologous recombination at the *his4* locus of chromosomal DNA (*Pichia* Expression Kit, Invitrogen Co., U.S.A.).

Relay Flask Culture

A single colony from guamerin-producing *P. pastoris* was inoculated to 50 ml of YPD medium and then cultured overnight in a shaking incubator at 30°C and at 150 rpm. This shake flask culture was used for an inoculum (5%, v/v) of the new flask culture. After the overnight culture of that flask, the culture was used again for an inoculum. This relay culture was repeated until the generation number reached about 70. Generation number was estimated by using the initial and final cell concentrations denoted by the optical density at 600 nm. Since the cell number increases by multiple factor of 2, the following equation could be deduced. Generation number (n)=ln (X_s/X_i)/ln2, where X_s=cell concentration at the time of sampling (O.D. at 600 nm) and X_i=cell concentration at inoculation (O.D. at 600 nm).

For the genomic DNA analysis, 10 ml of the culture broth was withdrawn from every relay flask culture just before the culture was used for inoculum. The total DNA of the cell was treated with *Sal*I, electrophoresed in 0.6% agarose gel, and then DNA was transferred to a nylon membrane for blotting. A probe against the structural gene for guamerin was prepared using a random prime labeling module (Gene Images™, Amersham International plc, U.K.).

High-Cell-Density Culture

For the high-cell-density culture of guamerin-producing *P. pastoris*, DO-stat fed-batch culture followed by the methanol induction was employed. Bioflo III fermentor (New Brunswick Scientific Co., NJ, U.S.A.) was used with an initial culture volume of 1.0 l. Temperature, pH, and aeration were

controlled at 30°C, 5.0, and 2.0 vvm, respectively. To prepare inoculum for the batch culture, 50 ml of the seed culture was cultured for 18 h in a YPD medium at 30°C and 200 rpm. The minimal salt medium containing yeast trace metal solution was used for the batch culture [1]. After glycerol in the medium was completely exhausted, the fed-batch culture was performed consecutively, in which the medium feeding was controlled by DO-stat mode with 50% of air-saturation as a set point. The feed medium was composed of 50% (w/v) glycerol and 12 ml/l yeast trace metal solution. After approximately 500 ml of feed medium was fed, the medium feeding by DO-stat mode was stopped, and then the culture was starved for 0.5 h to completely deplete the residual glycerol. Finally, the medium containing 50% methanol and 12 ml/l yeast trace metal solution was fed to induce the guamerin production, and its feeding rate was increased stepwise from 22 to 48 ml/h for 40 h. Cell growth was determined by examining the absorbance at 600 nm (Ultrospec II, LKB Biochrom, U.K.).

The amount of guamerin (mg/l) in the culture supernatant in high-cell-density culture was also estimated using the correlation between the inhibition unit (iu) and protein quantity (mg) of purified guamerin. The relationship between the inhibition unit and guamerin quantity could be shown with a simple equation; guamerin (mg/l)=(1000/897.222) × iu.

DNA Sequencing

Primers corresponding to 5'AOX (GAC-TGG-TTC-CAA-TTG-ACA-AGC) and 3'AOX (GCA-AAT-GGC-ATT-CTG-ACA-TCC) were used for amplification of the guamerin gene from chromosomal DNA of *Pichia* producing guamerin by polymerase chain reaction. Amplified guamerin gene was transformed to *E. coli* using the transformation kit (pGEM easy transformation kit, Promega, U.S.A.). The procedure for DNA sequencing is depicted in Fig. 2.

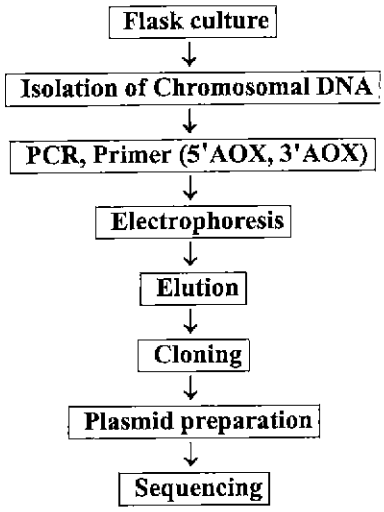

Fig. 2. Procedure flow diagram for genomic DNA sequencing of guamerin gene integrated into recombinant *P. pastoris* having a generation number of about 70, and into the end of production cells (EOPC).

Results and Discussion

Clone Selection

Using the shake flask culture, several hundreds of colonies selected in the agar plate were preliminarily evaluated for the elastase inhibition activity in the culture supernatant, in which the methanol induction was performed only once (data not shown). Two clones, #3-32 and #12-44, were selected as the candidate clones for guamerin production. As shown in Fig. 3, during the methanol induction process for three days, the clone #3-32 exhibited a higher elastase inhibition activity than the clone #12-44, therefore, clone #3-32 was chosen for the production clone and it was used for preparing MCB.

Homogeneity of MCB

To assure the culture homogeneity, 360 colonies were isolated from one vial of MCB and tested for the phenotype for auxotrophic selection marker, *his4*. Colonies isolated from the non-selection media containing L-histidine were picked and then transferred to the selection media lacking L-histidine. All colonies picked formed new colonies in the selection media after incubation at 37°C for 18 h (data not shown). It was concluded that all the cells in MCB harbored the expression cassette homogeneously in its genome.

Determination of Expression Cassette Number and Restriction Analysis with Genome of *Pichia* Producing Guamerin

To confirm the number of gene insertion events, the isolated genomic DNA of *P. pastoris* was restricted and

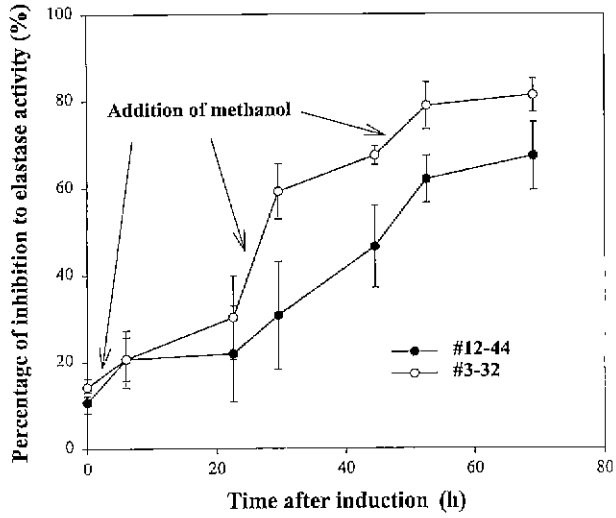

Fig. 3. Comparison of elastase inhibition activity between guamerin-producing clones, #3-32 and #12-44.

blotted with several combinations of restriction enzymes and probes. Table 1 shows the expected size of restricted DNA fragments detected by Southern blotting when the expression cassettes were integrated by homologous recombination at the *his4* site, as described in Materials and Methods. As shown in Table 1, multiple gene insertion into genomic DNA can be detected quantitatively by Southern blotting analysis. To confirm how many expression cassettes were integrated, the *Bgl*II restriction enzyme and *HIS4* probe were used. Fragments of 3.7, 4.7, and 5.8 kb were detected with the ratio of intensity of 1:1:1 (Fig. 4), suggesting that two copies of expression cassettes were inserted at the *his4* locus of the *Pichia* genome. Figure 5 shows the schematic diagram of the expected restriction map around the *his4* locus when two copies of these expression cassettes were inserted.

Besides the combination of *Bgl*II/*HIS4*, restriction analyses of genomic DNA using the combinations of *Eco*RI/*HIS4*, *Bgl*II/guamerin, and *Eco*RI/guamerin were also performed to prove that a correct recombination did indeed take place, as shown in Fig. 5. If two copies of the expression vector were integrated, the additional DNA fragments of 5.8 kb in the case of using *Bgl*II and of 8.2 kb in the case of using *Eco*RI (marked with bold type in Table 1) could be detected. As shown in Fig. 6, it was the case in all the combinations of restriction enzymes and probes, evidenced by the expected sizes of DNA fragments including additional 5.8 kb and 8.2 kb DNA sizes. These results were shown in Fig. 5 and Table 1. Although it was known that multiple gene insertions at a single locus in a cell occur with a low frequency, it was observed that the clone selected for manufacturing the cell bank harbored two expression cassettes of guamerin gene in its genome at the *his4* locus.

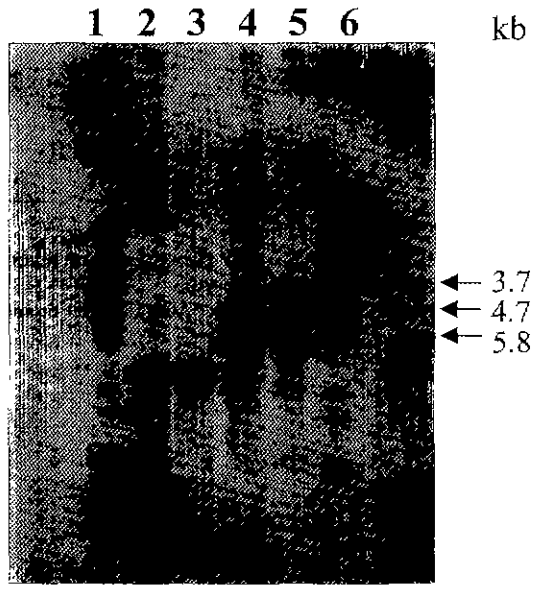

Fig. 4. Southern blot for cassette number estimation of recombinant *P. pastoris*.
Isolated genomic DNA of recombinant *P pastoris* was restricted with *Bgl*II and probed with *HIS4*. Lanes 1 and 6, λ/*BstE*II; lanes 2 and 3, host strain GS115; lane 4, Control strain integrated single cassette of guamerin gene, lane 5, strain #3-32.

Genetic Stability of the Structural Gene of Guamerin during Relay Culture

As shown in Fig. 7, it was found that the DNA fragment containing the structural gene of guamerin was stable up to the generation number of about 70. For achieving a reliable and a high yield production, the structural gene must be stable throughout the production process, including the preparation of working cell bank (WCB) and several passages of the seed culture in a large-scale fermentation (International Conference on Harmonisation, ICH harmonised tripartite guideline on quality of biotechnological products: Analysis of the expression construct in cells used for production of r-DNA derived protein products. Yokohama, Japan, 29 November - 1 December 1995). The generation number elapsed to prepare WCB from MCB was about 5-7 in our culture system. Thus, the stability of structural gene up to a 70 generation number would be enough for the entire lifetime of production, since one vial of MCB could produce several hundreds of WCB vials. Although a large-scale fermentation undergoes more number of generations and encounters more stressful conditions than that with a small lab-scale fermentation, since it needs more steps of culture including pre-seed and seed cultures for enlarging the volume of culture, its generation number does not exceed 20. As a results, by the relay culture test, it could be shown that the genetic stability of the structural gene was stable beyond the normal generation number required for the production process.

High-Cell-Density Culture of Guamerin-Producing *P. pastoris*

As shown in Fig. 8, the cell density reached about 250 of $OD_{600\,nm}$ after DO-stat fed-batch culture. During the methanol feeding, a slight increase in the cell density was observed.

Table 1. The expected size of DNA fragments detected by Southern blot using various combinations of restriction enzymes and probes in the #3-32 strain.

Restriction enzyme probe	*Bgl*II		*Eco*RI	
	HIS4	guamerin	*HIS4*	guamerin
Single integration (kb)	3.7, 4.7	4.7	3.8, 7.4	7.4
Double integration (kb)	3.7, 4.7, **5.8**	4.7, **5.8**	3.8, 7.4, **8.2**	7.4, **8.2**

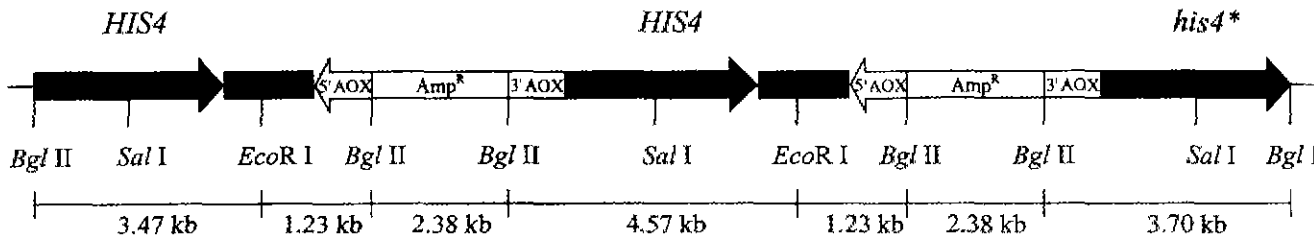

Fig. 5. Expected restriction map of the genome of recombinant *P. pastoris* after a second insertion event of expression vector had occurred. rG: recombinant guamerin gene.
S: α-factor signal sequence TT: terminator. 5AOX1: AOX1 promoter. *: mutation. The *his4** was mutated because it originated in the mutated *his4* gene from the host genome.

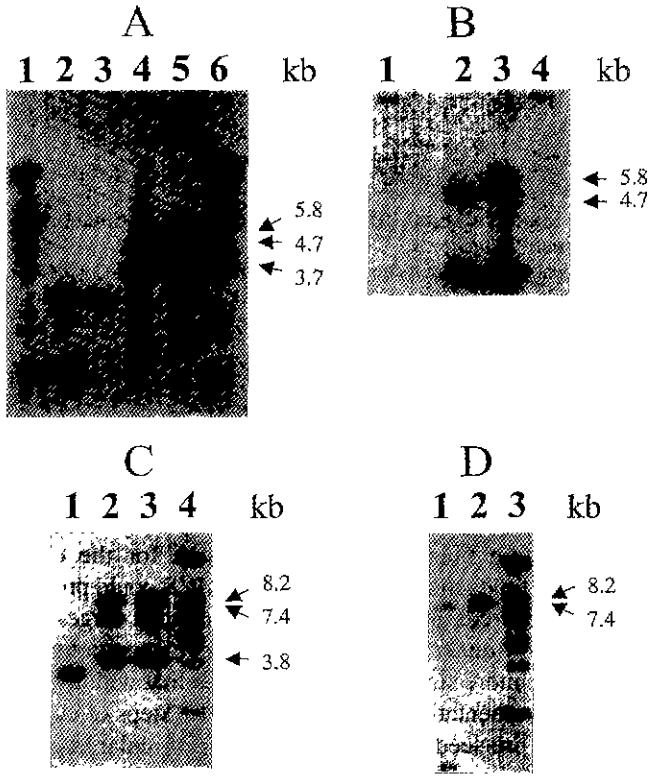

Fig. 6. Southern blot analysis of restriction fragments. Isolated genomic DNA of recombinant *P. pastoris* was restricted and probed with combinations of *Bgl*II/*HIS4* (A), *Bgl*II/guamerin (B), *Eco*RI/*HIS4* (C), and *Eco*RI/guamerin (D).
A Lanes 1 and 6. DNA size marker (λ/*Bst*EII); lanes 2 and 3, host strain GS115; lane 4, control strain integrated single cassette of guamerin gene; lane 5, strain #3-32 B: Lanes 1 and 4, DNA size marker (λ/*Bst*EII), lane 2, control strain integrated single cassette of guamerin gene, lane 3, strain #3-32 C: Lane 1, host strain GS115; lane 2, control strain integrated single cassette of guamerin gene; lane 3, strain #3-32, lane 4, DNA size marker (λ/*Bst*EII) D: Lane 1, control strain integrated single cassette of guamerin gene; lane 2, strain #3-32; lane 3, DNA size marker (λ/*Bst*EII).

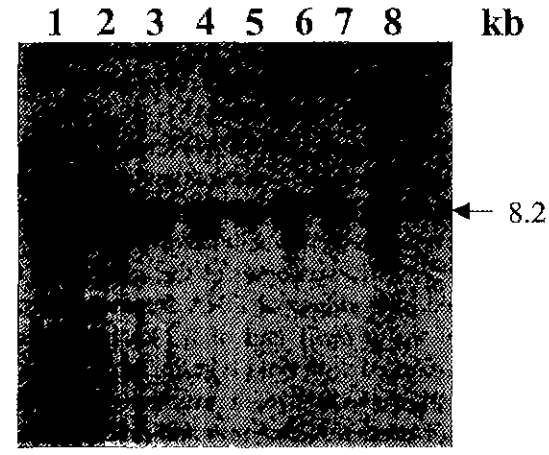

Fig. 7. Southern blot of the integrated gene in recombinant *P. pastoris* producing guamerin with increased number of generation for test of genetic stability.
Genomic DNA isolated from cells having a certain generation number was restricted with *Sal*I and probed with the guamerin gene. Lane 1, generation no. 7.5; lane 2, generation no 18.3; lane 3, generation no. 28.6, lane 4, generation no 40.1, lane 5, generation no. 47.4, lane 6, generation no. 58.8; lane 7, generation no 70.1; lane 8, size marker (λ/*Bst*EII).

Recombinant guamerin was secreted into the culture broth up to about 260 mg/l by the methanol induction process. The total generation number at the end of product cells (EOPC) in a high-cell-density culture was 10.4, which was smaller than that by a large-scale fermentation as mentioned above. During the induction phase, guamerin was produced by the manner of first order function, while the generation number increased by only 0.1. Based on the result, it could be interpreted that the expression cassettes were preserved and worked stably, not only during the growth phase but also in the induction phase.

DNA Sequencing

In order to examine whether or not the DNA sequence of the structural gene of guamerin was maintained correctly, DNA sequencing for the structural gene of guamerin was performed three times for each sample. Samples for DNA sequencing were prepared from MCB, the last sample of the relay flask culture, and the EOPC in a high-cell-density culture. All of the DNA sequences of the structural gene of *guamerin were identical to that of the expression vector* (data not shown). The results confirmed the fact that the increase of generation number during MCB preparation and a high-cell-density culture did not induce any change in the DNA sequence of the integrated guamerin structural gene.

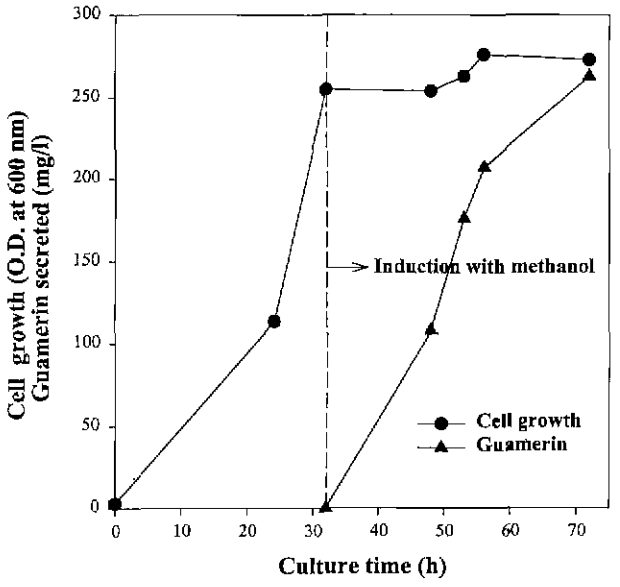

Fig. 8. High-cell-density culture of *P. pastoris* producing guamerin.

Acknowledgments

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