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Production of Toxin Protein by Recombinant *Escherichia coli* with a Thermally Inducible Expression System

HAN, SE JONG, HO NAM CHANG, YONG KEUN CHANG*, AND SEONG LYUL RHIM[1]

Department of Chemical Engineering, KAIST, Taejon 305-701, Korea
[1]Department of Genetic Engineering, Hallym University, Chuncheon, Kangwon 200-702, Korea

Physiological studies on the expression of *Bacillus thuringiensis* subsp. *tenebrionis* (*Btt*) gene coding for insecticidal protein in recombinant *Escherichia coli* 537 were carried out to identify optimal culture condition. It was necessary to shift culture temperature from 30 to 42°C to express the gene. Expression of the *Btt* toxin gene by recombinant *E. coli* 537 began within one hour after induction. Complex nitrogen sources increased production of the insecticidal protein. The total insecticidal protein was 0.5 g/l when using yeast extract as a complex nitrogen source. Soybean hydrolysate showed apparently the highest induction efficiency. After induction, the cellular content of the insecticidal protein was 5.4 times higher than it had been before induction. The optimal cultivation strategy was found to grow cells for 7 hours at 30°C and then 5 – 8 hours at 42°C. The optimal cultivation pH for the production of insecticidal protein was 6.5. The *Btt* toxin produced by the recombinant *E. coli* 537 was found to have the same level of potency against Colorado potato beetle as the original toxin.

Bacillus thuringiensis (*B.t.*) toxins are the only type of bioinsecticides that are manufactured on an industrial scale and made available on the market at prices which farmers can afford. Due to their selectivity, more *B.t.* insecticides based on different strains will be developed and marketed. Nevertheless, *B.t.* insecticides are still expensive in comparison to synthetic chemical insecticides. More work on fermentation optimization as well as development of formulation and quality control procedures is required. Streamlining the development process in order to minimize cost and time will be a major challenge. Recombinant DNA technology is a potentially powerful tool for improving the quality and economics of *B.t.* insecticides.

Genetic engineering techniques for the production of proteins by means of fast growing microorganisms such as *E. coli* are already used in many different areas. The first step in expressing foreign proteins in bacteria is to choose an expression vector that carries a strong, inducible promoter (8). The strong P_L promoter of bacteriophage λ is one of the most useful genetic elements for controlled expression of heterologous proteins in *E. coli*. If the host strain or the vector contains the *cI*857 gene, encoding a thermo-sensitive repressor of the P_L promoter, the production of the desired proteins can be accomplished by thermal induction of the culture (3, 10). Under non-inducing conditions, transcription from the λP_L promoter is repressed. This allows the plasmid to maintain stability for the production of toxic proteins at the later phase. Rhim *et al.* (7) greatly improved the expression in a recombinant *E. coli* of the *B.t.* toxin gene by controlling its transcription with the λP_L promoter and *cI*857 gene system. They reported that the host *E. coli* strain was able to express the engineered toxin gene from *Bacillus thuringiensis* subsp. *tenebrionis* at the same level as its original toxin gene.

In this study, the same recombinant *E. coli* strain developed by Rhim (7) was cultivated under various conditions to produce *B.t.* toxins. Physiological studies for the expression of the *B.t.* toxin gene in recombinant *E. coli* 537 were carried out to find optimal culture condition.

MATERIALS AND METHODS

Microorganism and Plasmids

The microorganism used in this study, *E. coli* 537, contains two plasmids, pEX-Btt (7, 10) and pcI857 (6). The toxin gene in plasmid pEX-Btt produces two proteins of 67 kDa and 85 kDa. The expression of *B.t.* gene in plasmid pEX-Btt is repressed by the *cI* gene in pcI857. It

*Corresponding author
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was necessary to adjust culture temperature from a growth temperature of 30°C to 42°C to express the insecticidal protein gene.

Cultivation

The seed culture was prepared as follows. A loop of cells from Luria-Bertani (LB) plates was grown at 30°C for ca. 9 h in a shaking incubator. One ml of this culture was then used to inoculate 100 ml of modified M9 or LB medium of the composition shown in Table 1 contained in a 250 ml shake flask. Effects of complex nitrogens and pH on cell growth, insecticidal protein production, and the cultivation strategy were evaluated.

Cell Growth Monitoring

Growth of the microorganism was monitored by measuring the optical density of the culture broth at 600 nm with a spectrophotometer (DU-65 Spectrophotometer, Beckman, USA). The cell concentration was also determined by measuring dry cell weight. The ratio of dry cell weight (g/l) to optical density was 0.4.

Protein Analysis

After a 3 – 20 h of second-stage incubation at 42°C, *E. coli* cells were harvested from a 1 ml sample of the culture broth, washed with distilled water, and then centrifuged. The pelleted cells were resuspended with 100 μl of TE buffer (10 mM Tris-Cl + 1 mM EDTA, pH 7.5) and disrupted by sonication (2×20 sec.) after freeze-thaw treatments. To remove the cell debris, brief centrifugation was carried out for 5 seconds. After centrifugation, 30 μl of the supernatant was boiled for 10 min to denature the polypeptides. And 6 μl of loading buffer (8) was added to the boiled sample and well mixed. This mixed sample was analyzed by SDS polyacrylamide gel (10%) electrophoresis (PAGE) (5). Proteins were stained with Coomassie Brilliant Blue. For analysis of the 67 and 85 kDa insecticidal proteins (IP) contents, the gels were scanned with a densitometer (2202 Ultrascan laser densitometer, LKB Bromma, Sweden). The total protein concentration was determined according to the method of Bradford (2) using bovine gamma globulin as a standard. Western blots (8) were carried out with a monospecific *B. thuringiensis* subsp. *tenebrionis* toxin antibody to identify the insecticidal protein.

Table 1. The composition of modified M9 medium and LB medium.

Component	Concentration (g/l)
Modified M9 medium	
Na_2HPO_4	6.0
KH_2PO_4	3.0
NaCl	0.5
NH_4Cl	1.0
$MgSO_4$	0.5
Glucose	2.0
$CaCl_2$	0.01
Complex N source	5.0
LB medium	
Tryptone	10.0
Yeast extract	5.0
NaCl	10.0

Culture Broth Analysis

One ml of the culture broth was centrifuged at 14,000 rpm for 10 min, and the supernatant was mixed with 0.1% H_2SO_4 and filtered through a 0.22 μm filter (P. J. Cobert Associates, Inc., USA). The acetic acid and glucose concentrations were determined using a high performance liquid chromatograph (Hitachi Co., Japan) with an Aminex HPX-87H column (Bio-Rad Laboratories, California, USA). The flow rate of the mobile phase, a 0.01 N H_2SO_4 solution, was 0.8 ml/min and the operating temperature was 60°C.

Bioassay

The bioassay of toxin activity was performed in quantified cell extracts which were spread on a 2 cm^2 piece of potato leaf in a petri dish. A known number of 10 second instar larvae were reared at 25°C. The number of surviving larvae was counted at various times to check the lethality of the protein.

RESULTS AND DISCUSSION

Expression of Insecticidal Protein Gene

To confirm the expression of the insecticidal protein gene, SDS-PAGE and western blots were carried out after cells were cultivated in LB medium. Fig. 1 shows that insecticidal protein gene was expressed successfully and the toxin proteins were produced within one hour after induction.

Effect of Complex Nitrogen Sources

E. coli 537 was cultivated in test tubes, in LB or modified M9 medium containing several complex nitrogen sources such as yeast extract, tryptone, soybean hydrolysate, and peptone. Shimizu *et al.* (9) reported that insecticidal protein production was elevated by some complex nitrogen sources. In their study, soybean hydrolysate was the most effective and the insecticidal protein content was 18.5% of the total protein. In this study, the insecticidal protein content after induction was 35.0% of the total cellular protein with soybean hydrolysate and this value was 5.4 times higher than that before induction (Table 2).

When yeast extract was used as the complex nitrogen source, the maximum specific growth rate was 1.31 h^{-1}, which was the highest value among the tested nitrogen sources. Fig. 2 shows that insecticidal protein concentration also has its maximum value of about 0.5 g/l when yeast extract was used. Consequently, yeast extract was selected as the complex nitrogen source in the subsequent runs of experiments.

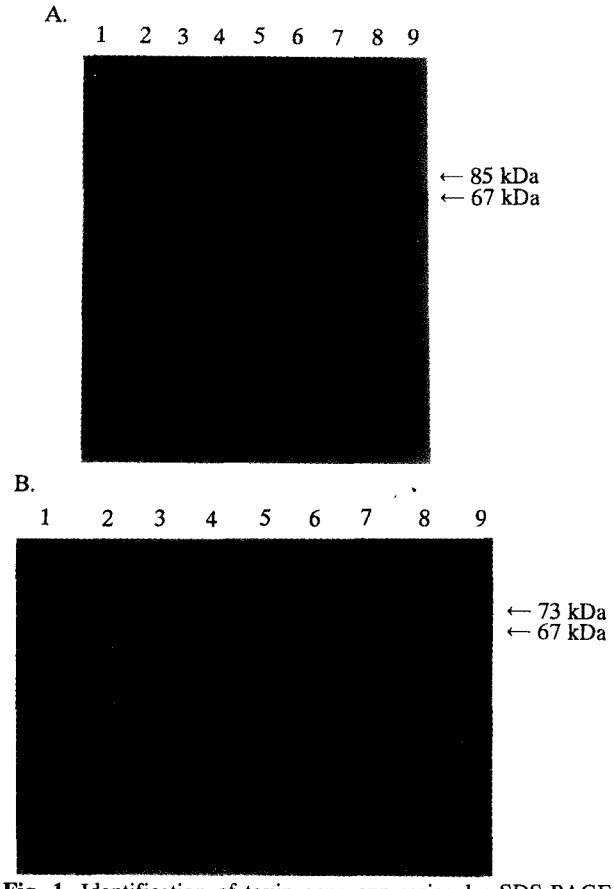

Fig. 1. Identification of toxin gene expression by SDS-PAGE and western blot.

A. SDS-PAGE. B. Western blot. Lane 1, control; lane 2, before induction; lane 3, after 1 h of induction; lane 4, 2 h; lane 5, 3 h; lane 6, 5 h; lane 7, 8 h; lane 8, 11 h; lane 9, marker 67, 73 kDa.

Table 2. Effect of nitrogen sources on specific growth rate and inseticidal protein production.

Nitrogen source	μ_{max} (h^{-1})	*IP content (%)	
		before induction	after induction
LB	0.94	6.0	22.2
Yeast extract	1.31	10.6	29.4
Tryptone	0.89	5.5	23.6
Soybean hydrolysate	0.83	6.4	35.0
Bacto peptone	0.68	9.9	38.5

*IP denotes insecticidal protein.
IP production was expressed as % of total cellular protein.

Effect of Cell Growth Stage

To test the effect of growth stage on the production of insecticidal protein, induction was performed after 2, 4, 7, and 12 h of incubation at 30°C, respectively. Induction was performed by shifting the cultivation temperature from 30°C to 42°C and growing cells for 3 h. Fig. 3 shows that for both LB and the modified M9 media the largest amount of insecticidal protein was produced

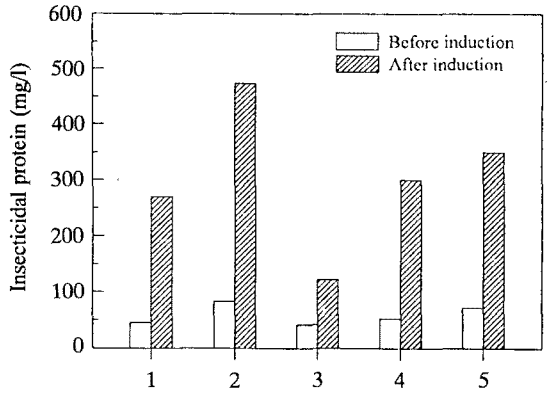

Fig. 2. Effect of complex nitrogen sources on content of insecticidal protein.

1, LB; 2, modified M9 containing yeast extract; 3, modified M9 containing tryptone; 4, modified M9 containing soybean hydrolysate; 5, modified M9 containing Bacto peptone.

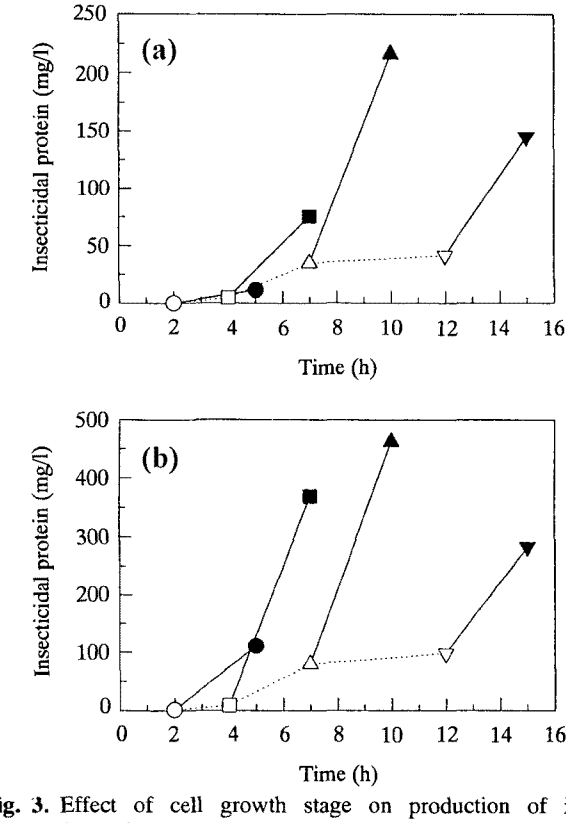

Fig. 3. Effect of cell growth stage on production of insecticidal protein.

(a) LB medium. (b) Modified M9 medium containing yeast extract. ···, without induction; —○—, 2 h at 30°C; —●—, 2 h at 30°C and 3 h at 42°C; —□—, 4 h at 30°C; —■—, 4 h at 30°C and 3 h at 42°C; —△—, 7 h at 30°C; —▲—, 7 h at 30°C and 3 h at 42°C; —▽—, 12 h at 30°C; —▼—, 12 h at 30°C and 3 h at 42°.

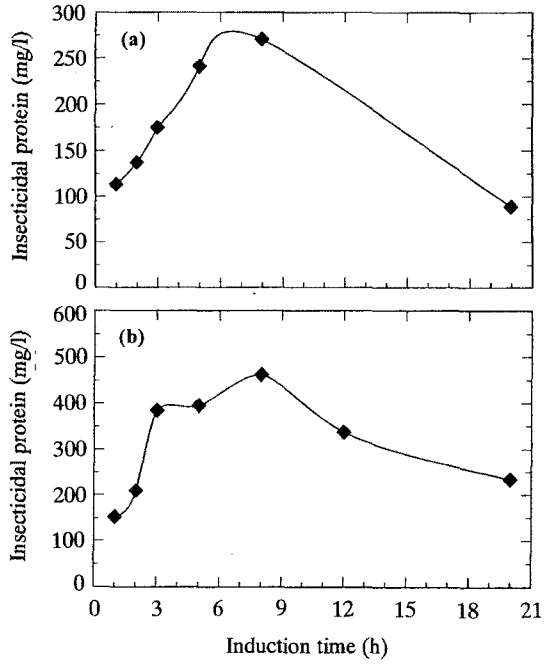

Fig. 4. Effect of induction time on production of insecticidal protein (Preincubation time at 30°C: 7 h).
(a) LB medium. (b) Modified M9 medium containing yeast extract.

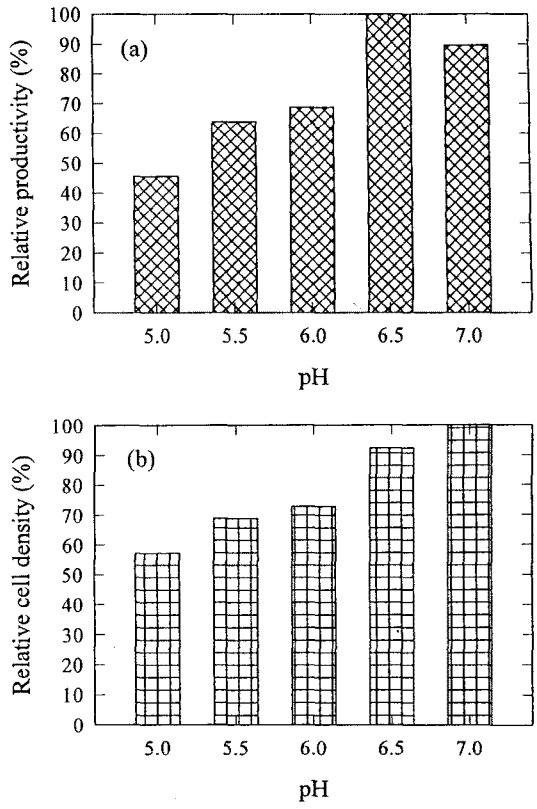

Fig. 5. Effect of pH on production of insecticidal protein and cell growth.
(a) Relative productivity. (b) Relative cell density.

when the temperature was shifted after 7 h of incubation at 30°C. When temperature induction was not performed, insecticidal protein production slowly increased with cultivation time at 30°C. It seems that the unregulated cells producing the insecticidal protein increased with cultivation time. After 7 h of incubation at 30°C, cells reached their late-exponential or early-stationary phase. In this stage, there are larger amounts of inducible cells producing insecticidal protein after induction. After 12 h of incubation at 30°C, cells reached late-stationary phase and cell death started in this phase.

Effect of Incubation Time at 42°C

The effect of incubation time at 42°C on the production of insecticidal protein was tested. Cells were grown for 7 h at 30°C followed by 1, 2, 3, 5, 8, 12, and 20 h at 42°C. When cells were grown for 5 – 8 h at 42°C, the highest value of insecticidal protein concentration was obtained in both cases of the LB and the modified M9 medium (Fig. 4). After 8 h of incubation at 42°C, the insecticidal protein started to break into two smaller size proteins.

Effect of pH

Horiuchi *et al.* (4) reported the effects of pH on β-galactosidase expression with a thermally-inducible P_L promoter. In their tests, the optimal pH for induction ranged from 5.4 – 5.8. In this study, however, the expression of insecticidal protein was not strongly promoted by lowering culture pH (Fig. 5(a)). Insecticidal protein productivity was not good at lower pH's, since cell growth was depressed at these values of pH (Fig. 5(b)).

While setting the pH at 7 is good enough to increase the cell density, it is not good enough to actually increase the protein production, since the base used to control pH could inhibit the production of insecticidal protein. High salt concentrations from NaOH or KOH could also reduce the expression rate of a recombinant protein (1). In this study, *E. coli* 537 had an optimal pH of 6.5 for the production of insecticidal protein.

Bioassay

Larvicidal activity of the *E. coli* extract was determined and compared to that of the original *Btt* toxin for 1.0, 0.2, 0.1, 0.05, and 0.02 g/l of total protein concentrations. The bioassay was carried out against the Colorado potato beetle, *Leptinotarsa decemlineata*. Mortality of the larvae was recorded after every 1 day up to a maximum period of 4 days. It was found that the minimum protein concentration was 0.02 g/l among the tested concentrations. In this case, toxic activity reached to 100% after 4 days (Table 3).

Table 3. Results of bioassay with *E. coli* extract transformed with pEX-Btt.

		Number of dead larvae in 10 second instar larvae				
		1st day	2nd day	3rd day	4th day	Mortality (%)
Btt extract (positive control)		0	9	10		100
E. coli extract (negative control)		0	0	0	0	0
Extract of *E. coli* (pEX-Btt)	1.0 g/l	0	9	10		100
	0.2 g/l	0	7	10		100
	0.1 g/l	0	7	10		100
	0.05 g/l	0	5	10		100
	0.02 g/l	0	4	7	10	100

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