J Food Sci Nutr
Vol 14, p 90~93 (2009)
DOI: 10.3746/jfn.2009.14.1.090

J Food Science and Nutrition

Production of *Cheonggukjang* by Using a Recombinant *Bacillus licheniformis* Strain

– Research Note –

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Abstract

***Cheonggukjang* was prepared from soybean inoculated with *B. licheniformis* ATCC 10716 cells transformed with pHY3-5 carrying a fibrinolytic enzyme gene. During the 54 hr of fermentation at 37°C, fibrinolytic activities of *cheonggukjang* were significantly higher than *cheonggukjang* fermented with *B. licheniformis* 10716 control cells. The plasmid, pHY3-5 was stably maintained during the 54 hr without antibiotic selection and more than 52% of cells retained the plasmid.**

Key words: *Bacillus licheniformis*, fibrinolytic enzymes, gene expression, *cheonggukjang*, *Bacillus subtilis*

INTRODUCTION

Bacillus licheniformis is one of the most common *Bacillus* species isolated from fermented soy foods such as *cheonggukjang* and *doenjang*, and also the most important species producing commercially important enzymes such as amylases and proteases (1-3). Some *B. licheniformis* strains produce fibrinolytic enzymes like some strains of closely related *B. subtilis* and *B. amyloliquefaciens* (4). Such organisms and their fibrinolytic enzymes are useful sources for the production of functional foods or medicines preventing or curing vascular diseases caused by fibrin accumulation (5). Nattokinase, produced by some strains of *B. subtilis*, is the most well known example of fibrinolytic enzymes. There have been increasing interests to develop similar products based on bacilli enzymes. It is expected that a fibrinolytic enzyme as efficient as Nattokinase will be characterized from other *Bacillus* species from fermented foods such as *cheonggukjang* and utilized for the production of functional foods or drugs (6). In this note, we report production of *cheonggukjang* from soybean inoculated with a recombinant *B. licheniformis* strain.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacillus licheniformis ATCC 10716 was cultivated in LB medium at 37°C with vigorous shaking and strains with pHY3-5 were cultivated in LBTc medium (tetracycline, 10 μg/mL). *B. licheniformis* competent cell preparation, electroporation procedure, selection of transformants (TFs), and fibrinolytic activity measurements were done as described previously (7).

SDS-PAGE and zymography

B. licheniformis ATCC 10716 cells were cultivated in LB broth for 36 hr at 37°C. Cells were recovered by centrifugation, resuspended in 50 mM PBS (phosphate buffered saline, pH 7.0), and disrupted by ultrasonication (30 sec, three times). Disrupted cells were centrifuged and the supernatant was used as the sample for soluble cytoplasmic proteins. Secreted proteins in the culture supernatant were concentrated by TCA (trichloroacetic acid) precipitation. Equal volume of cold 20% TCA solution was added to supernatant and the mixture was kept on ice for 20 min. TCA pellet was obtained by centrifugation at 5,000×*g* for 15 min, washed twice with cold 100% ethanol followed by 70% ethanol wash, and dissolved in 50 mM Tris-HCl (pH 7.4). SDS-PAGE (15% acrylamide gel) and fibrin zymography (10% acrylamide gel) were done for both samples as described previously (7).

Cheonggukjang fermentation

Soybean (Tae-Kwang, 2004 crop year, Gyeongbuk) washed previously was soaked in water at room temperature for 10 hr and autoclaved at 121°C for 20 min. Then soybean was inoculated with 2% (v/w) of *B. lichen-*

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iformis ATCC 10716 [pHY3-5], which previously grown in LBTc until the absorbance (600 nm) reached 1.7. Inoculated soybean was incubated for 54 hr at 37°C.

Plasmid stability

Stability of pHY3-5 in *B. licheniformis* 10716 during *cheonggukjang* fermentation was examined as follows (8). At each time point, 10 g of *cheonggukjang* was taken out and homogenate was prepared by using a stomacher. After serial dilutions, aliquots were spread onto LBTc and LB plates to count viable cells. The percentage of cells harboring pHY3-5 was calculated as follows.

$$\text{Percentage of cells keeping plasmid (\%)} = \frac{\text{Number of cells on LBTc}}{\text{Number of cells on LB}} \times 100$$

RESULTS AND DISCUSSION

Transformation of *B. licheniformis* 10716

B. licheniformis ATCC 10716 was transformed with pHY3-5, a pHY300PLK (Takara, Shiga, Japan) containing *aprE2* (7). pHY3-5 DNA was prepared from *E. coli* DH5α [pHY3-5] cells using plasmid mini prep kit (Nucleogen, Korea). *B. licheniformis* ATCC 10716 was selected as a host for *aprE2* expression because the strain lacked inherent fibrinolytic activities and plasmids (data not shown). TFs were selected from LBTc (10 μg/mL) plates after 24 hr incubation at 37°C and the presence of pHY3-5 was confirmed by restriction mapping (data not shown). The transformation efficiency, however, was low, ranging $10^1 \sim 10^2$ TFs/μg DNA.

Expression of *aprE2* in *B. licheniformis* ATCC 10716

SDS-PAGE results showed that the 29 kDa AprE2 band was observed only from concentrated culture supernatant sample from *B. licheniformis* [pHY3-5] (Fig. 1 A. lane 2), and not from cytoplasmic fraction. It indicated that AprE2, successfully synthesized in a heterologous host, secreted into culture medium efficiently. No distinct 29 kDa band was observed on a fibrin zymogram but a big smear was observed at the top of the gel (Fig. 1 B. lane 2). This indicated that AprE2 molecules were not able to migrate through an acrylamide gel to the point corresponding to the molecular weight. Instead they might form large-sized aggregates or tightly bound to fibrin in the gel. This phenomenon, so-called binding mode, was frequently observed, especially when a fibrinolytic gene was overexpressed (9,10).

***Cheonggukjang* fermentation**

During the 54 hr of fermentation, viable cell numbers of *B. licheniformis* ATCC 10716 increased rapidly during the first 6 hr and then gradually increased until 18 hr (Fig. 2). Then, cell numbers remained relatively constant, 10^{10} cells/g of sample. No significant difference in cell number was observed between *cheonggukjang* inoculated with 10716 and 10716 carrying pHY3-5. The fibrinolytic activities of *cheonggukjang* fermented with 10716 TF were 2.3 times higher than those of *cheonggukjang* fermented with 10716 control (Fig. 3). At 54 hr, the former had 8.9 U/g of dried sample whereas the latter had only 3.8 U/g of dried sample. *B. licheniformis* 10716 control cells showed weak fibrinolytic activities after 12 hr. Fibrinolytic activities often increased when bacilli cultivated for extended period. Cell lysis and re-

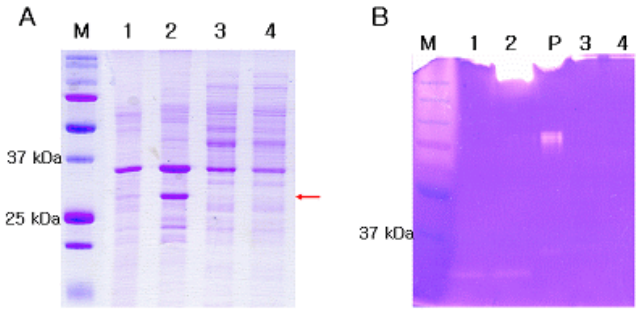

Fig. 1. SDS-PAGE (A) and fibrin zymography (B). A. Concentrated culture supernatants from *B. licheniformis* 10716 (lane 1) and *B. licheniformis* 10716 [pHY3-5] (lane 2); soluble cytoplasmic fractions from *B. licheniformis* 10716 (lane 3) and *B. licheniformis* 10716 [pHY3-5] (lane 4). M, protein size marker (BioRad, USA); P, plasmin (Sigma, USA, 0.6 mU). An arrow indicates the location of AprE2. For SDS-PAGE, 5 μg of protein sample was loaded for each lane. For zymogram, 1.7, 1.6, 44, and 36 μg of protein samples were applied onto lane 1, 2, 3, and 4, respectively.

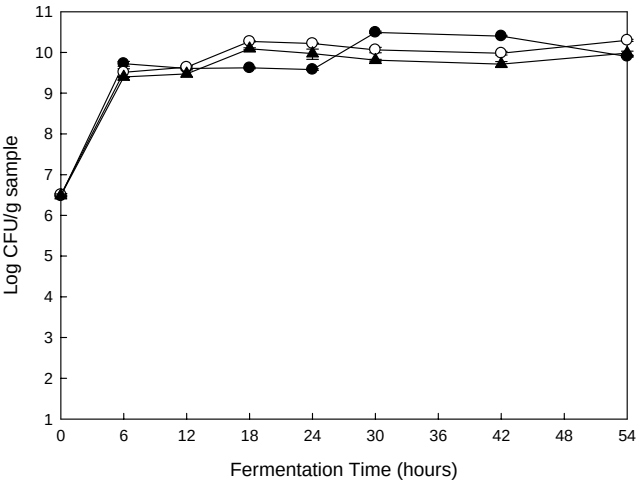

Fig. 2. Viable cell counts of *B. licheniformis* during *cheonggukjang* fermentation. For *cheonggukjang* fermented with *B. licheniformis* 10716 (control), serially diluted samples were spread onto LB plates (●). For *cheonggukjang* fermented with *B. licheniformis* 10716 TF, samples were spread onto LB plates (○) and LBTc (10 μg/mL) plates (▲), respectively.

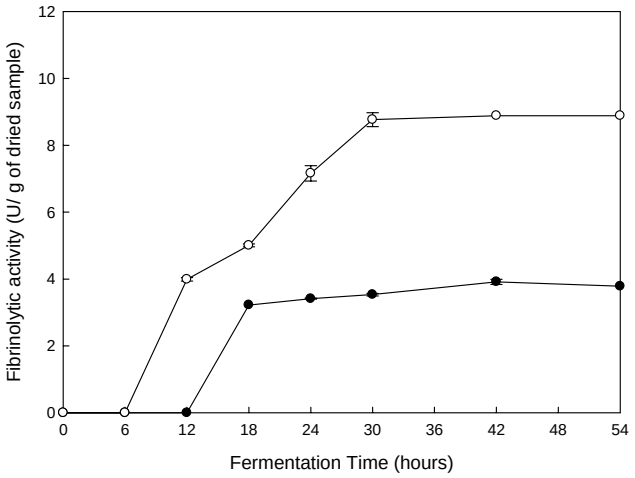

Fig. 3. Fibrinolytic activities of *cheonggukjang*. *Cheonggukjang* fermented with *B. licheniformis* 10716 (●) and *B. licheniformis* 10716 TF (○) for 54 hr at 37°C. Fibrinolytic activities were measured by comparing the area of lysis zone on a fibrin plate with those caused by different concentrations of plasmin (Sigma) as standards (7).

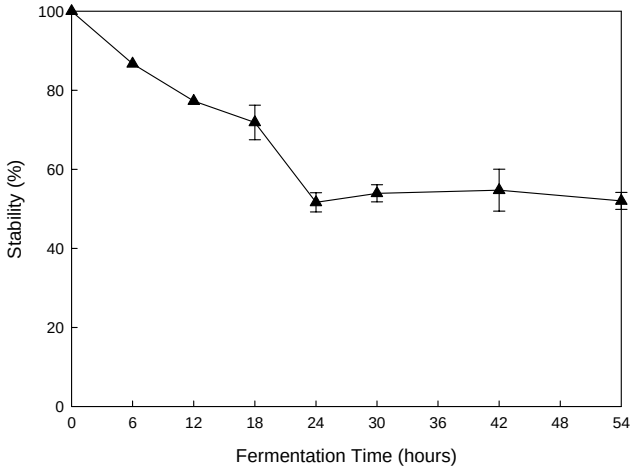

Fig. 4. Stability of pHY3-5 in *B. licheniformis* during *cheonggukjang* fermentation.

lease of proteolytic enzymes or activation of some proteases during stationary growth phase might be the reason. *Cheonggukjang* produced by recombinant strain was not seemingly different from that produced by control strain. Since the recombinant strain used in this study was not optimized for the maximum expression of *aprE2*, the level of fibrinolytic activity could be improved if careful modifications will be done for the vector and host. In this work, plasmid with an antibiotic resistance gene (Tc^R) was used but a food grade vector should be used for the commercialization of functional *cheonggukjang* products. Although the recombinant *B. licheniformis* strain constructed in this work is not suitable for food applications, it is the first construct from which food-grade recombinant strains will be derived. Deletion of Tc marker or integration of *aprE2* into the chromosome of *B. licheniformis* ATCC 10716 is under investigation.

Stability of pHY3-5 during fermentation

The stability of pHY3-5 in *B. licheniformis* during *cheonggukjang* fermentation was measured (Fig. 4). Cells rapidly lost pHY3-5 during the first 24 hr and 52% of cells maintained pHY3-5 at 24 hr. After that, cells stably maintained pHY3-5 until 54 hr. After 78 hr, cells lost pHY3-5 rapidly and 12.3% of cells retained pHY3-5 at 100 hr (data not shown). Since plasmids tend to be unstable in nature, especially without selection pressure, integration of *aprE2* into host chromosome might be a better way in terms of gene stability. In summary, we successfully showed a method for enhancing the fibrinolytic activities of *cheonggukjang* by using a recombinant *B. licheniformis* ATCC 10716 strain. Food-grade vectors based on pHY3-5 or recombinant strains where *aprE2* is integrated into the host chromosome will be useful for the production of *cheonggukjang* with enhanced functionalities.

ACKNOWLEDGMENT

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (No. R01-2007-000-20024-0). Gun Hee Kwon, Woo-Ju Jeong, Ae Ran Lee, Mee-Ryung Lee, and Jiyeon Chun were supported by the 2nd stage Brain Korea 21 program from the Korean Ministry of Education & Human Resources Development. The authors are grateful for the financial supports.

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(Received November 3, 2008; Accepted February 2, 2009)