Food Sci. Biotechnol. 20(6): 1585-1591 (2011)
DOI 10.1007/s10068-011-0219-y

RESEARCH ARTICLE

Improved Menaquinone (Vitamin K_2) Production in *Cheonggukjang* by Optimization of the Fermentation Conditions

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Received: 7 July 2011 / Revised: 9 September 2011 / Accepted: 14 September 2011 / Published Online: 31 December 2011

Abstract To improve the content of menaquinone (MK) in *cheonggukjang* by using *Bacillus amyloliquefaciens* KCTC11712BP, the fermentation conditions were optimized. The rate of sporulation was found inversely correlated with MK productivity in soybean extract medium during the fermentation process. The best sensory quality of *cheonggukjang* was appeared at 36 h of fermentation. The synthesis rate of MK was slowed down after fermented for 36 h, which may due to the accumulation of aromatic amino acids (phenylalanine, tyrosine, and tryptophan). Especially, tryptophan was found to be the most sensitive feedback inhibitor of MK biosynthetic pathway. The optimum temperature for MK production was 43ºC, and supplement of 4% glycerol could significantly increase the yield of MK. The content of MK in *cheonggukjang* fermented by using *B. amyloliquefaciens* KCTC11712BP under the optimum condition reached as high as 12.47 μg/g, which was about 4-fold higher than that of commercial *cheonggukjang* and *natto* products.

Keywords: *Bacillus amyloliquefaciens, cheonggukjang*, feedback inhibition, menaquinone, sporulation

Introduction

Menaquinone (MK) has been proved to play an important role in blood coagulation and bone metabolism (1). The number of isoprene units on the side chain of MK ranges from 1 through 14, thus individual MK is presented with the number of isoprene units, MK-1 through MK-14 (2). MK acts as an essential cofactor in the conversion of glutamic acid residues into γ-carboxyglutamic acid (Gla) in the Gla-containing proteins (3). In the human body, 3 primary types of Gla-containing proteins are found: the blood coagulation factors are synthesized in liver, osteocalcin is uniquely synthesized in bone tissue, and matrix Gla-protein is expressed in the arterial vessel wall (4). These findings indicate that MK is quite necessary for maintaining the human health in liver, bone, and arterial vessel. Extensive researches on the mechanism of MK synthesis in *Escherichia coli* and *Bacillus subtilis* have been carried out (5). Furthermore, studies to increase the production of MKs by using *Flavobacterium*, lactic acid bacteria, and *B. subtilis* also have been reported (6). A mutant strain of *Flavobacterium* was reported to produce as much as 182 mg/L MKs (MK-4 and MK-6) (7). In the case of lactic acid bacteria, 123 μg/L of MK (MK-7) was accumulated under the optimum conditions (8). *B. subtilis* that has been used to produce *natto* was widely investigated as a potential superior MK producer. A previous study reported that the concentration of total MK, mainly composed of MK-7 and MK-8, reached more than 60 mg/L under the optimum conditions with a mutant strain of *B. subtilis* (9). However, all of these fermentation researches were based on liquid medium. As to the solid medium, *natto*, a fermented soybean product traditionally consumed by Japanese, has been regarded as a high content of MK source (about 6-9 μg/g) and is found in everyday products. *Natto* produced by a mutated *B. subtilis* strain showed a much higher content of MK up to 12.98 μg/g (10).

As a similar product to *natto*, the Korean traditional fermented soybean, *cheonggukjang*, could also be speculated as MK-rich food. However, researches to improve the MK content in *cheonggukjang* have not been reported yet. If a kind of *cheonggukjang* with high content of menaquinone could be developed, it will bring about numerous benefits

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to humans especially for Koreans to maintain the bone health and reduce coronary heart disease. Thus, a preliminary studied *cheonggukjang* producing strain *B. amyloliquefaciens* KCTC11712BP with relatively high MK-productivity was used in the present study to optimize the conditions for the production of *cheonggukjang* with a high MK content.

Materials and Methods

Materials The *Bacillus amyloliquefaciens* strain KCTC 11712BP used in the present study has been previously isolated and identified with API kits and *gyr*B sequence analysis by the authors (11), and deposited at Korea Collection for Type Cultures (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). Authentic MK-4 and MK-7 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ChromaDex Inc. (Santa Ana, CA, USA), respectively. Glycerol and maltose were bought from Junsei Chemical Co. (Tokyo, Japan). Glucose, mannose, and starch were purchased from Yakuri Pure Chemical Co. (Kyoto, Japan). L-Phenylalanine, L-tyrosine, and L-tryptophan were obtained from Sigma-Aldrich. Methanol, *n*-hexane, 2-propanol, and other reagents used were of analytical grade.

Culture media and condition Soybean extract (the boiled soybean soup, 10%, w/v), a byproduct of the *cheonggukjang* manufacturing process, was used as the basal liquid medium in which the pre-culture was carried out. Twenty mL of the soybean extract medium was autoclaved and inoculated with 0.2 mL of the spore suspension (4×10^7 cells/mL) in a 50-mL flask, then incubated at 40°C and 120 rpm.

***Cheonggukjang* manufacture and sensory evaluation** Soybeans purchased from a local supermarket were washed 3 times with tap water and soaked for 12 h. They were autoclaved at 121°C for 30 min, and then cooled to 40°C before inoculation at 1%(v/w) with pre-culture cell suspension. Traditionally, *cheonggukjang* was fermented for 48 h (12). The quality of *cheonggukjang* was scored by 5 panelists according to the 5-point scale method, based on 5 viewpoints of appearance, flavor, savory taste, stringiness, and total quality (13).

Menaquinone (MK) analysis by HPLC The analysis of MK was performed according to the established procedure (9). 2-Propanol (4.8 mL) and *n*-hexane (9.6 mL) were mixed with 4 g of *cheonggukjang* product (or 4 mL of cultured soybean extract). The mixture was vigorously shaken and kept standing for 1 h. After phase separation, the hexane layer was collected and evaporated under nitrogen gas. The oil residue was dissolved in methanol. After filtering with 0.45-μm syringe filters (Millipore, Billerica, MA, USA), the organic solvent layer was subjected to HPLC system (model 1200 Series; Agilent Technologies, Wilmington, DE, USA), equipped with a Nova-Pak C_{18} column (Part No. WAT086344, 3.9×150 mm, Waters, Dublin, Ireland). After reduction by platinum black (reduction column, 4.6×10 mm, JIScience, Seoul, Korea), the peaks were detected with a fluorescence detector (1200 Series; Agilent Technologies) at 40°C, and detection wavelengths were as follows: excitation at 320 nm and emission at 430 nm. The mobile phase was 100% methanol at a flow rate of 0.8 mL/min, and injection volume of the sample was 5 μL. The concentration of MK in the medium was expressed as μg/g fermented *cheonggukjang* (or μg/mL of soybean extract).

Analysis of amino acids To extract the free amino acid, 0.1 g of *cheonggukjang* was mixed with 10 mL of distilled water, and extracted for 3 h at 25°C. After centrifugation for 10 min at 5,000×*g* and 4°C (VS-24SMTi; Vision Scientific, Bucheon, Korea), the supernatant was dissolved in 5 mL lithium citrate dilution buffer (0.2 M, pH 2.2) and filtered with a 0.2-μm syringe filter. Then, all the samples were injected directly into an amino acid analyzer Sykam S-433 (Eresing, Germany) to determine the amino acids amount.

Effect of carbon source on MK production The selection of carbon sources was based on the carbohydrate utilization capabilities studied in the preliminary research using API kits (data not shown). Glycerol, maltose, glucose, mannose, and starch, which showed positive utilization capabilities by *B. amyloliquefaciens* KCTC 11712BP were used as the carbon sources to determine their effects on MK production. Each carbon source was added to the soaked soybean before being autoclaved; the percentage was based on the soaked soybean weight. The MK content was assayed by HPLC after fermentation for 36 h.

Determination of sporulation rate The colony forming unit (CFU/mL) of total viable cells was obtained by plating cells on the surface of nutrient agar plate. The number of heat-resistant spore was also determined by plating cells on the surface of nutrient agar plate after heat treatment at 80°C for 30 min to remove the residual vegetative cells (14).

Statistic analysis Data were analyzed using a SPSS statistical software (SPSS 12.0k; SPSS Inc., Chicago, IL, USA), and a significant difference of samples were determined by Duncan's multiple test at the level of $p\leq0.05$.

Results and Discussion

Pre-culture time of soybean extract The pre-culture was conducted in 20 mL soybean extract under the growth condition described above to determine the optimal shaking culture time. After reciprocal shaking of the culture, the flask was kept static for a total culture time of 3 days, and the yield of MK was determined by HPLC. Total viable cell counts at the exponential phase increased with prolonging shaking time up to 6 h, reaching the maximal value of 18.57×10^8 cells/mL (Table 1). The content of MK-7 also increased as the shaking culture time increased, reaching the peak value at 6 h. These results suggest that shaken culture induces a rapid growth of the cells, thereby allowing immediate access of the strain to the MK-producing phase. After the cell content arrived at the maximum point, the necrocytosis was stimulated and some of the cells formed spores. Sato *et al*. (9) reported that the sporulation of cells progressed more rapidly in the shaken culture than in the static culture. In addition, MK, implicated as an electron transport cofactor in the bacterial species, was also found to play an important role in cell respiration (5). After sporulation, the need of MK by the cells decreased due to the lower respiration rate of the spores, resulting in the decrease of the MK productivity of the cells, indicates that rapid formation of spore has an inverse correlation with MK-7 production. Thus, to enhance the MK content in *cheonggukjang*, the shaken pre-culture should be terminated before the massive formation of spores and the content of living cells reaches the maximum point, which has an advantage to the cell rejuvenation in *cheonggukjang*.

Table 1. Effect of pre-culture shaking time on MK-7 production in soybean extract[1)]

Length of shaking time (h)	Total viable cells (10^7/mL)	Spore (10^2/mL)	MK-7 (μg/mL)
0	2.77±0.95$^{d2)}$	0.00±0.00^{c}	3.44±0.39^{b}
4	94.00±25.94^{b}	0.33±0.58^{c}	3.77±0.24^{ab}
6	185.67±23.75^{a}	7.00±1.00^{c}	4.22±0.06^{a}
8	55.33±5.86^{bc}	10.33±2.62^{c}	3.66±0.31^{b}
10	16.00±4.58^{cd}	104.00±65.48^{b}	3.70±0.26^{ab}
12	30.00±16.64^{cd}	758.00±98.80^{a}	3.25±0.29^{b}

[1)]Cells were cultured by reciprocal shaking at 120 rpm at 40°C for various periods to give rapid growth, then were kept stationary for total culture time of 3 days.
[2)]Data are mean±SD (n=3); Means with different letters within a column are significantly different at $p\leq0.01$ as determined by Duncan's multiple range test.

Changes of sensory quality, MK-7 content, and free amino acid amount during fermentation The sensory evaluation results of *cheonggukjang* during the fermentation are shown in Table 2. The appearance score is gradually decreased during the fermentation process, from an initial golden yellow color to black gray after 60 h fermentation, while the stringiness were increased during all the fermentation periods. However, the flavor, taste, and overall acceptability at 36 h were uniformly better than those at other fermentation times.

Changes of free amino acids and MK-7 content during *cheonggukjang* fermentation are shown in Fig. 1. The content of MK-7 increased remarkably from the initial amount of 1.13 (12 h) to 7.40 μg/g at the end of fermentation (60 h). The free amino acid contents also notably increased during the fermentation process (Fig. 1). The production rate of free amino acid remarkably enhanced before 36 h of culture, while thereafter, it slightly slowed down.

Meanwhile, the biosynthetic pathway of MK was presented at Fig. 2 (15). Three kinds of free aromatic amino acids (phenylalanine, tyrosine, and tryptophan) would participate in the feedback inhibition of the up-stream of MK biosynthetic pathway (16). A previous study investigated the feedback inhibition by aromatic amino acids on the biosynthesis of menaquinones by using *B. subtilis* (*natto*), reported that relative MK-7 productivity per cell in the cultivation using a liquid medium with addition of 3 aromatic amino acids together (300 mg/L; phenylalanine, tyrosine, and tryptophan 100 mg/L, respectively) was dramatically decreased by 1/5 of that in the case without them (16). However, within these aromatic amino acids, which one was the most efficient feedback inhibitor has not been revealed. Therefore, the separate effect of aromatic amino acids on the biosynthesis of MK was carried out with an addition of various amounts

Table 2. Sensory quality of *cheonggukjang* during fermentation by using *B. amyloliquefaciens* KCTC11712BP

Fermentation time (h)	Appearance	Flavor	Savory taste	Stringiness	Overall acceptability
12	4.80±0.43$^{a1)}$	3.60±0.89^{a}	3.00±1.00^{b}	1.60±0.89^{b}	2.60±1.41^{bc}
24	4.20±0.25^{ab}	3.20±0.84^{a}	3.80±0.45^{ab}	3.40±1.14^{a}	3.40±0.55^{ab}
36	4.00±0.71^{b}	4.00±0.71^{a}	4.20±0.45^{a}	3.80±0.84^{a}	3.80±0.84^{a}
48	3.20±0.35^{c}	3.20±0.84^{a}	3.60±0.89^{ab}	4.00±0.71^{a}	3.40±0.55^{ab}
60	1.80±0.41^{d}	1.60±1.41^{b}	3.40±0.55^{ab}	4.40±0.55^{a}	2.40±0.55^{d}

[1)]Data are mean±SD (n=5); Means with different letters within a column are significantly different at $p\leq0.05$ as determined by Duncan's multiple range test; Sensory evaluation score: 1, very bad; 2, bad; 3, standard; 4, good; 5, very good

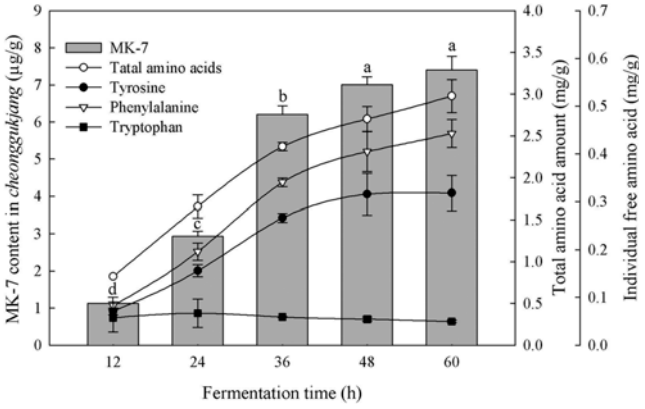

Fig. 1. Changes of free amino acids and MK-7 contents in *cheonggukjang* during fermentation. Results are expressed as the mean±SD of 3 replicates; Diffierent letters are significantly different at $p \leq 0.01$ as determined by Duncan's multiple range test.

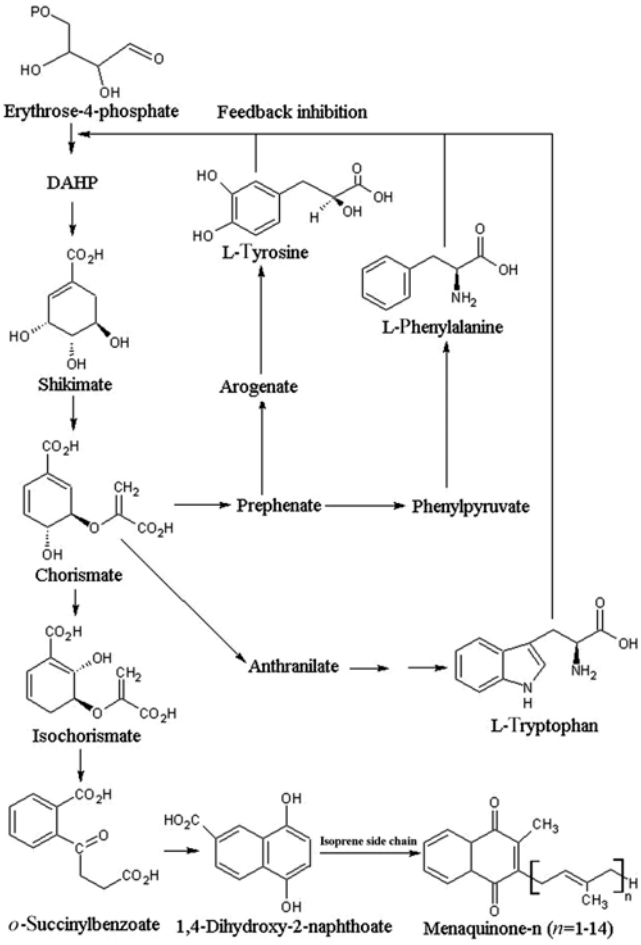

Fig. 2. Biosynthetic pathway of menaquinones and feedback inhibition of free amino acids.

of these 3 amino acids (from 1 to 5 mg/100 g *cheonggukjang*), respectively. Consequently it was found that the yield of MK-7 was strikingly decreased with the addition of each of these 3 aromatic amino acids (Fig. 3). In addition, the content of MK-7 could be constantly reduced by increasing the additive amount of tyrosine, tryptophan, and phenylalanine, especially tryptophan, which was found to be the most sensitive feedback inhibitor of MK biosynthesis pathway, followed by phenylalanine, while tyrosine was the weakest. When the addition of tryptophan arrived at a concentration of 5 mg/100 g, the yield of MK-7 was decreased from 6.39 to 1.63 μg/g. It is suggested that a longer fermentation time may lead to a great accumulation of free amino acids, which would strongly inhibit the production of MK-7. Considering changes of sensory quality, MK-7 content, and free amino acid amount during the *cheonggukjang* fermentation process, it was most appropriate to terminate the fermentation at 36 h.

Optimum temperature for menaquinone production The optimum temperature for menaquinone production was 43ºC as determined by the experiment carried out from 37 to 46ºC, and the content of MKs reached the maximum value of 8.21 μg/g (Table 3). In the preliminary study, the optimum temperature for the cell growth of *B. amyloliquefaciens* KCTC11712BP was 40ºC (11), and in *cheonggukjang* the highest amount of total viable cells was obtained at 40ºC, which was 2-fold higher than that at 43ºC. However, the yield of MKs at 43ºC was 1.3-fold higher than that at 40ºC. Sato *et al*. (9) reported that the yield of MK-7 in static culture at 45ºC was about twice as high as that in the shaken culture at 37ºC, although the cell growth rate under the latter condition was higher. These results may be attributed to the enzymatic activity. The results also indicated that *B. amyloliquefaciens* KCTC11712BP produce 2 types of MK (MK-4 and MK-7) in *cheonggukjang* (Table 3), whereas in soybean extract only MK-7 was produced (Table 1). A previous study reported that the menaquinone composition depended markedly on the stage of growth by *Streptomyces cyaneus* NCIB 9616 (17). The phenomenon in this study could be due to various reasons, but principally the nutrient availability of the medium (soybean itself versus soybean extract) and the form of medium (liquid or solid) may be the most important factors contributing to the observed difference in MK composition. Further investigations need to be carried out in the future to clarify the factors affecting the isoprene side chain length.

Effect of carbon source on MK production Soybean is well known to be the good source of nitrogen content, but the carbon content may not be so sufficient. Thus, the effect of carbon source on MK production was determined with the addition of various carbohydrates during the *cheonggukjang* manufacturing process. The productivity of MKs was significantly increased with the addition of carbon sources, and the highest concentration of MKs in

Table 3. Effect of temperature on MK production in *cheonggukjang* fermented by *B. amyloliquefaciens* KCTC11712BP for 36 h

Temperature (°C)	MK-4 (μg/g)	MK-7 (μg/g)	Total (μg/g)	Total viable cells (10^7/mL)
37	0.84±0.06[a1)]	4.61±0.32[c]	5.45±0.28[b]	62.4±12.3[ab]
40	0.71±0.07[b]	5.58±0.66[bc]	6.29±0.72[b]	86.9±14.2[a]
43	0.75±0.06[ab]	7.46±0.72[a]	8.21±0.77[a]	41.7±5.5[bc]
46	0.64±0.02[c]	5.67±0.33[b]	6.31±0.32[b]	22.3±6.3[c]

[1)]Data are mean±SD (n=3); Means with different letters within a column are significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test.

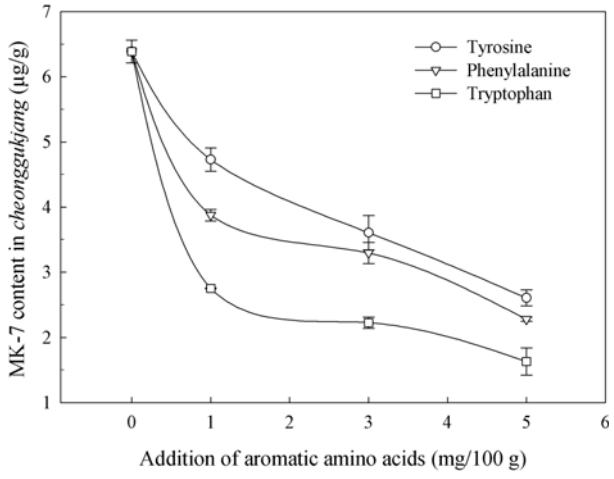

Fig. 3. Inhibitory effects of free amino acids on productivity of MK-7 in *cheonggukjang*. *Cheonggukjang* was fermented by *B. amyloliquefaciens* KCTC11712BP at 40°C for 36 h without or with addition various amount of phenylalanine, tyrosine, and tryptophan, respectively. Results are expressed as the mean±SD of 3 replicates.

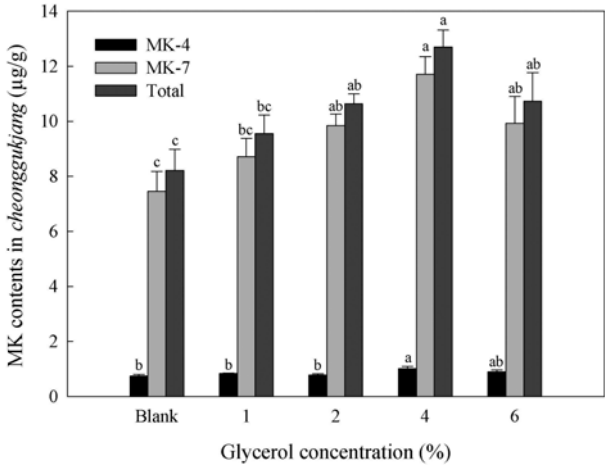

Fig. 4. Effect of glycerol percentage on menaquinone production in *cheonggukjang* fermented for 36 h. Results are expressed as the mean±SD of 3 replicates; Different letters are significantly different at $p \leq 0.01$ as determined by Duncan's multiple range test.

Table 4. Effect of additive carbon sources on MK production in *cheonggukjang*

Carbon source (2%)	MK-4 (μg/g)	MK-7 (μg/g)	Total (μg/g)
Control	0.62±0.09[b1)]	7.35±0.73[c]	7.97±0.82[d]
Glucose	0.69±0.07[b]	7.77±0.07[c]	8.46±0.01[cd]
Glycerol	1.02±0.08[a]	11.13±0.64[a]	12.15±0.62[a]
Maltose	0.69±0.05[b]	9.77±1.10[b]	10.45±1.04[b]
Mannose	0.71±0.09[b]	9.41±0.29[b]	10.12±0.20[b]
Starch	0.95±0.11[ab]	8.67±0.64[bc]	9.61±0.56[bc]

[1)]Data are mean±SD (n=3); Means with different letters within a column are significantly different at $p \leq 0.01$ as determined by Duncan's multiple range test.

cheonggukjang (12.15 μg/g) was attained with the addition of glycerol, followed by maltose (10.45 μg/g) and mannose (10.12 μg/g) (Table 4). Previously glycerol has been reported to be the most effective carbon source for both the growth and MK production by *Flavobacterium meningosepticum* (18). Another report also indicated that glycerol was very effective for MK production by *B. subtilis*, though it was not effective for the cell growth (9). In the present study with *B. amyloliquefaciens* KCTC11712BP, we found that glycerol was the most effective carbon source for MK-7 production, whereas the effect on the productivity of MK-4 was not so significant. The optimal glycerol concentration in the *cheonggukjang* was 4%(w/w), and the content of MKs increased by a 1.5-fold compared with the control (Fig. 4). As the production of menaquinone decreased at 6% of glycerol, we speculated that the increase of glycerol content might make the higher osmotic pressure, which may play a restrictive role during the synthetic progress of menaquinone in the cells.

MK production under optimum condition When *cheonggukjang* was made by using cooked soybeans supplemented with 4% of glycerol and cultured at 43°C for 36 h, the content of MK reached to as high as 12.47 μg/g. Comparison with the commercial *cheonggukjang* and *natto* products showed the MK content of our product was about 4-fold higher (Table 5). Previously, the highest content of MK production (36.6 μg/g) was found in the Chinese *natto* strain (*Unnan* SL-001), followed by (in μg/g of okara-*natto* wet mass): 14.2 in *Naruse*, 11.9 in *Asahi*, 6.8 in *Takahashi*, 5.2 in *Nitto*, 1.9 in *Miyagino,* and 1.9 in *Meguro* after incubation for 4 days at 37°C (19). Compared with these *natto* products, our *cheonggukjang* can be judged to be a high quality soybean fermentation product in terms of MK

Table 5. Comparison of MK content in different fermented soybean foods

Fermented soybean food[1)]	MK-4 (μg/g)	MK-7 (μg/g)	Total (μg/g)
Final product	0.76±0.02[a2)]	11.71±0.61[a]	12.47±0.63[a]
Fermented by *B. subtilis*	0.74±0.08[a]	2.71±0.15[bc]	3.45±0.09[bc]
Cheonggukjang-A	ND	2.79±0.12[bc]	2.79±0.12[bc]
Cheonggukjang-B	ND	1.12±0.15[c]	1.12±0.15[c]
Cheonggukjang-C	ND	4.61±0.31[b]	4.61±0.31[b]
Natto-A	ND	0.87±0.21[c]	0.87±0.21[c]
Natto-B	ND	1.02±0.11[c]	1.02±0.11[c]

[1)]Final product and fermented by *B. subtilis*; fermented by *B. amyloliquefaciens* KCTC11712BP with 4% glycerol under the optimum under the optimum condition and by *B. subtilis* (KCTC3239) with traditional method, respectively; *Cheonggukjang*-A, B, C: purchased from Pulmuone Food Co., Smile Food Co., and Yukbeomsu Food Co. in Korea, respectively; *Natto*-A, and B: purchased from Hokkaido and Kyoto in Japan, respectively

[2)]Data are mean±SD (n=3); Means with different letters within a row are significantly different at $p<0.05$ as determined by Duncan's multiple range test; ND, not detected (too less to be detected)

content. It should also be noted that the fermentation process of the present study is less time-consuming (36 h). As an MK-containing product, *cheonggukjang* is considered to have the same functions as *natto*. Therefore, the MK-containing *cheonggukjang* may also play important roles in the reduction of the risk of bone fracture (20), protection of the skin health (21), as well as contribution to the brain health (22). To our knowledge, this is the first report to determine the MK content in *cheonggukjang*, and develop a way to increase its content. All summed up, the present findings showed that produce *cheonggukjang* by using *B. amyloliquefaciens* KCTC11712BP under optimum conditions, not only remarkably increased the MK content but also reduced the production time compared to *natto*, and thus should receive attention from the food industry.

Acknowledgments This work (Grants No. 38006) was supported by Business for Cooperative R&D between Industry, Academy, and Research Institute funded by Korea Small and Medium Business Administration in 2009.

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