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Efficient production of menaquinone (vitamin K_2) by a menadione-resistant mutant of *Bacillus subtilis*

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Efficient production of menaquinone (MK) by *Bacillus subtilis* was achieved. An edible strain of *B. subtilis*, isolated from the traditional Japanese food natto, was mutated to improve MK productivity. A menadione-resistant mutant producing 30% more MK than its parent strain was obtained. Soybean extract and glycerol were the best nitrogen and carbon sources, respectively, among the sources tested. Addition of yeast extract also increased MK productivity. The maximum concentration of MK reached about 35.0 mg/l after 4 days of culture in a jar fermenter. The pH of the medium decreased to 5.5 after the start of cultivation, then spontaneously increased to 7.7–8.0. This pH change might be important in the production of MK because only small amounts of MK were obtained when pH was controlled at 5.7, 6.0, 7.0, 7.5 or 8.0. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 115–120.

Keywords: vitamin K_2; menaquinone; *Bacillus subtilis (natto)*

Introduction

There are two naturally occurring forms of vitamin K: vitamins K_1 and K_2. The latter (menaquinone, MK) is synthesized mainly by bacteria and has a variable side chain length of 4–13 isoprene units. They are referred to as MK-*n*, where *n* denotes the number of isoprenoid residues. Vitamin K is a cofactor required for posttranslational conversion of specific glutamic acid residues into γ-carboxyglutamic acid (Gla) in Gla-containing proteins which include blood coagulation factors II, VII, IX, X, osteocalcin (bone Gla protein) and matrix Gla protein [19].

Recently, it was reported that the vitamin K intake of most individuals is closer to the current recommended dietary allowance (RDA) in the US of 1 μg/kg body weight/day, but that many individuals fail to meet even this lower level of intake on a daily basis [3]. The RDA is based on the amount required for maintenance of plasma prothrombin concentrations. Absorption in the lower bowel of MK-*n* derived from intestinal flora is limited [21]. A decrease in the dietary intake of vitamin K causes an increases in the frequency of femoral neck fractures [6]. Supplementation of vitamin K_1 [5], MK-4 [20] and MK-7 [26] decreased serum undercarboxylated osteocalcin and increased γ-carboxylated osteocalcin in humans, suggesting that vitamin K levels in humans are not sufficient for full γ-carboxylation of osteocalcin. In addition to the conversion of specific glutamic residues to Gla residues in mature Gla proteins, MK has direct effects on bone formation and resorption [1,11,27]. The homologue, MK-4, with four isoprene units, has been used to prevent bone loss in osteoporosis [26]. However, MK-4 is highly counteractive of anticoagulants in vitamin-K-deficient rats induced by anticoagulants [2,4]. If the findings can be extrapolated to patients receiving anticoagulant therapy, MK-4 may influence the stability of orally administered anticoagulants [4]. On the other hand, MK-7, which may exert less influence on the stability of orally administered anticoagulants, is highly active in the maturation of proteins in liver [14]. Furthermore, MK-7 stimulates calcification in femoral metaphyseal tissues obtained from normal rats [18] and has a preventive effect on ovariectomy-induced bone loss [27].

Rowland and Taber [15], Rowland *et al.* [16] and Taber *et al.* [22] have extensively studied the mechanism of MK formation in *Bacillus subtilis*. However, studies to increase production of MK by *B. subtilis* have not been reported. Tani and Sakurai [23] and Tani and Taniguchi [24] reported on the efficient production of MK-4, MK-5 [25] and MK-6 [24] by *Flavobacterium* and that the maximum concentration of MK produced reached 192 mg/l [24]. On the other hand, industrial production of MKs with longer isoprene side chains was not reported until recently by Morishita *et al.* [12]. In their study, 29–123 μg/l of MK-7 was produced by lactic acid bacteria. The fermented soybean "natto," whose production requires *B. subtilis*, is popular in Japan and contains an exceptionally large amount of MK (600–900 μg/100 g) [17]. Since the strains of *B. subtilis* used for manufacturing natto are edible, they are among the most advantageous sources of MK in the food industry. In this study, we determined suitable conditions for production of MK by a menadione-resistant *B. subtilis* mutant, strain K3-176.

Materials and methods

Bacterial strains

B. subtilis (natto) strain MH-1 was used as a parent strain in this study. This strain was isolated from commercially available natto and produced the highest quantity of MK among the strains tested.

Materials and medium

Bacto-peptone, tryptone, nutrient broth, soytone, beef extract and yeast extract were purchased from Difco (Detroit, MI, USA). 3-Methyl-3-butanol and 3-methyl-3-butane-1-ol were purchased

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from Aldrich Japan (Tokyo, Japan). MK-4, polypeptone and carbohydrates were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn steep liquor was obtained from Honen (Tokyo, Japan). Soybean extract was obtained from Asahimatsu Foods (Osaka, Japan). All media used were adjusted to pH 7.3 before autoclaving them. In this study, 10% soybean extract, pH 7.3, was used as the basal medium, which contains 0.3% nitrogen, 6.4% carbohydrates and 1.7% ash.

Culture conditions

Spore suspensions (0.1 ml, 5×10^8 cells/ml) were inoculated into 50 ml of the medium containing 10% soybean extract, pH 7.3, in a 500-ml flask. The culture was incubated at 37°C with reciprocal shaking at 120 rpm for 3 days. For comparison of the parent and mutant strains, a medium which consisted of only 10% (w/w) soybean extract was used.

Alternatively, a 7-l jar fermenter (Takasaki, Tokyo, Japan) was used. After spore suspensions (0.1 ml, 5×10^8 cells/ml) were inoculated into 50 ml of the medium (10% soybean extract, 5% glycerol, pH 7.3) in a 500-ml flask, the flask was incubated at 37°C with reciprocal shaking at 120 rpm for 1 day. All of the culture was added to 5 l of medium containing 10% soybean extract, 5% glycerol, 0.5% yeast extract and 0.05% K_2HPO_4, pH 7.3. Temperature, agitation rate and aeration rate were maintained at 37°C, 250 rpm and 0.5 vvm, respectively.

Derivation of mutants

Menadione (vitamin K_3)-resistant mutants were derived by *N*-methyl-*N*′-nitro-*N*-nitroso-guanidine (NTG) treatment. Cells at the logarithmic growth phase were washed and suspended in physiological saline at a concentration of 10^8 cells/ml. Cell density was determined from the optical density at 660 nm. Cells were shaken in physiological saline containing NTG (250 μg/ml) for 30 min at 37°C with a survival ratio of 1%. The cells were washed twice with the same buffer and harvested by centrifugation at $8000\times g$ for 15 min and then spread on a plate containing the medium (0.25% yeast extract, 0.5% tryptone, 0.1% glucose, 1.5% agar) supplemented with menadione (20 mg/l). Colonies appeared within 3–5 days of incubation at 35°C.

Analysis of MK by high-performance liquid chromatography (HPLC)

The bacterial suspension (3 ml) was added to 4 ml of 2-propanol and 8 ml of *n*-hexane, mixed vigorously and centrifuged. The resultant organic solvent layer was filtered, and a small portion of the filtrate was injected into Shimadzu LC-10A HPLC system equipped with a fluorescence detector. The sample was separated on an ODS column (250×4.6 mm^2, L-column; Chemical Inspection and Testing Institute, Tokyo, Japan) and peaks were detected using a fluorescence detector after reduction by platinum black (10×4.6 mm^2 reduction column; Toa, Tokyo, Japan). Detection was carried out at an excitation wavelength of 320 nm and an emission wavelength of 430 nm. The mobile phase contained 200 ml of 2-propanol and 800 ml of methanol. The flow rate was 1.2 ml/min. When necessary, samples were diluted prior to analysis.

The concentration of MK in the cultured medium was expressed as milligrams per liter of medium volume.

Table 1 Effect of menadione on MK-7 production by *B. subtilis* strain MH-1

Menadione (mg/l)	OD 660	MK-7 (mg/l)
0	13.3	9.5
1	13.5	5.7
3	9.8	0.6
5	0	0
10	0	0

Cells were cultured at 37°C for 3 days with shaking at 120 rpm in a medium containing 10% soybean extract, pH 7.3.

Table 2 Comparison between *B. subtilis* strains MH-1 and K3-176 with respect to MK production

Strain	MK-6 (mg/l)	MK-7 (mg/l)	Total (mg/l)
MH-1	0.37±0.06	9.0±0.2	9.37±0.25
K3-176	0.66±0.10**	11.5±0.8**	12.16±0.90**

A menadione (20 mg/l)-resistant mutant, *B. subtilis* strain K3-176, was obtained from strain MH-1 by treatment with NTG. Culture conditions are as described in . Experiments were carried out in triplicate and values are expressed as mean±SD. Statistical analysis was carried out by Student's Table 1*t*-test.
**$P<0.01$ as compared with the value of MH-7.

Purification and identification of MK-5, MK-6, MK-7 and MK-8

To 1 l of the whole culture of *B. subtilis*, 1.2 l of 2-propanol and 2.4 l of *n*-hexane were added. The mixture was vigorously shaken and allowed to settle for 1 h, the *n*-hexane layer was separated and *n*-hexane was allowed to evaporate. An oily product (approximately 800 mg) was obtained. Before this product was subjected to column chromatography, it was extracted with 5 ml of *n*-hexane and insoluble materials were removed by centrifugation. The resultant oily product in *n*-hexane was loaded on a 400-ml silica gel column (650×45 mm^2). After washing the column with 500 ml of *n*-hexane, MK was isolated using toluene/*n*-hexane (1:2, v/v). Each 30-ml fraction was analyzed by HPLC and the MK fractions were obtained. About 10 mg of this MK fraction was loaded onto a 50-ml ODS silica gel column (500×22 mm^2). The compounds MK-5, MK-6, MK-7 and MK-8 were separated in pure form using methanol/acetonitrile (1:1, v/v). The structures of MK-5, MK-6, MK-7 and MK-8 were confirmed by mass spectrometry (MS-QP1100EX; Shimadzu, Kyoto, Japan).

Determination of the concentration of glycerol

The concentration of glycerol was determined using a commercially available kit (Boehringer Mannheim, Mannheim, Germany).

Results

Derivation of menadione-resistant mutants

B. subtilis (natto) strain MH-1, which produced the highest concentration of MK among the strains tested, was mutated to improve MK productivity. Homologues of vitamin K share a common chemical structure consisting of a naphthoquinone nucleus. MKs (vitamin K_2) have unsaturated side chains composed of 4–13 isoprene units. Menadione (vitamin K_3) lacks

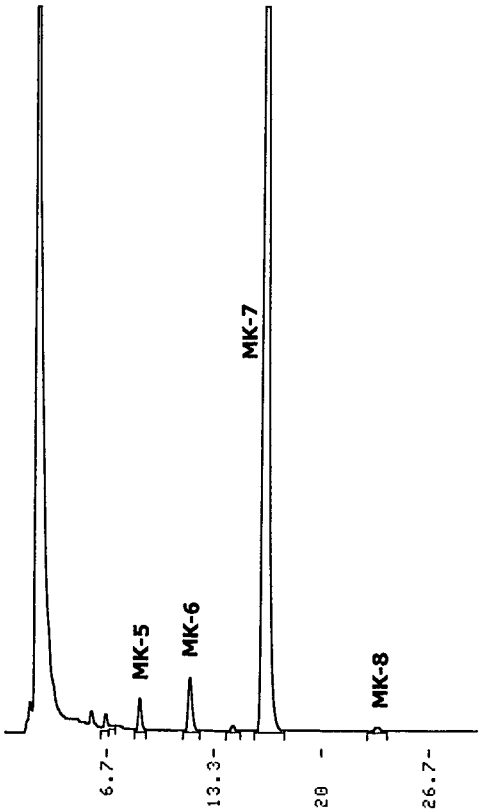

Figure 1 Chromatogram of MK in the culture of *B. subtilis* strain K3-176.

a side chain. Menadione inhibited MK synthesis and growth of *B. subtilis* (Table 1). Twenty-one mutants out of 80 menadione (20 mg/l)-resistant mutants revealed relatively high MK productivity. The MK productivity of the best MK-producing mutant was improved and stabilized after monocell selection processes. Then we obtained a menadione-resistant mutant of *B. subtilis* MH-1, strain K3-176. This mutant produced 30% more MK than its parent strain MH-1 (Table 2). A typical HPLC chromatogram of MK produced by the *B. subtilis* strain, K3-176, is shown in Figure 1.

Culture conditions

Although the shaking speed and temperature did not greatly affect the production of MK by *B. subtilis*, shaking at 120 rpm and at 37°C yielded the highest MK productivity and cell growth rate over the range from 60 to 180 rpm and from 30°C to 45°C (Figure 2).

Effects of media on MK production

The effect of the nitrogen source on MK production was examined in the culture with reciprocal shaking. Soybean extract was used as the nitrogen source, but it contains large quantities of carbohydrates. To compare the effect of the nitrogen source, nitrogen content was adjusted to 0.3% and sucrose was added to adjust final carbohydrate concentration to 6.4%. Under these conditions, among the nitrogen sources tested, soybean extract was the best medium for MK production (Table 3A).

The effect of the carbon source on MK fermentation was examined using 10% soybean extract, pH 7.3, as the basal medium (Table 3B). After cultivation, the OD 660 and the yield of MK-7 were measured. The highest concentration of MK-7 (about 21 mg/l) was produced by the addition of 5% glycerol to the medium. Glycerol did not affect cell growth, but increased MK-7 production on a per-cell basis (Table 3B).

About 100 compounds including surfactants, emulsifiers, minerals, amino acids, yeast extract and precursors of MK, i.e., geraniol, farnesol, 3-methyl-3-butanol, 3-methyl-3-butene-1-ol, chorismic acid and shikimic acid, were tested for their effects on MK production. Yeast extract (0.2% and 0.5%) increased MK production, although the addition of 1.5% yeast extract had a negative effect on the production (Table 3C). 3-Methyl-3-butanol slightly increased MK production; however, MK-6, but not MK-7, was increased (data not shown). Other precursors of MK, such as geraniol, farnesol, 3-methyl-3-butene-1-ol, chorismic acid and shikimic acid, did not increase MK production.

Secretion of MK by B. subtilis

After 3 days, the culture of *B. subtilis* strain MH-1 and K3-176 in a reciprocal flask was centrifuged at 12,000×*g* for 10 min at 4°C. The concentration of MK in the resultant supernatant and the collected cells was measured (Table 4). Forty to sixty-five percent of the MK produced by *B. subtilis (natto)* strain K3-176 and by MH-1 was found in the supernatant medium. The difference in secretion ratio between the two strains, K3-176 and MH-1, was not significant.

Production of MK by B. subtilis in a jar fermenter

The production of MK by *B. subtilis* in a jar fermenter using a medium containing 10% soybean extract, 5% glycerol, 0.5% yeast extract and 0.3% K_2HPO_4 at pH 7.3 was investigated.

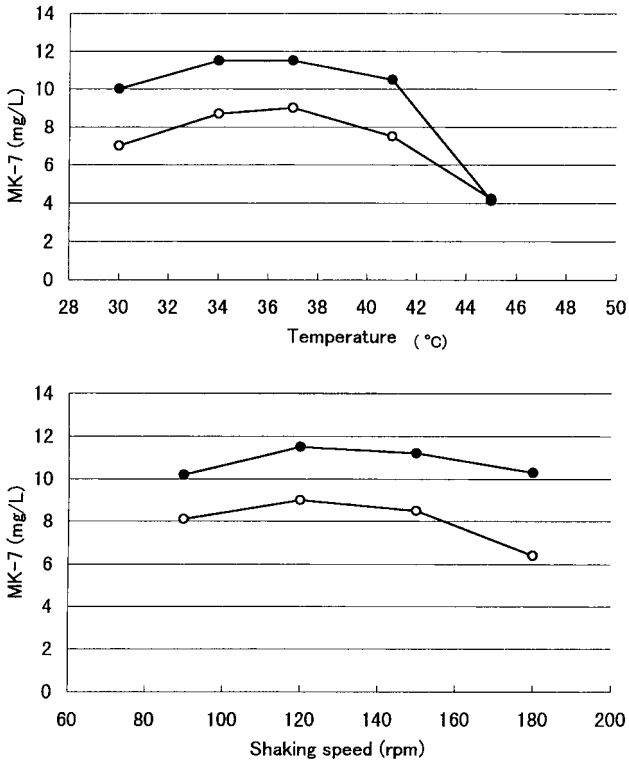

Figure 2 Effect of shaking speed and temperature on MK production by *B. subtilis* strain K3-176 (●) and MH-1 (○) was examined. Soybean extract (10%), pH 7.3, was used as the medium.

Table 3 Effects of media on MK-7 production by *B. subtilis* strain K3-176

Medium 1 (w/w)	Medium 2 (w/w)	Medium 3 (w/w)	Concentration of nitrogen (%)[a]	Concentration of carbohydrates (%)[a]	MK-7 (mg/l)[b]	OD 660[b,c]	MK-7/ OD 660
(A) Effects of nitrogen source							
Soybean extract, 10%			0.30	6.4	11.1±1.76	17.6±0.97	0.63
Tryptone, 1.9%	Sucrose, 6.3%		0.30	6.4	2.5±1.27	5.4±2.43	0.46
Peptone, 1.9%	Sucrose, 6.3%		0.30	6.4	3.1±0.90	9.0±1.03	0.34
Nutrient broth, 2.0%	Sucrose, 6.3%		0.30	6.4	1.4±0.51	3.3±0.76	0.42
CSL[d], 7.4%	Sucrose, 6.3%		0.30	6.4	7.5±0.41	15.2±0.64	0.49
Soytone, 3.2%	Sucrose, 5.6%		0.30	6.4	9.8±0.62	18.1±0.80	0.54
Phytone, 3.2%	Sucrose, 5.6%		0.30	6.4	8.5±0.41	16.5±1.13	0.52
Polypeptone, 2.3%	Sucrose, 6.1%		0.30	6.4	4.6±1.23	7.1±0.28	0.65
Beef extract, 2.1%	Sucrose, 6.4%		0.30	6.4	2.2±0.85	3.0±0.33	0.73
Skim milk, 5.3%	Sucrose, 3.6%		0.30	6.4	3.7±0.29	5.5±0.57	0.67
(B) Effects of carbon source							
Soybean extract, 10%			0.30	11.4	10.4±0.29	18.8±0.47	0.55
Soybean extract, 10%	Glycerol, 5%		0.30	11.4	21.2±1.40	19.7±0.25	1.08
Soybean extract, 10%	Glucose, 5%		0.30	11.4	13.2±0.24	25.3±0.62	0.52
Soybean extract, 10%	Mannose, 5%		0.30	11.4	12.3±0.47	24.5±0.41	0.50
Soybean extract, 10%	Sucrose, 5%		0.30	11.4	16.2±1.20	27.2±0.56	0.60
Soybean extract, 10%	Lactose, 5%		0.30	11.4	12.3±0.43	19.1±0.57	0.64
Soybean extract, 10%	Maltose, 5%		0.30	11.4	14.2±0.25	26.3±0.61	0.54
Soybean extract, 10%			0.30	6.4	10.4±0.29	18.8±0.47	0.55
Soybean extract, 10%	Glycerol, 1%		0.30	7.4	11.3±0.25	18.9±0.21	0.60
Soybean extract, 10%	Glycerol, 3%		0.30	9.4	16.7±0.87	19.5±0.62	0.86
Soybean extract, 10%	Glycerol, 5%		0.30	11.4	21.2±1.40	19.7±0.25	1.08
Soybean extract, 10%	Glycerol, 7%		0.30	13.4	18.6±0.66	19.8±0.94	0.94
Soybean extract, 10%	Glycerol, 10%		0.30	16.4	14.3±0.47	18.7±0.21	0.76
(C) Effects of additives							
Soybean extract, 10%	Glycerol, 5%		0.30	11.4	21.1±0.83	20.5±0.33	1.03
Soybean extract, 10%	Glycerol, 5%	Yeast extract, 0.2%	0.30	11.4	23.3±0.53	22.1±0.59	1.05
Soybean extract, 10%	Glycerol, 5%	Yeast extract, 0.5%	0.30	11.4	26.7±1.70	24.6±0.36	1.09
Soybean extract, 10%	Glycerol, 5%	Yeast extract, 1.5%	0.30	11.4	12.0±2.45	10.7±1.89	1.12

Cells were cultured by shaking at 37°C for 3 days. The experiment was carried out in duplicate or triplicate and values are expressed as mean±SD.
[a]Values are based on data sheets obtained from the medium supplies.
[b]Data are expressed as mean±SD.
[c]OD 660: optical density at 660 nm.
[d]CSL: corn steep liquor.

First, cultivation was carried out without controlling the pH which decreased from 7.3 to 5.5, then spontaneously increased to 7.7–8.0 (Figure 3). The concentration of MK-7 began to increase after the cell growth rate reached its maximum level, reaching 33.0–37.0 mg/l after 4 days.

The effect of pH on MK production was then examined. When the pH was controlled at 5.7, 6.0, 7.0, 7.5 and 8.0, only

Table 4 Secretion of MK in supernatant medium by *B. subtilis* strains MH-1 and K3-176

Media	Strain	Secreted MK	Total MK	Secreted MK/ Total MK (%)
10% soybean extract, pH 7.3				
	MH-1	4.3±0.75	9.0±0.23	47.0±7.3
	K3-176	6.5±1.60	11.4±0.95	56.2±9.4
10% soybean extract, 5% glycerol, pH 7.3				
	MH-1	8.9±1.59	17.4±0.50	51.2±9.6
	K3-176	11.0±1.92	21.2±0.30	52.0±8.6
10% soybean extract, 5% glycerol, 0.5% yeast extract, pH 7.3				
	MH-1	12.7±2.02	22.9±1.43	55.5±8.9
	K3-176	15.8±2.80	27.2±0.76	57.9±8.7

Cells were cultured by shaking at 37°C for 3 days. The experiment was carried out in triplicate and values are expressed as mean±SD.

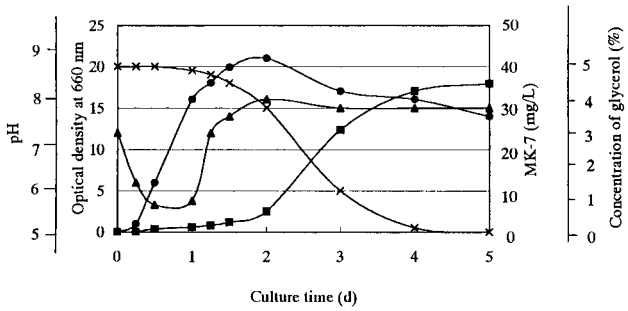

Figure 3 Time course of fermentation in a jar fermenter. Fifty milliliters of precultured medium was added to 5 l of the medium containing 10% soybean extract, 5% glycerol and 0.5% yeast extract in a jar fermenter. Temperature and agitation and aeration rates were maintained at 37°C, 250 rpm, and 0.5 vvm, respectively. The optical density at 660 nm (•), concentration of MK-7 (■), pH (▲) and concentration of glycerol (×) were measured.

Table 5 Effects of pH on MK-7 production by *B. subtilis* strain K3-176 in a jar fermenter

pH	MK-7 (mg/l)	Maximum OD 660	MK-7/ OD 660
Not controlled	30.5	30.4	1.00
5.7	10.4	16.6	0.63
6.0	13.9	20.2	0.69
7.0	16.6	30.8	0.54
7.5	17.2	31.4	0.55
8.0	13.5	24.0	0.56

Culture conditions were as described in . pH was controlled at the values shown. Maximum concentration of MK-7 and optical density at 660 nm (OD 660) are listed. Experiments were carried out in duplicate and average values are shown.Figure 3

small quantities of MK-7 were obtained despite good cell growth (Table 5).

Discussion

We investigated the production of MK-7 by an edible strain of *B. subtilis* isolated from fermented soybeans, *natto*, a traditional Japanese food. Tani *et al.* [25] reported that a menadione-resistant mutant of *Flavobacterium* produced increased levels of MK. We obtained a menadione-resistant mutant of *B. subtilis*, strain K3-176, which produced 30% more MK than the parent MH-1 (Table 2).

A medium containing 10% soybean extract and 5% glycerol was optimum for MK production. Glycerol was reported to be the most effective carbon source for both growth and MK productivity of *Flavobacterium* [25]. In the case of *B. subtilis*, the effect of glycerol was not the promotion of cell growth, but an increased yield of MK on a per-cell basis (Table 3).

Ikeda and Doi [10] showed that cells of *B. subtilis* lyse easily and about half of the MK produced is present in water-soluble micelles, whose components are derived from the cell membrane. *Flavobacterium* secreted MK into a culture medium supplemented with detergent resulting in a high yield of MK [24]. This secretion of MK was expected to increase MK productivity by easing constraints or intracellular capacity which normally results in metabolic repression of MK biosynthesis. In our study, 40–65% of the MK produced by *B. subtilis* strain K3-176, as well as MH-1, was secreted and present in water-soluble micelles. This characteristic of *B. subtilis* may be related to its ability to produce MK-7 in high concentrations. Correlation of the secretion and productivity of MK must be studied further in detail.

In a jar fermenter, during cultivation, the pH of the medium decreased from 7.3 to 5.5, then spontaneously increased to approximately 8.0. While the pH was controlled at a certain value throughout the culture period, only small quantities of MK-7 were produced (Table 5). Hill *et al.* [7] showed that the promoter of MK biosynthetic genes was activated when the extracellular pH was 5.7. However, in our study, controlling the pH at 5.7 did not increase MK production and cells did not grow well at this pH. Therefore, we assumed that a gradient change in pH, as outlined in Figure 3, would be suitable for MK production.

The maximum concentration of MK reached approximately 35 mg/l after 4 days of culture in a jar fermenter. This concentration is much higher than those produced by other microorganisms reported [12]. MK-7 can have direct effects on bone formation [18] and can prevent bone loss induced by ovariectomy [27], as well as have effects on the synthesis of Gla proteins [8,13]. In fact, MK-7 is the most predominant homologue of MK in blood [8] and bone [9] in humans. However, MK-7 has not been extensively utilized for dietary supplementation in the food industry because until now, efficient production has not been achieved. Thus, the method of production of MK-7 described herein would be of great benefit to the food industry.

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