Research in Molecular Medicine

Optimization of Culture Condition for the Production of Menaquinone-7 by *Bacillus Subtilis Natto*

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ABSTRACT

Background: Vitamin K2 refers to a series of naphthoquinone derivatives, which have a variety of physiological and pharmacological functions for the human body. The most important type of vitamin K2 is menaquinone-7 (MK-7), an expensive raw material with no local manufacturers in Iran.

Objectives: Since there was no report on the yield of MK-7 produced by the currently available *Bacillus subtilis natto* species in Iran, this study aims to optimize the culture condition for the production of MK-7 using this Bacillus species.

Materials and Methods: The base medium (BM) for MK-7 production contained glycerol (6.3%), soybean peptone (3%), and yeast extract (0.51%). The selected factors for optimizing the MK-7 production included the incubation temperature (30, 37, and 40°C) and incubation time (72, 96, and 120hr) with/without the addition of K_2HPO_4 to the fermentation medium. Three sets of experiments with six modes in each set were designed based on these parameters. MK-7 content was analyzed by the HPLC method.

Results: Two experiments showed the highest MK-7 production yields of 0.319 and 0.3158 mg/L. The culture condition for both of these yields was as follows:120 hours incubation time in the presence of K_2HPO_4. However, the incubation temperature was different in these two experiments. The incubation temperature of 30°C resulted in 0.319 mg/L MK-7 concentration, and 37°C yielded 0.3158 mg/L.

Conclusion: *B. subtilis natto* (IBRC-M 11153) is suitable to be used as a basic platform for the mutation and production of a high-producer species. Optimizing the culture conditions using the wild-type species is not beneficial in increasing the production ability of the bacterium. It is necessary to use different methods for enhancing the production yield of MK-7 to lower the cost of microbial production and make the industrial process economic.

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Introduction

Vitamin K is a group of vitamins with a 2-methyl-1,4-naphthoquinone ring called menadione (K3) as the common structure among this family members [1-3]. Based on the side chain structure at position 3 of the menadione structure, vitamin K is classified into four types: K1, K2, K3, and K4. Vitamin K1 (phylloquinone) and vitamin K2 (menaquinone) are two natural types of vitamin K. Phylloquinone (PK) has a monounsaturated side chain (phytyl) with four prenyl units and is commonly found in plants and photosynthetic algae [1, 4, 5]. Menaquinone (MK) mainly has bacterial sources and contains an unsaturated aliphatic side chain with variable numbers (1 to 14) of prenyl units which determines the respective MK type (i.e. MK-n). Accordingly, vitamin K2 is classified as short-chain (e.g. MK-4) and long-chain (e.g. MK-7, MK-8, and MK-9), with the most known types, MK-4 and MK-7 [5, 6]. Among MKs, the most remarkable biological activity belongs to MK-7, leading to its fast absorption by the intestine and long blood half-life [7]. Compared to MK-4, MK-7 has a substantially higher impact on lowering the levels of PIVKA-II (protein induced by vitamin K absence) [8]. PIVKA-II is a biomarker for hepatocellular carcinoma. Furthermore, MK-7 has been reported as the major MK homolog in the human bone and blood and is very efficient in vitamin K-dependent γ-carboxylation and bone metabolism [8-12].

MK-7 is a precious vitamin K family member with a noteworthy outcome in preventing osteoporosis and cardiovascular diseases (CVDs) and helpful roles in blood coagulation [7, 11, 13, 14]. Furthermore, MK-7 has been regarded as a member of fourth-generation anti-osteoporosis medications [5, 15]. Due to the increasing number of osteoporosis and CVD patients worldwide, and the high care cost for the management of these health complications [16, 17], research in recent years has focused on the development of preventive products. Furthermore, additional roles of vitamin K2 have been reported in many studies, including prevention of cancer by proliferation inhibition [15], the rescue of the mitochondrial dysfunction leading to Parkinson's disease [18], helping the liver functional recovery [19], type-2 diabetes mellitus risk reduction [20], and reduction of vascular calcification as a potential therapy for Alzheimer's disease [21] which represents vitamin K2 promising critical roles in the future.

MK-7 can be prepared by extraction from natural sources, chemical synthesis, and microbial fermentation. Due to the low MK-7 content in natural sources, extraction from these sources is not a suitable method for the industrial production of vitamin K2. The challenges in the chemical synthesis of vitamin K2 include various complex stages, low yields, production of many different isomers with low activity, and production of many by-products with undesirable effects on the environment [22]. Therefore, microbial fermentation seems to be a suitable method to produce vitamin K2 in its natural form.

According to previous studies, *Bacillus subtilis natto* is the most suitable subspecies for the industrial production of vitamin K2 [23]. Therefore, in this study, *B. subtilis natto* (IBRC-M 11153) was selected as the producer microorganism to optimize the production yield of vitamin K2. It was the only currently available species of *B. subtilis natto* in IBRC, and there was no report on the yield of MK-7 produced by this bacterial species in Iran.

Materials and Methods

Pure MK-7 was purchased from Gnosis (Gnosis, Desio, Italy). Peptone, agar, acetone, n-hexane, n-propanol, glycerol, and glucose were purchased from Merck (Germany), and yeast extract was purchased from Ibresco (Iran). $NaNO_3$ and K_2HPO_4 were purchased from Chem-Supply (Australia).

Microorganism, cultivation, and maintenance

B. subtilis natto (IBRC-M 11153) was purchased from the Iranian Biological Resource Center (IBRC) and used in this study to produce vitamin K2. This strain was cultivated in Luria-Bertani (LB) medium and stored at 2-8°C. The strain was first cultivated in LB liquid medium for long storage of the bacteria. Then, glycerol (15%) stocks of the bacteria were prepared and transferred to -20/-80°C after cooling at 4°C for 45 minutes.

Preculture preparation

The preculture medium (25 mL, glucose (3%), Soybean peptone (4%), NaCl (0.5%), yeast extract (0.5%), meat extract (0.5%)) was prepared in 100 mL culture flasks. The pH of the medium was adjusted to 7, and the medium was sterilized by autoclaving (121°C, 15 psi, 15min). Three preculture flasks were prepared for the inoculation. These preculture flasks were inoculated with 24-hour-plate cultures of *B. subtilis*. Shaking fermentation was conducted at 37°C with shaking at 120 rpm for 24 hours using a shaking incubator.

Fermentation process

To produce vitamin K2, *B. subtilis natto* was incubated on a rotary shaker in the base medium (BM) containing glycerol (6.3%), soybean peptone (3%), and yeast extract (0.51%). The composition of the BM was selected as the optimal base medium for the production of vitamin K2 based on the previous studies [24].

To evaluate the production of vitamin K2, three parts of the BM (47.5 mL) were first transferred to three 250 mL culture flasks, and the pH of the media was adjusted to 7. Then, three BM-containing flasks were sterilized at 121°C and 15 psi for 20 minutes. Finally, the inoculation of each BM-containing flask was performed with 2.5 mL (5%) of the 24-hour *B. subtilis*-containing preculture medium, incubated on a rotary shaker at 210 rpm, and grown under the designed experimental conditions.

Design of the experiments

The following parameters were chosen to optimize MK-7 production. In this study, the selected factors for optimizing vitamin K2 production included the incubation temperature (30, 37, and 40°C) and incubation time (72, 96, and 120 hours) with/without the addition of K_2HPO_4 to the fermentation medium. Three sets of experiments (Experiment Set 1, 2, and 3; ES1, ES2, and ES3) with six modes in each were designed based on the parameters mentioned above. Details of the designed experiments are illustrated in Table 1.

Vitamin K2 extraction

MK-7 separation was done according to the extraction methods of previous studies [24]. After incubation and bacterial growth, vitamin K2 was extracted as follows. The media were transferred to sterile 50 mL falcons and centrifuged at 6000 rpm for 10 minutes to separate the biomass and the supernatant. The supernatant (20 mL) was then transferred to a clean flask, and 40 mL of the extraction solvent (n-hexane 26.6 mL and isopropanol 13.4 mL) was added to this flask. The flask containing the extraction mixture was then placed in a shaker incubator at 30°C for 1 hour with a shaking rate of 160 rpm. Then, the flask content was transferred to a clean falcon and centrifuged at 6000 rpm for 10 minutes to separate the phases.

Twenty milliliters of the upper layer (solvent) were transferred to a clean flask, and the solvent was evaporated using a rotary and vacuum evaporator (10 minutes, 60°C). Finally, 1 mL of the HPLC solvent was used to wash the flask and used for the quantitative analysis.

MK-7 analysis

High-Performance Liquid Chromatography HPLC Agilent (Infinity 1260, Germany) equipped with a UV detector and C18 column (4 mm×25 cm, 5 µm) was used at 25°C for the analysis of MK-7 concentration. Dehydrated alcohol was used as the mobile phase with a flow rate of 0.7 mL/min. The wavelength of 280 nm was selected for calibration and analysis. Eight standard solutions (1, 5, 10, 20, 40, 60, 80, and 100 ppm) were prepared to draw the standard curve. The standard and sample solutions were protected from light and injected immediately after preparation. Agilent ChemStation software was used for data acquisition.

Results

Standard curve development

Eight standard solutions (1, 5, 10, 20, 40, 60, 80, and 100 ppm) were prepared using the USP standard MK-7 to draw the calibration curve (supplementary Figure 1 and Figure 1). The HPLC chromatogram of the 1 ppm standard solution is shown in Figure 2.

MK-7 analysis

The extracted samples were analyzed using the HPLC method. The concentration of the MK-7 in the samples was calculated using the following formula: Y=22.9x+3.9424, where the "y" represents the area under the curve (AUC) and the "x" represents the MK-7 concentration in the sample. The results are illustrated in Table 1. Figure 1 shows the MK-7 concentrations in the samples produced under the designed experiments. Based on the results shown in Table 1 and Figure 1, the highest MK-7 concentrations were observed in ES1-3, ES2-1, ES3-5, and ES3-6. The HPLC chromatograms of these samples are shown in Figures 3-6.

Effect of incubation time on MK-7 production

To find the optimum time of incubation for MK-7 production, three incubation times (72, 96, and 120 hours) were investigated in this study. Based on the results of HPLC analysis, MK-7 concentrations after 72, 96, and 120 hours were 0.259, 0.2492, and 0.319 mg/L, respectively, which indicated 120 hours as the optimum incubation time. However, increasing the in-

Table 1. Design of the experiments sets and vitamin K2 concentration

Experiment No.	Variables	MK-7 Concentration (mg/L)
	Experiment Set 1	
ES1-1	K_2HPO_4, 72 h, 40°C	0.1872
ES1-2	Without K2HPO4, 72 h, 30°C	0.2459
ES1-3	K_2HPO_4, 72 h, 37°C	**0.259**
ES1-4	without K_2HPO_4, 72 h, 40°C	0.156
ES1-5	without K_2HPO_4, 72 h, 37°C	0.18406
ES1-6	K_2HPO_4, 72h, 30°C	0.1809
	Experiment Set 2	
ES2-1	K_2HPO_4, 96 h, 37°C	**0.2492**
ES2-2	Without K_2HPO_4, 96 h, 30°C	0.1651
ES2-3	K_2HPO_4, 96 h, 30°C	0.1907
ES2-4	K_2HPO_4, 96 h, 40°C	0.199
ES2-5	Without K_2HPO_4, 96 h, 37°C	0.103
ES2-6	Without K_2HPO_4, 96 h, 40°C	0.186
	Experiment Set 3	
ES3-1	Without K_2HPO_4, 120 h, 40°C	0.1268
ES3-2	K_2HPO_4, 120 h, 40°C	0.1627
ES3-3	Without K_2HPO_4, 120 h, 30°C	0.179
ES3-4	Without K_2HPO_4, 120 h, 37°C	0.234
ES3-5	K_2HPO_4, 120 h, 30°C	**0.319**
ES3-6	K_2HPO_4, 120 h, 37°C	**0.3158**

cubation time from 72 hours to 96 hours had no substantial effect on MK-7 production yield.

Effect of incubation temperature on MK-7 production

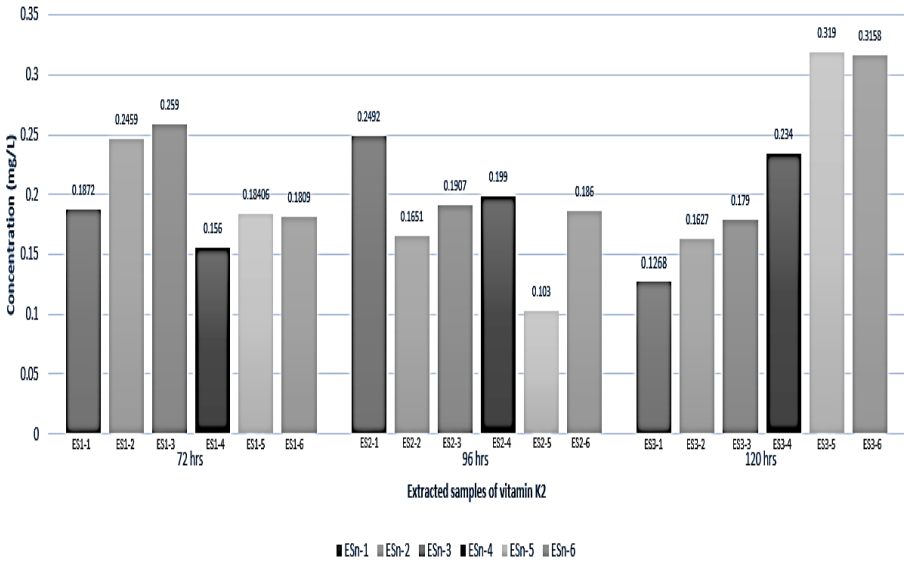
To find the optimum incubation temperature for MK-7 production, three temperatures (30, 37, and 40°C) were investigated in this study. The results of the HPLC analysis showed that MK-7 concentrations after 30, 37, and 40°C were 0.319, 0.3158, and 0.199 mg/L, respectively, which represented 30 and 37°C as the optimum incubation times. The results showed that the 40°C temperature is not a suitable incubation temperature for the production of MK-7 using *B. subtilis natto* (IBRC-M 11153).

Effect of K_2HPO_4 Addition on MK-7 Production

Two conditions, including media with and without K_2HPO_4, were investigated to study the effects of K_2HPO_4 addition on MK-7 production. The results of the HPLC analysis showed MK-7 concentrations with and without K_2HPO_4 were 0.3158 and 0.2459 mg/L, respectively, which showed K_2HPO_4 has a substantial positive effect on MK-7 production yield using *B. subtilis natto* (IBRC-M 11153).

Optimized culture condition for MK-7 production

Two experiments showed the highest MK-7 production yields of 0.319 and 0.3158 mg/L. The culture



Supplementary **Figure 1.** Comparison of MK-7 concentration produced under the designed experiments

Three sets of experiments (Experiment Set 1, 2, and 3; ES1, ES2, and ES3) with six modes in each set were designed based on three selected factors (incubation time, incubation temperature, and presence of K_2HPO_4 in the medium).

condition for both of these yields was as follows: 120 hours incubation time in the presence of K_2HPO_4. However, the incubation temperature was different in these two experiments. The incubation temperature of 30°C resulted in 0.319 mg/L MK-7 concentration, and 37°C yielded 0.3158 mg/L.

Discussion

Studies showed that human cells could not produce MK-7; therefore, it is necessary to use exogenous sources of MK-7, including food and dietary supplements. The natural sources have a low content of MK-7, and that is why vitamin K2 is called the "platinum vitamin". Consequently, developing a dietary supplement rich in MK-7 is highly desirable [5].

MK-7 can be prepared by extraction from natural sources, chemical synthesis, and microbial fermentation. Considering the low MK-7 content in the natural sources, and the challenges in the chemical synthesis of vitamin k2 [22], microbial fermentation seems to be a suitable method to produce vitamin K2 in its natural form. According to previous studies, *B. subtilis natto* is the most suitable subspecies for industrial production of vitamin K2 [23]. Therefore, in this study, *B. subtilis natto* (IBRC-M 11153) was selected as the producer microorganism to optimize the production yield of MK-7. It was the only currently available species of *B. subtilis*

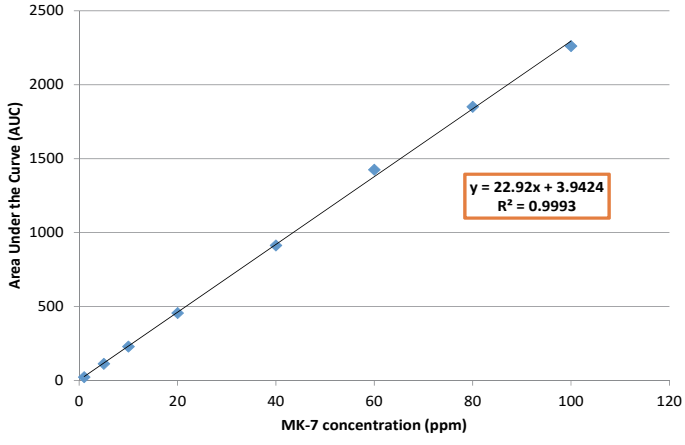


Figure 1. Calibration curve using eight concentrations of USP standard MK-7

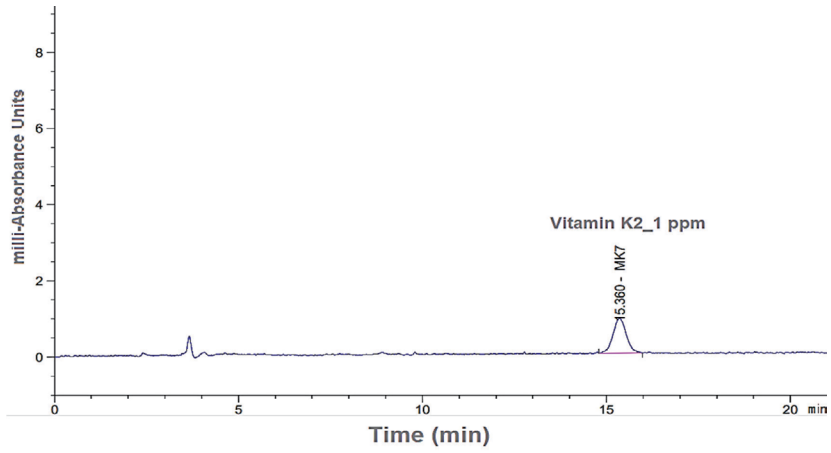


Figure 2. HPLC chromatogram of vitamin K2 standard solution (1 ppm)

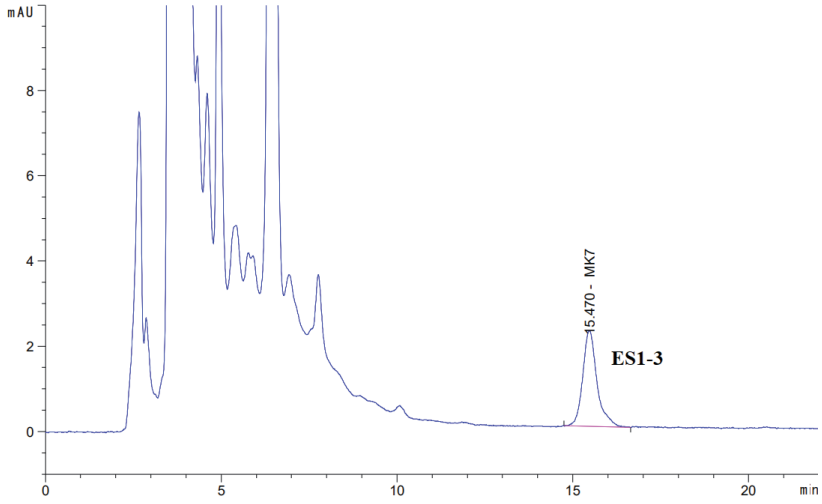


Figure 3. HPLC chromatogram of ES1-3 sample (K_2HPO_4, 72 hours, 37°C)

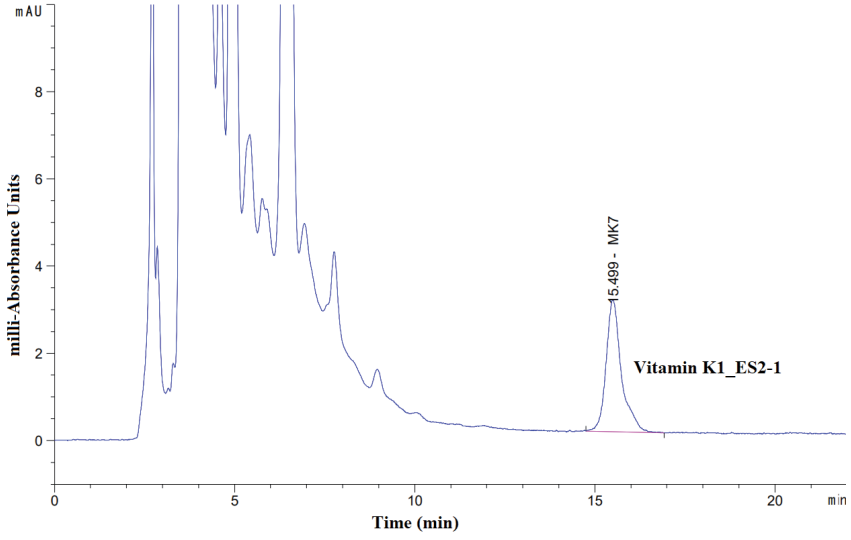


Figure 4. HPLC chromatogram of ES2-1 sample (K_2HPO_4, 96 hours, 37°C)

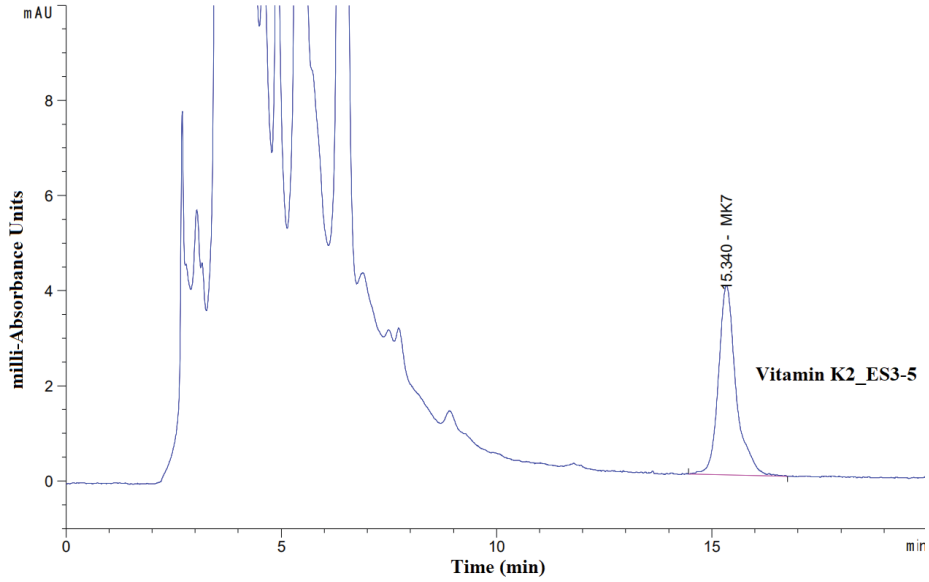


Figure 5. HPLC Chromatogram of ES3-5 Sample (K_2HPO_4, 120 hours, 30°C)

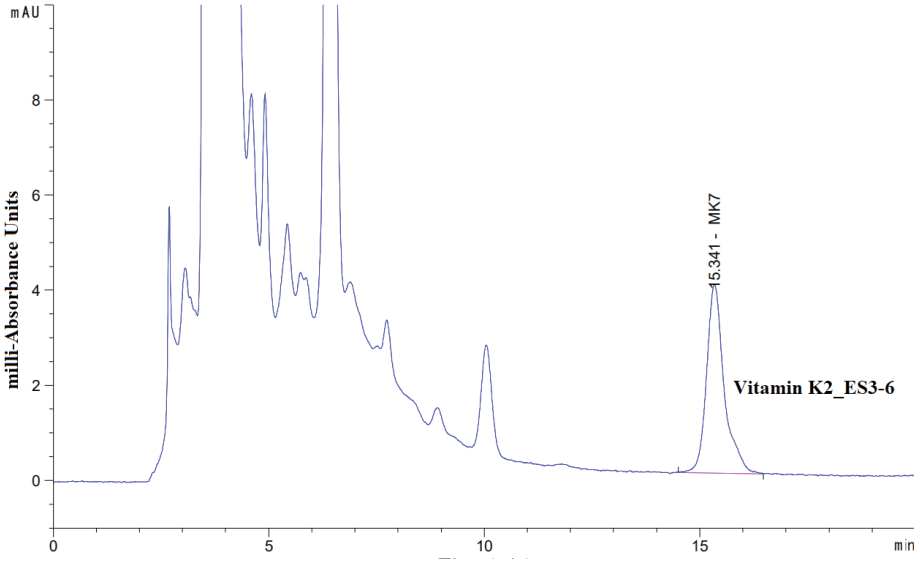


Figure 6. HPLC chromatogram of ES3-6 sample (K_2HPO_4, 120 hours, 37°C)

natto in IBRC, and there was no report on the yield of vitamin K2 produced by this species in Iran.

The biosynthetic pathway of vitamin K2 is very complicated, and the level of this vitamin produced by microorganisms is very low. In addition, the downstream processing of this vitamin includes many complicated steps, which increases the price of the final product. Many new developments have been achieved in the MK-7 bioprocessing technologies. However, this vitamin is still not available at an affordable price for industrial production. Accordingly, many efforts have been focused on optimizing the upstream and downstream processing of the microbial production of vitamin K2 [23, 25, 26].

This study investigated the optimization of the upstream processing of MK-7 using *B. subtilis*. Generally, the strategies used to optimize vitamin K2 production using microbial fermentation can be categorized into five groups: 1) genetic modification methods, including blocking or weakening of a biosynthesis pathway, 2) mutagenesis methods using structural analogs, 3) innovative designs in the bioreactors, 4) optimization of the fermentation process, including optimization of the medium components or the cultivation conditions,

and 5) increase in the secretion of the vitamin K2 by the addition of the surfactants or detergents [5]. In this study, the optimization of MK-7 production yield was investigated using the optimization of the fermentation process, including the optimization of one of the medium components' concentrations (K_2HPO_4) as well as the cultivation conditions, including incubation temperature and time.

One of the main factors that substantially affects the concentration of MK-7 in the bacteria is the media composition. This factor also affects the final cost of the product, which is considered one of the main challenges in the industrial production of vitamin K2.

The carbon source, as one of the main components of the media, has many critical roles in bacterial growth, including the preparation of the energy needed for bacterial activities and the construction of the bacterial cell components. In addition, the carbon source influences the metabolites produced by the bacteria. Glycerol was used in many studies as the carbon source for vitamin K2 production and showed satisfying effects on production yield [24]. Therefore, in this study, glycerol was used as the main source of carbon.

The other main media component is the nitrogen source which has many critical roles in bacterial functions, including synthesizing nucleic acid and protein. Previous studies reported beneficial effects of yeast extract and soybean peptone on the vitamin K2 production [27, 28]. Therefore, these materials were selected as the nitrogen sources in our study.

Previous studies used different organic salts in the media including NaCl (0.5 g/L), $MnCl_2$ (0.5 g/L), $FeCl_3$ (0.5 g/L), $MgSO_4$ (0.5 g/L), $CaCl_2$ (0.5 g/L), and $ZnSO_4 7H_2O$ (0.5 g/L) for the vitamin K2 production [6]. In this study, K_2HPO_4 is the media component which was selected to evaluate its effect on MK-7 production yield, since there were controversial reports on the evaluation of its effect on MK-7 production. Chao Zhang et al. used K_2HPO_4 in the fermentation medium and their results showed beneficial effects of this organic salt on vitamin K2 production [24]. In another study, vitamin K2 was produced in the absence of K_2HPO_4 [29]. K_2HPO_4 is an ionic compound, a highly water-soluble salt, and a source of potassium and phosphorus, and also used as a buffering agent in the media. The nutrient requirements of the bacteria are simple and different but among the most well-known necessary elements for bacteria, phosphorus reported to be very critical. Phosphorus is necessary for the storage and transfer of the biological information, integrity of the membrane, and energy metabolism. Phosphorus is essential in the formation of critical bacterial molecules including DNA and RNA [30]. In this study, two modes were designed including media with and without K_2HPO_4, and the results showed that the production yield was low in the absence of K_2HPO_4. This might be related to the fact that if a nutrient in the media is in short supply, it can affect the growth rate of the organism. Therefore, in the media containing K_2HPO_4, the bacteria have high growth rate leading to an increase in the vitamin K2 production yield.

Many environmental factors including the shaking rate, incubation time, incubation temperature, static vs shaking cultivation, and dissolved oxygen might influence the vitamin K2 production yield. First attempts for vitamin K2 production were based on solid-state fermentation systems. However, these systems needed long cultivation time and led to low productivity. Therefore, the liquid fermentation systems were substituted to substantially enhance the rate of the bacterial growth and reduce the fermentation time. It has been demonstrated that vitamin K2 production under a shaking condition leads to higher yields compared to a static culture [31]. In this study, incubation time and temperature under a shaking culture condition were used as the environmental factors to find the optimum values for vitamin K2 production. There were controversial results from previous studies. Sato et al. evaluated the effects of three temperatures (30°C, 37°C, and 45°C) on vitamin K2 production yield, and their results showed that the production yield may improve with an increase in the incubation temperature. They reported 45°C as the optimum temperature for vitamin K2 production [32]. However, the results of Zhang et al. showed the incubation temperature of 37°C as the optimum temperature for vitamin K2 production [24]. Based on the previous studies, *B. subtilis* can tolerate temperatures from 28°C to 45°C. Therefore, in this study, three temperatures (30, 37, and 40°C) were investigated for production of vitamin K2, and the results showed 30 and 37°C as the optimum incubation temperatures. The temperature of 30°C was not previously reported as an optimum temperature for vitamin K2 production.

There were also controversial reports on the optimum incubation time for vitamin K2 production. Luo et al. reported 60 hours, as the incubation time for maximum vitamin K2 production yield. However, Wang et al. reported 108 hours as the optimum incubation time for production of vitamin K2 [5]. In this study, three incubation times (72, 96, and 120 hours) were investigated to evaluate the effect of incubation time on the

vitamin K2 production yield. The results showed 120 hours as the optimum incubation time which showed the highest production yield.

The maximum MK-7 concentration was observed under the following condition as the optimum culture condition: media containing 6.3% glycerol, 3% soybean peptone, 0.51% yeast extract, and 0.05% K_2HPO_4 after 120 hours at 30 and 37°C.

Conclusion

Since there was no report on the yield of MK-7 produced by the currently available *B. subtilis natto* species in Iran (IBRC-M 11153), the goal of this study was to optimize the culture condition for the production of MK-7 using this Bacillus species. The results of this study showed that *B. subtilis natto* (IBRC-M 11153) is suitable to be used as a basic platform for mutation and production of a mutated species with better characteristics (high-producer species). In addition, this study showed that the optimization of the culture conditions using the currently available Bacillus species in Iran (the wild-type) could not be beneficial to increase the ability of the bacterium for a high production yield. This study would serve as a critical experimental basis for microbial production of MK-7 in Iran. However, it is necessary to use different methods for enhancing the production yield of MK-7 to lower the cost of microbial production of MK-7 and make the industrial manufacturing process economic.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article.

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This article is extracted from a thesis with the ethical code of IR.IUMS.REC.1400.374.

Authors contribution's

Data collection and analysis: Israa Khalid AL-Yasari, Sara Noroozi Eshlaghi, Negar Mottaghi-Dastjerdi and Mohammad Soltany-Rezaee-Rad; draft preparation, review, and editing: Mohammad Sharifzadeh, Negar Mottaghi-Dastjerdi and Marjan Shariatpanahi; conceptualization: Mohammad Soltany-Rezaee-Rad; and supervision: Negar Mottaghi-Dastjerdi.

Conflict of interest

The authors declare no conflict of interest.

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