PREPARATION OF VITAMIN K₂ MK-7 IN A PROCESS OF FERMENTATION OF DIFFERENT SEEDS AND CEREALS BY BACTERIA *BACILLUS SUBTILIS*

– Research paper –

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Abstract: In this study, 10 different plant materials (seeds/beans) were fermented by *Bacillus subtilis* var. natto. The influence of the process on vitamin K₂ MK-7 content during different fermentation periods was assessed. Fermented plant samples were analyzed by the procedure using HPLC UV/DAD. The fermented sunflower seeds, mung beans and peas appeared to be the most promising plants, reaching values of K₂ of 1080.18±55.11 µg/100g, 806.45±60.95 µg/100g and 636.92±59.86 µg/100g, respectively. The experiments showed that extending of the fermentation time to 5-6 days was favorable for the menaquinone-7 yield. The results show that almost all fermented seeds/beans, apart from soybean, can be good source of vitamin K₂ MK-7 and represent a new perspective, especially in terms of lower the phytoestrogen content.

Keywords: *Bacillus subtilis*(natto), bacterial fermentation, seeds/beans, HPLC/UV-DAD, menaquinone-7, vitamin K₂ MK-7

INTRODUCTION

Vitamin K is a very important component of human health, and it is essential for ensuring the normal functioning of the body. The World Health Organization and the Food and Agriculture Organization, specified the recommended daily allowance (RDA) for vitamin K at 65 g/day for men and 55 g/day for women, assuming 1 g/day/kg body weight (WHO & FAO, 2004). In living organisms, a group of these compounds has a function of a cofactor for the enzyme responsible for the post-translational carboxylation of glutamic acid (Glu) residues incorporated into the polypeptide chain, leading to formation of gamma-carboxyglutamate (Gla) residues. It was found that about 8 protein molecules obtained during this process are associated with blood clotting, while the role of the remaining eight proteins goes beyond the circulatory system (Chu et al., 2006), (Kucharz et al., 2018), (Schwalfenberg, 2017). In a consequence of this post-translational modification, the protein is able to bind calcium ions in bones and tissues, which implies a vital role

of that vitamin in bone metabolism (Adams & Pepping, 2005), (Palermo et al., 2017), (Wu et al., 2015). Furthermore, vitamin K-dependent proteins participate in the metabolism of blood vessels, prevention of vascular calcification (Vermeer et al., 2004), (Willems et al., 2014) and calcification of soft tissues, in the processes of cell growth and apoptosis (Erkkilä & Booth, 2008), as well as cell adhesion (Vermeer et al., 2004). In addition to already indicated benefits associated with taking vitamin K, other advantages are also worth mentioning, e.g. its anti-cancer effect and reduced cancer mortality risk (Dahlberg et al., 2017), (Zwakenberg et al., 2017), (Lamson & Plaza, 2003), because vitamin K participates in the sphingolipids synthesis, and in the anti-oxidation processes. Furthermore, vitamin K effectively reduces development of functional disorders of the nervous system (Ferland, 2013), as well as reduces the risk of diabetes (Manna & Kalita, 2016), (Suksomboon et al., 2017). In contrast, in plants the role of the Vitamin K is associated with the process of photosynthesis - Vitamin K₁ is an electron acceptor at the first stage of that process. Vitamin K is not one substance, but rather a whole group of substances having a similar structure and activity, known as vitamins K₁, K₂, and K₃

Received: 24.01.2021

Accepted in revised form: 20.05.2021

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(Figure 1). A characteristic feature of group K vitamins is the 2-methyl-1,4-naphthoquinone ring found in all C-3 isoprenoid groups attached to the C-3 position (Vermeer & Braam, 2001).

Figure 1. Structure of vitamins K1, K2 and K3.

Vitamin K1, i.e., phylloquinone (also known as phytomenadione), contains one unsaturated and three saturated isoprenoid groups, and it is synthesized only by plants. It is mostly found in leaf vegetables, such as lettuce, spinach, and cabbage, as well as in cucumbers, avocado, broccoli, potatoes, turnips, celery, and vegetable oils (Booth, 2012).

Vitamin K2 is a whole set of compounds known as menaquinones, and their structure depends on the number of isoprenoid groups in the side chain (MK-n, where n is the number of unsaturated isoprenoid groups in position 3, and ranges from n = 1 to n = 13). They are synthesized by bacteria and occur mainly in animal products (Kucharz et al., 2018), (Vermeer & Braam, 2001) such as cheese and other fermented dairy products. Meat and eggs contain their slightly smaller amounts, but their highest levels are found in natto, the famous Japanese breakfast made from fermented soybeans (Kucharz et al., 2018), (Walther et al., 2013).

Vitamin K3, i.e., menadione, is a synthetic vitamin K analogue which does not contain the aliphatic side chain and acts as a provitamin. Vitamins K1 and K2 are lipophilic, while vitamin K3 is a compound soluble in water.

In humans, sources of vitamin K2 include not only the diet, but also vitamin K1 (phytoquinone), which is converted, with the participation of bacteria, to the MK-4 form (containing 4 isoprenoid groups in the side chain) (Kucharz et al., 2018) in the testes, pancreas and artery walls. It

is assumed that up to 90% of vitamin K1 can be transformed in this way. The absorption of vitamins K1 and K2 varies, and these differences are associated with the structure of the isoprenoid side chain of phylloquinone and menaquinone, which determines the way of their transport or absorption by target tissue cells in animals, or their excretion by source cells (Shearer & Newman, 2008). Vitamin K2 is almost completely absorbed and has a long biological half-life, while K1 absorption accounts for only 10-15% of the total amount of this vitamin ingested with food (Schurgers & Vermeer, 2000). Similarly, K2 MK-4 is a less active biological form, when compared to MK-7 (containing 7 isoprenoid residues in the side chain) (Karmańska & Karwowski, 2015).

As reported by numerous studies, menaquinones are also synthesized by intestinal bacteria; for example *Bacteroides fragilis* produces MK-10 and MK-12, and *Eubacterium lentum* produces MK-6. Lactic acid bacteria, *Lactococcus lactis* ssp. *Lactis* and *L. lactis* ssp. *Cremoris* (used in the food industry), synthesize menaquinones from the MK-8 and MK-9 group, and also can convert MK-7 to MK-10. Propionobacteria are responsible for production of MK-9 (Beulens et al., 2013).

As mentioned earlier, soybean fermentation products (natto) are especially rich in menaquinones, where the dominant form of the vitamin is MK-7, synthesized by *Bacillus subtilis* (Schurgers & Vermeer, 2000), (Karmańska & Karwowski, 2015). Natto is a product obtained from soybeans subjected to many hours of cooking and a natural fermentation process. This well-known Japanese food supports circulation, maintains normal blood pressure, lowers cholesterol and sugar levels, improves metabolism, and supports the overall health of the body, because it contains high levels of nattokinase, isoflavones, saponins, unsaturated fatty acids, probiotics regulating the composition of the intestinal bacterial flora, fiber, vitamins, minerals, folic acid and pantothenic acid, as well as antioxidants (Tsubura S., 2012), (Nagai, 2015), (Swain et al., 2014), (Tamang et al., 2016), (Jensen et al., 2016), (Wintergerst et al., 2007). Furthermore, natto contains calcium and vitamin K2, contributing to the strengthening and health of bones (Maresz, 2015), (Tsukamoto et al., 2000). Regular consumption of natto may have other benefits as well as it may reduce the risk of certain cancers (Tse & Eslick, 2016), (Douglas et al., 2013) and improve brain health (Wang et al., 2016), (Kim et al., 2016).

A major disadvantage of soy could be the presence of phytoestrogens. Some studies have shown that

these compounds are structurally and functionally analogous to the estrogens, and their excessive intake may affect the hormonal balance of consumers, causing disturbances in the functioning of the thyroid gland, affecting fertility and sexual development, and resulting in feminization (Jargin, 2014), (Kim & Park, 2012), (Divi et al., 1997).

So far, numerous studies on the process of obtaining vitamin K2 MK-7 have focused mainly on soybean, and no information is available

whether it can be replaced with other grains or edible seeds, which may also contribute to evolution of new taste qualities.

The aim of this study was to obtain a natural form of vitamin K2 (menaquinone-7) as a result of fermentation by *Bacillus subtilis* var. natto, using 10 different substrates (pulses/cereals) as an alternative to the soybean. The course of the fermentation process was monitored by determining the vitamin concentration.

MATERIALS AND METHODS

Microorganism and culture conditions

The *Bacillus subtilis* ATCC 15245 culture was received from the American Type Culture Collection (USA). Bacteria were kept as spores suspended in 50% glycerol and stored at -18°C . Culture reagents, such as lysogeny broth (LB MIX) and yeast extract, were purchased from BTL sp. z o. o. (Poland), and sucrose and soy protein isolate were obtained from Chempur, (Polska) and Exeller® Polska Sp. z o.o., respectively.

Research material

The research material consisted of ten plants belonging to both cereals and beans, including: true millet (*Panicummiliaceum* L.), common maize (*Zea mays*), sunflower (*Helianthus annuus* L.), green lentil (*Lens culinaris* Medik.), chickpeas (*Coronilla* L.), mung beans (*Vignaradiata*), soybeans (*Glycine* Willd.), field beans (*Vicia faba* L.), peas (*Pisumsativum* L.), and white lupine (*Lupinus albus* L.). Plants came from organic farming and they were GMO free.

Chemical and reagents

All solvents were of high purity: n-hexane and 2-propanol for vitamin extraction, as well as acetonitrile, methanol and orthophosphoric acid for chromatographic analyses were purchased from Chemsolve, WITKO Sp. z o.o. (Poland). Certified vitamin K2-(MK-7) analytical standards were purchased from Carbosynth Ltd (United Kingdom).

Seed culture preparation

The *Bacillus subtilis* ATCC 15245 suspension was made from a single colony from actively growing slants. The bacteria were inoculated on the fresh LB MIX seed culture medium (1% v/v) composed of peptone K 10.0 g/L, sodium chloride 10.0 g/L, yeast extract 5.0 g/L, and distilled water. The liquid cultures were incubated at 37°C and 170 rpm in an orbital shaker for 24 hours, to acquire a

seed culture with the OD 550 nm of ~ 1.75 . Next, the 5% (v/v) seed culture was introduced into the production medium containing 3% soy extract, 0.5% sucrose, and 1.5% yeast extract, thereby obtaining a concentration of 1×10^8 cells/mL.

Nutraceutical production

The fermentation of plant products (10 types of seeds /cereal grains, with different protein and fat content (Table 1) was conducted in the solid state using *Bacillus subtilis* ATCC 15245. Plant products (weighing 50 g) were prepared for fermentation by washing, soaking, cooking and sterilization (Figure 2). 300 mL shaking flasks with this culture medium were inoculated with 10 mL of production medium, then the contents of the flask were mixed and closed with a cellulose stopper. The fermentation process took place in an incubator under controlled conditions of temperature and relative humidity (37°C and approx. 75%, respectively) for 24 hours, and then first samples of the plant material were collected. Then, at daily intervals, further samples were taken and the fermentation was continued until 144 hours elapsed. All microbiological experiments were performed in a sterilized environment.

After the end of the process, the fermented seeds/beans were stored at 4°C for 7 days to simulate the aging process (Rishipal et al., 2016), (Maruo & Yoshikawa, 1989). In the next step, the plant material was extracted for the quantification of vitamin K2 MK-7.

Vitamin extraction from fermented products

The sample preparation followed principles of the procedure described by Rishipal et al. (Rishipal et al., 2016). The fermented plants were ground in a mortar, then an analytical portion of 5 g was collected and introduced into a 50 mL centrifuge tube, next 15 mL of propan-2-ol and n-hexane (1:2 v/v) mixture were added. The contents of the tube were shaken vigorously for 10 min, and then vortex mixed (BenchMixer™, Benchmark, Edison, NJ, USA). The sample was then

centrifuged at 3000 rpm for 5 min, and the (top) layer of organic material was removed and filtered through a 0.45 µm membrane filter. However, to allow the final determination of vitamin K2, the procedure was modified by replacing the solvent

with acetonitrile directly before the high-performance liquid chromatography (HPLC) analysis, after a prior evaporation of the sample under a nitrogen stream.

Table 1. The content of protein, fat and carbohydrates in each type of plant fermented by *Bacillus subtilis* (the table data was compiled on the basis of the labels of the purchased products).

Plant	Protein content/100 g of product (g)	Fat content/100 g of product (g)	Carbohydrate content/100 g of product (g)
Chickpeas (<i>Coronilla</i> L.)	19.0	6.0	61.0
Common maize (<i>Zea mays</i>)	9.4	4.7	74.3
Field beans (<i>Vicia faba</i> L.)	25.0	1.5	18.0
Green lentil (<i>Lens culinaris</i> Medik.)	9.0	0.4	20.1
Mung beans (<i>Vigna radiata</i>)	24.0	1.2	63.0
Peas (<i>Pisum sativum</i> L.)	23.3	1.4	45.2
Soybeans (<i>Glycine</i> Willd.)	36.0	20.0	30.0
Sunflower (<i>Helianthus annuus</i> L.)	21.0	51.0	20.0
True millet (<i>Panicum miliaceum</i> L.)	11.0	4.2	72.9
White lupine (<i>Lupinus albus</i> L.)	40.0	4.4	40.4

Figure 2. A diagram presenting preparation and fermentation of plant products by *Bacillus subtilis* (natto).

Chromatographic analysis

All analyses were performed on an HPLC Ultimate 3000 system (Dionex, Germering, Germany) equipped with a photo-diode array detection (UV-DAD) detector. The data was processed with Chromeleon 6.8 software (Dionex Corporation, Sunnyvale, California, United States). The chromatography was conducted at 25°C. Sample separation was carried out on an ACE 5 C18 column packed with 5 µm (250 mm x 4.6 mm) shell particles. The chromatographic conditions were as follows: in the gradient elution mode, a mixture of water and methanol (1:1 v/v) acidified to pH 3.0 with orthophosphoric acid (A) and acetonitrile (B) was used as the mobile phase, the eluents flow rate was 1.2 mL. The sample injection volume was 20 µl. Each sample was analyzed in

three replicates. The total time of the analysis was 15 min. Absorbance measurements for vitamin K2 MK-7 were conducted at a wavelength of 248 nm. Vitamin K2 was identified on the basis of the retention time, and the quantification was performed by the standard curve method. For this purpose, six dilutions of the vitamin K2 MK-7 analytical standard were prepared in acetonitrile, with concentrations ranging from 1.95 to 250 µg/mL. The limit of detection (LOD) and the limit of quantification (LOQ) were verified experimentally on the basis of the established calibration curve for a series of standard solutions (Konieczka P., 2003). The working range and linearity of the method were estimated using a range of concentration values determined with the assumed precision, accuracy and uncertainty.

RESULTS AND DISCUSSIONS

In this study, a procedure of alternative fermentation by *Bacillus subtilis* (natto) of different plant materials, with subsequent extraction of vitamin K2 MK-7 was elaborated.

Validation parameters

The analysis was preceded by several preliminary experiments using the HPLC/UV-DAD system. To improve the selectivity and ensure high accuracy, the assessment focused on the scope of the method, precision, linearity, LOD and LOQ as validation parameters. Six different solutions of an analytical standard of vitamin K2 MK-7 (1.95, 7.0, 31.25, 62.5, 125, and 250 µg/mL) were analyzed by chromatography. Figure 3 shows an example of a chromatogram for an analytical standard.

Each sample was injected three times and the following regression equation was obtained: $y = 0.4136x + 0.3738$. The detector response linearity was established using the square correlation coefficients (R^2) determined for the calibration curves, and at the same time it was demonstrated that the regression line was linear within the range of all tested concentrations ($R^2 = 0.9991$).

The chromatographic analysis precision was described by the percent relative standard deviation (%RSD); its estimation was done by repeatability measurements for three replicates of the standards solution. The %RSD value of 0.09 was established. The limit of detection (LOD) (Konieczka P., 2003) and the limit of quantification (LOQ) were determined according to the formulas: $3.3 \times \sigma / S$, and $10 \times \sigma / S$, respectively, where σ is the response/signal standard deviation, while S is the calibration curve slope. In this study, LOD and LOQ were 0.718 and 2.176 µg/mL, respectively.

Fermentation products

In this study, the content of menaquinone-7 was determined for 10 different plant matrices, including legumes and cereals, fermented by *Bacillus subtilis*. The samples were analyzed in triplicate ($n=3$). The obtained results are showed in Figure 4. The solid-state fermentation under the same conditions was carried out in parallel for soybeans, as it is known that soybeans are the best source of K2 MK-7; therefore this plant was treated as a control material for the whole process. The fermentation was performed according to the scheme as indicated in the Methods section.

For sunflower seeds, the vitamin K2 MK-7 content was very high on the first day (835.85 ± 97.21 µg/100 g), on the next day it increased to 1080.18 ± 55.11 µg/100 g, and then decreased to 45.18 ± 15.34 µg/100 g after the sixth day. For all samples, excluding sunflower, the vitamin content increased during fermentation. The best yield of K2 MK-7, with a significant logarithmic growth of concentration on the fifth day of fermentation, was obtained for mung beans (891.94 ± 55.49 µg/100 g) and peas (673.22 ± 45.07 µg/100 g). The test substance was found at a similar level in field beans, lupine and lentils, ranging from 246.75 ± 15.27 to 289.44 ± 20.45 µg/100 g of the product. The lowest menaquinone-7 content in the entire fermentation process was recorded for maize (the maximum vitamin concentration was 154.17 ± 9.34 on the sixth day). According to the presented research, by extending the fermentation time to 120 – 144 h the vitamin K2 MK-7 production from plant products, excluding sunflower seeds, can be increased.

Figure 3. The chromatogram of menaquinone-7: concentration - 10 µg/ml, retention time - 1.307 min.

Nowadays, we witness an intensive development of new types of fermented foods of animal and plant origin. It was proven that the transformation of ingredients of food raw materials taking place during the bacterial fermentation process results in a change in certain bioactive ingredients and a formation of others, with new bioactivity, e.g. increased absorption of free amino acids from dietary proteins, being a rich source of free elements faster and easier absorbed by organisms, or with the increased content of some vitamins, γ -aminobutyric acid, and natural phenols. It should also be emphasized that these are products with probiotic properties (Steinkraus, 1996), (Szydłowska & Kołożyn-Krajewska, 2010), (Gan et al., 2017), (Adebo, 2017). All these properties of fermented products mean that they can certainly be recommended for consumption as forming a vital part of the human diet. They can also be developed into functional food helping to prevent some chronic disorders (Gan et al., 2017).

Bioprocessing, especially the solid state fermentation (SSF), considered to be a safe and environmentally friendly process, has recently attracted a growing interest (Bhanja Dey et al., 2016), (Rosales et al., 2018), (Chen et al., 2020). Numerous microorganisms, including yeast, lactic acid bacteria and molds (Zhang et al., 2015), (Lai et al., 2013), (Gamboa-Gómez et al., 2016), (Xiao et al., 2014), (Dordević et al., 2010), (Shin et al., 2019), (Gan et al., 2017) are used in fermentation, and their variety or arrangement can improve the fermentation efficiency.

The available literature lacks research on the possibility of using different plant matrices in the process of *Bacillus subtilis* (natto) fermentation for the biosynthesis of vitamin K2 MK-7. Following these considerations, we attempted in our research

to ferment various different edible seeds and cereals (field beans, true millet, peas, chickpeas, mung beans, white lupine, green lentils, maize, sunflower and soybeans) under the same fermentation conditions. The process was monitored by determining the concentration of menaquinone-7.

Menaquinone-7 (MK-7) extraction from fermented samples was conducted using 2-propanol and n-hexane (2:1, v/v) as extraction solvents. Vitamin levels were determined with the HPLC-UV/DAD method. MK-7 underwent gradient chromatographic separation on a C18 ACE column, with a mixture of water and methanol (1:1 v/v) acidified to pH 3.0 by addition of orthophosphoric acid (A) and acetonitrile (B) used as a mobile phase, with a flow rate of 1.2 mL/min. The UV detection was conducted within the range of 200 to 400 nm, and the chromatogram was acquired at a wavelength of 248 nm. Before the analysis the solvent was replaced with acetonitrile, and this change represents a modification of the method. The developed methodology is characterized by linearity in a wide range of concentrations from 1.95 to 250 µg/mL, high precision (%RSD) of 0.09, and low detection and quantification limits of 0.718 and 2.176 µg/mL, respectively. Under the described conditions, the MK-7 analysis was completed in less than 15 minutes, and a retention time was 1.307 minutes. The use of the method based on the extraction with 2-propanol and n-hexane, with the further chromatographic analysis using the HPLC system with ultraviolet detection is popular and has been successfully used in the determination of vitamin K2 MK-7 by other scientists (Rishipal et al., 2016), (Ranmadugala et al., 2018), (Berenjian et al., 2011).

Figure 4. Effect of plant samples fermentation during 144 h of fermentation by *Bacillus subtilis* (natto).

The results of conducted analyses showed the presence of K2 MK-7 in all fermented plant materials, the obtained data is presented in Figure 4. After the fermentation started, on the first day, the highest content of MK-7 in 100 g of product was found in sunflower seeds ($835.85 \pm 97.21 \mu\text{g}$), soybean ($472.31 \pm 80.03 \mu\text{g}$), lentil ($235.74 \pm 23.34 \mu\text{g}$), and in peas ($250.34 \pm 45.22 \mu\text{g}$). On the next day, the vitamin content increased in all the samples (with the greatest increase of up to $1080.18 \pm 55.11 \mu\text{g}$ in sunflower). In turn, on the third day, the highest increase in MK-7 levels was observed for the samples of chickpeas ($258.37 \pm 13.95 \mu\text{g}$), peas ($693.99 \pm 45.44 \mu\text{g}$), millet ($245.86 \pm 14.87 \mu\text{g}$), and lentils ($387.96 \pm 11.54 \mu\text{g}$). These values were the highest concentrations achieved for these plants during the menaquinone-7 synthesis. On the following days, the vitamin content in these products gradually decreased, except for soybeans, white lupine, field beans, mung beans, and maize, where the concentration increased almost until the end of fermentation, i.e. Day 6. Nevertheless, the highest content of K2 MK-7 was found in soybeans ($1526.89 \pm 120.01 \mu\text{g}$), followed by a definitely lower amount in mung beans ($806.45 \pm 60.95 \mu\text{g}$) and peas ($636.92 \pm 59.86 \mu\text{g}$). Maize, on the other hand, showed the lowest content of the tested substance, both at the initial and further stages of fermentation. Concluding, the extension in the fermentation time from 120 to 144 h leads to an increase in the production of vitamin K2 MK-7 in edible seeds and cereals. The exception is sunflower seeds, which reached the maximum concentration of the tested substance after the second fermentation day. Maize turned out to be the least efficient plant in terms of the menaquinone-7 biosynthesis.

The chemical composition of the medium and the fermentable material is of course of paramount importance for determining the fermentation yield. For the proper growth of microorganism cultures, it is necessary to ensure the appropriate amount of sugars, B vitamins and amino acids, as well as their availability. The menaquinone-7 content in soybeans is much higher than in other crops, therefore further studies can be conducted aiming at optimizing the fermentation process conditions, to increase the production of this vitamin in plants

CONCLUSIONS

The use of the biotechnological method in the production of natural vitamin K2 MK-7 from seeds of edible plants and cereals, as an alternative to

other than soy. Our experience shows that soybeans were characterized by the highest efficiency of K2 MK-7 synthesis (20 g of total fat content, 36 g of protein and 30 g of carbohydrates, Table 1.). In our study, soy was treated as a control material for other fermented plants. As shown by the presented data, mung beans (with significantly lower fat content of 1.2 g, lower protein content of 24 g, and higher carbohydrate content of 63 g, versus soybean) and peas (1.4 g of fat, 23.8 g of protein, and 45.2 g of carbohydrates) proved to be the most promising plant materials. On the other hand, sunflower seeds (51 g of total fat, 21 g of protein and 20 g of carbohydrates, Tab. 1) showed the highest efficiency of the menaquinone biosynthesis on the first days of the fermentation, and then a decrease to the level of $45.18 \pm 15.22 \mu\text{g}$. Sunflower is a source of mono- and polyunsaturated fatty acids, which could affect the process speed, and after reaching the maximum yield and exhausting food reserves, a decrease in the concentration was observed. Maize, containing only 9.4 g of protein, 4.7 g of fat and with the highest carbohydrate content among all tested types of seeds/cereals, showed the lowest menaquinone-7 synthesis. In this case, the addition of hydrolytic enzymes cleaving carbohydrates, such as cellulases, α -amylase, β -glucosidase, and pectinases, which can efficiently hydrolyze glycosidic, ester, or ether linkages (Bhanja Dey et al., 2016), (Chen et al., 2020), could be considered as an advantageous solution. In the conducted research, the used media included a culture medium consisting of peptone, sodium chloride, and yeast extract, and a production medium of soybean extract and sucrose, which are easily digestible sources of nitrogen and carbon accelerating the bacterial growth. A similar medium composition was used in those study settings by other scientists, including Liu et al. (Liu et al., 2005), Tian et al. (Tian et al., 2016), Luo et al. (Luo et al., 2016), and Mahdinia et al. (Mahdinia et al., 2019). Moreover, other conditions of the fermentation process suitable for *Bacillus subtilis*, such as temperature (37°C), or humidity (75%), were consistent with data obtained from literature sources (Rishipal et al., 2016), (Luo et al., 2016), only the process time was extended to 144 hours (preferably 24-122 hours).

soy, is undoubtedly possible. The optimized extraction and quantification procedure using HPLC UV/DAD was successfully applied in the analysis of fermented plant samples. The applied method was characterized by the linearity in the

range from 1.95 to 250 µg/mL, high precision (% RSD) of 0.09, LOD of 0.718, and LOQ equal to 2.176 µg/mL. *Bacillus subtilis* (natto), used in our experiments, is considered a safe strain, with no toxicogenic and pathogenic features (de Boer Sietske & Diderichsen, 1991). For this reason, it is a source of many products synthesized by the agri-food industry. In this study, the results obtained in the experiments clearly demonstrated that the extension of the fermentation time to 5-6 days (depending on the type of plant material) is advantageous for the menaquinone-7 yield. The results show that besides soy other fermented seeds/beans can be a good source of vitamin K2 MK-7 and provide a new perspective, especially in terms of phytoestrogen content. Mung beans and

peas, with the values of 806.45 ± 60.95 µg/100 g and 636.92 ± 59.86 µg/100 g, respectively, proved to be the most promising plants. The fermented sunflower seeds reached the maximum content of K2 (1080.18 ± 55.11 µg/100 g) already on the second day from the beginning of the process. Maize turned out to be the least suitable plant matrix. To obtain a competitive product with the best properties, the fermentation process should be refined within the key conditions, i.e. substrates, the inoculum optimization, and other technical parameters. Apparently, this may be a new direction in the development of a functional food product of a plant origin; therefore this will be an excellent area for further research.

Funding

This study was supported by Polish Ministry of Science and Higher Education [project number 2019/03/X/NZ9/00235 “Preparation of vitamin K2 (menaquinone-7) by fermentation with bacteria *Bacillus subtilis* - optimization and modification of the process”].

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