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REVIEW

Production and application of menaquinone-7 (vitamin K2): a new perspective

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Abstract Menaquinone-7, a highly valuable member of the vitamin K series, has significant effects on preventing osteoporosis and cardiovascular disease besides its positive effects on blood coagulation. In this review, chemical and biological aspects of menaquinone-7 are briefly summarized followed by a critical review on upstream and downstream processing developments for its production and recovery, including solid versus liquid fermentations, static versus agitated fermentations and online versus post-production recovery. Latest research outcomes for improving industrial scale production of menaquinone-7 are summarized and recommendations are given for areas of future research.

Keywords Vitamin K · Menaquinone · Fermentation · Production · Separation · Nutritional benefits

Introduction

Vitamins are generally organic compounds that are vital nutrients, which organisms need in often minor amounts. Vitamins are compounds that the organism cannot synthesize in sufficient quantities, and must be obtained through the diet; thus, the term "vitamin" is conditional upon the circumstances and the particular organism (Lieberman and Bruning 1990).

Vitamins are classified by their biological and chemical activity, not their structure. Thus, each vitamin refers to a number of compounds that all show the biological activity associated with a particular vitamin. Vitamins are grouped under an alphabetized vitamin generic descriptor title, such as vitamin A, which includes the compounds retinal, retinol, and four other known carotenoids (α-carotene, β-carotene, γ-carotene and cryptoxanthin). Vitamers by definition are convertible to the active form of the vitamin in the body, and are sometimes inter-convertible to one another (Maton 1993). To date, thirteen vitamins are universally recognized.

In 1935, Henrik Dam, who later on shared the 1943 Nobel Prize in medicine with Edward Doisy for their work on vitamin K, discovered a fat soluble anti-hemorrhagic factor with similar physical properties to vitamin E, but with a different physiological clotting function from any known vitamin (Dam 1935). Basically, Dam implemented fat-free regimens to chicks and he observed that soon the chicks developed serious hemorrhages. Moreover, by introducing different nutrients to the diet regimen, he discovered the natural sources of this new vitamin that could suppress the symptoms. Among these sources were hog liver oil, hemp seed, and certain vegetables such as kale and tomatoes (Dam 1935). Dam called this new vitamin "the anti-hemorrhagic vitamin" which finally got the name "vitamin K" based on the German and Scandinavian spelling of ''Koagulations'' (Dam 1935).

Soon after the discovery of vitamin K by Dam, it was found out that there are two major forms of vitamin K known as vitamin K1 (Fig. 1a) and K2 (Fig. 1b). Vitamin K1, also known as phylloquinone, phytomenadione, or phytonadione, is naturally produced by plants, and is found

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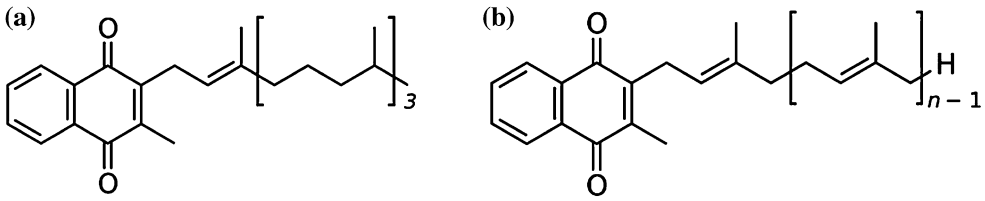

Fig. 1 Vitamin K1 (phylloquinone) **a** and Vitamin K2 (menaquinone), **b** (Berenjian et al. 2015)

in highest amounts in green leafy vegetables, since it is directly involved in photosynthesis (Widhalm et al. 2012). Vitamin K1 is also active in animals and performs the classic functions of vitamin K, including its activity in the production of blood-clotting proteins (Davidson et al. 1998). On the other hand, vitamin K2 has several subtypes, which differ in isoprenoid chain length. These vitamin K2 homologues, called menaquinones, are produced by microorganisms. They are characterized by the number of isoprenoid residues in their side chains as shown in Fig. 1 (Binkley et al. 1939). These vitamers are abbreviated as MK-n, where M stands for menaquinone, K stands for vitamin K, and n represents the number of isoprenoid side chain residues.

All K vitamins contain 2-methyl-1,4-naphthoquinone, which is also known as menadione, as a common ring structure, but differ from each other in the length and saturation degree of the polyisoprenoid side chain attached to the 3-position (Schurgers and Vermeer 2001). Basically, phylloquinone has a singular form but menaquinones include fourteen compounds (MK-1 to MK-14), yet the most common ones are MK-4 and MK-7 (Fig. 2). Unlike phylloquinone, menaquinones are produced in animal and bacterial cells. For example, large intestinal bacteria, such as *Escherichia coli*, can synthesize vitamin K2 in the form of MK-7 to MK-11 (Bentley and Meganathan 1982). In these bacteria, menaquinone transfers electrons among oxygen-independent metabolic reactions (anaerobic respiration) (Haddock and Jones 1977). However, such bacteria in the microbial ecosystem of the human intestine are not able to provide a dietary source of vitamin K2. Structural differences in the isoprenoid side chain in Vitamin K1 and K2, govern many facets of metabolism of K vitamins including the way they are transported, taken up by target tissues, and subsequently excreted (Shearer and Newman 2008).

Compared to the other forms of vitamin K, MK-7 (Fig. 2b) has much longer half-life in human blood and also can be produced by variety of bacterial species (Weber 2001; Suttie 1995; Geleijnse et al. 2004; Howard and Payne 2006; Yamaguchi 2006; Gast et al. 2009; Schurgers et al. 2007). Thus, it has attracted researchers to investigate bioprocess engineering technologies for industrial production of MK-7, during the last decade (Berenjian et al. 2015). The section below will be summarizing the studies for fermentation and recovery for the production of MK-7.

MK-7 fermentation

MK-7 fermentation processes can be performed by liquid or solid state fermentation, although a rigorous line cannot be drawn between them. Solid State Fermentation (SSF) processes can have up to 80% and as low as 12% water content, whereas for Liquid State Fermentations (LSF), water content of fermentation medium is typically between 90 and 95% (Mitchell et al. 2000). Also, MK-7 production is associated with low productivities and concentrations; therefore its production is a costly process (Berenjian et al. 2015). Thus, research has been conducted in the past decades to enhance the MK-7 production.

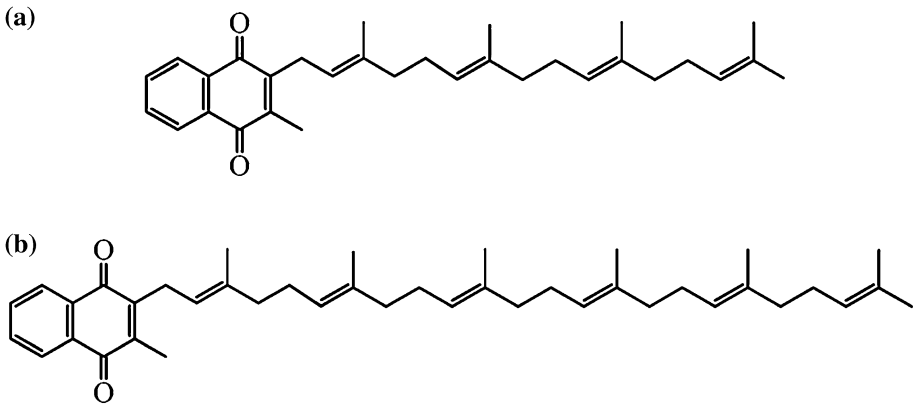

Fig. 2 Molecular structures of MK-4 (**a**) and MK-7 (**b**)

Solid state fermentation (SSF) for MK-7 production

Generally, SSF has been successful in production of secondary metabolites, since mycelial morphology of the microorganisms mainly used for secondary metabolites production, suits growth on a solid substrate (Krishna 2005). Yet, SSF processes for MK-7 production have certainly received less attention as compared to LSF (Pandey 2003). For instance, Natto, a traditional Japanese food, is made by a solid state fermentation of *Bacillus subtilis natto* on soybeans. Several studies have utilized a similar concept for production of MK-7 rich products. In this fashion, Mahanama et al. (2011), isolated the highest MK-7 yielding strain of *Bacillus subtilis natto* from commercial natto foods. Using SSF on corn grits and soy protein, fermentation parameters have been optimized using RSM and CCF techniques. A maximum amount of 67.01 mg/kg of MK-7 has been reported (Mahanama et al. 2011).

In another recent study by Singh et al. (2015), *B. subtilis* was used in SSF and several medium components were investigated to increase MK-7 concentrations. Among these components, glycerol, mannitol, yeast extract, malt extract, and calcium chloride were identified and optimized by RSM. Eventually a highest concentration of 39 μg of MK-7 per g of medium has been reported.

Bacillus amyloliquefaciens has also been used in fermentation of cheonggukjang, a Korean traditional fermented soybean (Wu and Ahn 2011). Similar to *B. subtilis natto*, supplementing 4% of glycerol has shown a significant increase on the MK-7 concentration. The content of MK-7 under the optimum condition is reported to reach as high as 11.13 mg MK-7 per kg of fermented food (Wu and Ahn 2011).

Generally, the major factors that affect MK-7 production by SSF systems are the selection of microbial strain, suitable substrate, pre-treatment, particle size, water activity (a_w) of substrate, size and type of inoculum, temperature and fermentation time during SFF (Pandey 2003). Selecting a substrate for MK-7 production in an SSF process mainly depends upon cost and availability and therefore usually involves screening several solid substrates.

Commonly, unprocessed raw substrates are used in SSF, such as corn and soy for MK-7 production. However, simultaneous substrate pre-treatment and fermentation have been used to increase the yield of vitamin and to reduce the fermentation time. For instance, pretreatment using α-amylase which increases the availability of sugar monomers at the first stage of fermentation, seems to increase MK-7 yield (Mahanama et al. 2011).

Selection of reactor type mainly depends on the fermentation volume. Commonly, tray type fermenters that operate in a static mode have been used for MK-7 production (Berenjian et al. 2014). A tray bioreactor consists of a chamber in which air is circulated with controlled temperature and relative humidity around a number of trays. Trays contain a thin layer of substrate, typically between 5 and 15 cm deep, and usually has an open top and perforated bottom. Since tray operations are very simple, they are ideal for low volume productions with low levels of technology (Mitchell et al. 2000). Using a static deep bed bioreactor, amyloglucosidase has been produced to a maximum of 8035 (units/g of dry mouldy bran) using *Aspergillus niger* (Ghildyal et al. 1993). Their results have indicated that temperature gradients in the bed play a key role in enzyme biosynthesis. Also, in production of alkaline protease by *Aspergillus flavus*, scaling up the production from Erlenmeyer flasks to tray fermenters and further to Koji rooms has improved enzyme production yields (Malathi and Chakraborty 1991).

Tray fermenters have been successful in lab-scale, pilot and large scale fermentations due to simplicity; however, the large space required for the installation of trays and more generally static mode fermenters and the risk of high temperature and oxygen gradients are the major concerns for the static mode reactors (Krishna 2005).On the other hand, dynamic mode fermenters, such as rotating drums, are among more suitable types for SSF processes and production of vitamins (Yang 2007). In a rotating drum bioreactor, the substrate bed is held within a horizontal or near horizontal drum, with or without baffles and the drum is continuously rotated. Air is not blown forcefully through the bed itself, but rather across the top of the substrate bed through the headspace. These bioreactors have a long history, having been used for α-amylase production in the early 1900s and penicillin after WWII (Mitchell et al. 2000). Moreover, these bioreactors have been successfully used for Vitamin B and E productions (Stahmann et al. 2000; Berenjian et al. 2015). However, the major issue in using dynamic mode fermentations is the high level of moisture content that may result in particle agglomeration (Yang 2007).

Although, SSF processes can offer alternatives to the conventional LSF, requiring less preprocessing energy, producing less wastewater and improving product recovery (Uyar and Baysal 2004), the complexity of SSF scale up, lack of devices to measure relevant operating variables inside the reactor (i.e. pH, DO, a_w, biomass) and difficulty in metabolic heat removal are limiting factors for impeding the technological development of SSF. Therefore, LSF provides more advantages for production of MK-7.

Liquid state fermentation (LSF) for MK-7 production

Among bacterial strains, *B. subtilis* and *B. licheniformis* are the well-studied strains for MK-7 LSF fermentation. *B. licheniformis* is an organism with well-characterized membranes,

contains menaquinone as the sole quinone, and possesses the ability to grow anaerobically. MK-7 exists in both wild-type and mutant strains playing a similar respiratory quinone role as MK-4 and MK-6 in *Flavobacterium* sp. 238-7 (Tani and Taguchi 1989). The highest amount of MK-7 produced was reported by Goodman as 0.25 μg/mg of dry weight of cells (Goodman et al. 1976).

Morishita et al. (1999) focused on production of menaquinones using lactic acid bacteria. The results have indicated that *Lactococcus lactis* ssp. *cremoris* (three strains), *Lactococcus lactis* ssp. *lactis* (two strains), and *Leuconostoc lactis* were all potent producers of quinones. These strains, when grown in a soymilk medium, produced a significant amount of MK-7. The quinones were presumed to be MK-7 to MK-10 by high performance liquid chromatography. Specifically for MK-7, the highest concentration observed was 12.3 mg/L in *Lactococcus lactis* ssp. *Cremoris*. Therefore, the authors concluded that these strains would be useful as starter cultures for dairy and other food fermentation or dietary supplements which may include dietary sources of menaquinones. Nonetheless, the focus of LSF studies has been on MK-7 production by *B. subtilis natto*. Berenjian et al. (2011, 2012, 2013, 2015) have carried out several studies on MK-7 production by LSF using *B. subtilis natto*. The effect of medium nutrients for *Bacillus subtilis natto* MK-7 was studied to enhance the MK-7 production. Maximum MK-7 concentration of 62.32 mg/L has been reported in the media containing 5% (w/v) yeast extract, 18.9% (w/v) soy peptone, 5% (w/v) glycerol and 0.06% (w/v) K_2HPO_4 (Berenjian et al. 2011).

In another study, Berenjian et al. (2012) determined the effect of fed-batch glycerol addition in the production of MK-7 during the fermentation in both small (25-mL) and bench scale (3-L) fermenters. The results of their study have demonstrated that the addition of glycerol in a fed-batch process considerably enhanced the MK-7 production. Maximum MK-7 has been produced when 2% (w/v) glycerol was added to the fermentation media in the second day of fermentation. The results have indicated a 40% increase in MK-7 concentration (86.48 mg/L) as compared to the batch culture. The authors have also suggested that adjusting the concentration and feeding strategy of essential nutrients may be considered as an efficient approach for enhancing MK-7 production (Berenjian et al. 2012).

Later on, Berenjian et al. (2013) have investigated the effect of suspended pellicle formation by *B. subtilis natto* on MK-7 fermentation. Surprisingly, by switching from static to agitated fermentation, they observed that pellicle formation had insignificant effect on MK-7 production. That is, when pellicle formation was inhibited, MK-7 production was slightly inhibited. At the same time, agitation improved biomass production. Glucose as the carbon source, mixture of soy peptone and yeast extract for nitrogen sources and temperature of 45 °C were found to be optimal for maximum cell density. Thus, it has been concluded that introducing agitation in MK-7 production might address the problem, which are mostly mass and heat transfer issues in nature, surrounding static fermentation for industrial applications (Berenjian et al. 2015). It is also demonstrated that the dynamic fermentation involving high stirring and aeration rates enhances the fermentation yield significantly as compared to the static system (Berenjian et al. 2014). Table 1 summarizes LSF and SSF menaquinone fermentations with different strains.

Extraction and recovery

MK-7, like all other menaquinones, is fat soluble and insoluble in water. Since it is extracellular in *Bacillus* species and bound to a protein, it needs to be extracted from the fermentation broth (Schurgers and Vermeer 2001). Liquid–liquid extraction is commonly used for extracting fat-soluble metabolites. Fermentation media containing vitamin is contacted with an immiscible or semi miscible solvent. The solubility of a vitamin compound in a solvent, the selectivity of solvent toward solute, and the dielectric constant of solvent has significant impacts on the extraction efficiency (Perry 2007). By the use of an organic mixed solvent composed of 2-propanol and n-hexane, MK-7 can be extracted from fermentation broth robustly (Berenjian et al. 2011). Different compositions have been evaluated and mixture of 1:2 2-propanol and n-hexane seemed to be most commonly used (Table 2). Sato et al. (2001) and Berenjian et al. (2011) have added 1:2 (v/v) propanol:n-hexane mixture to fermentation broth containing vitamin K. After vigorously shaking the mixture and settling down, the organic layer has been separated and evaporated; leaving vitamin K solid residues. Then, Sato et al. (2001) has extracted the residues with n-hexane and after centrifugation, the resultant solution has been washed through a silica gel column using 1:2 (v/v) toluene:hexane mixture. Finally, the fractions have been analyzed by HPLC (Sato et al. 2001). Berenjian et al. (2011), however, has dissolved the residues after evaporation step in methanol and analyzed the resultant solution by HPLC with methanol as the mobile phase (Berenjian et al. 2011). On the other hand, Tsukamoto et al. (2001) has first added 2-propanol to vitamin K samples and after 15 min of shaking, has added hexane and mixed again. Then the mixture has been dried and dissolved in 2-propanol and finally analyzed by HPLC.

After extraction, organic solvents can be separated from the aqueous fermentation media and evaporated under vacuum to recover the extracted MK-7. On the other hand, Berenjian et al. (2014) have investigated the use of

Table 1 LSF and SSF menaquinone fermentations

Strain type	State of fermentation	Menaquinone type	Maximum MK-7 concentration	References
Lactic acid bacteria	LSF	MK-7, MK-8, MK-9 and MK-10	12.3 mg/L	Morishita et al. (1999)
Flavobacterium	LSF	MK-4 and MK-6	–	Tani and Taguchi (1989)
Bacillus subtilis licheniformis	LSF	MK-7	0.25 μg/mg dry weight of cell	Goodman et al. (1976)
Bacillus subtilis natto	LSF	MK-7	62.3 mg/L	Berenjian et al. (2011)
Bacillus subtilis natto	LSF	MK-4, MK-5, MK-6, MK-7 and MK-8	45.1 mg/L	Sato et al. (2001)
Bacillus subtilis natto	LSF	MK-7	29.8 mg/L	Sumi (2004)
Bacillus subtilis natto	LSF	MK-7	50 mg/L	Benedetti et al. (2009)
Bacillus subtilis amyloliquifaciens	SSF	MK-4 and MK-7	7.5 μg/g	Wu and Ahn (2011)
Bacillus subtilis natto	SSF	MK-7	1719 μg/100 g natto	Tsukamoto et al. (2001)
Bacillus subtilis natto	SSF	MK-7	30 μg/g dry	Takenaka et al. (2002)
Bacillus subtilis natto	SSF	MK-7	8 μg/g	Wu and Chou (2009)
Bacillus subtilis natto	SSF	MK-7	67 mg/kg	Mahanama et al. (2011)
Bacillus subtilis natto	SSF	MK-7	39 μg/g	Singh et al. (2015)

Table 2 Aqueous: organic and 2-propanol: n-hexane ratios for MK-7 extraction

Aqueous: organic (v:v)	Propanol: hexane (v:v)	References
1: 2	1: 1	Morikawa et al. (2011)
1: 6.5	1.5: 5	Sumi (2004)
1: 4	1: 2	Sato et al. (2001), Berenjian et al. (2011), Schurgers and Vermeer (2001)
5: 11	5: 6	Tsukamoto et al. (2001)

vegetable oil (Long chain triglyceride) for extracting MK-7. Although, the oil has originally been added as an anti-foam agent, Berenjian et al. observed that at the end of the fermentation nearly 80% of the produced MK-7 was recovered in oil phase and the rest remained in the fermentation media. This tendency can help to develop an in situ recovery system for MK-7 fermentation. The amount of oil used in the fermentation broth was significant (over 16%) which may indeed interfere with media composition, microbial metabolism and fermentation dynamics. Therefore, this method may not be as robust as organic solvents. Yet, the use of vegetable oil eliminates the need of organic solvents, which helps to create a more environment friendly process. It seems that more thorough studies must be carried out to investigate the robustness of the mentioned liquid–liquid extraction compositions and also comparisons between different techniques in MK-7 recovery yields and purity.

Future studies

Apart from the extraordinary achievements in designing and developing new bioprocessing technologies for MK-7 production (Ebrahiminezhad et al. 2016; Puri et al. 2015), still MK-7 is not readily available at an affordable price for the industrial applications. This mainly is due to the low fermentation yields and the presence of several tedious downstream unit operations. Therefore, there is a need for novel and innovative technologies to overcome the existing challenges.

B. subtilis natto has a high tendency to form pellicles during fermentation, which is operationally problematic and undesirable during fermentation, but it can be effective on enhancing the MK-7 production (Berenjian et al. 2013). Thus, it seems that there is an opportunity here to simultaneously address such undesirability and improve the MK-7 production. Therefore, *B. subtilis natto* can be utilized in

biofilm reactors to improve MK-7 production as the future study.

Biofilm formations are usually undesirable contamination sources, since microorganisms are significantly able to tolerate the harsh conditions in mature biofilm form compared to planktonic one (Xu et al. 2011). Such extraordinary characteristics can be utilized in a bioreactor by allowing microorganisms to form biofilms under controlled conditions, which turns it into a "biofilm reactor". This can be achieved through microbial cells attaching to the support structure without the use of chemicals and thus forming biofilm. Biofilm reactors have been used in the biotechnology industry to improve the productivity and stability of the process (Ercan and Demirci 2013a). They have been studied to increase the effectiveness of waste treatment, production of alcohol, enzyme (Khiyami et al. 2006), organic acid, antibiotics (Pongtharangkul and Demirci 2006; Ercan and Demirci 2013b), biopolymers (Cheng et al. 2010), starter cultures and many other value-added products.

Conclusions

In the past several decades, many researches have attempted to enhance the yield of MK-7 production in both liquid and solid state fermentations by various species of bacteria. Among these species, however, *B. subtilis natto* seems to be the most promising microorganism. Studies on media optimization, fed-batch fermentation, in situ extraction protocols in LSF in conjunction with implementing dynamic and static bioreactors have reported significant improvements in MK-7 production to date. However, there is always a need to enhance the vitamin production further to make it more economical for commercial applications. Therefore, biofilm reactors may be able to open a new angle for the future MK-7 fermentation studies in order to further improve the productions.

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