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REVIEW ARTICLE

Vitamin K series: current status and future prospects

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Abstract

The Vitamin K series, particularly menaquinone, have been attracting research attention, due to the potential in reducing both osteoporosis and cardiovascular diseases. This review provides an overview of the types of vitamin K and their health benefits. This is followed by a critical review of the various biotechnological approaches used in the production of menaquinone, including solid and liquid state fermentations, extraction and recovery. The currently available market information is summarized and future growth prospects are discussed. Recommendations are also given for areas of future research in order to improve the production process for menaquinone and reduce costs.

Keywords

Biotechnology, extraction, fermentation, menaquinone, phylloquinone, Vitamin K

History

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Introduction

In 1935, Dam discovered an anti-hemorrhagic factor, with similar physical properties to vitamin E, but with a separate physiological clotting function from any known vitamin. He suggested the name vitamin K, based on the German and Scandinavian spelling of ''Koagulations'' (Dam, 1935). The discovery of extra hepatic vitamin K-dependent proteins has suggested an expanded physiologic function for the vitamin K, besides its known role in blood coagulation (Ferland, 1998, 2012). Most recently, epidemiological and intervention studies focused on the translational effect of vitamin K, phylloquinone and the menaquinones, with respect to cardiovascular and bone health (Ferland, 2012; Knapen et al., 2013). Several studies, primarily by researchers in Japan and the Netherlands, have shown that vitamin K is an essential nutrient for prevention of calcification in arteries and other soft tissues and also reducing the risk of bone fractures and bone loss by carrying calcium transport throughout the body (Gast et al., 2009; Kaneki et al., 1995; Schurgers et al., 2007; Tamatani et al., 1998; Tsukamoto et al., 2000a; Yamaguchi et al., 1999).

There is strong epidemiological evidence for the effects of vitamin Ks on prevention of osteoporosis and cardiovascular diseases (CVDs) (Shea & Holden, 2012; Vermeer, 2012). However, due to conflicting data from clinical trials, there is still debate on the therapeutic dosage and daily supplementation of these vitamins for prevention of these diseases. It is possible that daily consumption of vitamin Ks substantially reduces the risk of osteoporosis and CVDs (WHO, 2011). Subsequently, it can reduce the healthcare costs for their treatment and management. In the United States alone, the annual cost of treatment for osteoporotic fractures and CVD was estimated to be $22 billion (Blume & Curtis, 2011) and $502 billion (Lloyd-Jones et al., 2010), respectively. The objective of this review was to provide an insight for the current status of vitamin K production, its emerging pharmaceutical significance and the techniques that have been developed for enhancing its production. The potential of available technologies for the production of this compound(s) was also assessed to produce low cost supplementary food products. Sources, recommended intake, extraction and chromatography techniques for vitamin K have been thoroughly discussed in the supplementary material section.

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Types of vitamin K

Vitamin K occurs naturally in two forms known as vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone) (Shearer & Newman, 2008) as shown in Figure 1. All vitamin Ks are fat soluble compounds, which have a common 2-methyl-1,4-napthoquinone nucleus but differ in the structure of a side chain at the 3-position.

Dam found that hemp seed oil and hog liver fat were both rich in vitamin K. In fact, they contained different vitamers with the hemp seed oil containing phylloquinone and the hog liver fat containing menaquinone (specifically MK-4) (Dam, 1966; Dam et al., 1939).

Phylloquinone is present in the green leafy parts of plants where it functions as an electron receptor during photosynthesis. The pure extracted form of phylloquinone is a viscous yellowish oil (Binkley et al., 1978). Whilst phylloquinone is a single compound, menaquinone is a series of vitamers with multi isoprene units at the 3-position of the naphthoquinone ring structure (Brodie & Ballantine, 1960a,b). Menaquinone has 4–13 repeating isoprene units indicated by MK-4 to MK-13.

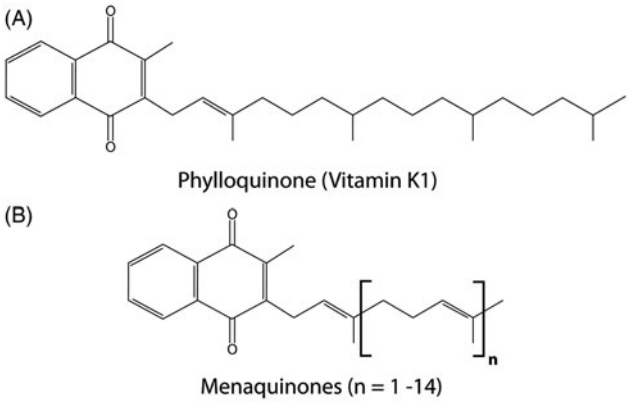

Figure 1. Molecular structure of phylloquinone (A) and menaquinone (B).

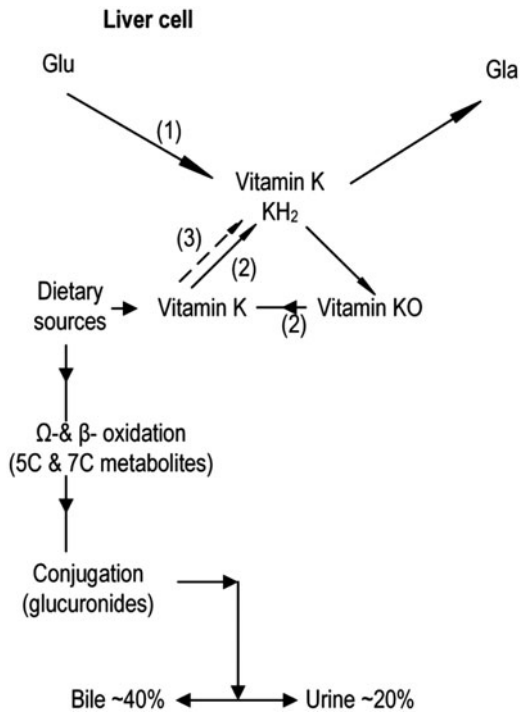

Figure 2. Schematic diagram of hepatic metabolism of vitamin K. Vitamin K functions as a cofactor in the glutamic acid (Glu) to g-carboxyglutamic acid (Gla). Vitamin K recovers from its epoxide (KO) for reuse in carboxylation. The active form of vitamin K needed by the g-glutamyl carboxylase is the reduced-form vitamin K quinol (KH2). The dose is excreted via bile and urine.

The menaquinones are produced by both Gram-positive and Gram-negative bacteria such as *Bacillus subtilis* and *Escherichia coli* to serve as an electron carrier in the electron transport chain required for respiration (Bentley & Meganathan 1982; Brodie & Ballantine 1960a,b; Meganathan 2001; Morishita et al., 1999; Tsukamoto et al., 2001). Although, menaquinone compounds are generally produced by bacteria, Skattebol & Aukrust (2010) have produced synthetic MK-7 by a chemical process that involves several stages.

Health benefits of vitamin K

Vitamin K has long been known for its key role in coagulation and more recently has been recognized for its potentially important role in maintaining bone strength and in the prevention of soft tissue calcification (Vermeer, 2012). It is generally accepted that naphthoquinone is the main functional group of vitamin K, although some researchers have suggested that the side chain may also play a role (Li et al., 2003). It is, therefore, expected that all vitamin Ks exhibit similar properties. Characteristics of the side chain, however, have shown significant impact on lipophilicity of a vitamer and may also result in substantial differences in properties such as intestinal absorption, transport, tissue distribution and bioavailability (Skattebol & Aukrust, 2010).

Inside the body, vitamin K functions as a cofactor in the γ-glutamyl carboxylation of certain glutamic acid (Gla) residues of vitamin K-dependent proteins for their activation as shown in Figure 2 (Booth, 2009). This mechanism can be achieved by all types of vitamin K though with varying affinities. The carboxylation reaction is essential for calcium binding ability of vitamin K-dependant proteins. K-dependent protein carboxylation has been used for the assessment of vitamin K nutritional status (Geleijnse et al., 2004). The hepatic vitamin K-dependent proteins involved in coagulation are factors II (prothrombin), VII, IX and X, and proteins C, S and Z, all of which need vitamin K for physiological activation. Other K-dependent proteins have also been identified in extrahepatic tissues namely osteocalcin and matrix γ-carboxyglutamic acid proteins (Truong & Booth, 2011).

Vitamin K, mainly in phylloquinone form, after solubilization into mixed micelles is absorbed from the proximal intestine (80% absorption) (Shearer & Newman, 2008). The vitamin is then incorporated into chylomicrons and enters the blood (Shearer & Newman, 2008). Although phylloquinone is the main circulating form of vitamin K, menaquinone also has lipoprotein distribution similar to phylloquinone, which makes it available in plasma. The human liver stores about 10% phylloquinone and 90% menaquinone (Usui et al., 1990). Phylloquinone liver stores are extremely labile, which results in a 25% reduction of their initial levels through excretion within 3 days (Usui et al., 1990). Bioassay data suggested that menaquinones as compared to phylloquinone were more efficient in reversing vitamin K deficiency, which indeed is due to their longer duration of the biological response and slower hepatic turnover (Shearer, 1992; Suttie, 1995). It should be noted that the bioavailability of phylloquinone from green leafy vegetables is lower than free form of phylloquinone. This poor absorption can be explained by its tight association with the thylakoid membrane and location in chloroplasts. In comparison, the bioavailability of menaquinone (MK-4) was more than two-fold higher than that of phylloquinone (Shearer, 1992; Suttie, 1995).

Both phylloquinone and menaquinone may play an important role in osteoporosis and CVDs (pathogenesis and progression). However, each of these compounds had distinct activity due to differences in their bioavailability and biological half lives. The current knowledge on the effect of vitamin K on the prevention of osteoporosis and CVDs are summarized in the following sections.

Vitamin K and osteoporosis

Osteoporosis is a skeletal disorder characterized by reduced bone density and weakness of bones, which increase the risk

of fractures most likely in hip, wrist and vertebrae (Christodoulou & Cooper, 2003). To date, several studies have been conducted to investigate the effect of phylloquinone, menaquinone and their combination on bone health and the risk of fractures.

Correlations between phylloquinone deficiencies and the risk of fractures have been reported extensively. Feskanich et al. (1999) used a food-frequency questionnaire test for their epidemiological study and collected information from 72,327 women with ages between 38 and 63 years. The results of this survey shows that low intake of phylloquinone (<109 μg/day) increases the risk of hip fractures in women. In a human trial conducted by Braam et al. (2003), 1 mg/day of phylloquinone was added to the diet of a group of people for a period of 3 years. It was observed that this diet reduces the risk of bone loss of the femoral neck. Booth et al. (2008) also observed that low phylloquinone intake (<56 μg/day) increases the incidence of hip fractures among 335 men and 553 women participants in their epidemiological study with an average age of 75.2 years. Cheung et al. (2008) reported that 5 mg/day of phylloquinone supplementation for 2–4 years may protect against fractures in postmenopausal women with osteopenia ($p < 0.04$), but does not change an age-related decline in bone mineral density (BMD). Consumption of 500 μg/day phylloquinone for 3 years among 474 elderly US men and women resulted in significantly lowering undercarboxylated osteocalcin (ucOC) levels. No effect was, however, reported on BMD in the treatment group (Booth et al., 2008).

Apart from phylloquinone, several studies have been attempted to evaluate the effect of menaquinone on osteoporosis. An early epidemiological study by Tamatani et al. (1998) showed that the amount of bone loss is higher when there is less MK-7 in the body among elderly men. In another case, Tsukamoto et al. (2000a) suggested that the intake of dietary MK-7 plays an important role in bone formation in normal individuals. In a human trial, a daily consumption of 45 mg MK-4 showed a clear increase in the OC serum level and preventing the occurrence of osteoporotic fractures (mainly vertebral fractures) (Shiraki et al., 2000). The results of this study show a positive effect of menaquinone treatment in preventing new fractures by enhancing γ-carboxylation of osteocalcin molecule (Shiraki et al., 2000). In another study, Vermeer & Braam (2001) reported that menaquinone is important for normal bone development and may contribute to a decrease in postmenopausal bone loss. Moreover, in two 48-week studies on menopausal Japanese women supplemented with 45 mg/day MK-4, ucOC levels showed a decrease without any effect on lumbar BMD (Ozuru et al., 2002). Rapid conversion of ucOC to carboxylated osteocalcin following vitamin K_2 treatment of 45 mg/day was confirmed in elderly osteoporotic women with vertebral fractures (Miki et al., 2003). In yet another clinical study by Iwamoto et al. (2006), it is demonstrated that daily intake of menaquinone reduces osteocalcin (OC) serum levels and improves bone strength indices in the femoral neck and reduces the incidence of clinical fractures. Emaus et al. (2010) conducted a placebo controlled trial with a dose of 360 μg/day of MK-7 for 1 year and found no difference in BMD status but a rise in OC and a decrease in ucOC in the treatment group. The number of fractures was five in each group; and it has been suggested that whilst the dose was high, the trial duration was not sufficient to observe any beneficial effects of MK-7 (Emaus et al., 2010). More recently, the results of a 3-year placebo or MK-7 (180 μg MK-7/day) supplementation of healthy postmenopausal women ($n = 244$) showed that bone strength was favorably affected by MK-7 (Knapen et al., 2013).

Reviewing the available literature shows that vitamin K status has mainly been correlated to BMD and OC serum levels as markers of bone health. However, the effect of vitamin K on the skeleton mediates by mechanisms other than BMD and as a result the correlation between BMD and fracture rates may not be reliable, while OC serum levels proved to be a better indicator of bone health.

Number of studies also attempted to compare the effect of phylloquinone and menaquinone supplementation on osteoporosis. The level of vitamin K was measured in the plasma of elderly Japanese women, 24 with and 36 without osteoporotic spinal fractures. In this epidemiological study, significant differences were observed between the levels of MK-7 in these two groups, while the levels of phylloquinone were nearly similar in both groups (Kaneki et al., 1995). In a review conducted by Cockayne et al. (2006), it has been reported that both bone loss and fractures were significantly lower for Japanese patients who used menaquinone compared to the group who had phylloquinone. Though Gundberg et al's (2012) review found that clinical trials did not provide overall support to vitamin K supplementation reducing bone loss risk in the general population.

In summary, bone fractures are significantly reduced when menaquinone was added in a daily dose compared to groups that taken phylloquinone. This effect was due to the fact that the half life of MK-7 is 68 hrs, while the short half life of phylloquinone leads to an 86% drop in concentration within 4 hrs (Schurgers et al., 2007). Therefore, the high half life of MK-7 results in more complete and effective γ-carboxylation of OC and significant improvement on bone fractures (Schurgers et al., 2007).

Vitamin K and CVD

CVD includes disorders of the heart and blood vessels and is one of the major causes of death worldwide (Dahlöf 2010; Sennerby et al., 2009). Research suggests that osteoporosis and some forms of CVD have common etiological mechanisms (Jie et al., 1996; McFarlane et al., 2004; Tankó et al., 2005).

Jie et al. (1996) in their epidemiological study has shown that low bone mass in atherosclerotic women was correlated with the level of vitamin K (defined by OC status) in a group of 113 postmenopausal women. This finding supports the hypothesis that vitamin K affects the mineralization processes in both bone and atherosclerotic plaques. A preventive role for vitamin K in CVD has been proposed based on its role as an activating matrix Gla protein, a calcification inhibitor that is expressed in vascular tissue (Shea & Holden, 2012). Vermeer & Braam (2001) reported that vascular vitamin K-dependent proteins appear to be potent inhibitors of arterial calcification. Beulens et al. (2009) in their epidemiological study compared the dietary intake of vitamins K_1 and K_2 on calcification of the coronary arteries. They investigated the association of

intake of phylloquinone and menaquinone, MK-4 to MK-10, among 564 post-menopausal women with coronary calcification. This study shows that high dietary menaquinone intake is significantly associated with reduction of coronary calcification as compared to phylloquinone (Beulens et al., 2009). These data were in agreement with the epidemiological study of Gast et al. (2009) with 16 057 women aged between 49 and 70 years. In another example, Geleijnse et al. (2004) reported similar results while examining 4807 subjects with dietary data. Shea & Holden's (2012) study came to a conclusion that menaquinone is more effective than phylloquinone in protecting against arterial calcification. Gast et al. (2009) concluded that MK-7, more specifically, is involved in coronary calcification reduction and could prevent the risk of CVDs. MK-7 is suggested to remove calcium deposits from arteries, increase deposition in bones (Howard & Payne, 2006) and could be more effective in carboxylation of Gla-protein, which is a major inhibitor of CVD (Gast et al., 2009; Tsukamoto et al., 2000b; Yamaguchi et al., 1999).

The review of the available epidemiological literature suggests that intake of menaquinone can more effectively prevent against coronary heart disease as compared to phylloquinone. Higher dietary menaquinone is associated with less calcification as compared to phylloquinone. It is likely that vitamin K contributes to reducing vascular calcification in certain patient populations, especially in those with chronic kidney disease (Shea & Holden, 2012). The results of large scale randomized controlled trials are required to determine whether supplementation is beneficial and to determine the dose. An overview for the outcomes of studies conducted using fermentation technology to produce menaquinone is given in the next section.

Menaquinone production

Liquid and solid state fermentation (LSF and SSF) processes have both been used for the production of menaquinone. The major difference between these two bioprocesses is the amount of free liquid in the substrate during the fermentation. Nutrient broth in a typical liquid fermentation might contain around 50–100 g/L of solutes, and therefore has a water content of 90–95% (Mitchell et al., 2000). In contrast, the water content of substrate in solid phase fermentation could conceivably range from ~70% as low as 12% (w/w) (Mitchell et al., 2000). However, there are circumstances where higher solute concentrations are used in LSF, which can be regarded as semi-solid fermentation systems, and the boundary between LSF and SSF is less clear. Generally, bacterial strains in SSF systems require higher moisture contents (>50%) than fungal strains (Mitchell et al., 2000). The production of menaquinone *via* fermentation processes are critically reviewed in the following sections.

Liquid state production of menaquinone

The *Bacillus* species, due to a long history of safe use, continues to be one of the dominant bacteria in liquid state microbial fermentations (Sonenshein et al., 2002). The ability of different *Bacillus* species to ferment in wide pH ranges, combined with the presence of thermophiles in the genus (Schallmey et al., 2004), and capacity to produce and secrete large quantities of menaquinone has placed them among the most important industrial vitamin K producers. Studies that reported using LSF techniques for menaquinone production are summarized in Table 1. To date, the highest level of MKs in general has been reported to be produced by *Bacillus subtilis natto* compared to

Table 1. List of studies that used LSF for the production of menaquinone.

Bacteria type	MK type and concentration (mg/L)	Shaking speed	Media composition	Temp (°C)	Time (d)	References
Flavobacterium sp. 238-7-K3-15	1 MK-4 (155 mg/L); 2 MK-6 (27 mg/L)	120 rpm	Glycerol 60 g/L; peptone 23 g/L; yeast extract 3 g/L; K_2HPO_4 7 g/L; NaCl 0.8 g/L; $MgSO_4.7H_2O$ 5 g/L; Rikanon UA 5012 0.1 g/L; cedar wood oil 0.1 g/L	28	7	(Tani & Taguchi, 1989)
Lactic acid bacteria	MK-7; MK-8; MK-9; MK-10; (0.123 mg/L)	–	Soymilk	30	2	(Morishita et al., 1999)
Bacillus subtilis natto B.S.D200-41	MK-4 (0.6 mg/L); MK-5 (0.12 mg/L); MK-6 (0.43 mg/L); MK-7 (60.1 mg/L); MK-8 (1.2 mg/L)	120 rpm for one day followed by five days static	Soybean 100 g/L; Glycerol 50 g/L; yeast extract 5 g/L; K_2HPO_4 0.5 g/L	37 (shaking) and 45 (static)	6	(Sato et al., 2001a)
Bacillus subtilis natto Miyagino	MK-7 (1.4 mg/L)	100 rpm	Polypeptone 2 g/L; glycerin 3 g/L	37	2	(Sumi, 2004)
Bacillus subtilis natto GN13/72	MK-7 (50 mg/L)	100 rpm	Soypeptone 12 g/L; yeast extract 0.5 g/L; dextrin 60 g/L; K_2HPO_4 0.05 g/L; NaCl 5 g/L	37	6	(Benedetti et al., 2009)
Bacillus subtilis natto	MK-7 (62.3 mg/L)	Static	Soypeptone 189 g/L; yeast extract 50 g/L; glycerol 50 g/L; K_2HPO_4 0.6 g/L	40	6	(Berenjian et al., 2011)

other bacteria. *Bacillus subtilis natto* showed the ability to produce a range of menaquinone homologues (MK-4, MK-5, MK-6, MK-7 and MK-8) with the major component being MK-7 (96%) (Sato et al., 2001b). Fermentation processes, nutrients and operating conditions are factors that have significant impacts on menaquinone production. The effect of fermentation conditions for the production of menaquinone will be discussed in following section.

Selection of fermentation temperature and nutrients

The review of available literature shows that the range of 28–45 °C has been frequently used for the production of menaquinone, as shown in Table 1. However, several studies show that there is not necessarily a correlation between increasing the *Bacillus subtilis* growth rate and menaquinone biosynthesis and that the maximum rates for both occur at different temperatures (Berenjian et al., 2011, 2013; Sato et al., 2001a). Most recently, it has been reported that 40 °C appears to be the optimum temperature for high menaquinone production (Berenjian et al., 2011, 2013).

Menaquinone production requires both carbohydrates and nitrogen sources (Seto et al., 2008). As shown in Table 1, glucose, dextrin and glycerol have been used as the main carbon sources, while soy peptone and yeast extract are among the frequently used nitrogen sources for menaquinone production.

It has been reported that soy peptone, yeast extract and glycerol have significant effect on enhancing menaquinone production (Berenjian et al., 2011, 2013; Sato et al., 2001a). The production of menaquinone is significantly increased with the use of glycerol in fermentation media of *Flavobacterium* and *Bacillus subtilis* (Berenjian et al., 2011, 2012; Sato et al., 2001a; Tani et al., 1984). Another study shows that the addition of cedar wood oil into fermentation media increases the productivity of MK-4 by a strain of *Flavobacterium* (Tani & Taguchi, 1989).

Lactic acid bacteria can also be a source of vitamin K in various fermented products. Successful production of menaquinone in reconstituted non-fat dry milk and a soymilk medium has been reported. The forms of menaquinone in the soymilk media ranged from MK-7 to MK-10. Therefore, it has been suggested that fermented products of lactic acid bacteria can be considered as a contributor to human health diet (Morishita et al., 1999).

More recently, a fed-batch process has been used for the incremental addition of glycerol to the fermentation of *Bacillus subtilis natto* to produce menaquinone, more specifically MK-7 (Berenjian et al., 2012). Our study demonstrates that addition of 2% (w/v) glycerol results in 86 mg/mL MK-7 biosynthesis, which is significantly higher than previously reported data in Table 1. The fed-batch addition of glycerol, therefore, resulted in a 40% increase of MK-7 production compared to the batch fermentation (Berenjian et al., 2012). This study also shows that the optimum conditions are different for achieving high concentrations of MK-7 and approaching maximum cell density.

Static fermentation vs agitation

Cells present a specific genetic expression and morphologies within biofilms, resulting in the production of different molecules and secondary metabolites as compared to their planktonic cells (Branda et al., 2005; Kuchma & O'Toole 2000). To date, studies on fermentation mode to increase the production of menaquinone are very limited. It has been reported that menaquinone production by *Bacillus subtilis* is associated with the early stage of sporulation. After completing spore formation, menaquinone was no longer produced (Farrand & Taber, 1974). Therefore, it was considered that the slow progress of spore formation in *Bacillus subtilis* within the biofilm in the static culture resulted in enhanced menaquinone production. For this reason, most of the studies on the production of menaquinone used static or relatively low stirring speeds to avoid the elimination of biofilm and consequently increase the production of menaquinone (Table 1). Production of relatively high yields of menaquinone in static conditions during 6 days fermentation has been reported by several researchers (Berenjian et al., 2011; Sato et al., 2001a). Sato et al. reported a total 60 mg/L MKs composed of MK-6 to MK-8 within the fermentation media.

We recently demonstrated that high shear force in a stirred fermentor reduces the biofilm formation and can dramatically increase the MK-7 production; this is a break-through in the fermentation of microorganisms that involve biofilm formation (Berenjian et al., 2013, Dehghani et al., 2013). Therefore, static fermentation is not crucial for menaquinone production by *Bacillus subtilis natto*. Agitated fermentation and elimination of biofilm production address the mass and heat transfer issues that might arise in large scale fermentation process for menaquinone production. In addition, we demonstrate that high speed stirring and aeration can significantly increase the growth rate of bacteria and the production of MK-7. For instance, it is viable to produce 226 mg/L MK-7 at 1000 rpm, 5 vvm, 40 °C after 5 days of fermentation (3-L fermentor).

Solid state production of menaquinone

Natto has been produced traditionally *via* SSF for centuries. Hence it is not surprising that during the past decade, researchers have used SSF techniques for menaquinone production, predominantly using *Bacillus subtilis*, more specifically *Bacillus subtilis natto* (Table 2). In these fermentations, commonly legumes, cereals and simple carbohydrates are used as the nutrients to produce MK-7 varying from 0.53 to 140 mg/kg. To date, the highest reported concentration of 140 mg/kg was achieved using *Bacillus subtilis natto* on an equal mixture of corn and soy as the substrate (initial moisture of 70% (w/w) at 37 °C) (Mahanama et al., 2012a). Some studies measured MK-4 (Chen et al., 2012; Wu & Ahn, 2011a) and also total MKs (MK-7 and MK-4) in SSF using *Bacillus spp*. Wu et al. (2011) reported a total MKs yield of 7.54 mg/kg in static fermentation of soybeans using *Bacillus amyloliquefaciens* at 40 °C in 2 days. MK-4 yield of 0.79 mg/kg were obtained using *Bacillus amyloliquefaciens* in similar fermentation conditions supplemented with 2% glycerol (Chen et al., 2012; Wu & Ahn, 2011b), again showing MK-7 comprises >90% of total MKs production in *Bacillus* species.

Physicochemical and biochemical parameters such as particle size, initial substrate moisture content, pH, pretreatment of the substrate, relative humidity, incubation

temperature, size of the inoculum, supplementation of trace elements and additional nutrients are factors that are considered in a SSF. However, for production of menaquinone by SSF fermentation, only a few of these factors are contemplated that are discussed in detail in this section.

Temperature and water activity

The temperature range considered for the production of MK-7 from fermentation of *Bacillus* species in SSF is between 37 °C and 45 °C (Table 2). Optimum incubation temperature for MK-7 production is similar to LSF; however, other factors such as bacterial strain, the water content of media, and bed thickness can significantly affect the optimum temperature in a SSF. Therefore, care must be taken when comparing data in Tables 1 and 2 to determine the impact of temperature. The temperature control in a SSF system is a challenge due to the metabolic heat generation and poor heat removal. This issue is more paramount in a large scale SSF, in using evaporative cooling to remove metabolite heat in the packed beds (Weber et al., 1999, 2002), which is commonly used as the most effective cooling system. In our study, a tray type SSF was used for the fermentation of *Bacillus Subtilis* on 1:1 weight ratio of corn:soy substrate. In this system, increasing the temperature from 35 °C to 45 °C reduced the yield of MK-7 production by 40% (Mahanama et al., 2012a).

The moisture level in SSF media has an impact on the physical properties of solid matrix. The low moisture content causes a reduction in the solubility of nutrients, low degree of substrate swelling and results in osmotic stress on bacteria due to dehydration. It is, therefore, critical to control the moisture content in the solid phase and humidity in the incubator to adjust the water activity during the fermentation period (Zadražil & Brunnert, 1981). The minimum level of water content is a function of microbial strain that grows on SSF. Fungi can grow at 50% moisture content; typically, higher moisture is required for the metabolism of bacteria (Pandey 1992). We investigated the effect of water content on MK-7 yield (Mahanama et al., 2011a) and found higher initial moisture content (70%) favors static mode fermentation (Mahanama et al., 2011a). In dynamic fermentation, moderately high moisture content (60–70%) decreases the MK-7 yield as limited biomass production due to particle agglomeration occurs when increasing water content (Mahanama et al., 2011b). The majority of studies have used wet substrates moisturized to the maximum water holding capacity as the initial moisture content. However, a few studies have measured the moisture content or humidity over time to assess the impact of moisture content on MK-7 production (Mahanama et al., 2011b). The results of our simulation study indicate that MK-7 cannot be produced at less than 50% moisture in the solid phase (Mahanama et al., 2012a). Similarly, these simulations predicted that a gradual drop of moisture content within the range of 10–20% dramatically decreased MK-7 production up to 80%, which are consistent with experiments.

Substrate and pre-treatment

Factors such as cost, stickiness and pre-treatment methods are considered in substrate selection as they have an impact on SSF process efficiency. Substrate physical characteristics, such as particle size, may affect mass and heat transfer during the SSF process. Our study shows that maximum MK-7 concentrations are higher in substrates comprising of a broader range of particle size distribution (Mahanama et al., 2011b). Size of soy granules and milled wheat was distributed from 150 μm to 2 mm and from 150 μm to 0.5 mm, respectively. After 3 days static fermentation using *Bacillus subtilis natto* at 37 °C (RH 85%, 3 g and 3 days) 28 mg/kg and 20 mg/kg of MK-7 were produced using soy and wheat, respectively. However, in dynamic fermentation (30 rpm) of soy granules and milled wheat at the same processing conditions, we observed 10-fold and 87-fold reductions in yield of MK-7,

Table 2. Solid state studies for the production of menaquinone ordered by date of publication.

Bacteria type	MK type and concentration (mg/L)	Shaking speed	Media composition	Temp (°C)	Time (d)	References
Bacillus subtilis OUV23481,	MK-7 17.2	Static	Soy beans	37	0.75	(Tsukamoto et al., 2001)
Bacillus subtilis natto	MK-7	Static	Okara	40	–	(Takenaka et al., 2002)
Bacillus natto BN-1	MK-7 68	Static	Raw wheat Urea	37	1	(Sumi et al., 2005)
Chinese natto strain (Unnan SL-001)	MK-7 36.6	Static	Okara	37	4	(Survase et al., 2006)
Naruse,	MK-7 14.2					
Asahi	MK-7 11.9					
Takahashi,	MK-7 6.8					
Miyagino	MK-7 1.9					
Meguro (natto bacilli for medicine)	MK-7 1.9					
B. subtilis BCRC 14715	MK-7 8	–	Black soy beans	45	0.75	(Wu & Chou, 2009)
Bacillus subtilis natto	MK-7 67 ± 0.2	Static	Soy:corn 1:1	40	4	(Mahanama et al., 2011a)
Bacillus subtilis natto	MK-7 106.4 ± 2.1	Static	Soy granules	37	8	(Mahanama et al., 2012b)
Bacillus subtilis natto	MK-7 28.3 ± 0.1	Static	Soy granules	37	3	(Mahanama et al., 2011b)
	MK-7 27.8 ± 0.1	Dynamic	Corn			
	MK-7 5.3 ± 0.6		Soy granules			
Bacillus amyloliquefaciens	MK-7 11.7 ± 0.6	Static	Soybean	40	2	(Wu & Ahn, 2011a)
KCTC11712BP	MK-4 0.8		glycerol 4%			
Bacillus amyloliquefaciens	MK-4 0.7 ± 0.1	Static	Soy beans	43	1.5	(Wu & Ahn, 2011a)
	MK-7 7.5 ± 0.7		4% glycerol			
Bacillus amyloliquefaciens KCTC11712BP	MK-4 0.8	Static	Soy beans	43	2	(Chen et al., 2012)
	MK-7 11.7 ± 0.6		2% glycerol			
Bacillus subtilis natto	MK-7 140	Static	Corn:soy	37	7	(Mahanama et al., 2012a)

respectively. This adverse effect of higher moisture levels and descending particle sizes was due to intense particle agglomeration during dynamic fermentation of MK-7.

In fermentation processes, particularly SSF that has lower water content than LSF, it is critical to have a balance on nutrient concentration and water activity to achieve high yield. For example, Wu & Ahn (2011a) studied the effects of glycerol concentration on menaquinone production. It was found that when the glycerol content was increased from 2% to 4% (w/w), menaquinone production was increased 1.5-fold after 36 hrs of fermentation. However, when the concentration of glycerol was increased to 6% (w/w), the menaquinone production was decreased due to the adverse effect on water activity and high osmotic pressure on cell membrane (Wu & Ahn, 2011a).

Raw (unprocessed) substrates are commonly used in SSF. However, simultaneous substrate pre-treatment and fermentation has been used to increase the yield of vitamin production and to reduce fermentation time (Mahanama et al., 2011a). The yield of MK-7 from fermentation of *Bacillus subtilis natto* approaches 67 mg/kg after 4 days (35 °C, 70% initial moisture) when using an equal mixture of corn and soy that are treated with an amylase loading of 10 μL/g. This effect is due to enzymatic pre-treatment by α-amylase that increases the availability of sugar monomers at the first stage of fermentation (Mahanama et al., 2011a). In another study, Tsukamoto et al. (2001) showed that amino acids including phenylalanine, tyrosine and tryptophan participate in the feedback inhibition upstream of the menaquinone biosynthetic pathway in LSF. Subsequently, Wu & Ahn (2011a) also demonstrated that in SSF, tryptophan is the most efficient amino acid in feedback inhibition, where MK-7 yield is decreased from 6.39 to 1.63 mg/kg at a concentration of 0.005% w/w. It has been suggested that a longer fermentation time may lead to a great accumulation of free amino acids, which strongly inhibits the production of MK-7 (Wu & Ahn, 2011b).

Reactor type

In SSF, the selection of reactor type mainly depends on the mass of fermentation media. Commonly, tray type fermentors that operate in a static mode are used for the menaquinone production. Dynamic mode fermentors, such as rotating drum, are also suggested for the SSF and production of vitamins such as vitamin B and K. It should be noted that one of the major issues in using dynamic mode fermentation is the high level of moisture content resulting in particle agglomeration (Mahanama et al., 2011b; Yang, 2007). On the other hand, the large space required for the installation of static mode fermentors and the risk of large temperature and oxygen gradients are among the major concerns for using a static mode reactor (Krishna, 2005; Rajagopalan & Modak, 1995). Further studies are required to design the best possible reactor type for vitamin K production.

Potential market, approvals and registration

The costs of treating osteoporosis and CVDs are very high and will likely increase as populations age. Estimates for the global osteoporosis drug market in 2009 are $8–9.4 billion (Commercial Insight:Osteoporosis, 2009); the market for cardiovascular drugs is larger with estimates of $110–140 billion (GCDM, 2010). There is, therefore, a large potential market for menaquinone. Judging from the limited available data, to date, menaquinone has only captured a very small fraction of the market with Natto Pharma (Høvik, Norway) reporting revenues of $1.8 million (NattoPharma, 2012) and Eisai's (Tokyo, Japan) sales of Glakay decreasing from $81 million in 2007 to $53 million in 2010 (Eisai, 2010). Shahidi reported that sales of MK-4 ($95 million) are less than one-third of pharmaceutical Ubiquinone ($288 million) (Shahidi, 2006). The US wholesale vitamin K market in 2010 was estimated to be $10 million (Daniells, 2010).

The safety, side effects, registration, regulatory approvals and marketing of phylloquinone have been reviewed previously (IARC, 2000). However, the use and regulatory status of MK-4 and MK-7 varies between countries. In Japan, MK-4 is marketed for the treatment of osteoporosis by Eisai Co. Ltd. (Eisai, 2010) and is acknowledged as a method to reduce fractures (Orimo et al., 2012), particularly for elderly generation with osteoporosis. MK-7 is registered by the US Food and Drug Administration (Quinlan, 2007) and also for sale in the Europe by European Food Safety Authority (European Safety, 2008). A few companies such as Danisco and NattoPharma offer a natural MK-7 for supplementation, however, at a very high price of $1200 per kg of 0.1% formulation. Current retail prices for menaquinone range from $3.50 to $13.30 per mg, which equate to $3.5–$13.3 million per kg. Given that the fermentation concentration achieved industrially is ∼100–150 mg/L, the prices are relatively high compared to the typical relationship between concentration and cost shown by Van Brunt's study (Van Brunt, 1988). Hence, in the current status of fermentation and synthesis, menaquinone appears to be still expensive. The aim for many menaquinone manufacturers is to decrease the cost by increasing the concentration of the product, which can be affordable for consumption by large population to minimize the risk of osteoporosis and CVDs. Therefore, it is beneficial to conduct further research to increase the concentration of menaquinone in fermentation media and develop new manufacturing processes to reduce the processing cost in future.

Conclusions and future perspective

This review has provided an overview of the recent development in vitamin K production. The dietary source of phylloquinone is green leafy vegetables and menaquinone is meat, dairy and fermented foods. Menaquinone appears to be most effective cofactor in bone metabolism and treatment and prevention of atherosclerosis and calcified arterial plaque; though, the therapeutic dosages are still the subject of ongoing research. Research conducted in the past decade to enhance the yield of production from both LSF and SSF. Contribution of initial fermentation media optimization, fed-batch addition of nutrients, *in-situ* extraction protocols in LSF and novel rotating and packed bed bioreactors for the production of directly consumable feed supplements in SSF resulted in significant improvements in menaquinone production to date. These strategies will aid in designing processes for bulk production of low cost products rich in vitamin Ks that can be

accessible by large populations in order to decrease the healthcare costs.

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Declaration of interest

The authors report no conflicts of interest. All authors are responsible for the content and writing of the paper.

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