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RESSEARCH PAPER

TITLE:

BIOENGINEERING OF THE OPTIMIZED BIOSYNTHESIS OF COMMERCIALLY VITAL MENAQUINONE (MK) - TECHNO-ADVANCED APPLICATIONS

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BIOENGINEERING OF THE OPTIMIZED BIOSYNTHESIS OF COMMERCIALLY VITAL MENAQUINONE (MK) - TECHNO-ADVANCED APPLICATIONS

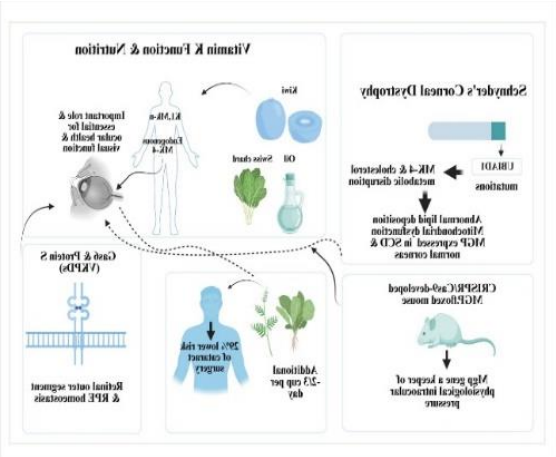
ABSTRACT

Menaquinone, also known as Vitamin K2, is a crucial fat-soluble vitamin for blood clotting and osteoporosis prevention. It is extensively utilized in the food and pharmaceutical industries and has attracted a lot of research interest.

Menaquinone is found in fish, meat, and vegetables, and can be produced through fermentation by bacteria, which can improve blood coagulation and bone health. Modern genetic and fermentation techniques allow for precise control over the production process, optimizing yields and developing high-quality products. Advances in genetic engineering also offer opportunities to enhance the productivity of *Bacillus* strains in vitamin K biosynthesis. Liquid fermentation has been developed to improve cell development pace and reduce fermentation duration. Researchers have improved the production of menaquinone through mutagenesis, screening techniques, optimizing culture conditions, and product secretion. Novel oxygen supply systems and innovative bioreactor designs could enhance menaquinone synthesis and address foaming during liquid fermentation. This study will provide an overview of its functions, biosynthesis routes, enzymes, challenges in large-scale microbial production, techniques like strain mutagenesis, genetic alteration, growth modes, fermentation, and separation procedures. Menaquinone is highly promising in the market, with several companies successfully industrializing its manufacturing. The future potential of microbial menaquinone synthesis should be examined considering existing advancements and obstacles.

KEYWORDS: Strain, Downstream processing, Vitamin K2, Bioengineering, Biosynthesis, Fermentation, *Bacillus*

GRAPHICAL ABSTRACT



INTRODUCTION

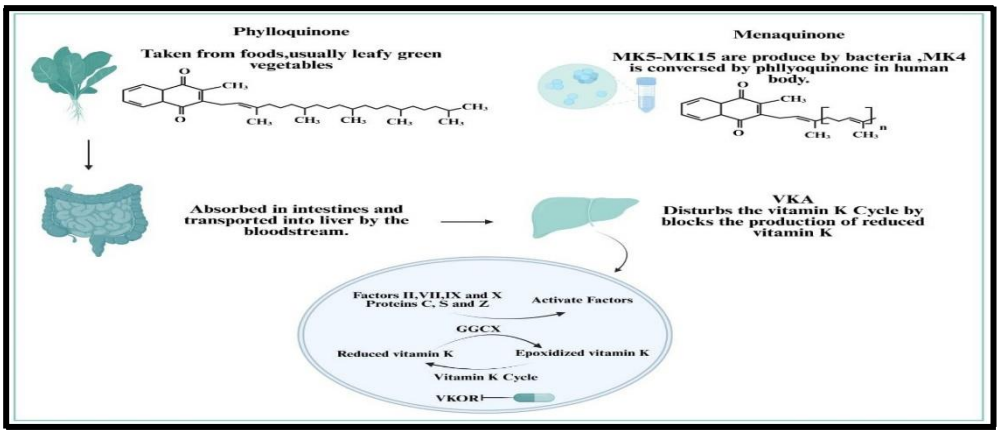
Vitamin K, a fat-soluble vitamin, is essential for bone health and blood coagulation. It is found naturally as menaquinone or phylloquinone, with vegetable green leaves containing phylloquinone (Kang et al., 2022a). Menaquinone absorption is primarily from fermented foods like cheeses, Korean

soybean products, and Japanese natto, and small levels can be found in animal-based foods like meat and eggs. Vitamin K's structural variation impacts biological processes, with its half-life and intestinal absorption influenced by its isoprene side chain **(Walther & Chollet, 2017).**

Phylloquinone and menaquinone are two essential vitamins that are present in different amounts in food and have different bioavailability levels. Phylloquinone is present in sufficient amounts in foods but has low bioavailability (Raseetha et al., 2023). Menaquinone is, however, readily available in the body indicating a hundred percent bioavailability (Walther & Chollet, 2017). Phylloquinone, contrary to menaquinone, is metabolized by the liver before entering the bloodstream. However, menaquinone is readily dispersed in body tissues and bones. Phylloquinone, plays a role in blood clotting and strengthens bones and teeth. Menaquinone, has been reported to play a pivotal significance in improving cardiovascular health **(Raseetha et al., 2023)**.

Vitamin K is essential for human health, as higher animals cannot produce it. It is obtained through dietary sources like leafy green vegetables, legumes, and vegetable oils like rapeseed and soybean oil. Vitamin K1 is also found in fish, meats, cereals, and drinks(Sun X. F. et al., 2020). Anaerobic bacteria produce vitamin K2, including some found in the gut lining. Hydrogenating vegetable oils produce dihydroxyvitamin K1, which is another potential source of vitamin K. Foods containing hydrogenated vegetable oil contain significant amounts of DHA, and human plasma contains dihydroxyvitamin K1, although its accessibility remains uncertain**(Lyytinen & Linneberg, 2023)**.

Figure 1: A schematic pathway indicating the sources of phylloquinone and menaquinone production and their subsequent absorption and transportation pathways in the human body.



Menaquinone, a rare natural compound, has been synthesized through chemical methods due to its limited availability. The first production of vitamin K2 utilized Friedel-

Craft's method of alkylation, but its selectivity was suboptimal. A new one-step technique was developed to synthesize several vitamin K compounds with over 60% yield **(Kang et al., 2022a)**. Researchers successfully produced MK-4 with a high selectivity of 96% using ethyl acetoacetate, and all-trans MK-7 with a purity of 99.9% utilizing isoprene, menadione, and trans-farnesol as substrates. Despite its widespread industrial production, chemical synthesis techniques often involve intricate procedures, resulting in limited product yields and by-products. Despite the industrialization of vitamin K2 synthesis, there is still a need for research on novel production methods due to the limitations of current chemical procedures **(Ren et al., 2020a).**

Microbial manufacturing is an eco-friendly and economical method for producing high-purity all-trans vitamin K2, despite concerns over its safety for GRAS dietary supplements. Chemical synthesis involves strong acids, bases, organic solvents, and heavy metals, while microbial fermentation uses inexpensive sources of strength, carbon, ions, and trace minerals. However, genetic instability can lead to poor yield and productivity in local vitamin producers **(Rusu et al., 2023)**. Metabolic engineering can help achieve high yield and stability through vitamin fermentation, overcoming these challenges. Bioprocess engineering techniques can enhance product production by adjusting fermentation conditions and medium composition. Strain engineering procedures have been used to improve vitamin production in microbial cell factories, particularly lactic acid bacteria (LAB). Modern genome editing techniques like CRISPR/Cas9 have recently boosted vitamin production in *E. coli* **(Raseetha et al., 2023)**.

Menaquinone-7, also known as MK-7, is only available to humans through diet or supplements due to its inability to be generated by human cells. The typical diet contains very little vitamin K2, with only 1 mg in pork or eggs. MK-7 is available from three sources: extraction from living things, chemical synthesis, and microbial production. Japan is known for its pioneering role in the production and utilization of vitamin K2, largely due to its traditional natto diet. Natto contains around 800-900 µg of vitamin K2 and other bioactive substances. Vitamin K2 analogs have also been found in dairy products, cheese, pork, yogurt, honey, and milk. Though the major source of menaquinone extraction is low productivity, additional sources may disclose structural analogues with different physiological activities and provide fresh perspectives on menaquinone supplementation. **(Ren et al., 2020a).**

Menaquinone Industrial and Chemical Synthesis

MK-7, also known as the "platinum vitamin," is a crucial nutrient for human health, as it cannot be produced by human cells. It is primarily obtained through food or dietary supplements, with a small amount found in regular meals. The source of MK-7 can be sourced from botanical, zoological, or culinary sources, chemical synthesis, or microbial cultivation. Japan is a pioneer in

the development and use of vitamin K2, primarily due to its consumption of natto, a traditional dish with a fibrous consistency and unique taste **(Sato et al., 2020).** Natto contains around 800-900 µg of vitamin K2 per 100g and other beneficial substances like nattokinase and γ-polyglutamic acid. Trace quantities of vitamin K2 analogs have been detected in various dietary sources, including cheese, honey, beef, yogurt, milk, and dairy products. However, the primary issue with vitamin K2 extraction is poor productivity. Other sources may offer insights into dietary supplements and structurally analogous compounds with different activities and physiological purposes **(Popa et al., 2021)**.

Menaquinone, a rare natural compound, has been chemically synthesized using various methods. The first synthesis of vitamin K2 was achieved using Friedel-Crafts alkylation, but its selectivity remained unsatisfactory. An efficient method was introduced for synthesizing vitamin K series compounds, resulting in high yields of over 60%. Scientists have developed methods to synthesize all-trans MK-7, achieving a purity level of 99.9% using substrates like menadione, isoprene, and trans-farnesol, and achieving a selectivity of 96% for the trans isomer. This has led to the industrialization of MK-7 **(Braasch-Turi & Crans, 2020).** In China, the process involves distillation, followed by extraction, and column chromatography, which is further processed by recrystallization, and drying.

However, these methods often involve complex procedures with limited productivity, yield cis isomers with limited reactivity, create large by-products, and contribute to environmental contamination. Despite the industrialization of vitamin K2 chemical synthesis, there is still a need to explore novel production methods to address these limitations **(Q.-W. Zhang et al., 2018a)**. Around 10 global businesses produce vitamin K2, with Kappa Bioscience and Gnosis being the top producers. NattoPharm, Viridis Biopharma, and Sungen are leading the global market for vitamin K2, producing natural MK-7 products using microbial fermentation techniques. They sell vitamin K2 at a high price of $1200 per kilogram. Intel Mint's research shows a 183% increase in vitamin K2-supplemented food and drinks in the last five years. In the US, treating cardiovascular disease and osteoporotic fractures costs $22 billion apiece. **(Lyytinen & Linneberg, 2023).**

Biosynthetic Pathways Involved in MK Production

Menaquinone, a microbial synthesis of vitamin K2, is produced through fermentation of various carbon sources like monosaccharides and glycerol. The MEP and isopentenyl diphosphate pathways generate the isoprenoid side chain, whereas the MK process forms the naphthoquinone ring.

Glycerol is converted into glycerol 3-phosphate and glyceraldehyde-3-phosphate (G-3-P) by glycerol kinase. This compound enters the glycolytic pathway to produce pyruvate, which undergoes decarboxylation to produce acetyl-CoA, which then enters the tricarboxylic acid (TCA) cycle. Pyruvate is then condensed with glyceraldehyde-3-phosphate, resulting in the synthesis of 1-

deoxyD-xylose-5-phosphate, which enters the MEP route to produce IPP **(Ren et al., 2020b)**.

Menaquinone is a compound produced by the biosynthesis of a substance, which requires the presence of a pyrophosphate (HPP) unit, which may be involved in the pentose phosphate (HMP) route. Shikimic acid is used as the first building block, forming the quinone structure of 2-hydroxy2-1,4-naphthalene formic acid (DHNA). The HPP unit is moved to the carboxyl group of DHNA by the enzyme 1,4-dihydroxy2-naphthoate octaprenyl transferase, which is made by the gene menA. **(Kloska et al., 2023)**.

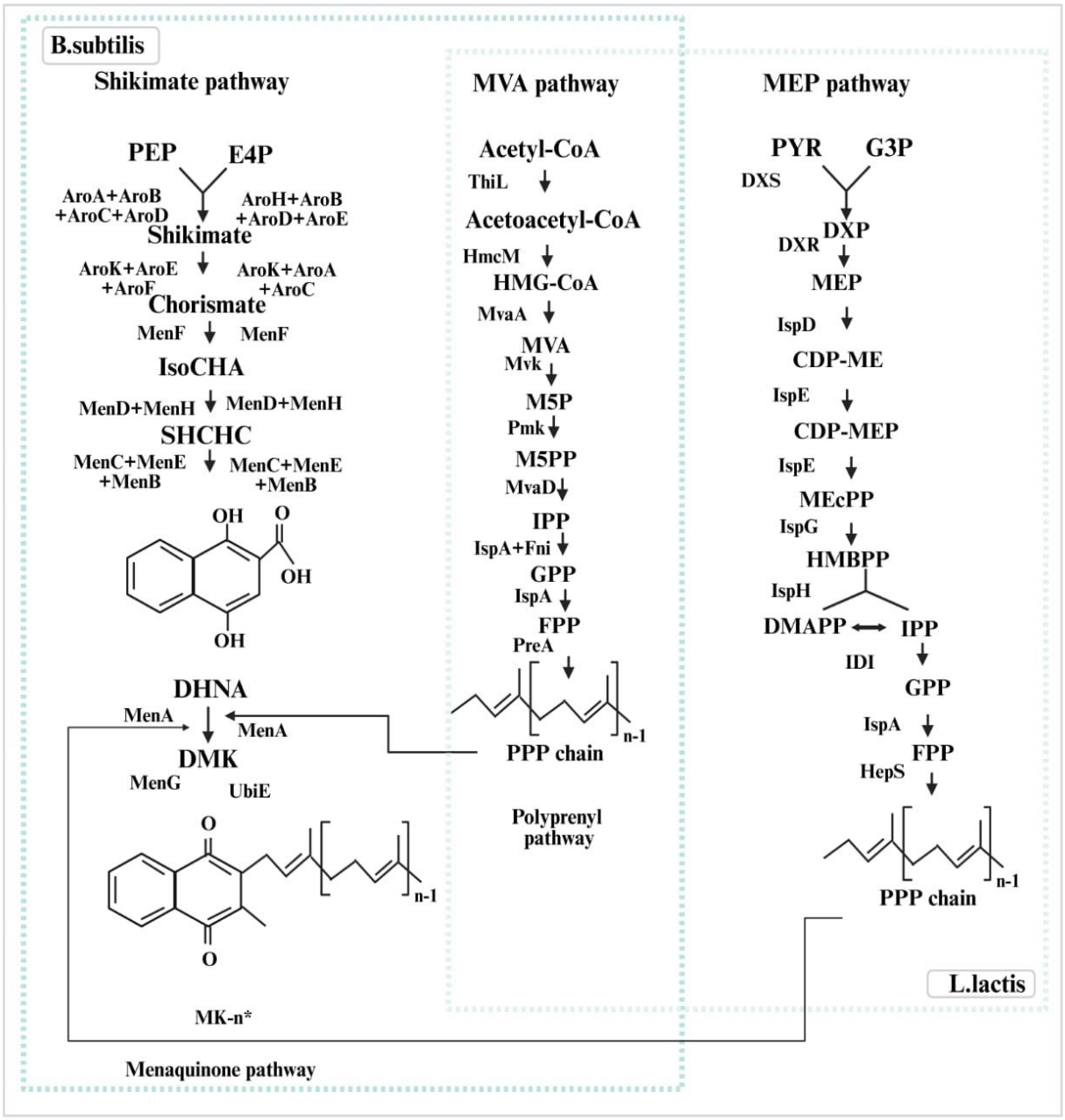


Figure 2 : Biochemical synthetic pathways involved in Menaquinone (Mk-2) Production.

The methylation process, resulting in the production of menaquinone, is facilitated by the enzymatic action of UbiE/menG. The men gene cluster, which includes genes involved in the shikimate pathway and menaquinone synthesis, has been extensively studied. *Bacillus subtilis* is considered the most extensively studied producer of MK-7,

with 22 genes essential for respiration. Polyprenyl pyrophosphate synthetase is an essential enzyme that alters the length of the isoprene side chain **(J. Wu et al., 2021).**

The futalosine pathway is an alternative method for menaquinone generation, involving the conversion of chorismate into futalosine and 1,4-dihydroxy6-naphthoate. Four enzymes that are encoded by the mqnABCD gene cluster assist this process. The exact process of this innovative approach for menaquinone production is still unknown, but it offers valuable insights for drug research **(Zhi et al., 2014)**.

Microbial biosynthesis of Menaquinone

Vitamin K production is primarily facilitated by bacteria, with several strains playing crucial roles. *Bifidobacterium* species, commonly found in the human gut, have demonstrated the ability to produce menaquinones, contributing to the microbial synthesis of vitamin K. *Propionibacterium freudenreichii*, found in fermented foods, is used for producing vitamin K2, particularly menaquinone-7 (MK-7). *Corynebacterium glutamicum*, engineered for enhanced vitamin K production, has been manipulated for specific genetic modifications**(Kang et al., 2022b).** *Enterobacter* species, including some strains, are being explored for their ability to produce vitamin K, with ongoing research focusing on optimizing conditions for increased synthesis. *Rhodobacter* species, a genus of purple bacteria, have also been investigated for their potential to produce vitamin K. Microalgae have also been explored for their capacity to synthesize vitamin K. The selection of microbial strains depends on efficiency, scalability, and safety, with genetic engineering techniques often employed to enhance their synthesis capabilities**(Ren et al., 2020b)**.

Bacillus subtilis

Bacillus subtilis var. natto, a GRAS (generally regarded as safe) bacterium, is used to produce menaquinone, a type of vitamin K. They are produced through a sequence of enzymatic processes within the bacterial cell, with Geranylgeranyl transferase being one of the enzymes involved. Menaquinones are synthesized using precursor compounds like isoprenoid units, and genes governing the expression of enzymes involved in menaquinone production often influence the synthesis process. The study investigated the use of various *Bacillus* strains for menaquinone production, with the KCTC 11712BP strain being the most commonly used. The *B. velezensis* ND strain was also tested in Cheonggukjang, a region with high menaquinone concentration. Different fermentation methods resulted in different synthesis rates, with the wild-type Bacillus strain producing the highest MK-7 titer after 100 hours**(Kang et al., 2022b).**

Here is a simplified overview of the production process of vitamin K through wild-type *Bacillus*. *Bacillus* strains are isolated from naturally occurring sources where they are frequently found, including soil or fermented foods. Following that, strains are identified using their morphological, biochemical, and molecular features. Various *Bacillus* strains are screened for their ability to produce vitamin

K2. This is often done by assessing their growth in a medium that promotes vitamin K2 production. A pure culture of the selected *Bacillus* strain is grown in a nutrient-rich medium to obtain a high-density inoculum. After that, the inoculum is moved to a bigger fermentation vessel with an appropriate growth media inside **(Kang et al., 2022b)**. The fermentation process is carefully controlled, optimizing conditions such as temperature, pH, and aeration to promote the growth and vitamin K2 production of the *Bacillus* strain. Once the fermentation is complete and the *Bacillus* strain has produced asufficient amount of vitamin K2, the culture is harvested. Cells are separated from the culture medium using methods such as centrifugation **(Lu et al., 2020)**.

The harvested cells are subjected to extraction processes to isolate vitamin K2. Various methods, including solvent extraction or chromatography, can be employed to extract and purify the vitamin. The purified vitamin K2 is then formulated into a suitable product, often in theform of a powder or oil. The final product is packaged in a manner that preserves the stability and bioavailability of the vitamin **(J. Wu et al., 2021)**. To make sure the vitamin K2 product satisfies the requirements forpurity, potency, and safety, it is put through extensive quality control testing. The final product is stored under appropriate conditions to maintain its stability. It is then distributed to manufacturers of dietary supplements, pharmaceuticals, or food fortification products. It is important to note that the specific details of the production process can vary based on the Bacillus strain used, the fermentation conditions, and the desired form of vitamin K2 (e.g., menaquinone-4 or menaquinone-7). Additionally, advancements in biotechnology and genetic engineering may be applied to enhance vitamin K2 production in Bacillus strains **(Ren et al., 2020b).**

Bifidobacterium spp.

Lactic acid bacteria may be the source of menaquinone, which has been detected in fermented dairy products in a variety of forms (LAB). Menaquinones have been reported to be produced during the fermentation of cheese by Propionibacteria and Lactococcus species. Vitamin K can be produced and absorbed by the human body from food-related LAB and components of the gutmicrobiota, such as *Bifidobacterium*. LAB are generally not known for synthesizing significant amounts of vitamin K**(Y. Liu et al., 2019)**. Vitamin K synthesis is more commonly associated with bacteria like *Escherichia coli*, *B acillus subtilis*. However, certain LAB, particularly *Bifidobacterium* species, have been found to possess some enzymatic activity related to the synthesis of menaquinones, a form of vitamin K2. Some *bifidobacterium* strains possess genes associated with menaquinone biosynthesis **(M. Hu et al., 2024; Y. Liu et al., 2019)**.

LAB may have enzymes involved in certain steps of menaquinone synthesis. This includes enzymes related to the prenylation of menaquinones. LAB might utilize precursor molecules, such as isoprenoid units, similar to other bacteria involved in

menaquinone synthesis. The synthesis pathway in LAB is likely regulated by specific genes that controlthe expression of enzymes involved in menaquinone synthesis. It's important to note that the synthesis of vitamin K, especially menaquinones, in LAB is not aswell-studied or established as in other bacteria. The quantities produced by LAB may be relatively small compared to other sources of vitamin K, and dietary intake of vitamin K from various food sources is still considered more significant. While LAB are crucial for the fermentation of certain foods and the production of some B vitamins, including certain forms of vitamin K, obtaining sufficient vitamin K through a balanced diet is essential. Foods like green leafy vegetables, broccoli, and fermented foods can contribute to vitamin K intake (Schöpping et al., 2021).

Flavobacterium meningosepticum

A bacterium that makes menaquinones is called *Flavobacterium meningosepticum*. *F. meningosepticum* is a gram-negative aerobic, nonmotile, oxidase-positive bacillus that inhabits freshwater, soil, and the ocean. Compared to other model bacteria, *F. meningosepticum* lacks biotechnological tools, making design challenging. several extraction techniques have been used to boost *F. meningosepticum*'s menaquinone production. An ultrasonic fermenter and a surfactant have been reported to be used to increase the amount of extracellular menaquinone produced during fermentation **(H. Wei et al., 2018a)**. To increase the productivity of the targeted products from biotechnological activities, surfactant, and ultrasound were employed to increase cell membrane permeability withoutthe need for chemical agents. Menaquinone synthesis increased with the addition of POE to the ultrasonic therapy, reaching apeak of 30.03 mg/L in an aqueous solution. Our results suggest that the temporary creation of pore-like structures in the cell membrane, which would increase cellular permeability, could increase the release of target metabolites from cells using physical and chemical techniques like ultrasound and surfactant. Menaquinone was extracted from *F. meningosepticum* grown cells using a variety of organic solvents and was reported to produce an excessive amount of the substance. Before collecting, freezing, and drying the cells. After all, it was shown that methanol was the most efficient organic solvent since it could extract menaquinone from *F. meningosepticum* up to 1.88 mg/g DCW. Many searches have been conducted in an attempt to find a distinct microbial strain that naturally generates vitamin K, other than *F. meningosepticum* **(Lee et al., 2022).**

Serratia marcescens and Enterococcus faecium

Two fecal flora strains *Serratia marcescens* and *E. faecium* were among the bacteria that could produce vitamin K and were extracted from the neonatal fecal flora. Using LC/MS analysis, the authors verified that the recently acquired bacteria could produce MK-4 in a fermentation medium. In a similar vein, the intestine anaerobic bacteria that can produce vitamin K have beenidentified based on their isolation from fecal samples **(Cooke et al.,**

2006). Commensal bacteria such as *Bacteroides, Eubacterium, Veillonella, Wolinella, Actinomyces, Arachniapropionica,* and *Propionibacterium* have been found to create menaquinone. These findings implied that a range of bacterial species might be involved in the production of vitamin K. However, satisfactory titer and host strain productivity are necessary for the commercial fermentation of vitamin K to be produced at a reasonable cost **(Lee et al., 2022)**.

Bio-engineered microbial-assisted Menaquinone production

The synthesis of vitamin K through the utilization of designed microorganisms represents a promising avenue in biotechnology. Engineered microbial strains, such as *Bacillus subtilis*, *Escherichia coli*, and Bifidobacterium, have been strategically modified to enhance the production of vitamin K, specifically menaquinones. This comprehensive review delves into the methodologies, challenges, and potential applications of generating vitamin K using geneticallyengineered microorganisms. The discussion covers genetic modifications, fermentation processes, and the optimization of growth conditions, highlighting the biotechnologicalbreakthroughs that pave the way for sustainable and scalable vitamin K production(Liao et al., 2021) .

Table 1: Menaquinone (Vitamin K2) biosynthesis by using various strains, extraction types, and fermentation conditions and/or methods.

Strain	Fermentation Type	Menaquinone types	Fermenter conditions (Temp/Pressure /duration/DO/pH/humidity%)	Extraction Method/s	Menaquinone Production	References
Bacillus velezensis ND	SSF - involves microorganisms to grow on a solid surface, producing desired products.	MK-7	37^{0}C/7 days /30 % humidity	MK-7 was extracted using n-hexane and a iso-propanol (2:1) volume-to-solvent ratio.	LSF- 52.90 BF - 73.30 SSF -150.02 (mg/L or mg/Kg)	**(Zhao et al., 2021a)**
Escherichia coli	Three modular biosynthesis of MK7: MVA, DHNA, and pathway, and MK-7 pathway (All of them were found improved).	MK-7/DMK-7	37 ^{0}C/52h / 20%–60% DO/ pH 7.0	*E. coli* cells were collected and extracted using n-Hexane, a chemical compound, and a solution of 2-propanol in a 2:1 ratio. (Berenjian et al., 2011)	2074 µM (1350 mg/L)	**(Gao et al., 2021)**
Bacillus amyloliquefaciens KCTC 11712BP	Optimized fermentation conditions.	Vitamin K2	43^{0}C/36h	The menaquinones of cultured cells were extracted using a heptane mixture.	12.47 µg/g	**(W.-J. Wu & Ahn, 2011)**
Escherichia coli	HepPPS[1]-synthesizes heptaprenyl pyrophosphate.	Menaquinone-7	37^{0}C/40-50h/ 40% DO / pH 7.0/ 254 OD	Naphthoquinone and Ubiquinone- extracted using n-hexane/iso-propanol (v:v/2:1).	(8.8 mg/L) 13.6 µM	(Gao et al., 2020)
Bacillus subtilis	metabolic engineering approaches	Menaquinone-7	37^{0}C/132h/ 60% DO	Fermentation Broth	281.4 ± 5.0 mg/L	**(S. Yang et al., 2020)**
Bacillus subtilis natto	base medium	vitamin K2 menaquinone-7	30 ^{0}C-37^{0}C//24h/ pH 7 (incubation duration 72, 96, and 120 hours)	The MK-7 separation was conducted using the extraction methods (C. Zhang et al., 2020)	0.319 and 0.3158 mg/L	**(Sharifzadeh et al., 2022)**
Bacillus subtilis	Shake flask culture	Vitamin K2 (menaquinone)	41^{0}C/6 days/ pH 7.0/ OD600nm	The MK-7 was extraction from 4:1 extracting agent, iso-propanol :n-hexane (v/v:1:2).	415 mg/l -242 mg/l	**(T. Chen et al., 2020)**
Bacillus subtilis natto.	Alkaline stress monitoring on fermentation	Menaquinone-7	37^{0}C/72h/ pH 5.5/ OD254nm	The MK-7 quantification fermentation broth - by methodology outlined by (Sun X. F. et al., 2020).	290.19 ± 1.12 mg/L	**(X. Chen et al., 2022)**
Lactococcus lactis	The process involves cloning and expressing the gene from strong promoters.	MK-8 and MK-9	30^{0}C/20h/ pH below 5.0	Heptane mixture -as extraction mixture - following a method (Koivu-Tikkanen et al., 2000)	(500 ng/g) 700 nmol/L fermented milk) is a significant concentration.	**(Bøe & Holo, 2020)**
Bacillus subtilis var. natto.	The procedure used HPLC UV/DAD to analyze fermented plant samples.	vitamin K2 MK-7	37^{0}C/120-144h/ OD 550 nm	The sample preparation adhered to the procedures outlined by (S et al., 2016)	154.17± 9.34	**(Słowik-Borowiec et al., 2021)**
Bacillus natto ALE-25– 40	MK-7 biosynthesis in *Bacillus natto* utilized chemical modulator (by an adaptive evolution)	Menaquinone-7 (MK-7	37^{0}C/6days/ – OD600	The method was used to measure the MK-7 yield. (Ma et al., 2019)	The MK-7 concentration was recorded at 0.42 mg/(L h). productivity. 62 mg/L,	**(B. Zhang et al., 2022)**

B. subtilis	A global gene expression - comparative transcriptomics analysis - MK-7 synthesis- induced by shake and static culture.	MK-7 production	127%/ 4days/ OD600	Biofilm and Fermentation Liquid Analysis • Analyzed MK-7 concentration. • Extracted from supernatant and cell.	360 to 410 mg/L	**(Cui et al., 2020)**
B. subtilis natto	Bioinformatic analysis- used to characterize MenA - physicochemical properties	menaquinone (MK)	25 °C/72h / pH 8.0/ OD660 nm	The DMK and MK were analyzed using a method from a previous report on cell debris extraction. (Y. Liu et al., 2018)	7.1 ± 0.2 mg/L	**(L. Hu et al., 2020)**
Bacillus subtilis	*Bacillus subtilis* - A regulatory pathway (quorum-sensing) - to enhance MK-7 production in BS168 .	MK-7	37 ° C/6 days /pH = 7.0//OD 600 nm	The MK-7 extraction procedure involves centrifuging mixed fermentation broth, adding extractant, shaking, centrifuging, and reserving the mixture, which is a mixture of n-hexane and isopropyl alcohol.	102.47 mg/L	**(huang et al., 2023)**
B. amyloliquefaciens MK50-36	*B. amyloliquefaciens* MK50-36 Acclimatization Success	MK-7	50 °C/ 7.2 ± 0.2 pH with 20% (w/v) NaOH/12 h/OD600 nm	The iodine-starch chromogenic assay was used for qualitative analysis of a-amylase activity.	52.7 ± 4.6 mg L–1	**(N. Liu et al., 2021)**
E. coli DH5α and *E. coli* BL21 (DE3	"Identification, Expression, Purification of EmOPPS from *E. meningoseptica* sp. F2"	MK-n (n=4, 5, 6, 7, 8)	37°C/ 24 h /pH 7/	Wei et al.'s work was used for the extraction and measurement of vitamin K2 concentration. **(H. Wei et al., 2018b**)	32 mg/L	**(Q. Yang et al., 2022)**

[1]HepPPS: heterogeneous enzyme; SSF: Solid-state fermentation;

Problems associated with the Menaquinone Biosynthesis

Microorganisms maintain menaquinone levels at micromolar concentrations, resulting in a complex and controlled production process. The presence of multiple enzymes, cofactors, and reactions in both biosynthesis routes contributes to the limited efficiency of menaquinone production. Pyruvate is used in the MEP route to produce 1-deoxy-dxylose-5-phosphate, while phosphoenolpyruvate is required for shikimate production. α-ketoglutaric acid serves as a precursor in the production route of menaquinone**(Z. Zhang et al., 2021)**. Synchronizing multiple metabolic processes is necessary for menaquinone creation. The manufacture of vitamin K2 is limited by feedback inhibition of important enzymes, such as pyruvate kinase, DAHP synthase, and polyprenyl pyrophosphate synthetase. The diminished amounts of enzymes responsible for MK-7 production may also contribute to the limited rates of menaquinone biosynthesis**(Liao et al., 2021)**.

Strategies to accelerate Menaquinones Industrialization

Numerous research efforts aim to enhance the vitamin K2 production by improving biosynthesis capacity, optimizing the

production process, and designing new bioreactors to enhance efficiency and expedite the industrialization process, with specific details to be discussed in the next section.

Strain mutagenesis :

The performance of a particular strain during fermentation has a substantial effect on the concentration and productivity of the end product. Menaquinone production encompasses several metabolic pathways and enzymes, which are controlled by various substances. These events impact the manufacturing process and provide valuable information for the strain mutagenesis. Yoshiki group pioneered the use of structural analogs to augment the synthesis of vitamin K2. HNA, a structural analogue of 1,4-dihydroxy-2-naphthoate, was used to conduct a mutation screening in *Flavobacterium meningosepticum* **(Bhagchandanii et al., 2020)**. This screening resulted in an increased production of MK-6 and MK-5, as well as enhanced synthesis of vitamin K2. Sulfonamides impede the synthesis of folic acid and promote the accumulation of chorismate in *Brevibacterium flavum*. In bacteria, Diphenylamine (DPA) suppresses the synthesis of menaquinone and carotenoids such as *Bacillus megaterium* and *Staphylococcus aureus*. The process of developing resistance to structural analogs has resulted in the creation of efficient producers of vitamin K2, but it requires a significant amount of time and manual effort **(Xu & Zhang, 2017a)**.

Genetic modification of strains:

Strain mutagenesis has been used to enhance production capacity in bacteria, but it has limited efficacy. The limited amounts of vitamin K2 in wild-type strains may be due to low levels of expression of crucial enzymes responsible for menaquinone production. Scientists are working to improve efficiency in *Bacillus* by altering metabolic pathways or creating new pathways in other model bacteria **(Xu & Zhang, 2017b).** Overexpression of enzymes involved in precursor molecule production led to a significant increase in MK-8. Overexpression of MenA or MenD led to a five-fold increase in menaquinone concentration, while inhibiting the ubiquinone-8 pathway resulted in a 30% increase. In a separate study, the primary enzyme involved in the ubiquinone-8 pathway was rendered inactive using site-directed mutagenesis, resulting in a 130% increase in MK content. Menaquinone levels increased by a factor of 11 by simultaneously expressing rate-limiting enzymes and adding substrate precursors. In a genetically modified strain of B. subtilis, the strain overexpressed various combinations of enzymes, leading to a significant rise in MK-7 titer and strong genetic stability **(Salazar-Cerezo et al., 2023).**

Modular pathway engineering:

Modular engineering pathway is a successful method for enhancing the production of certain compounds. *B. subtilis* 168 was used as a foundation for producing MK-7 and this biosynthesis pathway was further divided into four modules. They improved each module individually to investigate critical processes that restrict MK-7 biosynthesis. Inducing higher levels of menA in the MK-7 route and seven enzymes in the MEP pathway resulted in a 2.1-fold rise and an 82% improvement, respectively.

Nevertheless, the shikimate pathway negatively affected the MK-7 synthesis because of the inhibitory activity of chorismate. Another study focused primarily on enzymes encoded by the male gene cluster. In a separate strain of *Bacillus amyloliquefaciens* Y2, the overexpression of the HepS gene resulted in a more significant rise compared to other enzymes. This finding revealed insights into the rate-limiting processes in distinct MK-7 manufacturers. However, static metabolic engineering solutions can disrupt or overload the normal metabolic networks, leading to metabolic imbalance.

Bioengineering modes of cultivation:

Solid state fermentation (SSF) and liquid state fermentation (LSF) are the primary methods used for the synthesis of menaquinone. The old method of producing natto involved cultivating synthesis *B. subtilis* natto on boiling soybeans, which was straightforward and uncomplicated. Various methods have been documented to enhance fermentation yield by using legumes, cereals, and raw wheat as a substrate. MK-7 yield, however, varies from 0.53-to-140 mg/kg, depending on parameters such as strain variations, medium components, starting moisture levels, and ambient conditions.

Solid-state fermentation eliminates the need for costly organic solvent extraction and can be dried and formed into pellets for consumption as a nutritional supplement. However, controlling the process parameters is challenging, and inadequate humidity can significantly impact the solid matrix's physical characteristics. Liquid fermentation, on the other hand, enhances cellular proliferation, decreases fermentation duration, and minimizes bioreactor capacity requirements.

An optimal glycerol concentration of 4% (v/w) is recommended, whereas a suitable Ca2+ concentration enhances the synthesis of MK-7. Berenjian's research group enhanced the cultivation of menaquinone-7 (MK-7) by B. subtilis natto by the use of a glycerol concentration of 50 g/L and soybean peptone concentration of 189 g/L. This optimization led to the production of MK-7 reaching a level of 62.3 mg/L. Other fed-batch fermentation procedures, including the introduction of glycerol at a later stage, have also been suggested for improving MK-7 production.

Optimization of environmental conditions

Bacillus fermentation environment can influence the development of cells and the concentration, yield, and productivity of vitamin K2. The ideal temperature for high vitamin K2 synthesis is 40 degrees Celsius. Bacillus produces spores when exposed to unfavorable environmental conditions, which impacts the production of vitamin K2. Studies have shown that agitated fermentation can significantly increase the production of menaquinone. A maximum yield of MK-7 (226 mg/L) was achieved after 5 days of cultivation under highly oxygenated conditions. The rate of sporulation and the concentration of MK-7 were found to correlate. The synthesis of MK-7 is boosted under conditions of high oxygen supply due to increased pyruvate kinase activity, a highly efficient tricarboxylic acid (TCA) route, and a reduced G-3-P buildup **(Q.-W. Zhang et al., 2018b)**. Current methods for enhancing menaquinone production include techniques such as cell

immobilization, magnetic nanoparticles, and iron oxide nanoparticles. Cell immobilization confines the cell inside a restricted area, enhancing production efficiency. Magnetic nanoparticles do not impact mass transfer, and iron oxide nanoparticles were found to be advantageous for menaquinone-7 production without harmful effects on the cells. Altering the iron oxide nanoparticles with a coating of either 3-aminopropyltriethoxysilane or L-lysine resulted in a twofold increase in the generation of menaquinone-7**(Salazar-Cerezo et al., 2023)**.

Increased Secrretion of Menaquinone:

Menaquinone, a coenzyme involved in the process of respiration, is released into the liquid medium during fermentation, with 20-60% being excreted. The released vitamin K2 is present as a soluble complex composed of 20% carbohydrate and 80% peptide. This peptide mixture contains a 10 kDa peptide and 3 kDa amphiphilic peptides. Inducing the release of vitamin K2 to promote uninterrupted synthesis during fermentation can significantly enhance overall production of vitamin K2. Surfactants, including polymeric, ionic, non-ionic, and edible surfactants, can increase membrane permeability and promote the release of products from the cell**(Xu & Zhang, 2017a)**. Soybean oil, an edible surfactant, was found to be an efficient addition for promoting the biosynthesis of MK-7. The addition of 20 g/L soybean oil during the logarithmic phase in Bacillus resulted in a 61.1% rise in the secretion ratio of MK-7 and allowed for the recovery of 80% of the generated MK-7 on-site **(Xu & Zhang, 2017b).**

An approach to generate a concentration of 226 mg/L MK-7 was used during a 5-day fermentation period, along with an oxygen supply method of high intensity. Non-ionic surfactants span 20 may also enhance product production. The presence of amine surfactants, including betaine, did not significantly impact the secretion of MK-7**(Xu & Zhang, 2017a; Z. Zhang et al., 2021).**

Bioreactor Design:

Bacillus strains often form biofilms in broth while being cultivated, which may have an impact on the viscosity of the broth and the efficiency of mass transfer. In order to tackle this problem, innovative bioreactors, such as biofilm reactors, have been created for the purpose of producing vitamin K2. Passive immobilized cell reactors use supports such as lignocellulosic materials, metallic alloys, or plastic composites to attach microbial cells, therefore facilitating biofilm development and achieving high cell concentrations (Shaikh et al., 2023). Over the last several decades, biofilm reactors have been used to augment the production of antibiotics, biopolymers, and enzymes **(Xu & Zhang, 2017a).** A clear correlation between biofilm formation and vitamin K2 synthesis in stagnant cultures has been discovered. The researchers examined four distinct plastic composite supports and assessed both batch and fed-batch fermentation techniques in a biofilm reactor, leading to a 2.3-fold enhancement in the concentration of the end product**(Shaikh et al., 2023)**. The use of biofilm reactors in the synthesis of vitamin K2 is now in its nascent phase, necessitating more investigation to enhance reactor configurations for the menaquinones production **(Zhao et al., 2021b).**

Downstream Processes:

Extraction and refining operations are crucial for the manufacturing cost of certain biochemicals, often contributing over 50%. Vitamin K2, found in membranes, is typically isolated from culture broth using organic solvent extraction after breaking down cells. Acid heating and homogenization are effective strategies for enhancing the extraction process **(Cooke et al., 2006)**. The disturbed cell is then submerged in the extract's solvent. A mixture of organic solvents, such as 2-propanol and n-hexane, is used for extraction. Tsukamoto et al. introduced distinct solvents, mixing them for 15 minutes and then adding hexane. The concoction is dehydrated and reconstituted for HPLC examination. In some studies, several menaquinones isolated by using a single organic solvent, but the organic solvents must be thoroughly eliminated to ensure safety, leading to complex subsequent steps and higher processing costs **(W. Wei et al., 2021).**

Scientists have devised an in situ extraction method to retrieve 80% of menaquinone-7 from the oil phase, using soybean oil as an anti-foaming agent. This technique obviates the need for organic solvents and enables the direct synthesis of oil high in menaquinone-7. During the production of commercial menaquinone, the fermentation broth undergoes acid treatment, filtration, and concentration using an ultrafiltration membrane **(Nagarajan et al., 2022)**. The concentrated combination is then removed using soybean oil to provide an oil abundant in menaquinone. The powdery form of Vitamin K2 may be synthsiz by microencapsulation with the use of supplementary ingredients. Nevertheless, the cost of synthesizing vitamin K2 is greatly impacted by downstream processing techniques like as filtration, extraction, drying, and sub-packaging, which poses a major barrier to its widespread use**(W. Wei et al., 2021)**.

Mutation of Wild strain of Bacteria:

By randomly mutating from the wild-type strain of bacteria, the mutant strain can create vitamin K, which can subsequently be given to food. While the random mutagenesis method increased the amount of vitamin K produced, it took a lot longer and more work to choose and screen the changes than it did with rational engineering. By employing high-throughput screening methods,it might be able to select strains from the mutant library that produce an excessive amount of vitamin K more quickly improving the production of vitamin K involves optimizing the conditions for the growth of bacteria that naturally synthesize this vitamin **(Wang et al., 2023)**.

The process of producing vitamin K involves several steps. First step involved the identification of the bacterial strains indicating high production capabilities. This is followed by the optimization of fermentation conditions, like temperature, pH, and oxygen levels, to enhance growth and vitamin K production **(Pertics et al., 2023)**. To ensure the optimum growth medium must provide sufficient nutrients, including precursors which are required for vitamin K production. Exploration of genetic modification techniques is considered necessary to enhance the production capabilities of bacterial strains. It is essential to provide co-factors and precursors which are essential for the vitamin K synthesis

pathway. The optimal fermentation duration was determined and balanced increased production with overall efficiency. The strain stability is ensured over successive generations to maintain consistent and reliable production. This is followed by the optimization of bioreactor design to facilitate efficient growth and vitamin K synthesis. Development of efficient downstream processing methods to extract and purify vitamin K from the bacterial culture is followed by the implementation of rigorous quality control measures to monitor and ensure the consistency and purity of the produced vitamin K**(Wang et al., 2023)**.

It's important to note that these strategies might vary depending on the specific bacteria used for production (e.g., *Bacillus subtilis*), and considerations for regulatory compliance should be considered when employing genetic modification techniques. Additionally, collaboration with experts in microbiology, biotechnology, and fermentation processes can be valuable for optimizing vitamin K production**(Zhao et al., 2021b).**

Contemporary Genetic and Fermentation Methods For Vitamin K Production:

Modern genetic and fermentation techniques have allowed for the optimization of vitamin K production by Bacillus strains. Here is a detailed process that combines genetic engineering and fermentation for the production of vitamin K: A *Bacillus* strain with a high potential for vitamin K production is selected. Genetic engineering techniques, such as recombinant DNA technology, are employed to modify the strain for enhanced vitamin K production. This may involve introducing or overexpressing key genes involved in the biosyntheticpathway of vitamin K**(huang et al., 2023)**. The genes responsible for vitamin K biosynthesis are identified and cloned from the *Bacillus* strain or other sources. These genes are then introduced into the selected *Bacillus* strain using genetic transformation methods, such as electroporation or plasmid transfer. A specialized fermentation medium is designed to support the growth and vitamin K production of the genetically modified Bacillus strain. The composition of the medium is optimized for nutrient availability, pH, temperature, and other parameters **(Zhao et al., 2021b)**.

The genetically modified strain is inoculated into a larger fermentation vessel forscale-up production. Fermentation conditions are carefully controlled to maximize vitamin K production while maintaining cell viability. Advanced monitoring techniques, such as online sensors and analytics, areemployed to track the progress of the fermentation process in real-time**.** Control strategies, such as feedback control loops, are implemented to adjustconditions based on the observed data**.** When the fermentation is complete, cells are harvested using methods like centrifugation**.** Cell disruption techniques, such as mechanical or enzymatic methods, are applied to release intracellular vitamin K**.** Purification steps, including filtration and chromatography, are employed toisolate and concentrate vitamin K from the cell extract**.** Downstream processing is optimized to remove impurities and obtain a high-purity vitamin K product **(huang et al., 2023)**. The purified vitamin K is formulated into the desired product form (e.g., powder, oil, or liquid)**.** The final product is packaged in a

way that ensures stability and bioavailability**.** The vitamin K product undergoes thorough quality control testing, including assays for potency, purity, and safety**.** The final product is stored under controlled conditions to maintain its quality**.** It is distributed for use in various applications, such as dietary supplements, pharmaceuticals, or food fortification**(Q.-W. Zhang et al., 2018b).**

Genetic and fermentation techniques tend to enhance the titer, productivity, and vitamin K production of microorganisms. Generating strains with high vitamin K production is made easier by systems metabolic engineering, which combines engineering techniques from synthetic biology, systems biology, and evolution (huang et al., 2023) . Furthermore, machine learning and in silico metabolic modeling may make the microbial hosts that produce vitamin K more competitive in the industrial market.

Registration and Marketing of Fermented Goods:

Certain bacteria that generate vitamin K are not GRAS microorganisms, hence there are restrictions on their registration and marketing. The microorganisms that create vitamin K need to be safe and classified as GRAS in order for it to be utilized as a dietary supplement. Although there is no risk involved in using GRAS wild-type bacteria, they frequently produce very little vitamin K. The composition and fermentation conditions were modified to maximize the wild-type bacteria's capacity for vitamin K production. Ultrasound and surfactant therapy are two more chemical and physical techniques that have been utilized to increase the synthesis and release of vitamin K **(Fischer & Titgemeyer, 2023).**

Conclusions and Future Perspectives

Menaquinne, a dietary supplement with potential to prevent osteoporosis and cardiovascular disease, has been researched for 90 years since its discovery in 1929. The primary strain used for producing menaquinone-7 is *Bacillus subtilis* natto. Solid-state fermentation methods have limitations, such as lengthy culture periods and poor volumetric output. Liquid fermentation has been developed to improve cell development pace and reduce fermentation duration. Scientists have improved vitamin K2 manufacturing through mutagenesis, screening methods, cultivation conditions, and secretion. Metabolic engineering approaches have also improved efficiency. However, unresolved issues remain, including the complex metabolic network and limited synthetic output, due to the limited production of vitamin K2. Currently, vitamin K2 is highly promising in the market, with several companies successfully industrializing its manufacturing. Future potential of microbial vitamin K2 synthesis should be examined considering existing advancements and obstacles. Methods to enhance production capacity include developing novel strain mutagenesis techniques, enhancing biosynthetic pathways or suppressing competing pathways, and adapting strains to enhance resistance to environmental influences.

Manaquinone plays a crucial role in the electron transport chain, impacting cell respiration and oxygen consumption. Controlling menaquinone production in the presence of oxygen could be advantageous. Novel oxygen supply systems and innovative

bioreactor designs could enhance vitamin K2 synthesis and address foaming during liquid fermentation. Despite being classified as genetically modified organisms (GMOs), modified host strains can now be easily identified thanks to advancements in genome editing technologies such as the CRISPR/Cas9 system. Utilizing free recombinant strains enables the secure and eco-friendly production of foodcomponents. Advancements in synthetic biology have made genetic modification technologies accessible for non-model microorganisms, enabling the production of many compounds.

ABBREVIATIONS:

G-3-P	glyceraldehyde-3-phosphate
TCA	Tricarboxylic acid
DMK	Demethylmenaquinone
Mk	Menaquinone
UV/DAD	UV/Diode Array Detector
OD	Optical Density
DO	Dissolved Oxygen
LSF	Liquid-state Fermentation
SSF	Solid State Fermentation
BF	Biofilmbased Fermentation
GMOs	Genetically modified Organisms

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