CHAPTER II
 REVIEW OF LITERATURE
2.1. VITAMIN K

Vitamin K is an essential group of fat-soluble vitamins required for a unique posttranslational chemical modification of proteins with calcium-binding properties, which is known as vitamin K-dependent proteins (VKDPs) or Gla-proteins. Vitamin K is essential for blood coagulation but it is also known to be involved in metabolic pathways in bone and other tissue. In the early 1930s, Vitamin K was discovered by the Danish biochemist Henrik Dam by investigating the role of cholesterol by feeding chickens a cholesterol-depleted diet. After several weeks, he noticed that the animals developed hemorrhages and started bleeding which could not be restored by adding purified cholesterol to the diet. In the later years during 1935, Dam observed and described the nature of this anti-hemorrhagic factor whose absence was responsible for bleeding (Dam 1935) and he proposed to name the new factor vitamin K (for Koagulation from the German and Scandinavian languages). This was led to the identification of chemical structure of the new component. The significant step was the isolation, which was reported in 1939, of a pure sample of vitamin K prepared from green leaves. Consequently, a different vitamin K type was also isolated from putrefied fish meat (Dam 1946). Finally, in the same year, the American scientist Edward Doisy not only solved the chemical structure of both substances as different 1, 4-naphthoquinone derivatives, but was also able to synthetically prepare them under laboratorial conditions (Dam 1946; Doisy 1976). Since then, these two compounds have been known as vitamin K\textsubscript{1} and vitamin K\textsubscript{2}. The vitamin K\textsubscript{1} is found in green vegetables and chemically is a 2-methyl-3-phytyl-1, 4-naphthoquinone, or phylloquinone (PhQ), whereas the vitamin K\textsubscript{2} is of eubacterial origin and structurally is a 2-methyl-3-prenyl-1, 4-naphthoquinone, or
menaquinone (MQ) (Plaza 2003). Dam was awarded the Nobel Prize in Physiology or Medicine in 1943 for the discovery of vitamin K; together with Doisy for the purification, characterization and synthesis of the molecule. Several laboratories synthesized the compound(s) in 1939 (Fieser 1939).

For several decades, the only method to quantify vitamin K in various foods was the vitamin K-deficient chick model: the vitamin K-deficient chicks were made and later they were fed with vitamin K-containing food of known amounts. The extent to which blood coagulation was restored by the diet was taken as a measure for its vitamin K content. Three groups of physicians autonomously found this: Biochemical Institute, University of Copenhagen (Dam and Johannes Glavind), University of Iowa Department of Pathology (Emory Warner, Kenneth Brinkhous, and Harry Pratt Smith), and the Mayo Clinic (Hugh Butt, Albert Snell, and Arnold Osterberg) (Dam Henrik (December 12, 1946). In 1938, Smith, Warner, and Brinkhous, published the first report on successful of a life-threatening hemorrhage in a jaundiced patient with prothrombin deficiency treatment was made by with vitamin K (Warner et al., 1938).

During the next 20 years, it was noted that very little research on vitamin K was published. Until the late 1950s, the structures of the other K vitamins (menaquinones) were not published and were demonstrated that it was bacteria, as conflicting to green vegetables, that producing menaquinones with the same naphthoquinone ring as phylloquinone, but with different side chains. After the initial discovery, to find the exact role of vitamin K as a cofactor for post-translational protein modification to elucidate it took more than two decades.

Until the 1970s, it was not revealed that the role of vitamin K as a substrate for γ-glutamyl carboxylase for the post-translational modification of certain proteins (Gla-
proteins) and the recycling of vitamin K through the vitamin K cycle. Thus, until then, vitamin K was considered to be a coagulation protein. The significant factor for bone and cardiovascular health recognized only after the identification of the important Gla-proteins in certain tissues was the acknowledgement for vitamin K (http://www.nutraceuticalbusinessreview.com).

The exact function of Vitamin K in the body was not discovered until 1974. Scientists across three laboratories (Stenflo et al., 1974; Nelsestuen et al., 1974; Magnusson et al., 1974) were able to isolate prothrombin, a vitamin K-dependent coagulation factor, from cows that had been given a high dose of the vitamin K inhibitor warfarin. Among the cows which were treated with warfarin, the prothrombin had ten glutamate (Glu) residues at the amino terminus, while in case of untreated cows had ten unusual residues were then identified as gamma-carboxyglutamate (Gla). This addition of a carboxyl group to the glutamate showed to have an important role of vitamin K in converting Glu to Gla.

2.2. STRUCTURE AND PROPERTIES

Vitamin K is a group name for a number of related compounds, which have a methylated naphthoquinone ring structure in common, and which vary in the aliphatic side chain attached at the 3-position (Figure 1). Two natural occurring forms are well known, namely phylloquinone (also known as vitamin K1) and the menaquinones (vitamin K2). Phylloquinone (2-methyl-3-phytyl-1,4-naphtoquinone) is found in plants and has the same phytol side chain as chlorophyll (Lichtenthaler 1993). The menaquinones (2-methyl-3-multi-prenyl-1, 4-naphtoquinone) are generally denoted as MK-n, where n stands for the number of unsaturated isoprenyl residues which may vary between 1 and 14. The forms most common in food are menaquinone-4 (MK-4,
containing 4 isoprenoid residues) and the long-chain menaquinones MK7, MK-8 and MK-9. The another form of vitamin K is vitamin K3 also called as menadione, however it is synthetically synthesized and do not have vitamin K activity by itself. This form does not contain a side chain. Menadione is also known as provitamin and it can be converted to MK–4 in the body (Ritzl 1970 and Dialameh et al., 1971). Apart from this, it can also be used as a supplement in animal food. Because of its toxic side effects it is not used anymore in humans (Allison 1955 and Meyer and Angus, 1956).

Due to the close structural relationship of different vitamin K forms, it can be noticed that most of their chemical and physical properties are similar. Phylloquinone is liquid at room temperature whereas menadione and menaquinones are solids (Parrish 1980). K vitamins are insoluble in water, slightly soluble in alcohol and readily soluble in non-polar organic solvents, for example in \(n\)-hexane, ether, and chloroform. They are sensitive to light and alkaline conditions, but stable in slightly acidic media and under oxidizing conditions. They also have relatively high thermostability (Lambert and de Leenher, 1992). All K vitamins have an ultraviolet spectrum characteristic of the naphthoquinone nucleus with four distinct peaks between 240 nm and 280 nm. The extinction coefficient decreases as the length of side-chain increases (Parrish 1980).

2.3. DIETARY SOURCES AND IMPORTANCE

Menaquinones generally are of microbial origin. Important dietary sources are cheese, curd and natto (a traditional Japanese food composed of fermented soya beans) (Schurgers and Vermeer, 2001) while dietary phylloquinone is mainly found in green vegetables, notably spinach, broccoli, kale and Brussels sprouts (Bolton-Smith et al., 2000; Shearer and Bolton-Smith, 2000).
Figure 1. Chemical structures of menadione (vitamin K3), phylloquinone (Vitamin K1), and menaquinone (Vitamin K2). The (n) stands for number of isoprene residues.

Vitamin K2 is suggested to play a role in preventing age-related bone loss and it is essential for the $\gamma$-carboxylation of osteocalcin, a bone matrix protein containing $\gamma$-carboxyglutamic acids, which is synthesized in osteoblasts of bone tissues (Hauschka et al., 1975 and Price 1985). Menaquinone-7 (MK-7) with seven isoprene units, an analog of vitamin K2, is abundant in fermented soybean (natto) (Tsukamoto 2004).

The level of menaquinone-7 (MK-7) is found to be very low in different food products except for fermented soybean foods such as natto (9.39 μg/g), Hikiwari natto (chopped natto, 8.27 μg/g) and black soybean natto (796 μg/g) (Kamao et al., 2007). It is shown that, an appropriate amount of MK-7 may be significant in preventing age-related bone loss, though, it is been not yet determined the biological effect of this substance. The prolonged dietary intake of MK-7 has a preventive effect on bone loss induced by ovariectomy in rats was demonstrated recently.
Recent research has demonstrated that MK-7 consumption significantly reduces the risk of bone fractures (Schurgers et al., 2007; Truong and Booth, 2011) and cardiovascular disorders (Gast et al., 2009; Theuwissen et al., 2012). Recommended daily intake of vitamin K by European experts suggested that, preferably in the form of vitamin K2 is 200–500 μg/day which is required for optimal carboxylation of extrahepatic GLA proteins. Natto, a Japanese fermented food, has traditionally been obtained from cooked soybean fermented with Bacillus species under SSF (Sakano et al., 1988). In Japan, natto has received approval as a nutritional food containing high amount of MK-7.

There is growing evidence that vitamin K, which is a nutritional factor, may play a role in the regulation of bone metabolism. Vitamin K2 (menatetrenone, MK-4) is essential for the γ-carboxylation of osteocalcin, a calcified tissue protein containing γ2-carboxyglutamic acids that is synthesized in osteoblasts of bone tissue (Hauschka et al., 1975 and Price 1985). Noncarboxylated osteocalcin cannot bind to hydroxyapatite in mineralized tissues (Hauschka and Carr, 1982; Price 1985). Vitamin K role in bone metabolism has been paid much attention, because its supplementation may be considered as an important therapeutic tool for osteoporosis. (Hart 1985; Knapen et al., 1989).

Recent studies have shown that MK-7 can stimulate calcification in the femoral-metaphyseal tissues obtained from normal rat’s in vitro (Ehara et al., 1996 and Sato et al., 1996). The action of MK-7 on bone calcification has been shown to have the same effect as menaquinone-4 (MK-4) (Sato et al., 1996). Natural MK-7 is highly contained in the fermented soybean (Sato et al., 1996). It has been shown that the intake of dietary MK-7 can prevent ovariectomy-induced bone loss in rats (Yamaguchi et al., 1999 and Yamaguchi et al., 2000) suggesting a role in the prevention of osteoporosis (Tsukamoto et al., 2000(a); Tsukamoto et al., 2000(b).
2.4. MICROORGANISMS PRODUCING MENAQUINONES

Biological synthesis of vitamin K2 (menaquinones) is restricted only to microorganisms. The plants are not known for the synthesis of vitamin K2 as they are known only to produce phylloquinones (vitamin K1). Among the microorganisms MK can be produced by Archaea and bacteria, such as green sulfur bacteria, *flavobacteria* and Gram-positive bacteria (Nowicka and kruk, 2010). There are several genera and species known among bacteria for the production of menaquinones. The important microorganisms involved in the production of menaquinones are as follows.

2.4.1. BACTERIA

Several genera of bacteria are well recorded for the production of vitamin K2. *Bacillus subtilis* (Sato et al., 2001a) was the predominantly used bacteria for the large scale production of vitamin K2. As described previously, MK is mainly synthesized by bacteria such as *Bacillus cereus*, *Bacillus mycoides*, *Escherichia coli*, *Flavobacterium*, *Lactic acid bacteria*, *Mycobacterium tuberculosis*, *Sarcinia lutea* and *Serratia marcescens* (Bentley and Meganathan, 1982; Hiratsuka et al., 2008). In addition to this, *Bacillus amyloliquefaciens* BY01 has been reported with high productivity of menaquinones for cheongukkjang production (Wu and Ahn, 2011).

2.5. PATHWAYS OF SYNTHESIS OF MENAQUINONES

During menaquinone-7 biosynthesis, the isoprene side chain and quinone skeleton (1, 4-naphthoquinone) is dependent on the presence of carbon sources such as glucose, fructose and glycerol in the fermentation media. The presence of mono, di and polysaccharides and glycerol in the fermented medium can be used in glycolysis process by *B. subtilis* strains which enhance the production of MK-7 (Sonenshein et al., 2002).
The MK biosynthetic pathway is initiated by isomerization of chorismate to isochorismate catalysed by MenF (Daruwala et al., 1996). In the subsequent reaction conducted by MenD, the isochorismate condensates with the thiamine pyrophosphate (TPP) anion of succinic semialdehyde, resulting in the formation of 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) (Palaniappan et al., 1992). This is dehydrated by MenC to the aromatic benzenoid compound o-succinylbenzoate (OSB) (Sharma et al., 1993). The OSB is converted by MenE to a CoA thioester (Sharma et al., 1996), followed by cyclization of the naphthalene aromatic ring and thioesterolysis of the CoA by sequential action of MenB and MenH enzymes, generating 1,4-dihydroxy-2-naphthoate (NA) (Sharma et al., 1992; Meganathan, 2001). In the last steps, coordinated by MenA and MenG proteins, NA is prenylated and methylated giving the end product MK (Koike-Takeshita et al., 1997; Suvarna et al., 1998) (Figure 2).

![Figure 2. Biosynthetic pathway of menaquinone-7 (MK-7)](image-url)
2.6. MECHANISM OF ACTION OF VITAMIN K2

The importance of vitamin K to coagulation is well known as it activates coagulation factors synthesized in the liver. Vitamin K is an essential cofactor for the posttranslational conversion of glutamate (Glu) residues into gamma-carboxyglutamate (Gla). In these reactions the reduced form of vitamin K (KH2 – hydroxyquinone or quinol) de-protonates glutamate via gamma-glutamylcarboxylase enzyme. The epoxide (KO) formed is recycled via vitamin K epoxide reductase and quinone reductase, and glutamic acid containing proteins such as coagulation factors II, VII, IX and X, protein C, and protein S, are carboxylated.

![Figure 3. Mechanism of action of vitamin K2](image)

Carboxylation of vitamin K-dependent proteins conveys ability to bind calcium ions, which is essential for their biological activity. Compared to other vitamin K analogues, vitamin K2 has the most potent gamma-carboxylation activity. Elevated levels
of under carboxylated osteocalcin (OC), a vitamin K-dependent protein involved in bone
metabolism, may result from subclinical vitamin K deficiency and are frequently
observed in the elderly. It is hypothesized that an inadequate dietary intake of vitamin K
may similarly result in under carboxylation of vascular matrix Gla protein (MGP) leading
to enhanced calcification of atherosclerotic lesions and, consequently, an increased risk of
coronary heart disease (Figure.3).

2.7. PRODUCTION OF VITAMIN K2

2.7.1. SUBMERGED FERMENTATION

The process of production of vitamin K2, like any other products was mainly by
microbial fermentation. The major process of production of vitamin K2 was in aqueous
systems indicating submerged fermentation. Several reports have been made on the
production of vitamin K2 in submerged system by various researchers. Sato et al.,
(2001a), have reported on the production of vitamin K2 in submerged condition
employing *Bacillus subtilis*, isolated from commercially available natto. Soyabean extract
was used as a basal medium in their study. Sato et al., (2001b), studied on the production
of vitamin K2 in submerged condition by *Bacillus subtilis* (natto) strain MH-1 as a parent
strain and menadione (vitamin K3)-resistant mutants were derived by N-methyl-\(\text{N}\)-nitro-
N-nitroso-guanidine (NTG) treatment.

Morishita et al., (1999), studied the production of menaquinones (MK) K2 in
submerged condition by lactic acid bacteria using synthetic media i.e., Rogosa medium
and then produced a beneficial quantity for dietary supplement using those strains grown
either in reconstituted nonfat dry milk or a soymilk medium. Recently, Berenjian et al.,
(2011a), have extensively worked and reported on the production of vitamin K2 (MK-7)
in submerged condition employed to optimize the effective factors for the extraction of
MK-7 using the design of experiments (DOE).
Berijean et al., (2011b), studied the effect of addition the limiting nitrogen and carbon sources for enhancing MK-7 production in submerged fermentation. Berenjian et al., (2012), reported on the enhanced production of MK-7 by the addition of glycerol in a fed-batch process in submerged condition. Both submerged and solid state systems were generally being followed for the production of MK-7.

2.7.2. SOLID STATE FERMENTATION

Solid-substrate fermentation (SSF) has been practiced for many centuries especially for the production of fermented foods. Indigenous SSF describes the microbiological transformation of plant raw materials into highly nutritious foods and flavor enhancing ingredients including Koji, Tempeh, Sake, Soy sauce etc. The scientific studies of principles behind SSF, identification of the essential microorganisms, development of suitable and versatile equipment, control of the process, and quality control of the substrate and final product can have significant impacts on the availability and consumption of these fermented foods (Paredes-Lopez et al., 1988).

Vitamin K2 is predominantly being produced by using natto as a chief substrate by microbial fermentation, expected to be under solid state conditions. The Japanese fermented food natto contains Menaquinone-7(MK-7). Natto is traditionally produced via the solid state fermentation of soybeans by Bacillus subtilis natto (Mahanama et al., 2011). Natto is made from soybeans, typically natto soybeans. Smaller beans are preferred, as the fermentation process will be able to reach the center of the bean more easily. The beans are washed and soaked in water for 12 to 20 hours to increase their size. Next, the soybeans are steamed for 6 hours. The beans are mixed with the bacterium Bacillus subtilis, known as nattō-kin in Japanese. From this point on, care will be taken to keep the ingredients away from impurities and other bacteria. The mixture is fermented at 40 °C (104 °F) for up to 24 hours. Afterward the natto is cooled, then aged in a
refrigerator for up to one week to allow the development of stringiness. (https://en.wikipedia.org/wiki/Natt%C5%8D) Natto.in. 2004. Retrieved 2013-09-15). The crude fermented product i.e., natto can be used directly as a food supplement as it is known for rich in Menaquinone-7(MK-7) (Sakano et al., 1988).

Mahanama et al., (2011), assessed both the methods, static and dynamic solid state fermentation by using different solid substrates and various moisture levels to obtain highest MK-7 concentrations.

Wu and Ahn, (2011), reported in their studies with high productivity of Menaquinone for cheonggukjang production by Bacillus amyloliquefaciens BY01. Cheonggukjang, a Korean traditional fermented food, which is made from cooked soybeans fermented with microbes including Bacillus sp. for over two days. It is regard as a good source of protein, hydrolyzed peptides and lipids, and is popularly consumed for its health benefits (Cho et al., 2011).

Wu and Ahn, (2011), reported in their studies about improved menaquinones (vitamin K2) production in cheonggukjang by optimization of the fermentation conditions. Mahanama et al., (2012), revealed in their research, where they carried out to cater the need for industrial production of supplementary MK-7 by mimicking the solid state fermentation conditions of natto to produce superior MK-7 concentrations as most of the published research has been carried in liquid state fermentation (LSF). Their study was involved in modeling the effect of bed height and particle size for vitamin K2 production in a static bed fermenter. They evaluated the suitability and utility of soy protein granules as best substrate for optimization of key fermentation factors including incubation time, size and substrate bed thickness.
2.8. PROCESS OPTIMIZATION FOR THE PRODUCTION OF VITAMIN K2 (MK-7)

The role of various physicochemical factors and nutritional components are very important and critical to achieve the maximum product in any biological process in general and microbiological process in particular. Several reports have been recorded on the influence of pH, temperature, carbon sources, nitrogen sources and phosphate sources for the production of vitamin K2 in both submerged and solid conditions in different media.

2.8.1. OPTIMIZATION OF FACTORS IN SUBMERGED FERMENTATION

A range of various pH was assessed and reported by several researchers for the maximum production of vitamin K2 under submerged conditions in various media employing different organisms. Sato et al., (2001a), examined the production of vitamin K2 at the pH range of 7.3 of the medium. It was found suitable pH for MK production. Sato et al., (2001b), assessed the influence of pH on the production of vitamin K2 in submerged condition, they first carried out cultivation without controlling the pH which decreased from 7.3 to 5.5, then spontaneously increased to 7.7-8.0. The concentration of MK-7 began to increase after the cell growth reached its maximum level, reaching 33.0-37.0 mg/L after 4 days. The effect of pH on MK production was examined. When the pH was controlled at 5.7, 6.0, 7.0, 7.5 and 8.0, only small quantities of MK-7 were obtained despite good cell growth.

Effect of temperature on the production of vitamin K2 was assessed by several researchers, from 30°C to 48°C in submerged conditions wide range of temperature and their influence on the maximum production of vitamin K2 were recorded by Sato et al.,
A wide spectrum of carbon sources were employed by different researchers to achieve the maximum production of vitamin K2 under submerged conditions. (Sato et al., 2001a) have studied as many as eleven different carbon sources to understand the maximum production of vitamin K2. They have examined the effect of glucose, mannose, galactose, fructose, sucrose, ribose on the production of vitamin K2.

Various nitrogen sources were employed by different researchers for the maximum production of vitamin K2 under submerged conditions. Sato et al., (2001a) have tested as many as nine different nitrogen sources for the production of vitamin K2. Sato et al., (2001b), have tested soyabean extract, tryptone, peptone, nutrient broth, corn steep liquor, soytone, phytone, polypeptone, beef extract, skim milk as different nitrogen sources for the maximum production of vitamin K2.

2.8.2. OPTIMIZATION OF FACTORS IN SOLID STATE FERMENTATION

Similar to the studies on the influence of various factors and nutritional components for the maximum production of vitamin K2, under submerged conditions, few reports have also been made under solid state conditions for the maximum production of vitamin K2 employing variety of natto as substrate.

Mahanama et al., (2011), investigated the effects of static and dynamic solid state fermentation on the production of MK-7 using polenta (milled corn substrate), nixtamalized corn grits, semolina (milled wheat substrate) and soy protein granules. In addition, the effect of initial moisture content on MK-7 concentration was measured for both dynamic and static SSF and all substrates. During the experiment the following
process parameters were kept constant, incubation temperatures (37°), substrate wet weight (39), incubation time (3 days) and relative humidity (~8.5%).

Mahanama et al., (2012), reported that soy granules were used as a substrate for microbial production of vitamin K2 using Bacillus subtilis, static bed “tray type” fermentation was carried out and the operation of this fermentation was optimized using full factorial statistical method of analysis. The optimum parameters were obtained as: bed thickness; particle size, incubation time.

Ahmad et al., (2013), their research was to maximize production of menaquinone-7(MK-7) by solid state fermentation process. Menaquinone-7 (MK-7) was produced by Phaseolus vulgaris in presence of a co-cultured by Bacillus subtilis. Three separate nutritional parameters were screened for each experiment design. Parameters were optimized by Box-Behenken design of response surface methodology for the production of menaquinones-7(MK-7).

Glycerol, mannitol, sorbitol, maltose, yeast, soybean extract, Urea, (NH4)2SO4 and MnCl2 were the nine medium constituents selected for the study.

Wu and Ahn, (2011), reported to improve the content of menaquinones (MK) in cheonggukjang by using Bacillus amyloliquefaciens KCTC11712BP, the fermentation conditions were optimized. The optimum temperature for menaquinones production was 43°C as determined by the experiment carried out from 37 to 46°C. The effect of carbon sources on MK production was determine with the addition of various carbohydrates during the cheonggukjang manufacturing process. i.e., glucose, glycerol, maltose, mannose and starch.
2.9. EMPLOYING OF RESPONSE SURFACE (RSM) METHODOLOGY

The conventional ‘one parameter at-a-time’ is the most common method being used for the production of bio-molecules of microbial origin (Prakasham et al., 2006). In this type of study optimum parameter can be determined but it may fail to determine the critical level of parameter. To overcome from this problem a statistical method response surface methodology (RSM) is being employed since from last one and half decade. RSM is consists of a group of mathematical and statistical techniques used in the development of adequate functional relationship between a response of interest and a number of associated control (or input) (Andre and Siuli 2010). This optimization technique gained significant interest owing to its application towards the determination of critical process variables and their relation in achieving optimum productivity in fermentation process. There is very limited literature on the usage of RSM for the production of MK-7.

A face centered central composite design of RSM was employed to investigate the interactive effects of five variables namely glycerol, mannitol, yeast extract, malt extract and calcium chloride which were identified as optimal sources earlier by one-factor-at-a-time approach for the production of MK-7 by Bacillus subtilis. Employing of this statistical approach contributed significant enhancement in MK-7 production (Singh et al., 2015).

In a study the Plackett-Burman experimental design of RSM for eleven variables i.e., nine nutritional components (independent variables) and two dummy variables were used to evaluate the relative importance of various nutrients for a higher production of MK-7 production by (Ahmad et al., 2013) using Bacillus subtilis NCIM 2708 strain.

RSM was also employed in solid state fermentation to investigate different process variables i.e., Bed thickness, particle size and incubation time, for the production
of MK-7 by *Bacillus subtilis* using the Full factorial design with three independent variables (Mahanama *et al.*, 2012). On the other hand plenty of research reports are available on implementation of RSM designs for optimizing process variables in many bioprocesses which involved in the production of various bio-molecules from microbes. The Box-Behenken Design of RSM was used to determined the optimum level of key process variables to obtain the maximum MK-7 concentration. In this study, three independent variables effecting MK-7 production such as fermentation medium pH, fermentation temperature and inoculums volume were studied at different levels. (Singh *et al.*, 2015). There is much more evidences are available on the contribution of RSM approach towards the improvement of several bioprocess methods for the production of microbial products.

### 2.10. ENHANCED PRODUCTION OF VITAMIN K2

#### 2.10.1. STRAIN IMPROVEMENT STRATEGIES

For the industrial production of vitamin K2 it has been a common strategy to use random mutagenesis in order to generate strains that produce vitamin K2 in high yields. Generally this has been achieved by treating the appropriate microorganisms with mutagenic agents like, N-methyl-N-nitro-N-nitroso-guanidine (NTG) or UV light, and selecting the strains with practical advantages, such as productivity, genetic stability, reasonable growth rates and resistance to high concentrations of toxic intermediates present in the medium (Khovsky *et al.*, 1998).

These programs consist essentially of the treatment of the productivity microorganisms with a mutagenesis agent and for the selection of the strains bringing mutations leading to some practical advantage (higher productivity, good genetic stability, resistance to higher concentrations of substances present in the medium, higher
rate of growth, etc). As this kind of mutant is extremely rate (of the order of $10^{-5}$), their selection obtained by direct examination of all the strains deriving from the mutagenesis treatments is a very tedious and long lasting task. As soon as knowledge on the biosynthetic pathway progresses, this essentially random technique is implemented and more rational and productive techniques are adopted.

Sato et al., (2001a) reported that the cells were treated with N-methyl-N-nitro-N-nitroso-guanidine (NTG) at a concentration of $10^8$ cells/ml for the production of menaquinones (vitamin K2) by Bacillus subtilis. Another study by Sato et al., (2001), was the menadione (vitamin K3)-resistant mutants were derived by NTG, treatment for the efficient production of menaquinones (vitamin K2) by a menadione-resistant mutant of Bacillus subtilis.

Tsukamoto et al., (2001), reported that the strain was treated with ultraviolet rays (UV) as a physical method or NTG as a chemical one was used for the high productivity of vitamin K2 (menaquinone-7) by Analog resistance using Bacillus subtilis (natto).

Song j et al., (2014), reported the study to enhance the production of vitamin K2 by using N-methyl-N-nitro-N-nitroso guanidine (NTG) and low energy ion beam implantation and optimizing the fermentation medium.

2.11. PURIFICATION OF MK-7

In bioprocess practices, the menaquinones produced are always mixed with lipid content of bacterial cell wall. Hence recovery and purification of menaquinones is difficult task. Therefore, based on the production technique, downstream process can be designed with multi step process and usually it can be achieved by common methods such as, various chromatographic techniques i.e., thin layer chromatography, column chromatography and high performance liquid chromatography, etc.
Several research findings are available on many microbial sources from which menaquinones have been isolated and purified. An equal volume of petroleum ether or diethyl ether was added to the acetone mixture, followed by 10 volumes of water. The petroleum ether or diethyl layer containing the lipids was separated from the aqueous phase, dried over anhydrous sodium sulfate, and evaporated to dryness under nitrogen. The material was resuspended in petroleum ether and chromatographed on a column of acid-washed silicic acid (100 to 200 meshes, Clarkson Chemical Company, Inc., Williamsport, Pennsylvania) or Permutit, Folin (Fisher Scientific Company). The column was washed with petroleum ether, and the quinine was eluted with 3% diethyl ether in petroleum ether from silicic acid or 4% diethyl ether in petroleum ether from Folin (Dunphy et al., 1968). Sato et al., (2001b), purified the menaquinones by using 2-Propanol (1.2 L) and n-hexane (2.4 L) was added to the culture (1 L) of Bacillus subtilis. After this mixture was vigorously shaken and allowed to settle for 1 h, the n-hexane layer was removed and n-hexane was evaporated. An oily product (about 800 mg) was obtained. The oily product was extracted with 5 ml of n-hexane, and insoluble materials were removed by centrifugation. The resultant solution was charged on 400 ml of silica gel in a column (650mm*45mm). The column was washed with 500 ml of n-hexane and MK was separated using toluene/n-hexane(1:2,v/v). Each 30 ml fraction was analyzed by HPLC, and the fractions containing MK were obtained. About 10 mg portions of MK-rich compounds were charged on 50 ml of ODS-silica gel in a column (500 mm*22mm). MK-5, MK-6, MK-7 and MK-8 were separated using methanol/ acetonitrile (1:1, v/v). Morishita et al., (1999), separated menaquinones extracts by HPLC equipped with a postcolumn electrochemical reducer (Environmental Sciences Associates, Chelmsford, MA, (USA), a fluorometric detector as mentioned by (Hirauchi K et al., 1986 and Hirauchi K et al., 1991) in their studies and a reverse –phase column (Inertsil ODS-3;4.6
i.d.*250mm., GL-Science Co. Ltd., Tokyo, Japan). Many other researchers also purified and analysed by HPLC (Kroppenstedt; 1982; Cooke et al., 2006).

2.12. CHARACTERIZATION OF MK-7

MK-7 was characterized using HPLC. Yamamoto et al., (1998), reported in their studies by HPLC profile of standards of menaquinones. Standard MK-6, MK-7, MK-8 and MK-9 eluted at retention times of 8.91 min, 11.76 min, 15.33 min and 20.47 min, respectively. The major menaquinones extracted from 10 species eluted at 11.76 min, which was identified as MK-7 by the HPLC profile of standard MK-7 prepared from *M. thermaacetica* DSM 521^T^. These results shown that all studied thermophilic clostridia with the exception of *Thermoanaerobacterium thermosaccharolyticum* DSM571^T^ contained MK-7 as the major component of the isoprenoid quinone system. The sample eluted at the same retention time with MK-7 standard was also identical with MK-7 by photodiode-array detector scanning from 200 to 400 nm. Berenjian et al., (2011a) and Mahanama et al., (2012), confirmed the molecular weight of MK-7 using LC-MS system (LCMS-2010EV, Shimadzu, Japan) with atmospheric pressure chemical ionization (APCI) ion source for the ionization in negative ion mode at 2KV and 250 °C. Compound UV spectra were acquired by collecting the entire wavelength was set at 360 nm±20 nm bandwidth. For the structural elucidation of MK-7 variants the mass spectrometer was operated in scan mode covering the mass range of 50-1000 m/z. The CDL voltage was 0V, Q-array DC voltage at -5V and RF was set at 150V. Nitrogen was used as a nebulising gas and was delivered at a flow rate of 2 l/min. Other researchers also reported the use of mass spectrometer for confirmation of MK-7 (Sato et al., 2001a; Sato et al., 2001b). Yamamoto et al., (1998), reported the details of study of their work, the mass spectrum obtained for the compound from a sample of *Caloramator fervidus* ATCC
The spectra shown a base peak at \( m/z \) 225 and another prominent peak at \( m/z \) 187. Those peaks are indicative of menaquinone. A relatively strong peak at \( m/z \) 648 shows that the multiprenyl side chain is 7 isoprene units long and is fully unsaturated. In addition, lower-intensity peaks were observed at \( m/z \) 239, 307, 375, 443, 511 and 579, pointing to the presence of a mutiprenyl side chain. Sato et al., 2001, confirmed the structure of MK-7 by \(^1\)H-NMR (JNM-EX400, JEOL Ltd., Tokyo). Furthermore, all bands observed in the proton NMR spectrum of the isolated MK-7 (δ 8.05, 7.75, 5.08, 3.37, 2.19, 2.00, 1.80, 1.66 and 1.59 ppm) corresponded to those of standard MK-4. Similar results were shown by Das et al., (1989).

2.13. APPLICATION STUDIES OF MK-7

There is growing evidence that vitamin K2 may play a role in the regulation of bone metabolism. Vitamin K2 is essential for the \( \gamma \) – carboxylation of osteocalcin, a calcified tissue protein containing \( \gamma\text{-carboxyglutamic acids} \), which is synthesized only in osteoblasts (Hauschka et al., 1975; Price 1985). Non carboxylated osteocalcin cannot bind to hydroxyapatite in mineralized tissues (Hauschka and Carr 1982; Price 1985). Much attention has been paid to the role of vitamin K in bone metabolism, because its supplementation may be important as a therapeutic tool for osteoporosis.

2.13.1. VITAMIN K AND OSTEOPOROSIS: ANIMAL STUDIES

The effects of vitamin K on bone remodeling were studied in rats in which osteoporosis was experimentally induced by different means such as ovariectomy, orchietomy, glucocorticoids, removal of the sciatic nerve, tail suspension and calcium or magnesium deficient diet (Giammanco et al., 2012)
2.13.1.1. EFFECTS OF VITAMIN K2 ON OVARIECTOMIZED RATS

Recently, it has been demonstrated that vitamin K2 (Menaquinone-7) can directly stimulate calcification in the femoral metaphyseal tissues obtained from normal rat’s in vitro (Ehara et al., 1996; Sato et al., 1996). The action of menaquinones-7(MK-7) on bone calcification has been shown to have the same effect as menaquinones-4(MK-4) (Sato et al., 1996). Therefore, Yamaguchi et al., (1999), investigated the preventive effect of dietary MK-7 and the fermented soybean (natto) containing MK-7 on ovariectomy (OVX) – induced bone loss.

Ma et al., (2001), revealed in their studies, that the intake of supplement containing zinc and MK-7 caused a significant increase in femoral dry weight, alkaline phosphatase activity, DNA, calcium and zinc contents in the femoral-diaphyseal and metaphyseal tissues of elderly rats, indicating that the intake can reveal an anabolic effect on bone metabolism. This finding suggested that the supplemental intake of zinc and MK-7 has a preventive effect on bone loss with ageing.

2.13.1.2. EFFECTS OF VITAMIN K2 IN RATS TREATED WITH GLUCOCORTICOIDs

It is well established that glucocorticoids (GCs) treatments may cause to numerous clinical complications, including bone loss and increased risk of fracture (Feng and McDonald, 2011). The administration of glucocorticoids in rats causes a decrease of the osteoblastic activity (Feng and McDonald, 2011). This phenomenon leads to a decreased osteogenesis and, consequently, may cause the onset of cortical and spongy osteopenia of the tibia and femoral length and a decrease of bone density, bone strength and calcium content. These effects can be prevented by vitamin K2 treatment (Hara et al., 1993; Hara et al., 2002). In line with these studies, Iwamoto et al., (2009), have recently shown that
vitamin K2 reduces the suppression in glucocorticoids treated rats. The mechanism by which vitamin K2 induced GC bone loss appears to be due to its ability in preventing the reduction of osteoprotegerin (OPG) a cytokine which inhibits both the differentiation and functions of osteoclasts (Sasaki et al., 2005; Boyce and Xing, 2007). These finding further support the clinical value of vitamin K2 in the treatment and prevention of GC induced osteoporosis.