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VITAMIN K2 (MENAQUINONE-7) PRODUCTION BY *BACILLUS SUBTILIS NATTO* IN A BIOFILM REACTOR

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ABSTRACT

Menaquinone-7 (MK-7), a subtype of vitamin K, has received significant attention due to its effect on improving bone and cardiovascular health. Current fermentation strategies, which involve static fermentation without aeration or agitation, are associated with low productivity and scale-up issues and hardly justify the commercial production needs of this vitamin. Previous studies indicate that static fermentation is associated with pellicle and biofilm formations, which are critical for MK-7 secretion while posing significant operational issues. Therefore, the present study is undertaken to evaluate the possibility of using biofilm reactors as a new strategy for MK-7 fermentation.

In the first phase of the study, 15 *Bacillus* species namely *Bacillus subtilis natto*, *Bacillus licheniformis*, and *Bacillus amyloliquifaciens* as well as 4 plastic composite supports (PCS) were investigated in terms of MK-7 production and biofilm formation. Results show the possibility of using a biofilm reactor for MK-7 biosynthesis. *Bacillus subtilis natto* strain NF1 and soybean flour yeast extract PCS (SFY) in glucose medium were found as the most efficient combination for production of MK-7.

In the following phase, biofilm reactors were constructed using the selected Plastic Composite Support (PCS) and *B. subtilis natto* strain NF1 for MK-7 production. Using response surface methodology (RSM), optimum growth parameters including temperature, pH, and agitation were determined in a glycerol-based medium. Results were presented in a statistical model (R^2=0.90), leading to optimum growth conditions of temperature (35°C), agitation (200 rpm) and pH (6.58). Model predicted MK-7 concentration was validated and MK-7 concentration of 12.1±1.2 mg/L was produced in the biofilm reactor. The obtained concentration was 58% higher as compared to the suspended-cell culture (7.7±1.5 mg/L).

Then, in order to optimize the same fermentation growth parameters for MK-7 production in the glucose-based medium, Central Composite Design (CCD) was carried out along with supplementary runs to determine the optimum conditions. The biofilm

reactors were able to produce a maximum concentration of 18.4±0.8 mg/L of MK-7, which was 237% higher than the suspended-cell fermentation.

The next phase was undertaken to utilize biofilm reactor by optimizing the components in the glucose-based medium. Response Surface Methodology (RSM) was used to determine optimum concentrations of three major medium components (glucose, yeast extract, and casein). Maximum MK-7 concentration in biofilm reactors was achieved as 20.46±0.51 mg/L, which was 344% higher compared to the suspended-cell reactors containing the same optimum media composition.

Later, optimization phases were undertaken to utilize biofilm reactors in investigating and optimizing different media components in the glycerol-based medium. By using Response Surface Methodology (RSM), the effects of glycerol, yeast extract, and soytone were studied in the fermentation medium on MK-7 production in biofilm reactor. With a composition of 45 g/L of glycerol, 5 g/L of yeast extracts, 10 g/L of soytone and 0.06 g/L of K_2HPO_4, MK-7 concentrations could reach 14.7±1.4 mg/L in biofilm reactors, which was 57% higher compared to the MK-7 concentration achieved in suspended-cell reactor under similar conditions. While glycerol was depleted by the end of the fifth day in the biofilm reactor, it was never depleted in the suspended-cell reactor. Evidently, biofilm reactors present a reliable strategy likely to mitigate the operational issues with MK-7 biosynthesis at the industrial scale.

Then, fed-batch strategies were investigated for glucose and glycerol-based media, as carbon source addition seemed crucial in batch fermentations. Results indicated that fed-batch additions can be significantly effective in glucose-based medium, increasing the end product concentrations to 28.7±0.3 mg/L of MK-7 which renders the biofilm reactors a potential replacement for static fermentation strategies with a maximum 32.5±0.4 mg/L of MK-7. Moreover, morphological changes of the applied *B. subtilis* strain was tracked during the 12 day long runs and finally, SEM investigations confirmed robust biofilm and extracellular matrices formed on the Plastic Composite Supports (PCS) in the biofilm

reactors. In conclusion, biofilm reactors especially with fed-batch fermentation regimes seem to be an effective tool to enhance MK-7 productions on industrial scales.

In order to reach the desired MK-7 quality, several downstream processing including extraction, drying, ultrasonication, etc. must be carried out after its biosynthesis in the broth. These processes, however, need to be carried out in such way to ensure least amount of losses and maximum recovery into the end-product. Therefore, in this phase of the study, drying, storage, and ultrasonication steps were evaluated under different conditions. Results showed that drying under forced air flow is not only fastest, but also demonstrated a better preservation of the vitamin and should replace the vacuum drying. Ultrasonication for 15 minutes seem to be harmless and sufficient for phase transition in analysis. Also, storage at refrigerated temperatures seem to preserve MK-7 at least for one week. Static liquid fermentations were conducted in McCartney bottles to explore the maximum MK-7 secretion potentials in different glycerol and glucose-based media compositions that were optimized in our previous studies. Maximum 32.5±0.4 mg/L and 14.6±0.4 mg/L concentrations were achieved in glycerol and glucose-based media respectively. Furthermore, fermentations in deeper culture tubes indicated how the MK-7 concentrations are distributed in different zones of the static liquid broth. Results in general, showed a clearer road map to ensuring better quality and preservation of the valuable end-product and enlightened more the path to further scaling up the fermentation process when compared with results obtained in optimized biofilm reactors in the previous steps.

Last phase in the study was to mathematically model the findings in batch fermentations in the biofilm reactors and thus further elucidate the conditions governing fermentation. The logistic equation was modified to correlate substrate consumption with fermentation time and was utilized to model the substrate consumption in the four batch fermentations. Results indicated very accurate fits and therefore there is no need for more complex equations ($R^2 > 0.953$). Then, this successfully modified-logistic equation was inserted into the basic Luedeking-Piret equation modifying it to model MK-7 production based on substrate consumption. Furthermore, modified-Gompertz model was also used

for the same purpose. Results indicated more accurate fits by the modified Luedeking-Piret equation ($R^2 = 0.9705, 0.943, 0.970, and\ 0.959$) compared to the modified-Gompertz ($R^2 = 0.914, 0.943, 0.949\ and\ 0.860$). Yet, the modified Luedeking-Piret equation was a more complex model compared to the modified-Gompertz.

In summary, bench-top biofilm reactors were successfully constructed using suitable PCS and *B. subtilis natto* combination. Growth parameters including temperature, pH and agitation along with medium components including carbon and nitrogen sources in glycerol and glucose-based media were optimized using RSM. Results indicated significant enhancement of MK-7 production in biofilm reactors compared to suspended-cell bioreactors by up to 344%. Furthermore, the fermentation profiles indicated robust carbon source consumptions leading to substrate depletions around halfway of the fermentations. Thus, fed-batch substrate additions were investigated and results indicated up to 28.7±0.3 mg/L MK-7 concentrations in the glucose-based medium with fed-batch additions. These concentrations are comparable with maximum concentrations coming from static fermentation counterparts (32.5±0.4 mg/L). Finally batch results were mathematically modeled and optimum processing procedures were investigated. The result from this study show a potential for larger pilot scale fermentations in biofilm reactors and investigating downstream and in-situ recovery techniques on pilot scales.

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Chapter 1

Introduction

Vitamin K refers to a group of fat-soluble vitamins the human body needs for complete synthesis of proteins required for blood coagulation, as well as calcium binding in bones and tissues. The vitamin K-related modification of the proteins allows them to bind calcium ions, which they cannot do otherwise. Without vitamin K, blood coagulation is seriously impaired, and hemorrhaging occurs. Low levels of vitamin K also weaken bones and promote calcification of arteries and other soft tissues, and thus cause heart and vascular diseases. Vitamin K occurs naturally in two forms known as vitamin K1 (phylloquinone) and vitamin K2 (menaquinone) (Shearer and Newman, 2008). Phylloquinone is present in the green leafy parts of plants, where it has a direct function as an electron receptor during photosynthesis. The pure extracted form of phylloquinone is a viscous yellowish oil (Binkley et al., 1939).

Specifically menaquinone-7 (MK-7), a highly valuable member of the vitamin K family, has a significant effect on preventing osteoporosis and cardiovascular diseases (CVD) besides its positive effects on blood coagulation (Gast et al., 2009; Schurgers et al., 2007). In the United States alone, the annual cost of treatment for osteoporotic fractures and CVD was estimated to be $22 billion (Blume and Curtis, 2011) and $502 billion (Lloyd-Jones et al., 2010), respectively. Due to the increasing number of patients with osteoporosis and CVD, researchers seek an alternative approach to preventing these health complications. A therapeutic dose of MK-7 to be effective in treatment of osteoporosis and CVD from MK-7 rich dietary sources (i.e., cheese, meat, and fermented soybean) would require the consumption of impractically large quantities of these food. For instance, hard cheeses typically have about 1.5 μg of MK-7 per 100 gram of the

cheese, which is quite insignificant (Howard and Payne, 2006). 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 (Berenjian et al., 2015). Therefore, it is desirable to develop a rich source of MK-7 as a dietary supplement. In order to accomplish this goal, MK-7 needs to be produced by an economical approach at commercial scales.

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 (Meganathan 2001; Morishita et al., 1999; Tsukamoto et al., 2001). Moreover, menaquinones have also been produced by *Flavobacterium*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and lactic acid bacteria such as *Lactococcus lactis* ssp. *cremoris* and *Leuconostoc lactis* (Tani and Taguchi, 1989; Goodman et al., 1976; Morishita et al., 1999). However, *Bacillus subtilis natto* is the major microorganism for the industrial production of MK-7. It is originally used in natto, a traditional Japanese food, which is made from soybeans fermented by *Bacillus subtilis natto.* Thus, *Bacillus subtilis natto* is an excellent source for producing MK-7 (Berenjian et al., 2013a).

The fermentation of *Bacillus subtilis natto* in static culture is associated with pellicle formation, suspended in liquid media. On the other hand, *B. subtilis* is able to form biofilm, a microbial community, which is formed by the attachment of the microbial community on biotic or abiotic surfaces and embedment in an extracellular matrix, which is self-produced by microbial community (Burmolle et al., 2006 ; Xu et al., 2011). In comparison with planktonic cells, several types of microorganisms have the ability to form a biofilm due to a specific genetic expression and morphologies resulting in production of different molecules and secondary metabolites (Branda et al., 2005; Kuchma and O'Toole, 2000). Biofilm formation has been reported to increase, several-fold, the production of enzymes (Villena and Gutiérrez-Correa, 2006) microbial exopolysaccharides (Cheng et al., 2010), and antibiotics (Rahman et al., 2007), compared to the suspension fermentation via certain genetic changes that cells undergo during passive immobilization. Berenjian et al. (2013) concluded that the static fermentation of *B. subtilis natto* results in the formation of

both pellicle and MK-7 and that temperature and nutrients have significant impacts on pellicle and MK-7 formation. They have called the suspended and floating microbial formations in static fermentation, biofilm, but it seems the term "pellicle" is more suitable for such formations as biofilms are much denser and need to stick to a surface (Kuchma and O'Toole, 2000). In the same study, they also found out that agitation during fermentation reduces the pellicle generation considerably, but only has a small effect on MK-7 production. Therefore, pellicle formation was found not to be crucial for MK-7 production by *B. subtilis natto*. It has been suggested that elimination of pellicle formation will address the mass and heat transfer issues that might arise in large scale fermentation process for MK-7 production (Berenjian et al., 2013b).

Thus, although it seems that pellicle formation by *B. subtilis natto* is not essential for MK-7 production, biofilm formation may improve production conditions significantly. This may be achieved by cell immobilization, which can be achieved by active and passive immobilization methods. Active immobilization is entrapment in a polymer matrix (agar, alginate, polyacrylamide, chitosan, gelatin, and collagen) and covalent binding to surfaces using coupling agents. Furthermore, passive immobilization occurs by natural adsorption and multilayer growth of cells around or within the solid support materials, which is essentially a biofilm (Demirci et al., 2007).

In summary, *Bacillus subtilis natto*'s tendency to form a biofilm along with extracellular nature of MK-7 in *Bacillus subtilis natto* presents the opportunity of utilizing a biofilm reactor for MK-7 production. Therefore, the purpose of this study was to enhance MK-7 production efficiency and productivity by utilizing a biofilm reactor.

Chapter 2

Literature review

This chapter presents general knowledge on different aspects related to this research including: What are vitamins generally and more specifically what is vitamin K? Also, what is special about it? What are vitamin K types and sources? What are the functions and how does it work? Furthermore, chemical and biological characteristics, the role of vitamin K in improving human health and more specifically the special nature of MK-7 are illustrated. Then, past studies on vitamin K production and the future trends for MK-7 production are discussed.

2.1 Vitamins

Vitamins are generally organic compounds that are vital nutrients, which often needed in limited amounts. Vitamins are organic compounds which cannot be synthesized in sufficient quantities by the humans and animals and must be obtained through the diet (Lieberman and Bruning, 1990).

Vitamins do not include either other essential nutrients, such as dietary minerals, essential fatty acids, or essential amino acids (which are not usually synthesized by human metabolism) or the great number of other nutrients that promote health, and are required less often to maintain the health of the organism. Thirteen vitamins are universally recognized at present (Table 2.1). Vitamins are classified by their biological and chemical activity, not their structure. Thus, each vitamin refers to a number of compounds that all show the biological activity associated with a particular vitamin. Some vitamins are named under an alphabetized vitamin generic descriptor such as vitamin A, which includes the

compounds retinal, retinol, and four other known carotenoids as vitamers. Vitamers by definition are convertible to the active form of the vitamin in the body, and are sometimes inter-convertible to one another, as well (Maton 1993).

Vitamins are mainly categorized into water soluble and fat soluble vitamins. Vitamins A, D, E and K of all types are among the fat soluble vitamins and others such as Vitamins B and C are the water soluble vitamins.

Obviously, all vitamins are highly crucial for human health. Vitamin A (Retinol) is best known to be essential for vision in human body, but it is also important in gene transcription and skin health. Vitamin B1 is one of the best known members of the Vitamin B family. Vitamin B1 (Thiamine) deficiency causes Beriberi, a neurological and cardiovascular disease that can be lethal in severe cases. Vitamin B2 (Riboflavin) deficiency known as ariboflavinosis leads to stomatitis including painful red tongue with sore throat, chapped and fissured lips (cheilosis), and inflammation of the corners of the mouth. Although riboflavin deficiency may not be as severe as thiamine deficiency or lethal but it may also cause anemia. Vitamin B3 (Niacin or Nicotinic acid) is the third member of Vitamin B family. Niacin chronic deficiency is the cause of Pellagra, a disease, which was very common during the early decades of the twentieth century in the United States due to the corn consumption, which is the only grain low in digestible niacin. Although Vitamin B5 (Pantothenic acid) deficiency is quite rare and unstudied, it is known to be associated with impaired energy production, due to low CoA levels, which could cause symptoms of irritability, fatigue, and apathy. Vitamin B6 (Pyridoxine) deficiency is also associated with neurological disorders such as neuropathy and confusion in severe cases. Vitamin B7 (Biotin) is the cofactor for numerous enzymatic reactions and its deficiency may cause hair loss, conjunctivitis (pink eyes), depression in adults and many metabolic disorders. Vitamin B9 (Folic acid) deficiency during pregnancy can cause severe damage to the fetus including neural tube defects. Therefore, high uptake of folic acid is recommended to mothers during pregnancy. High uptakes may also reduce the risk of strokes in adults as well. Vitamin B12 (Cobalamins) deficiency can potentially cause severe and irreversible damage, especially to the brain and nervous system. At levels only slightly lower than normal, a range of symptoms such as fatigue, depression, and poor memory may

be experienced. Vitamin C (Ascorbic acid) deficiency is commonly known with scurvy, a disease, which was very common among sailors due to lack of access to fresh vegetables and fruits. Today vitamin C deficiency is rarely seen and despite the common belief that it prevents or reduces severity of common colds, it may just reduce the duration of illness. A diet deficient in vitamin D group in conjunction with inadequate sun exposure causes osteomalacia (or rickets when it occurs in children), which is a softening of the bones. Today, in most developed countries commercial milk is often supplemented with calciferol as vitamin D deficiency has become a worldwide issue in the elderly and remains common in children and adults. As an antioxidant, vitamin E acts as a peroxyl radical scavenger, preventing the propagation of free radicals in tissues, by reacting with them to form a tocopheryl radical, which will then be reduced by a hydrogen donor (such as vitamin C) and thus return to its reduced state. Vitamin E also has an effect on gene expression, plays a role in neurological functions and inhibition of platelet aggregation and also protects lipids and prevents the oxidation of polyunsaturated fatty acids. As a result, it is obvious that vitamin E deficiency can and will cause numerous health risks in human body.

On the other hand, vitamin K is a fat soluble vitamin. Average diets are usually not lacking in vitamin K, and primary deficiency is rare in healthy adults. Newborn infants are at an increased risk of deficiency. In cases of deficiency, symptoms include anemia, bruising, and bleeding of the gums or nose in both sexes, and heavy menstrual bleeding in women. However, osteoporosis and coronary heart disease are strongly associated with lower levels of K2 (menaquinone). Vitamin K2 (MK-7) deficiency is also related to severe aortic calcification and all-cause mortality (Maton, 1993). Vitamin K includes two natural forms, vitamin K1 and vitamin K2; both are fat soluble vitamins and they are found in distinct food sources. vitamin K1 is mostly found in green leafy vegetables while vitamin K2 is only found in animal source foods such as meat, cheese and liver oils (Binkley et al., 1939).

Table 2.1: The discovery dates of the vitamins and their sources and functions*.

Vitamin	Year of discovery	Food source	Solubility	Function
Vitamin B1 (Thiamine)	1910	Rice bran	Water	Nervous system, Beriberi
Vitamin A (Retinol)	1913	Cod liver oil	Fat	Vision, gene transcription
Vitamin C (Ascorbic acid)	1920	Citrus, most fresh foods	Water	Collagen synthesis, Scurvy
Vitamin D (Calciferol)	1920	Cod liver oil	Fat	Bone health, Osteomalacia
Vitamin B_2 (Riboflavin)	1920	Meat, dairy products, eggs	Water	FAD synthesis, Ariboflavinosis
Vitamin E (Tocopherol)	1922	Wheat germ oil, vegetable oils	Fat	Enzymatic activities, gene expression
Vitamin B12 (Cobalamins)	1926	Liver, eggs, animal products	Water	Brain and nervous system
Vitamin K1 (Phylloquinone)	1929	Leafy green vegetables	Fat	Heart and bone health
Vitamin B5 (Pantothenic acid)	1931	Meat, whole grains, in many foods	Water	Coenzyme A synthesis, cell metabolism
Vitamin B7 (Biotin)	1931	Meat, dairy products, eggs	Water	Cell growth, production of fatty acids
Vitamin B6 (Pyridoxine)	1934	Meat, dairy products	Water	Cell metabolism, gene expression
Vitamin B3 (Niacin)	1936	Meat, grains	Water	Pellagra
Vitamin B9 (Folic acid)	1941	Leafy green vegetables	Water	DNA and cell division

*Lieberman and Bruning, 1990.

2.1.1 Vitamin K

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

Soon after the discovery of vitamin K by Dam, it was found out that there are two major forms. One had the smaller molecular weight, was less effective in treatment of symptoms and was mostly found in certain vegetables. This form was called vitamin K1. The other one had larger molecular weight, was more effective and was mostly found in animal sources such as fish meals and liver oils and was called vitamin K2 (Figure 2.1) (Binkley et al., 1939).

Vitamin K1, also known as phylloquinone, phytomenadione, or phytonadione, is synthesized by plants, and is found in highest amounts in green leafy vegetables, because it is directly involved in photosynthesis (Widhalm et al., 2012). It may be thought of as the "plant" form of vitamin K. It is active as a vitamin in animals and performs the classic functions of vitamin K, including its activity in the production of blood-clotting proteins. Animals may also convert it to vitamin K2 (Davidson et al., 1998).

On the other hand, vitamin K2, the main storage form in animals, has several subtypes, which differ in isoprenoid chain length. These vitamin K2 homologues are called menaquinones, and are characterized by the number of isoprenoid residues in their side chains. Menaquinones are abbreviated MK-n, where M stands for menaquinone, the K stands for vitamin K, and the n represents the number of isoprenoid side chain residues.

For example, menaquinone-4 (abbreviated MK-4) has four isoprene residues in its side chain, which is the most common form of vitamin K2 in animals.

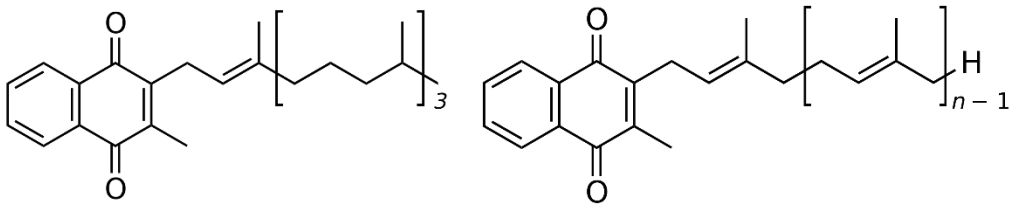

Figure 2.1: Vitamin K1 (phylloquinone) (on the left) and vitamin K2 (menaquinone) (on the right) (Berenjian et al., 2015).

Moreover, many bacteria, such as *Escherichia coli* found in the large intestine, can synthesize vitamin K2 (MK-7 up to MK-11) (Bentley and Meganathan, 1982). Menaquinone transfers two electrons between two different small molecules, during oxygen-independent metabolic energy production processes (anaerobic respiration) (Haddock and Jones, 1977). However, such bacteria in the microbial ecosystem of the human intestine cannot provide a non-dietary source of vitamin K2. Structural differences in the isoprenoid side chain in Vitamin K1 and K2, govern many facets of metabolism of K vitamins including the way they are transported, taken up by target tissues, and subsequently excreted (Shearer and Newman, 2008).

2.1.1.1 Menaquinone-7 (MK-7)

As mentioned earlier, vitamin K2 (menaquinone) includes several subtypes. The two subtypes most studied are menaquinone-4 (MK-4) and menaquinone-7 (MK-7) (Figure 2.2).

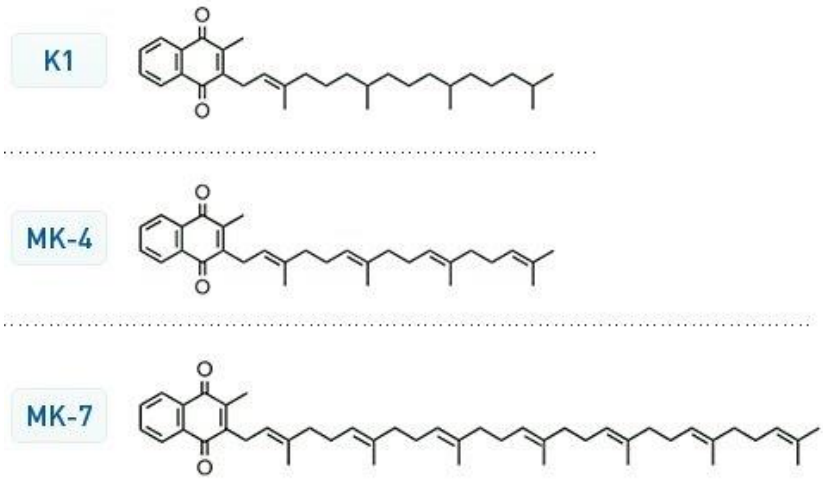

Figure 2.2: Molecular structures of phylloquinone, MK-4 and MK-7.

Menaquinone-7 is different from MK-4 in that it is not produced by human tissue. However, it can be produced from a variety of bacterial sources(Berenjian et al., 2015). Bacterially derived menaquinones (MK-7) appear to contribute minimally to overall vitamin K status (Weber 2001; Suttie 1995). MK-4 and MK-7 are both found in the United States in dietary supplements for bone health.

Schurgers et al. (2007) compared vitamin K1 and MK-7 *in vivo*. They demonstrated that after oral ingestion, MK-7 is more effective in both catalyzing osteocalcin carboxylation in bone and counteracting coumarin anticoagulants in the liver. The mechanism underlying this observation may be much longer half-life time of MK-7 during the circulation and its reported 6-fold higher *in vitro* cofactor activity. Since vitamin K is a cofactor in the production of blood coagulation factors (in the liver), osteocalcin (in bone), and matrix Gla protein (cartilage and vessel wall). The results of this study depict a superiority of MK-7 over its counterpart, vitamin K1.

2.2 Health and Vitamin K

Osteoporosis and coronary vascular disease (CVD) are strongly associated with lower levels of vitamin K2 (Yamaguchi, 2006); Gast et al., 2009). Vitamin K2 (MK-7)

deficiency is also related to severe aortic calcification and all-cause mortality (Geleijnse et al., 2004). Also, recently researchers discovered antitumor activities associated with MK-7 in vivo (Shi et al., 2017).

2.2.1 Vitamin K and Bone Health

Although there is no good evidence that vitamin K supplementation helps prevent osteoporosis or fractures in postmenopausal women (Hamidi et al., 2013), MK-7 has been demonstrated to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption (Yamaguchi, 2006).

On the other hand, based on the national MCBS to estimate the prevalence and costs of fractures and osteoporosis in the US, the national medical cost of osteoporosis and fractures has been over $16 billion in 2002 (Blume and Curtis, 2011). A cost estimate for 2008, accounting for the 8% growth in Medicare elderly enrollees and for 27% medical inflation, would be $22 billion (Blume and Curtis, 2011).

2.2.2 Vitamin K and Heart Health

Lloyd-Jones et al. (2010) estimated the direct and indirect annual costs of heart diseases and strokes throughout the US. The total staggering amount is over $502 billion per year.

Gast et al. (2009) examined the relationship between dietary vitamins K1 and K2 intake, and their subtypes, and the incidence of CVD. The study shows that a higher dietary intake of vitamin K2 was significantly associated with a lower incidence of CVD. The association was mainly driven by vitamin K2 subtypes MK-7, MK-8 and MK-9. However, no association between vitamin K1 intake and CVD is observed.

2.2.3 Vitamin K and Cancer

In recent years, several experimental studies have demonstrated that menaquinones may inhibit tumor cell growth. It has been demonstrated that menaquinones can inhibit the growth of various tumor cell lines, such as hepatocellular carcinoma, lung tumor, colorectal tumor, gastric tumor, breast tumor, bladder tumor, ovarian tumor, oral epidermal tumor, nasopharyngeal carcinoma, and leukemia cell lines. Recent research has demonstrated that tyrosine kinases associated with cyclins have been shown to be affected by menaquinones, which can lead to cell cycle arrest and cell death, and are likely to play a role in any form of solid tumor (Shi et al., 2017).

2.3 Vitamin K Production

Vitamin K1 has been extracted and purified from the natural sources, which are mostly leafy vegetables (Binkley et al., 1939). On the other hand, pure menaquinones (Vitamin K2) are rarely purified from their animal sources. However, it is much easier to obtain them from microbial sources. Various studies have been carried out on the subject of menaquinone producing microorganisms, especially MK-7 producing strains (Berenjian et al., 2013a).

2.3.1 MK-7 Fermentation

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

(Berenjian et al., 2013b). Thus, research has been conducted in the past decades to enhance the MK-7 production.

2.3.1.1 Solid State Fermentation (SSF) for MK-7 Production

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

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

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

Generally, the major factors that affect MK-7 production by SSF systems are the selection of microbial strain, suitable substrate, pre-treatment, particle size, and water activity (a_w) of substrate, size, and type of inoculum, temperature, and fermentation time

during SFF (Pandey 2003). Selecting a substrate for MK-7 production in an SSF process mainly depends upon cost and availability and therefore usually involves screening several solid substrates.

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

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

Tray fermenters have been successful in lab-scale, pilot, and maybe even large scale fermentations due to simplicity; however, the large scale requires the installation of a high number of trays and more generally static mode fermenters and the risk of high temperature and oxygen gradients are the major concerns for the static mode reactors (Krishna, 2005). To overcome this, dynamic mode fermenters, such as rotating drums, can be recommended for SSF processes and production of vitamins (Yang, 2007). In a rotating drum bioreactor, the substrate bed is held within a horizontal or near horizontal drum, with or without baffles and the drum is continuously rotated. Air is not blown forcefully through the bed itself, but rather across the top of the substrate bed through the headspace. These bioreactors have

a long history, having been used for α-amylase production in the early 1900s and penicillin after WWII (Mitchell et al., 2000). Moreover, these bioreactors have been successfully used for Vitamin B_2 (20 g/L) (Stahmann et al., 2000; Berenjian et al., 2015). However, the major issue in using dynamic mode fermentations is the high level of moisture content that may result in particle agglomeration (Yang, 2007).

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

2.3.1.2 Liquid State Fermentation (LSF) for MK-7 production

Among bacterial strains, *B. subtilis* and *B. licheniformis* are the well-studied strains for MK-7 LSF fermentation.

B. licheniformis is an organism with well-characterized membranes, contains menaquinone as the sole quinone, and possesses the ability to grow anaerobically. MK-7 exists in both wild-type and mutant strains playing a similar respiratory quinone role as MK-4 and MK-6 in *Flavobacterium* sp. 238-7(Tani and Taguchi, 1989). The highest amount of MK-7 produced was reported by Goodman as 0.25 µg/mg of dry weight of cells (Goodman et al., 1976). Table 2.2 summarizes SSF and LSF strategies applied for vitamin K2 fermentation.

Table 2.2. LSF and SSF menaquinone fermentations.

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

Morishita et al. (1999) focused on production of menaquinones using lactic acid bacteria. The results have indicated that *Lactococcus lactis* ssp. *cremoris* (three strains), *Lactococcus lactis* ssp. *lactis* (two strains), and *Leuconostoc lactis* were all potent producers of quinones. These strains, when grown in a soymilk medium, produced a significant amount of MK-7. The quinones were presumed to be MK-7 to MK-10 by high performance liquid chromatography. Specifically for MK-7, the highest concentration observed was 12.3 mg/L in *Lactococcus lactis* ssp. *Cremoris*. Therefore, the authors

concluded that these strains would be useful as starter cultures for dairy and other food fermentation or dietary supplements which may include dietary sources of menaquinones.

Nonetheless, the focus of LSF studies has been on MK-7 production by *B. subtilis natto*. Berenjian et al. (2011, 2012, 2013a and 2013b) have carried out several studies on MK-7 production by LSF using *B. subtilis natto*.

The effect of medium nutrients for *B. subtilis natto* MK-7 was studied to enhance the MK-7 production. Maximum MK-7 concentration of 62.32 mg/L has been reported in the media containing 5% (w/v) yeast extract, 18.9% (w/v) soy peptone, 5% (w/v) glycerol and 0.06% (w/v) K_2HPO_4 (Berenjian et al., 2011a).

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

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

enhances the fermentation yield significantly as compared to the static system (Berenjian et al., 2014).

2.4 Recovery and Extraction

MK-7, like all other menaquinones, is fat soluble and insoluble in water. Since it is extracellular in *Bacillus* species and bound to a protein, it needs to be extracted from the fermentation broth (Schurgers and Vermeer, 2001). Liquid-liquid extraction is commonly used for extracting fat-soluble metabolites. Fermentation media containing vitamin is contacted with an immiscible or semi miscible solvent. The solubility of a vitamin compound in a solvent, the selectivity of solvent toward solute, and the dielectric constant of solvent has significant impacts on the extraction efficiency (Perry, 2007). By the use of an organic mixed solvent composed of 2-propanol and n-hexane, MK-7 can be extracted from fermentation broth robustly (Berenjian et al., 2011a). Different compositions have been evaluated and mixture of 1:2 2-propanol and n-hexane seemed to be most commonly used (Table 2). Sato et al. (2001) and Berenjian et al. (2011) have added 1:2 (v/v) propanol:n-hexane mixture to fermentation broth containing vitamin K. After vigorously shaking the mixture and settling down, the organic layer has been separated and evaporated; leaving vitamin K solid residues. Then, Sato et al. (2001) has extracted the residues with n-hexane and after centrifugation, the resultant solution has been washed through a silica gel column using 1:2 (v/v) toluene:hexane mixture. Finally, the fractions have been analyzed by HPLC (Sato et al., 2001b). Berenjian et al. (2011), however, has dissolved the residues after evaporation step in methanol and analyzed the resultant solution by HPLC with methanol as the mobile phase(Berenjian et al., 2011a). On the other hand, Tsukamoto et al. (2001) has first added 2-propanol to vitamin K samples and after 15 minutes of shaking, has added hexane and mixed again. Then the mixture has been dried and dissolved in 2-propanol and finally analyzed by HPLC. Table 2.3 summarizes the compositions utilized for menaquinone extraction.

Table 2.3. Aqueous : organic and 2-propanol : n-hexane ratios for MK-7 extraction.

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

After extraction, organic solvents can be separated from the aqueous fermentation media and evaporated under vacuum to recover the extracted MK-7. On the other hand, Berenjian et al. (2014) have investigated the use of vegetable oil (Long chain triglyceride) for extracting MK-7. Although, the oil has originally been added as an antifoam agent, Berenjian et al. observed that at the end of the fermentation nearly 80% of the produced MK-7 was recovered in oil phase and the rest remained in the fermentation media. This tendency can help to develop an in situ recovery system for MK-7 fermentation. The amount of oil used in the fermentation broth was significant (over 16%) which may indeed interfere with media composition, microbial metabolism and fermentation dynamics. Therefore, this method may not be as robust as organic solvents. Yet, the use of vegetable oil eliminates the need of organic solvents, which helps to create a more environment friendly process. It seems that more thorough studies must be carried out to investigate the robustness of the mentioned liquid-liquid extraction compositions and also comparisons between different techniques in MK-7 recovery yields and purity.

2.5 Biofilm Reactors

In this study, biofilm reactors have been suggested to improve Vitamin K fermentation. Therefore, this section provides a background on biofilm reactors, which include agitated reactors, rotary reactors, fixed-bed reactors, trickling filters, rotating disk

reactors, and membrane biofilm reactors. Robust biofilm formations on suitable support materials are the common link in all such biofilm reactors that are used for various purposes from wastewater treatment processes to biofuel and vitamin productions.

2.5.1 Biofilm Formations

Microorganisms often construct and live within surface-associated multicellular communities known as biofilms. The precise structure, chemistry and physiology of the biofilm all vary with the nature of its resident microbes and local environment (Branda et al., 2005). In response to specific environmental cues, planktonic cells initiate cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. In response to developmental signals, microcolonies undergo differentiation to form the mature biofilm characterized by pillar-like structures surrounded by extracellular polymeric substance (EPS) and interspersed with fluid-filled channels (Figure 2.3). The developmental progression leading to a mature biofilm requires changes in gene expression (Kuchma and O'Toole, 2000).

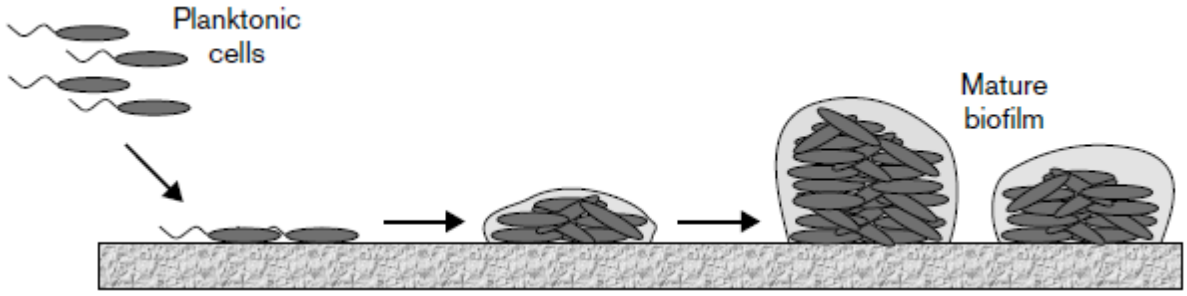

Figure 2.3. Model of biofilm development (Kuchma and O'Toole, 2000).

Often, biofilm formations are undesirable since microorganisms are significantly harder to kill in mature biofilm form compared to planktonic one. As a result, biofilm formation has been a major concern to food processors and medical device manufacturers (Xu et al., 2011).

Furthermore, researchers have discovered interactions and possible synergy that may exist between different strains that may form biofilm together. They observed that different species may interact synergistically in biofilms. As one result, biofilm biomass increases. Moreover, it has been observed that the mixed-species biofilm matrix may render the species more resistant to chemicals, antibiotics, and predator invasion (Burmolle et al., 2006). Such results could unveil the motives behind biofilm formation by microorganisms.

2.5.2 Biofilm Compositions

The biofilm matrix is composed of 50 to 90% of the extracellular polymer matrix (Characklis and Marshall, 1990). The matrix also consists of protein and inorganic substances (Flemming and Wingender, 2010). Each one has a role in biofilm formation and has an effect on the property of the biofilm. The extracellular polymer matrix is mainly responsible for the irreversible adhesion and integrity of the biofilms (Characklis and Marshall, 1990). Polysaccharides and proteins act in the adhesion and aggregation of bacterial cells. Amphiphilic molecules also have a role in reversible adhesion. Neutral and charged polysaccharides, proteins (amyloids and lectins) play a role in the cohesion of biofilms.

They form a hydrated polymer network, mediate the mechanical stability of the biofilm, determine the biofilm architecture and allow cell-cell communication. Hydrophilic polysaccharides help the retention of water inside the biofilm. This provides a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments (Flemming and Wingender, 2010). Polysaccharides and proteins also act as a protective barrier, which has resistance to host defenses during infection and tolerance to antimicrobial agents. Charged or hydrophobic polysaccharides and proteins accumulate the nutrients from the environment. In addition to charged polysaccharides and proteins, organic substituents, such as phosphate and sulfate, provide ion exchange, mineral formation and promote polysaccharide gel

formation. Enzymes inside the biofilm make the exogenous macromolecules available for digestion. Exopolysaccharides are also a potential nutrient source, which includes carbon, nitrogen and phosphorous for the microbial community inside the biofilm. Polysaccharides act as a sink for excess energy by storing excess carbon under an unbalanced carbon to nitrogen ratio (Flemming and Wingender, 2010).

The extracellular polysaccharides can be divided into two groups: specific and nonspecific polysaccharides (Characklis and Marshall, 1990). The specific polymers, such as glucose, galactose, mannose, glucuronic acid, galacturonic acid, are specific to individual bacterial strains. The nonspecific polysaccharides can be secreted from a variety of bacterial strains. Most of the nonspecific polysaccharides are homopolysaccharides (Characklis and Marshall, 1990). Homopolysaccharides contain only one type of monosaccharide, whereas when the repeating unit contains two or more different monosaccharides, it is called heteropolysaccharides. Some of the examples of homopolysaccharides are dextrans (a-1,6 and a-1,3-linked glucose polymers), glucans (b-1,3- glucose polymers), fructans (b-2,6-linked D-fructose polymers), polygalactans, which are produced by *Leuconostoc mesenteroides*, *Pediococcus spp.*, *Lactobacillus sanfranciscensis*, *Lactococcus lactis*, respectively (Hutkins, 2006). Most of the heteropolysaccharides contain a combination of glucose, galactose and rhamnose. Moreover, lesser amounts of fructose, acetylated amino sugars, ribose, glucuronic acid, glycerol, phosphate, pyruvyl and acetyl groups are found in heteropolysaccharides (Hutkins, 2006). The composition, structure and physicochemical properties of heteropolysaccharides produced by each bacterium may differ between strains. In addition, an organism may produce both types of polysaccharide (Characklis and Marshall, 1990; Hutkins, 2006).

In addition to the function in biofilm formation, the polymers (mostly polysaccharides) also affect the physical property of the biofilm. The shape of the polymer has an influence on the physical property of the biofilm. The shape of the biofilm is dependent on the primary structure, solvent composition, pH, temperature, and inorganic salts (Characklis and Marshall, 1990). The aqueous solutions of most bacterial polysaccharides are viscous and non-Newtonian. Phenotypic adaptation may occur in the

biofilm, because it has been reported that shear rate affects the viscosity of the biofilm Flemming and Wingender, 2010). Overall, the biofilm shows viscoelastic properties. Biofilms react to the forces on the exoploysaccharide matrix by both reversible elastic responses and irreversible deformation (Flemming and Wingender, 2010).

Matrix stability may be enhanced by entanglement of biopolymers via shaping network structures (Flemming and Wingender 2010). Because biofilms consist mainly of polysaccharides, which contain many hydrophilic sugar residues, biofilms are generally considered as hydrophilic (Characklis and Marshall, 1990). The thermodynamic properties of the biofilm are dependent on the biofilm mass and thickness. Because the biofilm is largely composed of water, the thermal conductivity of the biofilm has been reported to be the same as that of water (Characklis and Marshall 1990. In addition, the diffusion coefficient of the biofilm is 80% of the diffusion coefficient of the water and diffusional length is affected by the biofilm thickness (Characklis and Marshall 1990). It has been reported that mixed culture biofilms are usually thicker than pure culture biofilms (Murga et al., 1995). For example, the biofilm thickness of a mixed culture including *Pediococcus aeruginosa* and *Klebsiella pneumoniae* was reported as 400 mm, while the thicknesses of pure culture biofilms of *P. aeruginosa* and *K. pneumoniae* were 29 and 100 mm, respectively (Murga et al., 1995). Biofilm density varies based on environmental conditions and microbial species. As another example, limited oxygen may trigger the filamentous growth and that affects the density of the biofilm. Moreover, increasing shear stress causes increases in biofilm density. Similarly, increasing calcium concentration, inorganic salts with low solubility can increase the biofilm density significantly (Characklis and Marshall, 1990).

It has been also reported that biofilm density increases with biofilm age. Moreover, the biofilm densities on the surface and in the intermediate are different from each other (Characklis and Marshall 1990). The geometric properties of the biofilm are not uniform. The surface morphology as well as the environment interaction may affect the geometric properties. The roughness of the biofilm is dependent on the age of the biofilm and filamentous microbial growth (Characklis and Marshall 1990).

Such extraordinary characteristics mentioned above can be utilized in a bioreactor by allowing microorganisms to form biofilms under controlled conditions, which turns it into a "biofilm reactor". This can be achieved through microbial cells attaching to the support structure without the use of chemicals and thus forming biofilm. This process is called passive immobilization. Biofilm reactors provide improved productivity and stability (Ercan and Demirci, 2013).

2.5.3 Biofilm Reactors for Value-added Products

Biofilms have been used for fermentation to improve the productivity and stability of the process. Colonization through biofilm formation provides microorganisms the ability to better metabolize the nutrients and produce metabolites. Usually, in response to environmental stress, planktonic cells adapt into biofilm formation in order to gain better chances of survival (Kuchma and O'Toole, 2000). These adaptations and changes on the cellular and genetic levels, in the case of production of value-added products in biofilm reactors, lead to enhanced production of these products; especially secondary metabolites such as enzymes and antibiotics. Biofilm reactors have been studied to increase the effectiveness of waste treatment, alcohol, enzyme, organic acid, antibiotics, polymers, starter cultures production and many other value-added products. However, diffusion of oxygen and substrate into the cell or release of the extracellular product into the medium can be limited in biofilm reactors (Ercan and Demirci, 2013a). Value-added products have been reported to be produced several folds more efficiently in biofilm reactors.

For instance, biofilm reactors have been studied extensively for alcohol production. Zhang et al. (2009) used corn stalk as a solid support for the production of acetone, butanol, and ethanol by using *Clostridia beijerinckii*. They used a reactor, in which growing media was circulated for cell immobilization, and then media was fed and the product was discharged. While the productivity and yield in a suspended cell bioreactor were reported as 0.22 g/L/h and 0.24 g/g, respectively, the productivity and yield were achieved in the reactor with the corn stalk support at the level of 0.6 g/L/h and 0.32 g/g, respectively.

Acetone, butanol, and ethanol production was also investigated in a cell immobilized reactor with corn steep liquor as a medium ingredient, which is a by-product of the corn wet-milling process. Acetone-butanol-ethanol (6.29 g/L) was produced during fermentation (Zhang et al., 2009).

Chen et al. (2008) used packed bed bioreactor with loofa sponge as a support for the production of bioethanol from uncooked raw starch. The microorganisms used in the process were an engineered yeast strain of *Saccharomyces cerevisiae* with *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase on the cell surface. Although the concentration of ethanol was obtained at the level of 42 g/L in 3 days, 55% drop of initial ethanol concentration was observed after five cycles due to a decrease in cell mass and cell viability in the bioreactor. Therefore, they proposed addition of cells to the bioreactor, which cause 75% restoration of initial ethanol production (Chen et al., 2008).

Weuster-Botz et al. (1993) developed a continuous fluidized bed reactor operation system for ethanol production by *Zymomonas mobilis* using hydrolyzed β-starch without sterilization. Two phases were applied in the operation system. Macroporous glass carriers, filled with a monoculture of *Z. mobilis* in the first phase, were used as a solid support. In the second phase, a short residence time was employed for an unsterile fermentation of hydrolyzed β-starch to ethanol to prevent penetration of contaminant into the glass carriers. Ninety-nine percent of the unsterile hydrolyzed β-starch was converted to glucose and 50 g/L ethanol was produced when the 120 g/L glucose was used in the substrate (Weuster-Botz et al., 1993).

Ozmihci and Kargi (2009) evaluated ethanol production from cheese whey solution in a packed-column bioreactor. For biofilm formation, the reactor was fed with a concentrated culture of *Kluyveromyces marxianus* (DSMZ 7239) and sterile cheese whey solution and the medium was circulated and 90% of the sugar was consumed. This process was repeated three times. As a result of this process, 22.5 g/L ethanol was produced when the feed sugar content was 100 g/L (Ozmihci and Kargi, 2013).

Biofilm reactors with plastic composite supports were also studied for ethanol production. Kunduru & Pometto (1996a; 1996b) evaluated a biofilm reactor with plastic composite support chips for ethanol production. First, Kunduru & Pometto (1996a) tested

different compositions for plastic composite support chips in packed bed reactors. The supports consisted of polypropylene and up to 25% (w/w) of cellulose, soy hulls, soy flour, zein, oat hull, corn starch, corn hull (Kunduru and Pometto, 1996a). Moreover, they tested different cultures, which are pure and mixed cultures of *Z. mobilis* or *S. cerevisiae* and mixed cultures of either of these ethanol-producing microorganisms and the biofilmforming *Streptomyces viridosporus*. Maximum ethanol productivity was achieved at the level of 374 g/L/h (44% yield) on polypropylene composite supports of soybean hullzein-polypropylene by using *Z. mobilis*. Then, continuous ethanol fermentations were evaluated for 60 days with *Z. mobilis* or *S. cerevisiae* in packed bed reactors with plastic composite supports (Kunduru and Pometto, 1996b). The compositions of plastic composite-supports were polypropylene (75%) with ground soybean-hulls (20%) and zein (5%) for *Z. mobilis*, or ground soybean-hulls (20%) and soybean flour (5%) for *S. cerevisiae*. Maximum productivity was reported as 499 g/L/h (37% yield) with *Z. mobilis* while the productivity without support was 124 g/L/h with *Z. mobilis*.

Later, Demirci et al. (1997) also evaluated ethanol production in a biofilm reactor with plastic composite support. As a result, 40% soybean hull, 5% soybean flour, 5% yeast extract-salt and 50% polypropylene was selected as the best composite composition. They also reported that *S. cerevisiae* produced 30 g/L ethanol on PCS with ammonium sulfate medium, which had lowered nitrogen content, in repeated batch fermentation. This production level was found 2 to 10 times higher than on polypropylene-alone support (Demirci et al., 1997).

Increase in enzyme production by the application of biofilm reactors has been reported in many studies. Thermostable amylase production was increased fivefold by using immobilized recombinant *E. coli* (EC147) on silicone foam support compared to suspended cell (Oriel, 1988).

Spouted bed reactor with 6mm diameter, spherical, stainless steel biomass support particles was applied for continuous cellulase production by *Trichoderma viride* QM 9123. The broth was recycled, causing a jet at the base of a bed of particles was created to spout and circulate the particles. Steady-state conditions were reported due to prevention of excess biomass as a result of high shear near the jet inlet. Overall, the amount of cellulase

produced in the biofilm reactor was more than three times that of production in suspended cell reactor (Webb et al., 1986).

Cellulase production using a woven nylon pads biofilm reactor was reported by Hui et al. (2010). They reported similar cultural and nutritional requirements, except temperature, for free cell and immobilized cells. The temperature requirement was found to be 40^{o}C for immobilized cells while the optimum temperature for free cells was reported as 30^{o}C. The surface area of the support material affected the production of cellulolytic enzymes. Cellulase production in the woven nylon pads biofilm reactor was achieved 4.5 times more than the production in suspended cell reactors for a much longer period (Hui et al., 2010).

Kang et al. (1995) evaluated the production of cellulase and xylanase by *Aspergillus niger* KKS, which was immobilized on Celite and polyurethane foams. The enzyme productivities by immobilized cells were twice as high as the productivities by shake flask culture (Kang et al., 1995).

A circulating bed reactor with biomass support particles was utilized for the fed-batch and batch production of intracellular lipase by *Rhizopus chinensis* (Nakashima et al., 1988). Cubic polyurethane foam with a porosity of around 0.97 and a pore size of 40 pores per linear inch was used as a biomass solid support. Productivity in fed-batch operations was 1.3–1.5 times more than that obtained in batch cultivation.

Extracellular lignin peroxidase enzyme has been used to transform a wide range of hazardous compounds. A biofilm membrane stirred tank reactor was used for the improved production of lignin peroxidase and treatment of pentachlorophenol by *Phanerochaete chrysosporium*. They reported that lignin peroxidase production was affected by the surface area for biofilm growth and glucose consumption. As a result, 10.5 mg/L/day of pentachlorophenol, which is fivefold more than the rate in the flask-scale experiment, was disappeared in the biofilm reactor by the production of lignin peroxidase (Venkatadri et al., 2009).

Various organic acid production systems have been studied in biofilm reactors. A multi-stage biofilm reactor was developed for acetic acid production by liquid surface culture of *Acetobacter aceti* M7. The biofilm reactor, which consisted of 10 shallow flow

horizontal reactors of laboratory scale, had a total liquid surface area of 2957 cm^2, a total normal length of 18.9 m, working volume of 860 mL and a liquid depth of 2.8 mm on average. The production temperature was held constant at 30^oC and it was aerated by natural ventilation through the opening between the top edge of the composite horizontal reactors and the lids on them. A production rate of 4.3 g/L/h was achieved in the biofilm reactor with the application of step feeding of the ethanol-rich medium (Park and Toda, 1992).

Horiuchu et al. (2000) also investigated the acetic acid production in the biofilm reactor. They used a packed bed bioreactor for continuous acetic acid production. The pellets, which have a high specific surface area (200m2/g) with numerous micropores (2–10 mm), were produced from waste mushroom medium by thermal carbonization. The production conditions had no pH control, at constant temperature (30^oC) and supplementation of oxygenenriched air with a 40% O_2 content. The fermentation process took 120 day and 6.5 g/L/h productivity was obtained (Horiuchi et al., 2000).

Andrews and Fonta (1989) used a fluidized bed biofilm reactor with activated carbon for lactic acid fermentation by *Streptococcus thermophilus*. Substrate inhibition was eliminated due to adsorption of substrate in the carbon. The productivity level was 12 g/L/h without pH control (Andrews and Fontat, 1989).

Demirci et al. (1993) evaluated 12 different bacteria, including *Bacillus*, *Pseudomonas*, *Streptomyces*, *Thermoactinomyces* and *Thermomonospora* species and solid supports, including pea gravels, 3M®-macrolite ceramic spheres and polypropylene mixed with 25% of various agricultural materials (e.g., corn starch, oat hulls), which were extruded to form chips (polypropylene composite) for biofilm formation in continuous lactic acid production. The polypropylene composite was selected as the best support for biofilm formation with *Pseudomonas fragi*, *Streptomyces viridosporus* T7A and *Thermoactinomyces vulgaris*. The biofilm producer strains were mixed with lactic acid producer strains. *Streptomyces viridosporus* T7A and *Lactobacillus casei* on pp-composite chips produced the highest amount of lactic acid (13.0 g/L lactic acid) among other combinations in the biofilm reactor without pH control (Demirci et al., 1993).

Demirci and Pometto (1995) later evaluated repeated batch fermentations for lactic acid production in a biofilm reactor. The repeated batch operation included reactor medium change every 3 days for 24 batches. The pH was controlled at 5 for *Streptomyces viridosporus* T7A for biofilm formation and *L. casei* subsp. *rhamnosus* (ATCC 11443) for L-lactic acid production were used in the system. The PCS provided much greater yield than polypropylene-alone supports. Pure culture in the biofilm reactor produced more lactic acid than a mixed culture and free cell fermentation (Demirci and Pometto, 1995).

Ho et al. (1997a) also investigated the effects of different agricultural components on biofilm formation and lactic acid production. The highest biofilm population ($2.3x10^9$ CFU/g of support) and lactic acid concentration (7.6 g/L) in minimal medium were obtained with the plastic composite supports containing soybean hulls, yeast extract, soybean flour, bovine albumin and mineral salts (Ho et al., 1997a). The leaching rate of nutrients inside the plastic composite support and lactic acid accumulation on the PCS were also studied. The leaching rates in the PCS with dried bovine albumin, dried bovine erythrocytes and/or soybean flour were lower than the rate in PCS blended with only yeast extract. No correlations were observed between lactic acid accumulation in the support and lactic acid production or biofilm formation (Ho et al., 1997b).

Vassilev et al. (1993) evaluated the production of gluconic acid by cells of *Aspergillus niger* immobilized on polyurethane foam in repeated-batch shake-flask and bubble-column fermentations. The polyurethane foam cubes were 1.0– 0.3 cm^3 and had an average pore size of 0.6–0.8 mm. They reported that immobilized cells were able to be maintained for 65–70 h with high productivity. About 143 g/L gluconic acid was produced with foam cubes in the bubble column (Vassilev et al., 1993).

Many studies have also been conducted for citric acid production in biofilm reactors. Sanroman et al. (1994) investigated adsorption and entrapment techniques in polyurethane foams for citric acid production by *A. niger* in a fluidized bed reactor were compared. The adsorption technique was provided to possess more durable activity than the entrapment technique (Sanroman et al., 1994).

The production of citric acid in the biofilm dipping reactor was observed by Sakurai et al. (1999). This reactor was composed of a horizontal glass cylinder with segment-

shaped stainless steel meshes. The temperature was maintained constant at 30°C and the optimum dipping period was determined as in the range below 20 s to hold the critical dissolved oxygen level, which produced in the range of 0.9–1.3 mg/L of citric acid (Sakurai et al., 1991).

Jianlong (2000) used a rotating disk reactor for citric acid production by *A. niger*. Plastic disks mounted on a horizontal shaft with polyurethane foam, which is a porous biomass support, were used as a solid support for biofilm formation. The disks were exposed to both air and the medium. The volumetric productivity was obtained (0.896 g/L/h) three times higher than that obtained with a stirred-tank fermenter (0.33 g/L/h). The immobilized biofilm provides a stable bioactivity for over eight cycles of fermentation (Jianlong, 2000).

Citric acid production was also studied with the immobilization on cellulose microfibrils (Sankpal et al., 2001). In this study, both continuous and batch production of citric acid from sucrose were investigated using *A. niger* NCIM 588. Mycelia of *A. niger* formed a uniform biofilm on cellulose microfibrils under conditions rich in oxygen. A recycle reactor was used in fed-batch mode and the volumetric productivity was improved to 1.85 g/L/h of citric acid, which is 15-fold greater than the productivity in shake-flasks and 1.6-fold greater than in a conventional aerated batch reactor. In continuous fermentation, 2.08 g/L/h of volumetric citric acid productivity for 26 days was achieved without loss of productivity.

Pramod and Lingappa (2008) employed polyurethane foam for the immobilization of *A. niger* strains MTCC 281 and KLP20. As a carbon source, carob pod extract was used. As a result, immobilized *A. niger* strains MTCC 281 and KLP20 were able to produce 33 and 38 g/L citric acid while free cells produced 23 and 27 g/L citric acid, respectively (Pramod and Lingappa, 2008).

Rotating disk reactors with self-immobilized mycelia of *Rhizopus oryzae* on the plastic disks were used to produce fumaric acid. The rotary biofilm reactor included six plastic disks (125 cm^2/disk) as a solid support for biofilm formation. The disks were placed on a horizontal shaft and exposed to the fermentation medium and the air space with rotation at 22 rpm. The productivity was achieved at the level of 3.78 g/L/h within 24 h in

the rotary biofilm reactor with the supplement of calcium carbonate. The volumetric productivity level was reported as threefold higher than with a stirred-tank fermenter with calcium carbonate. The duration of fermentation in the rotary biofilm reactor became shorter than the stirred-tank system, and the immobilized biofilm remained active for more than 2 weeks (Cao et al., 1997).

Cao et al. (1996) also developed an integrated system of simultaneous fermentation-adsorption for the production and recovery of fumaric acid from glucose by *R. oryzae*. A nitrogen-rich medium was used for the self-immobilization *R. oryzae* on to the plastic disks of a rotary biofilm contactor. A coupled adsorption column was used to remove the fumaric acid continuously produced, to eliminate the inhibition, moderate the pH decrease, enhance the fermentation rate and sustain cell viability. As a result, an average yield of 85 g/L was obtained by repetitive fed-batch cycles, which took 20 h. The productivity level was achieved at the level of 4.25 g/L/h, while the productivity was reported as 0.9 g/L/h in a stirred-tank reactor (Cao et al., 1996).

Urbance et al. (2003; 2004) investigated succinic acid production using *Actinobacillus succinogenes* in a biofilm reactor with plastic composite support. Fifty percent (w/w) polypropylene, 35% (w/w) dried ground soybean hulls, 5% (w/w) dried bovine albumin, 2.5% soybean flour, 2.5% yeast extract, 2.5% dried red blood cells and 2.5% peptone were selected as the best composition of plastic composite support for succinic acid production. The medium was modified by the substitution of industrial yeast extract for vitamins and fatty acid (Urbance et al. 2003). The yield of succinic acid production increased to 70% in the biofilm reactor from 64% in suspended cell fermentation. Then, continuous and repeated batch fermentation modes were applied for succinic acid production in the biofilm reactor with plastic composite support. In continuous fermentation, 71.6% yield was achieved at a dilution rate of 0.2 h_1 while the yield for fed-batch fermentation was reported as 86.7% with an initial glucose concentration of 40 g/L (Urbance et al., 2004).

Pongtharangkul and Demirci (2006) compared nisin production by *Lactococcus lactis* subsp. *lactis* (NIZO 22186) in a biofilm reactor and suspended-cell. Also, due to the substrate inhibition that takes place at high levels of carbon source, fed-batch fermentation

has been proposed and tested as a better alternative for nisin production. Fed-batch fermentation has enhanced nisin production for both suspended-cell (4,188 IU/ml) and biofilm (4,314 IU/ml) reactors; 1.8- and 2.3-fold higher nisin titer than their respective batch fermentations. This study concludes that fed-batch fermentation can be successfully used to enhance nisin production for both suspended-cell and biofilm reactors and using a biofilm reactor not only shortens the lag phase of nisin production, but also enhances the production.

Khiyami et al. (2006) achieved lignin peroxidase and manganese peroxidase production up to 50.0 and 63.0 U/L by *Phanerochaete chrysosporium* in defined medium in plastic composite support biofilm stirred tank reactors. They have reported that the PCSs stimulate the formation of a *Ph. chrysosporium* biofilm and in each batch; the PCS biofilm becomes significantly noticeable on day 2 and thick on day 6. By utilizing the biofilm reactor, the authors have succeeded to perform fourteen repeated batches fermentation without contamination.

Organic acids such as butyric, acetic and propionic were produced concurrently with the production of hydrogen through photosynthesis and fermentation from wastewater as the raw material. Horizontal packed bed bioreactor with expanded clay beads as a support material was used. Optimum production was obtained when the operation was conducted without a buffer agent (Leite et al., 2008).

Cheng et al. (2010) produced pullulan in a biofilm reactor by using *Aureobasidium pullulans*. The study was designed not only to evaluate the potential use of biofilm reactor for pullulan production but also to enhance the pullulan production through pH profile optimization. PCS with soybean hulls, defatted soy bean flour, yeast extract, dried bovine red blood cells, and mineral salts has been selected for biofilm reactor fermentation through test tube fermentations, due to its high biomass attachment and pullulan production. The pullulan produced in the study from different PCS types are reported varying from 4.2 to 5.8 g/L. However, the authors have reported no specific relationship between biomass attached on PCS and pullulan production. Within 7 days of fermentation they reached the concentration of 32.9 g/L of pullulan, which is staggeringly 180% higher than the suspended fermentation. In the end, the authors have concluded that PCS biofilm reactor

system has several advantages for pullulan production, such as advanced pullulan production, and easy-to-process fermentation since no potential hazard of operation at low pH environment and apart from their well-known benefits of being highly stable systems with lower capital cost, biofilm reactors also possess great potential to be developed into a more productive process, such as continuous fermentation.

As an example of antibiotics production in biofilm reactors, Ercan and Demirci (2014) increased human lysozyme production by *Kluyveromyces lactis* K7 to 173 U/ml in a biofilm reactor, which is about 57% improvement compared to the suspended-cell fermentation. Authors have aimed to evaluate the fermentation medium composition for enhanced human lysozyme production by *Kluyveromyces lactis* K7 in biofilm reactor with Plastic Composite Supports (PCS). Batch fermentations have been conducted to select the best medium composition in the biofilm reactor, which had already stable biofilm in it. The conditions were reported to be 25°C, no pH control, 150 rpm, and no aeration. Time for fermentation of each batch was 80 h. The results indicate that the optimum fermentation media consists of 16.3% lactose, 1.2% casamino acid, 0.8% yeast nitrogen base for maximum human lysozyme production. The biofilm reactor used, had 12 PCS tubes bound to the agitator shaft in a grid-like fashion, with six rows of two parallel tubes. The authors observed that after five repeated-batch fermentations, stable biofilm on the supports were formed.

Izmirlioglu and Demirci (2016) determined the optimum culture conditions for ethanol production in biofilm reactor by *S. cerevisiae* while using waste potato mash as a carbon source for bioethanol fermentation. They identified that pH 4.2, 34°C and 100 rpm were the optimum conditions for *S. cerevisiae* for ethanol production in biofilm reactors in the waste potato mash medium, yielding 37.05 g/L of ethanol with 92.08% theoretical yield and 2.31 g/L/h ethanol productivity (Izmirlioglu and Demirci 2016). Furthermore, in another study, they determined optimum conditions in biofilm reactors for maximum ethanol production of SSF of potato waste by cocultures of *A. niger* and *S. cerevisiae* to be 35°C, pH 5.8 and no aeration (0 vvm). Under these conditions they reported a maximum of 37.93 g/L ethanol was produced under optimum conditions, with 0.410 g ethanol/g

starch yield and 1.044 g/L/h productivity. The results suggested that PCS biofilm reactors can be used for co-immobilization of co-culture systems (Izmirlioglu and Demirci, 2017).

2.6 The State-of-the-Art in Menaquinone-7 (Vitamin K2) Production by *Bacillus subtilis natto* in a Biofilm Reactor

Research has been conducted in the past decade to enhance the yield of production of MK-7 from both liquid fermentation and solid state fermentation (SSF) by various species of mostly bacteria. Among these species however, *Bacillus subtilis natto* seems to be the key microorganism. Contribution of initial fermentation media optimization, fed-batch addition of nutrients, *in-situ* extraction protocols in liquid fermentation 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 (Mahdinia et al., 2017a).

Moreover, it has been concluded that MK-7 production by *B. subtilis natto* is associated with pellicle formation, which causes operational issues in static fermentation. On the other hand, it contributes to higher MK-7 production. Thus, this study was carried out to simultaneously harness the advantages of pellicle and biofilm formation and address these issues, by conducting *B. subtilis natto* fermentation in a biofilm reactor. In this regard, almost every aspect of this task was investigated, from media composition and operational variables to biofilm support material. Since *B. subtilis natto* is a food-grade microorganism and is consumed raw as a food source, there are no concerns regarding production of MK-7 from this strain for food supplementation (Berenjian et al., 2015). Hopefully, results presented here can lead to more production efficiency and reaching higher concentrations on an industrial scale so that miraculous MK-7 can be produced less costly and more abundantly.

Chapter 3

Strain and plastic composite support (PCS) selection for vitamin K (Menaquinone-7) production in biofilm reactors*

3.1 Abstract

Menaquinone-7 (MK-7), a subtype of vitamin K, has received a significant attention due to its effect on improving bone and cardiovascular health. Current fermentation strategies, which involve static fermentation without aeration or agitation, are associated with low productivity and scale-up issues and hardly justify the commercial production needs of this vitamin. Previous studies indicate that static fermentation is associated with pellicle and biofilm formations, which are critical for MK-7 secretion while posing significant operational issues. Therefore, the present study is undertaken to evaluate the possibility of using a biofilm reactor as a new strategy for MK-7 fermentation. Bacillus species, namely, *Bacillus subtilis natto*, *Bacillus licheniformis*, and *Bacillus amyloliquifaciens* as well as plastic composite, supports (PCS) were investigated in terms of MK-7 production and biofilm formation. Results show the possibility of using a biofilm reactor for MK-7 biosynthesis. *Bacillus subtilis natto* and soybean flour yeast extract PCS in glucose medium were found as the most potent combination for production of MK-7 as high as 35.5 mg/L, which includes both intracellular and extracellular MK-7.

Keywords: MK-7, Menaquinone-7, Vitamin K, Biofilm reactor, *Bacillus subtilis natto*

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3.2 Introduction

Henrik Dam, who shared the 1943 Nobel Prize in medicine with Edward Doisy for their work on vitamin K, discovered a fat soluble anti-hemorrhagic factor, which had physical properties similar to vitamin E (Dam, 1935). They called this new vitamin "the anti-hemorrhagic vitamin", which finally got the name "vitamin K". A few years after the discovery of vitamin K by Dam, it was found that this vitamin has two major forms. One had the smaller molecular weight, was less effective in treatment of symptoms and was mostly found in certain vegetables. This form was called vitamin K1. The other one had larger molecular weight, was more effective and was mostly found in animal sources such as fish meals and liver oils and was called vitamin K2 (Binkley et al., 1939).

Vitamin K1, which is also known as phylloquinone, is synthesized in plants, and is found dominantly in green leafy vegetables, because it is directly involved in photosynthesis (Widhalm et al., 2012). Animals may also convert it to vitamin K2 (Davidson et al., 1998). On the other hand, vitamin K2, the main storage form in animals, has several subtypes, which differ in isoprenoid chain length. These vitamin K2 homologues are called menaquinones, and are characterized by the number of isoprenoid residues in their side chains. For example, menaquinone-4 (MK-4) has four isoprene residues in its side chain, which is the most common form of vitamin K2 in animals.

Many bacteria, such as E. coli found in the large intestine, can synthesize vitamin K2 types; MK-7 up to MK-11(Bentley and Meganathan, 1982). The two most studied vitamin K2 subtypes are menaquinone-4 (MK-4) and menaquinone-7 (MK-7). MK-7 can be produced from a variety of bacterial sources (Berenjian et al., 2015). MK-4 and MK-7 are both allowed in the United States in dietary supplements for bone health. Yet, MK-7 can be superior in the case of health benefits as compared to its counterparts which is due to the fact that MK-7 stays active in human blood circulatory system for a longer time (Schurgers et al., 2007; Howard and Payne, 2006).

Osteoporosis and coronary vascular disease (CVD) are strongly associated with low levels of vitamin K2 (Yamaguchi, 2006; Gast et al., 2009). Vitamin K2 deficiency is also related to severe aortic calcification and all-cause mortality (Geleijnse et al., 2004).

Vitamin K2 has also been demonstrated to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption (Yamaguchi, 2006).

Industrial production of MK-7 currently relies on solid state fermentations using mostly tray systems on an industrial scale. Simultaneously, due to its great effects on bone and heart health, the market for supplementary MK-7 pills have grown significantly over the last few years; in some cases over 500% (Berenjian et al., 2015). Research has been conducted in the past decade to enhance the yield of production of MK-7 from both liquid fermentation (LSF) and solid state fermentation (SSF) by various species of mostly bacteria (Survase et al., 2015). With *B. subtilis natto* being in the center, LSF strategies have been reported to produce several types of menaquinone (MK-4, MK-5, MK-6, MK-7, MK-9 and MK-10). Yet, most of the studies have been focused on MK-7 production (Sato et al., 2001b).

B. subtilis strains have a high tendency to form pellicle and biofilm during fermentation, which is operationally problematic and undesirable during fermentation, but it can be effective on enhancing the MK-7 production (Berenjian et al., 2013). Presumably, since MK-7 plays a critical role in *B. subtilis* respiration, MK-7 concentration involved in respiration possibly increases to encounter the restricted aeration within the proliferated biofilm matrix (Ikeda and Doi, 1990). Also since MK-7 biosynthesis usually shows a linear correlation with cell density and yet it reaches maximum concentrations in prolonged fermentations, it is safe to assume that MK-7 is a mixed-type metabolite (Berenjian et al., 2013). On the other hand, this tendency can be domesticated in a controlled environment in the form of a biofilm reactor. While there have been numerous studies in the past decades to enhance MK-7 production by fermentation, the idea of using a biofilm reactor for this purpose is still unexplored. Despite the fact that there have been several extraordinary accomplishments in designing and developing new bioprocessing technologies for MK-7 production, MK-7 production levels are not yet robust enough to provide an affordable price for the industrial applications. This mainly is due to low fermentation yields (40-60 mg/L) and several tedious down streaming unit operations(Mahdinia et al., 2017a). Therefore, there seems to be a need and opportunity for a novel technology to address these challenges. The mechanism of how pellicle or biofilm formation effect extracellular MK-

7 secretion is still debatable. Whether it has a role in extracellular matrix carboxylation (Ikeda and Doi, 1990) or it is because biofilm and pellicle formations alter physiology and gene expressions, resulting in enhanced production of required metabolic molecules (Berenjian et al., 2012) is not definite. What is known is that *B. subtilis natto* has a high tendency to form pellicles during fermentation, which is operationally problematic and undesirable during fermentation, but as our previous studies have indicated, it can be effective on enhancing the MK-7 production ca. Thus, it seems that there is an opportunity here to simultaneously address such undesirability and improve the MK-7 production by the use of biofilm reactors.

Similar to naturally occurring cases, in biofilm reactors, microorganisms gain more resistance to desiccation, grazing, and antimicrobial agents in the biofilm structure. Biofilms have been used in the biotechnology industry to improve the productivity and stability of the process (Ercan and Demirci, 2013a). Many value-added products were reported to be produced several folds more efficiently in biofilm reactors including antibiotics (Pongtharangkul and Demirci, 2006; Ercan and Demirci, 2013b), biopolymers (Cheng et al., 2010b), and enzymes (Khiyami et al., 2006). Similarly, biofilm reactors can have a potential to enhance MK-7 production. This could be possible through microbial cells migrating to the support structure through what is called “passive immobilization”. Therefore, this study has been undertaken to evaluate MK-7 producing species and Plastic Composite Supports (PCS) for MK-7 production in biofilm reactor. The bacterial strain produces MK-7 molecules as a metabolite used in respiration and possibly other functions, while the PCS provides a suitable surface for the bacterial cells to attach and form biofilms. Since one of the objectives of this study is to provide MK-7 fermentation with robust agitation and aeration, it utilizes PCS which is designed to provide a suitable scaffold for biofilm formations under such conditions. Therefore, PCS selection is carried out under robust agitation and aerobic conditions. Collectively, this research will help to investigate and develop a new production strategy for production of MK-7.

3.3 Materials and methods

3.3.1 Microorganisms and media

Fifteen strains of *Bacillus subtilis natto* (NRRL NRS-352, NRRL B-14201, DSM 17766), *Bacillus licheniformis* (NRRL B-65066 and NRRL NRS-745), *Bacillus amylolyquifaciens* (NRRL B-14394) and natto isolates F1, F2, F3, F4, F5, NF1, NF2, NF3 and NF4 were grown at 40°C for 24 h in TSB (Difco, Detroit, MI) as base medium at static fermentation. Natto isolates were obtained from commercially available natto by using Tryptic Soy Agar (TSA) plates. Then, the cultures were stored at 4°C and sub-cultured monthly in order to maintain viability. For long-term storage, stock cultures were maintained at −80°C in a 20% glycerol solution. Two fermentation media were utilized; TSB and a synthetic medium "SM" consisting of 189 g of soytone (Difco), 50 g of yeast extract (Difco), 50 g of glycerol (EMD Chemicals, Gibbstown, NJ) and 0.6 g of K2HPO4 (VWR, West Chester, PA) per liter of deionized water at 37°C (Berenjian et al., 2011a).

For strain selection, 3 mL of TSB or SM was prepared in 50 mL amber McCartney bottles, covered with cotton and aluminum foil and autoclaved at 121°C for 15 min. Consequently, bottles were inoculated with 3% (v/v) 24-h grown inoculum of bacteria from each strain. After 96 hours of static fermentation at 37°C, samples were analyzed for MK-7 concentration. Fermentations were carried out in 3 replicates. Random suspended-cell samples were Gram stained by using safranin, iodine, crystal violet, and decolorizing solutions obtained from BD (Becton, Dickinson and Company, Sparks, MD). Gram stained bacterial cells were observed morphologically under the microscope using a ZEISS Axio Scope.A1 light microscope (ZEISS, Ontario, CA).

3.3.2 PCS selection

PCS tubes were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw co-rotating Brabender PL2000 extruder

(model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Ho et al (Ho et al., 1997a). Polypropylene and other ingredients of PCS were mixed together before being extruded at 13 rpm through a medium pipe die with barrel temperatures of 220 and 200°C and a die temperature of 165°C.

The resulting tubes had a wall thickness of approximately 2.5 mm and an outer diameter of 10.5 mm. For testing and selection of PCS in test tube fermentations, the PCS tubes were cut into small disks with a thickness of 3 mm using a band saw.

Four types of PCS with different compositions (Table 3.1) were evaluated for both biofilm formation and MK-7 production using test tube fermentation with three replicates. For each replicate, 3 g of PCS disks was autoclaved in a 75-mL test tube for 1 hour at 121°C. After cooling, 15 mL of sterile baseline medium was added aseptically to the test tube containing PCS and incubated at 37°C overnight in order to hydrate the PCS. The medium was then aseptically decanted, and fresh sterile medium was added. After inoculating with 3% (v/v) of 24-h grown bacteria cell culture, the test tubes were incubated at 37°C for 96 h at 200 rpm. Then, the cell populations on the PCS and MK-7 concentration were determined. The best PCS type was selected according to the biofilm formation on PCS (colony-forming unit per gram, PCS) and MK-7 production (miligrams per liter).

Table 3.1. Types of PCS and their ingredients.

PCS Type	Ingredients
SF	Polypropylene (50%), soybean hulls (45%), soybean flour (5%), salt
SFY	Polypropylene (50%), soybean hulls (40%), soybean flour (5%), salt, yeast extract (5%)
SFYB	Polypropylene (50%), soybean hulls (35%), soybean flour (5%), salt, yeast extract (5%), bovine albumin (5%)
SFYR	Polypropylene (50%), soybean hulls (35%), soybean flour (5%), salt, yeast extract (5%), bovine red blood cell (5%)

3.3.3 Biofilm determination on PCS

For evaluating the biofilms formed on PCS rings after incubation, sand stripping method was used (Cheng et al., 2010a). Briefly, once incubation was completed, media was decanted aseptically. The rings were rinsed with sterile 0.9% peptone water twice to remove all biomass that is not in the form of biofilm attached on the rings. Then, the rings were transferred aseptically to sterile 75 mL culture tubes with sterile 15mL of 0.1% peptone water and 5 g of sand. The sand and peptone water were autoclaved for 30 minutes prior to sand-stripping. Then, the PCS rings were vortexed vigorously for two periods of 30 sec to detach the biofilm on the rings and suspend them into the peptone water. Then, the supernatant was serially diluted and spirally plated on TSA plates using a Spiral Biotech Autoplater 4000 (Spiral Biotech, Norwood, MA). After incubation at 37°C and formation of visible colonies, the colonies were counted using a Q-CountTM counter (Version 2.1, Spiral Biotech) and Colony Forming Units (CFU) were determined per one gram of PCS rings.

3.3.4 MK-7 analysis

MK-7 was extracted from the fermentation broth using 2:1, v/v n-hexane:2-propanol mixture (Berenjian et al., 2011a). In this study, n-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used for MK-7 extraction. MK-7 has both intracellular and extracellular forms in *B. subtilis* strains and the extracellular form is conjugated with a protein-based binding factor that renders it soluble in water. The liquid-liquid extraction used here is reliable to extract both forms of MK-7 (Ikeda and Doi, 1990). The mixture was vigorously shaken with a vortex mixer for 3 min and then the organic phase was separated and evaporated under forced air flow. MK-7 residues were dissolved in methanol in a Biosonic (Cuyahoga Falls, OH) ultra-sonication bath for 15 min at ambient temperature. The samples were then filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY). High performance liquid chromatography (HPLC) (Waters, Milford, MA) equipped with a 2489 UV/Visible detector and a Supelcosil C18

column (15 cm×4.6 mm, 5µm, Supelco Analytical, Bellefonte, PA) was used at 40ºC for the analysis of MK-7 concentration. Methanol (EMD Chemicals, Gibbstown, NJ) was used as mobile phase with the flow rate of 1 mL/min. The wavelength of 248 nm was selected for calibration and analysis. The MK-7 calibration curve was linear between 0.1 mg/L and 30 mg/L (R^2 = 0.999).

3.3.5 Statistical analysis

Using Minitab 17.0 ANOVA (Minitab Inc., State College, PA) with general linear model with Box-Cox transformation and adjustable λ, the effect of strain, PCS and media along with combined effects were obtained,(Rahimi et al., 2017a and 2017b). Yield was calculated as the amount of MK-7 in mg/L produced per one million CFU/g PCS. CFU, Media (TSB or SM), PCS type and strain were the variables. A confidence level of 99.167% was implemented throughout the analysis procedures that contained the 5 variables with Tukey's pairwise comparison module; therefore, the overall confidence was 95% (Ahmadian et al., 2011).

3.4 Results and discussion

3.4.1 Media selection

In past studies, among the different carbon sources tested, glycerol has found out as the most prominent carbon source for MK-7 production. Thus, the glycerol-based media was composed which has been also used in the present study (Berenjian et al., 2011a). However, this glycerol-based medium contains very high compositions of soytone and yeast extract, which not only is costly, but also creates high viscosity which can be problematic in an agitated and aerated biofilm reactor. Moreover, the morphology of Gram stained-bacterial cells growing in SM (the glycerol-based medium) and TSB were

distinctively different once observed under the microscope, which indicates also distinctive morphologies in the two media (Figure 3.1).

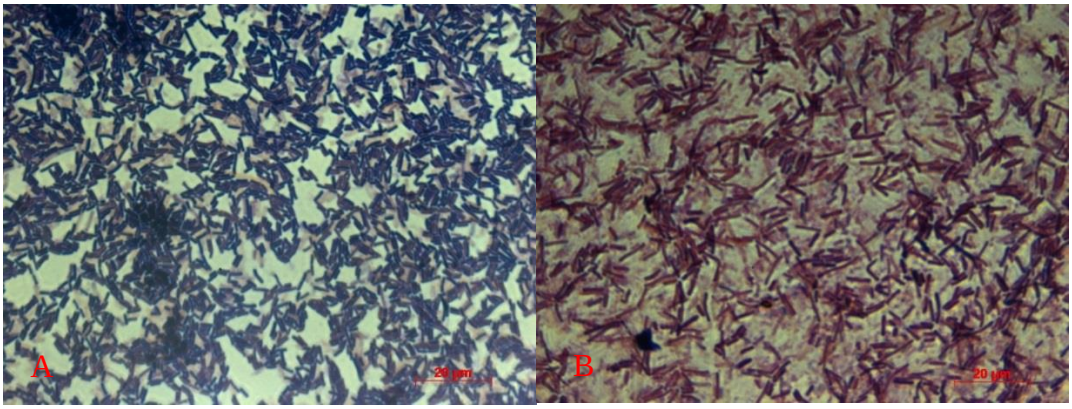


Figure 3.1. Mature *B. subtilis natto* cells growing in TSB (A) and SM (B).

SM medium (as richer medium in nitrogen and carbon sources) showed much higher MK-7 production as compared to TSB medium (as a more dilute medium). As it is obvious in Figure 3-1B, *B. subtilis* cells were longer in length and typically larger. Also, after Gram staining, cells grown in glycerol medium demonstrated a more pinkish color compared to cells grown in glucose medium that were dominantly violet (Figure 3.1A). It is known that in *B. subtilis* the majority of phospholipids consist of phosphatidylglycerol, cardiolipin and phosphatidylethanolamine (Berenjian et al., 2012). Also, it has been reported that glycerol addition at different stages of cell density significantly influences the phospholipids composition of cell membranes (Berenjian et al., 2012). Thus, crystal violet-iodine complexes are less likely to stay entrapped inside the bacterial cell walls and hence the pinkish color dominance in glycerol grown cells (Figure 3.1B). Such effects from glycerol in the medium can also be beneficiary to MK-7 secretion as membrane composition alterations due to glycerol presence occur, the protein-MK-7 complex is perhaps more easily permeated out of the cell membrane and thus glycerol presence enhances extracellular MK-7 concentrations. However, Tryptic Soy Broth (TSB) medium, which is a glucose-based medium, was also used in parallel to the glycerol-based medium. Although, glucose has been found inferior to glycerol in MK-7 production (Sato et al.,

2001b), TSB is much cheaper and much less viscous than the rich glycerol-based medium and thus was incorporated in this study.

3.4.2 Strain selection

Since higher concentrations of MK-7 were expected in the static fermentations, McCartney bottles for strain selection were incubated in static format and thus pellicles were allowed to form freely in the broth. As mentioned earlier, pellicle and biofilm formations significantly enhance MK-7 secretion in *B. subtilis* as MK-7 plays a key role in forming these colonial formations (Berenjian et al., 2013). For comparing different strains of bacteria and selecting superior strains, MK-7 concentration was measured after four days of fermentations. A total of 15 bacterial strains were investigated for MK-7 and biofilm studies. Six of these strains namely NRRL NRS-352, NRRL B-14201, DSM 17766, NRRL B-65066, NRRL NRS-745 and NRRL B-14394 were obtained as pure cultures, five strains were isolated from one source of natto (F1, F2, F3, F4 and F5) and 4 more from another natto source (NF1, NF2, NF3 and NF4). Based on the results, F-type (except for F1) and NF-type (except for NF2) strains produced over 25 g/L of MK-7 in SM (Figure 3.2). On the other hand, *B. subtilis natto* NRS-352 produced comparable amounts of MK-7 (23.7 mg/L) and the others were significantly less potent in MK-7 biosynthesis as shown in Figure 3-2. Thus, four strains of F2, F5, NF1 and NF3 would be the top four strains in SM. This is in alignment with previous studies where *B. subtilis natto* strain isolated from commercial natto was utilized (Berenjian et al., 2011b).

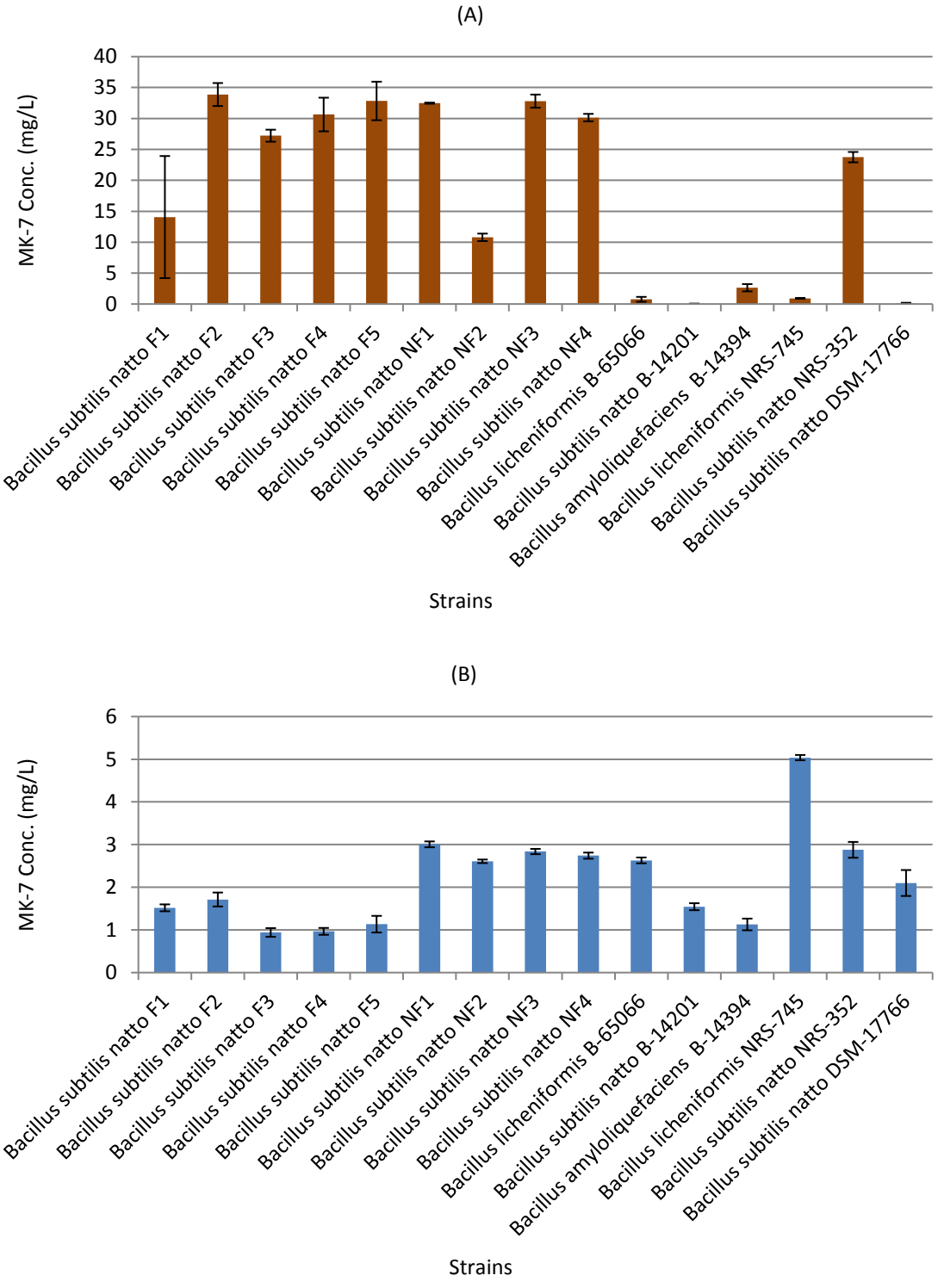


Figure 3.2. MK-7 concentrations of different strains of bacteria in SM (A) and TSB (B) media after 4 days of static fermentation at 37°C.

As can be concluded from Figure 3.2, fermentation in SM showed concentrations as high as 35.5 mg/L; while fermentation in TSB could hardly reach 5 mg/L. Although F3 and F5 strains produced prominent amounts of MK-7 in SM medium; generally F-type strains were not as efficient in TSB medium. At the same time, NF-type strains were significantly more efficient in TSB as compared to F-types. Based on the results, NRS-352 produced relatively high concentrations of MK-7 in TSB. Furthermore, *B. licheniformis* NRS-745 did not produce significant amounts of MK-7 in SM, results in TSB were unrivaled. Conclusively, *B. subtilis natto* strains NF1, NF3, NRS-352 and *B. licheniformis* NRS-745 were selected for the next phase for PCS selection. These four strains were selected for prominent MK-7 secretion not just in TSB or SM media, but in both at the same time. Four strains were selected instead of just one to be tested in combination with four PCS types available to provide a more statistically reliable selection in the end.

3.4.3 PCS selection

Although many different support materials have been utilized in bioreactors such as lignocellulosic materials (sawdust, wood chips/shavings, rice husks, cotton towels and straw), metallic alloys and plastic composites (Demirci et al., 2007), PCS types used in this study have been mostly yielding best properties to support different biofilm growths in our previous studies (Ercan and Demirci, 2013a). This is due to the fact that these PCS types favor microorganism adhesion, have a high mechanical resistance to liquid shear forces and particle collision which makes them ideal for the purpose of this study.

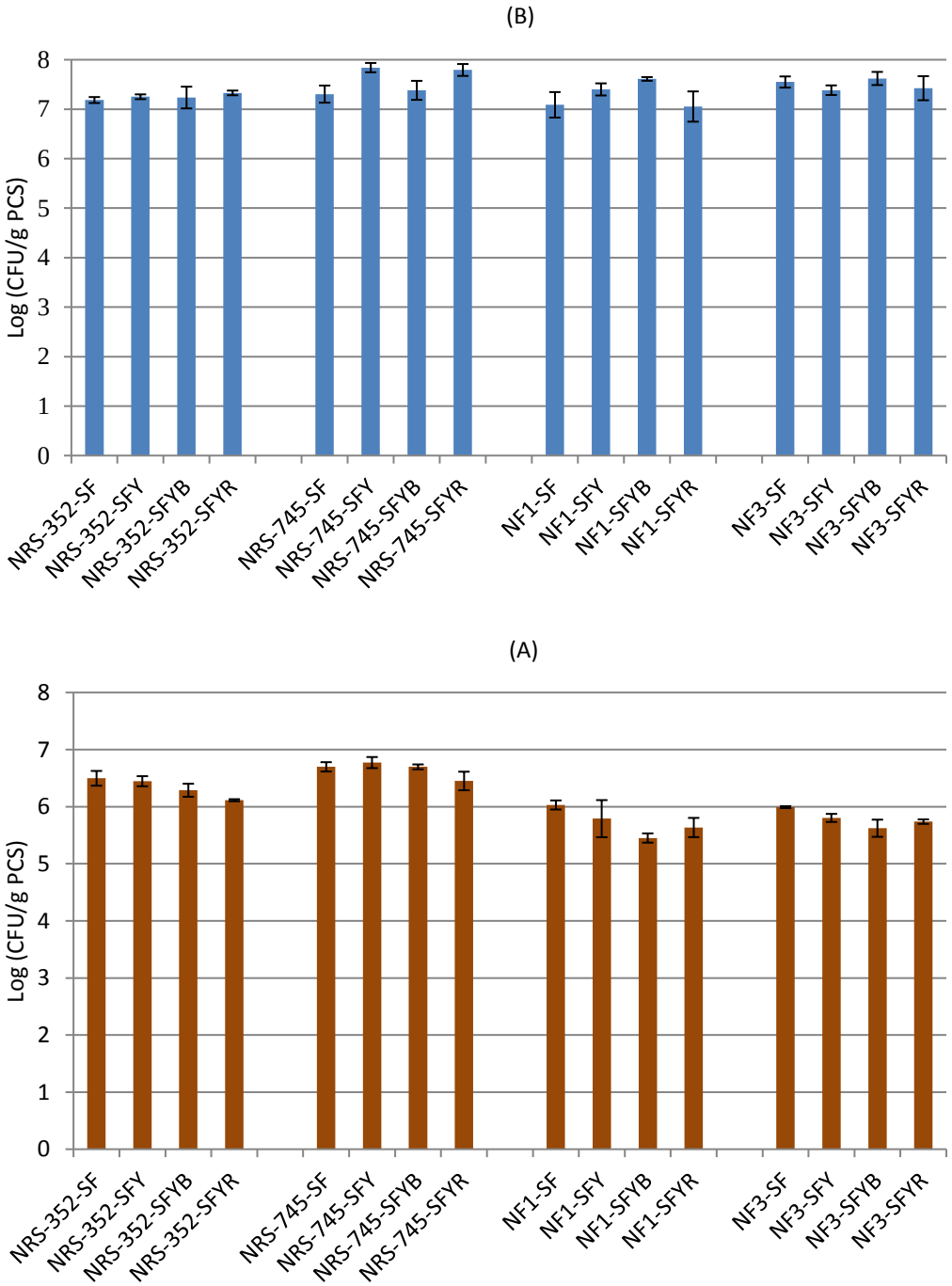


Figure 3.3. Biofilm formations for four top strains on four PCS types in SM (A) and TSB (B).

In the next step while the addition of PCS rings, pellicles were not freely formed anymore. Once agitation and PCS rings were introduced into the fermentation, bacterial colonies generally tended to grow better on PCS rings in TSB medium rather than SM medium (Figure 3.3). Biofilm formations were significantly more populated on the PCS rings in TSB as compared to SM (maximum 7.84 and 6.77 on the log scale respectively), regardless of the PCS type. Since TSB is low in nutrient, these results strengthen the conception that planktonic cells tend to form biofilms in order to enhance survival. However, since CFU counts on the PCS rings in SM were not too low (minimum 5.45 on the log scale), despite the fact that differences in CFU counts in TSB and SM were significantly different, CFU counts were not considered as a crucial factor in selections and thus only MK-7 concentrations were considered as the sole response. In this regard, however, the presence of the PCS rings along with agitation resulted in almost inhibiting MK-7 excretion in SM medium they did so less significant in TSB (Figure 3.4).

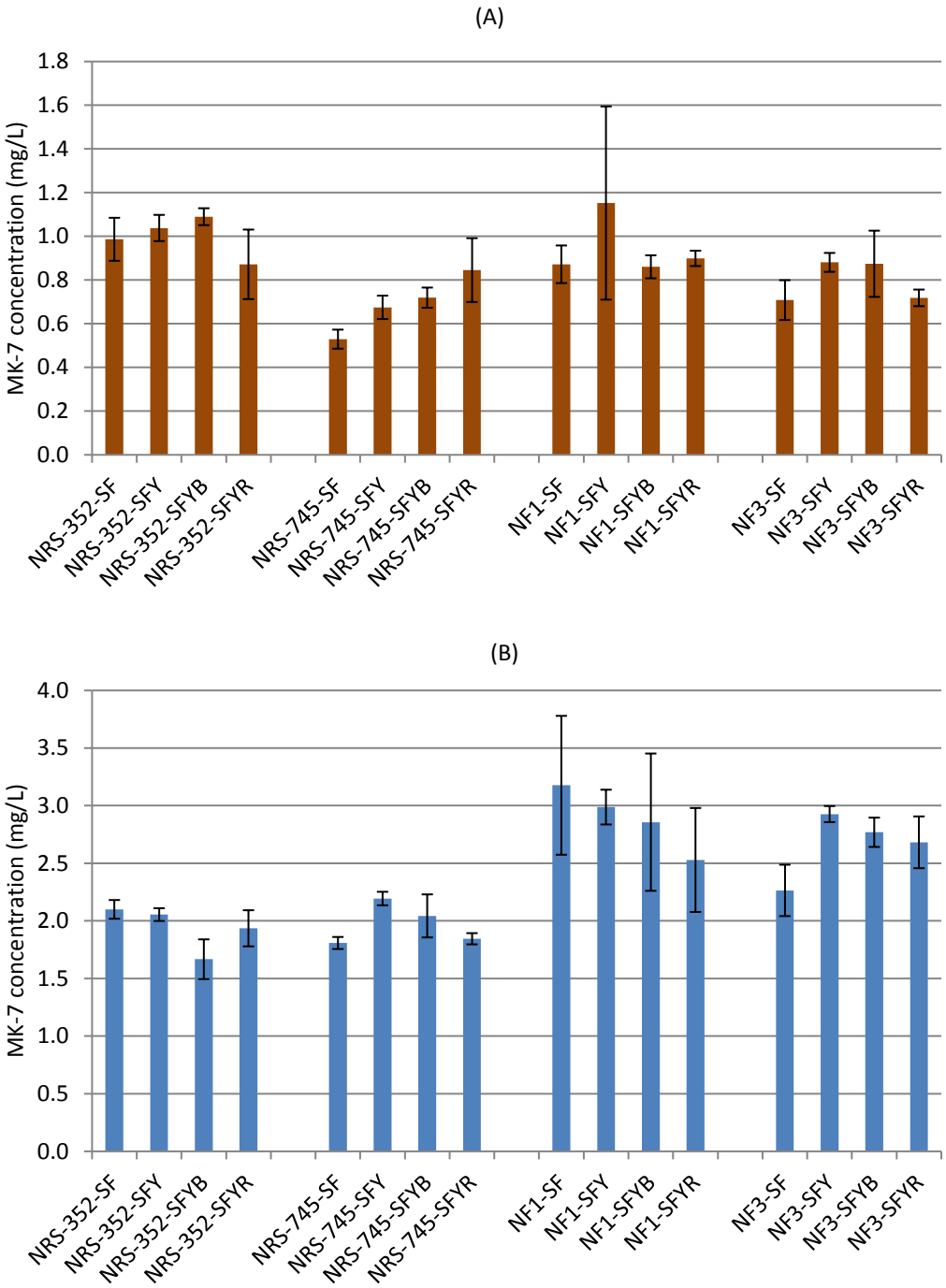
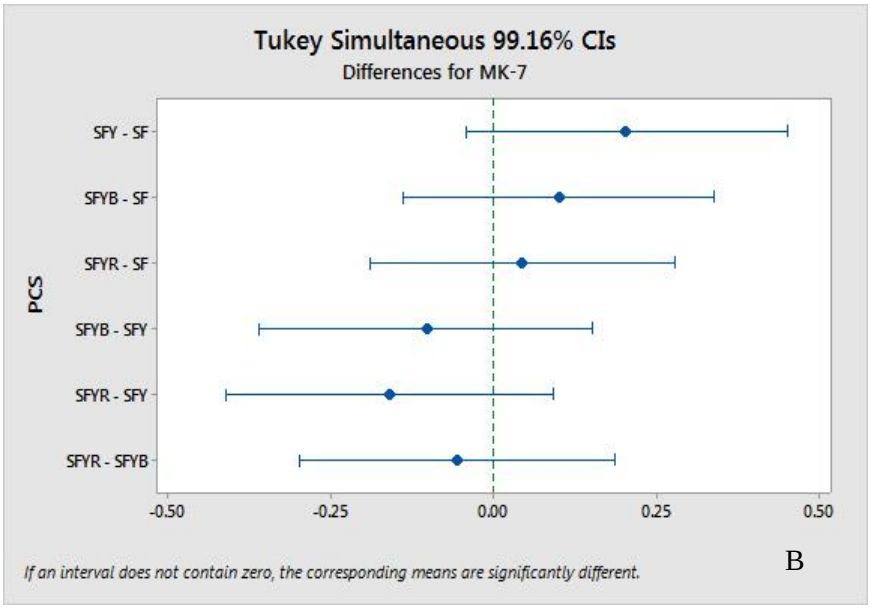


Figure 3.4. MK-7 concentrations in SM (A) and TSB (B) media in presence of PCS rings and agitation after 4 days of fermentation at 37°C and 200 rpm.

When the results in Figure 3.2 are compared to those in Figure 3.4, it indicates that concentrations in static fermentation are up to 37 mg/L of MK-7 in SM without PCS rings which are much higher than those in agitated fermentations with PCS rings where no concentrations higher than 1.6 mg/L were observed. This was once again aligned with our hypothesis that pellicle and biofilm formations are crucial for MK-7 metabolism (Berenjian et al., 2013). Possibly, the presence of PCS rings has even helped to eliminate pellicle formations, as PCS rings enhance turbulence in the mixture. Thus, applying bioreactors to the glycerol-based medium seems to require significant improvement through fermentation optimization to compensate for this suppression of MK-7 expression while using the biofilm reactors addresses scale-up and heat and mass transfer issues associated with static fermentations (Mahdinia et al., 2017a). In TSB however, MK-7 concentrations were not as drastically decreased. NF-type strains NF1 and NF3 produced significantly higher amounts of MK-7 as compared to NRS-352 and NRS-745.



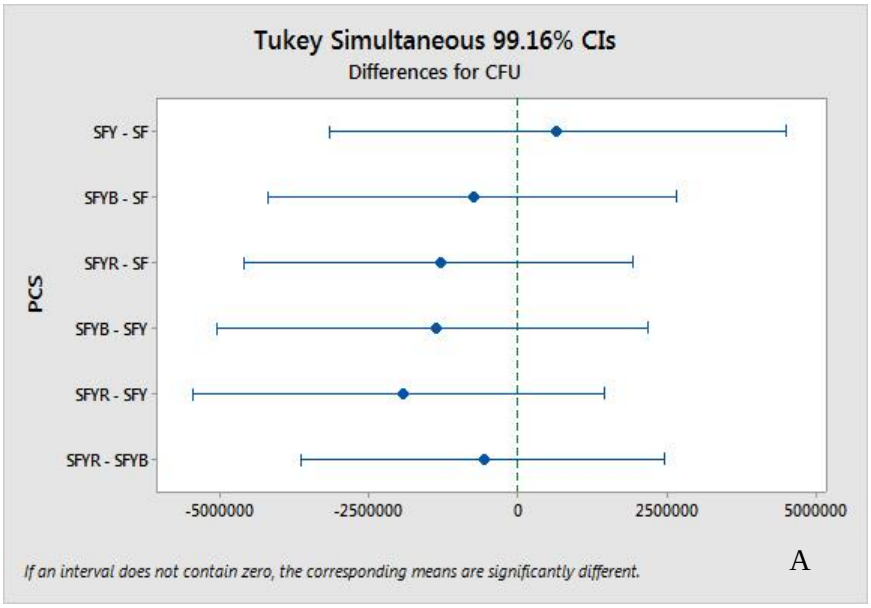


Figure 3.5. Pairwise comparisons of different PCS types on CFU formation (A) and MK-7 concentrations (B).

Pairwise comparisons (overall $p>0.05$) of CFU counts and MK-7 concentrations between different PCS types in Figures 3-2 and 3-3 indicated that none of the PCS types are significantly superior to the others in either media (Figure 3.5). The direct conclusion is that all 4 PCS types are interchangeable. These results enable the use of any of the four PCS types for constructing the biofilm without effecting the MK-7 concentrations. Thus,

the other practical considerations may be taken into account for selecting the PCS type for constructing biofilm reactors. Similarly, in the past study on pullulan production by *Aureobasidium pullulans*, three top PCS types namely SFY, SFYB and SFYR were also statistically insignificant (Cheng et al., 2010a). Yet, the 4 bacterial strains were significantly different as shown in Figure 3.6.

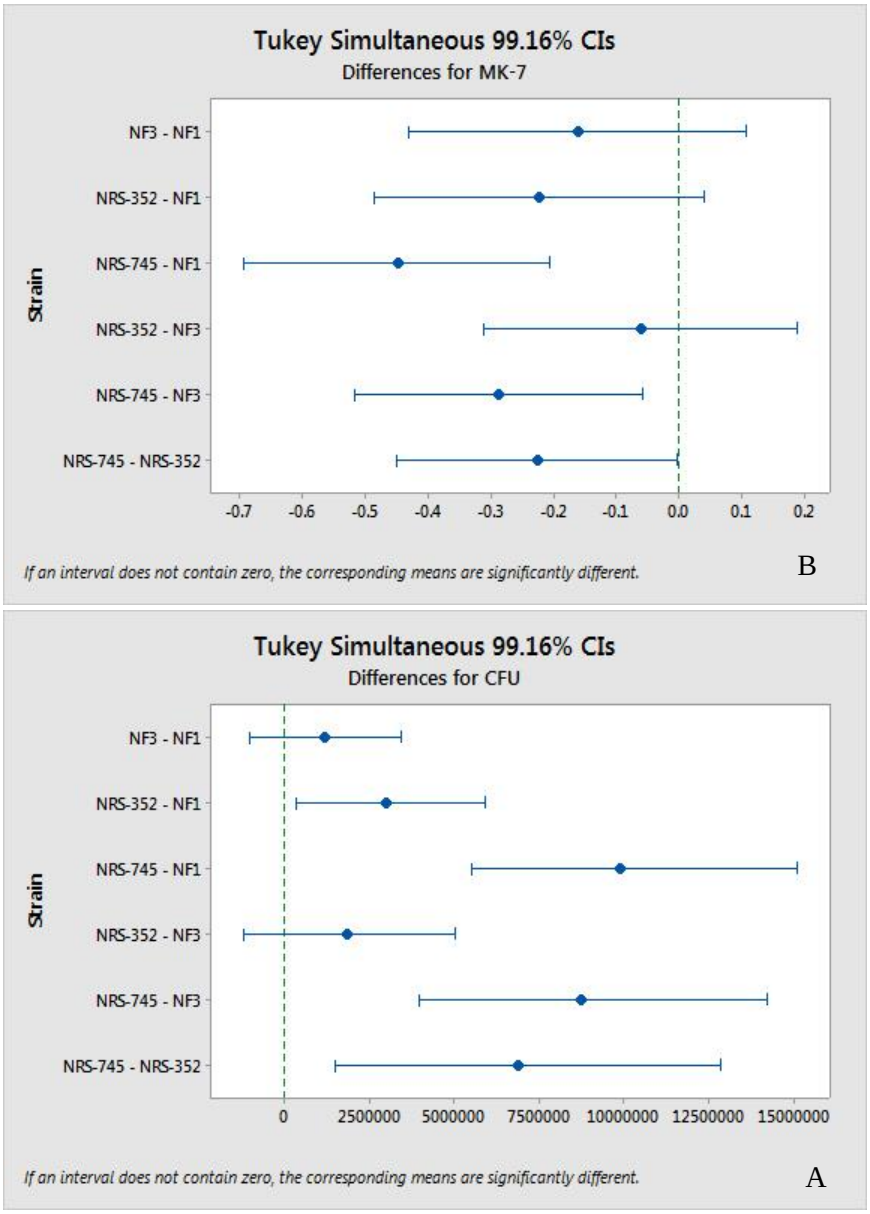


Figure 3.6. Pairwise comparisons of different bacterial strain types on CFU formation (A) and MK-7 concentrations (B).

Once again, although *B. licheniformis* NRS-745 showed significantly higher potential in CFU formations on the PCS rings, since CFU formation of other strains were not critically low, CFU formations were checked but not responded to. Therefore, it seems that NF1 is superior for MK-7 production in TSB. Analyzing the results in the optimizer software in Minitab with MK-7 concentrations as a sole response confirms this conclusion. Optimization using Minitab rendered the strain NF1, the PCS type SFY and the TSB medium to be the most potent combination for MK-7 production, although the PCS type is interchangeable with the other types, as none of the PCS types were statistically superior (Figures 3.5 and 3.7).

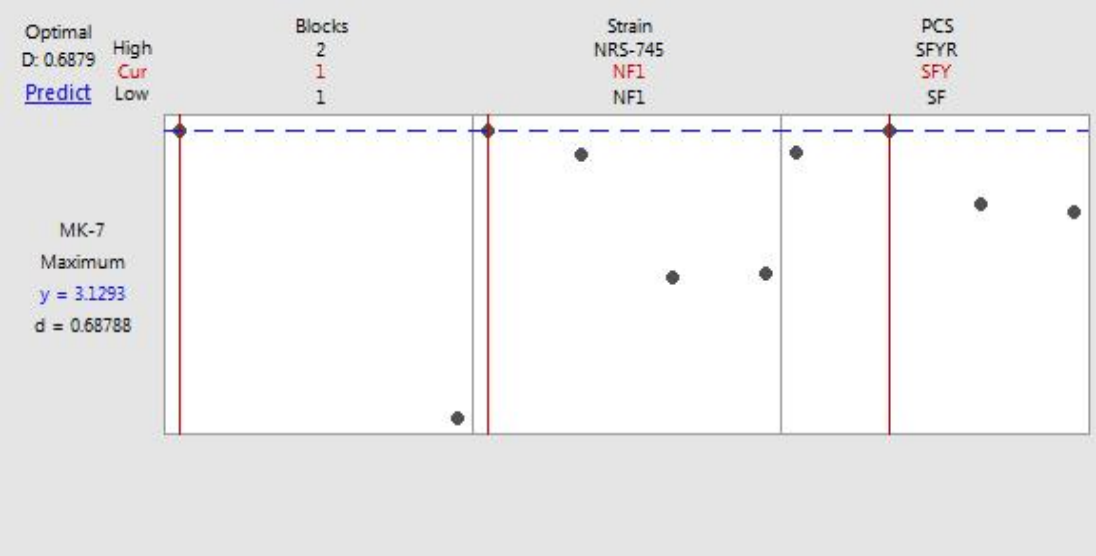


Figure 3.7. Optimization plot for maximum MK-7 concentration (block 1 for TSB and block 2 for SM).

3.5 Conclusions

Bacterial strains of *Bacillus*, especially *Bacillus subtilis natto*, have a key role in MK-7 production. On the other hand, their high tendency to form pellicles and biofilms on

surfaces controls MK-7 excretion. In this fashion, using a biofilm reactor to produce MK-7 may be a worthy effort and the first step to constructing a biofilm reactor is to find a suitable PCS type and strain. Thus, among the different *Bacillus* strains, 4 were selected to undergo PCS selection tests. Statistical analysis of PCS selection tests indicated that PCS types may not have a significant impact on MK-7 production and can be used interchangeably. However, different strains showed different potentials. Thus, pairwise comparisons of different combination possibilities and optimization targeting maximum MK-7 concentration suggest a combination of a natto derived *B. subtilis natto* strain (NF1) with the PCS type SFY in TSB medium to be the promising combination. The next practical step shall be implementing such viable combination in real-time biofilm reactors to design a production process for possible industrial production of MK-7. Thus, although biofilm reactors using PCS are anticipated to produce less amount of MK-7 compared to static fermentations, but through fermentation optimization, this gap in concentration may be filled. In that case hopefully, applying biofilm reactors that utilize robust agitation and aeration in liquid state will have none of the scale-up or heat transfer and mass transfer issues associated with current static fermentation strategies.

3.6 Acknowledgements

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Chapter 4

Optimization of *Bacillus subtilis natto* growth parameters in glycerol-based medium for vitamin K (Menaquinone-7) production in biofilm reactors*

4.1 Abstract

Menaquinone -7 (MK-7) is the key form of vitamin K used as a dietary supplement and its production revolves around *Bacillus subtilis natto*. Current fermentation strategies, which suggest static fermentations without aeration and agitation, can be problematic for large scale MK-7 production due to biofilm formation. The use of biofilm reactors, therefore, is proposed in the present study, which could utilize both agitation and aeration without interrupting MK-7 secretion. In this study, biofilm reactors were constructed using the selected Plastic Composite Support (PCS) and *B. subtilis natto* strain for MK-7 production. Using response surface methodology (RSM), optimum growth parameters including temperature, pH, and agitation were determined in a glycerol-based medium. Results were presented in a statistical model (R^2=0.90), leading to optimum growth conditions of temperature (35°C), agitation (200 rpm) and pH (6.58). Model predicted MK-7 concentration was validated and MK-7 concentration of 12.1 mg/L was produced in the biofilm reactor. The obtained concentration was 58% higher as compared to the suspended-cell culture (7.7 mg/L). The results of this study will provide a critical step towards improved industrial scale production of MK-7.

Keywords: MK-7, Menaquinone-7, Vitamin K, Biofilm reactor, *Bacillus subtilis*, RSM Optimization

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4.2 Introduction

Since 1935, when Henrick Dam studied on a fat soluble anti-hemorrhagic factor led to the discovery of vitamin K, numerous studies have been carried out until today to characterize different types and better understand the effects and metabolism of it (Dam, 1935). It did not take scientists long to discover that this vitamin has two major forms. The one that had smaller molecular weight, was less effective in treatment of symptoms and was mostly found in certain vegetables was called vitamin K1. The other one with typically larger molecular weight, was more effective and was mostly found in animal sources such as fish meals and liver oils, was called vitamin K2 (Binkley et al., 1939). The K1 type, also known as phylloquinone, has a direct role in photosynthesis in plants and therefore is abundant in most green leafy vegetables as a food source for vitamin K (Widhalm et al., 2012). Phylloquinone has a singular form and animal cells are capable to convert it to K2 subtypes (Davidson et al., 1998). K2 types are main storage forms in animals and have several subtypes that are characterized by the number of isoprenoid residues in their side chains, which is 4 residues in MK-4 and 7 residues in MK-7 (Mahdinia et al., 2017a).

Later on, scientists discovered that many bacterial strains can produce K2 types especially long-chain subtypes (MK-7 to MK-11) as they play the same role of electron transports as other quinones in respiration (Bentley and Meganathan, 1982). This discovery opened a new window to produce other MK types through fermentation and to utilize them as dietary supplements. Among these subtypes, MK-7 has definitely been most profoundly studied for this purpose as compared to the other common subtypes such as MK-4 (Berenjian et al., 2015). Both of these subtypes are used in the US as dietary food supplements but MK-7 has superior effects in human metabolism (Schurgers et al., 2007; Howard and Payne, 2006).

Health benefits of MK-7 for humans have been studied for several decades (Shi et al., 2017). There are two major areas where MK-7 has shown promising effects; in reducing the risk of cardiovascular diseases (Gast et al., 2009; Geleijnse et al., 2004) and osteoporosis (Yamaguchi, 2006). Therefore, such extraordinary benefits associated with

MK-7 has expanded the demand for its industrial production through microbial fermentation (Mahdinia et al., 2017a).

When it comes to MK-7 fermentation, there are only a few strains of the *Bacillus* genus including *B. subtilis natto* (Berenjian et al., 2011b), *B. licheniformis* (Goodman et al., 1976) and *Bacillus amylolyquifaciens* (Wu and Ahn, 2011) that have been studied for this purpose. All of these bacteria are aerobes and have a potent tendency to form pellicles and biofilms (Mahdinia et al., 2017a). Solid State fermentation (SSF) strategies have been studied for MK-7 production (Singh et al., 2015; Wu and Ahn, 2011), but they are basically problematic to scale up, although they are generally associated with less operating costs (Pandey, 2003). On the other hand, Liquid State Fermentation (LSF) strategies without agitation or aeration preserve pellicle and biofilm formations that are beneficial to MK-7 biosynthesis, since MK-7 secretion is hypothesized to be effected by the precursors engaged in the formation of the extracellular matrices in the pellicle and biofilm formations (Ikeda and Doi, 1990); but creates operational issues including heat and mass transfer problems (Berenjian et al. 2013). Thus, the ideal case is to create a suitable condition for biofilm formations to form and at the same time being able to robustly agitate and aerate the system. This is efficiently possible by the use of biofilm reactors (Ercan and Demirci, 2013a).

In general, biofilm reactors harness the same extraordinary features that microbial cells gain by switching from planktonic cell form to mature biofilm form through passive immobilization (Demirci et al., 2007). Gaining more resistance to desiccation, grazing, and antimicrobial agents in the biofilm structure as well as boosted expression of certain metabolites through gene expression changes that occur due to immobilization, are among such features (Kuchma and O'Toole, 2000). Production of many value-added products have been enhanced by the use of biofilm reactors and formation of biofilms (Ercan and Demirci, 2013b; Ercan and Demirci, 2014; Ho et al., 1997; Izmirlioglu and Demirci, 2016).

In the past studies, different carbon sources have been investigated, namely glucose, mannose, sucrose, etc (Berenjian et al., 2011a). However, glycerol-based media have been recommended due to the fact that glycerol is not only metabolized by *B. subtilis* as a cheap carbon source, but also may improve MK-7 biosynthesis and secretion

through cell membrane and media alterations (Berenjian et al., 2011b).Therefore, the aim of this study was to form biofilm reactors and evaluate and optimize the key growth factors effecting MK-7 production in *Bacillus subtilis natto*.

4.3 Materials and methods

4.3.1 Microorganisms and media

As previously described (Chapter 3), *Bacillus subtilis natto* (NF1) were isolated from commercially available natto by using tryptic soy agar plates and were grown at 40°C for 24 h in Tryptic Soy Broth (TSB) (Difco, Detroit, MI) supplemented with 0.8% yeast extract (TSBYE) (Difco) as a base medium at static fermentation for inoculum preparation. TSB medium including 10% (w/v) glucose (Tate & Lyle, Decatur, IL) and 0.8% yeast extract (Biospringer, Milwaukee, WI) (TSBGYE) was used for biofilm formation. Main fermentation media consisted of 100 g of soytone (Difco), 35 g of yeast extract (Difco), 45 g of glycerol (EMD Chemicals, Gibbstown, NJ) and 0.6 g of K_2HPO_4 (VWR, West Chester, PA) per liter of deionized water, as concluded in previous studies with some modifications (Berenjian et al., 2011a). The cultures were stored at 4°C and sub-cultured monthly in order to maintain viability. For long-term storage, stock cultures were maintained at −80°C in a 20% glycerol solution.

4.3.2 Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA) equipped with 2-L vessels were used. During the fermentation, sterile 4N sulfuric acid (EMD) and 4N sodium hydroxide (Amresco, Solon, OH) along with antifoam B emulsion (Sigma-Aldrich, Atlanta, GA) were automatically added to the bioreactors to maintain pH and suppress foaming as needed. Plastic Composite Support (PCS) tubes type SFYB (50%

Polypropylene, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% bovine albumin and salts) were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Ho et al. (Ho et al., 1997a). PCS tubes were cut in about 6.5 cm lengths and the grid-like fashion was formed on the agitator shafts (Figure 4.1) as described in previous studies (Ercan and Demirci, 2014; Izmirlioglu and Demirci, 2016).

Figure 4.1. PCS grid formation in bioreactors.

4.3.3 Biofilm formation

In order to form the biofilms on the PCS grids, bioreactors were set up as described above and were autoclaved at 121°C for 45 min containing 1.5 L of DI water, as the working volume of the bioreactors were 1.5 L each. After sterile TSBGYE medium replaced aseptically the initial water, bioreactors were inoculated with 3% (v/v) 24-h grown suspended-cell culture at 25°C. Then, TSBGYE medium was refreshed every 48 h for 4

times to allow biofilm formations on the PCS. Once the biofilm was in place, the fermentation broth was sampled and Gram-stained to verify a pure culture.

4.3.4 Experimental design

Response Surface Methodology (RSM) was used to investigate the effects of three growth variables each with 3 levels being temperature (35-45ºC), pH (6-8) and agitation (100-200 rpm) chosen in this study on MK-7 concentrations. A total of 15 consecutive batch fermentations were carried out and each run was 144h long and samples were taken every 12 hours before the medium was refreshed for the next set of conditions of the next batch. After each batch completion, medium was completely pumped out aseptically so that only biofilm formations on the PCS would remain and then quickly, fresh sterile medium was pumped into the fermenters to start the next batch. Maximum MK-7 concentration was treated as the sole response (Table 4.1). After optimization by using the responses, repeated validation runs in biofilm reactors were performed by under suggested optimum growth parameters. Also, repeated suspended-cell fermentations as a control were also performed and compared with the biofilm reactors. Furthermore, repeated bioreactors implemented with PCS tubes the same as biofilm reactors were carried out; but in this case, the biofilm formation steps were not carried out in order to provide comparison to illustrate the effects of the biofilm formation steps.

4.3.5 MK-7 measurement

MK-7 was extracted from 3 mL of fermentation broth using 2:1, v/v n-hexane:2-propanol mixture (Berenjian et al., 2011a). In this study, n-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used for MK-7 extraction. The mixture was vigorously shaken with a vortex mixer for 3 min and then the organic phase was separated and evaporated under forced air flow. Then, dried pellets were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH) for 15 min at ambient

temperature. After the pellets were completely suspended in methanol, the mixtures were filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY). High performance liquid chromatography (HPLC) (Waters, Milford, MA) equipped with a 2489 UV/Visible detector and a Supelcosil C18 column (15 cm×4.6 mm, 5µm, Supelco Analytical, Bellefonte, PA) was used at 40ºC for the analysis of MK-7 concentration. Methanol (EMD) was used as mobile phase with the flow rate of 1 mL/min. The wavelength of 248 nm was used for calibration and analysis. The MK-7 calibration curve obtained from 99.9% pure MK-7 (Chroma Dex Corporation, Irvine, CA) was linear between 0.1 mg/L and 30 mg/L ($R^2 = 0.999$).

4.3.6 Glycerol measurement

Glycerol is the primary carbon source in fermentations in this study. Also, glycerol is believed to affect MK-7 biosynthesis through altering cell membrane composition in *B. subtilis* and medium viscosity (Berenjian et al., 2011b). Therefore, it is essential to investigate and monitor the consumption of glycerol concentration throughout the runs. In this fashion, fermentation broth was centrifuged at 9000 g for 5 min (Microfuge 20 Series, Beckman Coulter Inc., Brea, CA) and then filtered through 0.2 µm cellulosic filters (PALL). Then, without dilution, the clear broth was analyzed by HPLC (Waters) equipped with a 2414 Refractive Index detector and an HPX-87H Aminex column (300×7.8 mm, 9 µm, Bio-Rad, Hercules, CA) at 50ºC and 410 nm. A 0.05 M sulfuric acid (EMD) solution was used as the mobile phase. Samples were kept at 4°C during the injections. The glycerol calibration curve obtained from 99.3% pure glycerol (EMD) was linear between 1 g/L and 60 g/L ($R^2 > 0.999$).

4.3.7 Statistical analysis

Using Minitab 17.0 Analysis of Variance (ANOVA) (Minitab Inc., State College, PA) with statistical model and regression analysis with Box-Cox transformation optimal λ,

the effects of temperature (°C), pH and agitation (rpm) along with 2nd order and two-way interaction effects were obtained. Minitab is able to design RSM tables and analyze the sole or several responses (in this case MK-7) that are measured in the experiments through ANOVA or Analysis of Covariance (ANCOVA) to determine statistically significant differences in the responses and through optimization determine the optimal conditions (in this case temperature, pH and agitation) for extremum (in this case maximum) response(s). Since it was hypothesized that higher levels of interactions are not significantly effective, ANOVA was selected instead of ANCOVA. A confidence level of 95% was implemented throughout the analysis procedures to distinguish significant parameters (Rahimi et al., 2017a and 2017b).

4.4 Results and discussions

Temperature, pH and agitation were chosen as the key factors to optimize *B. subtilis natto* growth in glycerol based medium for MK-7 production in biofilm reactors. Table 1 shows the factorial design used and the observed MK-7 values in the analysis and predicted values from the model. As mentioned above, these responses were the maximum MK-7 concentrations observed in each run in the design, which were almost entirely obtained towards the end of the runs in the sixth day of fermentation. The maximum observed value in the entire runs was 12.65 mg/L at 35ºC, pH 7.5 and 200 rpm (Table 4.1). Following ANOVA of these observed values rendered a statistical model to explain the effect of each variable on MK-7 concentrations.

Table 4.1. Response Surface Methodology design including variables temperature (°C), pH, agitation (rpm) and 1 vvm aeration in predicting MK-7 concentrations (mg/L) in biofilm reactors.

Run	Growth factors			MK-7 Concentration (Observed) (mg/L)	MK-7 Concentration (Predicted) (mg/L)
	Temperature (°C)	pH	Agitation (rpm)		
1	40	7.5	150	11.92	10.36
2	45	8.0	150	9.14	8.59
3	45	6.0	150	3.69	4.11
4	35	7.5	200	12.65	12.52
5	45	7.5	100	7.88	8.195
6	40	6.0	200	10.96	9.82
7	40	6.0	100	6.82	6.80
8	40	8.0	200	9.19	9.58
9	35	6.0	150	11.75	12.50
10	40	7.5	150	10.03	10.36
11	45	7.5	200	10.97	11.21
12	40	8.0	100	6.85	6.56
13	35	7.5	100	10.16	9.51
14	35	8.0	150	7.09	7.55
15	40	7.5	150	8.87	10.36

4.4.1 Temperature

In this study, it was concluded that temperature affects MK-7 concentrations in biofilm reactors in a different way as compared to the results reported in previous studies, despite the fact that similar *B. subtilis natto* strains were used in both studies. In the respective studies, 40°C and 45°C were deemed best temperature for MK-7 secretion (Berenjian et al., 2013; Sato et al., 2001), but in this study 35°C was found to be the optimum (Figure 4.2).

Figure 4.2. Optimal conditions and the optimal MK-7 concentration predicted by the model optimizer in biofilm reactors with 1 vvm aeration.

Although similar medium compositions and bacterial strain were utilized in these studies, it must be taken into consideration that fermentation in shake flasks and biofilm reactors are distinctive enough to cause such differences. In this case, temperature effects were statistically most significant as compared to agitation and pH (p-value < 0.000). The RSM design temperature range was 35-45°C and ANOVA analyses indicated a constant increase in MK-7 secretion as temperature decreased (Figures 4.3B and 4.3E); rendering 35ºC to be optimum (Figure 4.2).

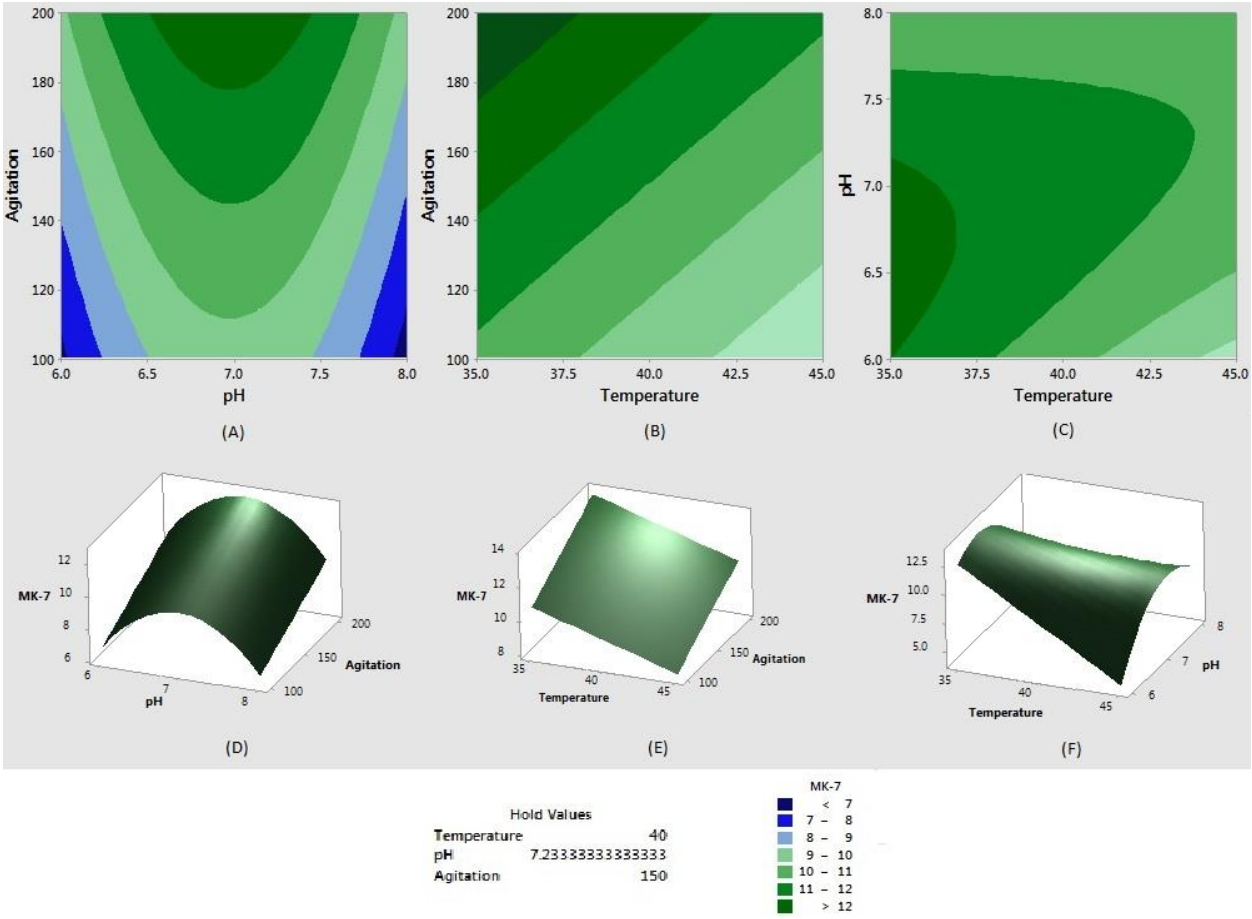

Figure 4.3. Contour and surface plots generated by the model for MK-7 concentration (mg/L) behaviors with response to different temperature (°C), pH and agitation (rpm) in biofilm reactors with 1 vvm aeration.

However, further tests in lower temperatures (25, 28, and 30°C) indicated that 35°C is in fact the optimum temperature in this case (Figure 4.4). Temperatures less than 30°C are not really expected to work anyway since glycerol as the primary carbon source is elaborative to metabolize for *Bacillus* strains (Lindgren and Rutberg, 1974) and low temperatures certainly do not help. Especially going down from 28°C to 25°C hindered the metabolism as glycerol consumptions were declined by around 80% which explains the insignificant MK-7 concentrations in those temperatures (Figure 4.4).

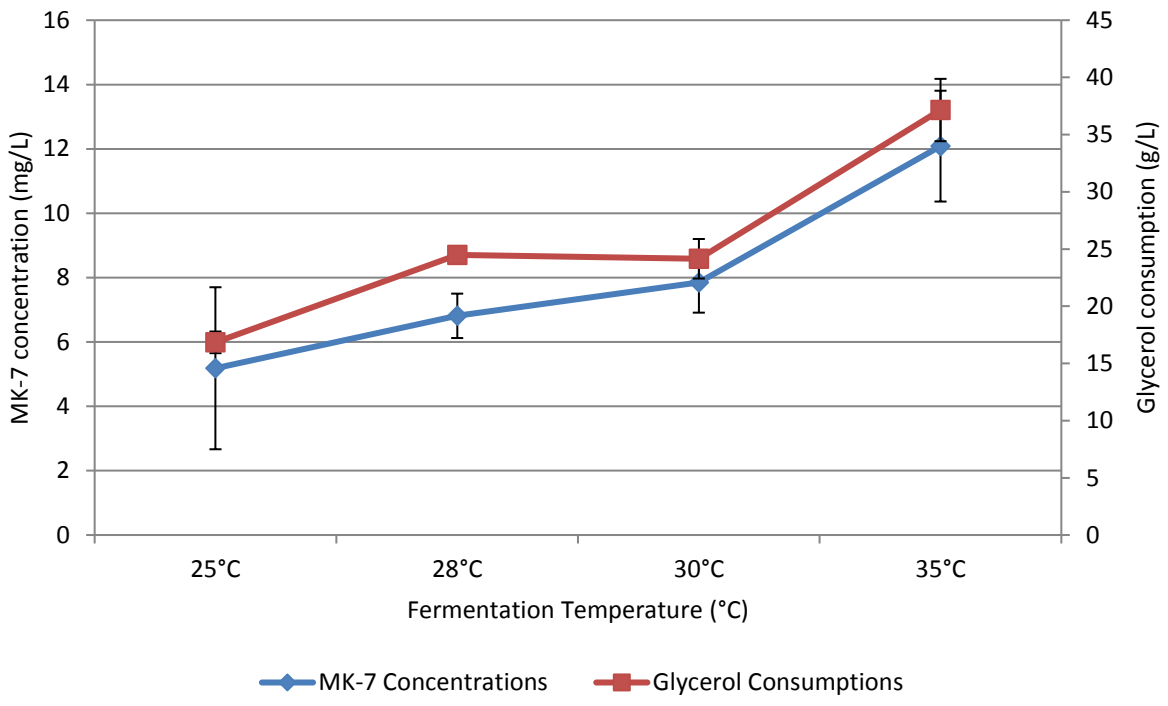

Figure 4.4. MK-7 concentrations (mg/L) (repeated) and glycerol consumptions (g/L) (repeated) observed under pH 6.58 and agitation 200 rpm and temperatures equal and below 35°C in biofilm reactors with 1 vvm aeration.

4.4.2 pH

In previous studies in the literature, pH was never controlled as it was believed that significant pH changes that occur during the fermentation is important for MK-7 secretion (Sato et al., 2001a; Berenjian et al., 2011a). However, in biofilm reactors pH controlling seems to be beneficial to MK-7 secretion despite the fact that without pH control, a similar pH profile is observed throughout the fermentation as compared to Sato and Berenjian's findings (Figure 4.5). pH itself was found statistically significant by second order (p-value < 0.002) (Table 4.2) and RSM plots confirmed a significance of pH-temperature interaction (Figures 4.3C and 4.3F) that was originally predicted by ANOVA (Table 4.2).

Table 4.2. ANOVA output for MK-7 concentrations (mg/L) versus temperature (°C), pH, agitation (rpm) and 1 vvm aeration in biofilm reactors.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Regression	5	73.31	89.98%	73.31	14.66	16.17	0.000
Temperature	1	12.42	15.25%	28.51	28.51	31.43	0.000
pH	1	1.08	1.32%	4.40	4.40	4.85	0.055
Agitation	1	18.18	22.31%	18.18	18.18	20.04	0.002
pH*pH	1	16.60	20.38%	16.60	16.60	18.31	0.002
Temperature*pH	1	25.03	30.72%	25.03	25.03	27.60	0.001
Error	9	8.16	10.02%	8.16	0.91		
Lack-of-Fit	7	3.42	4.20%	3.42	0.49	0.21	0.952
Pure	2	2.00	474.20%	4.74	2.37		
Total	14	81.475	100.00%				

Thus, pH and temperature two-way interaction was statistically significant and incorporated into the finalized model (p-value < 0.001) and an optimum pH of 6.58 was determined by RSM (Figure 4.2). Furthermore, MK-7 concentrations decreased when pH was not controlled as compared to the pH controlled fermentation at the optimum pH (6.58) (Figure 4.5). This observation is yet another proof of how significantly scaling up production can effect MK-7 secretion, in this case from shake flask fermentations to 1.5 liter bench-top bioreactors. Similar to low temperatures, extremely high pH conditions seem to have similar effects; maintaining pH conditions above 8 completely inhibits metabolism although under extreme nutrient deficiencies pH may naturally reach pH 9 in the fifth or sixth day (data not shown).

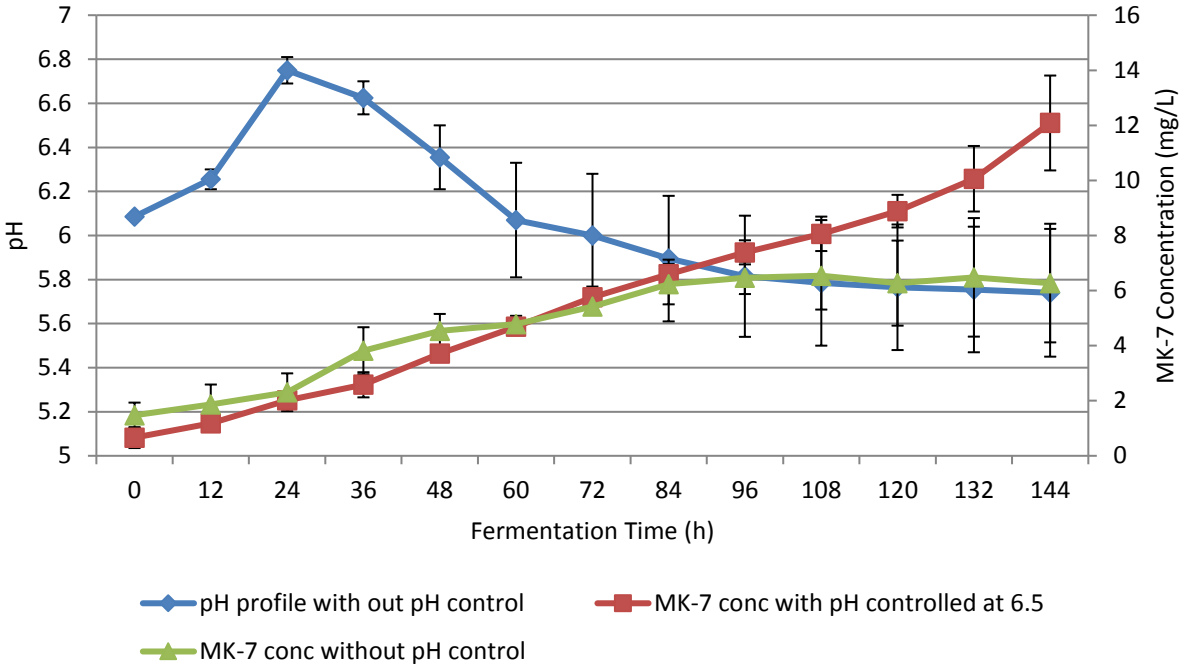

Figure 4.5. MK-7 concentrations (mg/L) (repeated) with pH controlled at 6.58 and without pH control both in biofilm reactors under optimal temperature (35°C), agitation (200 rpm) and 1 vvm aeration.

4.4.3 Agitation

Generally for aerobic fermentations in biofilm reactors, oxygen transfer is a challenge (Ercan and Demirci, 2013a). Therefore, higher agitation and aeration rates are usually believed to be favorable in many cases. Similarly, higher agitation rates were also found to be favorable in this study for MK-7 fermentation (Figures 4.3A and 4.3D). While aeration rates were kept at a constant 1vvm throughout all the experiments in this study and it is practically the highest feasible in the utilized bioreactors in the long-term, agitation rates were kept below 200 rpm. Higher agitation rates were possible, but not recommended since they may cause overstress on the PCS sticks on the propellers that may lead to biofilm detachment. Naturally, agitation was a significant factor (p-value < 0.002) and the highest

evaluated agitation rate (200 rpm) was recommended by the optimizer software as a constant MK-7 concentration enhancement was observed as agitation rates increased (Figures 4.2, 4.3B, and 4.3E).

4.4.4 Optimization of evaluated growth factors

Among these variables all 3 were found significantly effective on MK-7 concentration (Table 4.2). Using ANOVA, the effective terms of the model were obtained as shown in Table 2. Despite the fact that the first order pH term was less significantly affecting the response (p-value > 0.055), it was kept in the model to maintain hierarchy. The other terms, temperature and agitation were more significantly effective (p-value < 0.000) (Table 4-2). The regressed model is displayed in Eqn. 4.1.

$$\left[MK-7\ Concentration\ \left(\frac{mg}{L}\right)\right] = [8.6] - [3.669] \times [Temperature\ (°C)] + [22.6] \times [pH] + [0.03015] \times [Agitation\ (rpm)] - [2.971] \times [pH] \times [pH] + [0.4717] \times [Temperature\ (°C)] \times [pH] \quad \text{Eqn. 4.1}$$

As also shown in Figure 4-6, regression of the predicted values versus observed values renders a slope of 1.0 and R^2=0.90 with no significant abnormalities.

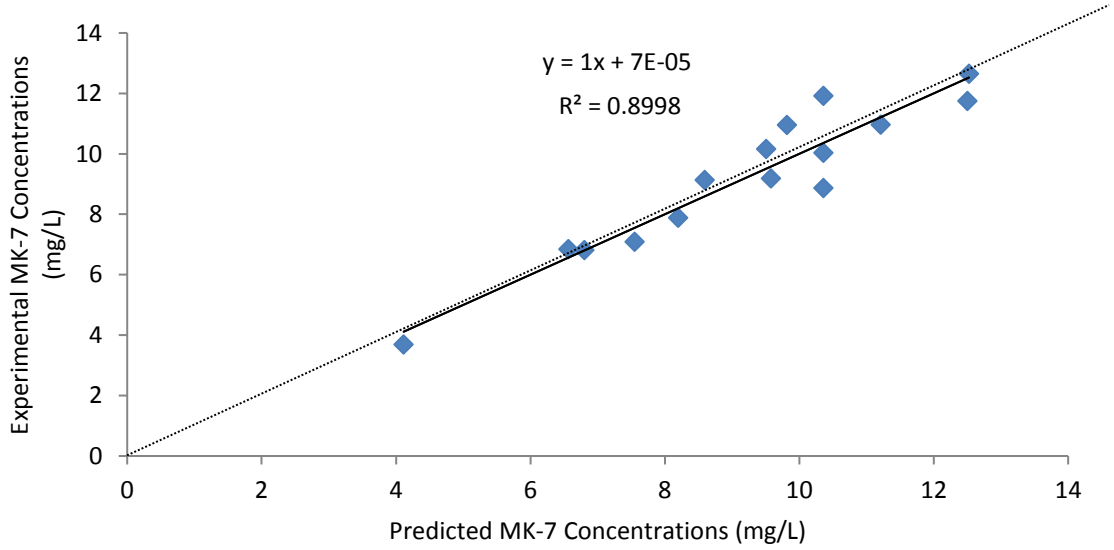

Figure 4.6. Experimental MK-7 concentrations (mg/L) in biofilm reactors at pH 6.58, 200 RPM, 35°C and 1 vvm plotted vs predicted values generated by the model.

Temperature of 35ºC, pH 6.5 and agitation of 200 rpm were predicted by the model to be optimum. Optimizing conditions targeting maximum MK-7 concentration, a maximum MK-7 concentration of 15.02 mg/L was predicted (Figure 4.2). Thus, validating this prediction, a maximum concentration of 12.09±1.72 was achieved, which is within a 75% accuracy range (Figure 4.7). This accuracy is close to the prediction accuracy that the statistical model suggests (70.8%). However, 12.09±1.72 mg/L concentration in the biofilm reactors is still 58% higher than the ones in suspended-cell reactors under the same optimum conditions (7.67±2.15 mg/L) (Figure 4.7), which clearly shows the contribution of biofilm reactors. Also, increasing agitation rate from 200 to 250 RPM does not seem to meaningfully make a difference in MK-7 concentration or even glycerol metabolism (Figure 4-8). Also running PCS implemented bioreactors resulted in glycerol consumption patterns similar to suspended-cell reactors, although MK-7 profile seemed different. As figure 4-8 depicts, there is a clear gap between the glycerol consumption pattern in biofilm reactors and all the controls (suspended-cell at 200 or 250 rpm and PCS implemented), where glycerol is almost depleted in biofilm reactors but barely goes below 20 g/L in the controls (Figure 4.8). This proves that biofilm formations on the PCS significantly boost the metabolism that also leads to enhanced MK-7 biosynthesis. As figure 4.7 indicates, again there is a meaningful gap between MK-7 biosynthesis profile in biofilm reactors compared to suspended-cell reactors. However, PCS implementation created a pattern in MK-7 biosynthesis similar to suspended-cell reactors until 96h but then there was a sudden increase that led to concentrations similar to the ones in biofilm reactors by the end of the sixth day (Figure 4.7).

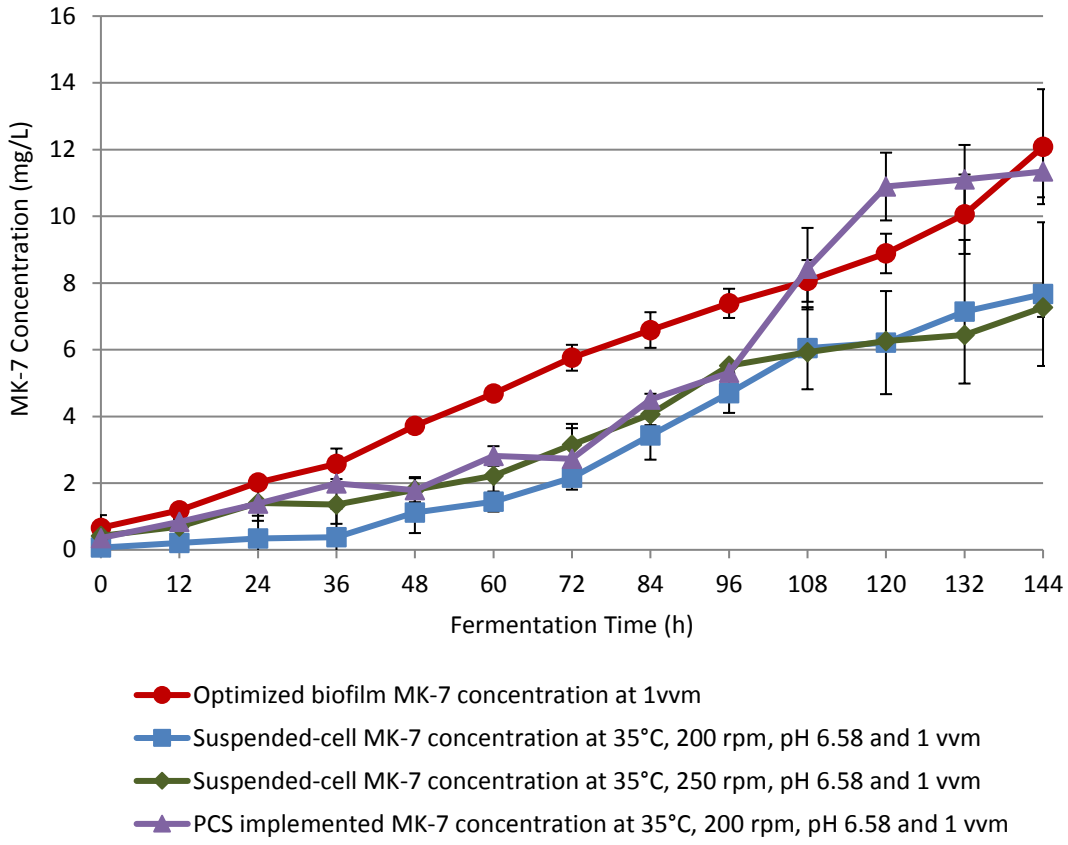

Figure 4.7. MK-7 profile for fermentation in biofilm reactors, suspended-cell bioreactors and PCS implemented bioreactors.

This is possibly due to the fact that biofilm formations are slowly forming on the PCS and kick in after four days of fermentation in the PCS implemented bioreactors and hence the sudden jump in concentrations. This only confirms how efficient biofilm formations are in enhancing metabolism in *B. subtilis* and consequently MK-7 biosynthesis. Therefore, this can be a critical step towards improved industrial scale production of MK-7 by using biofilm reactors.

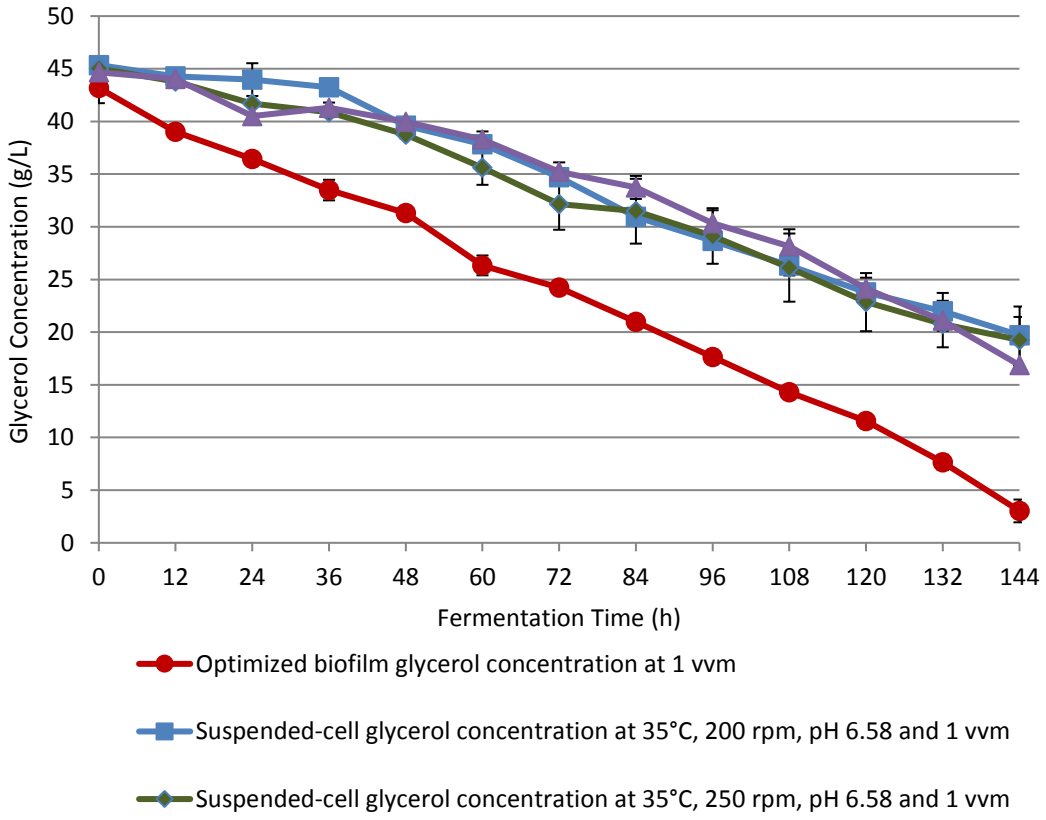

Figure 4.8. Glycerol profile for fermentation in biofilm reactors, in suspended-cell bioreactors and PCS implemented bioreactors.

4.5 Conclusions

In this study, biofilm reactors were successfully constructed and used to produce MK-7. Using RSM with a general factorial design, temperature, agitation and pH were optimized to achieve the highest MK-7 biosynthesis in biofilm reactors. Maximum MK-7 concentration was achieved under the optimum conditions which was significantly higher that its counterpart in suspended-cell reactors. This finding promises a potential of using biofilm reactor in MK-7 production and addressing current issues. Also, the results give a

perspective towards future studies to optimize media components and investigate different media to even further enhance the concentrations.

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Chapter 5

Utilization of glucose-based medium and optimization of *Bacillus subtilis natto* growth parameters for vitamin K (Menaquinone-7) production in biofilm reactors

5.1 Abstract

Menaquinone -7 (MK-7) is the most potent form of vitamin K prescribed as a dietary supplement. MK-7 is commonly produced via static fermentation of *Bacillus subtilis natto*. The fermentation of this bacterium is associated with formation of large amounts of pellicles and biofilm, which are effective in MK-7 production, but also result in significant heat and mass transfer challenges during the scale-up process. Thus, the objectives of this study were to develop and evaluate the possibility of using a biofilm reactor for MK-7 production. In this research, biofilm reactors were constructed using the Plastic Composite Supports (PCS). In order to optimize the fermentation parameters for MK-7 production, Central Composite Design (CCD) was carried out along with supplementary runs to determine the optimum temperature, pH, and agitation. The biofilm reactors were able to produce a maximum concentration of 18.45±0.76 mg/L of MK-7 which was 237% higher than the suspended-cell fermentation. Therefore, the present work suggests the possibility of using biofilm reactors as a new and effective fermentation strategy to address the issues associated with MK-7 fermentation.

Keywords: MK-7, Menaquinone-7, Vitamin K, Biofilm reactor, *Bacillus subtilis*, RSM Optimization

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*Mahdinia, E., Demirci, A., & Berenjian, A. (2018). Utilization of glucose-based medium and optimization of *Bacillus subtilis natto* growth parameters for vitamin K (menaquinone-7) production in biofilm reactors. *Biocatalysis and Agricultural Biotechnology*. 13, 219-224.

5.2 Introduction

First time in 1935, Henrick Dam carried out a series of studies on a fat soluble anti-hemorrhagic factor in chicken which later led to the discovery of vitamin K (Dam, 1935). Since its discovery, many more studies have been conducted to characterize different types of vitamin K and to better understand its effects and metabolism. Soon, it was discovered that this vitamin has two major forms. The one with smaller molecular weight, less effective in treatment of symptoms and abundant in green leafy vegetables was named phylloquinone or vitamin K1 (Widhalm et al., 2012). Phylloquinone has a unique form and some animal cells are capable of converting it to K2 subtypes (Davidson et al., 1998). The other type with larger molecular weights, more effective in human metabolism and available in animal source foods such as red meats and cheese were named menaquinones or vitamin K2 (Binkley et al., 1939; Mahdinia et al., 2017a).

More recently, scientists realized that many bacterial strains are capable to produce menaquinones, especially MK-7 to MK-11. These longer chains subtypes play the same electron transporting role as other quinones do in cell respiration (Bentley and Meganathan, 1982). Thus a new window of opportunity was opened to produce vitamin K through fermentation and to utilize it as dietary supplement. Among these subtypes, MK-7 is the one that has been most profoundly studied for this purpose as compared to all the other common menaquinones due to its unique effects on reducing the risk of cardiovascular disease and osteoporosis (Berenjian et al., 2015; Schurgers et al., 2007; Howard and Payne, 2006). In this fashion, studies surrounding MK-7 have indicated that higher doses being supplemented to diets, significantly reduces the risk of cardiovascular diseases (Gast et al., 2009; Geleijnse et al., 2004) and may help prevent osteoporosis as well (Yamaguchi, 2006). Moreover, recent studies have shown that MK-7 has significant antitumor potentials as well (Shi et al., 2017). These extraordinary benefits of MK-7 have created a greater demand for its industrial production through microbial fermentation (Mahdinia et al., 2017a).

For fermentation purposes, only a few strains of the *Bacillus* genus including *Bacillus subtilis natto* (Berenjian et al., 2011a), *Bacillus licheniformis* (Goodman et al., 1976) and *Bacillus amylolyquifaciens* (Wu and Ahn, 2011) have been investigated during

the last decade. Besides being aerobic, all of these strains have a potent tendency to form pellicles and biofilms (Mahdinia et al., 2017a). Applying Solid State Fermentation (SSF) strategies for MK-7 production (Singh et al., 2015), (Wu and Ahn, 2011) due to the formation of large amounts of biofilm that are beneficial to MK-7 biosynthesis(Ikeda and Doi, 1990), results in serious operational issues including heat and mass transfer limitations and thus, scale-up issues (Pandey, 2003). More or less the same scenario occurs by applying Liquid State Fermentation (LSF) strategies without agitation or aeration (Mahdinia et al., 2017a). Therefore, there is a need for a fermentation process that can simultaneously address the heat and mass transfer issues and allow for microbial biofilm formation. Such conditions can exist in biofilm reactors (Ercan and Demirci, 2013a).

Microbial cells can survive hash environments by switching from planktonic cells into mature biofilms through passive immobilization (Kuchma and O'Toole, 2000). Biofilm reactors provide the means to implement and harness such extraordinary characteristics for fermentation purposes (Demirci et al., 2007). Production of numerous value-added products have been enhanced in the past studies by using biofilm reactors (Ercan and Demirci, 2013b; Izmirlioglu and Demirci, 2016; Ho et al., 1997; Khiyami et al., 2006). There are two main components needed for constructing a biofilm reactor: a suitable surface for the biofilms to form on and a suitable strain. For the former purpose Plastic Composite Supports (PCS) are used in this study. The most potent combination of PCS and strain for MK-7 production in biofilm reactors have been determined (Chapter 3). Also similar to any type of bioreactors, biofilm reactors require a suitable medium for biofilm formation and perhaps a different medium for producing the target product. In this case, most of the past studies have focused on glycerol as the sole pure carbon source for MK-7 fermentation, since glycerol is believed to have a positive effect on extracellular expression of MK-7 in *B. subtilis* (Berenjian et al., 2011b). However, the glycerol-based media typically contain a significant amount of soy peptone, which in some cases reaches nearly 20% of the medium's mass (Berenjian et al., 2012, 2013; Mahdinia et al., 2017c). Such high concentrations make the fermentation broth highly viscous and therefore may pose operational and downstream difficulties and hinder effective mass transfer and thus prolong efficient metabolism as well. Since *B. Subtilis* strains have demonstrated robust

growth, biofilm formation and metabolism in glucose-based media (Chapter 4), therefore, the aims of the present study are to: (i) form biofilm reactors and utilize and investigate a glucose-based medium, and (ii) optimize the key growth factors affecting MK-7 production with *B. subtilis natto* using glucose-based medium. In this fashion, it is possible to produce comparable amounts of MK-7 with less fermentation and downstream processing costs.

5.3 Materials and methods

5.3.1 Microorganisms and media

Bacillus subtilis natto (NF1) were isolated from commercially available natto by using tryptic soy agar plates as described previously (Chapter 3). TSB medium including 10% (w/v) glucose (Tate & Lyle, Decatur, IL) and 0.8% yeast extract (Biospringer, Milwaukee, WI) was used for biofilm formation. Main fermentation media consisted of 150 g of glucose (Tate & Lyle), 17.5 g of tryptone (Marcor, Carlstadt, NJ), 8 g of yeast extract (Biospringer), 3 g of soytone (Marcor), 5 g of NaCl (EMD Chemicals, Gibbstown, NJ) and 2.5 g of K_2HPO_4 (VWR, West Chester, PA) per liter of deionized water. The cultures were stored at 4°C and sub-cultured monthly in order to maintain viability. For long-term storage, stock cultures were maintained at −80°C in a 20% glycerol solution.

5.3.2 Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA) equipped with 2-L vessels were used for fermentation studies. Sterile 4N sulfuric acid (EMD) and 4N sodium hydroxide (Amresco, Solon, OH) along with antifoam B emulsion (Sigma-Aldrich, Atlanta, GA) were automatically added to the bioreactors to maintain pH and suppress foaming as needed. PCS tubes type SFYB (50% Polypropylene, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% bovine albumin and salts) were manufactured in the

Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Ho et al. (1997). PCS tubes were cut in about 6.5 cm lengths and the grid-like fashion was formed on the propellers as described in previous studies (Ercan and Demirci, 2014; Chapter 4; Izmirlioglu and Demirci, 2016).

5.3.3 Biofilm formation

In order to form the biofilms on the PCS grids, bioreactors were set up and were autoclaved at 121°C for 45 min containing 1.5 L of DI water. After sterile TSBGYE medium replaced aseptically the initial water, bioreactors were inoculated with 3% (v/v) 24-h grown suspended-cell culture at 25°C. Then, TSBGYE medium was refreshed every 48 h for 4 times to allow a robust biofilm formation on the PCS. Once the biofilm was in place, the fermentation broth was sampled and Gram-stained to verify a pure culture. Gram staining solutions including safranin, iodine, crystal violet and decolorizing solutions were purchased from BD (Becton, Dickinson and Company, Sparks, MD). Post Gram staining bacterial cells were observed under the microscope using a ZEISS Axio Scope.A1 light microscope (ZEISS, Ontario, CA).

5.3.4 Experimental design

Response Surface Methodology (RSM) was used to investigate the effects of temperature (35-45ºC), pH (6-8) and agitation (100-200 rpm) each with 3 levels on MK-7 biosynthesis. A total of 20 runs were carried out and each run was 144-h long and samples were taken every 12 hours before the medium was refreshed for the next set of fermentation experiment. Maximum MK-7 concentration was treated as the sole response (Table 5.1). Optimum conditions were validated by duplicated validation runs in biofilm reactors. Also,

duplicated suspended-cell fermentations were also performed under same conditions as a control and results were compared with the biofilm reactors.

Table 5.1. Response Surface Methodology Central Composite Design including variables temperature (°C), pH and agitation (rpm) in predicting MK-7 concentrations (mg/L).

Run Order	Temperature (ºC)	pH	Agitation (rpm)	MK-7 (mg/L)
1	35.0	8.00	200	2.6
2	40.0	7.00	150	8.0
3	45.0	6.00	100	3.0
4	40.0	8.68	150	2.3
5	45.0	8.00	100	2.0
6	35.0	8.00	100	6.4
7	48.4	7.00	150	3.1
8	45.0	6.00	200	7.0
9	31.6	7.00	150	3.5
10	40.0	5.32	150	2.9
11	35.0	6.00	100	6.3
12	40.0	7.00	234	10.2
13	40.0	7.00	66	3.8
14	40.0	7.00	150	5.5
15	45.0	8.00	200	5.0
16	40.0	7.00	150	5.8
17	40.0	7.00	150	5.7
18	35.0	6.00	200	10.8
19	40.0	7.00	150	3.3
20	40.0	7.00	150	3.4

5.3.5 Analysis

5.3.5.1 MK-7 concentration

A mixture of n-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used for MK-7 extraction (Berenjian et al., 2006, 2014). The mixture was vigorously shaken

with a vortex mixer for 3 min and then the organic phase was separated and evaporated under forced air flow. Then, dried pellets were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH) for 15 min at ambient temperature. The mixtures were then filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY). High performance liquid chromatography (HPLC) (Waters, Milford, MA) equipped with a 2489 UV/Visible detector and a Supelcosil C18 column (15 cm×4.6 mm, 5µm, Supelco Analytical, Bellefonte, PA) was used at 40ºC for the analysis of MK-7 concentration. Methanol (EMD) was used as mobile phase with the flow rate of 1 mL/min. The wavelength of 248 nm was used for calibration and analysis. The MK-7 calibration curve obtained from 99.9% pure MK-7 (Chroma Dex Corporation, Irvine, CA) was linear between 0.1 mg/L and 30 mg/L ($R^2 = 0.999$).

5.3.5.2 Glucose concentration

Fermentation broth was centrifuged at 9000 g for 5 min (Microfuge 20 Series, Beckman Coulter Inc., Brea, CA) and then filtered through 0.2 µm cellulosic filters (PALL). Then, without dilution, the clear broth was analyzed by HPLC (Waters) equipped with a 2414 Refractive Index detector and an HPX-87H Aminex column (300×7.8 mm, Bio-Rad, Hercules, CA) at 50ºC and 410 nm. A 0.05 M sulfuric acid (EMD) solution was used as the mobile phase. Samples were kept at 4°C during the injections. The glucose calibration curve obtained from 99.9% pure glucose (EMD) was linear between 1 g/L and 60 g/L ($R^2 > 0.999$).

5.3.5.3 Statistical analysis

Using Minitab 17.0 ANOVA (Minitab Inc., State College, PA) with statistical model and regression analysis with Box-Cox transformation optimal λ, the effects of pH and agitation (rpm) along with 2nd order and two-way interaction effects were obtained. A

confidence level of 95% was implemented throughout the analysis procedures to distinguish significant parameters (Rahimi et al., 2017a and 2017b)

5.4 Results and discussions

Temperature, pH and agitation are the three variables that were chosen by which to optimize the MK-7 production by *B. subtilis natto* in biofilm reactors. Table 5.1 shows the Central Composite Design (CCD) and the MK-7 concentrations. The highest MK-7 concentration that was observed in the CCD runs was 10.80 mg/L at 35ºC, pH 6, and 200 rpm (Table 5.1). After applying ANOVA to these results, the obtained model was unable to predict temperature effects and therefore temperature was studied independently.

5.4.1 pH

Due to its excellent fermentation properties, with high product yields (20 to 25 g/L), *Bacillus subtilis* strains are used to produce various enzymes, such as amylase and proteases. For such purposes of course, the optimum pH for production may be far from pH level for growth which is about the zwitterion point (Asgher, 2007). In the case of MK-7 biosynthesis and similar to glycerol-based media (Sato et al., 2001b; Berenjian et al., 2011a), although pH changes during the fermentation process were fast and usually drastic in the glucose-based medium, these pH changes were found not to be essential for MK-7 expression. Thus, adjusting the pH at a constant level was deduced to be beneficiary for this purpose. Originally, pH changes were effective in flask fermentations but not in biofilm reactors. This was once again emphasizing on how significantly scaling up the production can affect MK-7 secretion, in this case from shake flask fermentations to 1.5-liter bench-top bioreactors. pH was controlled within 6-8 since optimum pH was hypothesized to be in that range. Optimum pH was found to be 6.47 as shown in Figure 5.2. This is very similar to the optimum pH for glycerol-based medium in biofilm reactors (pH 6.58), which suggests that pH effects on MK-7 biosynthesis can be independent of

different media components used in fermentation (Mahdinia et al., 2017c). As shown in Table 5.2, first (pH) (p <0.119) and second-order terms (pH*pH) (p <0.129) were comparable and therefore both were incorporated into the model. Agitation and pH interaction terms were not deemed significant by ANOVA and thus were incorporated independently (Table 5.2); which can be inferred from RSM plots as well (Figure 5.1).

Table 5.2. ANOVA output for MK-7 concentrations (mg/L) versus temperature (°C), pH and agitation (rpm).

Source	DF	Seq SS	Seq MS	F-Value	P-Value
Regression	4	1.9437	0.4859	2.80	0.064
Agitation	1	0.6867	0.6867	3.96	0.065
pH	1	0.4724	0.4724	2.73	0.119
Agitation*Agitation	1	0.3376	0.3376	1.95	0.183
pH*pH	1	0.4469	0.4469	2.58	0.129
Error	15	2.5992	0.1733		
Lack-of-Fit	4	0.4716	0.1179	0.61	0.664
Pure Error	11	2.1276	0.1934		
Total	19	4.5429			

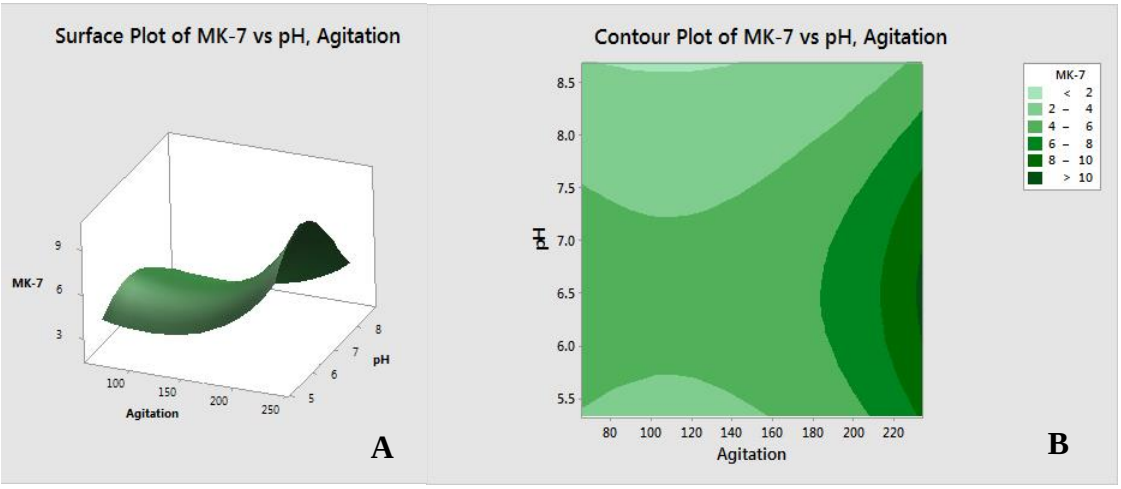

Figure 5.1. Response Surface Methodology (RSM) surface (A) and contour (B) plots indicating pH and agitation effects on MK-7 concentration.

5.4.2 Agitation

Diffusion of oxygen and substrate into the cell or release of the extracellular product into the medium can be limited in biofilm reactors. Since Oxygen Transfer Rates (OTR) are often limited in aerobic fermentations, higher agitation and aeration rates are normally desirable in biofilm reactors (Ercan and Demirci, 2013a). The number of the cell layers in the biofilm may vary due to the strain type, changes in environmental conditions, etc. Increase in the cell layer may cause an increase in biofilm thickness, which may create diffusion resistance to the substrate and nutrients. Thus, thin biofilm applications are usually desirable, but the biomass activity of the thin biofilm is at the same time a concern (Qureshi et al., 2005). In this case, biofilm formations on the PCS were assuredly thin enough after formation and since *Bacillus subtilis* strains are traditionally considered to be obligate aerobes, still higher agitation rates were also found to be favorable for MK-7 expression (Figure 5.1). Similar to glycerol-based medium, aeration rates in this study were kept constant at 1vvm throughout all the runs. Normally, the highest feasible agitation rate in bioreactors in the long-term is around 200 to 250 rpm, when the propellers are equipped with PCS shafts. Going for any higher agitation rates may be possible, but it is not recommended due to the fact that it may cause overstress on the PCS which may lead to biofilm detachment from PCS surface. Obviously, agitation rate was an effective factor (p <0.065) and even the second-order agitation term (Agitation*Agitation) was within the effective boundaries (p <0.183) and therefore were incorporated into the model (Table 5.2). Highest agitation rate tested in CCD runs at 234 rpm was selected by optimizer (Figure 5.2).

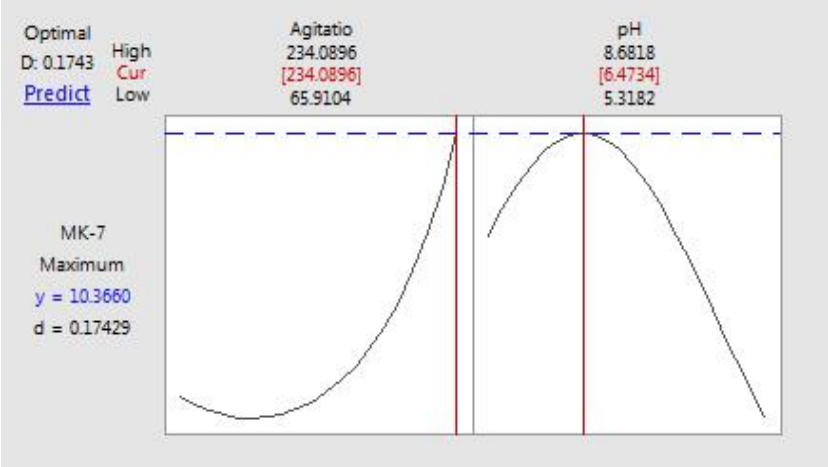

Figure 5.2. Optimum pH and agitation values for maximum MK-7 concentration predicted by RSM.

5.4.3 Temperature

Unlike previous studies where *B. subtilis natto* strains were used in suspended cultures, temperature effects were quite distinct in this study. In those studies, 35, 40 and 45°C were found to be optimum temperatures for MK-7 production (Berenjian et al., 2013; Sato et al., 2001a, Chapter 4). Yet, in this study it was at 30°C where highest concentrations of MK-7 were observed (Figure 5.3). As the CCD deign was unable to predict and model temperature effects in the temperature range of 35-45 ºC, agitation and pH were maintained at their optimum levels and temperature was studied from 48ºC down to 20ºC (Figure 5.3).

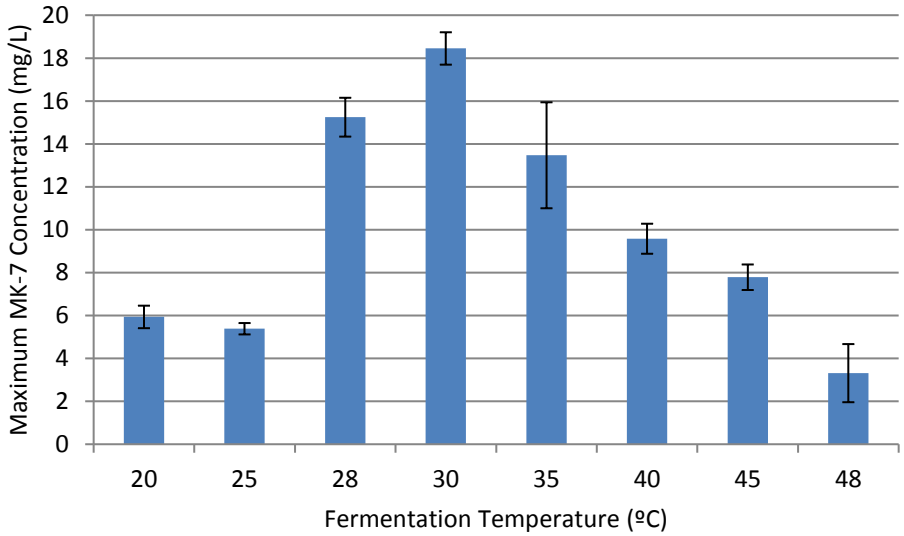

Figure 5.3. Temperature effects on MK-7 concentrations at optimum pH 6.47, agitation 234 rpm and 1 vvm (repeated).

As figure 5.3 indicates, temperatures below 28ºC were not able to sustain a robust metabolism and even a readily metabolized carbon source such as glucose was not well taken up by the bacterial cells as they did not run out of glucose. At the same time at higher temperatures, glucose was depleted within 72 or 96 hours of fermentation. Moreover, it is also obvious in figure 5.3 that the maximum glucose depletion rate occurred at 35ºC around after 60 hours while at 30ºC glucose depleted around 108th hour of fermentation. Thus, at 30ºC although metabolism does not seem to be as robust as it is at 35ºC, it seems to be robust enough to maintain good growth and at the same time not too fast for MK-7 biosynthesis to miss its best window of expression. On the other hand, changing the temperature from 30ºC to 35ºC resulted in 37% reduction in MK-7 biosynthesis. At 35ºC signifiant amounts of NaOH solution was needed to be applied during the fermentation to maintain the pH level at 6.48 (similarly at higher temperatures such as 40 or 45ºC), while much less NaOH solution was used at 30ºC. This observation could be justified by the fact that glucose is metabolised faster at higher temperatures, leading to oxygen deficiency and secretion of intermediate organic acids instead of carbon dioxide, although glucose consumption rates were comparable (Figure 5.4). Either way, 30ºC stands out as the

optimum temperature and temperatures below 28ºC simply could sustain a robust metabolism (Figure 5.4).

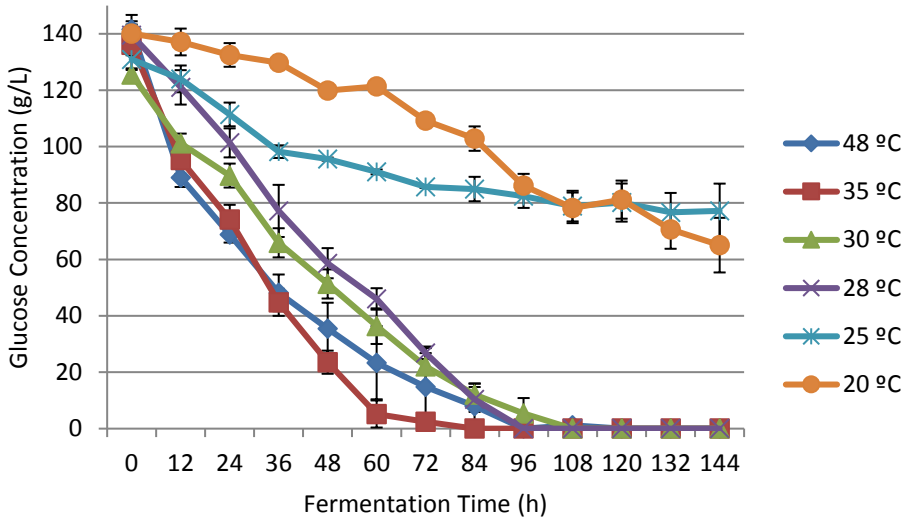

Figure 5.4. Temperature effects on glucose concentrations at optimum pH 6.47, agitation 234 rpm and 1 vvm (repeated).

5.4.4 Optimum conditions in biofilm reactors

The CCD design indicated that both agitation and pH were effective variables in MK-7 expression and can be modeled together as shown in Eqn. 5.1.

$$\ln(MK-7) = [-5.21] - [0.0118] * [Agitation] + [2.27] * [pH] + [0.000054] * [Agitation] * [Agitation] - [0.175] * [pH] * [pH] \quad \text{Eqn. 5.1}$$

Under the optimum conditions, a maximum of 18.45±0.76 mg/L of MK-7 was produced in biofilm reactors. This was 237% higher than production in suspended-cell reactors under same conditions where only 5.48±0.51 mg/L was achieved. Glucose consumptions were also very distinct (Figure 5.5). As figure 5.5 shows, in biofilm reactors, glucose depletion occured within 96 hours ; a window of time which seems to be optimal for maximum MK-7 biosynthesis in batch feremntation. Yet, in suspended-cell reactors under same conditions, glucose concentrations never declined to levels lower than 40 g/L within the six days of fermentation. This clearly indicates how much more robust

metabolism occurs in biofilm reactors in this case as compared to suspended-cell reactors. It was also observed that in suspended-cell fermentations, broth viscousity almost constantly increased whereas in biofilm reactors this phenamonea lasted only in the first 12 or 24 hours of fermentation (data not shown). This could be due to macromolecule expressions that are required for forming the extracellular matrices that are associated with passive immobilization and biofilm formations (Berenjian et al., 2013). In other words, in the suspended-cell reactors, it seemed that *B. subtilis* cells struggled to form biofilms yet were unable to, due to robust agitation and aeration and therefore metabolism including glucose consumptions and MK-7 expressions were impaired. Yet in biofilm reactors, cells already had the infrastructure for robust metabolism. This amount of production is also a significant improvement when compared with the amounts produced in glycerol-based medium (12.09±1.72 mg/L), not to mention that the glycerol-based medium is much richer in nutrients and therefore a more expensive medium as compared to the glucose-based medium used in this study (Chapter 4).

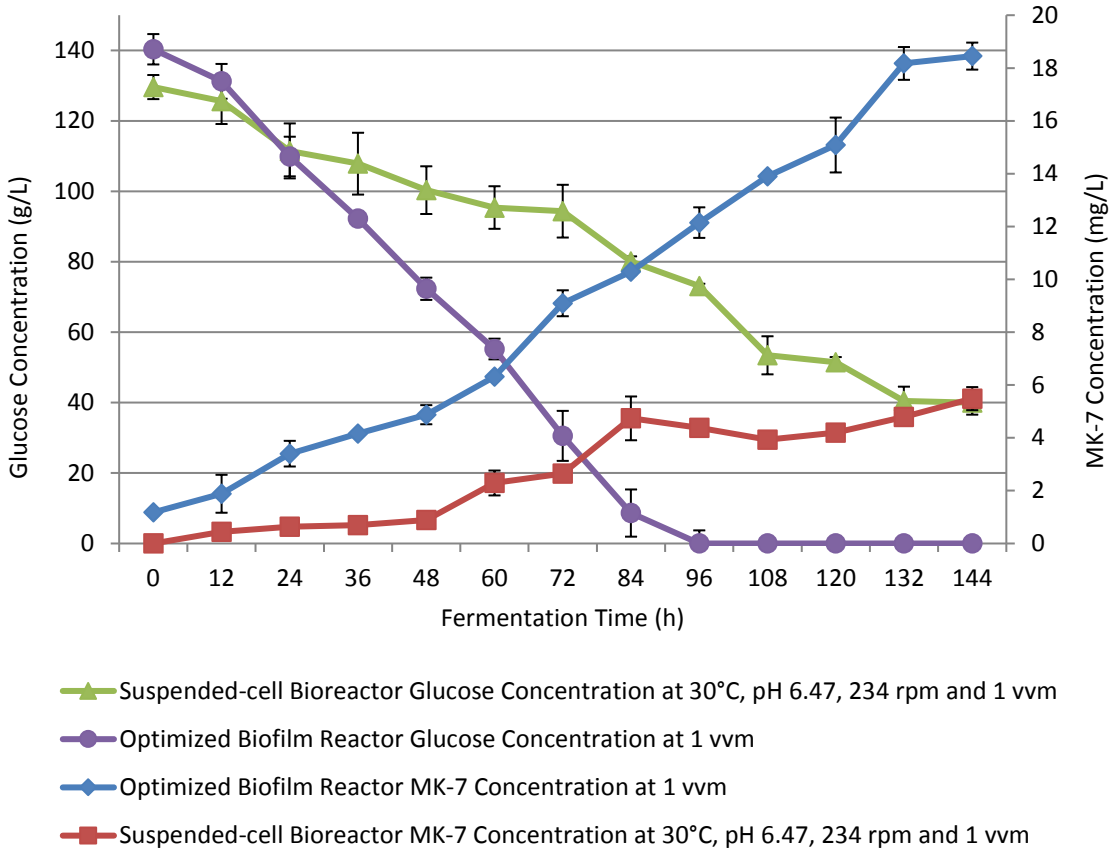

Figure 5.5. MK-7 and Glucose concentration in biofilm reactors vs suspended-cell reactors at pH 6.47, agitation 234 rpm, 30ºC and 1 vvm (repeated).

5.5 Conclusions

Biofilm reactors were utilized in this study to investigate the effects of growth parameters including temperature, pH and agitation on MK-7 expression in a glucose-based medium and obtained optimum conditions for maximum MK-7 biosynthesis. This study demonstrated the feasibility of using a biofilm reactor for MK-7 production to address scale-up and engineering issues associated with current fermentation strategies. Glucose consumption and MK-7 biosynthesis took place much more robustly in biofilm reactors as compared to suspended-cell reactors under the same conditions. Furthermore,

using these optimum growth conditions, by optimizing media components in the future studies, even higher concentrations and more robust production may be achieved.

5.6 Acknowledgments

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Chapter 6

Enhanced vitamin K (Menaquinone-7) production by *Bacillus subtilis natto* in biofilm reactors by optimization of glucose-based medium

6.1 Abstract

Benefits of vitamin K have been reported by many studies recently, due to its miraculous effects in reducing the risk of cardiovascular diseases and its potential benefits against osteoporosis. Specifically, menaquinone-7 (MK-7), being the most potent form of vitamin K, has definitely received most of the attention. Currently, solid or static liquid fermentation strategies are utilized for industrial production of MK-7 by *Bacillus* strains. However, these strategies face fundamental operational and scale-up issues as well as intense pellicle and biofilm formations which is problematic in static liquid fermentation, due to heat and mass transfer inefficiencies they create. In this fashion, biofilm reactors seem to be a practical solution to overcome these issues and enhance the production in agitated liquid fermentation. Thus, this study has been undertaken to utilize biofilm, reactor by optimizing glucose-based medium. Response Surface Methodology (RSM) was used to determine optimum concentrations of three major medium components (glucose, yeast extract, and casein). Maximum MK-7 concentration in biofilm reactors was achieved as 20.46±0.51 mg/L, which was 344% higher compared to the suspended-cell reactors containing the same optimum media composition. These results promise the potential of utilizing biofilm reactors for MK-7 production on an industrial scale.

Keywords: MK-7, Menaquinone-7, Vitamin K, Biofilm reactor, *Bacillus subtilis*, RSM Optimization

6.2 Introduction

Shortly after Henrick Dam's discovery of vitamin K (Dam, 1935), it was discovered that vitamin K naturally exists in two biological forms. Vitamin K1, also known as phylloquinone (PK), is the plant form of vitamin K and has a direct role in photosynthesis (Widhalm et al., 2012). It is found abundant in leafy green vegetables, such as cabbage, spinach, lettuce, and specially kale (Booth, 2012; Binkley et al., 1939). The other form known as vitamin K2, is referred to as menaquinones (MK) and is found in animal sources and also predominantly of microbial origin (Mahdinia et al., 2017a). Hence, vitamin K2 is mainly present in fermented foods such as cheese and natto (Japanese traditional fermented soybeans).Also, gut microbiota are also able to synthesize vitamin K2 (Davidson et al., 1998). Despite the fact that intestinal microflora synthesize significant amounts of menaquinones, the bioavailability of these menaquinones is poor, and thus, diet is the major source of functionally available vitamin K2 for human metabolism (Walther et al., 2017). Among all forms of vitamin K, MK-7 is the most potent form with numerous beneficial effects on human health (Shi et al., 2017; Howard and Payne, 2006; Gast et al., 2009; Geleijnse et al., 2004; Yamaguchi, 2006; Weber, 2001)

Although presence of menaquinones in bacteria has been investigated in numerous studies, only few strains of the *Bacillus* genus such as *Bacillus subtilis natto* (Berenjian et al., 2011a), *Bacillus licheniformis* (Goodman et al., 1976) and *Bacillus amylolyquifaciens* (Wu and Ahn, 2011). However, these strains are aerobes and have a potent tendency to form pellicles and biofilms (Mahdinia et al., 2017a). Both Solid State Fermentation (SSF) and Liquid State Fermentation (LSF) strategies have been investigated for MK-7 production (Singh et al., 2015; Wu and Ahn, 2011; Berenjian et al., 2015). However, SSF strategies face serious operational issues including heat and mass transfer limitations, while LSF strategies without agitation or aeration face similar problems (Mahdinia et al., 2017a; Pandey, 2003). Therefore, both face with scale-up issues for industrial MK-7 production (Pandey, 2003). However, it is possible to prevent pellicle and biofilm formations in LSF with robust agitation and aeration; yet, such formations are beneficial to MK-7 biosynthesis (Ikeda and Doi, 1990). In this fashion, simultaneously addressing these operational heat

and mass transfer issues and allowing microbial biofilm formations is the challenge for improving MK-7 production. Therefore, biofilm reactors can be utilized to provide solutions for these issues.

Naturally, microbes tend to undergo certain genetic changes and switch from planktonic cells into mature biofilm formations through passive immobilization in order to enhance survival and boost metabolism through colonization (Kuchma and O'Toole, 2000; Ercan and Demirci, 2013a). Microorganisms in biofilm reactors also possess such extraordinary characteristics, in this case for fermentation purposes (Demirci et al., 2007). Many value-added products have been produced more efficiently in the past studies by utilizing biofilm reactors (Ercan and Demirci, 2013b; Izmirlioglu and Demirci, 2016; Ho et al., 1997; Khiyami et al., 2006). Moreover, biofilm reactors require a suitable medium for both biofilm formation and product formation. Therefore, it is essential to optimize fermentation medium for biofilm reactors. In past studies, different media compositions have been studied to enhance extracellular expression of MK-7 in *B. subtilis* (Berenjian et al., 2011b). Several carbon sources such as glycerol, mannitol, and glucose, have been studied (Sato et al., 2001) and glycerol has been usually recommended (Berenjian et al., 2011b). However, the glycerol-based media typically contain significantly high amounts of soy peptone reaching nearly 20% (Berenjian et al., 2012; Berenjian et al., 2013; Mahdinia et al., 2017c). Such a medium composition is not only expensive, but may also result in operational and downstream difficulties. Moreover, although glycerol is believed to enhance MK-7 by *B. subtilis*, it can have a growth inhibitory effect as well, prolonging the fermentation time due to the richness and high density of the medium (Berenjian et al., 2011a). On the other hand, *B. subtilis* strains showed robust growth, biofilm formation and metabolism in glucose-based media in past studies (Mahdinia et al., 2017b, 2017c), therefore, the aim of this study was to investigate the effect of different components that constitute the glucose-based media in order to optimize and thus enhance MK-7 production by *B. subtilis natto* in a biofilm reactor.

6.3 Materials and methods

6.3.1 Microorganisms and media

Bacillus subtilis natto (strain NF1) were isolated from commercial natto as described in our previous study (Chapter 3). Biofilm formations were formed on PCS tubes in a modified Tryptic Soy Broth (TSB) medium including 10% (w/v) glucose (Tate & Lyle, Decatur, IL) and 0.8% yeast extract (Biospringer, Milwaukee, WI) (Chapter 4). The fermentation media consisted of 3 g of soytone (Marcor), 5 g of NaCl (EMD Chemicals, Gibbstown, NJ), 2.5 g of K_2HPO_4 (VWR, West Chester, PA) and variable levels of glucose (Tate & Lyle), casein (tryptone) (Marcor, Carlstadt, NJ) and yeast extract (Biospringer), per liter of deionized water. The cultures were stored at 4°C and sub-cultured monthly in order to maintain viability. For long-term storage, stock cultures were maintained at −80°C in a 20% glycerol solution.

6.3.2 Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA) equipped with 2-L vessels were utilized in this study. In order to maintain pH and suppress foam formation in the vessels, sterile 4N sulfuric acid (EMD) and 4N sodium hydroxide (Amresco, Solon, OH) along with antifoam B emulsion (Sigma-Aldrich, Atlanta, GA) were automatically added to the bioreactors. PCS tubes type SFYB (50% Polypropylene, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% bovine albumin and salts) were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Ho et al. (1997). PCS tubes were cut in about 6.5 cm lengths and were installed in 6 rows on the propellers in the grid-like fashion (Chapter 4).

6.3.3 Biofilm formation

After setting up the bioreactors, the vessels were autoclaved at 121°C for 45 min containing 1.5 L of DI water. Then, the water was replaced with sterile TSBGYE medium aseptically and bioreactors were inoculated with 3% (v/v) 24-h grown suspended-cell culture at 40°C. TSBGYE medium was refreshed every 48 h for four repeated batches to reach robust biofilm formations on the PCS. With biofilm in place, the fermentation broth was sampled and Gram-stained to verify the pure culture. Post Gram staining bacterial cells were observed under the microscope using a ZEISS Axio Scope.A1 light microscope (ZEISS, Ontario, CA).

6.3.4 Experimental design

In order to investigate the effects of glucose (100-200 g/L), yeast extract (5-15 g/L) and casein (10-25 g/L) concentrations on MK-7 biosynthesis, Response Surface Methodology (RSM) with a factorial design was used with at least three levels of variables (Table 6.1). A total of 15 runs were carried out with each run being 144-h long. Fermentation samples were taken every 12 hours before the medium was refreshed for the next set of fermentation experiment and were kept in 4°C. The sole response for ANOVA analysis was the maximum MK-7 concentration that was observed throughout each fermentation run. Optimum conditions predicted by the model were validated by duplicated validation fermentation runs in biofilm reactors. Furthermore, repeated suspended-cell fermentation runs were also carried out under same conditions as a control to provide comparison with the biofilm reactors.

Table 6.1. RSM factorial design including variables glucose, yeast extract and casein concentration (g/L) in predicting MK-7 concentrations (mg/L).

Run	Medium Component (g/L)			MK-7 Concentration (Observed) (mg/L)	MK-7 Concentration (Predicted) (mg/L)
	Glucose	Yeast Extract	Casein		
1	100	5	17.5	11.56	12.17
2	200	5	17.5	14.19	13.58
3	100	15	17.5	8.79	5.76
4	200	15	17.5	4.16	7.17
5	100	10	10.0	4.51	4.60
6	200	10	10.0	6.10	6.00
7	100	10	25.0	2.53	4.86
8	200	10	25.0	8.59	6.27
9	150	5	10.0	10.52	11.06
10	150	15	10.0	5.18	4.65
11	150	5	25.0	11.88	11.33
12	150	15	25.0	4.37	4.915
13	150	8	17.5	19.44	21.08
14	150	8	17.5	22.66	21.08
15	150	8	17.5	21.15	21.08

6.3.5 Analysis

6.3.5.1 MK-7 concentration

To extract MK-7 from fermentation broth, a mixture of n-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used as describe in previous studies (Berenjian et al., 2011c; Berenjian et al., 2014). Vigorous shaking with a vortex mixer for 3 min was applied and then the organic phase was separated and evaporated under forced air flow at ambient temperature. Dried residues containing the MK-7 were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH) for 15 min at room temperature. The solution was then filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY). To analyze the MK-7 in the solutions, High performance liquid chromatography (HPLC) (Waters, Milford, MA) equipped with a UV/Visible detector and

a Supelcosil C18 column (15 cm×4.6 mm, 5µm, Supelco Analytical, Bellefonte, PA) was used at 40ºC. Methanol (EMD) was used as mobile phase with an isocratic flow rate of 1 mL/min. At a wavelength of 248 nm, MK-7 calibration curve obtained from 99.9% pure MK-7 (Chroma Dex Corporation, Irvine, CA) was linear between concentrations of 0.1 mg/L and 30 mg/L ($R^2 > 0.999$).

6.3.5.2 Glucose concentration

Samples were centrifuged at 9,000 x *g* for 5 min (Microfuge 20 Series, Beckman Coulter Inc., Brea, CA) and then filtered through 0.2 µm cellulosic filters (PALL). Without diluting the samples, the clear broth was then analyzed by HPLC (Waters) equipped with a 2414 Refractive Index detector and an HPX-87H Aminex column (300×7.8 mm, Bio-Rad, Hercules, CA) at 50ºC and a wavelength of 410 nm. A 0.05 M sulfuric acid (EMD) solution was used as the mobile phase. Samples were kept at 4°C during the injections to preserve them throughout the analysis. Glucose calibration curve obtained from 99.9% pure glucose (EMD) was linear between 1 g/L and 60 g/L ($R^2 > 0.999$).

6.3.5.3 Statistical analysis

For statistical analysis, Minitab 17.0 ANOVA (Analysis of variance) (Minitab Inc., State College, PA) with statistical model and regression analysis with Box-Cox transformation at optimal λ was utilized. Effects of glucose, yeast extract and casein concentrations (g/L) on MK-7 concentration along with 2^{nd} order and two-way interaction effects were evaluated at a confidence level of 95% throughout the analysis procedures (Rahimi et al., 2017a and 2017b)

6.4 Results and discussions

The three variables that were evaluated in this study and their levels of initial concentrations are shown in Table 6.1. With ANOVA applied to responses and obtaining the most parsimonious model, the predicted values by the model are also depicted in Table 6.2.

Table 6.2. ANOVA output for MK-7 concentrations (mg/L) versus glucose, yeast extract and casein initial concentrations (g/L).

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Regression	6	552.653	93.85%	552.653	92.109	20.35	0.000
Glucose	1	3.973	0.67%	161.151	161.151	35.61	0.000
Yeast Extract	1	176.736	30.01%	31.588	31.588	6.98	0.030
Casein	1	0.140	0.02%	243.878	243.878	53.88	0.000
Glucose*Glucose	1	104.657	17.77%	157.535	157.535	34.81	0.000
Yeast Extract *Yeast Extract	1	19.994	3.40%	51.924	51.924	11.47	0.010
Casein*Casein	1	247.152	41.97%	247.152	247.152	54.61	0.000
Error	8	36.208	6.15%	36.208	4.526		
Lack-of-Fit	6	31.001	5.26%	31.001	5.167	1.98	0.372
Pure	2	5.207	0.88%	5.207	2.604		
Total	14	588.862	100.00%				

6.4.1 Glucose

Glucose was used as the sole carbon source for fermentation in three levels of 100, 150 and 200 g/L (Table 6.1). ANOVA showed that glucose concentration is very effective in MK-7 biosynthesis as shown in Table 6.2 ($p < 0.000$). However, with 100 g/L concentration of glucose, depletion occurred within 60 hours of fermentation which is not even halfway through fermentation in this case (data not shown) while with 150 or 200 g/L concentration depletion did not occur within 96 hours. Unlike glycerol, glucose as the carbon source promotes faster growth for *B. subtilis*, which enhances metabolism for MK-

7 production (Berenjian et al., 2011b). However, as shown in Figure 6.1, high concentrations (near 200 g/L) are undesirable probably due to inhibition and low concentrations (near 100 g/L) are not adequate to sustain a robust metabolism for MK-7 secretion towards the end of the fermentation cycle. In a previous study, a maximum concentration of 15.4 mg/L MK-7 (which is about 25% lower compared to maximum amounts achieved in this study) was achieved in 25 mL bottle static cultures with 100 g/L glucose concentration as sole carbon source (Berenjian et al., 2011b). Considering the benefits of static fermentation compared to agitated bioreactors in regards of MK-7 biosynthesis (Ikeda and Doi, 1990), it seems that the strain used in this study along with biofilm reactor efficacy, show prominent results (over 20 mg/L MK-7 concentrations here compared to about 30 mg/L concentrations in static fermentations) (Chapter 9). Also, Sato et al. (2001) achieved a maximum concentration of 23.5 mg/L MK-7 in shaking flasks which is comparable to the amounts achieved in this study; yet shake-flasks were utilized in that study with only 10% working volume (Sato et al., 2001). *B. subtilis* utilizes glucose as the preferred carbon source for energy (Stülke and Hillen, 1999). Glucose is taken up from medium and phosphorylated by the glucose permease of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) and then catabolized by glycolysis and the pentose phosphate pathway, similar to other sugars and polyols (such as glycerol) that are phosphorylated and converted to intermediates of either catabolic pathway (Blencke et al., 2003). However, MK-7 biosynthesis takes longer to reach maximum concentrations (Berenjian et al., 2011a; Chapter 4). On the other hand, high concentrations of glucose and other readily metabolized simple sugars can be associated with inhibition of complex metabolites biosynthesis such as vitamins and enzymes in *B. subtilis* (Blencke et al., 2003); yet this does not seem to be the case for MK-7 biosynthesis in the conventional glucose concentration around 15% and lower (Chapters 3 and 5). Therefore, a range of 10-20% glucose concentration was elected for RSM as preliminary runs showed robust growth and metabolisms in that range.

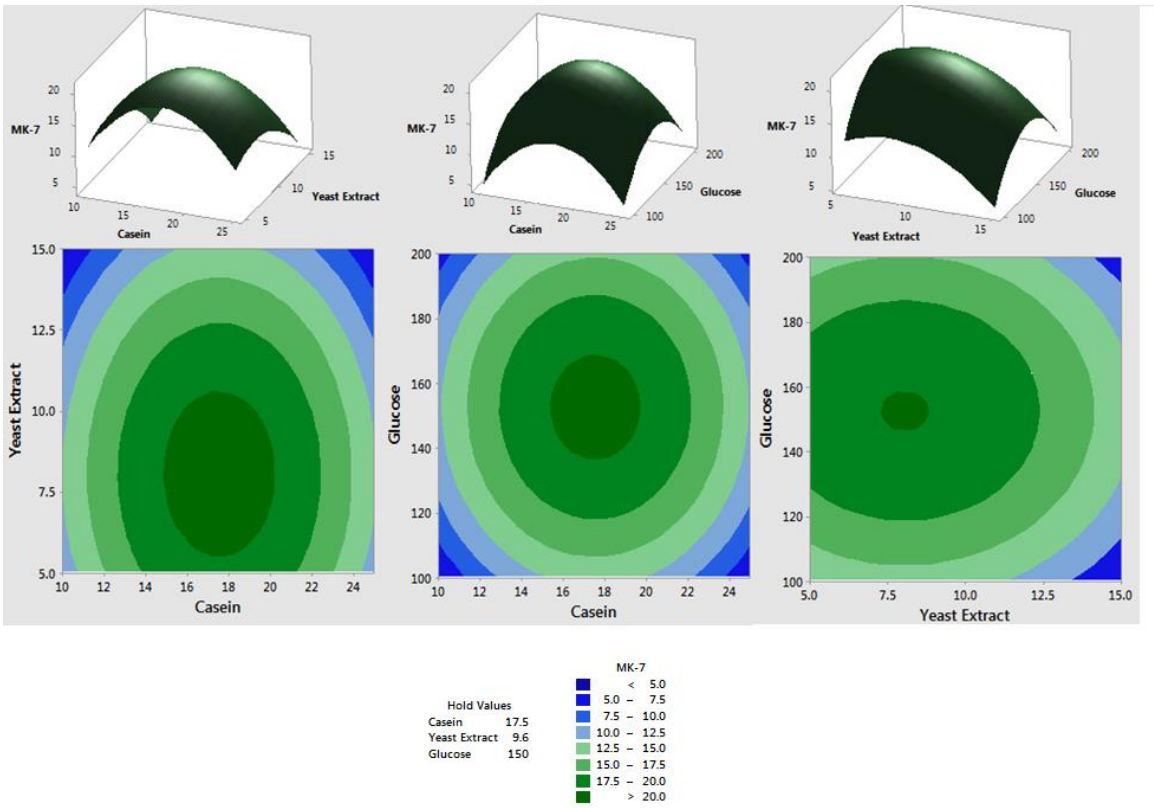

Figure 6.1. RSM surface and contour plots glucose, yeast extract and casein effects on MK-7 concentration.

6.4.2 Yeast extract

As Table 6.2 indicates, yeast extract concentration was effective in MK-7 biosynthesis in biofilm reactors ($p < 0.03$). Figures 6.1 and 5.2 indicate that the spectrum of readily metabolized proteins that exist in yeast extract are essential for MK-7 biosynthesis, high concentrations have a clear suppressing effects. It is established that nitrogen metabolism genes in *B. subtilis* strains are regulated by the availability of such rapidly metabolizable nitrogen sources. Sporulation is triggered with nitrogen starvation whereas protein-dependent repression occurs in cells growing with excess nitrogen (Fisher, 1999). On the other hand, extracellular MK-7 in *B. subtilis* is secreted attached to a protein known as vitamin K2 Binding Factor (KBF), rendering MK-7 soluble in the aqueous broth

and accessible to the extracellular matrices (Ikeda and Doi, 1990). In this fashion, it is understandable that as shown in Figure 6.1, MK-7 biosynthesis showed a steep repression with yeast extract concentrations higher than 10 g/L and yet, the optimum concentration of yeast extract was 8 g/L (Figure 6.2). Yeast extract provides a spectrum of nitrogen compounds that are usually readily metabolized by microorganisms. Nitrogen source is essential for *B. subtilis* growth and MK-7 biosynthesis since nitrogen sources are required in production of heme, which subsequently is involved in reducing menaquinone to menaquinol as a key reaction of respiration in cell membrane (Berenjian et al., 2011b).

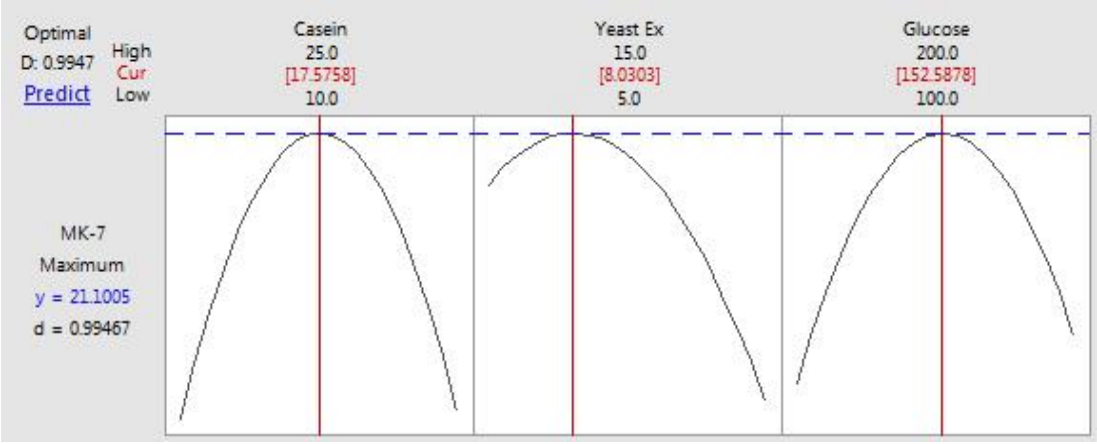

Figure 6.2. Optimized concentrations of glucose, yeast extract and casein for maximum MK-7 concentration.

6.4.3 Casein

As casein (tryptone) was also highly effective in Mk-7 biosynthesis as shown in Table 6.2 ($p < 0.000$); a relatively low concentration of soytone (3 g/L) was incorporated in the media compositions in order to avoid possible interactive effects with casein and possibly yeast extract. Since casein is originally predominant to soytone in TSB medium, its effects were chosen to be investigated. A concentration of 17.6 g/L was found optimum by the optimizer (Figure 6.2). Similar to yeast extract, casein (tryptone) is another excellent source of nitrogen for bacterial growth. Tryptone used in this study is a digestion product

of casein by the protease trypsin and due to digestion; it is liable to have significant amounts of short-chained and simple amino acids. Therefore, high concentrations casein had similar repressing effects on MK-7 biosynthesis as yeast extract (Figure 6.1); yet both yeast extract and casein are essential to enhance metabolism. However, Low levels of casein were also unfavorable perhaps because access to such easily metabolized nitrogen sources is essential to sustain a robust metabolism including growth and subsequently MK-7 synthesis. In past studies presence of soy peptone (which is a digested soybean protein nitrogen source) was deemed to be critical for MK-7 expression (Sato et al., 2001; Berenjian et al., 2011b) and therefore was included in the media compositions in this study as well.

6.4.4 Optimum conditions in biofilm reactors

ANOVA of the RSM responses indicated that all three variables along with their second-order effects were significantly effective ($p < 0.05$) on MK-7 concentration (Table 6.2) and therefore the corresponding terms were incorporated in the model.

$$[MK-7] = [97.5] + [5.216] * [Casein] + [2.65] * [Yeast\ Extract] + [0.815] * [Glucose] - [0.1485] * [Casein] * [Casein] - [0.1645] * [Yeast\ Extract] * [Yeast\ Extract] - [0.002668] * [Glucose] * [Glucose] \quad \text{(Eq. 6.1)}$$

Eq.1 parsimoniously predicts MK-7 concentration in regards of the effective terms with an $R^2_{(pred)}$ of 76.95%. Also, using the model, predicted values of MK-7 concentration were obtained at the observed conditions (Table 6.2). Plotting the predicted versus observed concentrations gives and R^2 of 93.8%, which along with a good lack-of-fit ($p > 0.372$) indicate an accurate model (Figure 6.3).

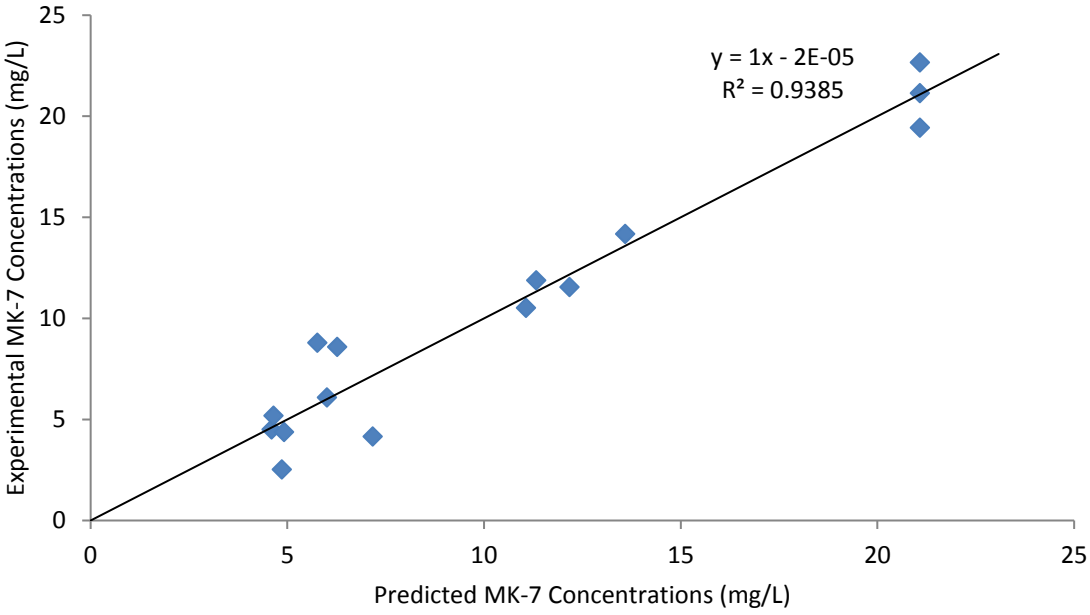

Figure 6.3. Experimental values vs values predicted by the model for MK-7 concentrations (mg/L).

Under the optimum concentration of glucose (152.6 g/L), yeast extract (8 g/L) and casein (17.6 g/L), a maximum MK-7 concentration of 20.46±0.51 mg/L was obtained in biofilm reactors for the validation runs compared to 21.08 mg/L concentration that was predicted by the model. Under optimum conditions, glucose depleted at 96 hours and yet, MK-7 profile pushed on rising until the end of fermentation cycle (144h). However, under the same conditions in suspended-cell reactors, glucose did not deplete at all and MK-7 concentrations never reached beyond 4.61±0.44 mg/L which indicates that biofilm reactors were able to produce MK-7 344% more competently. This is also a significant improvement compared to maximum concentrations that were achieved in TSB medium in McCartney bottles in static fermentations and shaking culture tubes (14.6±0.2 mg/L) (Chapter 9). Also, as maximum concentration are achieved within 6 days of fermentations in biofilm reactors (Figure 6.4), it is good improvement in productivity compared to static LSF or SSF strategies with glycerol where fermentation runs can take two weeks or more to complete (Berenjian et al., 2012). Also, since the glucose-based medium used is this study is relatively less rich in nutrients compared to the glycerol-based media used in

previous studies, MK-7 yield can be improved by switching to this glucose-based medium. Overall, Such distinct improvements are yet again due to the fact that metabolism is much more robust in biofilm reactors in which colonies are able to metabolize carbon and nitrogen sources more efficiently and as a result produce value-added products also more efficiently (Demirci et al., 2007).

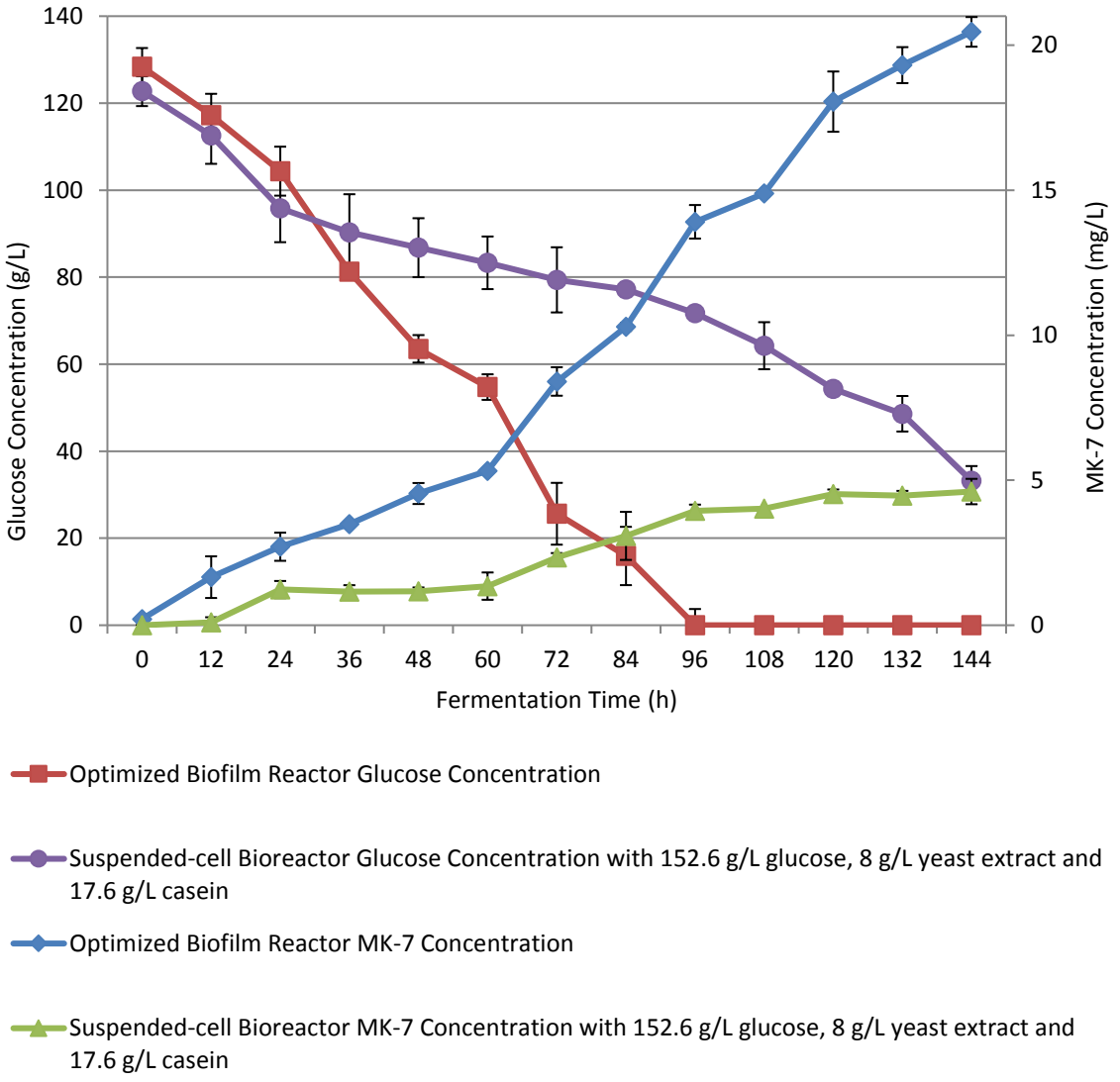

Figure 6.4. MK-7 and Glucose concentration in biofilm reactors vs suspended-cell reactors.

6.5 Conclusions

Using biofilm reactors, the effects of glucose, yeast extract and casein on MK-7 biosynthesis were investigated in this study using RSM. Optimum conditions were obtained by ANOVA and the model indicated 97% accuracy under the optimum conditions. A maximum concentration of 20.46±0.51 mg/L was achieved with media optimization that shows significant improvements compared to suspended-cell reactors or static and shaking bottle fermentations. Since, glucose metabolism was robust and glucose depletion occurred before completion of fermentation cycles in bioreactors, there seems to be an opportunity for fed-batch fermentation strategies in the future studies where glucose depletion can be addressed for reaching even higher MK-7 concentrations without creating carbon source inhibitions.

6.6 Acknowledgments

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Chapter 7

Effects of components in a glycerol-based medium on MK-7 production by *Bacillus subtilis natto* in a biofilm reactor

7.1 Abstract

Menaquinone -7 (MK-7) as the most important form of Vitamin K has been reported to have miraculous benefits such as preventing Cardiovascular Diseases (CVD) and osteoporosis along with antitumor effects. Therefore, there have been numerous studies in the past decades to improve MK-7 production via microbial fermentation. Unfortunately, both solid and liquid state fermentation strategies that are utilized for MK-7 production, face fundamental operational and scale-up issues as well as intense heat and mass transfer problems during fermentation. In this regard, biofilm reactors seem to be a practical solution to overcome these issues and enhance the production in agitated liquid fermentation. Therefore, this study was undertaken to utilize biofilm reactors in investigating and optimizing different media components in a glycerol-based medium. By using Response Surface Methodology (RSM), the effects of glycerol, yeast extract, and soytone were studied in the fermentation medium on MK-7 production in biofilm reactor. With a composition of 45 g/L of glycerol, 5 g/L of yeast extracts, 10 g/L of soytone and 0.06 g/L of K_2HPO_4, MK-7 concentrations could reach 14.7±1.4 mg/L in biofilm reactors, which was 57% higher compared to the MK-7 concentration achieved in suspended-cell reactor under similar conditions, while glycerol was depleted by the end of the fifth day in biofilm reactor, but glycerol was never depleted in suspended-cell reactor. Evidently, biofilm reactors present a reliable strategy to address the operational issues that MK-7 biosynthesis on the industrial scale faces.

Keywords: MK-7, Menaquinone-7, Vitamin K, Biofilm reactor, *Bacillus subtilis*, RSM Optimization

7.2 Introduction

Vitamin K was first discovered as a fat-soluble cofactor, essential for blood clotting and avoiding hemorrhages in chickens (Dam, 1935). Not many years later, it was also discovered that vitamin K exists in two major forms (Widhalm et al., 2012). The plant form, known as phylloquinone, is found abundant in most leafy green vegetables such as spinach and kale (Booth, 2012; Binkley et al., 1939). The animal and microbial forms, known as menaquinones has several subtypes (designated MK-1 to MK-15) and include the predominant forms in microbial metabolisms (Mahdinia et al., 2017a). In this fashion, microbial flora present in human intestines are also able to synthesize vitamin K2; although there is no significant absorbance in this way due to poor bioavailability (Davidson et al., 1998; Walther et al., 2017). Thus, the only feasible method to produce vitamin K on an industrial scale is through microbial fermentations (Berenjian et al., 2015). Among all subtypes studied for this purpose, MK-7 stands out with extraordinary benefits for human health (Schurgers et al., 2007; Howard and Payne, 2006; Gast et al., 2009; Geleijnse et al., 2004; Yamaguchi, 2006).

In this fashion, several bacterial strains including *Bacillus subtilis natto* (Berenjian et al., 2011a), *Bacillus licheniformis* (Goodman et al., 1976) and *Bacillus amylolyquifaciens* (Wu and Ahn, 2011) *in* both Solid State Fermentation (SSF) and Liquid State Fermentation (LSF) strategies have been investigated for MK-7 production with *B. subtilis natto* as the dominant strain (Singh et al., 2015; Wu and Ahn, 2011). However, as pointed out in previos studies, both SSF and static LSF strategies with no robust agitation and aeration, face serious scale-up and operational issues (Pandey, 2003; Mahdinia et al., 2017a). This is besides the fact that pelicle and biofilm formations that create these issues are beneficial for the MK-7 biosynthesis in the bacteria (Ikeda and Doi, 1990). This situation presents an opportunity to utilize biofilm reactors to keep these benefits and at the same time have robust agitation and aeration.

Biofilm reactors host biofilms that result from migration of cells from planktonic form into biofilm formations through passive immobilization (Kuchma and O'Toole,

2000). With suitable microbial strain and support (Chapter 3), they are able to harness the extraordinary abilities that cells gain in the biofilm colonies (Demirci et al., 2007). Throughout recent years, production of many value-added products have been enhanced by biofilm reactors (Ercan and Demirci, 2013; Izmirlioglu and Demirci, 2016; Ho et al., 1997; Khiyami et al., 2006). Similarly, biofilm reactors seem to be able to enhance MK-7 production in *B. subtilis* as well (Chapter 4). Biofilm reactors also require an optimum media to produce MK-7. Different carbon and nitrogen sources have been investigated in past studies (Berenjian et al., 2011b; Sato et al., 2001). Sato et al. (2001) investigated several carbon sources including glucose, mannose, galactose, fructose, sucrose, lactose, maltose, glycerol, mannitol, sorbitol and ribose with *B. subtilis* and the top MK-7 producers were glycerol (45.1 mg/L) and mannitol (42.8 mg/L). Moreover, Berenjian et al. (2011) reported that among glucose, glycerol, starch and sucrose, only glycerol as a single carbon source had a significant effect on MK-7 production. Thus, glycerol-based media have been recommended by these studies and have been utilized most commonly (Berenjian et al., 2011b; Berenjian et al., 2012; Berenjian et al., 2013; Chapter 4). Nevertheless, effects in bench-top scale bioreactors need to be investigated and optimized for biofilm reactors as well, as non-optimal levels can suppress growth and MK-7 biosynthesis (Berenjian et al., 2011a).

7.3 Materials and methods

7.3.1 Microorganisms and media

Bacillus subtilis natto (NF1) was isolated from commercial natto, as previously described (Chapter 3). For biofilm formation on the Plastic Composite Support (PCS), TSB medium fortified with 10% (w/v) glucose (Tate & Lyle, Decatur, IL) and 0.8% yeast extract (Biospringer, Milwaukee, WI) was used. Main fermentation media consisted of 100 g of soytone (Difco), 35 g of yeast extract (Difco), 45 g of glycerol (EMD Chemicals,

Gibbstown, NJ) and 0.6 g of K_2HPO_4 (VWR, West Chester, PA) per liter of deionized water, as concluded in previous studies (Chapter 4).

7.3.2 Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA) equipped with 2-L vessels were utilized. Sterile 4N sulfuric acid (EMD) and 4N sodium hydroxide (Amresco, Solon, OH) along with antifoam B emulsion (Sigma-Aldrich, Atlanta, GA) were added automatically to maintain pH and suppress foaming as needed. Plastic Composite Support (PCS) tubes type SFYB (50% Polypropylene, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% bovine albumin and salts) were manufactured and implemented as described in previous studies (Ho et al., 1997; Ercan and Demirci, 2014; Izmirlioglu and Demirci, 2016).

7.3.3 Biofilm formation

For biofilm formations to form on the PCS grids, bioreactors were set up with grid-like fashion PCS formations as described above. Then, sterile medium was added to the bioreactors and refreshed for four times, as described in the previous study (Chapter 5). At the end of the four fermentation cycles, the fermentation broth was sampled and Gram-stained to verify a pure culture.

7.3.4 Experimental design

Effects of glycerol (30-60 g/L), yeast extract (20-50 g/L) and soytone (50-200 g/L) concentrations on MK-7 production were evaluated using Response Surface Methodology (RSM) Box-Behnken design with three levels of variables (Table 1). Each batch was carried out in144 hours (6 days). Samples were obtained every 12 hours before the medium

was refreshed for the next set of fermentation experiment and were kept in 4°C. ANOVA analysis was performed with maximum MK-7 concentration that was observed throughout each fermentation run as the only response (Table 7.1). In order to validate the effects of the biofilm formations, duplicated suspended-cell fermentation runs were also carried out under same conditions as a control.

Table 7.1. RSM factorial design including variables glycerol, yeast extract and soytone concentrations (g/L) in predicting MK-7 concentrations (mg/L).

Run	**Medium Component (g/L)**			**MK-7 Concentration (Observed) (mg/L)**	**MK-7 Concentration (Predicted) (mg/L)**
	Glycerol	**Yeast Extract**	**Soytone**		
1	30	20	125	6.731	6.544
2	60	20	125	5.922	6.081
3	30	50	125	4.663	5.724
4	60	50	125	3.952	5.291
5	30	35	50	10.976	9.843
6	60	35	50	7.713	6.389
7	30	35	200	3.107	3.288
8	60	35	200	5.115	5.011
9	45	20	50	8.528	9.895
10	45	50	50	6.599	6.348
11	45	20	200	3.533	3.529
12	45	50	200	5.998	4.723
13	45	35	125	7.693	5.901
14	45	35	125	3.734	5.901
15	45	35	125	6.891	5.901

7.3.5 Analysis

7.3.5.1 MK-7 analysis

Fermentation broth sample (3 ml) was mixed with 2:1, v/v n-hexane:2-propanol mixture to extract the MK-7 content (Berenjian et al. 2011a). N-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used. The mixture was vigorously shaken using a vortex mixer for 3 min and then the organic phase was separated and evaporated under forced air flow at ambient temperature. Then, dried pellets containing the MK-7 were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH) for 15 min at ambient temperature. After the pellets were completely suspended in methanol, the mixtures were filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY). MK-7 concentrations in the samples was then analyzed by High Performance Liquid Chromatography (HPLC) as described in previous studies (Chapter 4).

7.3.5.2 Glycerol analysis

Samples of the fermentation broth was centrifuged at 9000 x *g* for 5 min (Microfuge 20 Series, Beckman Coulter Inc., Brea, CA) and then filtered through 0.2 µm cellulosic filters (PALL). Then, with no dilution, the cell-free broth was analyzed by HPLC as described the previous studies (Chapter 4).

7.3.5.3 Statistical analysis

The effects of glycerol (g/L), yeast extract (g/L) and soytone (g/L) along with the 2^{nd} order and two-way interaction effects were obtained using Minitab 17.0 ANOVA (Minitab Inc., State College, PA) with statistical model and regression analysis with Box-Cox transformation optimal λ,. A confidence level of 95% was implemented throughout the analysis procedures to distinguish significant parameters (Rahimi et al., 2017a and 2017b)

7.4 Results and discussions

As shown in Table 1, the highest MK-7 concentration observed in the Box-Behnken design was 10.98 mg/L. This is fairly low and depicts possible inhibitory behavior from the components when compared with previous studies with glycerol-based medium (Chapter 4). With ANOVA applied to responses (Table 7.2), the predicted values obtained by the model are also depicted in Table 7.1.

Table 7.2. ANOVA output for MK-7 concentrations (mg/L) versus glycerol, yeast extract and soytone initial concentrations (g/L).

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Regression	5	1.85274	70.28%	1.8527	0.37055	4.26	0.029
Yeast Extract	1	1.30116	49.36%	0.7886	0.78865	9.06	0.015
Glycerol	1	0.05487	2.08%	0.2656	0.26559	3.05	0.115
Soytone	1	0.01703	0.65%	0.2780	0.27800	3.19	0.108
Yeast Extract *Soytone	1	0.21200	8.04%	0.2120	0.21200	2.44	0.153
Glycerol *Soytone	1	0.26767	10.15%	0.2677	0.26767	3.07	0.113
Error	9	0.78356	29.72%	0.7836	0.08706		
Lack-of-Fit	7	0.38034	14.43%	0.3803	0.05433	0.27	0.920
Pure Error	2	0.40322	15.29%	0.4032	0.20161		
Total	14	2.63630	100.00%	1.8527	0.37055		

7.4.1 Yeast extract

Figure 7.1 and 7.2 clearly indicate a significant negative effect of yeast extract concentration on MK-7 biosynthesis. As shown in Figure 7.1, increasing yeast extract concentration from 20 g/L to 50 g/L can decrease MK-7 concentration by about 50% (from 10 mg/L to 5 mg/L).

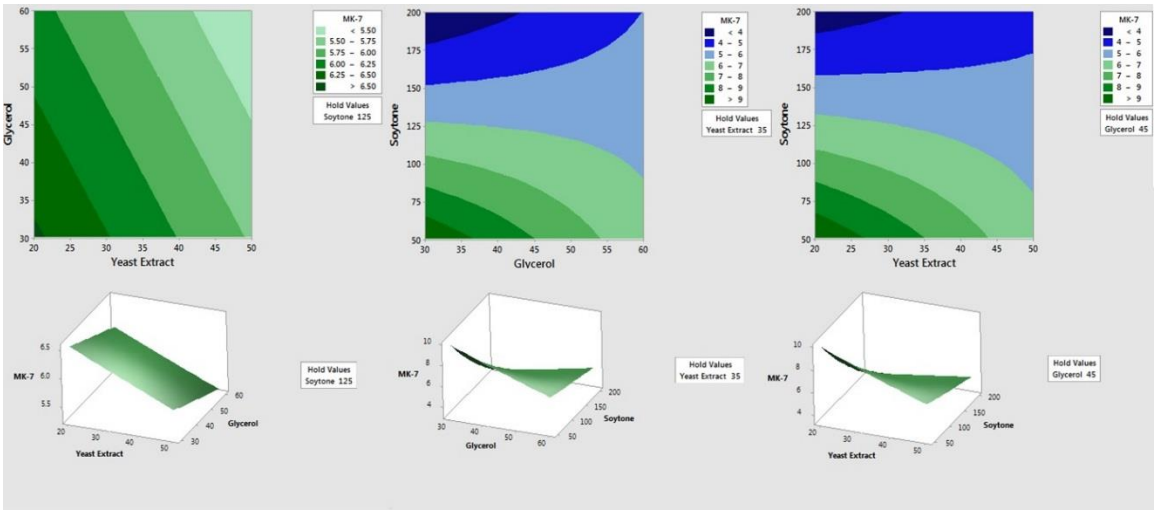

Figure 7.1. RSM surface and contour plots for glycerol, yeast extract and soytone effects on MK-7 production.

Although yeast extract is an excellent source of nitrogen source for any microbial fermentation due to containing a spectrum of readily metabolized proteins, *B. subtilis* protein synthesis pathways are regulated by such simple nitrogen sources (Fisher, 1999). Also, extracellular MK-7 is biosynthesized and secreted coupled with a protein known as vitamin K2 binding factor (KBF) that renders MK-7 soluble in the broth and accessible to the extracellular matrices (Ikeda and Doi, 1990). In this fashion, it is understandable that simple nitrogen sources such as yeast extract have a negative effect on MK-7 biosynthesis via inhibition of protein expressions; especially at rather high concentrations (Figure 7.2).

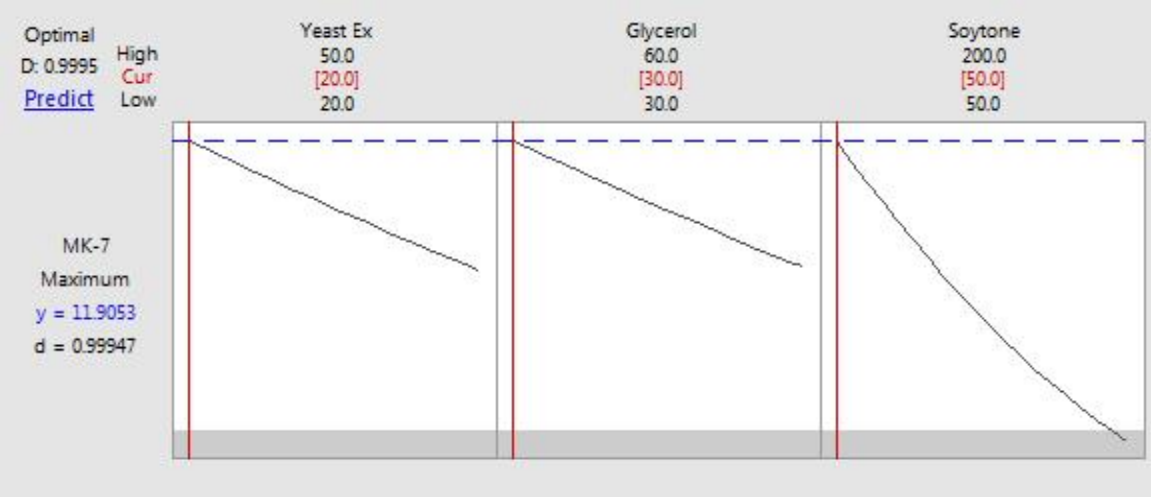

Figure 7.2. Optimized concentrations of glycerol, yeast extract and soytone for maximum MK-7 production.

Thus, it did not seem enough to just opt for the lower level of yeast extract, as the negative effect seemed to be more drastic compared to shake flask fermentations (Berenjian et al., 2011a, 2011b). Therefore, several studies independent of the Box-Behnken design were carried out to explore further lower concentrations of yeast extract compared to the lower level of the original design which was at 20 g/L (Figure 3). Although, even at these lower concentrations the negative effect did not seem to vanish completely, a level of 5 g/L seems to result in the highest amount of MK-7, which is superior to the conditions were yeast extract was eliminated and soytone was the sole nitrogen source (Figure 7.3).

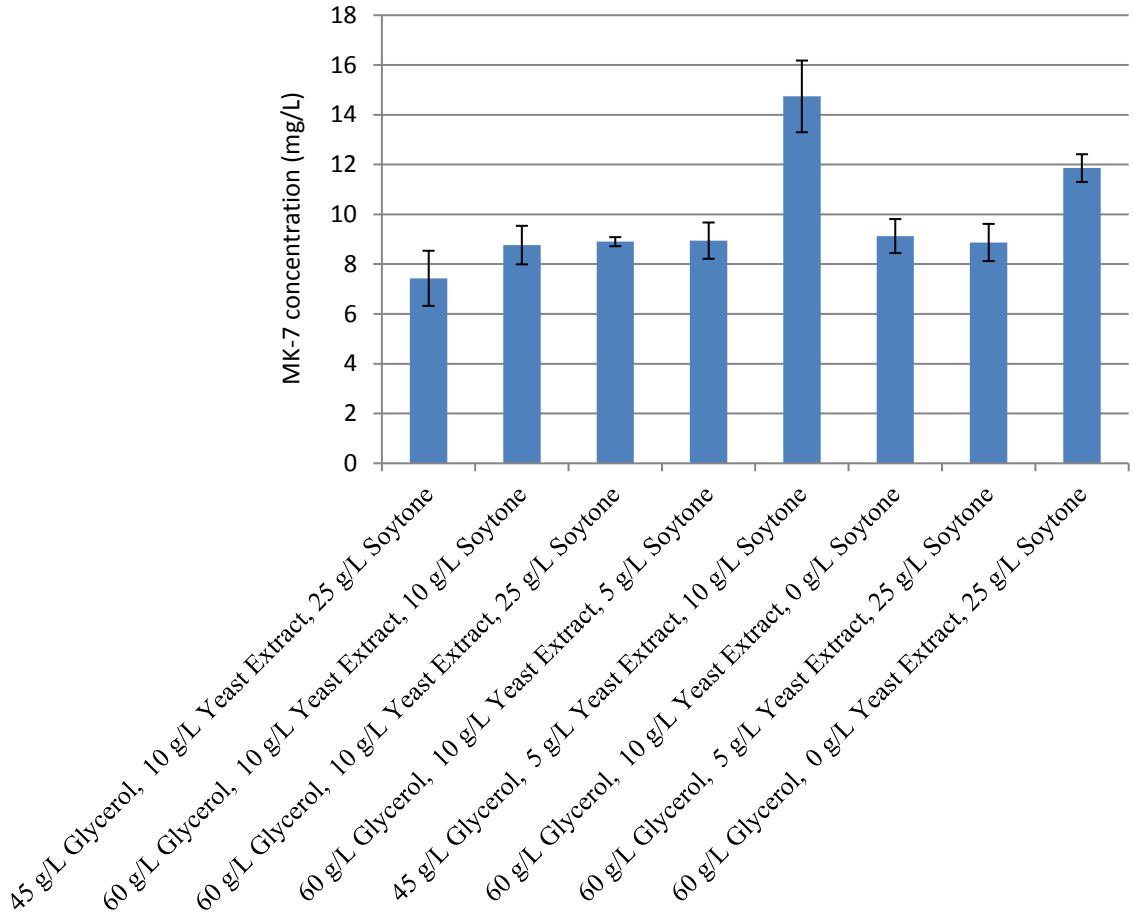

Figure 7.3. Effects of combinations with lower concentrations of yeast extract and soytone in the media on MK-7 biosynthesis.

7.4.2 Soytone

Similar to yeast extract, soytone also posed a negative effect on MK-7 synthesis in levels of 50-200 g/L. Naturally, soytone interaction with yeast extract was statistically significant on MK-7 biosynthesis (Table 7.2), since both are potent sources of amino acids for the fermentation. As figure 2 clearly illustrates, the negative effects associated with soytone are even greater than those of yeast extract. Increasing soytone concentrations from 50 g/L to 200 g/L may decrease MK-7 concentration from over 10 mg/L to less than 4

mg/L (over 60% decrease). On one hand, this makes sense since applied soytone concentrations were much higher than yeast extract. But, on the other hand, soytone effects were positive in previous studies in shake flasks and a rather high concentration of 189 g/L was selected for fermentation (Berenjian et al., 2011b). However, in biofilm reactors it seems that the negative effects appear since the role of soytone in the fermentation can be played with lower concentrations required. Yet, MK-7 biosynthesis is closely dependent on soy protein products being present in the medium. Either soytone or soybean extracts at rather high concentrations have been a key composition for MK-7 biosynthesis (Wu and Ahn, 2011; Sato et al., 2001; Berenjian et al., 2011a, 2011b). Soytone is an enzymatic digestion product of soybean proteins and soybean extracts are byproducts of natto manufacturing where soybeans are steamed before being inoculated with *B. subtilis*. Besides MK-7 dependency on KBF, heme expression is also critical, which requires amino acids coming from easily metabolized nitrogen sources such as yeast extract and soytone (Berenjian et al., 2011a). Furthermore, *B. subtilis* species are potent spore former strains and sporulation is triggered in them by nitrogen source starvation (Fisher, 1999). Since MK-7 biosynthesis continues until several days in LSF, it is essential to prevent nitrogen starvation and sporulation which would naturally terminate the biosynthesis. That seems to be the vital role that in this case soytone plays and the reason that soytone was favorable at concentrations not lower than 10 g/L (Figure 7.3).

7.4.3 Glycerol

As shown in Figure 7.1, glycerol concentration in interaction with yeast extract does not pose significant effects on MK-7 biosynthesis. However, glycerol interaction with soytone ($p < 0.113$) has significant effects, comparable to glycerol concentration itself ($p < 0.115$) (Table 7.2). This also makes sense, because unlike yeast extract, soytone has a significant content of carbohydrates which can and do combine effects with those of glycerol. That is simply why the glycerol-soytone interaction unlike glycerol-yeast extract interaction is rendered significant by ANOVA (Table 7.2). However, glycerol is the

primary carbon source in this case and is believed to have effects beyond a mere carbon source for MK-7 biosynthesis. It is known that in *B. subtilis* strains the majority of phospholipids consist of phosphatidylglycerol, cardiolipin and phosphatidylethanolamine. Also, it has been reported that glycerol addition at different stages of cell density significantly influences the phospholipids composition of cell membranes and as MK-7 is a membrane associated compound, one possibility is that its biosynthesis could be affected by the effect of glycerol addition on cell membranes (Berenjian et al., 2012).

Moreover, glycerol has been found to have suppressing effect on biopolymers production in bacteria such as poly(hydroxyalkanoate) in *Pseudomonas corrugate* (Ashby, 2005) and ε-poly-L-lysine in *Streptomycetaceae* (Nishikawa and Ogawa, 2006) and decrease the fermentation viscosity, which might boost the mass transfer and stimulate the uptake of extracellular substrates. The difference between the MK concentrations and cell growth rates in media with and without glycerol addition may be due to this medium composition phenomenon. Or as an alternative explanation glycerol might similarly suppress the production of glycopeptide biopolymers in *B. subtilis* strains and since extracellular MK is presumed to have a role in extracellular matrix carboxylation made of these biopolymers, it indirectly induces the MK expression. However, figure 2 indicates that at levels 30-60 g/L in biofilm reactors, glycerol has a constant negative effect. This is in agreement with previous evaluation of glycerol in a similar glycerol-based medium in shake flasks where a similar negative effect resulted in choosing the lower level of glycerol in the Central Composite Design (CCD) at 50 g/L (Berenjian et al., 2011b). Similarly, a 5% glycerol content, and not a higher concentration, was concluded to be optimal along with 10% soybean extract being implemented in the shake flasks (Sato et al., 2001). Naturally, as Figure 2 suggests, the lower concentration level of glycerol being 30 g/L is desirable for MK-7 expression. However, Figure 7.4 indicates that even when applying 45 g/L of glycerol in the medium, glycerol is depleted in the biofilm reactors by the middle of the fifth day (where MK-7 concentration is about 12.7 mg/L), whereas MK-7 concentrations continue to rise until the end of the sixth (to about 14.7 mg/L). Eventually, it is clear that despite the fact that the metabolic negative effects of glycerol persists at 45

and 60 g/L concentrations, concentrations lower than 45 g/L are not feasible simply, because they cannot sustain the metabolism long enough for the MK-7 concentration to peak. As a result, only 45 and 60 g/L glycerol concentrations were coupled with low concentrations of yeast extracts and soytone in Figure 7.3. As expected, 45g/L of glycerol was strong enough for a robust metabolism (i.e. does not deplete by the fifth day) and yet carried less negative effect on the biosynthesis clearly.

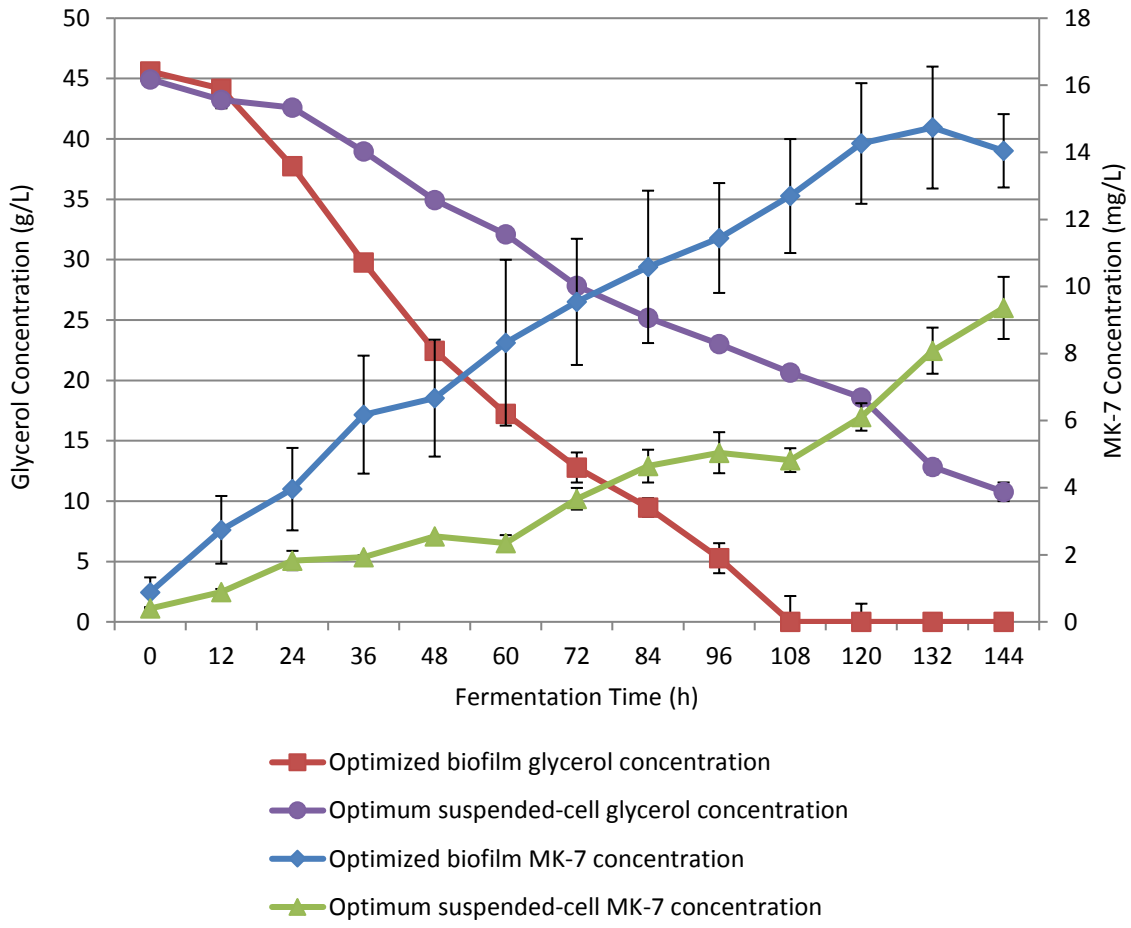

Figure 7.4. MK-7 and Glycerol concentrations in biofilm reactors vs suspended-cell reactors with medium composition of 45 g/L glycerol, 5 g/L yeast extracts, 10 g/L soytone and 0.06 g/L K2HPO4.

7.4.4 Optimum conditions in biofilm reactors

After the application of ANOVA on the Box-Behnken design, significant effects ($p < 0.15$) were incorporated in the model.

$$[MK-7]^{0.5} = [5.62] - [0.0311] * [Yeast\ Extract] - [0.0318] * [Glycerol] - [0.02289] * [Soytone] + [0.000205] * [Yeast\ Extract] * [Soytone] + [0.000230] * [Glycerol] * [Soytone] \quad \text{Eqn.1}$$

Eqn.1 indicates the most parsimonious statistical model that can explain the effects on MK-7 biosynthesis. The negative terms representing effects of yeast extract, glycerol and soytone concentration clearly demonstrate their suppressive effects at these high concentrations concluded above. Yet, this model has a fairly accurate capability in predicting the MK-7 concentrations within this range ($R^2 > 0.70$) with an insignificant lack-of-prediction effect ($p > 0.920$). By plotting the values for MK-7 concentration predicted by the model versus the observed values in the design, a precision of 78.6% is attained (Figure 7.5). The optimizer software suggests a highest concentration of MK-7 at 11.9 mg/L with the lowest levels of the components applied. However, this amount was still clearly suppressed by the negative effects coming from the medium components. Thus, at the lower concentrations studied independently, in the medium composed of 45 g/L glycerol, 5 g/L yeast extracts, 10 g/L soytone and 0.06 g/L K_2HPO_4, MK-7 concentrations were as high as 14.7±1.4 mg/L. These concentrations were also 57.4% higher than those achieved in suspended-cell reactors where MK-7 concentrations could not exceed 9.4±0.9 mg/L without the effects of biofilm formations. As figure 4 shows, there is a clear gap between MK-7 biosynthesis in biofilm reactors and suspended-cell reactors with same conditions. In biofilm reactors, MK-7 concentrations peak at concentrations above 14 mg/L by the 132th hour, whereas in suspended-cell reactors concentrations cannot even reach a peak by the end of the sixth day. This is due to the fact that glycerol is much more efficiently taken up and metabolized by the biofilm formations in biofilm rectors where the entire glycerol content is depleted within 108 hours; and as such efficient infrastructures do not exist in suspended-cell reactors, glycerol concentrations in them do not even go

below concentrations at 10 g/L at the end of the 144 hour period. Since in both biofilm reactors and suspended-cell reactors, MK-7 concentration follow an almost constant increase until the end of the six day period, since concentrations in the biofilm reactors are significantly higher (14.7±1.4 mg/L compared to 9.4±0.9 mg/L), biofilm reactors give higher productivities. On the other hand, glycerol is a waste byproduct of biodiesel production and quite abundant and cheap. Therefore, glycerol recycling does not make economic sense in this case and therefore suspended-cell reactors do not really present any higher yields. Furthermore, the higher productivity achieved in biofilm reactors seem to be much more imperative when considering operational costs. This is once again evidence of how biofilm reactors can boost the MK-7 biosynthesis (Chapter 6).

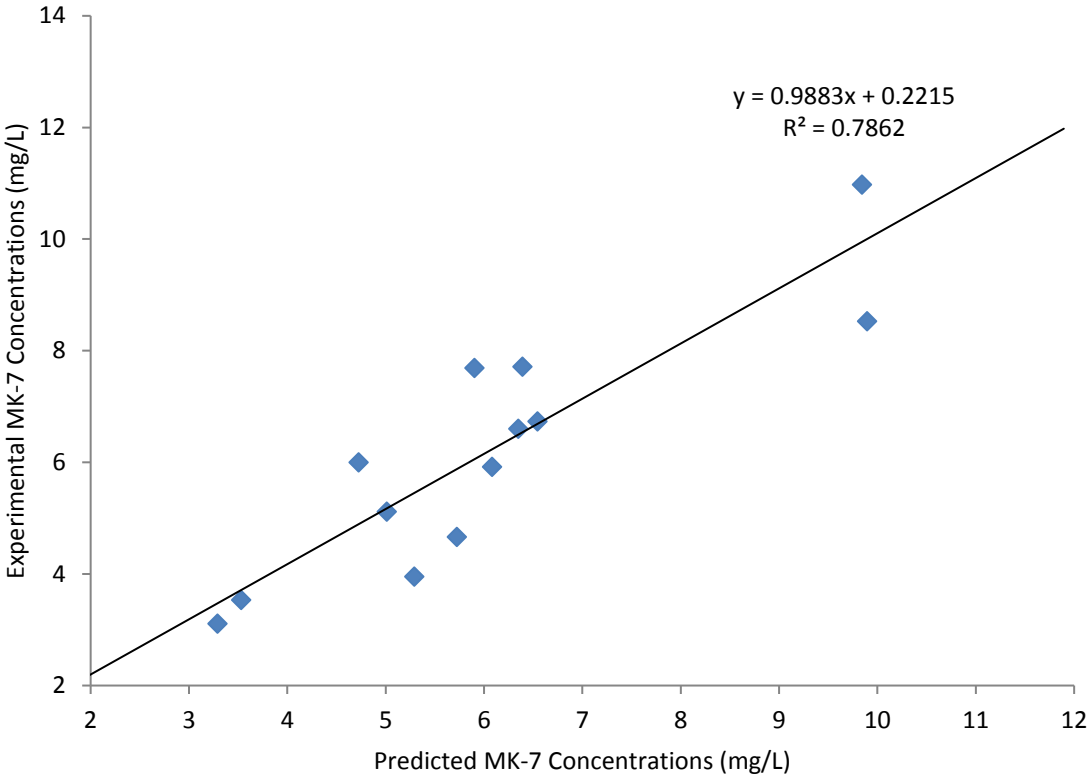

Figure 7.5. Experimental values vs values predicted by the model for MK-7 concentrations (mg/L).

7.5 Conclusions

Using a Box-Behnken design and RSM analysis, effects of glycerol, soytone and yeast extract were investigated on MK-7 biosynthesis in biofilm reactors. Parsimonious model with $R^2 > 0.70$ was obtained to predict the effects at optimum conditions; however, RSM analysis indicated that all three components have severe negative effects at the studied levels in biofilm reactors; unlike shake flasks. Thus, eight different compositions consisted of lower concentrations of soytone and yeast extract concentrations were studied to investigate the negative effects. Results indicated that in a medium composition of 45 g/L glycerol, 5 g/L yeast extracts, 10 g/L soytone and 0.06 g/L K_2HPO_4, MK-7 concentrations could reach 14.7±1.4 mg/L in biofilm reactors (which was 57.4% higher compared to suspended-cell reactors) and glycerol was depleted by the end of the fifth day. Under similar conditions in suspended-cell reactors, MK-7 concentration could not go beyond 9.3±0.9 mg/L and glycerol levels were not depleted. This was once again a sure sign how biofilm reactors can improve MK-7 production in LSF strategies. For future studies, it may be possible to further remedy the negative effects caused by carbon source inhibitions by implementing a fed-batch strategy for glycerol in the medium.

7.6 Acknowledgments

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Chapter 8

Implementation of fed-batch strategies for vitamin K (Menaquinone-7) production by *Bacillus subtilis natto* in biofilm reactors

8.1 Abstract

Recent studies show more and more essential health benefits associated with vitamin K, especially Menaquinone-7 (MK-7). These benefits include reducing risks of cardiovascular diseases, osteoporosis and even cancer. However, MK-7 production on an industrial level is only possible through bacterial fermentation and current static fermentation strategies are not potent enough with difficulties to scale up and critical heat and mass transfer issues. Biofilm reactors, however, may be a practical alternative. In this study, fed-batch strategies were conducted and investigated for glucose and glycerol-based media, as carbon source addition seemed crucial in batch fermentations. Results indicated that fed-batch additions can be significantly effective in glucose-based medium, increasing the end product concentrations to 28.7±0.3 mg/L of MK-7 which renders the biofilm reactors a potential replacement for static fermentation strategies with a maximum 32.5±0.4 mg/L of MK-7. Moreover, morphological changes of the applied *B. subtilis* strain was tracked during the 12 day long runs and finally, SEM investigations confirmed robust biofilm and extracellular matrices formed on the Plastic Composite Supports (PCS) in the biofilm reactors. In conclusion, biofilm reactors especially with fed-batch fermentation regimes seem to be an effective tool to enhance MK-7 productions on industrial scales.

Keywords: MK-7, Menaquinone-7, Vitamin K, Biofilm reactor, *Bacillus subtilis,* Fed-batch

8.2 Introduction

Not long after the discovery of vitamin K as an essential cofactor for blood clotting by Dr. Henrik Dam (Dam, 1935), it was discovered that vitamin K comes in two major forms in nature (Widhalm et al., 2012). The plant form, known as phylloquinone, is found abundant in most leafy green vegetables such as spinach and kale (Booth, 2012; Binkley et al., 1939). The animal and microbial forms, known as menaquinones has several subtypes (designated MK-1 to MK-15) and include the predominant forms in microbial metabolisms (Mahdinia et al., 2017a). The bacterial flora in human intestines do secrete significant amounts of menaquinones, yet due to the very low bioavailability of these sources, no significant absorption takes place unfortunately (Davidson et al., 1998; Walther et al., 2017). Therefore, microbial fermentation of MK-7 on an industrial scale and supplementing it via diet supplementary pills seem to be the only feasible way to boost vitamin K levels in human metabolism (Berenjian et al., 2015). Menaquinone-7 (MK-7) is the most potent form of all the Vitamin K subtypes that were studied for this purpose with extraordinary benefits for human health (Schurgers et al., 2007; Howard and Payne, 2006; Gast et al., 2009; Geleijnse et al., 2004; Yamaguchi, 2006).

The most common bacterial strains that were studied are *Bacillus subtilis natto* (Berenjian et al., 2011a), *Bacillus licheniformis* (Goodman et al., 1976) and *Bacillus amylolyquifaciens* (Wu and Ahn, 2011). Consequently, *B. subtilis natto* has been most common in the studies. Both Solid State Fermentation (SSF) and Liquid State Fermentation (LSF) strategies have been investigated for MK-7 production with *B. subtilis natto* (Singh et al., 2015; Wu and Ahn, 2011). However, both SSF and static LSF strategies with no robust agitation and aeration, face serious scale-up, and operational issues (Pandey, 2003; Mahdinia et al., 2017a). Nevertheless, pelicle and biofilm formations that create these issues are beneficial for the MK-7 biosynthesis in the bacteria (Ikeda and Doi, 1990). Thus there is an opportunity to use biofilm reactors to harness the biofilm formations and keep these benefits and at the same time have robust agitation and aeration.

In biofilm reactors, biofilm formations are created through passive immobilization of planktonic cells onto a suitable surface (Kuchma and O'Toole, 2000; Demirci et al.,

2007). In the past decades, many value-added productions have been enhanced by the use of biofilm reactors (Ercan and Demirci, 2013; Izmirlioglu and Demirci, 2016; Ho et al., 1997; Khiyami et al., 2006). Using the most potent combination of strain and PCS for MK-7 production (Chapter 3), biofilm reactors have been constructed and utilized to enhance MK-7 production in *B. subtilis* for batch fermentations for two different media; glycerol and glucose-based media. Batch optimizations of growth parameters including temperature, pH and agitation rates have resulted in 58% and 237% MK-7 concentration enhancements in biofilm reactors compared to suspended-cell bioreactors in glycerol and glucose-based media respectively (Chapters 4 and 5). Moreover, by optimizing carbon and nitrogen source compositions in the glycerol and glucose-based media, MK-7 concentrations were further enhanced with 57.4% and 344% improvements in biofilm reactors compared to suspended-cell bioreactors with same media composition, respectively (Chapters 6 and 7). However, as these batch fermentation studies have indicated, carbon source depletion occurs quite before MK-7 concentrations cease. Thus, this study was undertaken to investigate the possibilities of glucose and glycerol fed-batch injections into biofilm reactors and enhancing MK-7 concentrations through prolonging the stationary phase of the fermentation.

8.3 Materials and methods

8.3.1 Microorganisms and media

Bacillus subtilis natto (NF1) was isolated from commercial natto, as previously described (Chapter 3). For biofilm formation on the Plastic Composite Support (PCS), TSB medium fortified with 10% (w/v) glucose (Tate & Lyle, Decatur, IL) and 0.8% yeast extract (Biospringer, Milwaukee, WI) was used. Glycerol-based medium consisted of 10 g of soytone (Difco), 5 g of yeast extract (Difco), 45 g of glycerol (EMD Chemicals, Gibbstown, NJ) and 0.6 g of K_2HPO_4 (VWR, West Chester, PA) and the glucose-based medium consisted of 30 g Tryptic Soy Broth (TSB) composition, 8 g/L yeast extract (Biospringer) and 150 g/L glucose (Tate & Lyle) per liter of deionized water, as suggested by our previous chapters (Chapters 6 and 7).

8.3.2 Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA) equipped with 2-L vessels (1.5-L working volume) were utilized. Sterile 4N sulfuric acid (EMD) and 4N sodium hydroxide (Amresco, Solon, OH) along with antifoam B emulsion (Sigma-Aldrich, Atlanta, GA) were added automatically to maintain pH and suppress foaming as needed. Plastic Composite Support (PCS) tubes type SFYB (50% Polypropylene, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% bovine albumin and salts) were manufactured and implemented and biofilm reactors for glycerol and glucose-based media were operated at optimum conditions as described in previous studies (Ho et al. 1997; Chapters 4 and 5).

8.3.3 Biofilm formation

For biofilm formations to form on the PCS grids, bioreactors were set up with grid-like fashion PCS formations. Then, sterile medium was added to the bioreactors and refreshed for four times, as described in the previous study (Chapter 4). At the end of the four fermentation cycles, the fermentation broth was sampled and Gram-stained to verify a pure culture.

8.3.4 Experimental design

After the biofilm reactors were up and running, fed-batch fermentation runs were started with main fermentation media. Then, sterile glycerol solutions for 15, 30 or 45 g/L additions were prepared in 150 mL of total volume feeding and glucose for 50, 100 or 150 g/L additions solutions were prepared in 400 mL of total volume feeding at 72h and 144 h of fermentation Glucose and glycerol additions were implemented in glucose and glycerol-based media to investigate cross-effects. Samples were obtained every 12 hours until 288 hours for MK-7 and substrate analysis.

8.3.5 Analysis

8.3.5.1 MK-7 analysis

Three mL of fermentation broth was mixed with 2:1, v/v n-hexane:2-propanol mixture to extract the MK-7 content (Berenjian et al. 2011a). N-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used. The mixture was vigorously shaken using a vortex mixer for 3 min and then the organic phase was separated and evaporated under forced air flow at ambient temperature as described in previous studies (Mahdinia et al, 2017g). Then, dried pellets containing the MK-7 were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH) for 15 min at ambient temperature. After

the pellets were completely suspended in methanol, the mixtures were filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY). MK-7 concentrations in the samples was then analyzed by High Performance Liquid Chromatography (HPLC) using UV-Vis light as described in previous studies (Chapter 4, Shahami et al. 2017).

8.3.5.2 Substrate analysis

Samples of the fermentation broth was centrifuged at 9000 x *g* for 5 min (Microfuge 20 Series, Beckman Coulter Inc., Brea, CA) and then filtered through 0.2 µm cellulosic filters (PALL). Then, with no dilution, the cell-free broth was analyzed by HPLC as described the previous chapters (Chapters 4 and 5).

8.3.5.3 Statistical analysis

All observations were repeated and the average values were obtained and demonstrated with standard errors of the repetitions as error bars (Rahimi et al., 2018; Zhu et al. 2016)

8.3.6 Light microscopy

After Gram staining, *B. subtilis* cells from glucose and glycerol-based medium fermentations in biofilm reactors at ages of 28h, 144h and 288h were observed using a ZEISS Axio Scope Imager A1m light microscope equipped with an AxioCam MRm camera (ZEISS, Ontario, CA).

8.3.7 Scanning electron microscopy

Scanning Electron Microscopy (SEM) was utilized to observe and evaluate biofilm formation on the PCS tubes in comparison with the control before cell growth. The biofilm cells on the exterior and interior surfaces of the PCS tubes were maintained by chemical fixation of the cells. PCS tubes were soaked in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 7.2) with 0.02% Triton X-100. Then the fixative solution was decanted and samples were washed 3–5 times with the phosphate buffer and then were serially dehydrated with 25%, 50%, 70%, 85%, 95% and 100% (×3) ethanol for 5 min. Finally, the remaining moisture was eliminated using critical point drying for 3 hours (Figure 8.1). Zeiss Sigma Variable Pressured Field Emission Electron Scanning Microscope (VP-FESEM ZEISS, Ontario, CA) was used to observe the processed surfaces (Pashazanusi et al., 2017; Izmirlioglu and Demirci, 2017).

Figure 8.1. Processed PCS tubes cut and whole from control with no medium and cell encounters (A), glycerol-based medium bioreactors (B) and glucose-based medium bioreactors (C).

8.4 Results and discussions

For fed-batch fermentation in biofilm reactors, the target substrate concentrations must be sufficient to maintain the stationary phase as long as desired and at the same time the concentrations must not pose inhibitory and negative effects. For these purposes, several different combinations of fed-batch additions were implemented and the results are presented.

8.4.1 Effects of carbon source on fed-batch fermentations

8.4.1.1 Carbon source concentration

The optimum glucose-based medium starts the fermentation with an initial 150 g/L of glucose and the optimum glycerol-based medium starts with 45 g/L glycerol. Since it was stablished in previous chapters that higher concentrations for glucose and glycerol may result in severe inhibition of MK-7 secretion (Chapters 6 and 7); the fed-batch concentrations that were designed in this study targeted equal or less amounts of the starting concentrations. In other words, 50, 100 and 150 g/L glucose and 15, 30 and 45 g/L glycerol were the compositions that were implemented to evaluate the most efficient strategy (Figure 8.2).

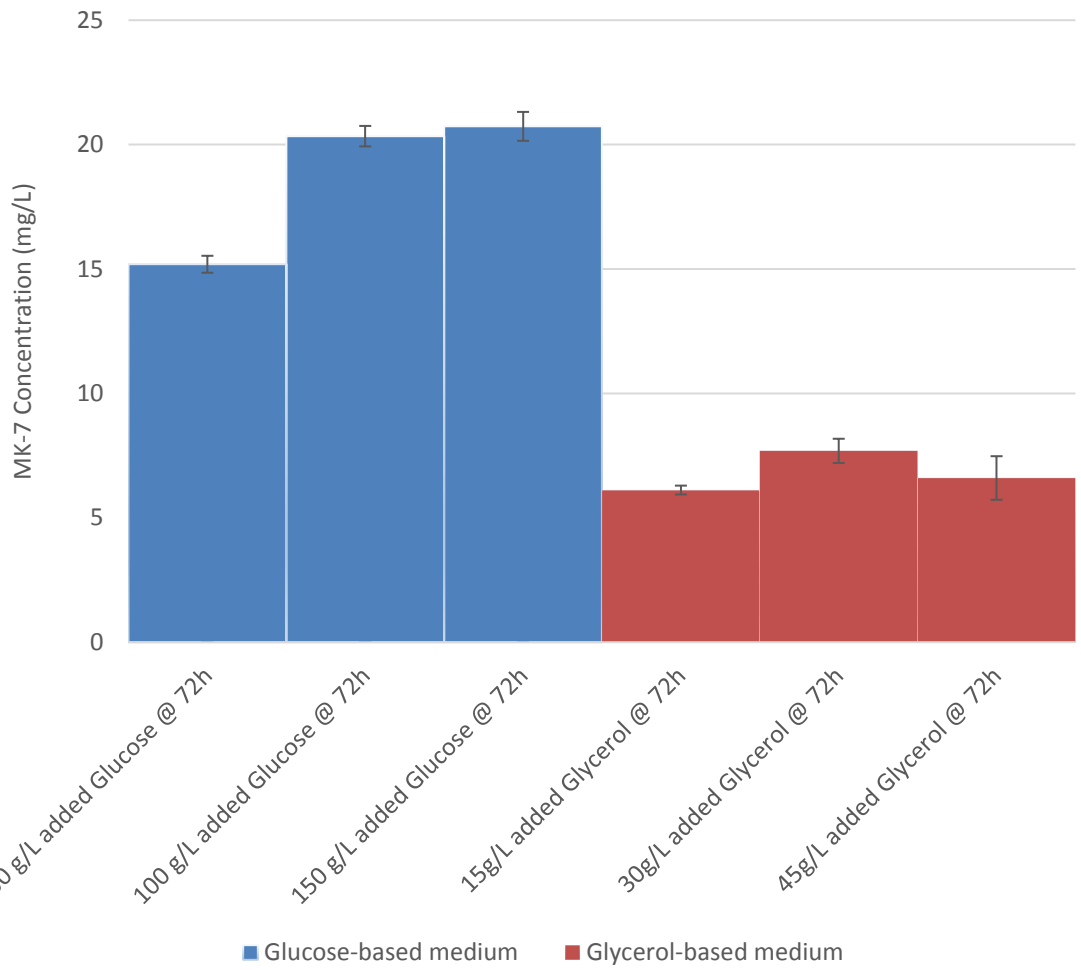


Figure 8.2. MK-7 fermentation in glucose and glycerol-based media with different concentrations of glucose or glycerol with fed-batch implemented.

Besides, as previous studies also indicated, carbon source depletion typically occurs on or around 72h of fermentation in both media (Figures 8.3 and 8.4). As observed in previous studies, glucose consumption in biofilm reactors happened more quickly at around 95% of the initial glucose is consumed within the first 72 h (Figure 8.3); whereas for glycerol, at 72 h still over 35% of the initial glycerol still exists in the broth (Figure 4). This makes perfect sense since glucose is a preferred and more readily metabolized carbon source compared to glycerol by *B. subtilis* strains (Stülke and Hillen, 1999). Concordantly, as it is obvious in Figures 8.3 and 8.4, in both cases carbon source consumption is

efficiently continued after the injections; yet in the case of glucose, consumption continues with a steeper slope compared to glycerol.

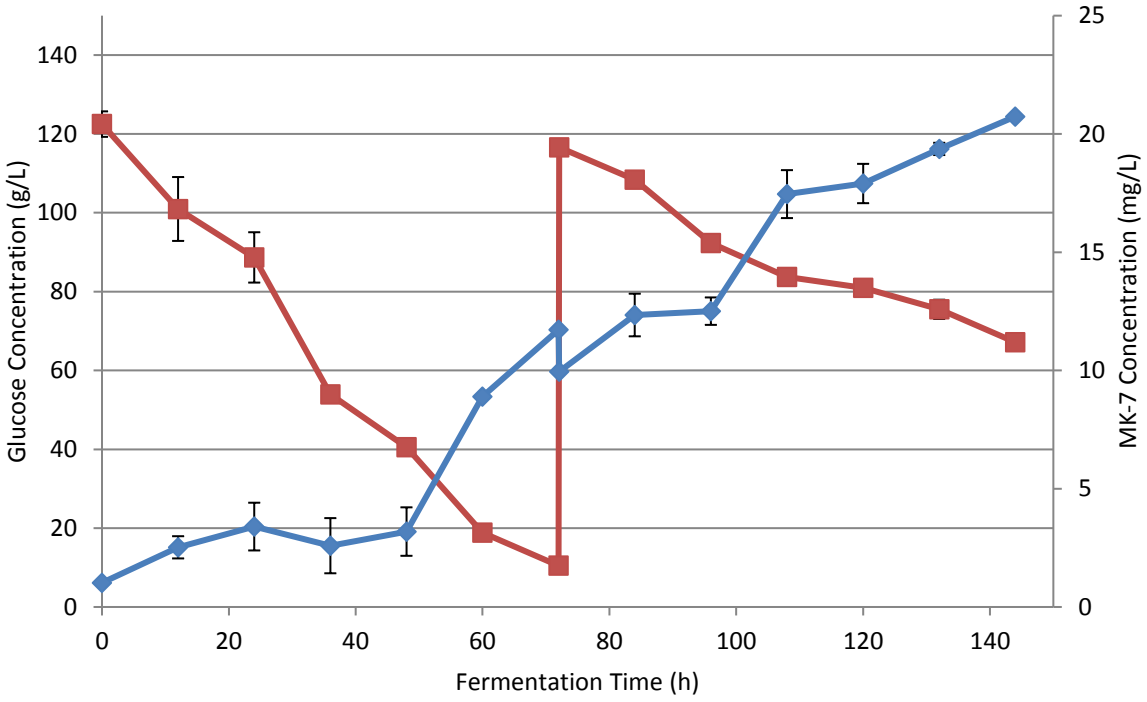


Figure 8.3. Maximum MK-7 profile in glucose-based medium obtained with 150 g/L glucose solution fed-batch added at 72 h of fermentation.

Thus, these concentrations were applied at the 72 h of fermentations. As Figure 8.2 indicates, for glucose the MK-7 profile is ascending with glucose concentration and the final MK-7 concentration of 20.7±1.2 mg/L achieved with 150 g/L glucose supplementation is in compliance with the 20.5±0.5 mg/L maximized concentrations in batch fermentations (Chapter 6). Thus, glucose does not seem to pose any inhibitory effects on MK-7 profile here and therefore, the higher concentration of 150 g/L is favorable. On the other hand, when glycerol applied in the glycerol-based medium, the story is different. As seen in Figure 8.2, the middle concentration of 30 g/L stands out compared to 15 and 45 g/L, which suggests an inhibitory effect. Furthermore, the highest concentration achieved here was 7.7±1.1 mg/L which is significantly lower than the concentrations achieved in batch fermentations (14.7±1.4 mg/L) (Chapter 7). This observation also

suggests a glycerol inhibitory effect in the glycerol-based medium. Although glycerol is believed to have beneficiary effects on MK-7 secretion and fed-batch glycerol addition in shake-flasks were has been more successful where fed-batch addition of glycerol at 48 h increased the final MK-7 concentrations by about 40% (Berenjian et al., 2012; Berenjian et al., 2011a); in biofilm reactors with fed-batch fermentation, it seems that the inhibitory effects persist just as in batch fermentation (Chapter 7). As a result the middle concentration of 45 g/L in consistence of the initial concentration was elected.

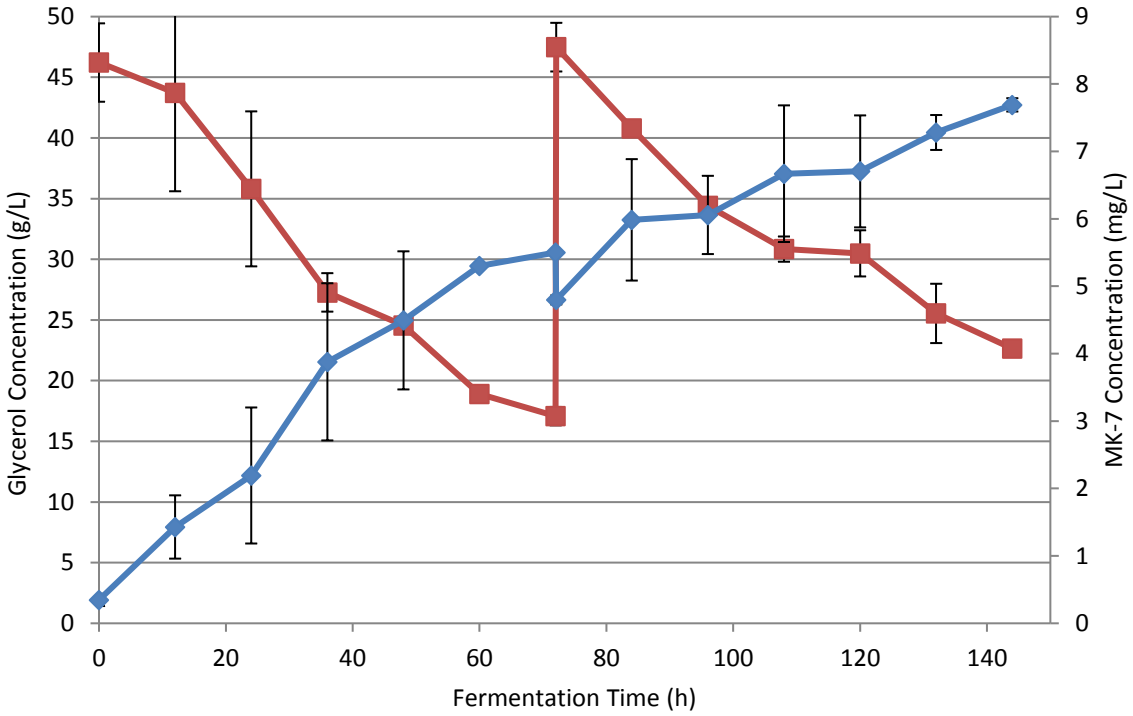


Figure 8.4. Maximum MK-7 profile in glycerol-based medium obtained with 30 g/L glycerol solution fed-batch added at 72 h of fermentation.

8.4.1.2 Glucose-based medium

As applying the 150 g/L glucose injection at 72 h into the glucose-based medium in previous section projected, with only this injection and continuing the fermentation until 288 h, the added glucose was not adequate to maintain the fermentation in good conditions

of the stationary phase for long and therefore the MK-7 profile plateaued and did not exceed 21 mg/L boundary (Figure 8.5).

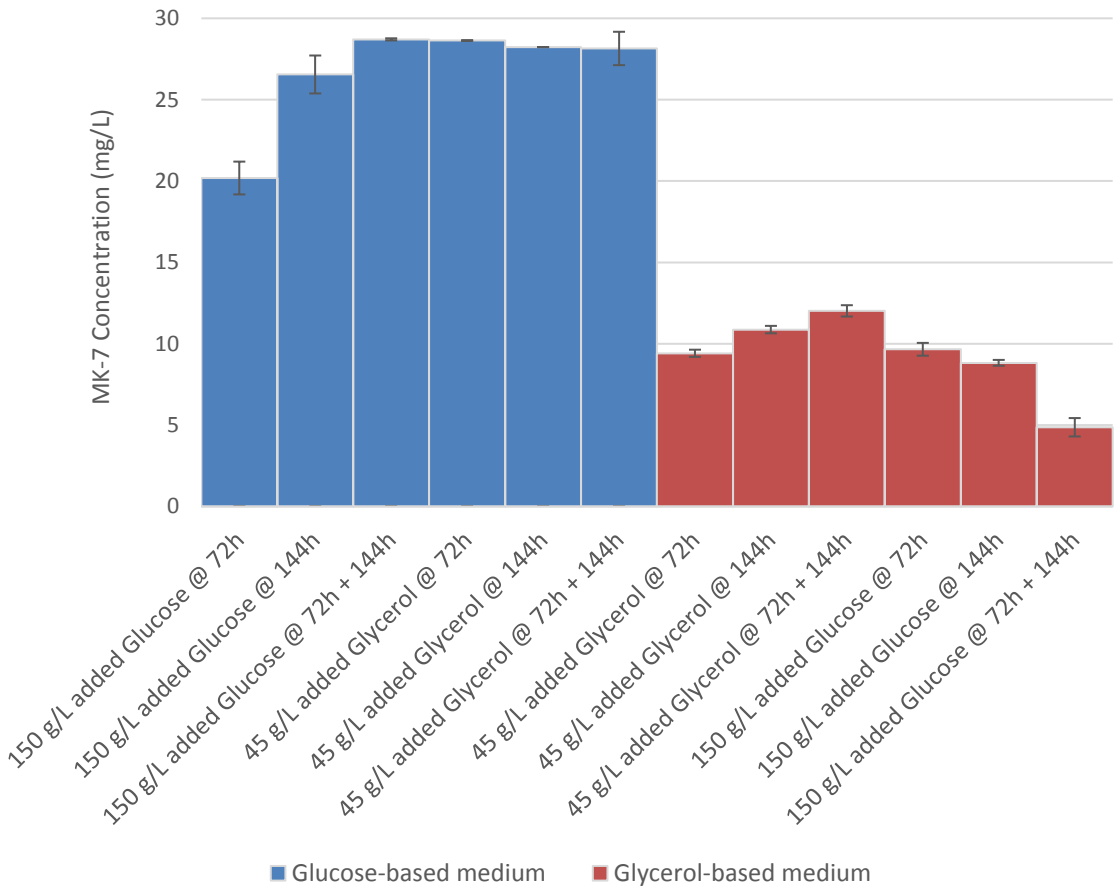


Figure 8.5. Maximum MK-7 concentrations in glucose and glycerol-based media with different fed-batch strategies implemented.

On the other hand, feeding at 144 h gave better results (26.5±1.8 mg/L) despite the glucose depletion that occurred between 72h and 144h and the fact that glucose depleted nevertheless by the 288 h (data not shown). The best results however, came from feeding at 72 and 144 h, which led to 28.7±0.3 mg/L MK-7 concentration. This was the highest amount observed in bioreactors so far, which puts the production in biofilm reactors quite comparable to the maximum concentrations in static fermentations (32.5±0.4 mg/L) (Chapter 9). Interestingly, single and double injections of glycerol solutions into the glucose-based medium led to similar results (28.6±0.1, 28.2±0.1 and 28.1±1.2 mg/L). The

explanation is simple. MK-7 seems to be a mixed metabolite; it starts right off the exponential phase and keeps flourishing as long as severe starvation does not occur and fermentation is maintained in the stationary phase. While the glycerol-based medium cannot support a metabolism as robust as the glucose-based one, glycerol fed-batch shots are quite adequate to preserve the fermentations in stationary phase long enough to reach such high concentrations. Possibly, some of the benefits of glycerol presence might have kicked in without inhibiting the secretion. As Figure 8.6 shows, the second feeding is slowly consumed with a lot of significant glucose left over at the end of the 12 day fermentation period, while the first feeding is consumed a lot faster and the initial concentrations are consumed even faster. Although significant amounts of glucose is used for the fed-batch fermentation, the high selling price of MK-7 and low-cost glucose may make fed-batch fermentation still cost effective.

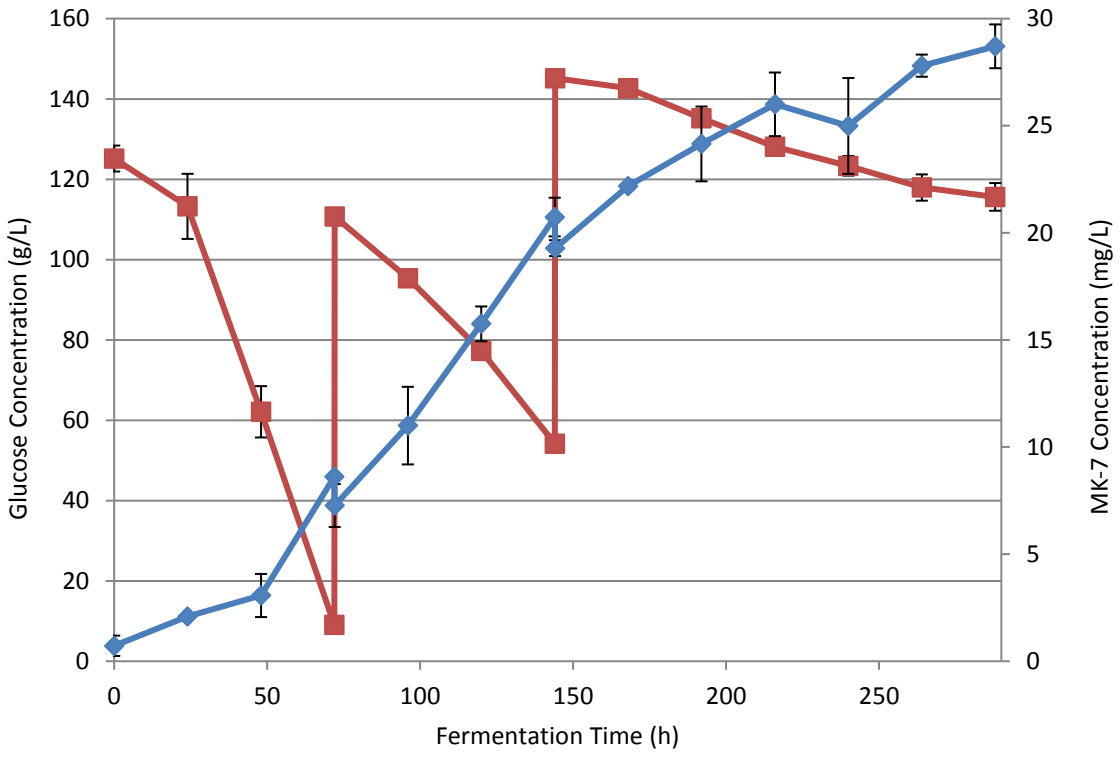


Figure 8.6. Highest MK-7 concentration profile observed in the glucose-based medium.

8.4.1.3 Glycerol-based medium

When fed-batch strategies were applied for the glycerol-based medium, inhibitory effects were obvious throughout all the experiments. As it can be seen in Figure 8.5, similar to the glucose-based medium, double feeding approach (72 and 144 h) was better than single feeding at 72h; possibly due to similar reasons. On the other hand, glucose feeding into the glycerol-based medium followed a reverse pattern. As it is shown in Figure 8.5, the double glucose feeding in this case has given out the lowest MK-7 concentrations. Therefore, not only the high glucose concentrations added do not seem to induce MK-7 secretion and redeem the inhibitory effects, they seem to amplify them. Furthermore, the highest concentrations achieved with these various fed-batch regimes are 12.0±0.5, which is again even lower than the 14.7±1.4 mg/L achieved in optimized batch biofilm reactor (Chapter 7). Thus, unlike the glucose-based medium, fed-batch strategies do not seem to be beneficial for MK-7 production in the glycerol-based medium in biofilm reactors; despite the robust metabolism observed in fed-batch biofilm reactors (Figure 8.7).

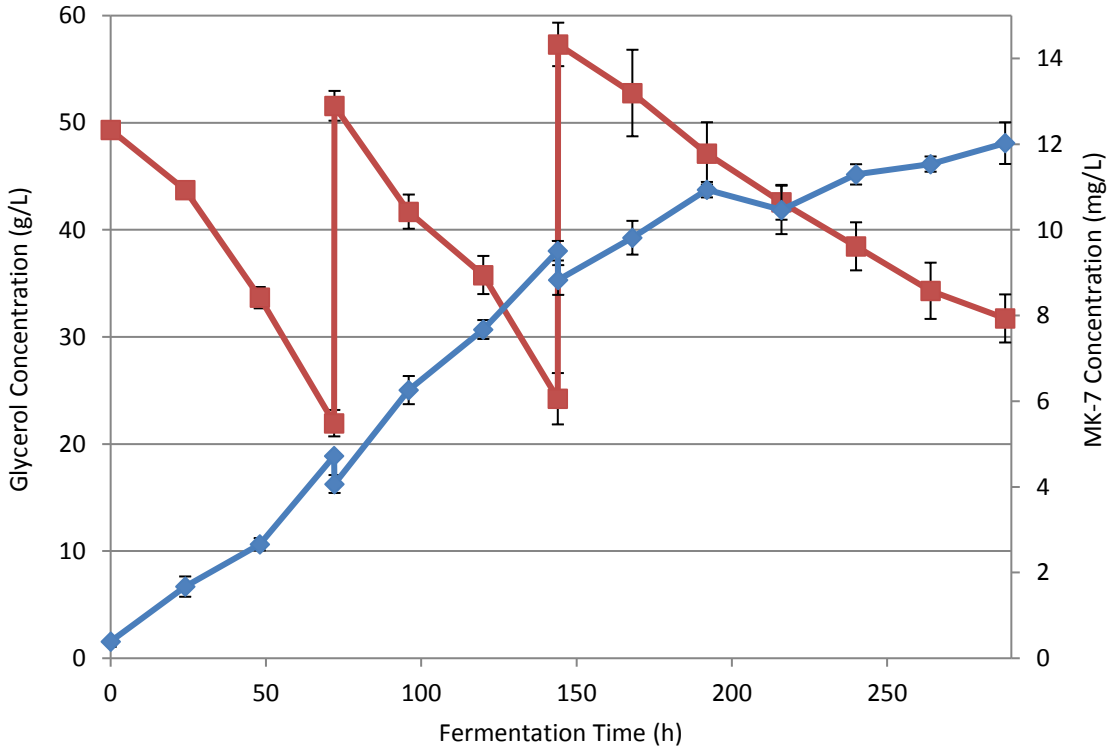


Figure 8.7. Highest MK-7 concentration profile observed in the glycerol-based medium.

8.4.2 Cell morphologies

Another important observation for both media in fed-batch fermentation was how *B. subtilis* cells morphed and changed during the long 12 days of fermentation. Figure 8.8 clearly indicates that as fermentation goes on in both media, young short cells that are observed at 24h morph into long aged *Bacilli* cells that no longer have a lot of resemblance to their young selves. It is known that *B. subtilis* species are potent spore former strains and sporulation is triggered by N-source starvation (Fisher, 1999). It is also known that sporulation and morphological and consequent gene expression changes are closely connected (Stragier et al., 1988). Since only carbon sources were fed here and nitrogen sources were only supplied in the beginning of the fermentation; nitrogen starvation may be possible, which can lead to such adaptations in morphology.

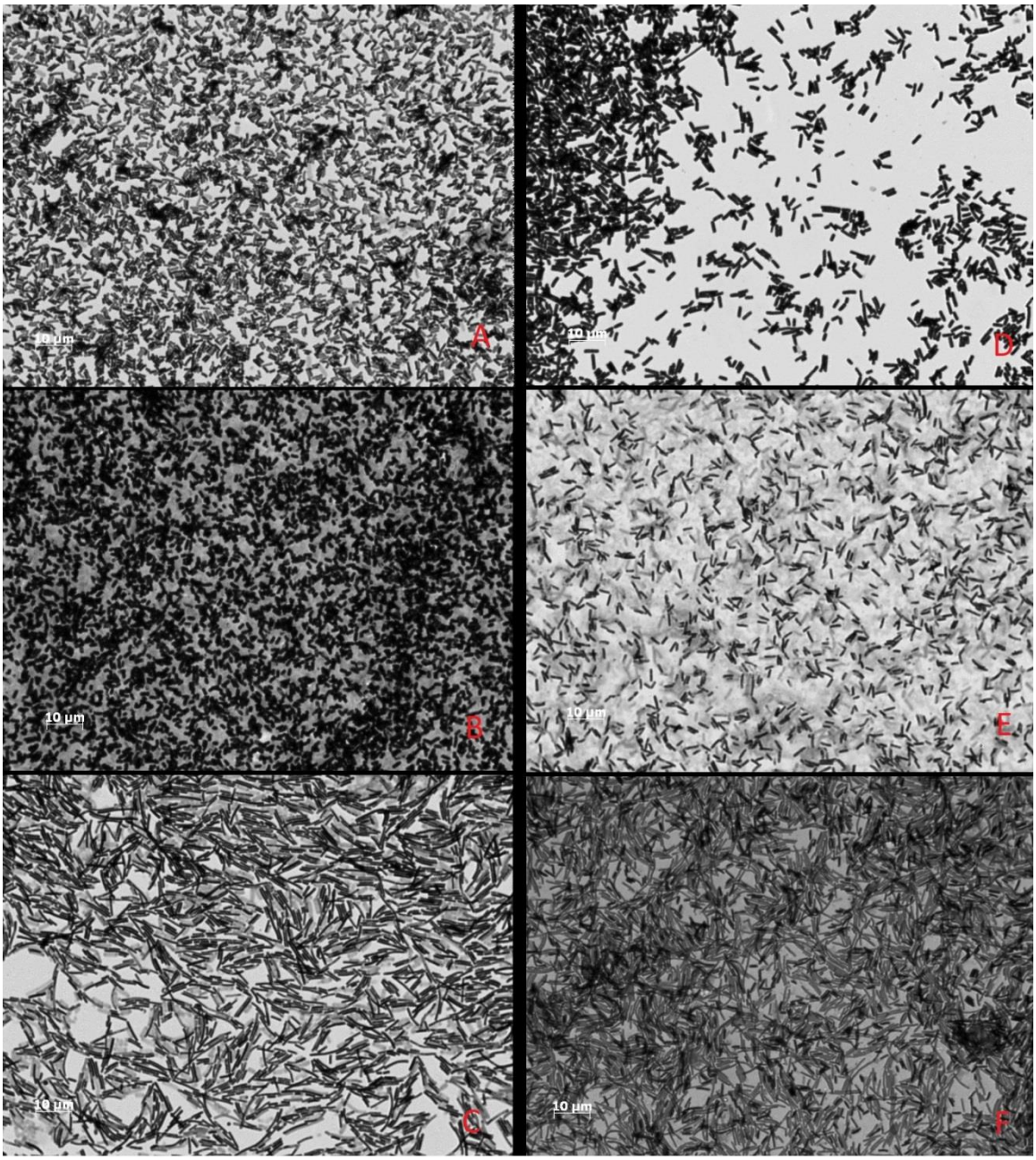


Figure 8.8. Morphology change in *B. subtilis* cells going from 24h old (A) to 144h (B) and 288h (C) in the glucose-based medium and 24h old (D) to 144h (E) and finally 288h (F) in the glycerol-based medium.

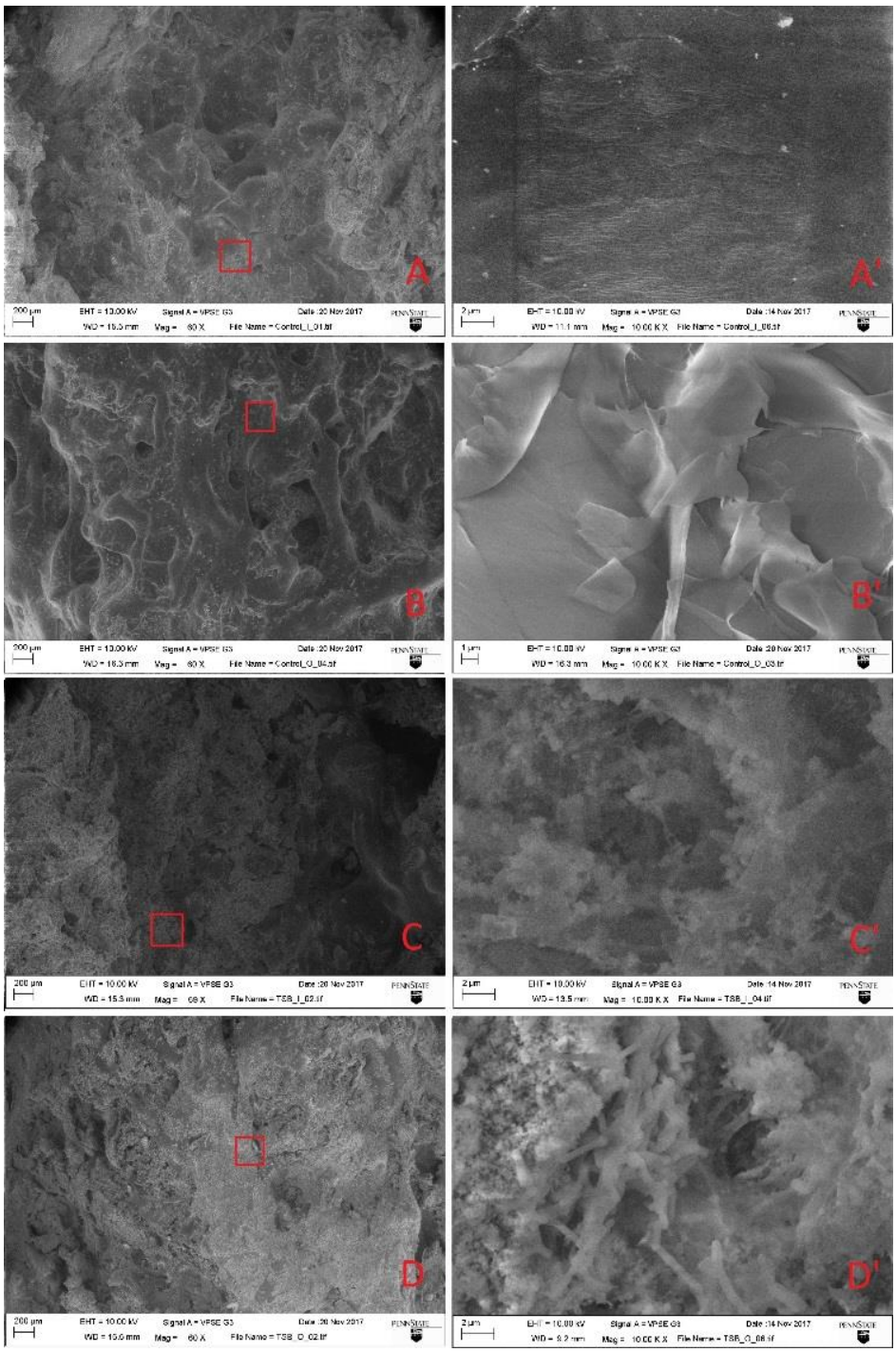
Another explanation could be considering the fact that biofilm reactors are based on the passively immobilized cells on the PCS that initiate the planktonic population in each batch. Also, biofilm reactors in this case are highly agitated and the shear stress on

the PCS is considerable. Thus, it is possible that the short tough cells are coming from the PCS-based biofilm, which are adapted to endure the stress and as these planktonic cells reproduce away from that stress in the following generations, the need to be short and tough goes away and the long relaxed cells are replaced.

8.4.3 SEM for biofilm formations

In order to clearly confirm the formation of biofilms on the PCS and to investigate the morphology on the PCS with the extracellular matrices, SEM was used. Figure 9 shows the low magnification captures of the PCS surfaces (60x magnifications) and the higher magnifications to observe the cells in the biofilm forms (10000 x magnifications). These clearly showed that immobilized cells in biofilm formations existed on the surface of the PCS tubes in both media (Figure 8.9D' and F"). Even some robust biofilm population was found inside the tube center hole when the tube was cut (Figure 8.9C' and E'). In the glucose-based media, the biofilm density on the surface was significantly more than the center of the tube of course. However, this was not the case for the PCS in the other medium, where the biofilm observed in the tube center was comparable and in some cases even more robust than the ones on the surface. This is surprising since *B. subtilis* is highly aerobic and tends to stay on the surface where oxygen is more available, unlike anaerobic microorganisms which may prefer to seep inside for more anaerobic conditions (Izmirlioglu and Demirci 2017). However, one explanation could be that the less nourishing glycerol medium could not enable the cells to strive on the surface and handle the physical stress as well as the glucose-based medium. It is hard to miss how much denser the biofilm formations are in glucose-based medium (Figure 8.9D') compared to the ones formed on the surface of the PCS in the glycerol-based medium (Figure 8.9F"). Also, the γ-polyglutamate extracellular matrices depositions are also clearly visible and also distinct in the two media and of course in comparison with the surface of the control (Figure 8.9A' and B'). Finally, Figure 8.9F' (5000x magnification) shows how the matrices look like

containing the less populated cells and figure 8.9F''' shows a close-up morphology of a singular *B. subtilis* cell attached onto the matrix.



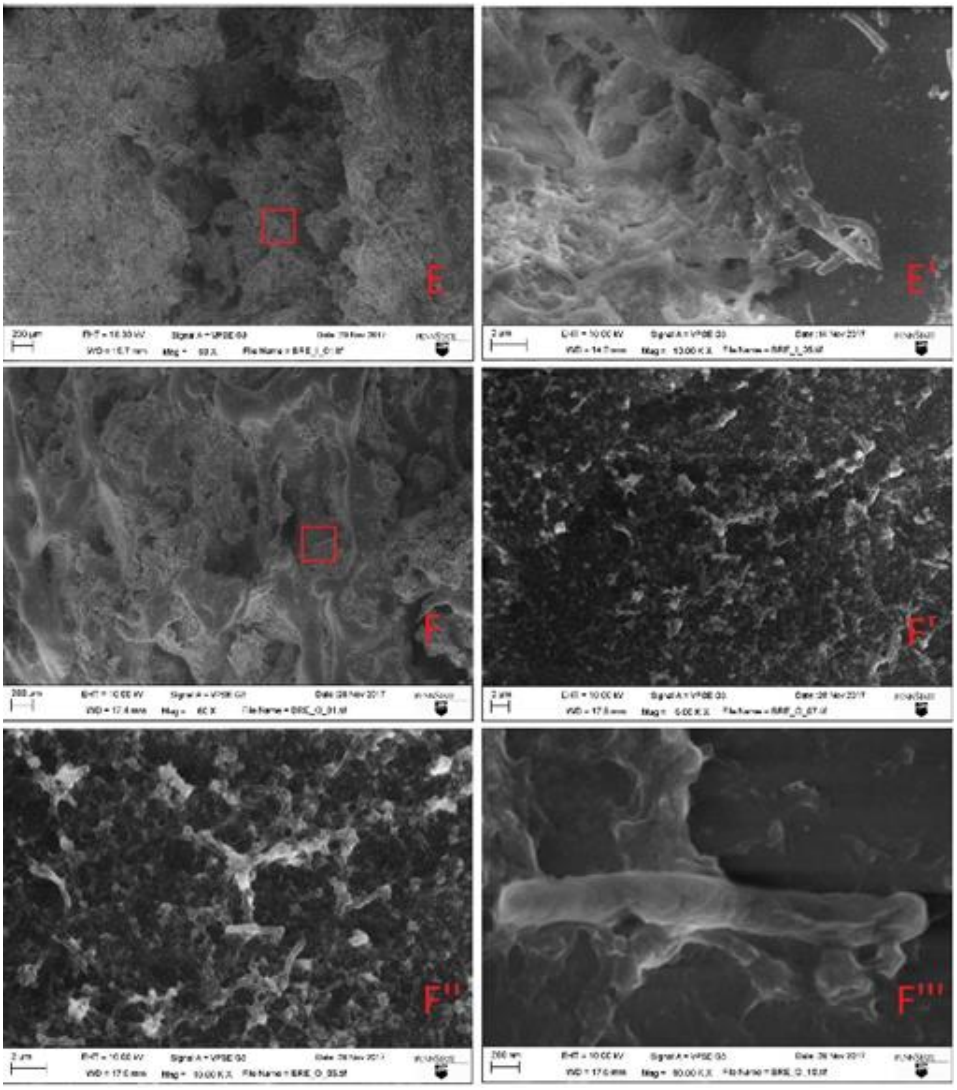


Figure 8.9. SEM images of the interior and the exterior of the PCS where figure A shows the interior of the control PCS at 60x magnification and A' is the 10000x magnification of the red square region in A. Similarly, B and B' show the exterior of the control; C and C' show the interior and D and D' show the exterior of the PCS in glucose-based medium, E and E' show the interior and F, F' (5000x) and F'' (10000x) show the exterior of the PCS in glycerol-based medium. F''' is a close-up view of a single *B. subtilis* cell attached to the exterior surface of the PCS via the γ-polyglutamate (γ-PGA) extracellular matrix at 80000x magnification.

8.5 Conclusions

As carbon source depletion occurs in both glycerol and glucose-based media in *B. subtilis* fermentation in biofilm reactors within 72h; one chance to increase final MK-7 concentrations is perhaps applying fed-batch C-source additions. For this purpose, biofilm reactors with glucose and glycerol-based media were supplemented with different concentrations of glycerol and glucose. The high concentration for glucose (150 g/L) and the middle for glycerol (30 g/L) was favorable in 6 day fermentations. These concentrations were implemented in different combinations at 72 and/or144 h and the product was analyzed until the end of the 12^{th} day. Results indicated that in glucose-based medium, double glucose feeding demonstrated MK-7 production of 28.7±0.3 mg/L, which is the highest concentrations reported in bioreactors and are quite comparable to the maximum amounts achieved with static fermentations of these strains (32.5±0.4 mg/L). This is perhaps the significant step necessary towards the introduction of biofilm reactors as a legitimate replacement for current static fermentation strategies, which are difficult to scale up and associated with serious mass and heat transfer issues.

8.6 Acknowledgments

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Chapter 9

Evaluation of vitamin K (menaquinone-7) stability and secretion in glucose and glycerol-based media by *Bacillus subtilis natto*

9.1 Abstract

Menaquinone-7 (MK-7) is a type of Vitamin K. MK-7 is commercially produced via bacterial fermentation and in order to reach the desired product quality, several downstream processing including extraction, drying, ultrasonication, etc. must be carried out. These processes, however, need to be carried out in such fashion to ensure least amount of losses and maximum recovery into the end-product. Therefore, in this study, drying, storage, and ultrasonication steps were evaluated under different conditions. Results showed that drying under forced air flow is not only fastest, but also demonstrated a better preservation of the vitamin and should replace the vacuum drying. Ultrasonication for 15 minutes seem to be harmless and sufficient for phase transition in analysis. Also, storage at refrigerated temperatures seem to preserve MK-7 at least for one week. Static liquid fermentations were conducted in McCartney bottles to explore the maximum MK-7 secretion potentials in different glycerol and glucose-based media compositions that were optimized in our previous studies. Maximum 32.5±0.4 mg/L and 14.6±0.4 mg/L concentrations were achieved in glycerol and glucose-based media respectively. Furthermore, fermentations in deeper culture tubes indicated how the MK-7 concentrations are distributed in different zones of the static liquid broth. Results in general, showed a clearer road map to ensuring better quality and preservation of the valuable end-product and enlightened more the path to further scaling up the fermentation process when compared with results obtained in optimized biofilm reactors in the past studies.

Keywords: MK-7, Menaquinone-7, Vitamin K, Stability, *Bacillus subtilis*, Secretion

9.2 Introduction

Vitamin K was discovered by Dr. Henrik Dam in his studies investigating an anti-hemorrhagic factor in chicken (Dam, 1935). Soon after Dam's discovery, it was established that vitamin K comes in two major forms in nature (Widhalm et al., 2012). Phylloquinone is one of the those forms with a singular chemical structure which is the plant form of vitamin K, found abundant in most leafy green vegetables such as spinach and kale with a direct role in photosynthesis (Booth, 2012; Binkley et al., 1939). The other form known as menaquinones have several subtypes (MK-1 to MK-15) with different isoprenoid repeats. Menaquinones constitute the vitamin K forms found animal sources foods such as red meat, egg yolk and cheese and also are expressed by several microbial strains as an electron carrier in respiration (Mahdinia et al., 2017a). As a result, the only feasible way to produce vitamin K on an industrial scale is through microbial fermentations (Berenjian et al., 2015). MK-7 is the subtype of vitamin K that stand out as the most potent form for benefits in human diet due to its relatively long half-life in the circulatory system and remarkable in vivo effects (Schurgers et al., 2007; Howard and Payne, 2006; Gast et al., 2009; Geleijnse et al., 2004; Yamaguchi, 2006).

Bacillus subtilis natto (Berenjian et al., 2011a), *Bacillus licheniformis* (Goodman et al., 1976) and *Bacillus amylolyquifaciens* (Wu and Ahn, 2011) strains in both Solid State Fermentation (SSF) and Liquid State Fermentation (LSF) strategies have been investigated for potential MK-7 production on an industrial scale (Singh et al., 2015). In this regard, fermentation in McCartney bottles and shake-flasks have been investigated in numerous past studies (Berenjian et al., 2014; Berenjian et al., 2012; Sato et al., 2001). Results of those studies sought to investigate best media compositions including effects of different carbon sources, nitrogen sources, salts, pH changes, effects of pellicle and biofilm formations and several other conditions affecting MK-7 biosynthesis. Also, our previous studies investigated the feasibility and conditions for scaling up MK-7 fermentation from bottles and flasks to bench-top biofilm reactors and indicated their superiority to suspended-cell bioreactors (Chapters 3, ,4 ,5, 6 and 7). However, the integrity and stability of MK-7 as it is produced in the fermentation process needs to be studied through analysis

or downstream processes. Also, the leap from fermentations in flasks and bottles to bench-top bioreactors having been carried out with different *B. subtilis* strains needs to be further scrutinized. In this fashion, this study focused on investigating MK-7 stability through those processes and also further looking into its biosynthesis in bottles and tubes with different glucose and glycerol-based media compositions that were optimized earlier in bioreactors.

9.3 Materials and methods

9.3.1 Microorganisms and media

Bacillus subtilis natto (NF1) was isolated from commercial natto, as previously described (Chapter 3). For working culture, Tryptic Soy Broth (TSB) medium (Difco, Detroit, MI) fortified with 5% (w/v) glucose (Tate & Lyle, Decatur, IL) and 0.8% yeast extract (Biospringer, Milwaukee, WI) was used (Chapter 4). Original TSB media consisted of glucose (Tate & Lyle), 17.5 g/L tryptone (Marcor, Carlstadt, NJ), 3 g/L of soytone (Marcor), 5 g/L of NaCl (EMD Chemicals, Gibbstown, NJ) and 2.5 g/L of K_2HPO_4 (VWR, West Chester, PA), and then different percentages of glucose (w/v) (0, 5, 10 or 15%) and 8 g/L of yeast extract (Biospringer) were added to fortify the TSB medium (Designated as TSB 5%, TSB 10%, etc.). Also, two compositions of the glycerol-based medium were evaluated. The Lesser concentration Glycerol-based Medium (LGM) contained 45 glycerol (EMD), 10 g/L soytone (Marcor), 5 g/L yeast extract (Biospringer) and 0.6 g/L K_2HPO_4 (VWR) (Chapter 7). The Higher Glycerol-based Medium concentration (HGM) was consisted of 50 g/L glycerol (EMD Chemicals, Gibbstown, NJ), 189 g/L soytone (Marcor), 50 g/L yeast extract (Biospringer) and 0.6 g/L K_2HPO_4 (VWR) (Chapter 4).

9.3.2 Fermentation

9.3.2.1 Inoculum preparation

Fermentations were carried out in either 30 mL McCartney amber bottles (VWR) with 3 mL of media in them or in 75 mL culture tubes (VWR) with 20 mL media. All fermentations were carried out in static form at 37°C in L-C incubators (Lab-line Thermal Scientific, Fort Worth, TX) for 96 hours. For inoculum, 150 mL of TSB medium with 15% (w/v) glucose (Tate & Lyle) and 0.8% yeast extract (Biospringer) was prepared in 250 mL Erlenmeyer flask and autoclaved for 20 min at 121°C and then inoculated with the working culture. After 24 hours of incubation at 37°C and 200 rpm, tubes (Figure 1) and bottles (Figure 2) were inoculated with a constant 3% inoculation ratio.

9.3.2.2 Fermentation in McCartney bottles

Amber McCartney bottles (30 ml) were used containing 3 mL of each medium (Figure 9.1). After autoclaving and inoculation, bottles were incubated at 37°C statically for 96 h. Then the entire mixture in the bottles were analyzed for MK-7 concentration.

Figure 9.1. Bacillus subtilis natto fermentation in McCartney bottles.

9.3.2.3 Fermentation in culture tubes

For tube fermentations, 75 mL culture tubes contained 20 mL of each medium composition (Figure 9.2). After autoclaving and inoculation, tubes were also incubated at 37°C statically for 96 h. Then, 3 mL samples were drawn from the top layer, the middle of the broth and from the bottom of the tubes and were analyzed for MK-7.

Figure 9.2. *Bacillus subtilis natto* fermentation in 75 ml culture tubes (HGM on the left, LGM in the middle and TSB 10% on the right).

9.3.3 Stability evaluations

9.3.3.1 Drying

In order to investigate the effects of drying on MK-7 stability, different methods of drying was applied. Thus, 10 mg/L MK-7 standard solutions in methanol were prepared. First, without any drying step, one set of samples were analyzed to confirm the original concentration. Others were dried under forced air or nitrogen flow (~5 L/min) for a few hours, vacuum (<3 psia) at 37°C overnight and under a fume hood without any forced gas flow or vacuum for up to 72h until all pellets were completely dried, all at ambient temperatures. Then, the organic mixture N-hexane:2-propanol (2:1, v/v) (EMD) with 1:4 (liquid:organic, v/v) was added to the dried pellets and dried again. Under forced gas flows the samples took a few hours to dry with a marginal gas exiting the nozzle to ensure no pellet disturbance or fluidization (<10 psig), in vacuum they were dried overnight

(Berenjian et al., 2011b), and in the fume hood they took about 72 hours to dry up completely. Then, dry pellets were resuspended in methanol and analyzed for MK-7 by HPLC.

9.3.3.2 Storage

For insuring proper storage, standard 10 mg/L MK-7 solutions were prepared in typical fermentation medium. Half of the solutions were extracted and then dried and the other half were stored at 4 and -20°C. After 7 days, MK-7 concentration was analyzed in all of them in comparison with the samples that were extracted and analyzed fresh.

9.3.3.3 Ultrasonication

To ensure optimum ultrasonication treatment of samples, standard 10 mg/L MK-7 solutions were prepared in the extracting organic mixture N-hexane:2-propanol (2:1, v/v) (EMD) with 1:4 (liquid:organic, v/v) and then dried under the forced air flow. After that, methanol was added to the pellets and then different periods of ultrasonication (5, 10, 15, and 30 min) were applied to the samples prior to HPLC analysis for MK-7 concentration.

9.3.4 Analysis

9.3.4.1 MK-7 analysis

Fermentation broth sample (3 ml) was mixed with 2:1, v/v, n-hexane:2-propanol mixture to extract the MK-7 content (Berenjian et al., 2011b). N-hexane:2-propanol (2:1, v/v) (EMD) with 1:4 (liquid:organic, v/v) was used. The mixture was vigorously shaken using a vortex mixer for 3 min and then the organic phase was separated and evaporated under forced air flow (or otherwise as mentioned) at ambient temperature. Then, dried

pellets containing the MK-7 were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH) for 15 min (or otherwise as mentioned) at ambient temperature. After the pellets were suspended completely in methanol, the mixtures were filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY). High Performance Liquid Chromatography (Waters, Milford, MA) was utilized then to analyze MK-7 concentrations dissolved in pure methanol in the samples using UV-Vis light as described in previous studies (Chapter 4; Shahami et al., 2017). Pure MK-7 (99.3%) was purchased from ChromaDex (Irvine, CA) and 100 mg/L liquor solution was prepared from pure MK-7. The MK-7 calibration curve was linear between 0.1 mg/L and 50 mg/L (R^2 = 0.999).

9.3.4.2 Statistical analysis

All observations were repeated at least three times and the average values were obtained and demonstrated with standard errors of the repetitions as error bars (Rahimi et al., 2018a; Feilizadeh et al., 2017).

9.4 Results and discussions

MK-7 stability and secretion under static liquid fermentations were the subjects for investigation in this study. First hypothesis was how MK-7 is impacted by drying and ultrasonication steps that are necessary prior to HPLC analysis and if MK-7 is stored at refrigerated or frozen conditions perhaps prior to downstream processing or analysis, what are the best conditions to minimize potential losses within the storage time? The second hypothesis was how is MK-7 secretion taking place at static fermentations in different media compositions that have been implemented in biofilm reactors? In order to answer the first question, drying, storage and ultrasonication were applied on standard MK-7 solutions at different conditions and MK-7 concentration was analyzed under those conditions to track changes.

9.4.1 MK-7 stability

9.4.1.1 Drying

Once MK-7 is secreted into the broth and the broth is harvested from bioreactors; it needs to be extracted and dried to be analyzed or extracted and then purified and freeze-dried to be pharmaceutically utilized. In either case, it is important to know how drying may effect MK-7 stability. For analysis, after extraction and separation of the phases, the organic phase is usually dried at ambient temperatures; yet, this can be done under vacuum and with or without forced gas flow onto the evaporating phase.

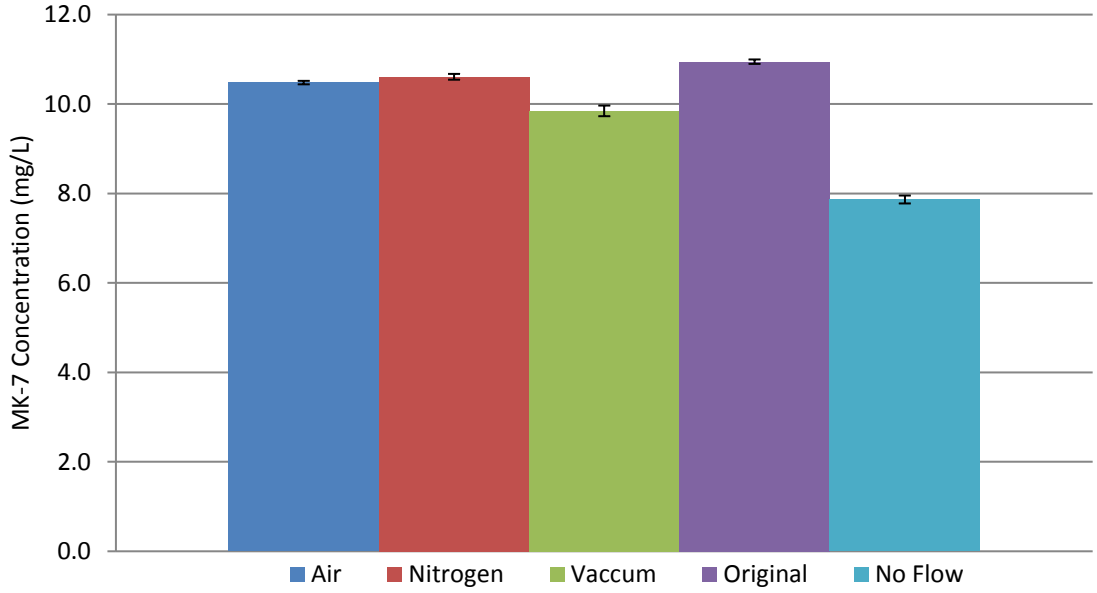

Figure 9.3. Effects of different drying methods on MK-7 concentration.

As Figure 9.3 indicates, under forced gas flow drying would not affect MK-7 significantly and the differences were only about 3 or 4%. However, vacuum drying leads to about 10% loss and drying in fume hoods leads to about %30 drop in MK-7 concentration. This is possibly due to the fact that with forced air or nitrogen the MK-7 is exposed to ambient conditions for only a few short hours and therefore preserved. The fact

that air did not give different levels than nitrogen states that exposure to ambient oxygen for that short period at least does not affect the MK-7 integrity; which is a good news that enabled the use of air, because it is economical compared to nitrogen gas. Since vitamin K is generally sensitive to light, although extraction and evaporation and injection steps are all carried out in amber vials; prolonged exposure to ambient conditions must be minimized (Okano et al., 2008). This is perhaps another benefit of using the forced air flow for drying besides saving processing time.

9.4.1.2 Storage

Storing the harvested MK-7 from the broth may be inevitable for a few days due to the fact that downstream processing steps may not be online as soon as the fermentation cycle is complete (Perry, 2007). Therefore, it is imperative to ensure that the harvested MK-7 is stored suitably so that potential losses are minimized. Figure 9.4 indicates that within 7 days of storage in refrigerator or freezer the MK-7 shows very strong stability and no detectable degradation is observed. The stored samples showed slight deviation with the original solution (11.1, 10.8, 11.2 and 10.4 mg/L as compared to 10.7 mg/L in the original solution); which suggest reasonable extraction and HPLC analysis error ranges. These observations reveal that low temperatures preserve the MK-7 chemical structure well and refrigeration is sufficient for that purpose possibly due to the fact in storage MK-7 is kept in the dark and away from any light exposure and degradation due to microbial degradation is significantly low.

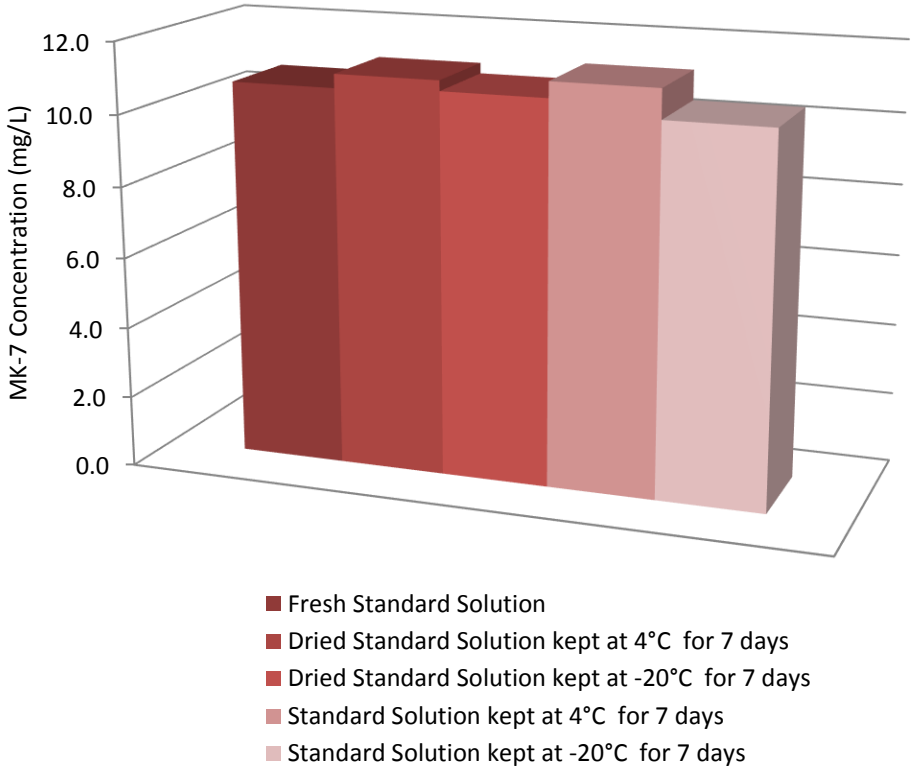

Figure 9.4. MK-7 concentration changes in 10 mg/L standard solutions stored under different conditions.

9.4.1.3 Ultrasonication

Once the harvested MK-7 is dried, in order for the analysis to be carried out, the MK-7 dry pellets need to be resuspended in pure methanol for HPLC analysis. Although this step is not required for downstream processing of MK-7, it is needed for analytical and quality control purposes. Since, MK-7 has fairly low solubility in methanol and is quite sensitive to high temperatures due to its fragile long isoprenoid side chain (Shearer and Newman, 2008), one practical way of dissolving the pellets quickly in methanol is to apply ultrasonication in ambient temperatures. Ultrasonication does a very good job in dissolving

the pellets; yet, there can be reasonable doubts about powerful ultrasonication waves damaging the chemical structure of MK-7 (Hasanian and Lissenden, 2017). Thus, it was imperative to ensure and determine safe usage of ultrasonication. As Figure 9.5 shows, ultrasonication for 5, 10, 15 and 30 minutes seems to be harmless to MK-7 integrity (10.0, 10.0, 10.0 and 10.1 mg/L MK-7 concentrations respectively as compared to the 10.0 mg/L original standard concentration). In fact, the small increased concentration in the samples treated with 30 minutes of ultrasonication is probably due to loss of a bit of solvent to evaporation. Therefore, 15 min of ultrasonication seems to be harmless and ample.

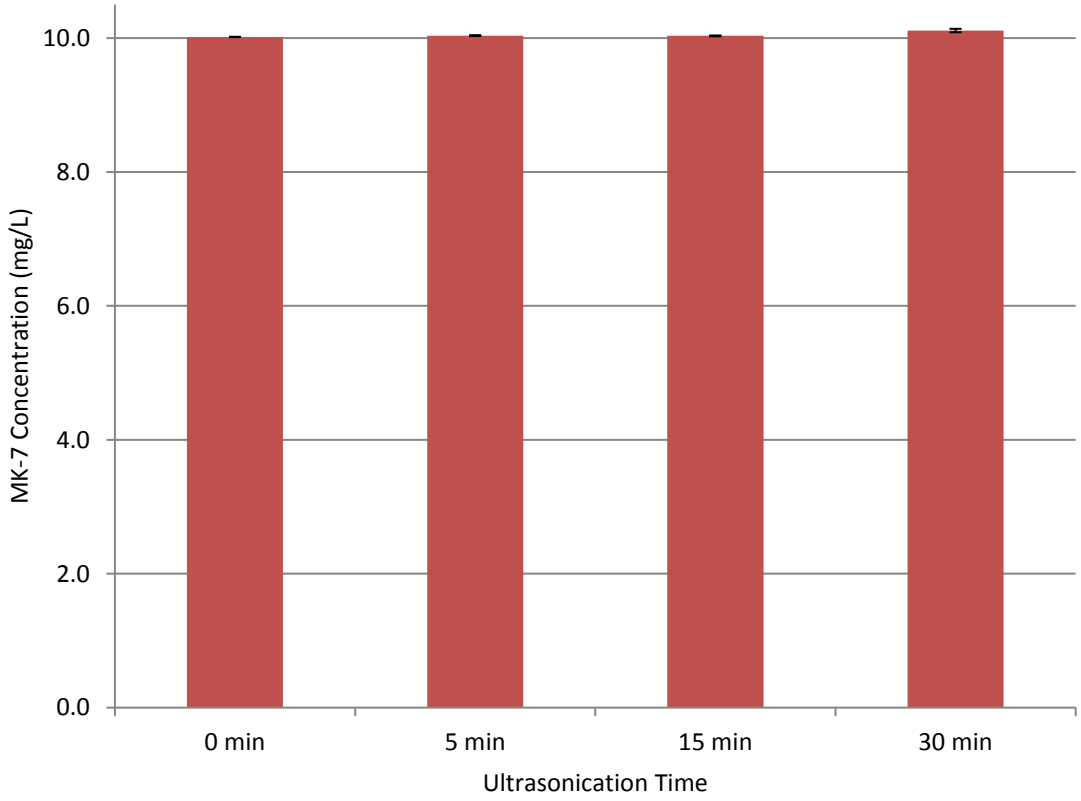

Figure 9.5. Effects of different periods of ultrasonication on MK-7 concentration in 8 mg/L standard solutions.

9.4.2 Fermentation in McCartney bottles

For the second hypothesis in this study, fermentation was carried out in different media compositions and in different vessels (tubes and bottles) to demonstrate. The hypothesis states that MK-7 fermentation similar to any fermentation process is highly dependent on the scale of the fermentation as well as the fermentation strategy.

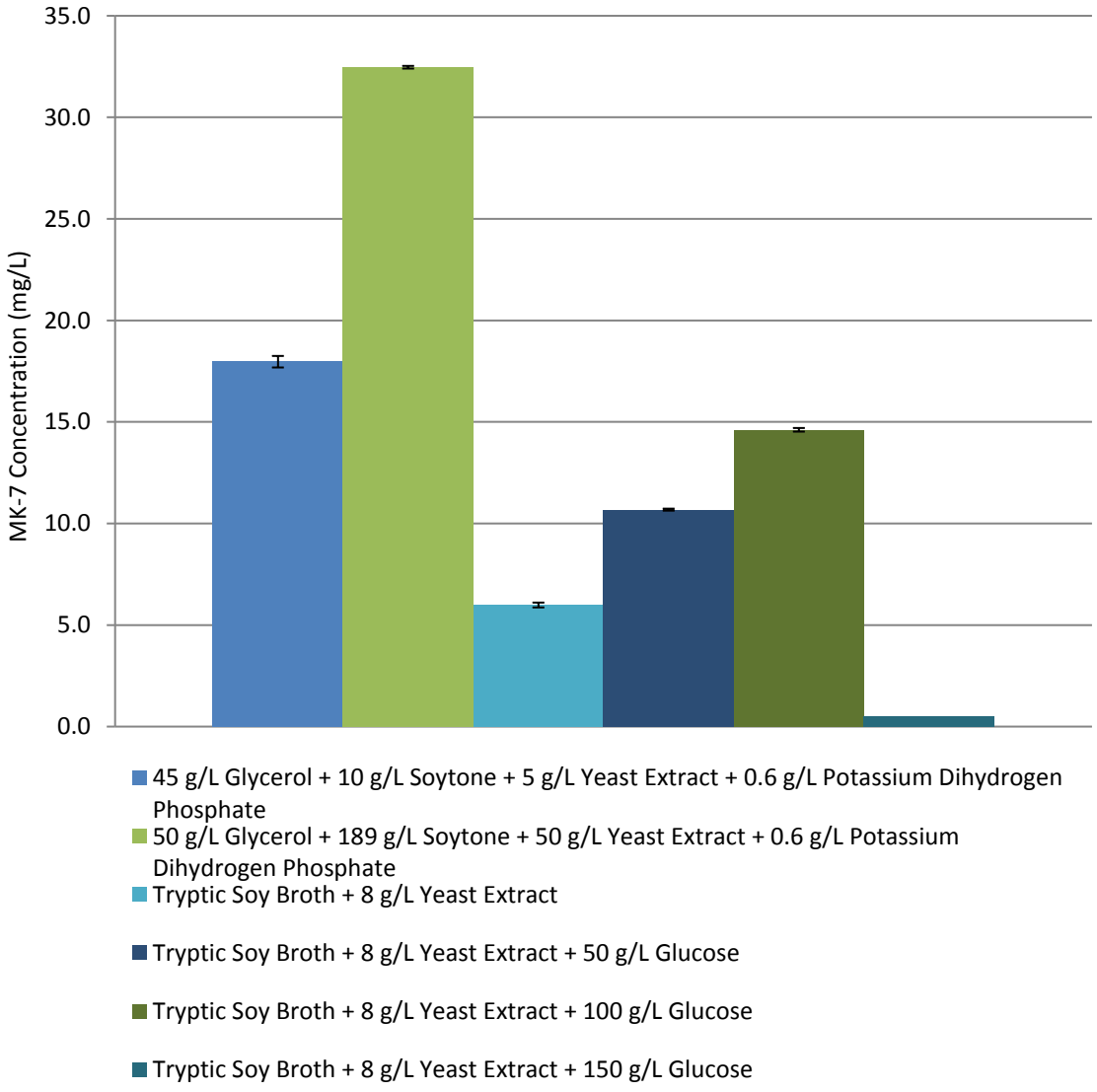

Figure 9.6. MK-7 concentration produced in different media and vessels all under static liquid state fermentations.

In other words, from McCartney bottles to culture tubes the fermentation is liable to be just as distinct as those in bioreactors. Fermentation in McCartney bottles as shown in Figure 9.1 is the conventional method for producing maximum MK-7 in *B. subtilis* strains (Chapter 3). Fermentation in McCartney bottles shows how much potential the *B. subtilis* strain has for MK-7 secretion. Figure 9.6 indicates that a maximum 32.5 ± 0.4 mg/L MK-7 concentration is achievable by the *B. subtilis natto* NF1 in the rich glycerol-based medium. This is basically the highest amount of MK-7 concentration observed in our recent studies in bottles, tubes or bioreactors. *B. subtilis* is a highly aerobic bacteria and as biofilm reactor studies indicated, high agitation and higher Oxygen Transfer Rates (OTR) are always favorable for MK-7 secretion (Chapter 5). Thus, it seems that the ample headspace provided in the McCartney bottles besides shallow broth create enough oxygen diffusion into the fermentation mixture for MK-7 secretion pathways to flourish (Figure 9.1). Furthermore, as it can be seen in Figure 9.1, pellicle formations formed by the bacteria which are predominantly consisted of γ-polyglutamate (γ-PGA) are less dense than the broth and therefore enable the dense microbial colonies in the extracellular matrices float on the surface of the broth and thus in closer contact with oxygen diffusing from air (Morikawa et al., 2006). On the other hand, the very rich glycerol-based medium provide the nutrients required for these robust colonies to expand quickly so that at the end of the 96 hour fermentation, the broth was almost solidified and the pellicle formation were significantly denser in the rich glycerol medium. This clearly explains why MK-7 is expressed also significantly more compared to other media compositions. Our previous studies indicated that these extracellular matrices are beneficial to MK-7 expression same as biofilm reactors being more efficient in this regard compared to suspended-cell bioreactors (Berenjian et al., 2013; Chapter 6). However, such rich medium did not seem to have as much efficiency in biofilm reactors; yet, the less rich glycerol medium produced comparable amounts of MK-7 under both regimes (14.7±1.4 mg/L in biofilm reactors compared to 18.0±0.3 mg/L in bottles) (Chapter 7). In the TSB media, fortifying the composition with more glucose from TBS without additional glucose to 5 and 10% added glucose significantly increased the MK-7 concentrations (6.0±0.1, 10.7±0.2 and 14.6±0.4 mg/L, respectively) and all these three compositions showed robust pellicle formations.

These concentrations are still lower than those obtained in optimized biofilm reactors with %15 added glucose (Maximum 20.46±0.51 mg/L). However, with 15% added glucose in the bottles, metabolism was inhibited and growth and therefore MK-7 secretion was very poor so that no pellicle formations were visible at the end of the 96 hours. One explanation could be that with 15% glucose simply created inhibition in the static fermentation perhaps due to higher oxygen demands and low oxygen availability, while in biofilm reactors this was not the case with robust agitation and aeration (Chapter 5 and 6).

9.4.3 Fermentation in culture tubes

Fermentation in culture tubes in this study can be considered a small scale-up step from McCartney bottles. Culture tubes contained 20 mL of broth with relatively less head space. Thus, as expected, in culture tube, the ideal conditions that existed in McCartney bottles do not exist anymore. The depth of the broth is simply too much for oxygen to diffuse deep and reach the bottoms. However, fermentation in the culture tubes provided enough depth to clearly see the three layers that occur in static fermentation. As it can be seen in Figure 9.2, at the end of the fermentation period and regardless of the media composition, there are three distinct layers formed in the tubes. On the top, there is a thick layer of pellicle formations, in the middle the broth is clearer and contains mostly planktonic cell and on the bottom there are sunken cells. Results in Figure 9.7 show that in glycerol-based media the settled down cells in the bottom zones are significantly MK-7 richer. One explanation could be that the cells that are dense enough on the surface layers sink to the bottom possibly as they die and thus release the intracellular MK-7 into the broth, as MK-7 is both extracellularly and intracellularly expressed in *B. subtilis* (Ikeda and Doi, 1990). However, in glucose-based media MK-7 expression is almost negligible when compared to McCartney bottles (barely reaching over 1 mg/L) and as oxygen diffusion is even more critical here, glucose richer compositions are much poorer in MK-7. This may also explain why MK-7 concentrations in the top layers are better than middle layers and also why the richer glycerol-based medium top and middle layers are richer than

those layers in the less rich medium; i.e. the rich medium requires more oxygen availability and therefore in the top layers does a better job with relatively more oxygen availabilities. Overall, the MK-7 secretion in the tubes are significantly lower than McCartney bottles (over 32 mg/L maximum concentrations in bottles whereas they are barely over 5 mg/L in bottles); however, the results in the tubes show how MK-7 distribution occurs in liquid static fermentations in different zones.

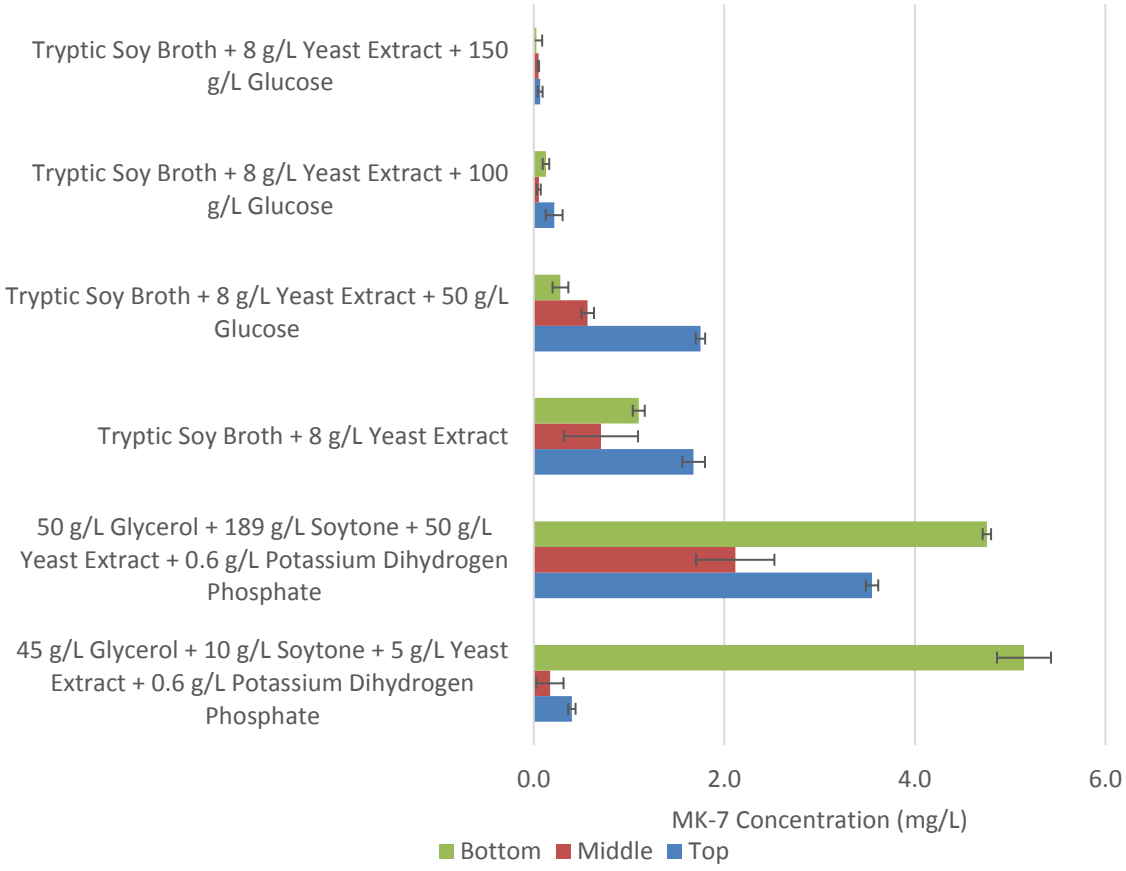

Figure 9.7. MK-7 production in different media compositions at the top, middle or bottom zones of the broth in culture tubes.

9.5 Conclusions

MK-7 stability and integrity was investigated under different conditions during the drying, storage and ultrasonication steps necessary for analysis and perhaps downstream processing. Results indicated that if the MK-7 produced is quickly dried using forced air flow after extraction, over 95% of it can be preserved. Also, refrigeration at 4°C will be sufficient to preserve the MK-7 product in the original broth for at least 7 days, should the downstream processing or analysis is delayed. Moreover, ultrasonication for 15 minutes seems to be harmless and sufficient prior to analysis. Besides, fermentations in McCartney bottles and culture tubes revealed that the conditions in the McCartney bottles are ideal to explore the maximum potential for MK-7 secretion, which occurred in the glycerol-rich medium (32.5±0.4 mg/L) seconded by TSB medium fortified with 10% glucose (14.6±0.4 mg/L). Finally, in culture tubes as expected, the MK-7 concentrations were much less than bottles (Maximum 5.1±0.2 mg/L); yet they provided good insight as of how MK-7 secretion is distributed in different zones of the static liquid fermentation. These results in comparison with those achieved in optimized biofilm reactors complete the last pieces of the picture of scaling up the MK-7 fermentation step by step and enlighten the road to maximum preservation of the end product.

9.6 Acknowledgments

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Chapter 10

Modeling vitamin K (menaquinone-7) fermentation by *Bacillus subtilis natto* in biofilm reactors

10.1 Abstract

Menaquinoe-7 (MK-7) is the most potent form of vitamin K with numerous benefits for human health such as reducing the risks of cardiovascular diseases and osteoporosis and also possesses valiant antitumor characteristics. Thus, MK-7 production via bacterial fermentation has been studied intensely in the last few decades. Recently, biofilm reactors were implemented to enhance production levels. Therefore, this study was undertaken to mathematically model the findings in batch biofilm reactors and thus further enlighten the conditions governing fermentation. The logistic equation was modified to correlate substrate consumption with fermentation time and was utilized to model the substrate consumption in the four batch fermentations. Results indicated that this modified logistic model fits well with the experimental data for substrate consumption. Therefore there was no need for more complex models ($R^2 > 0.953$). Furthermore, this successfully modified-logistic equation was inserted into the Luedeking-Piret model to modify for MK-7 production based on substrate consumption. Besides, modified-Gompertz model was also evaluated for the same purpose. Results indicated that the modified Luedeking-Piret model fits more accurately for MK-7 production ($R^2 = 0.971$, 0.943, 0.970, and 0.959) compared to the modified-Gompertz ($R^2 = 0.914$, 0.943, 0.949 and 0.86). Yet, the modified Luedeking-Piret equation is a more complex and less parsimonious model compared to the modified-Gompertz. Considering typical MK-7 profiles in batch fermentations, there is no doubt that MK-7 biosynthesis follows a mixed-metabolite pattern in *Bacillus subtilis natto*.

Keywords: Menaquinone-7, Vitamin K, Biofilm reactor, *Bacillus subtilis*, Modeling

10.2 Introduction

Shortly after the discovery of vitamin K (Dam, 1935), it was discovered two major forms of vitamin K exist (Widhalm et al., 2012). Phylloquinone is the plant form and is found in most leafy green vegetables (Booth, 2012; Binkley et al., 1939). The animal and microbial forms are menaquinones and besides their food sources such as red meat, egg yolk and cheese, they include the predominant forms in microbial metabolisms (Mahdinia et al., 2017a). Menaquinone-7 (MK-7) is the most potent form of Vitamin K with extraordinary benefits for human health (Schurgers et al., 2007; Howard and Payne, 2006; Gast et al., 2009; Geleijnse et al., 2004; Yamaguchi, 2006). Microbial fermentation on an industrial scale is the only feasible way to produce MK-7 (Berenjian et al., 2015).

Bacillus subtilis natto (Berenjian et al., 2011a), *Bacillus licheniformis* (Goodman et al., 1976) and *Bacillus amylolyquifaciens* (Wu and Ahn, 2011) are the most common strains studied for this purpose. Solid State Fermentation (SSF) and Liquid State Fermentation (LSF) strategies have been investigated for MK-7 production with *B. subtilis natto* (Singh et al., 2015; Wu and Ahn, 2011); yet, both SSF and static LSF strategies with no robust agitation and aeration, face serious scale-up, and operational issues (Pandey, 2003; Mahdinia et al., 2017a). On the other hand, pellicle and biofilm formations creating these issues are beneficial for the MK-7 biosynthesis (Ikeda and Doi, 1990). With the opportunity to use biofilm reactors to harness the biofilm formations and keep these benefits and at the same time have robust agitation and aeration, biofilm reactors were constructed with suitable PCS and strain combination and growth conditions and medium compositions were optimized using Response Surface Methodology (RSM) (Chapters 3-7).

However, there is a need to mathematically model the MK-7 production and substrate consumption in these batch biofilm reactors to further elucidate the larger scale fermentation strategies. Logistic function model is originally utilized to model biomass growth and Luedeking–Piret and Gompertz equations are used to correlate product formation to biomass growth and substrate consumption and also to model substrate consumption in relation to biomass production as well. Coban and Demirci (2016) used

modified versions of Gompertz and logistic mathematical models to predict lactic acid production and glucose consumption in lactic acid fermentation by *Rhizopus oryzae*. Also, Logistic equation was applied to calculate batch kinetics of microbial growth for polysaccharide production (Mohammad et al., 1995). Moreover, Mohammad et al. (1995) described pullulan production with the modified-Luedeking–Piret model and modified-Gompertz model. Similarly, Cheng et al. (2010) modified logestic, Gompertz, and Luedeking–Piret models to explain pullulan fermentation by using a color variant strain of *Aureobasidium pullulans* in biofilm reactors. Results indicated that the modified Gompertz models proposed in the study demonstrated its flexibility to fit all biomass production, pullulan production, and sucrose consumption. Mahanama et al. (2012) also assumed the Luedeking–Piret model in correlation with biomass growth on solid state substrate to explain MK-7 biosynthesis by *B. subtilis natto* in tray fermenters. In this study, however, direct correlation of MK-7 production with substrate consumption is aimed for in batch biofilm reactors rather than fed-batch biofilm reactors or bottle and tube fermentations as a further step towards scaling-up MK-7 fermentation in liquid state fermenters (Chapters 8 and 9).

10.3 Materials and methods

10.3.1 Microorganisms and media

Bacillus subtilis natto (NF1) was isolated from commercial natto, as previously described (Chapter 3). For biofilm formation on the Plastic Composite Support (PCS), Tryptic Soy Broth (TSB) medium fortified with 10% (w/v) glucose (Tate & Lyle, Decatur, IL) and 0.8% yeast extract (Biospringer, Milwaukee, WI) was used. Optimum fermentation media consisted of 10 g of soytone (Difco, Detroit, MI), 5 g of yeast extract (Difco), 45 g of glycerol (EMD Chemicals, Gibbstown, NJ) and 0.6 g of K_2HPO_4 (VWR, West Chester, PA) for the glycerol-based medium and 3 g of soytone (Marcor, Carlstadt, NJ), 5 g of NaCl (EMD), 2.5 g of K2HPO4 (VWR) and 152.6 g of glucose (Tate & Lyle), 17.6 g of casein

(tryptone) (Marcor) and 8 g of yeast extract (Biospringer), per liter of deionized water, as concluded by our previous studies (Chapters 6 and 7).

10.3.2 Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA) equipped with 2-L vessels (1.5-L working volume) were utilized. Sterile 4N sulfuric acid (EMD) and 4N sodium hydroxide (Amresco, Solon, OH) along with antifoam B emulsion (Sigma-Aldrich, Atlanta, GA) were added automatically to maintain pH and suppress foaming as needed. Plastic Composite Support (PCS) tubes type SFYB (50% Polypropylene, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% bovine albumin and salts) were manufactured and implemented and biofilm reactors for glycerol and glucose-based media were operated at optimum conditions as described in previous studies (Ho et al. 1997; Chapters 6 and 7).

10.3.3 Biofilm formation

For biofilm formations to form on the PCS grids, bioreactors were set up with grid-like fashion PCS formations. Then, sterile medium was added to the bioreactors and refreshed for four times, as described in the previous study (Chapter 4). At the end of the four fermentation cycles, the fermentation broth was sampled and Gram-stained to verify a pure culture.

10.3.4 Experimental design

Growth parameters (temperature, pH, and agitation) and medium components (glycerol, soytone, yeast extract, glucose, and casein) were optimized using Response

Surface Methodology and optimum conditions were validated as described in our previous studies (Chapters 4-7).

10.3.5 Analysis

10.3.5.1 MK-7 analysis

Fermentation broth sample (3 ml) was mixed with 2:1, v/v n-hexane:2-propanol mixture to extract the MK-7 content (Berenjian et al. 2011a). N-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used. The mixture was vigorously shaken using a vortex mixer for 3 min and then the organic phase was separated and evaporated under forced air flow at ambient temperature as described in previous studies (Mahdinia et al., 2017g). Then, dried pellets containing the MK-7 were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH) for 15 min at ambient temperature. After the pellets were completely suspended in methanol, the mixtures were filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY). MK-7 concentrations in the samples was then analyzed by High Performance Liquid Chromatography (HPLC) using UV-Vis light as described in previous studies (Chapter 3, Shahami et al., 2017).

10.3.5.2 Substrate analysis

Samples of the fermentation broth was centrifuged at 9000 x *g* for 5 min (Microfuge 20 Series, Beckman Coulter Inc., Brea, CA) and then filtered through 0.2 µm cellulosic filters (PALL). Then, with no dilution, the cell-free broth was analyzed by HPLC as described the previous studies (Chapters 4 and 5).

10.3.5.3 Statistical analysis

Growth and medium main effects along with the 2nd order and two-way interaction effects were obtained using Minitab 17.0 ANOVA (Minitab Inc., State College, PA) with statistical model and regression analysis with Box-Cox transformation optimal λ. A confidence level of 95% was implemented throughout the analysis procedures to distinguish significant parameters (Rahimi et al., 2017a and 2017b).

10.3.6 Mathematical models

MATLAB R2017a (MathWorks, Natick, MA) was used to carry out mathematical calculations and fits. Modified Logistic equation was applied for modeling the substrate consumptions. Modified Gompertz and modified Luedeking–Piret equations were derived and used to model MK-7 productions. Models for MK-7 productions and the model for substrate consumptions were developed using the average results of optimum growth conditions and medium compositions obtained in previous studies (Chapters 4- 7). Table 10.1 summarizes the nomenclatures used in this study.

Table 10.1. Nomenclatures.

Symbol	Name	Value	Unit	Equations
k_s	Specific substrate consumption rate		h^{-1}	(1),(2),(4)
k_p	Specific MK-7 production rate		h^{-1}	
$k_{p\text{-}m}$	Maximum specific MK-7 production rate		h^{-1}	(5)
$k_{s\text{-}A}$	Specific substrate consumption rate for glycerol growth	0.030	h^{-1}	
$k_{p\text{-}mA}$	Maximum specific MK-7 production rate for glycerol growth	0.133	h^{-1}	
$k_{s\text{-}B}$	Specific substrate consumption rate for glucose growth	0.062	h^{-1}	
$k_{p\text{-}mB}$	Maximum specific MK-7 production rate for glucose growth	0.179	h^{-1}	
$k_{s\text{-}C}$	Specific substrate consumption rate for glycerol medium	0.054	h^{-1}	
$k_{p\text{-}mC}$	Maximum specific MK-7 production rate for glycerol medium	0.175	h^{-1}	
$k_{s\text{-}D}$	Specific substrate consumption rate for glucose medium	0.059	h^{-1}	
$k_{p\text{-}mD}$	Maximum specific MK-7 production rate for glucose medium	0.120	h^{-1}	
S	Substrate concentration (glycerol or glucose)		g/L	(1),(2),(3),(4)
S_0	Initial substrate concentration			(1),(2), (4)
$S_{0\text{-}A}$	Initial glycerol concentration for glycerol growth	43.2	g/L	
$S_{0\text{-}B}$	Initial glucose concentration for glucose growth	140.3	g/L	
$S_{0\text{-}C}$	Initial glycerol concentration for glycerol medium	45.6	g/L	
$S_{0\text{-}D}$	Initial glucose concentration for glucose medium	128.4	g/L	
P	Product concentration		mg/L	(3),(5)
P_0	Initial product concentrations	0	mg/L	
P_m	Maximum product concentration		mg/L	(5)
$P_{m\text{-}A}$	Maximum product concentration for glycerol growth	12.1	mg/L	
$P_{m\text{-}B}$	Maximum product concentration for glucose growth	18.5	mg/L	
$P_{m\text{-}C}$	Maximum product concentration for glycerol medium	14.7	mg/L	
$P_{m\text{-}D}$	Maximum product concentration for glucose medium	20.5	mg/L	
T	Time		h	(1),(2),(3),(4),(5)
T	Time of maximum specific substrate consumption rate		h	(2),(4)
τ_A	Time of maximum specific substrate consumption rate for glycerol growth	72	h	
τ_B	Time of maximum specific substrate consumption rate for glucose growth	48	h	
τ_C	Time of maximum specific substrate consumption rate for glycerol medium	48	h	
τ_D	Time of maximum specific substrate consumption rate for glucose medium	48	h	
E	Constant	2.710		(2),(4),(5)
Λ	Lag time for MK-7 production		h	(5)
λ_A	Lag time of MK-7 production for glycerol growth	24	h	
λ_B	Lag time of MK-7 production for glucose growth	12	h	
λ_C	Lag time of MK-7 production for glycerol medium	12	h	
λ_D	Lag time of MK-7 production for glucose medium	0	h	
A	First Modified-Leudeking–Piret constant		mg/g	(3),(4)
B	Second Modified-Leudeking–Piret constant		mg/g/h	(3),(4)
α_A	First Modified-Leudeking–Piret constant for glycerol growth	-0.250	mg/g	
β_A	Second Modified-Leudeking–Piret constant for glycerol growth	0.00065	mg/g/h	
α_B	First Modified-Leudeking–Piret constant for glucose growth	-0.088	mg/g	
β_B	Second Modified-Leudeking–Piret constant for glucose growth	0.00041	mg/g/h	
α_C	First Modified-Leudeking–Piret constant for glycerol medium	-0.089	mg/g	
β_C	Second Modified-Leudeking–Piret constant for glycerol medium	0.00301	mg/g/h	
α_D	First Modified-Leudeking–Piret constant for glucose medium	-0.138	mg/g	
β_D	Second Modified-Leudeking–Piret constant for glucose medium	0.00010	mg/g/h	

10.3.7 Modeling for substrate consumption

Bacterial cells utilize the substrate available to them to grow, reproduce, and maintain and besides these, biosynthesize numerous metabolites. The substrate consumption curves, in which substrate concentrations are plotted versus time of

fermentation, usually exhibit sigmoid patterns including a lag phase followed by an exponential phase and finally a stationary phase. Although sigmoid functions such as the logistic equation have been originally developed and modified for modeling growth curves (Zwietering et al., 1990); yet further-modified versions of the Logistic equation have been developed for substrate consumptions (Kargi, 2009):

$$-\frac{dS}{dt} = kS\left(1 - \frac{S}{S_0}\right) \qquad \text{(Eqn. 10.1)}$$

In this fashion, k is the specific substrate consumption rate. Integrating Eqn. 10.1 considering $S_{(t=0)} = S_0$ and if $\tau \gg 0$ then assuming $S_{(t=\tau)} = \frac{S_0}{2}$ and finally $S_{(t=\infty)} = 0$ we will have:

$$S_{(t)} = \frac{S_0}{1+e^{k(t-\tau)}} \qquad \text{(Eqn. 10.2)}$$

In Eqn. 10.2, S_0 is the initial substrate concentration, τ is the time of fermentation when specific substrate consumption rate (k_s) is maximum and t is the time elapsed in fermentation. This integrated form (Eqn. 10.2) is the Logistic equation for substrate consumption independent of biomass concentrations. Such an equation is capable of presenting a sigmoidal curve of substrate (in this case glycerol or glucose) concentrations throughout fermentation period with empirical representation of the lag, log and stationary phases.

10.3.8 Modeling for product production

The product biosynthesis in this study (MK-7) is described by two models. First is the Luedeking–Piret kinetics. In this regard, product biosynthesis rate is considered dependent upon both instantaneous substrate concentration S and substrate consumption rate dS/dt in a linear fashion:

$$\frac{dP}{dt} = \alpha \frac{dS}{dt} + \beta S \qquad \text{(Eqn. 10.3)}$$

Where α and β are empirical constants that vary dependent on fermentation conditions and are determined to best-fit the experimental values.

Thus, a good estimation of the product biosynthesis would be to insert Eqn. 10.1 and Eqn. 10.2 into Eqn. 10.3 and integrate with the assumption that $P_{(t=0)} = P_0 = 0$:

$$P_{(t)} = \frac{\alpha S_0}{1+e^{k(t-\tau)}} - \beta S_0 \left[t - \frac{ln\left(e^{k(t-\tau)}+1\right)}{k} \right] - \frac{\alpha S_0}{e^{-k\tau}+1} + \frac{\beta S_0 ln\left(e^{-k\tau}+1\right)}{k} \qquad \text{(Eqn. 10.4)}$$

The Luedeking–Piret equation was originally developed to explain fermentation kinetics of glucose to lactic acid secretion based on instantaneous rate of bacterial growth and to the bacterial density (Luedeking and Piret, 1959). However, modifying it so that production rate correlates directly to instantaneous substrate consumption rate and substrate concentration was attempted here; since the Luedeking-Piret equation is effective in modeling mixed-metabolite products such as organic acids or in this case MK-7 (Leh and Charles 1989).

Moreover, a modified-Gompertz function that was previously successful in modeling bacterial cellulose was also evaluated in this study as well (Cheng et al. 2010c):

$$P_{(t)} = P_m exp\left\{-exp\left[\frac{k_{m-p}e}{p_m}(\lambda - t) + 1\right]\right\} \qquad \text{(Eqn. 10.5)}$$

Where P_m is the maximum product (MK-7) concentration in each batch, k_{m-p} is the maximum specific production rate in each batch and λ is the lag time for production.

10.4 Results and discussions

Models for glycerol or glucose consumptions and MK-7 production were constructed using the repeated data obtained from four batch fermentation runs under

optimum growth parameters in glycerol-based medium, growth parameters in glucose-based medium, medium components in glycerol-based medium and medium components in glucose-based medium. All models were developed in a way to reflect relevant biological parameters such as specific growth rate, specific production rate, lag time, etc. Using these terms, the models were implemented to fit the experimental values of substrate and product concentrations during the fermentations. In order to evaluate the models, R-squared values, Root-Mean-Square Errors (RMSE) and Mean Absolute Errors (MAE) between modeled values and experimental values were obtained. Moreover, experimental vales were plotted versus the corresponding values from the models and the slope of the best fitted trend-line was obtained.

10.4.1 Substrate consumption models

Modified-logistic model as described in Eqn. 10.2 was used to obtain predicted fits for glycerol and glucose concentrations in the four batches. These values along with the experimental values were plotted versus time (Figure 10.1). As it can be seen from Figure 10.1, the modified-logistic model fitted to the experimental values well ($R^2 > 0.953$) and since this model is a very simple and parsimonious one, unlike previous studies, there was no need to look into more complex models such as the Gompertz model (Cheng et al., 2010c). Also, it is shown in Figure 10.1 that the glycerol consumption in the glycerol growth plot is unlike the other three graphs. In this case, glycerol does not deplete at all (Figure 10.1A), whereas substrate completely depletes in the other cases by the fourth or fifth day of the fermentations (Figure 10.1B, C, and D). It is obvious from Figure 10.1 that the consumption pattern is unique for glycerol growth optimization run, possibly because the medium is much richer in nutrients compared to others (Chapter 6). Thus, $\tau = 72h$ was more proper for the glycerol growth case while $\tau = 48h$ was used for the other cases simply because maximum specific substrate consumption rates kicked in sooner in those cases. Concordantly, the specific substrate consumption rates applied for Figure 10.1A is considerably lower ($k = 0.030$) than the values for Figure 10.1B, C, and D, which were

very close (0.062, 0.054 and 0.059, respectively). Table 10.2 indicates that for glucose consumptions, RMSE (5.1 and 5.1 g/L) and the MAE values (4.1 and 3.5 g/L) are significantly higher than the amounts for glycerol consumptions (2.7, 2.4 and 2.4, 1.8 g/L, respectively); yet, the R^2 values for glycerol consumptions (0.953 and 0.981) are also lower than those of the glucose consumption models (0.991 and 0.989). In other words, although RMSE and MAE values for glycerol consumption model are lower than glucose model, glucose consumptions are modeled more accurately by the models.

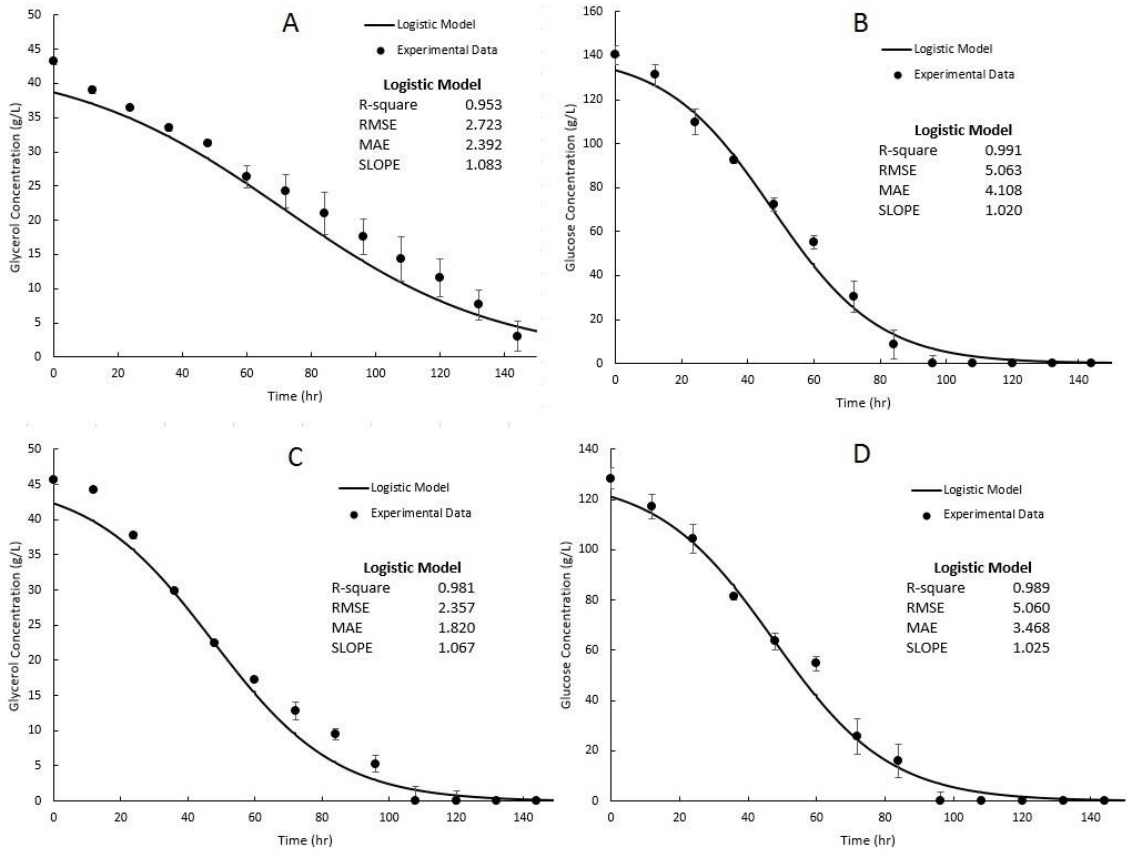

Figure 10.1. Experimental values and fitted Modified-Logistic models for substrate concentrations in batch fermentations for glycerol growth optimization (A), glucose growth optimization (B), glycerol medium optimization (C) and glucose medium optimization (D).

This is simply because the amount of glucose consumed are over or nearly 3 times the amount of glycerol and therefore the RMSE and MAE values are not directly comparable. Nonetheless, all models for substrate consumptions are very accurate and were sufficient to be applied for MK-7 production models.

Table 10.2. Model validations for substrate consumptions.

Model	RMSE (g/L)	MAE (g/L)	R^2	Slope
Modified-Logistic for glycerol growth	2.7	2.4	0.953	1.08
Modified-Logistic for glucose growth	5.1	4.1	0.991	1.02
Modified-Logistic for glycerol medium	2.4	1.8	0.981	1.07
Modified-Logistic for glucose medium	5.1	3.5	0.989	1.03

10.4.2 MK-7 production models

Experimental MK-7 concentrations and the model values from the modified-Luedeking–Piret and modified-Gompertz models for all four batches were plotted as shown in Figure 10.2. As it is depicted in Figure 10.2A, B, and C for glycerol and glucose growth and glycerol medium fermentations, the modified-Gompertz models showed better fit to the experimental values compared to the glucose medium fermentation (Figure 10.2D). The reason is more obvious in Table 10.3 where $R^2 = 0.836$ was obtained for Figure 2D, whereas for others, the R^2 values were significantly higher (0.914, 0.943 and 0.950). The RMSE and MAE and slope comparisons also indicate a significant difference. The RMSE for Figure 10.2D is equal to 2.5 g/L for the modified-Gompertz model but those values for Figure 10.2A, B and C are equal to 1.0, 1.4 and 1.0 g/L respectively. Same as MAE values where 2.0 g/L is compared to 0.9, 1.3 and 0.8 g/L and slope where 1.44 is compared with 0.87, 0.97 and 0.88. The RMSE and MAE values speak the truth this time because there are no differences in the scale of the MK-7 concentration in the fermentations. Such a lack of accuracy for Figure 10.2D is perhaps coming from the low maximum specific production rate and is also evident when the lag time is observed which is equal to zero in this case. It means that the MK-7 is being biosynthesized form the very beginning of the

fermentation, which is simply not true and some lag time always exist, even in biofilm reactors (Ercan and Demirci, 2013a).

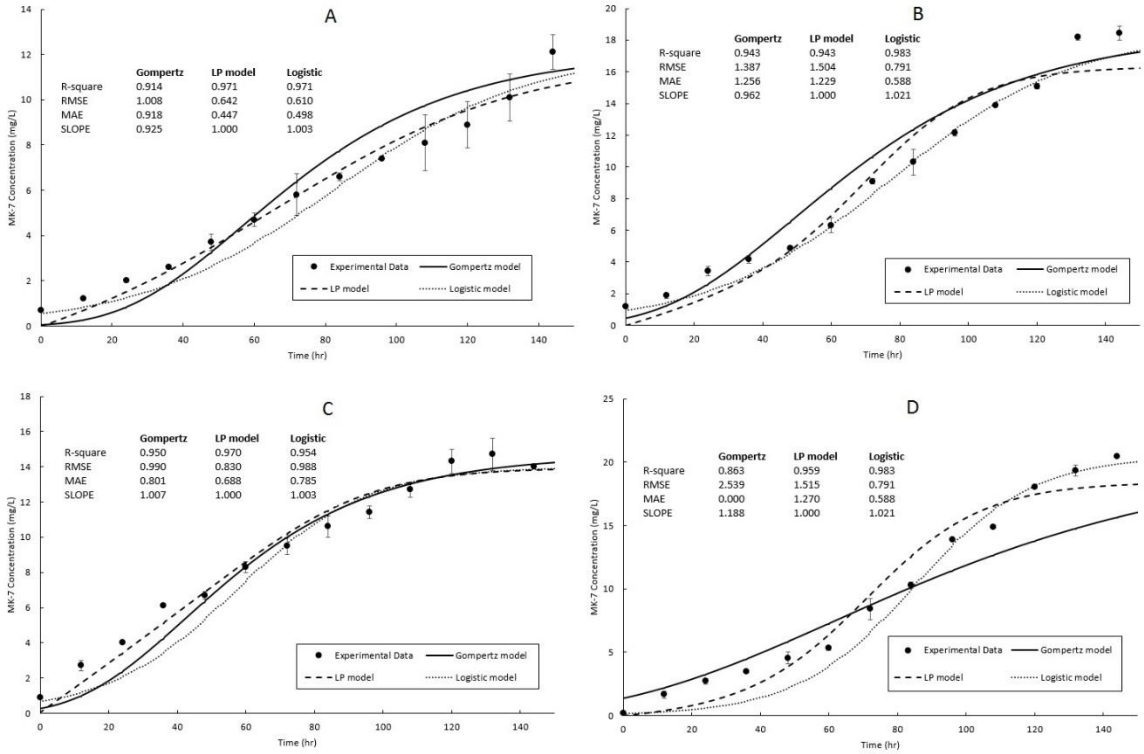

Figure 10.2. Experimental values and fitted Modified logistic, Modified-Gompertz and Modified-Leudeking–Piret models for MK-7 production in batch fermentations for glycerol growth optimization (A), glucose growth optimization (B), glycerol medium optimization (C) and glucose medium optimization (D).

On the other hand, the modified-Luedeking–Piret equation was fair to all four models. Once again, lower RMSE (0.6, 1.5, 0.8, and 1.5 mg/L) and MAE values (0.4, 1.2, 0.7, and 1.3 mg/L) meant higher R^2 values (0.971, 0.943, 0.970, and 0.959), which therefore suggest more accurate models. In general, modified-Luedeking–Piret equation was (in most cases significantly) more accurate than the modified-Gompertz equation in modeling MK-7 production as evident from R^2 values (0.971 vs 0.914, 0.943 vs 0.943, 0.970 vs 0.943 and 0.959 vs 0.863 for modified-Luedeking–Piret vs modified-Gompertz, respectively). This is naturally because the modified-Luedeking–Piret equation and the

Luedeking–Piret equation in general is more competent and even specific to modeling mixed-metabolites such as the MK-7 in this case (Luedeking and Piret 1959). Also, the modified-Luedeking–Piret equation possesses three fitting parameters (α, β and τ) whereas the modified-Gompertz equation only has one (λ). Thus, although modified-Gompertz model is a more parsimonious model, it is notaccurate and therefore effective for modeling MK-7 biosynthesis.

Table 10.3. Validation for MK-7 productions.

Model	RMSE (mg/L)	MAE (mg/L)	R^2	Slope
Modified-Leudeking–Piret for glycerol growth	0.6	0.5	0.971	1.00
Modified-Gompertz for glycerol growth	1.0	0.9	0.914	0.93
Modified-logistic for glycerol growth	0.6	0.5	0.971	1.00
Modified-Leudeking–Piret for glucose growth	1.5	1.2	0.943	1.00
Modified-Gompertz for glucose growth	1.4	1.3	0.943	0.96
Modified-logistic for glucose growth	0.8	0.6	0.983	1.02
Modified-Leudeking–Piret for glycerol medium	0.8	0.7	0.970	1.00
Modified-Gompertz for glycerol medium	1.0	0.8	0.949	1.01
Modified-logistic for glycerol medium	1.0	0.8	0.954	1.00
Modified Leudeking–Piret for glucose medium	1.5	1.3	0.959	1.00
Modified-Gompertz for glucose medium	2.5	2.0	0.863	1.19
Modified-logistic for glucose medium	0.8	0.6	0.983	1.02

10.5 Conclusions

In this study, substrate (glycerol and glucose) consumptions and product biosynthesis (MK-7) in four batch fermentations were modeled. RMSE, MAE, R^2 and slopes for predicted values versus experimental values were obtained to evaluate models. For substrate consumptions, a modified-Logistic equation was implemented and the accuracy of the models were high enough to overrule any needs for more complex models (R^2 values of 0.953, 0.981, 0.991, and 0.989). The modified-Logistic models were inserted in the modified-Luedeking–Piret equation and integrated, then with a modified-Gompertz equation, MK-7 productions were modeled. Results indicated that naturally, the modified-

Luedeking–Piret equation (R^2 values of 0.9705, 0.943, 0.970, and 0.959) was more accurate in modeling the MK-7 production compared to the modified-Gompertz model (R^2 values of 0.914, 0.943, 0.9425, and 0.863). Thus, considering typical MK-7 profiles in batch fermentations, there is no doubt that MK-7 biosynthesis follows a mixed-metabolite pattern in *Bacillus subtilis natto*.

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Chapter 11

Conclusions and future studies

In the past several decades, researches have attempted to enhance the yield of MK-7 production in both liquid and solid state fermentations by various species of bacteria. Among these species, however, *Bacillus subtilis natto* seems to be the most promising microorganism. Studies on media optimization, fed-batch fermentation, and in situ extraction protocols in LSF in conjunction with implementing dynamic and static bioreactors have reported significant improvements in MK-7 production to date. However, there is always a need to enhance the vitamin production further to make it more economical for commercial applications. *B. subtilis natto*'s tendency to form a biofilm along with extracellular nature of MK-7 in *B. subtilis natto* presents the opportunity of utilizing a biofilm reactor for MK-7 production. Thus, the purpose of this study was to enhance MK-7 production efficiency and productivity by utilizing a biofilm reactor.

In the first phase of the study, screening protocols were implemented to find a suitable microbial strain and Plastic Composite Support (PCS) to construct the biofilm reactor. Thus, among the different *Bacillus* strains, 4four were selected to undergo PCS selection tests. Statistical analysis of PCS selection tests indicated that PCS types may not have a significant impact on MK-7 production and can be used interchangeably. However, different strains showed different potentials. Thus, pairwise comparisons of different combination possibilities and optimization targeting maximum MK-7 concentration suggest a combination of a natto derived *B. subtilis natto* strain (NF1) with the PCS type SFY in TSB medium to be the promising combination. The next practical step shall be implementing such viable combination in real-time biofilm reactors to design a production process for possible industrial production of MK-7. Thus, although biofilm reactors using PCS are anticipated to produce less amount of MK-7 compared to static fermentations, but through fermentation optimization, this gap in concentration may be filled. Therefore,

applying biofilm reactors that utilize robust agitation and aeration in liquid state will have none of the scale-up or heat transfer and mass transfer issues associated with current static fermentation strategies.

Then, biofilm reactors were successfully constructed and used to produce MK-7. Using RSM with a general factorial design, temperature, agitation and pH were optimized to achieve the highest MK-7 biosynthesis in biofilm reactors in the glycerol-based medium. Maximum MK-7 concentrations of 12.1 mg/L was achieved under the optimum conditions which was significantly higher that its counterpart in suspended-cell reactors.

Next, the effects of same growth parameters in a glucose-based medium were investigated and optimum conditions for maximum MK-7 biosynthesis were obtained. Glucose consumption and MK-7 biosynthesis took place much more robustly in biofilm reactors as compared to suspended-cell reactors under the same conditions, producing a maximum of 18.5 mg/L of MK-7.

In the following phase, the effects of glucose, yeast extract and casein on MK-7 biosynthesis were investigated in the glucose-based medium using RSM. Optimum conditions were obtained by ANOVA and the model indicated 97% accuracy under the optimum conditions. A maximum concentration of 20.5 mg/L was achieved with media optimization that shows significant improvements compared to suspended-cell reactors or static and shaking bottle fermentations. Since, glucose metabolism was robust and glucose depletion occurred before completion of fermentation cycles in bioreactors, there seemed to be an opportunity for fed-batch fermentation strategies in the future steps where glucose depletion can be addressed for reaching even higher MK-7 concentrations without creating carbon source inhibitions.

As the last optimization phase, using a Box-Behnken design and RSM analysis, effects of glycerol, soytone and yeast extract were investigated on MK-7 biosynthesis in biofilm reactors in the glycerol-based medium. Parsimonious model with $R2 > 0.70$ was obtained to predict the effects at optimum conditions; however, RSM analysis indicated that all three components have severe negative effects at the studied levels in biofilm reactors; unlike shake flasks. Thus, eight different compositions consisted of lower concentrations of soytone and yeast extract concentrations were studied in biofilm reactors

to investigate the negative effects. Results indicated that in a medium composition of 45 g/L glycerol, 5 g/L yeast extracts, 10 g/L soytone and 0.06 g/L K2HPO4, MK-7 concentrations could reach 14.7 mg/L in biofilm reactors (which was 57.4% higher compared to suspended-cell reactors) and glycerol was depleted by the end of the fifth day. Under similar conditions in suspended-cell reactors, MK-7 concentration could not go beyond 9.3 mg/L and glycerol levels were not depleted. This was once again a sure sign how biofilm reactors can improve MK-7 production in LSF strategies.

As carbon source depletion occurred in both glycerol and glucose-based media in *B. subtilis* fermentation in biofilm reactors within 72 h; one chance to increase final MK-7 concentrations was perhaps applying fed-batch carbon source additions. For this purpose, biofilm reactors with glucose and glycerol-based media were supplemented with different concentrations of glycerol and glucose. The high concentration for glucose (150 g/L) and the middle for glycerol (30 g/L) was favorable in 6 day fermentations. These concentrations were implemented in different combinations at 72 and/or144 h and the product was analyzed until the end of the 12th day. Results indicated that in glucose-based medium, double glucose feeding demonstrated MK-7 production of 28.7 mg/L, which is the highest concentrations reported in bioreactors and are quite comparable to the maximum amounts achieved with static fermentations of these strains (32.5 mg/L). This was perhaps the significant step necessary towards the introduction of biofilm reactors as a legitimate replacement for current static fermentation strategies, which are difficult to scale up and associated with serious mass and heat transfer issues.

Next, MK-7 stability and integrity was investigated under different conditions during the drying, storage and ultrasonication steps necessary for analysis and perhaps downstream processing. Results indicated that if the MK-7 produced is quickly dried using forced air flow after extraction, over 95% of it can be preserved. Also, refrigeration at 4°C will be sufficient to preserve the MK-7 product in the original broth for at least 7 days, should the downstream processing or analysis is delayed. Moreover, ultrasonication for 15 minutes seems to be harmless and sufficient prior to analysis. Besides, fermentations in McCartney bottles and culture tubes revealed that the conditions in the McCartney bottles are ideal to explore the maximum potential for MK-7 secretion, which occurred in the

glycerol-rich medium (32.5 mg/L) seconded by TSB medium fortified with 10% glucose (14.6 mg/L). Finally, in culture tubes as expected, the MK-7 concentrations were much less than bottles (Maximum 5.1 mg/L); yet they provided good insight as of how MK-7 secretion is distributed in different zones of the static liquid fermentation. These results in comparison with those achieved in optimized biofilm reactors completed the last pieces of the picture of scaling up the MK-7 fermentation step by step and enlighten the road to maximum preservation of the end product.

In the final phase of this study, substrate (glycerol and glucose) consumptions and product production (MK-7) in the four batch fermentations were modeled. RMSE, MAE, R^2 and slopes for predicted values versus experimental values were obtained to evaluate models. For substrate consumptions, a modified-Logistic equation was implemented and the accuracy of the models were high enough to overrule any needs for more complex models (R^2 values of 0.953, 0.981, 0.991, and 0.989). The modified-Logistic models were inserted in the modified-Luedeking–Piret model and integrated, then with a modified-Gompertz model, MK-7 productions were modeled. Results indicated that naturally, the modified-Luedeking–Piret model (R^2 values of 0.9705, 0.943, 0.970, and 0.959) was more accurate in modeling the MK-7 production compared to the modified-Gompertz model (R^2 values of 0.914, 0.943, 0.9425, and 0.863). Thus, considering typical MK-7 profiles in batch fermentations, there remains no doubt that MK-7 biosynthesis follows a mixed-metabolite pattern in *B. subtilis natto*.

However, this is not the end of the long journey towards improving MK-7 fermentation on an industrial scale in liquid state fermentation in biofilm reactors; it is only the stepping stone. Future studies should focus on numerous possible improvements and investigations:

- Scaling up the bench-top 2-liter biofilm reactors used in this study to multi-hundred liter pilot-scale biofilm reactors and optimizing MK-7 production in them is the next check point. Challenges such as robust aeration or feasible and effective PCS grid formations are the main issues that must be investigated and addressed in those studies.

- To investigate and overcome the oxygen transfer limitations in biofilm reactors, perhaps 40% or higher purity oxygen can substitute air for aerating the reactors. This way, oxygen transfer rates may be improved while aeration and agitation rates are at their maximum operational rates. However, effectiveness and cost analysis of this approach must be studied.
- As vitamin K binding protein (KBF) is a crucial supplement to extracellular MK-7 expression, it is possible that the protein expression is limiting in the process. Thus, by genetically modifying the strain or physiochemical treatments, overexpression of KBF may lead to higher MK-7 secretions, which would definitely be worthy of investigation.
- So far, none of the past studies have attempted co-culturing MK-7 secreting strains. Two or more candidate strains may create synergy in MK-7 secretion and therefore enable higher concentrations.
- Although this study and few past studies indicate undisputable biofilm formation effectiveness on MK-7 biosynthesis; this effectiveness is only measured by comparison with suspended-cell reactors. An alternative comparison may be possible by addition of digestive enzymes (such as trypsin perhaps) to eliminate biofilm on the PCS and then analyzing MK-7 biosynthesis in the reactors; provided that the enzyme specifically targets the biofilm matrices and nothing else. In this way, the amount of MK-7 that may have been entrapped in the biofilm matrices can also be measured.
- Furthermore, continuous fermentation biofilm reactors are still an opportunity to look into and despite the fact that the slow MK-7 biosynthesis poses a threat to its feasibility, it is possible to overcome this hinder in larger pilot-scale biofilm reactors.
- Also, in-situ recovery techniques and optimum and less-costly and more effective downstream processing steps may be untouched and potent fields for future studies.
- Secondary models including temperature, pH and agitation should be studied.

- In the end, comprehensive cost analyses will be essential to evaluate the potential of the liquid state fermentation strategies in biofilm reactors for replacing current static fermentation strategies in industries.

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Curriculum Vitae

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Honors & Awards

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Best Paper Award: 14th Annual College of Engineering Research Symposium, April 4th, 2017
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Silver Medal: Fifth National Student Laboratory Olympiads, (2005) Yazd, Iran.
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