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

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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.

1. Introduction

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

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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 (Mahdinia et al., 2017b). 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 (Mahdinia et al., 2017c), 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.

2. Materials and methods

2.1. Microorganisms and media

Bacillus subtilis natto (NF1) were isolated from commercially available natto by using tryptic soy agar plates as described previously (Mahdinia et al., 2017b). Tryptic Soy Broth (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 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.

2.2. Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA) equipped with 2-L vessels were used for fermentation studies. Sterile 2 M sulfuric acid (EMD) and 2 M 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; Mahdinia et al., 2017c; Izmirlioglu and Demirci, 2016).

2.3. 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 deionized 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).

2.4. Experimental design

Response Surface Methodology (RSM) was used to investigate the effects of temperature (35–45 °C), 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 h before the medium was refreshed for the next set of fermentation experiment. Maximum MK-7 concentration was treated as the sole response (Table 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.

2.5. Analysis

2.5.1. MK-7 concentration

A mixture of n-hexane:2-propanol (2:1, v/v) with 1:4 (aqueous:

Table 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 (°C)	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