Effects of Inoculation Loading and Substrate Bed Thickness on the Production of Menaquinone 7 via Solid State Fermentation

Raja Mahanama, Aydin Berenjian, Andrea Talbot, Ray Biffin, Hubert Regtop, Fariba Dehghani, John Kavanagh

Abstract— Natto, the richest known source of menaquinone 7 (MK7), is traditionally produced by the solid state fermentation of Bacillus subtilis natto on cooked soy beans. In this work we used a mixture of nixtamalized corn grits and soy protein granules, to assess the effect of inoculation loading and critical bed thickness on MK7 production. The highest concentration of MK7 found was 46.98 mg/kg dry weight which, corresponded to the lowest inoculation loading of 8.4 logCFU/g. The overall trend shows that increasing spore loading adversely affects the MK7 production. This effect may be due to the amount of available oxygen and nutrients per bacteria in the first day of inoculation. Increasing CFU by 250 times can substantially decrease the amount of nutrients that were available for bacteria to grow on the surface of solid samples. Similarly, the production of MK7 was dramatically elevated by decreasing the bed thickness. When the bed thickness was changed from 2 mm to 10 mm, the amount of MK7 was decreased fivefold underlining the significance of bed thickness to promote oxygen diffusion rate for microbial growth, heat transfer and MK7 production.

Index Terms— Bacillus subtilis natto, **SSF, MK7**

I. Introduction

Vitamin K naturally exists in two major forms: vitamin K_1 and K_2 [1]. Vitamin K_1 is widely distributed in green and leafy vegetables, while vitamin K_2 is produced by bacteria during fermentation or contained in animal derived foods [2]. The predominant dietary form of vitamin K in most parts of the world is vitamin K_1. However, *Natto* is the major form of vitamin K_2 consumed in Japan [2] which is the richest known MK7 dietary supplement known to date (15.4-23.1 mg//kg) [3]. *Natto* is produced via Solid State Fermentation (SSF) for centuries where recent studies have shown that MK7 consumption in the form of *natto* substantially reduces the risk of bone fractures and cardiovascular disorders [4-10].

SSF can be of special interest in those processes where the crude fermented product may be used directly as food supplement like *natto*. SSF is best defined as the cultivation of microorganisms on solid substrates deficient in free water; however, the substrate must possess enough moisture to support growth and metabolism of microorganisms [11]. SSF offers a viable alternative to the conventional Liquid State Fermentation (LSF) which requires less pre-processing energy, low waste water output and improved product recovery than LSF [12]. The major factors that affect microbial synthesis in a SSF system include: selection of a suitable substrate and microorganism, substrate pre-treatment, particle size of the substrate; water activity (a_w), size and type of the inoculum, temperature and fermentation time [13].

In SSF inoculum must be distributed homogeneously and must be high enough to assure predominance of the strain. The microbial (fungal/bacterial) particles which initially will be on the outer surface of the substrate particles, will slowly grow, multiply and penetrate into the macro and micro pores of the solid [14]. They consume the available energy sources and secreted enzymes can also break down the starch and cellulose in the substrate to provide energy. Most of the previous studies observed a strong influence of high inoculum size on the production microbial metabolites [15-21].

The major challenge in the scale up of SSF is the transfer of O_2 into the substrate bed to obtain high cell densities where critical bed thickness plays a prominent role. In SSF, heat removal is also a major concern where it is more difficult to remove the waste metabolic heat from a bed of solids while preventing H_2O being evaporated [14]. In a substrate bed of a tray type fermenter; transport phenomena are of crucial importance in governing the concentration and temperature gradients.

The effect of temperature, incubation time, initial moisture, substrate mix and amylase loading has been optimized previously by same authors [22] on the production of MK7, and this continuation will study the influence of inoculum size and bed thickness on the biosynthesis of MK7 by *Bacillus subtilis* in solid culture.

II. Materials and Methods

Microorganism

Strain *Bacillus subtilis natto* isolated from commercially available *natto* after screening different types for highest MK7 producing strain as described in [23]. Bacterial cells were cultivated in a liquid culture constituting 0.5 %

Manuscript received June 28, 2011; revised July 07, 2011. This work was financially supported by the Australian Research Council and Agricure Scientific Organics through the ARC Linkage Project (LP100100347). Raja Mahanama is with the School of Chemical and Biomolecular Engineering Department, University of Sydney, NSW, Australia (corresponding author e-mail: raja.mahanama@sydney.edu.au).

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ISBN: 978-988-19251-7-6
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

Peptone 0.5 % Glucose 0.05 % Yeast Extract for 5 days before streaking on tryptic soy agar plates for asceptic spore genesis. Plates were scrapped after 5 days and harvested cells were suspended in a 0.9 % NaCl solution. The spore suspension was kept in a heated water bath (80 ^{o}C, 30 mins) in order to kill the residual vegetative cells, centrifuged at 3000 rpm for 10 mins to remove the cell debris, diluted using 0.9 % NaCl solution to obtain the standard spore solution (10.8±0.04 $\log_{10}$CFU/mL) of 0.31 Optical Density (OD) where wavelength (λ) =660 nm (Varian 50 scan UV-Visible spectrophotometer, USA).

Materials

Pure MK7 (99.3%) was purchased from ChromaDex (USA). Methanol, n-Hexane and 2-proponol were obtained from Merck (USA). The substrates, soy protein granules and nixtamalized corn grits (hominy) were kindly donated by Agricure Pty Ltd (Australia), and were of agricultural grade.

MK7 fermentation

The substrates were autoclaved in absence of free water for 20 minutes at 121 ^{o}C. All samples were soaked using sterilized water (50% moisture) for 24 hrs at 4 ^{o}C and inoculated with a spore loading of (8.4 ± 0.04) logCFU/g. All samples were inoculated with a spore loading of (8.4 ± 0.04) logCFU/g and final moisture of 70 %. Fermentations were carried out in square Petri dishes (120×120 ×17 mm, Greiner, Germany) and incubated at 37 ^{o}C inside an incubator in duplicate, where the relative humidity (RH) was maintained at 90–95%. The Relative humidity, temperature and dew point were measured throughout the incubation period using a data logger (LASCAR Electronics, UK). The production of MK7 was checked at the given day of fermentation via organic solvent extraction. Each sample was sacrificed during the each fermentation day of interest; which enabled the extraction of whole media directly to avoid error in sampling.

MK7 extraction and analysis

MK7 was extracted from the fermentation media using 12 mL *2-propanol: n-hexane* (v:v 1:2) [23]. In each run the mixture was vigorously shaken with a vortex mixer for 2 minutes then centrifuged at 6000 g/min for 10 minutes to separate two phases. The organic phase was then separated; filtered through 0.45 µm syringe driven filter (Whatman, UK) to obtain an organic solution free of any solid material, free of culture before evaporated under vacuum to recover extracted MK7. High performance liquid chromatography HP 1050 (Hewlett-Packard, USA) equipped with a photon diode array UV detector and XDB C8 ZORBAX column (5µm, 150 × 4.6 mm, Agilent, USA) was used at 40 °C for the analysis of MK7. Methanol 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 LC-MS system (LCMS-2010EV, Shimadzu, Kyoto) were used to confirm the structure of MK7. Atmospheric pressure chemical ionization (APCI) ion source was used for the ionization in negative ion mode and the interface voltage was 2KV. The interface temperature was held at 250 $^{\circ}$C, the heating block at 200 $^{\circ}$C and the CDL at 230 $^{\circ}$C. Nitrogen was used as a nebulizing gas and was delivered at a flow rate of 2 L/min. For the structural elucidation of MK-7 variants the mass spectrometer was operated in scan mode covering the mass range of 50-1000 m/z. The CDL voltage was 0V, Q-array DC voltage at -5V and RF was set at 150V. Compound UV spectra were acquired by collecting the entire wavelength range from 200-400 nm. The optical slit was fixed at 1.2 nm and the reference wavelength was set at 360 nm ± 20 nm bandwidth.

III. Results and Discussion

SSF is more competitive process, and it may be a viable option for the industrial production of microbial metabolites [24]. Success in achieving higher MK7 concentrations by SSF [22] encouraged us to further optimize selected process conditions prior to commercial production. In the present work, the study was undertaken to optimize the critical bed thickness and inoculum size in SSF for the production of MK7. Different bed thicknesses (2 mm-10 mm) were used for MK7 production in SSF. The results (Fig. 1) show that the MK7 concentration was maximal (110 mg/kg) in the culture comprised of 2 mm bed thickness. However, the MK7 concentrations were diminished with increasing bed thickness producing the lowest MK7 concentration of 21 mg/kg at the highest bed thickness of 10 mm. Reducing thickness resulted in increased surface area showed larger variations where reducing thickness from 10 mm to 2 mm increased MK7 concentration 1.2 to 4.8 kg/m^2. It has been reported that [14] at larger bed thicknesses, the oxygen concentration at the bottom of the bed falls to zero within first 24 hrs of fermentation depending on the height of the bed which leads not only to inefficient use of the reactor but also to undesirable situation like anaerobiosis and cell lysis [14]. Thus, for the bed of 8 cm thickness, oxygen concentration at the bottom of the tray falls to zero at 21.5 h itself. Mitchell *et al.,* (2003) also showed bed heights greater than critical bed height O_2 limitation occurring in deeper regions of bed [25]. Simulation studies show that with the progress of fermentation, the oxygen concentration falls to zero at some interior location in the substrate bed, resulting in a zone of zero oxygen concentration within the bioreactor. Studies have confirmed that scale-up cannot be achieved by increasing the bed height in the tray because this quickly leads to overheating problems [26], and scale-up can be achieved only by increasing the area of trays or by using more trays [27].

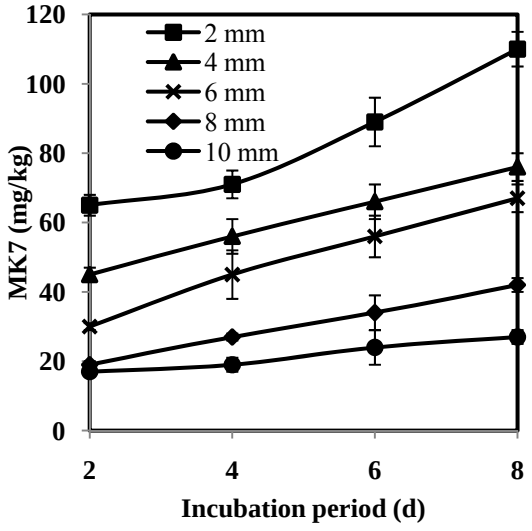

Fig. 1. MK7 concentration in different bed heights

ISBN: 978-988-19251-7-6
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

The effect of the inoculum size on MK7 production was also studied by adding different initial spore loadings at inoculation (8.4-10.8 logCFU/g) and fermentation was carried out for 6 days. The moisture content, relative humidity and incubation temperature were kept at constant levels.

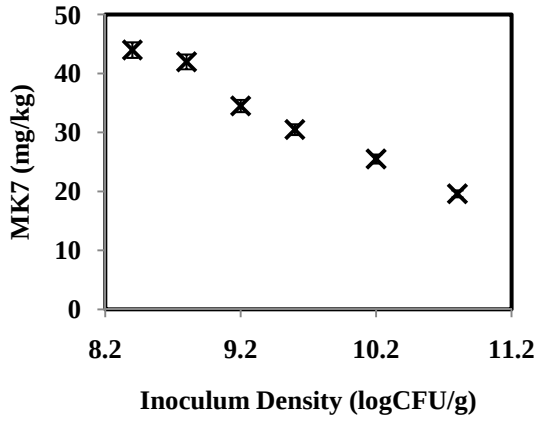

Fig. 2. MK7 concentration in different spore loadings

As it is presented in Figure 2 the highest concentration found was 46.98±3.1 mg/kg dry weight which, corresponded with the lowest inoculation loading 8.4 logCFU/g. High inoculum levels are inhibitory in nature where the overall trend shows that an increased spore loading adversely affect the MK7 production. This maybe correlated to the amount of available oxygen and nutrients per bacteria in the first day of inoculation; increasing CFU to 250 times can substantially decrease the amount of nutrients that were available for bacteria to grow on the surface of solid samples. This result is in agreement with the reports [28-31]. Mudgetti *et al*. 1992 reports where higher inoculum than optimum may produce too much biomass and may deplete the nutrients necessary for microbial metabolite production [32].

IV. Conclusion

The effect of inoculation loading and bed thickness on the production of MK7 was assessed using mix of corn and soy solid substrates. Low inoculum levels were favoured in SSF; where the uppermost MK7 concentrations were obtained at lowest bed thickness; due to the fact that elevated rate of biochemical processes. The uppermost concentration of lowest bed thickness fermentation was averagely 5 times higher than commercially available *natto*. When the height of the substrate be increases beyond the critical height increasing proportion of the bed will undergo oxygen starvation resulting in low MK7 yields. SSF process technique might provide a better choice for MK7 production than LSF, which may generate valuable by-products and significantly enhance the economies of the process.

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ISBN: 978-988-19251-7-6
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

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ISBN: 978-988-19251-7-6
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)