ABSTRACT

The Japanese fermented food *Natto* contains menaquinone 7 (MK7), which is known to increase bone mineral density and reduce bone fractures. *Natto* is traditionally produced via the solid state fermentation of soy beans by *Bacillus subtilis natto*. The aim of this study was to investigate the effects of static and dynamic solid state fermentation (SSF) on the production of MK7 using polenta (milled corn substrate), nixtamalized corn grits, semolina (milled wheat substrate) and soy protein granules. In addition, the effect of initial moisture content on MK7 concentration was measured for both dynamic and static SSF and all substrates. During the experiment the following process parameters were kept constant; incubation temperature (37 °C), substrate wet weight (3 g), incubation time (3 days) and relative humidity (~85 %).

Our initial hypothesis was that conducting dynamic fermentation should increase the production of MK7 due to enhancing the rate of microbial growth via reducing spatial temperature and moisture gradients, yielding better homogeneity. However, average MK7 yield decreased by 62.7 % (p < 0.05) when using dynamic fermentation. This result may be due to substrate lump formation, which was observed inside the bioreactor particularly at higher initial moisture levels, resulting in decreased surface area per unit volume. This reduces nutrient and oxygen availability for microbial consumption.

MK7 concentration increased with initial moisture levels in static fermentation where the highest MK7 concentration was ~20% greater than commercial *natto*. The highest concentrations 28.26±0.11 mg/kg and 27.80±0.06 mg/kg were obtained using soy protein granules at uppermost moisture levels, 65 % and 70 %, respectively; owing to the fact that elevated rate of biochemical process due to increase in solubility of nutrients of the substrate and high degree of swelling. The highest concentration obtained by dynamic fermentation was 5.3±0.54 mg/kg at 60 % moisture. This is ~81% less than that of highest MK7 yield obtained via static fermentation.
INTRODUCTION

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

SSF is best defined as the cultivation of microorganisms on solid substrates devoid or deficient in free water; however, the substrate must possess enough moisture to support growth and metabolism of microorganism (Pandey, 2003). SSF can offer a viable alternative to the conventional liquid state fermentation (LSF) as it may require less pre-processing energy, produce less waste water and have improved product recovery compared to LSF (Uyar and Baysal, 2004). A resurgence of interest has been witnessed in SSF processes in the last decade for the production of microbial metabolites such as fine chemicals, antibiotics and commodity enzymes which had waned with the advances in LSF during the 20th century (Pandey and Larroche, 2008, Mitchell et al., 2006). The complexity of SSF scale up, lack of devices to measure relevant operating variables inside the reactor (i.e. pH, DO, \(a_w\), biomass) and difficulty in metabolic heat removal are factors that impede the technological development of SSF.

The major factors that affect microbial synthesis in a SSF system include: selection of a suitable substrate and microorganism, substrate pre-treatment, particle size, water activity (\(a_w\)), size and type of the inoculum, temperature and fermentation time (Pandey et al., 1999). The selection of a substrate for MK7 production in a SSF process mainly depends upon cost and availability, and thus may involve screening of several solid substrates. It is critical to conduct screening of several solid substrates in a SSF due to the effect of substrate that supplies the nutrients to the microbial culture as well as serves as an anchorage for the cells.

SSF can be of special interest in those processes where the crude fermented product may be used directly as food supplement like Japanese fermented food natto rich in menaquinone 7 (MK7) (15.4 - 23.1 mg/kg)(Sakano et al., 1988). Recently research has demonstrated that MK7 may reduce the risk of bone fractures (Truong and Booth, 2011) and cardiovascular disorders (Gast et al., 2009). Menaquinone 7 is a nutritionally interesting long chain menaquinone (Schurgers et al., 2008) which belongs to the vitamin K group was discovered more than 75 years ago as an antihemorrhagic factor capable of correcting dietarily induced bleeding disorders in chickens (Dam, 1935).
Vitamin K is classified as Vitamin $\text{K}_1$ (Phylloquinone) and Vitamin $\text{K}_2$ (Menaquinone) where all the vitamers have a methylated naphthoquinone ring structure, and vary in the aliphatic side chain attached at the 3-position (Figure. 1) (Vermeer and Schurgers, 2000). Phylloquinone is commonly found in green plants where menaquinones are substances synthesized by bacteria (Gijsbers et al., 1996). All K vitamins have a similar function but differ in their pharmacokinetic behaviours and hence their physiological significance. Human intake of vitamin $\text{K}_1$ from green vegetables accounts for about 90% of total vitamin K consumed but absorption varies from 5% to 15%. Long chain menaquinones constitute only 10% of human intake but absorption is closer to 90%, due in part to a longer biological half life (Schurgers et al., 2008). The postulated therapeutic doses of 3mg/day (Morikawa et al., 2011) mean that dietary vitamin K sources such as broccoli, cheese, meat and even natto are not sufficient for therapeutic use.

The research herein was carried as a step towards the industrial production of supplementary MK7 by mimicking the solid state fermentation conditions of natto in contrast to the majority of published research that has been carried out in LSF.

**MATERIALS AND METHODS**

**Microorganism and inoculum preparation**

Strain *Bacillus subtilis natto* was isolated from commercially available natto after screening different types for highest MK7 producing strain as described in (Mahanama et al., 2011). Bacterial cells were cultivated in a liquid culture constituting 0.5 % 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 °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 logCFU/mL) of 0.31 Optical Density (OD) where wavelength ($\lambda$) $=660$ nm (Varian 50 scan UV-Visible spectrophotometer, USA).
Materials

Pure MK7 (99.3%) was purchased from ChromaDex (USA). Methanol, n-Hexane and 2-propanol were obtained from Merck (USA). Polenta (milled corn substrate) and Semolina (milled wheat substrate) were purchased from a local grocery store. The substrates, soy protein granules and nixtamalized corn grits (hominy) were of agricultural grade.

Particle size analysis

The dry substrate was sieved through standard mesh sieves to determine the particle size distribution in the substrate. Particles from nine individual mesh sizes were used (0.15 mm-2 mm) and results are reported as cumulative particle size by mass.

Substrate preparation and fermentation

The substrates were autoclaved in absence of free water for 20 minutes at 121 °C. All samples contained 3 g of wet substrate inoculated with a spore loading of $(8.4 \pm 0.04)$ logCFU/g. The inoculum was diluted using sterilized water in order to moisten the substrates to different initial moisture levels. Dynamic fermentation was carried out in 30 mL amber coloured Boston glass bottles on rollers (Stuart STR6D, USA) equipped with cotton plug, whereas static fermentation was carried out in Petri Dishes (100mm x 15mm). The rotary bottles were rotated at 5 revolutions per minute.

The samples were incubated at 37 °C inside an incubator in duplicate, where 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 day three 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.

Menaquinone 7 extraction and determination

MK7 was extracted from the fermentation media using 12 mL 2-propanol: n-hexane (v:v 1:2) Mahanama et al., 2011). 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 evaporate 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.

Liquid chromatography and mass spectroscopy (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 Nitrogen was used as a nebulizing gas. For the structural elucidation of MK7 variants the mass spectrometer was operated in scan mode covering the mass range of 50-1000 m/z.
RESULTS AND DISCUSSION

A detailed study of MK7 production in SSF using different substrates of dissimilar particle sizes was carried out to characterize the process. The goal of this study was to compare static vs. dynamic laboratory scale fermentation models and determine conditions suitable for scale-up. Four different solid substrates were studied to determine if these could be used to formulate a commercially attractive substrate. The results of the comparative study under identical conditions are depicted in Figure 2. The data indicate significant differences (p< 0.05) in productivities using different fermentation methods. In fact, the MK7 concentrations were higher in static fermentation conditions at all the initial moisture levels as compared to those in the dynamic fermentation.

The uppermost MK7 concentrations obtained were 28.26±0.11 mg/kg and 24.1±0.97 mg/kg using soy protein granules and nixtamalized corn grits, respectively (Figure 2). These yields obtained in 3 days of fermentation are 1.2-1.25 times higher than commercially available *natto*. Shorter fermentation time has been favoured in terms of industrial production as minimum number of days deemed to reduce the operating costs of the process. Polenta did not show excellent results in both fermentation modes, with a recorded highest yield of 3.24±1.11 mg/kg. The behavior of MK7 concentration in dynamic fermentation increased with ascending moisture levels up to 45-60 % (Figure 2) for all different substrates then decreased subsequently. The highest MK7 concentration obtained in dynamic fermentation was 5.3±0.54 mg/kg at 60% initial moisture using soy protein granules as the substrate. This is more than 5 fold reduction when compared to the MK7 yield obtained via static fermentation.

Polenta gave a very poor yield of MK7 compared with nixtamalized corn grits. The difference may relate to the production process (Mahanama *et al.*, 2011) where the grains are processed via nixtamalization (alkali cooking). This process increases the bioavailability of nutrients and proteins, thereby raising the nutritional value of the grain (Wall *et al.*, 1971) which may enhance the MK7 production. Whilst, nixtamalized corn grits and polenta are similar based on mean particle size, their size distributions are quite different with the nixtamalized grits having a much broader distribution see Figure 3. This may in turn affect packing/bulk density. Packing density is significant to SSF as it has a significant impact on process yield. An increase in packing density causes a reduction in the void space between particles and a concomitant reduction in the area of exchange with the surrounding atmosphere (Barrios-Gonzalez *et al.*, 1993). The bulk density of the substrates was measured, for polenta it is ~ 740 kg/m$^3$ which is slightly higher than that of nixtamalized corn grits ~ 730 kg/m$^3$. Hence whilst polenta would have less oxygen transfer than the corn grits, the difference in MK7 concentration would seem more likely due to nutrient availability.

SSF processes are distinct from SLF culturing, since microbial growth and product formation occurs at or near the surface of the solid substrate particles having low moisture contents (Pandey *et al.*, 1999). Thus, it is crucial to provide optimized water content, and control the water activity ($a_w$) of the fermenting substrate, as the availability of water in lower or higher concentrations affects microbial activity adversely. Results and visual observation indicated the significance of moisture content of solid substrate, the lower moisture level dries culture and decreases the growth rate, subsequently drop the MK7 production. According to Lonsane *et al.*, 1992 only limited water is used in SSF, but water exhibit profound effects on the physicochemical properties of solids, which in turn affects process productivities. There is a direct correlation between MK7 production and moisture level; high moisture levels favoured MK7 production. The production of MK7 was enhanced for all solid substrates with the initial moisture level of 70% when using static fermentations.
Fig. 2: MK7 concentration after 3 days of SSF in 4 different substrates (a) nixtamalized corn grits, (b) soy protein granules, (c) semolina and (d) polenta. Solid squares are the static fermentations, solid circles are dynamic fermentations.

The highest concentration obtained by dynamic fermentation was 5.3±0.54 mg/kg at 60% moisture which was ~80% less than that achieved using static fermentation. Sato et al. 2001 observed similar results in production of MK7 using a liquid medium that production was higher in stationary system compared with using agitation. The reduction of yield in dynamic mode can be attributed to aeration and mixing rate which disintegrate biofilm and microorganism’s colony. The effect of rotational speed on SSF performance has received most attention; in particular, the extent of the effect of shear caused by rotation is controversial (Stuart et al., 1999).

Smaller particle sizes and narrow distribution of particles showed a detrimental effect on MK7 production (Figure. 3); where polenta and semolina resulted in lowest MK7 yields. Maximum MK7 productivities were obtained in substrates comprising of mixture of large and small particles. The low yield of MK7 in dynamic fermentation may be due to particle agglomerations; which were dramatic in high moisture levels and descending particle sizes. Small particle size may increase lump formation and this might be one reason why semolina, which comprised with the finest particles (Figure. 3), showed more than a ~ 87% reduction in MK7 concentration (Figure. 2) obtained via dynamic fermentation. The magnitude of the reduction was decreased when using corn grits.
grits and soy granules where both showed broader range of PSD’s varying from 150 μm to 2 mm.

![Graphs showing cumulative particle size distribution in different solid substrates](image)

**Fig. 3:** Initial cumulative particle size distribution in different solid substrates: 4 different substrates (a) nixtamalized corn grits, (b) soy protein granules, (c) semolina and (d) polenta

As particle size decreased, there was lower surface area to volume ratio and void space between particles, causing adverse effects to the SSF performance (Saw et al., 2011); however, as particle size increase the air may tend to follow preferential routes in a bed of larger particles (the phenomenon of channelling). (Mitchell et al., 2000). Nevertheless the combination of small and large particles sizes may correlate to optimized nutrient availability and oxygen transfer. Substrate properties can be quite important in affecting how a SSF bioreactor performs; especially the size and shape of the substrate particles, along with the manner in which the bed is packed. This factor
determines the sizes of the inter-particle spaces and the degree of continuity between them (Mitchell et al., 2006).

The tray type bioreactors were scaled up for manufacturing secondary metabolites in 20th century (Lockwood, 1979), this technique requires the allocation of considerable work space and labour for large scale production (Krishna, 2005). The bed height of a tray reactor is limited due to heat and mass transfer limitations and heat transfer gradients (Rajagopalan and Modak, 1995). In contrast, rotary reactors deemed to have complex operational system, low fill volume (less than 30% typically) and shear in the substrate bed (Yang, 2007).

The maximization rate of the product formation and yield in the bioreactor is part of the production optimization process. Many factors may affect the bioreactor performance such as the amount of substrate, moisture content, aeration and temperature regulation.

The development of a simpler bioreactor, with low cost, higher substrate usage capacity and shorter cultivation period could increase the bulk MK7 production and commercialization. But further investigations should be carried out on lab scale reproducibility of MK7 production which may affected by phenomenon such as particle agglomeration and substrate packing density prior to scaling up.

CONCLUSION

Both static and dynamic solid state fermentation were assessed using different solid substrates and various moisture levels. High moisture levels were favoured in static fermentation; where the uppermost MK7 concentrations were obtained at highest initial moisture level using soy protein granules. Soy protein granules and nixtamalized corn grits showed the best results in solid state fermentation in static mode. MK7 concentration was significantly decreased when using dynamic fermentation. The uppermost concentrations achieved by static fermentation were 20% higher than commercially available natto. SSF might provide a better choice for MK7 production than LSF; however, the operational difficulties of large scale flat bed SSF will need to be overcome. The selection of a suitable substrate is a critical factor for a SSF process since it is not only nutrient material for growth of microorganisms, but also the supporter for microorganisms.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Australian Research Council through the ARC Linkage Project (LP100100347). LC-MS analysis was performed by Peter Valtchev, School of Chemical and Biomolecular Engineering, The University of Sydney, NSW, Australia. The contribution, guidance and technical assistance provided by Agricure Scientific Pty Ltd, Braemer, Australia is highly acknowledged.
REFERENCES


**Brief Biography of Presenter**

Raja Mahanama is currently a Masters by research student in the school of chemical and bio-molecular engineering at the University of Sydney. He pursued his Masters of Engineering (Sustainable processing) in the University Sydney and B.Sc (Hons) in Chemical and process engineering at The University of Moratuwa Sri Lanka. Raja is currently working on new fermentation and purification technologies for menaquinone 7 production.