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Advances in Menaquinone-7 Production by *Bacillus subtilis Natto*: Fed-Batch Glycerol Addition

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ABSTRACT

Menaquinone-7 was produced by fermentation mainly using *Bacillus subtilis* species. Due to the low concentration of menaquinone-7 in fermentation processes and the associated expense of recovering the menaquinone-7, it was desirable to improve the fermentation concentration. The aim of this study was to determine the effect of fed-batch glycerol addition in the production of menaquinone-7 during the fermentation of *Bacillus subtilis natto* in both small (25-mL) and bench scale (3-L) fermenters. Liquid phase fermentation was carried out aerobically at 40°C for a period of 6 days. The pH, bacterial growth, glycerol and menaquinone-7 concentrations were measured every day. Maximum menaquinone-7 was produced when 2% (w/v) glycerol was added to the fermentation media in the second day (48 h) of fermentation. The results were demonstrated 40% increase of menaquinone-7 concentration compared to batch culture. This study provides valuable data for further optimization and scale-up the menaquinone-7 fermentation process.

Key words: Menaquinone-7, glycerol, fed-batch, fermentation, *Bacillus subtilis natto*

1. INTRODUCTION

Menaquinone-7 (MK-7) a highly valuable member of the vitamin K family has a significant effect on preventing osteoporosis and cardiovascular diseases besides its positive effects on blood coagulation (Gast *et al*., 2009; Schurgers *et al*., 2007; Yamaguchi *et al*., 1999). Due to the increasing number of patients with osteoporosis and cardiovascular diseases researchers seek an alternative approach to preventing these health complications. The likely therapeutic dose of MK-7 for treatment of osteoporosis and cardiovascular diseases from MK-7 rich dietary sources (i.e., blue cheese, meat and fermented soybean) would require the consumption of impractically large quantities (Howard and Payne 2006). It is, therefore, desirable to develop a rich source of MK-7 as a dietary supplement.

Bacillus subtilis is used for production of many value added products including MK-7 (Berenjian *et al*., 2011; Jazeh *et al*., 2012). The results of previous studies demonstrate that glycerol is the most effective carbon source for enhancing MK-7 production (Berenjian *et al*., 2011; Sato *et al*., 2001). Menaquinone is produced from a complex series of pathways. The pathway for isoprene side chain and quinone skeleton (1,4-naphthoquinone) production relies on the presence of carbon sources such as glucose, fructose and glycerol in the fermentation media (Sonenshein *et al*., 2002). The pathway for the production of shikimate is based on conversion of carbon sources into menaquinone by condensation of phosphoenolpyruvate and erythrose-4-phosphate. Catabolite suppression has been observed in which in the presence of a rapidly metabolizable carbon source, greatly

reduces the expression of genes for the transport and metabolism of other carbon energy sources (Fisher and Sonenshein, 1991). For *Bacillus*, rapidly metabolizable carbon sources such as glycerol clearly alleviate the expression of enzymes required for utilization of other carbon sources (Sonenshein *et al*., 2002). The current challenge for MK-7 fermentation involves increasing the yield of fermentation to make large scale production viable. Optimization of substrate feeding strategy showed a significant impact on enhancing the formation of high value products (Tian *et al*., 2010). The glycerol feeding strategy may enhance the MK-7 biosynthesis. The aim of this study was to assess the effect of fed-batch glycerol addition to MK-7 production.

2. MATERIALS AND METHODS

2.1. Microorganism and Inoculum Preparation

Bacillus subtilis natto (Berenjian *et al*., 2011) was cultivated in the media composed of 0.5% (w/v) peptone, 0.05% (w/v) yeast extract and 0.5% (w/v) glucose. Cells were scrapped from tryptic soy agar plates after 5 days and harvested cells were suspended in a 0.9% (w/v) sodium chloride solution. The spore suspension was kept in a water bath at 80°C for 30 min, centrifuged at 3000 rpm for 10 min to remove the cell debris and diluted with 0.9% (w/v) sodium chloride solution to obtain the solution of $5.2\pm0.5\times10^{10}$ spores/mL.

2.2. Chemicals

Methanol, 2-propanol, *n*-hexane and dichloromethane were purchased from Sigma-Aldrich (Sigma-Aldrich Co., USA). Glycerol and K_2HPO_4 were supplied from a Chem-Supply (Chem-Supply Co., Australia). Pure MK-7 (99.3%) was purchased from ChromaDex (ChromaDex Co., USA). Soy peptone and yeast extract were purchased from Oxoid (Oxoid Co., UK) and BD (Becton-Dickinson Co., USA), respectively.

2.3. Fermentation Process

The initial fermentation media were comprised of 5% (w/v) yeast extract, 18.9% (w/v) soy peptone; 5% (w/v) glycerol and 0.06% (w/v) K_2HPO_4. *Bacillus subtilis natto* standard spore solution was added to the fermentation media using an inoculum size of 2% (v/v). Fermentation was aerobically conducted in round bottles (25-mL) at 40°C for the period of 6 days (Berenjian *et al*., 2011). The amount of 1 and 3% (w/v) glycerol was used to study the optimum glycerol addition time (day); concentrations of 1-8% (w/v) glycerol were used to study the most favorable concentration on MK-7 biosynthesis. Cell density, glycerol concentration and pH were also measured accordingly. Additionally, a 3 liter fermenter (BioFlo/CelliGen 115, New Brunswick Scientific Co., USA) was used to validate the optimized conditions acquired from the small scale and further investigate the effect of glycerol addition on MK-7 production. Temperature, agitation and aeration rate were maintained at 40°C, 50 rpm and 2vvm, respectively. All results were reported as mean ± Standard Deviation (SD) of three measurements. One-way Analysis Of Variance (ANOVA) with post-hoc mean comparison with Tukey-test was used to evaluate the experimental data with the statistical significance level of $p<0.05$.

2.4. MK-7 Extraction and Determination

MK-7 was extracted from the fermentation media with the addition of n-hexane:2-propanol in the following ratios (2:1, v/v) and 1:4 (liquid:organic, v/v). High performance Liquid Chromatography (HPLC) was performed to measure the concentration of MK-7. The HP 1050 (Hewlett-Packard, USA) with a photon diode array UV detector and C_{18} Gemini column (5 µm, 250×4.6 mm, Phenomenex, USA) was operated at 40°C for measuring MK-7 concentration. Mobile phase consisted of methanol:dichloromethane (9:1, v/v) with the flow rate of 1 mL min^{-1} (Mahanama *et al*., 2011; Berenjian *et al*., 2011).

2.5. Glycerol Measurement

The concentration of glycerol was determined enzymatically using free glycerol determination kit (Sigma-Aldrich Co., USA) following its own procedure. Briefly, 800 µL of the glycerol free reagent was inoculated with 10 µL of fermentation media and then incubated for 5 min at 37°C. The UV-absorbance of resulting solution was measured at 540 nm using the absorbance of water as the reference.

2.6. Cell Density Measurement

The optical density of each solution was measured at 600 nm with a UV-spectrophotometer (Cary Co., USA) to measure cell density.

3. RESULTS AND DISCUSSION

3.1. Glycerol Addition Point

We primarily investigated the effect of glycerol addition time on MK-7 biosynthesis in a small scale fermenter. Glycerol was added at different days, namely

day 1, 2, 3 and 4, to cover the whole *Bacillus subtilis* growth stages (Berenjian *et al*., 2011; Sato *et al*., 2001; Zohora *et al*., 2009). As shown in **Fig. 1**, MK-7 production had different responses to glycerol addition time. The addition of both 1% and 3% (w/v) glycerol at the second day (48 h) of fermentation had a negative effect on the concentration of MK-7 on days 3 and 4. However, glycerol showed a positive effect on the concentration of MK-7 on days 5 and 6. Glycerol addition possessed a negative effect on MK-7 production when it was added at first (24 h), third (72 h) and fourth (96 h) day of fermentation. The addition of glycerol on the second day of fermentation was, therefore, selected as the optimum time for enhancing the MK-7 production in the fermentation process.

Our results show that the addition time for glycerol had an impact on MK-7 production. In *Bacillus subtilis* the majority of phospholipids consist of phosphatidylglycerol, Cardiolipin and phosphatidylethanolamine (Sohlenkamp *et al*., 2003). However, it has been reported that glycerol addition at different stages of cell density significantly influences the phospholipids composition of cell membranes (Du *et al*., 2005). As MK-7 is a membrane associated compound its biosynthesis could be affected by the effect of glycerol addition on cell membranes.

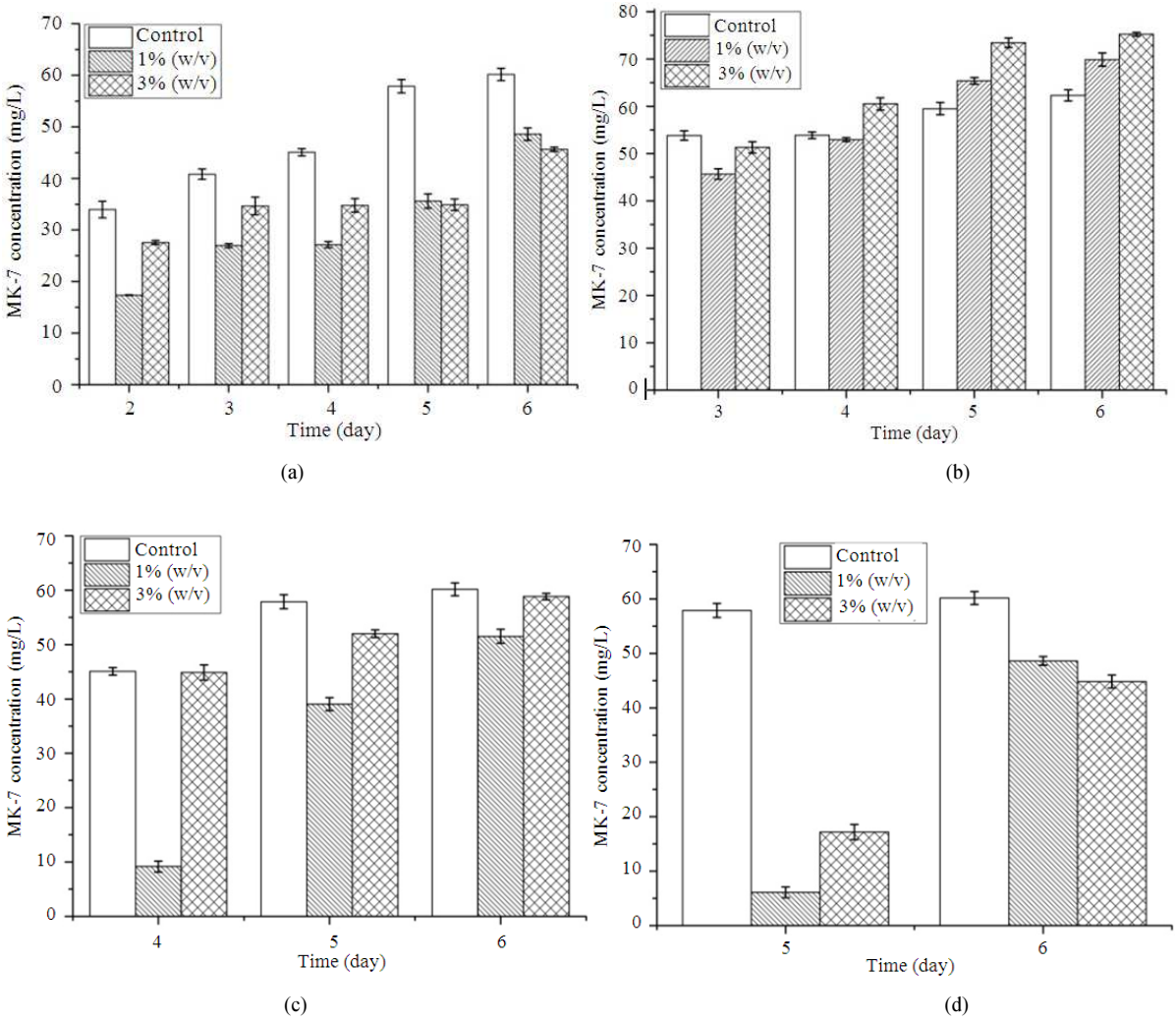


Fig. 1. Time profile of MK-7 production with 1% and 3% (w/v) glycerol addition after (a) 24, (b) 48, (c) 72 and (d) 96 h in comparison with control (no glycerol addition) in 25-mL fermenter

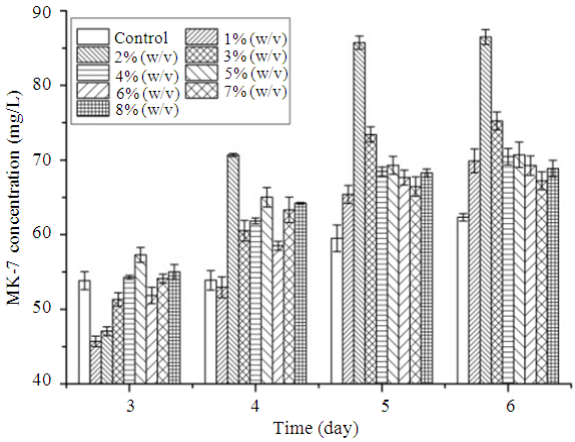


Fig. 2. Effect of glycerol concentration of MK-7 production on day 2 in 25-mL fermenter

3.2. Glycerol Concentration

The effect of adding various concentrations of glycerol on the second day of fermentation (48 h) on MK-7 production was investigated. As depicted in **Fig. 2** compared with control (in absence of adding glycerol) the MK-7 production was enhanced 40% at the end of fermentation process when adding the optimum glycerol concentration of 2% (w/v) in second day of fermentation. In comparison with the control the effect of fed-batch addition of glycerol on MK-7 production was negligible during the third and fourth day of fermentation. However, in the fifth day of fermentation, the yield of MK-7 showed at least 8% increase as compared with control media.

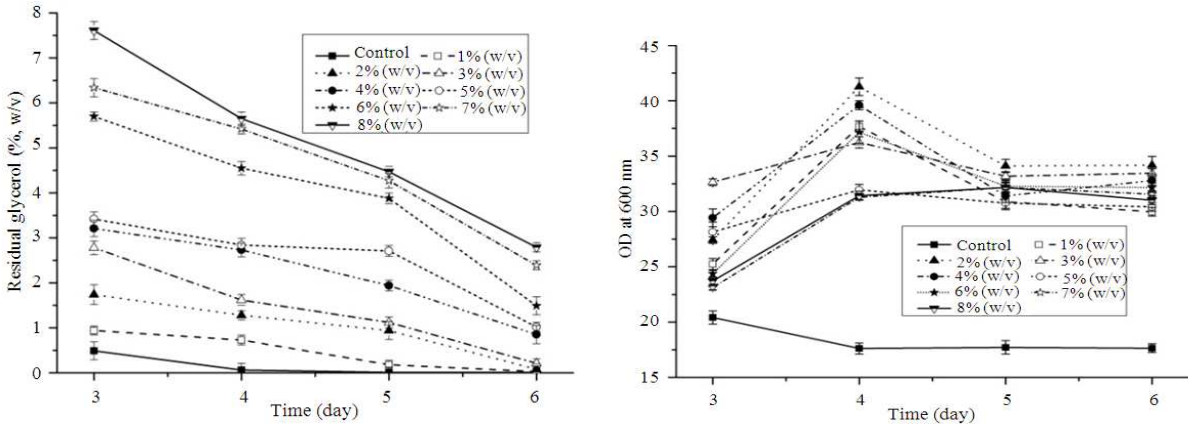
The concentration of glycerol was measured during the fermentation process. Glycerol concentration in the media was decreased, regardless of the initial concentrations throughout the incubation period (**Fig. 3a**). In addition, the MK-7 concentration was increased upon depletion of glycerol in the fermentation media. The addition of 2% (w/v) glycerol resulted in the gradual depletion of glycerol in the media and complete consumption at the end of fermentation. A further increase in the glycerol concentration from 4-8% (w/v) resulted in higher glycerol consumption. The glycerol consumption rate was increased from 0.48-4.82% (w/v) by elevating the glycerol concentration from 0-8% (w/v). However, MK-7 biosynthesis were only slightly increased when using higher concentration of glycerol (**Fig. 2**).

It has been reported that glycerol decreases the molecular weight of biopolymers, such as ε-poly-L-lysine and poly (hydroxyalkanoate) (Ashby *et al*., 2005; Nishikawa and Ogawa, 2006). This reduction results in decreasing the viscosity of the media, which can have a positive impact on stimulating the uptake of extracellular substrates and enhancing the mass transfer. The viscosity affects the diffusion of oxygen, consequently the growth of *Bacillus subtilis natto* and MK-7 production. As depicted in **Fig. 3b** the growth of bacteria was increased by the addition of glycerol into the fermentation media. Compared with controlled media (i.e., no glycerol addition), this effect was still pronounced when adding a low concentration of glycerol (1% (w/v)) at the second day. The addition of 2% (w/v) glycerol resulted in the highest peak cell density at the fourth day of fermentation. It was, therefore, concluded that at the conditions examined an optimum concentration of glycerol existed for achieving maximum cell density and MK-7 biosynthesis; although the optimum cell density and MK-7 concentration was not on the same day. In the fermentation of *Bacillus subtilis,* there is no linear correlation between glycerol concentration, cell density, water activity and solution viscosity (Luard, 1982; Mazurkiewicz *et al*., 2001). The presence of 2% (w/v) glycerol in the media may provided the optimum balance between water activity and viscosity resulting in recovering cell density and MK-7 biosynthesis.

Glycerol consumption, pH, bacterial growth and MK-7 production at the end of fermentation are summarized in **Table 1**. After addition of different glycerol concentrations the respective pH values of cultures on day 2, were varied between 7.8 and 8.2. At the end of the fermentation period (day 6) the pH values were changed between 6.7 and 7. However, within the range examined no clear correlation between the pH and MK-7 production was observed. The pH might have an impact on MK-7 biosynthesis; however, attempt to control the pH at a specific level (i.e., 5.7, 6, 7, 7.5 and 8) showed no significant effect on MK-7 production (Sato *et al*., 2001).

3.3. Production of MK-7 in a 3-L Bench Top Fermenter

The optimum small scale result was also studied in large scale (3-L) to further investigate the behavior of process on MK-7 production. The effect of glycerol addition to the concentration of MK-7, cell density and glycerol consumption were measured each day. The trend observed in 3-L vessel was consistent with the data acquired from the small volume fermenter (data not shown). Fed-batch glycerol addition resulted in increasing the MK-7 production as compared to the controlled media (e.g., no glycerol addition). The addition of 2% (w/v) glycerol in a fed-batch mode at the second day (48 h) of fermentation resulted in a 50% increase in MK-7 production compared to control. The concentration of MK-7 was increased steadily with time and approached to a maximum level on day 6.



(a) (b)

Fig. 3. The effect of fed-batch addition of various concentrations of glycerol on second day on (a) glycerol consumption and (b) optical density at different days of fermentation in 25-mL fermenter

Table 1. Effect of fed-batch glycerol addition at second day, on MK-7 production, glycerol consumption, optical density and pH after 6 days of fermentation

Added glycerol concentration% (w/v)	pH	Residual glycerol % (w/v)	OD_{600}	MK-7 (mg/L)
0	8.03±0.02a	0.01±0.01a	17.65±0.20a	62.32±0.15a
1	7.62±0.05a	0.03±0.04a	29.97±0.22b	69.88±1.11b
2	7.62±0.17a	0.08±0.08a	34.18±0.41c	86.48±0.52c
3	6.72±0.01b	0.21±0.09a	33.45±0.18d	75.24±0.73d
4	6.99±0.05b	0.85±0.12b	32.78±0.16d	70.45±0.62e
5	6.68±0.22b	1.02±0.09b	30.42±0.21d	70.69±1.23e
6	6.74±0.18b	1.49±0.11c	32.16±0.14d	69.25±0.85e
7	7.01±0.04b	2.39±0.11d	31.56±0.09d	67.21±0.68e
8	6.79±0.35b	2.79±0.08e	31.02±0.12d	68.86±0.74e

Values are reported as mean ± Standard Deviation (SD); Means with the same letter within a column are not significantly different ($p<0.05$)

During the fermentation, the amount of glycerol consumption in large scale fermenter was similar to data acquired for small scale. The amount of glycerol in the fermentation media was mostly consumed at the end of the fermentation process that maximum MK-7 was produced and the cell density was approached the stationary phase.

Based on the achieved results, fed-batch glycerol addition at the second day found to be an efficient strategy for enhancing MK-7 production. However, the metabolism and the pathway for the consumption of glycerol by *Bacillus subtilis natto* and production of MK-7 has not yet been identified. Nevertheless, our results demonstrate the significant impact of glycerol on MK-7 production can be considered as an efficient approach for enhancing the yield of MK-7 in a large scale fermentation process and further optimization of this vital vitamin.

4. CONCLUSION

The optimum concentration and conditions for glycerol addition was determined. The results of this study demonstrate that the addition of glycerol in a fed-batch process considerably enhances the MK-7 production by *Bacillus subtilis natto*. Adjusting the concentration and feeding strategy of essential nutrients may be considered an efficient approach for enhancing MK-7 production.

5. ACKNOWLEDGEMENT

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