

# The Effect of Amino-acids and Glycerol Addition on MK-7 Production

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

**Abstract—** *Bacillus subtilis natto* was fermented at 40 °C for six days in the media consisted of yeast extract 5% (w/v); soy peptone 18.9% (w/v); glycerol 5% (w/v) and K<sub>2</sub>HPO<sub>4</sub> 0.06% (w/v). Serine, arginine and asparagine as amino acid sources and glycerol as carbon supply were the limiting at the end of fermentation time while total free amino acids concentration increased 1.7 fold during the fermentation period. Addition of 2 % (w/v) glycerol on the second day of fermentation to the medium enhanced the MK-7 production whereas addition of limiting amino acids did not result in higher MK-7 production. This is the first report on identifying the deficient amino acids in MK-7 fermentation and contribution of additional glycerol feeds on enhancing the production in *Bacillus subtilis natto*.

**Index Terms—**amino acids, *Bacillus subtilis natto*, fermentation, glycerol, Menaquinone-7

## I. INTRODUCTION

Menaquinone-7 (MK-7) which is produced by bacteria mainly *Bacillus subtilis* species play an important role in electron transport, oxidative phosphorylation and active transport chain [1],[2]. MK-7 consumption showed an inhibitory effect on bone fractures and cardiovascular diseases [3],[4]. Gast et al [3], concluded that MK-7, reduces coronary/artery calcification and could protect against cardiovascular diseases. MK-7 could be more effective in carboxylation of Gla-protein, which is a powerful inhibitor of cardiovascular problems. Tamatani et al [5] found that the rate of bone loss is higher when there was lower MK-7 serum concentration in the body. Tsukamoto et al [6], suggest that the intake of MK-7 stimulates carboxylation of osteocalcin (an essential protein for normal bone metabolism) which plays a critical role in bone formation.

MK-7 is produced by fermentation mainly using *Bacillus subtilis* species. Fermentation media (carbon and nitrogen sources) have a critical effect on enhancing the MK-7 production. *Bacillus subtilis natto* have favoured carbon and

amino acid sources for MK-7 production. As far as rapidly metabolizable carbon sources are available in the fermentation media, expression of genes and enzymes required for transport and metabolism of other carbon energy sources is greatly reduced [7]. Amino acids are essential for production of proteins like heme, which are involved in the respiration process of *Bacillus subtilis*. Heme is involved in reducing menaquinone to menaquinol in the membrane [7]. When amino acid/s become limiting in the media, specific genes become activated and serve as a positive regulator for some other nitrogen metabolism genes [8]. Based on our previous study [9] effective carbon and nitrogen sources for higher MK-7 production was investigated and the substrate concentrations (amino acids and glycerol) were also measured during the fermentation process. Yeast extract and soy peptone mixture as the nitrogen source and glycerol as the carbon source were found to be the most effective sources among the factors examined for enhancing MK-7 production. The variation of amino acids concentration was not consistent for all of them. The levels of asparagine, arginine and serine showed 75.67, 11.08 and 7.59 fold decrease, respectively. While the other remaining amino acids showed increase or negligible changes in their concentrations during the fermentation time. Most of the decrease in glycerol and limiting amino acids concentrations in the media was over the first three days of the fermentation where the bulk of the cell growth and MK-7 formation occurred. The objective of this study was to study the effect of addition the limiting nitrogen and carbon sources on MK-7 production.

## II. MATERIALS AND METHODS

### Microorganism

*Bacillus subtilis natto* strain was isolated from commercial *natto* available in the Australian market. Bacterial cells were cultivated in a liquid culture constituting 0.5% (w/v) peptone, 0.5% (w/v) glucose and 0.05% (w/v) yeast extract for 5 days before streaking on tryptic soy agar plates for aseptic spore genesis. Plates were scrapped after 5 days and harvested cells were suspended in a 0.9% (w/v) NaCl solution. The spore suspension was kept in a heated water bath (80 °C, 30 min) in order to kill the residual vegetative cells, centrifuged at 3000 rpm for 10 min to remove the cell debris, diluted using 0.9% (w/v) NaCl solution to obtain the standard spore solution ( $5.2 \pm 0.5 \times 10^{10}$  spores/mL).

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### Materials

Pure MK-7 (99.3 %) was purchased from ChromaDex (USA). Yeast extract was purchased from BD (USA). Soy peptone was purchased from Oxoid (UK). Glycerol and  $K_2HPO_4$  were purchased from Chem-Supply (Australia). Methanol, dichloromethane, 2-propanol and n-hexane were obtained from Sigma-Aldrich (USA).

### MK-7 fermentation

The optimized media consisted of 5% (w/v) yeast extract; 18.9% (w/v) soy peptone; 5% (w/v) glycerol and 0.06% (w/v)  $K_2HPO_4$  inoculated with the *Bacillus subtilis natto* standard spore solution using an inoculum size of 2% (v/v). Fermentation was conducted six days aerobically at 40 °C as reported in our previous study [9]. Asparagine, arginine, glutamine and serine as amino acid sources and glycerol as the carbon source were found limited after second day of fermentation in the optimized media. To study the effect of these factors on MK-7 production, 2% (w/v) glycerol, 1.23 g/L asparagine, 2.44 g/L arginine and 1.86 g/L serine solutions (10% v:v) were added on second day of fermentation. The prepared concentrations are based on their initial concentrations in the optimized media.

### MK-7 extraction and analysis

MK-7 was extracted from the fermentation media using 2-propanol and n-hexane mixture, with n-hexane: 2-propanol (2:1, v/v) and 1:4 (liquid:organic, v/v) as previously reported [9],[10]. High performance liquid chromatography (HPLC) HP 1050 (Hewlett-Packard, USA) was used for the analysis of MK-7 concentration and the LC-MS system (LCMS-2010EV, Shimadzu, Kyoto) were used to confirm the structure of MK-7. Concentrations of free amino acids were determined by Waters Acquity™ Ultra Performance LC (UPLC) system. The description of each analysis and conditions were described in details previously [9].

### Analytical methods

Cell density was determined from the optical density at 600 nm with a spectrophotometer (Cary, USA) after suitable dilution with deionised water. The concentration of glycerol was assayed with free glycerol determination kit from Sigma-Aldrich (USA). The pH was measured directly in the cultivation medium by the laboratory standard pH meter (Hanna Instruments, USA).

## III. RESULTS AND DISCUSSION

Asparagine, arginine, glutamine and serine as amino acid sources and glycerol as the carbon source were found limited after second day of fermentation. To study the effect of these factors on MK-7 production glycerol, asparagines, arginine and serine solutions were added on second day of fermentation. When glycerol solution was added to the fermentation media, the yield of MK-7 showed 41% increase, from third day of fermentation in compare with the control media. Supplying the optimum media with asparagine, arginine and serine did not result in higher MK-7 production ( $p>0.05$ ) as shown in Figure 1. Glycerol enhanced the cell growth 1.7 fold one day after its addition

to the fermentation media compared to control sample (Figure 2). Addition of limiting amino acids (asparagines, arginine and serine) showed no significant effect on cell growth rate ( $p>0.05$ ). MK-7 concentration was increased between cell's mid-exponential growth and the stationary phase.

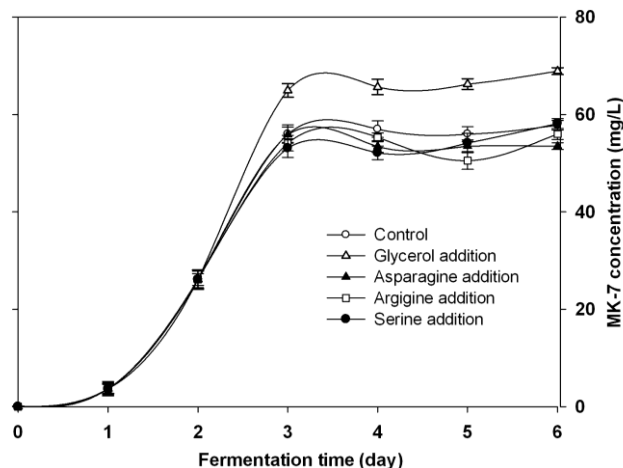


Fig. 1. Changes in MK-7 production while addition of glycerol, asparagine, arginine and serine during the fermentation period.

According to the results it can be concluded that MK-7 production is closely coupled with cell growth rate. It has been reported that glycerol addition to the medium could also decrease molecular weight of some biopolymers, such as poly(hydroxyalkanoate) and  $\epsilon$ -poly-L-lysine and decrease the fermentation viscosity which relieve the mass transfer limitation and stimulate the uptake of extracellular substrates. The difference between the MK-7 concentrations and cell growth rates in media with and without glycerol addition may be due to this medium composition phenomenon [11],[12]. This behaviour was a unique discovery in MK-7 production, as to our knowledge; there is no report about the increase of MK-7 production by the addition of glycerol in medium and identifying the limiting amino acids on MK-7 production.

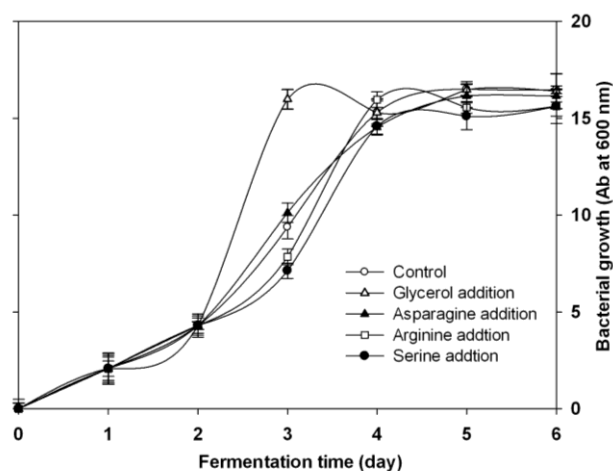


Fig. 2. Changes in bacterial growth while addition of glycerol, asparagine, arginine and serine during the fermentation period.

#### IV. CONCLUSION

Yeast extract and soy peptone mixture as nitrogen sources and glycerol as the carbon source were found to be the most effective nutrients for enhancing MK-7 production. Glycerol addition showed the ability to increase the rapid cell growth and MK-7 production. This was the first time to discover the contribution of glycerol addition on enhanced production of MK-7 by *Bacillus subtilis natto*. The improved yield of MK-7 reached  $68.6 \pm 1.3$  g/l which was at a relative higher level compared with our previous study. It was deduced that glycerol might influence to alter MK-7 biosynthesis. In fact, it was such a complex function of glycerol and was still unclear as to why the addition of glycerol resulted in MK-7 production, and more work should be further investigated to elucidate the biosynthesis mechanism of MK-7 with the presence of glycerol.

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