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Menaquinone-7 production from maize meal hydrolysate by *Bacillus* isolates with diphenylamine and analogue resistance*#

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Abstract: A menaquinone-7 (MK-7) high-producing strain needs to be isolated to increase MK-7 production, in order to meet a requirement of MK-7 given the low MK-7 content in food products. This article focuses on developing MK-7 high-producing strains via screening and mutagenesis by an atmospheric and room temperature plasma (ARTP) mutation breeding system. We isolated an MK-7-producing strain Y-2 and identified it as *Bacillus amyloliquefaciens*, which produced (7.1±0.5) mg/L of MK-7 with maize meal hydrolysate as carbon source. Then, an MK-7 high-producing strain *B. amyloliquefaciens* H.β.D.R.-5 with resistance to 1-hydroxy-2-naphthoic acid, β-2-thienylalanine, and diphenylamine was obtained from the mutation of the strain Y-2 using an ARTP mutation breeding system. Using strain H.β.D.R.-5, efficient production of MK-7 was achieved ((30.2±2.7) mg/L). In addition, the effects of nitrogen sources, prenyl alcohols, and $MgSO_4$ on MK-7 production were investigated, suggesting that soymeal extract combined with yeast extract, isopentenol, and $MgSO_4$ was beneficial. Under the optimized condition, the MK-7 production and biomass-specific yield reached (61.3±5.2) mg/L and 2.59 mg/L per OD_{600} unit respectively in a 7-L fermenter. These results demonstrated that strain H.β.D.R.-5 has the capacity to produce MK-7 from maize meal hydrolysate, which could reduce the substrate cost.

Key words: Menaquinone-7; *Bacillus amyloliquefaciens*; Analog resistance; Diphenylamine resistance; Maize meal hydrolysate

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1 Introduction

Menaquinone-7 (MK-7) is one part of family of menaquinone (MK-*n*) with a side chain of seven isoprene units (Walther *et al*., 2013). MK-7 can activate the osteocalcin in bone marrow to promote the formation of bone, and can improve the bone mineral density (BMD) in the aged so as to reduce the risk of haunch bone fracture (Howard and Payne, 2006). In addition, it can lower blood pressure in rats (Kim *et al*., 2011). Food products, such as sauerkraut, cheese, or fermented soybean, may be predominant sources of dietary MK-7 in many parts of the world (Berenjian *et al*., 2011). However, MK-7 content in food products is generally very low. Although traditional Japanese fermented soybean (i.e. "*natto*") is one good source of MK-7 (800–900 mg MK-7/100 g *natto*), it is not appreciated by many people because of its strong smell and taste (Berenjian *et al*., 2011). Hence, there is a need to increase the MK-7 content in the daily diet via the addition of MK-7. So it is desirable to be able to breed high-producing strains of MK-7, which can be used to biosynthesize MK-7 by fermentation processes.

According to the literature, many microorganisms can be used to biosynthesize MK-7, such as *Bacillus subtilis* and *Escherichia coli* (Howard and Payne, 2006; Berenjian *et al*., 2011). Most MK-7 is

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produced by *Bacillus* sp., which is isolated from *natto* (Berenjian *et al*., 2011; Kim *et al*., 2011). However, researchers have focused on genetic mutation to improve the productivity of the microorganism because of the low MK-7 content in fermentation processes. 3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, as the first regulatory enzyme in the shikimate pathway, is feedback-inhibited by 1,4-dihydroxy-2-naphthoate (DHNA), L-tyrosine, L-phenylalanine, and L-tryptophan (Fig. S1) (Tsukamoto *et al*., 2001). The desirable mutants with feedback-resistant intermediates and high MK-7 yield have been developed by breeding the mutants with resistance to 1-hydroxy-2-naphthoic acid (HNA), *p*-fluoro-D,L-phenylalanine (pFP), *m*-fluoro-D,L-phenylalanine (mFP), and β-thienylalanine (βTA) (Tsukamoto *et al.*, 2001; Liu *et al*., 2014). Some researchers have reported that diphenylamine (DPA) inhibits the synthesis of MK-*n* (Sato *et al*., 2001b). To obtain mutants with resistance to DPA and/or an analogue, the wild-type strains were treated by the conventional mutation methods, such as ultraviolet (UV) and *N*-methyl-*N*-nitro-*N*-nitroso-guanidine (NTG) (Tani *et al*., 1985; Sato *et al.*, 2001a; 2001b). Compared with these mutation methods, however, an atmospheric and room temperature plasma (ARTP) biological breeding system (Wuxi Tmaxtree Biotechnology Co., Ltd., Wuxi, China) has many outstanding features, such as high positive mutation rate, rapid mutation, and high operation flexibility (Zhang *et al*., 2014; 2015). For an ARTP breeding system, the DNA of microorganism is destroyed by different chemical reactive species (e.g. reactive oxygen species, reactive nitrogen species, and helium lines), which causes the greatest DNA damage to individual cells while maintaining their viability (Chen *et al*., 2008; Li *et al*., 2011; Zhang *et al*., 2015). Therefore, ARTP has become a popular mutation method and has been successfully applied to breed many kinds of compounds' high-yielding strains.

To produce MK-7, glycerol is selected as the major carbon resource (Sato *et al.*, 2001a; 2001b; Berenjian *et al*., 2011). However, it is costly and poisonous to humans, and is thus restricted to scaled fermentation. Rosa-Putra *et al*. (2001) have indicated that *Bacillus* sp. can utilize mono-, di-, and polysaccharides in glycolysis except for glycerol. Various renewable agricultural or raw materials, such as soybean extract (Sato *et al*., 2001a) and corn syrup (Wee *et al*., 2008), have been used as feedstock for MK-7 fermentation. Maize, also called corn, is widely planted in China, especially in northern China. Since maize contains not only large amounts of starch (about 70%–75%, dry basis), protein (about 10%), and lipid (about 4%–5%) but also multi-vitamins (vitamin A, vitamin B_1, vitamin B_2, etc.), maize is a promising feedstock for organic compound production by microbial fermentation, such as lactic acid and amino acid (Song *et al*., 2014; Xu *et al*., 2014). However, as far as we know there has been no research on MK-7 production from maize meal hydrolysate.

In this study, in order to obtain MK-7 high-producing strains, *Bacillus amyloliquefaciens* Y-2 with the ability to utilize maize meal hydrolysate was isolated from Chinese fermented bean (designated as *douchi*), a popular food in China. Afterwards, this isolate was further genetically mutated by multi-round mutagenesis to give it DPA and analogue resistance ability. We investigated the effects of nitrogen source, prenyl alcohols, and magnesium (Mg^{2+}) on MK-7 production with maize meal hydrolysate as substrate using the isolated strain and DPA- and analogue-resistant mutant. Finally, the production of MK-7 by DPA- and analogue-resistant mutant in a 7-L fermenter was also investigated.

2 Materials and methods

2.1 Chemicals

MK-7 (99.8% purity) was purchased from ChromaDex (Irvine, USA). βTA was purchased from Sigma-Aldrich (Colorado, USA). Isopentenol, farnesol, and geraniol were purchased from Tokyo Chemical Industry (TCI; Tokyo, Japan). DPA and HNA were obtained from Sinopharm (Shanghai, China). Maize meal was supplied by China Green Food (Holding) Co., Ltd. (Xiamen, China).

2.2 Environmental samples and media

Commercial *douchi* samples (one-pocket or one-tin) were directly purchased from different food companies of China. The medium used for screening MK-7-producing strains from *douchi* consisted of (per liter): 5 g fibrin, 15 g glucose, 15 g soy peptone, 1 g $KH_2PO_4{\cdot}12H_2O$, 2.5 g $K_2HPO_4{\cdot}3H_2O$, 2.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g natamycin, and 15 g agar.

Tryptone-yeast extract-glucose (TYG) medium comprising of 5 g/L tryptone, 2.5 g/L yeast extract, and 5 g/L glucose was used as seed medium. Glycerol-yeast extract-soy peptone (GYS) medium containing 70 g/L glycerol, 25 g/L yeast extract, 15 g/L soy peptone, and 0.5 g/L KH_2PO_4 was used as the basal medium (Fernandez and Collins, 1987). The glycerol in the GYS medium was replaced with maize meal hydrolysate and designated as MYS medium. All media were adjusted to pH 7.2±0.2 with 0.2 g/ml NaOH.

2.3 Isolation and identification of MK-7-producing strains

Each *douchi* sample (about 0.5 g) was incubated in sterile physiological saline containing glass beads at 80 °C and 120 r/min for 15 min. Subsequently, the above suspensions were diluted to different concentrations with sterile saline and then spread on the selected agar plates and incubated at 37 °C for 24 h. Those colonies, which showed large transparent zones around them, were purified as a single colony, and then MK-7 production was detected by thin-layer chromatography (TLC). Individual bacterial colonies, which showed high MK-7 production, were isolated for further investigation. The identity of the tested isolates was determined by 16S rDNA according to the description of Li *et al*. (2014). A 16S rDNA was amplified by polymerase chain reaction (PCR), and the resulting PCR products were purified and sequenced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Homology search of 16S rDNA of tested isolate was conducted using the Basic Local Alignment Search Tool (BLAST) program available from the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and analyzed for species identification.

2.4 Derivation of mutants

For the mutation procedure, a helium plasma jet was used as a physical method, and carried out in an ARTP mutation breeding system. The operating parameters were set as described by Zhang *et al*. (2015): input radio frequency (RF) power was 100 W, the available irradiation range was 2.0 mm, helium flow rate was 10 L/min, plasma jet temperature was 25–35 °C, and plasma treatment time was set at 60 s according to our pre-experimental result (Fig. S2).

The parent strain was inoculated from a fresh TYG-plate and cultivated at 37 °C and 120 r/min agitation to the logarithmic growth phase. The cells were harvested by centrifugation and washed three times with sterile physiological saline, and then suspended in saline at a concentration of 10^7–10^8 cells/ml. Bacterial suspension (15 μl) was spread on a sterilized sample plate and dried in pure nitrogen for a few minutes, and then exposed to the helium plasma jet for 60 s. All the samples treated by ARTP were then washed with 1.5 ml sterilized physiological saline, and the eluent was spread on TYG-plates containing an analogue (i.e. HNA as an analogue of DHNA and βTA as an analogue of L-tyrosine) or DPA and then cultivated at 37 °C for 3–5 d. According to our pre-experiment's data (unpublished results), the concentrations of HNA, βTA, and DPA were set at 60, 5, and 200 mg/L, respectively. Colonies appearing in the selected agar plate were purified as a single colony, and the ability of each colony for resistance to analogue or/and DPA was confirmed with the corresponding selected agar plate. Individual bacterial colonies showing the highest MK-7 production were isolated for further investigation.

2.5 Preparation of maize meal hydrolysate

To make the liquefied maize meal, 25.0 g maize meal and 12.5 mg thermostable α-amylase were added to 100 ml distilled water, and then the mixture was performed by heating starch mixture at 100 °C for 30 min. When the temperature drops to room temperature, the hydrolysate was added with water to a total of 100 ml. The dextrose equivalent (DE) value was detected according to the Chinese standard (AQSIQ and SAC, 2007), and the total sugar and total reducing sugars were detected by 3,5-dinitrosalicylic acid (DNS) assay according to Patil and Dayanand (2006).

2.6 Fermentation for MK-7 production

Batch cultivations of MK-7-producing strains were carried out in shake flasks. Unless otherwise stated, MK-7 fermentation was carried out in a 250-ml Erlenmeyer flask containing 50 ml of GYS medium or MYS medium. All experiments were performed at a constant temperature of 37 °C and a constant agitation cycle of 120 r/min.

Scale-up of MK-7 fermentation was carried out in a 7-L jar fermenter (KF-7 L, Korea Fermenter Co.,

Inchon, Korea) containing 3 L media. The initial pH of the fermentation medium was adjusted to 7.2±0.2 using 0.2 g/ml NaOH. The aeration rate was set to 2.0 L/min by the integrated gas flow controller. Dissolved oxygen was detected using a pO_2 (oxygen partial pressure) electrode (Oxferm FDA, Hamilton, New Zealand) and maintained at about 10% saturation level by variation of the stirrer speed. Fermentation process was carried out at 37 °C for 6 d, and cell growth, pH, and MK-7 accumulation were monitored over the course of the experiment.

2.7 Analytical methods

MK-7 extraction was carried out according to the method of Berenjian *et al.* (2011), and the detection of MK-7 was performed by TLC or high performance liquid chromatography (HPLC) equipped with a diode array UV detector (248 nm). When analyzing the MK-7 by TLC, extracts were spotted on a silica gel plate (60GF254 plate; Amresco, Ohio, USA) with hexane-diethylether (85:15, v/v) solvent as developing solvent. MK-7 reference standard was used to compare spots with extracts, and MK-7 was quantified by UV densitometry at 254 nm. For HPLC, separation was carried out on a Zorbax SB C_{18} column (250 mm×4.6 mm, Agilent, USA) at 40 °C using methanol-dichloromethane (9:1, v/v) solvent as mobile phase with a flow rate of 1 ml/min. The MK-7 calibration curve was set according to the method of Berenjian *et al*. (2011).

In addition, the cell growth was reflected by cell density, which was detected by a photometer at 600 nm after an appropriate dilution. The concentration of sugar was detected by DNS assay according to Patil and Dayanand (2006). pH was directly monitored in the cultivation medium by the fermenter's native pH electrode (Korea Fermenter Co., Inchon, Korea).

3 Results

3.1 Isolation and characterization of MK-7-producing strains

In this study, 10 *douchi* samples from different food companies in China were used to isolate the nattokinase-producing strains, and 56 nattokinase-producing strains were isolated from these samples. Among these strains, strains T-26 (isolated from samples of T company), S-33 (isolated from samples of S company), R-5 (isolated from samples of R company), Q-11 (isolated from samples of Q company), Y-2 (isolated from samples of Y company), and W-21 (isolated from samples of W company) showed large transparent zones around them, and were picked up to detect MK-7 production by HPLC. The production of MK-7 by strains T-26, S-33, R-5, Q-11, Y-2, and W-21 was (12.3±0.8), (9.4±1.0), (5.2±0.3), (1.1±0.3), (11.8±0.7), and (6.5±0.4) mg/L, respectively. Moreover, strain Y-2 had the highest capability of maize starch hydrolysis among these strains and could utilize the maize meal hydrolysate (80 g/L of total sugar with a DE value of 54%) as a carbon source for MK-7 production. Maize meal is an inexpensive and abundant available feedstock, but there is as yet no research on using maize meal hydrolysate as a carbon source for MK-7 production. Therefore, strain Y-2 was chosen as a preferable MK-7 producer for further investigations.

In order to further understand strain Y-2 and communicate with counterparts, strain Y-2 was identified by the 16S rDNA identification method. A 1516-bp 16S rDNA fragment was obtained by PCR amplification, and then the PCR product was purified and sequenced by Sangon Biotech (Shanghai) Co., Ltd., China. Phylogenetic relationships based on the 16S rDNA gene sequence were analyzed by Clustalx 1.83 and MEGA 5.0. As shown in Fig. 1, strain Y-2 had the closest relationship with the *B. amyloliquefaciens* subsp. strain FZB42 (99% identity in 16S rDNA), which was also supported by the morphological and physiological characterization. Therefore, strain Y-2 was designated as *B. amyloliquefaciens* Y-2.

3.2 Derivation of analogue-resistant mutants

In order to relieve the inhibition of DAHP synthetase by DHNA, we attempted to screen a mutant with resistance to HNA using the ARTP mutation breeding system. After mutagenic treatment, 76 mutants with resistance to HNA were isolated from the TYG medium containing 60 mg/L HNA. Then those isolated mutants were used to analyze their ability in MK-7 production, and 35 mutants from those NHA-resistant mutants revealed higher MK-7 productivity than the parent strain Y-2. Among the tested strains, *B. amyloliquefaciens* H.R.-13 reached the highest concentration of MK-7 ((14.4±1.3) mg/L), which was

103% higher than that of the parent strain (Table 1). It should be noted that the cell growth of this mutant was not so different from that of parent strain (optical density at 600 nm (OD_{600}) 30.8 vs. 32.5; Table 1). Therefore, strain H.R.-13 was selected as a promising MK-7-producing strain for further studies.

A further mutation procedure using TYG containing 5 mg/L of βTA was carried out with strain H.R.-13 as the parent strain. As a result, 50 mutants with analog resistance to βTA were isolated and then used to analyze their ability in MK-7 production. Compared with strain H.R.-13, roughly half the mutants exhibited increased production of MK-7. Among them, one mutant, designated as *B. amyloliquefaciens* H.β.R.-27, was found to produce the highest MK-7 production ((18.5±1.0) mg/L), which was 28% higher than that of strain H.R.-13 ((14.4±1.3) mg/L). Therefore, strain H.β.R.-27 was selected as a promising MK-7-producing strain for further study.

Table 1 Cell growth and MK-7 production of parent strain and mutants during growth on MYS medium

Strain	OD_{600}	MK-7 (mg/L)	$Y_{P/X}$[a] (mg/L per OD_{600} unit)
Y-2	32.5±2.1	7.1±0.5	0.218
H.R.-13	30.8±1.2	14.4±1.3	0.468
H.β.R.-27	29.4±0.6	18.5±1.0	0.629
H.β.D.R.-5	24.3±1.5	30.2±2.7	1.242

[a] $Y_{P/X}$ represents the biomass-specific yield. All data (except $Y_{P/X}$) are mean values of three determinations of three independent experiments with standard errors (±SE)

At this point, strain *B. amyloliquefaciens* H.β.R.-27 almost deregulated the inhibition of DAHP synthetase. This enabled access to the high carbon flux from erythrose-4-phosphate to MK-7 production via the biosynthetic pathway of a quinone skeleton (Tsukamoto *et al*., 2001).

3.3 Derivation of DPA-resistant mutants

In order to obtain a more potent MK-7 producer, *B. amyloliquefaciens* H.β.R.-27 was further mutated using the ARTP mutation breeding system to relieve the inhibition of polyprenyl pyrophosphate synthetase by DPA. As a result, 34 mutants with resistance to DPA were isolated from the TYG medium containing 200 mg/L of DPA. The isolated mutants were then used to analyze their ability in MK-7 production. One mutant exhibited the best MK-7 productivity among these tested mutants and was designated as *B. amyloliquefaciens* H.β.D.R.-5. The MK-7 production of strain H.β.D.R.-5 reached (30.2±2.7) mg/L, which was 63% higher than that of strain H.β.R.-27 ((18.5±1.0) mg/L). However, the cell growth of strain H.β.D.R.-5 was slightly lower than that of other tested strains (Table 1). In addition, the MK-7 production, cell growth, and sugar utilization of strain H.β.D.R.-5 had inheritable stability (Fig. S3).

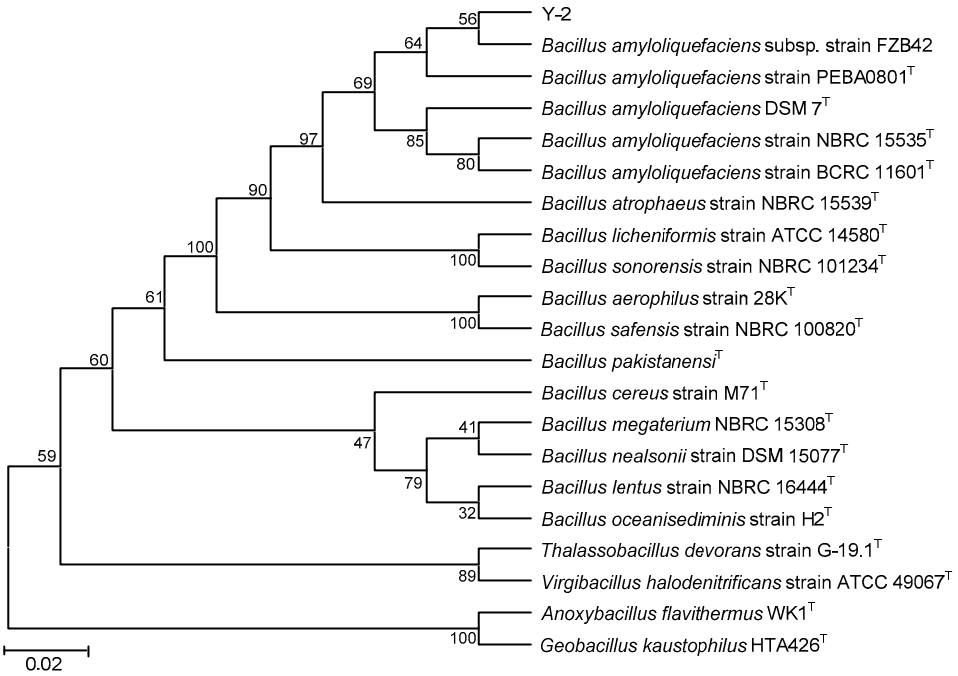

Fig. 1 Phylogenetic tree based on 16S rDNA sequence homology of the strain Y-2

3.4 Effect of nitrogen source on MK-7 production

According to a previous study, soy peptone was the most suitable nitrogen material for MK-7 synthesis, but it is much needed (189 g/L) (Berenjian *et al*., 2011). To make a balance between MK-7 production and cost, a middle stratagem was applied, which was 40 g/L soymeal extract as the major nitrogen source. In addition, yeast extract was used as additive to help to produce more MK-7. As can be seen from Fig. 2b, the MK-7 production and cell concentration (OD_{600}) were (31.7±2.5) mg/L and 25.7±1.8, respectively, during growth on improved MYS medium with 40 g/L soymeal extract and 25 g/L yeast extract as the nitrogen source, which were about 15.7% and 22.4% higher than those without yeast extract (27.4 mg/L of MK-7 and 21.0 of OD_{600}), respectively.

From the above data, it is clear that soymeal extract combined with yeast extract as nitrogen source is beneficial to MK-7 production and cell growth. To look for the optimum concentration of yeast extract for MK-7 production, yeast extract with the concentration from 5 to 25 g/L was used as additives in the following study. As shown in Fig. 2b, the highest yield of MK-7 ((41.9±2.4) mg/L) and biomass-specific yield ($Y_{P/X}$, 1.47 mg/L per OD_{600} unit) were obtained when the concentration of yeast extract was 10 g/L. However, a decrease in the biomass-specific yield was observed when the concentration of yeast extract exceeded 10 g/L (Fig. 2b). Therefore, 10 g/L of yeast extract was selected as the additional nitrogen source for subsequent experiments.

3.5 Effect of prenyl alcohols on MK-7 production

In order to analyze the effect of prenyl alcohols on the production of MK-7, different prenyl alcohols (including isopentenol, geraniol, and farnesol) with different concentrations (0.2, 1.0, 5.0, and 25.0 mmol/L) were supplemented to the MYS medium. As shown in Table 2, isopentenol was most effective for MK-7 production. The highest MK-7 concentration of (46.3±5.1) mg/L, which was about 10.5% higher than that of the control group ((41.9±2.4) mg/L MK-7), was achieved when 5.0 mmol/L of isopentenol was added to the MYS medium. At this condition, the $Y_{P/X}$ also reached a maximum (2.29 mg/L per OD_{600} unit). However, the cell concentration, MK-7 production, and $Y_{P/X}$ decreased when more than 5.0 mmol/L of isopentenol was added to the MYS medium, especially cell concentration and MK-7 production (Table 2). For two other prenyl alcohols (geraniol and farnesol), the highest MK-7 concentrations of (44.9±1.5) and (43.1±3.2) mg/L were achieved when adding 1.0 mmol/L of geraniol and farnesol, respectively (Table 2). As with adding excess isopentenol, the cell concentration, MK-7 production, and $Y_{P/X}$ decreased when adding more than 1.0 mmol/L of geraniol and farnesol (Table 2).

3.6 Effect of magnesium (Mg^{2+}) supplement on MK-7 production

Mg^{2+} as a cofactor for some key enzymes, especially for polyprenyl/solanesyl pyrophosphate synthetase, can control the enzyme activity, cell growth, and MK-*n* biosynthesis (Sagami *et al*., 1977; Fujii *et al*.,

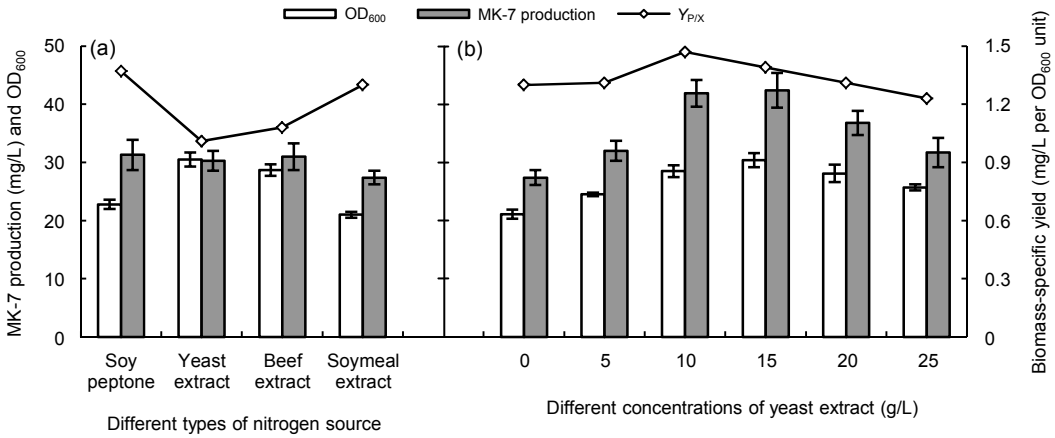

Fig. 2 Effects of nitrogen source (a) and concentration (b) on cell growth and MK-7 production by the strain H.β.D.R.-5
Each value represents mean with standard error (±SE) of three biological replicative experiments

Table 2 Effects of different concentrations of prenyl alcohols on the cell growth and MK-7 production by strain H.β.D.R.-5

Prenyl alcohol	*C* (mmol/L)	OD_{600}	MK-7 (mg/L)	$Y_{P/X}$ (mg/L per OD_{600} unit)
Isopentenol	0.0	28.4	41.9	1.47
	0.2	28.1	42.5	1.51
	1.0	25.8	44.2	1.71
	5.0	20.2	46.3	2.29
	25.0	7.7	7.8	1.03
Geraniol	0.0	28.5	41.5	1.46
	0.2	28.0	42.2	1.51
	1.0	22.5	44.9	2.00
	5.0	16.7	34.1	2.04
	25.0	6.6	6.4	0.97
Farnesol	0.0	28.3	41.7	1.47
	0.2	28.1	42.0	1.49
	1.0	21.9	43.1	1.97
	5.0	14.9	20.4	1.37
	25.0	4.5	3.6	0.80

C represents the concentration of prenyl alcohols. $Y_{P/X}$ represents the biomass-specific yield. All data are mean values of three determinations of three independent experiments with standard errors ≤7%

1980). In order to investigate the effect of Mg^{2+} supplementation on MK-7 fermentation, different concentrations of $MgSO_4$ ranging from 0.25 to 1.00 g/L were added to the MYS medium with 5.0 mmol/L isopentenol. As can be seen from Table 3, an increase in the MK-7 production was observed when the $MgSO_4$ concentration was lower than 0.50 g/L. However, when the concentration of $MgSO_4$ exceeded 0.50 g/L, the MK-7 production decreased. At the optimal concentration of $MgSO_4$ (0.50 g/L), the MK-7 production and $Y_{P/X}$ were (52.6±2.1) mg/L and 2.49 mg/L per OD_{600} unit, respectively, which were 13.6% and 8.7% higher than those of the control group. In addition, Mg^{2+} supplementation led to an increase in the utilization of sugar (Table 3).

3.7 Monitoring the MK-7 production in a jar fermenter

Under optimal conditions, the accumulation of MK-7 in the 500-ml scale broth reached (52.6±2.1) mg/L using maize meal hydrolysate as the carbon source and soymeal extract combined with yeast extract as the nitrogen source for 6-d fermentation. In order to evaluate the results on a larger scale, MK-7 fermentation by strain H.β.D.R.-5 was carried out in a 7-L jar fermenter, where the fermentation medium was the GYS medium or improved MYS medium containing 80 g/L maize meal hydrolysate, 5 g/L yeast extract, 40 g/L soymeal extract, 5.0 mmol/L isopentenol, 0.50 g/L $MgSO_4$, and 0.5 g/L KH_2PO_4. As shown in Fig. 3, MK-7 production dramatically increased after the logarithmic growth phase and steadily approached a maximum of (57.8±2.5) mg/L in the GYS medium and (61.3±5.2) mg/L in the improved MYS medium. In the logarithmic growth phase, however, there was only a small amount of MK-7 accumulation. In addition, the production of MK-7 during cultivation in the improved MYS medium was higher than that in the GYS medium (Fig. 3). Moreover, the pH values of both GYS medium and improved MYS medium were changed during the culture process and the change of pH in the GYS medium was sharper than that in the improved MYS medium (Fig. 3).

Table 3 Effects of different concentrations of $MgSO_4$ on the cell growth, sugar utilization, and MK-7 production by the strain H.β.D.R.-5

Mg^{2+} (g/L)	Initial total sugar concentration (g/L)	Final total sugar concentration (g/L)	Final reducing sugar concentration (g/L)	MK-7 production (mg/L)	OD_{600}	$Y_{P/X}$ (mg/L per OD_{600} unit)
0.00	80	22.5±1.2	14.2±1.1	46.3±3.8	20.2±1.2	2.29
0.25	80	20.7±0.7	11.5±0.8	48.7±2.6	20.7±1.2	2.35
0.50	80	17.2±1.5	7.6±0.2	52.6±2.1	21.1±1.7	2.49
0.75	80	15.9±1.0	6.8±0.5	47.0±1.4	20.7±2.0	2.27
1.00	80	15.2±1.5	6.3±0.5	38.8±1.9	20.4±1.3	1.90

$Y_{P/X}$ represents the biomass-specific yield. All data (except Mg^{2+} concentration, initial total sugar concentration, and $Y_{P/X}$) are mean values of three determinations of three independent experiments with standard errors (±SE)

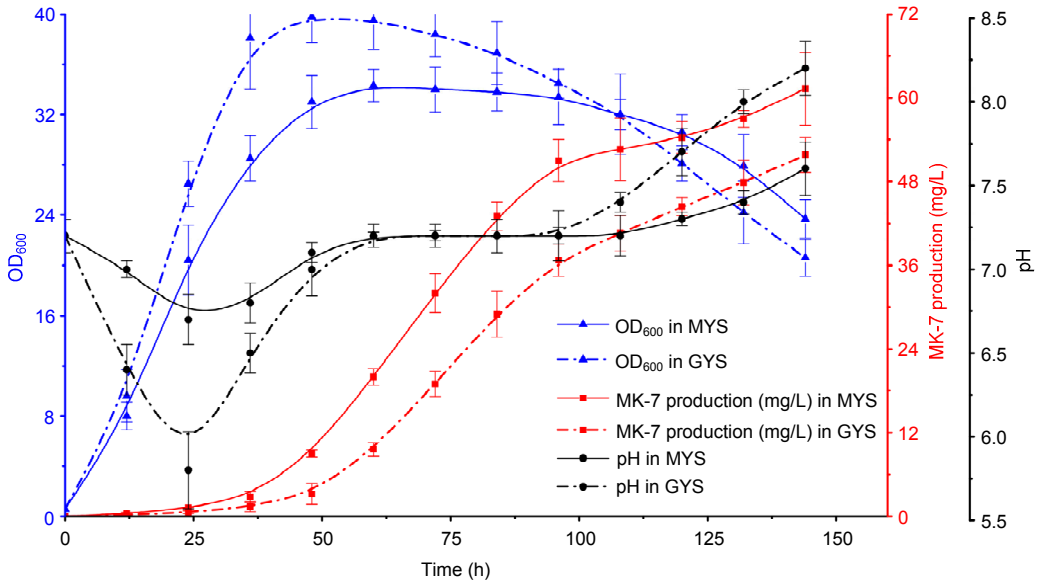

Fig. 3 Monitoring the cell growth at 600 nm, MK-7 production, and pH during the time course of fermentation in a 7-L jar fermenter, and carrying out the performance comparison between fermentation in GYS medium and fermentation in improved MYS medium

Each value represents the mean with standard error (±SE) of three biological replicative experiments

4 Discussion

MK-7 is one part of family of MK-*n*, and is popular among consumers because of its unique physiological functions (Howard and Payne, 2006). However, the content of MK-7 in traditional food is too low to meet people's needs. In order to resolve this problem, an MK-7 high-producing strain should be isolated and the fermenting condition should be optimized. In the present study, we focused on developing MK-7 high-producing strains via screening and mutagenesis by the ARTP mutation breeding system and evaluating the effects of nitrogen sources, prenyl alcohols, and Mg^{2+} on MK-7 production.

According to previous reports (Yanagisawa and Sumi, 2005; Armougom *et al*., 2009), *Bacillus* sp. with high fibrinolytic activity isolated from fermented soybean such as Chinese *douchi*, Japanese *natto*, and Korea *chungkookjang* also generally produced high MK-7 content. Among 56 nattokinase-producing strains, which were isolated from 10 *douchi* samples, strain Y-2 had a higher capability of MK-7 production and maize meal hydrolysis. Given that maize meal is an inexpensive and abundant available feedstock and has not been used to produce MK-7, we chose strain Y-2 as a potential MK-7-producing strain. After 16S rDNA identification analysis, strain Y-2 was designated as *B. amyloliquefaciens* Y-2 (Fig. 1).

The biosynthetic pathway of MK-*n* is composed of a couple of mutually exclusive pathways, the biosynthetic pathway of the quinone skeleton and the biosynthetic pathway of the isoprene side chain (Fig. S1) (Tsukamoto *et al*., 2001). However, the DAHP synthetase in the biosynthetic pathway of the quinone skeleton is feedback-inhibited by DHNA, L-tyrosine, L-phenylalanine, and L-tryptophan (Fernandez and Collins, 1987; Tsukamoto *et al*., 2001; Wee *et al*., 2008). Moreover, the polyprenyl pyrophosphate synthetase in the biosynthetic pathway of the isoprene side chain is inhibited by DPA (Takahashi *et al*., 1980; Sato *et al*., 2001b). In order to relieve the inhibition of DAHP synthetase by DHNA and L-tyrosine, the mutants with resistance to HNA and βTA were screened using the ARTP mutation breeding system. After mutagenic treatment, roughly half of mutants exhibited increased production of MK-7 compared with the parent strain (Table 1). This result once again shows that the ARTP mutation breeding system has a higher

positive genotoxic response than traditional mutation methods because of the highest DNA damage and cell viability (Tsukamoto *et al*., 2001; Zhang *et al*., 2015). Among these strains, strain H.β.R.-27 with resistance to HNA and βTA produces the highest MK-7 production ((18.5±1.0) mg/L), which are 160.6% higher than that of strain Y-2 ((7.1±0.5) mg/L). Takahashi *et al*. (1980) have pointed out that DPA inhibits the synthesis of MK-*n* because DPA controls the activity of polyprenyl pyrophosphate synthetase. In order to further relieve the inhibition of polyprenyl pyrophosphate synthetase by DPA, strain H.β.R.-27 was further mutated using the ARTP mutation breeding system. The resultant strain H.β.D.R.-5 shows more resistance to DPA and higher MK-7 production (Table 1). It should be noted that the cell growth of strain H.β.D.R.-5 was slightly lower than that of other tested strains (Table 1), and this is perhaps because of greater carbon source flux into the biosynthetic pathway of MK-7. In addition, strain H.β.D.R.-5 is a stable producer of MK-7, with steady cell growth and sugar utilization (Fig. S3), and it is indicated that strain H.β.D.R.-5 is genetically stable following mutation and has potential value for industrial application.

From the above results, the strain H.β.D.R.-5 could use maize meal as the sole carbon source to produce MK-7. Generally, maize has a high starch content (approximately 70%–75%) and small amounts of protein and lipid (less than 15%). However, a nitrogen source is one of the key elements, which influences cell growth, protein and MK-*n* production (Berenjian *et al*., 2011). In this study, we again showed that soy peptone is the best nitrogen source for MK-7 production from among yeast, beef, or soymeal extract (Fig. 2a). Previous studies showed that different nitrogen sources affect MK-7 production because of the different cell concentrations (Sato *et al*., 2001a; Berenjian *et al*., 2011). To make a balance between MK-7 production and cost, soymeal extract and yeast extract were selected as the nitrogen sources. As can be seen from Fig. 2b, the MK-7 production and cell concentration during growth on the improved MYS medium with soymeal extract and yeast extract are higher than those without yeast extract. This is because yeast extract is beneficial to cell growth and MK-7 production (Chen *et al*., 2010; Berenjian *et al*., 2011). However, the increase of cell growth is higher than the increase of MK-7 production, indicating that more carbon source flux flows into cell growth rather than into MK-7 biosynthesis on the addition of 25 g/L yeast extract. Based on an optimization test, 10 g/L of yeast extract was the best concentration for MK-7 production (Fig. 2b). Fermentation performance with high concentrations may be reduced due to the synergistic effect between microbial proliferation and their activity (Chen *et al*., 2010). Moreover, a high concentration of nitrogen sources might inhibit cell growth and MK-7 production because of high osmotic pressure and low water activity (Berenjian *et al*., 2011).

A membrane-associated enzyme, polyprenyl pyrophosphate synthetase, has been used to catalyze the substrate prenyl-donating compounds (Saito and Ogura, 1981). Thus, the chain length of MK in bacteria may be controlled by the availability of prenyl pyrophosphates in cells (Tani *et al*., 1985). Consistent with previous results, prenyl alcohols are beneficial to MK-7 production because they provide prenyl for MK-7 production (Tani *et al*., 1985). It should be noted that the cell concentration and MK-7 production were decreased when the concentration of prenyl alcohols exceeded a certain threshold level (Table 2). Some studies have shown that prenyl alcohols have a harmful effect on cell growth, such as exhibiting wide antimicrobial properties (Lorenzi *et al*., 2009) and inhibiting biofilm biosynthesis (Unnanuntana *et al*., 2009). Therefore, the toxicity of prenyl alcohols towards microorganisms may be used to interpret the problems mentioned above.

Our results indicate that Mg^{2+} affects the MK-7 production in the batch fermentation of strain H.β.D.R.-5 (Table 3). These results are consistent with findings reported by Sagami *et al*. (1977) and Fujii *et al*. (1980), while they are in contrast to previous results reported by Takahashi *et al*. (1980). Sagami *et al*. (1977) and Fujii *et al*. (1980) have reported that the carbon chain length of polyprenyl pyrophosphates produced by polyprenyl pyrophosphate synthetase was dependent on the concentration of Mg^{2+}. However, Takahashi *et al*. (1980) pointed out that polyprenyl pyrophosphate synthetase of *B. subtilis* catalyzed the synthesis of all-*trans* C_{35} prenyl pyrophosphates. These results indicated that the polyprenyl pyrophosphate synthetase of strain H.β.D.R.-5 produced different kinds of polyprenyl

pyrophosphates ranging in carbon chain length from C_{15} to C_{45} in various ratios, and the ratios were dependent on the concentration of Mg^{2+}. In addition, Mg^{2+} supplement led to an increase in the utilization of sugar (Table 3). This is because Mg^{2+} participates in the energy metabolism of the cell and activates more than 300 enzyme systems, including sugar utilization, protein synthesis, adenosine triphosphate (ATP) metabolism, etc.

The production performance of strain H.β.D.R.-5 was investigated in a fed-batch process. During fermentation in a fermentor, MK-7 production started at a logarithmic growth phase and steadily approached a maximum (Fig. 3). It should be noted that the stationary phase of cell growth is a major phase of MK-7 biosynthesis. This result was in contrast with the previous reports that the majority of MK-7 production was during the logarithmic growth phase of *B. subtilis natto* (Berenjian *et al*., 2011). Wu and Ahn (2011) have reported that the amount of the intracellular MK-7 is larger than that of extracellular MK-7. However, the intracellular MK-7 can be released into the extracellular fraction by forming an intracellular complex with protein during the culture process (Armougom *et al*., 2009). Therefore, the amount of MK-7 in broth will be dramatically increased after the stationary phase because of cell lysis. Consistent with the previous reports (Sato *et al.*, 2001b; Berenjian *et al*., 2011), the pH was changed during the culture process (Fig. 3). This may be due to the breaking of the cell and effusion of intracellular alkaline substances (Berenjian *et al*., 2011). In addition, because of the different fermentation substrate, especially the different carbon source, the change of pH in GYS medium is sharper than that in the improved MYS medium.

In conclusion, we have isolated an MK-7-producing strain *B. amyloliquefaciens* Y-2, and subsequently mutated it by an ARTP mutation breeding system. The mutant *B. amyloliquefaciens* H.β.D.R.-5 with resistance to HNA, βTA, and DPA was obtained, which could use maize meal hydrolysate as carbon source to produce MK-7. Moreover, the effects of nitrogen source, prenyl alcohols, and $MgSO_4$ on MK-7 production were investigated, and MK-7 production and $Y_{P/X}$ were (61.3±5.2) mg/L and 2.59 mg/L per OD_{600} unit, respectively, when strain H.β.D.R.-5 was used in a 7-L fermenter under optimized conditions. These results indicated that strain H.β.D.R.-5 has a superior capacity to produce MK-7 from maize meal hydrolysate.

Compliance with ethics guidelines

Jian-zhong XU and Wei-guo ZHANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Biosynthetic pathway of MK-7 and regulation mechanism by inhibition of aromatic amino acids and diphenylamine (Armougom *et al*., 2009)

Fig. S2 Mutation rate and lethality rate of *B. amyloliquefaciens* Y-2 by ARTP

Fig. S3 Cell growth, MK-7 production, and sugar utilization of the mutant *B. amyloliquefaciens* H.β.D.R.-5 after several generations

中文概要

题　目：带有二苯胺和结构类似物抗性的芽孢杆菌突变株以玉米水解液为底物合成甲萘醌-7 的研究

目　的：通过从自然界中筛选和传统诱变育种相结合的方法，获得一株以玉米水解液为底物且能高效合成甲萘醌-7（MK-7）的芽孢杆菌突变菌株。

创新点：首次在中国的发酵豆制品——豆豉中分离得到一株能以玉米水解液为底物合成 MK-7 的解淀粉芽孢杆菌 Y-2（*Bacillus amyloliquefaciens* Y-2），并通过传统诱变育种获得一株带有二苯胺和结构类似物抗性的、以玉米水解液为底物的、高产 MK-7 的菌株 *B. amyloliquefaciens* H.β.D.R.-5。

方　法：以来自中国不同省市地区的豆豉为分离样品，筛选高产纳豆激酶的菌株，再从中挑选出高产 MK-7 的菌株，并通过 16S rDNA 分析对其种属进行鉴定。采用常压室温等离子体（ARTP）系统，对分离到的高产 MK-7 菌株进行诱变处理，获得解除 3-脱氧-D-阿拉伯庚酮糖-7-磷酸合成酶（即结构类似物抗性）和聚丙烯焦磷酸合成酶（即二苯胺抗性）反馈调节的菌株。最后，考察不同氮源、乙戊烯醇和镁离子（Mg^{2+}）对突变菌株合成 MK-7 的影响，并分析在 7 L 发酵罐中合成 MK-7 的区别。

结　论：从中国豆豉中分离到了一株以玉米水解液为底物合成 MK-7 的菌株，经 16S rDNA 分析比对，鉴定为 *Bacillus amyloliquefaciens*（图 1）。通过比较 MK-7 产量，发现利用 ARTP 可以有效获得解除反馈调节作用的且高产 MK-7 的突变菌株 H.β.D.R.-5（表 1）。以大豆水解液和酵母水解液为氮源，异戊醇和 $MgSO_4$ 有利于突变菌株 H.β.D.R.-5 合成 MK-7（图 2、表 2 和表 3）。综上所述，利用 ARTP 处理从中国豆豉中分离到的以玉米水解液为底物的合成 MK-7 的菌株，可获得高产的 MK-7 菌株，该方法对选育工业化合成 MK-7 的菌株有重要参考价值。

关键词：甲萘醌-7；解淀粉芽孢杆菌；类似物抗性；二苯胺抗性；玉米水解液