Annals of Microbiology (2018) 68:655–665
https://doi.org/10.1007/s13213-018-1372-9

ORIGINAL ARTICLE

Coproduction of menaquinone-7 and nattokinase by *Bacillus subtilis* using soybean curd residue as a renewable substrate combined with a dissolved oxygen control strategy

Han Wang[1,2] · Xiaojuan Sun[1,2] · Li Wang[1] · Hefang Wu[1] · Genhai Zhao[1] · Hui Liu[1] · Peng Wang[1] · Zhiming Zheng[1]

Received: 4 May 2018 / Accepted: 23 August 2018 / Published online: 14 September 2018

Abstract

Numerous physiological functions of menaquinone-7 (MK-7) act to reduce vascular calcification, suggesting that MK-7 may be a potential therapy for Alzheimer's and Parkinson's disease, and in this study, we attempted to increase the concentration of MK-7 synthesized by *Bacillus subtilis* natto, a standard nattokinase (NK) producing strain. Different *Bacillus subtilis* isolates demonstrated positive correlations between MK-7 and NK concentrations. Response surface methodology (RSM) was employed to optimize a culture medium for the simultaneous production of these molecules; the optimized medium contained the following components (%, *w*/*v*): soybean curd residue, 12.2; soya peptone, 5.7; lactose, 2.6; and K_2HPO_4, 0.6. The fermentation process was subsequently optimized based on online feedback control of fermentation process parameters. The dissolved oxygen (DO) concentration played an important role in the production of MK-7 and NK. With increased DO concentrations, the cell growth rate and NK activity increased. In contrast, at low DO concentrations, the concentration of MK-7 rapidly increased during the late fermentation stage. Thus, in this study, the production of MK-7 and NK by *Bacillus subtilis* was accomplished using soybean curd residue through medium optimization and DO control. This novel coproduction strategy was developed by controlling the aeration rate during the fermentation process. The concentrations of MK-7 and NK achieved in this study reached 91.25 mg/L and 2675.73 U/mL, respectively.

Keywords *Bacillus subtilis* · Menaquinone-7 · Nattokinase · Optimization of fermentation · Process control · Response surface

Introduction

Menaquinones, also named vitamin K2, consist of a 2-methyl 1,4-naphthoquinone nucleus and an isoprene side chain. There are 14 types of menaquinones (MK-1 to MK-14), which are differentiated by the number of isoprene units on the variable side chains. As a constituent of plasma membranes, menaquinone plays an important role in oxidative phosphorylation, transfer activity, and electron transfer (Vossen et al. 2015; Zhu et al. 2017). In recent years, menaquinone has been reported to potentially prevent Alzheimer's and Parkinson's diseases (Vos et al. 2015; Bhatt et al. 2018). MK-7 can be extracted from animal-derived foods, cheese and *natto*, a traditional Japanese food. Notably, *natto* contains a comparatively high amount of MK-7 relative to other foods (Mandinia et al. 2017). In addition to MK-7, *natto* also contains nattokinase (NK, also known as subtilisin NAT), a potent fibrinolytic enzyme (Cai et al. 2017). Nattokinase exhibits four-fold greater fibrinolytic activity than plasmin. Researchers observed that nattokinase not only has plasminogen activator activity but also has the ability to digest fibrin directly (Dabbagh et al. 2014). However, because of their low concentrations in soybeans, the current economic benefits of using soybean products to produce MK7 and

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13213-018-1372-9) contains supplementary material, which is available to authorized users.

✉ Peng Wang
pengwang@ipp.ac.cn

✉ Zhiming Zheng
zhengzhiming2014@163.com

[1] Key Laboratory of High Magnetic Field And Ion Beam Physical Biology, Hefei Institutes of Physical Science, Chinese Academy of Sciences, P.O. Box 1138, Comprehensive Laboratory Building, 350 Shushanhu Road, Hefei, Anhui 230031, People's Republic of China

[2] University of Science and Technology of China, Hefei 230026, People's Republic of China

nattokinase are very low. Thus, recent studies have primarily focused on generating genetic mutations and optimizing fermentation conditions to improve the production of MK-7 (Song et al. 2014; Hu et al. 2017). Additionally, optimization of the culture medium and fermentation process for NK production have been performed to obtain high fibrinolytic activity (Narasimhan et al. 2015).

Most *Bacillus subtilis* have the ability to produce nattokinase, while *Bacillus subtilis* also has the potential to synthesize MK-7 efficiently (Mandinia et al. 2017). In *Bacillus subtilis*, nattokinase is encoded by the gene *aprN* (Cai et al. 2017), whereas MK-7 is synthesized by the enzyme octaprenyltransferase, encoded by the gene *menA*. Since both MK-7 and NK have no competitive metabolic pathways, it would be of great economic benefit if MK-7 and NK could be produced simultaneously by *Bacillus subtilis*. However, although many studies have investigated the production of MK-7 and nattokinase, less attention has been given to the simultaneous production of MK-7 and NK by *Bacillus subtilis*. Liquid-state fermentation has been successfully used in coproduction strategies to generate high value-added bioproducts (Sekar et al. 2017). For example, 3-propanediol and L-lactate were produced simultaneously from *Bacillus coagulans* by coupling their production with the 1,3-propanediol-producing pathway (Xin et al. 2017). Another successful example of a coproduction strategy is the use of *Clostridium acetobutylicum* to simultaneously produce butanol and acetoin (Liu et al. 2015). Due to the fat-soluble nature of MK-7 (Wei et al. 2018) and the water-soluble nature of NK (Ni et al. 2016), the addition of metal ions with a valency of two or more could allow for MK-7 to be separated from NK (Shimatani et al. 2006). By applying an optimized control strategy, fermentation can improve the utilization rate of raw materials, enhance production value, and enrich product variety (Xu et al. 2015).

Because MK-7 yields are typically low, production costs of this molecule are high. Protein-rich natural materials can be used to replace expensive raw chemicals (Dajanta et al. 2012). Soybean curd residue is a soybean by-product that is often treated as waste, and every kilogram of soybeans processed into soymilk or tofu produces approximately 0.1 kg of fresh soybean curd residue. Soybean curd residue is rich in fiber, fat, protein, vitamins, and trace elements. Furthermore, the soluble and insoluble protein content in soybean curd residue is much higher than that of other crop by-products, such as wheat straw, corn stover, brewer's grains, and rice straw (Reddy et al. 2016). As is typical of industrial food processing waste, soybean curd residue is used as raw material for energy creation during fermentation. Different types of valuable products, such as crude antioxidants, iturin A, antioxidants, and phenolic compounds, can be biosynthesized by *Bacillus subtilis* from soybean curd residue (Li et al. 2013b).

In this study, an optimal medium for *Bacillus subtilis* growth using soybean curd residue was developed. The effects of the type and concentration of medium, as well as dissolved oxygen (DO) levels, on MK-7 and NK production by *Bacillus subtilis* were systematically investigated. Based on different DO demands at different growth stages, a new coproduction strategy for NK, a traditional bioproduct, and MK-7, a new high-value drug, by *Bacillus subtilis* is provided (see Fig. 1 for the optimized process from flask to bioreactor). The production of two or more valuable products using a single production process is not only economically attractive but also produces less wastes and potentially decreases pollution.

Materials and methods

Chemicals and reagents

Soybean curd residue was purchased from a commercial domestic supplier and was dried and powdered before use. Culture media components and reagents ($NaNO_3$, glycerol, starch, glucose, yeast extract, and K_2HPO_4, etc.) and organic solvents (methanol, n-hexane, n-butyl alcohol, and isopropanol, etc.) were obtained from Sinopharm Chemical

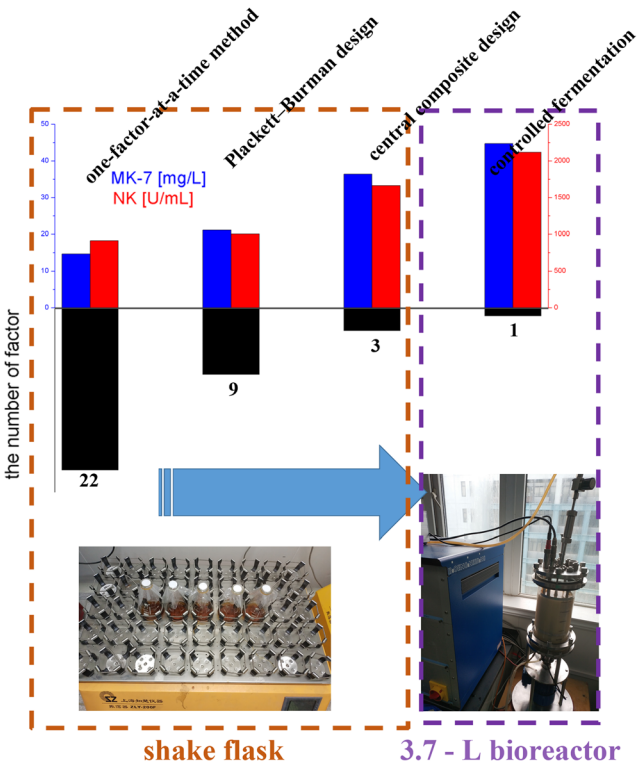

Fig. 1 The four optimization methods from the shake flask to the 3.7-L bioreactor. As the number of optimized factors decreased, the concentration of nattokinase and menaquinone increased

Reagent (Shanghai, China). MK-7 (98.0%) was provided by Wako Pure Chemical Industries (Japan). Fibrinogen and thrombin were purchased from Sigma-Aldrich (USA).

Microorganisms

Bacillus sp. 10262, 20637, and 20644 were purchased from the China Center of Industrial Culture Collection (CICC). *Bacillus* sp. 14255 was purchased from the China General Microbiological Culture Collection Center (CGMCC). *Bacillus* sp. BN-2-6, JA, and E8 were isolated from natto purchased from a market (Chen et al. 2008; Gong et al. 2009), and other strains were obtained through mutagenesis using an ion beam implantation device (Song et al. 2014). All strains were stocked in 20% glycerol and stored at − 80 °C.

Production of MK-7 and NK via fermentation

Seed medium was composed of 1.0% (*w*/*v*) soya peptone, 1.0% (*w*/*v*) glucose, 0.5% (*w*/*v*) NaCl, and water, with the final pH adjusted to 7.0. A 500-mL Erlenmeyer flask containing 100 mL of seed medium was inoculated with 5 mL of strain BN-P15-11-1, which was obtained from frozen stocks (− 80 °C, 25% glycerol). The culture was incubated on a rotary shaker (37 °C, 200 rpm) for 24 h. The fermentation culture was grown in a 500-mL flask containing 50 mL of liquid medium with 5 mL of inoculum on a rotary shaker (37 °C, 200 rpm). All experiments were replicated three times. MK-7 and NK production was measured after fermentation for 144 and 36 h, respectively (see in "Results and discussion").

Optimization research was carried out in a 3.7-L stirred bioreactor (Benchtop Fermenter KLF2000; Bioengineering AG, Wald, Switzerland). Agitation, temperature, pH, and DO were online controlled and recorded. Experiments were carried out at 37 °C with 2.0 L of culture medium. Pure cultured cells of *Bacillus subtilis* BN-P15-11-1 were inoculated in an initial medium volume of 200 mL (pH 7.0), which was subsequently added to the bioreactor. Filter-sterilized air was supplied to the bioreactors during the fermentation process. Experiments investigating DO control strategies were carried out in a 3.7-L bioreactor using the optimized medium (see in "Results and discussion"). The number of colony-forming units (CFUs) in cultures, the concentration of MK-7, and the activity of NK in the fermentation broth were measured every 12 h.

Analytical methods

The number of CFUs in culture was determined as previously reported by Berenjian et al. (2011).

MK-7 was extracted using a 2-propanol and n-hexane mixture by adjusting the protocol proposed by Berenjian et al. (2011). MK-7 was extracted from the fermentation medium with a 2-propanol:n-hexane (1:2, *v*/v) mixture, using 10 mL of the mixture for every 5 mL of medium. For each extraction, the mixture was mixed at a speed of 300 rpm for 30 min and then centrifuged at 15,000 rpm for 5 min to separate the two phases. The supernatant and n-butyl alcohol mixture was then shaken with a shaker at 300 rpm for 10 min. After incubating, the organic layer containing MK-7 was analyzed by high-performance liquid chromatography (HPLC, Shimadzu, Japan). A methanol/dichloromethane solution (4:1, *v*/*v*) was used as the mobile phase with a flow rate of 1 mL/min. A wavelength of 248 nm was selected for calibration and analysis. The LabSolutionsEssentia software was used for data acquisition (Berenjian et al. 2011). The MK-7 calibration curve was linear between 1 and 50 mg/L ($R^2 = 0.999$). The fibrinolytic activity of NK was determined by measuring the hydrolysis of fibrin (Anson 1938). The culture was centrifuged at 15,000 rpm at 4 °C for 5 min, and the clear supernatant was assayed for NK activity. First, 0.2 mL of a 0.3% fibrinogen solution was aliquoted into a test tube with 0.6 mL of 0.1 M Tris-HCl buffer (containing 10 mM $CaCl_2$, pH 7.8) and incubated at 37 °C for 5 min. After incubating, 0.1 mL of a 20-U/mL thrombin solution was added. The solution was incubated at 37 °C for 10 min, and 0.1 ml of the sample enzyme solution was added, after which the incubation continued at 37 °C for 60 min. The reaction was stopped by adding 1 mL of 0.2 M trichloroacetic acid (TCA). After the reaction mixture was centrifuged at 15,000 rpm for 5 min, 1 mL of the supernatant was collected, and the absorbance at 275 nm was measured. In this assay, 1 unit of enzyme activity is defined as the amount of enzyme needed to produce an increase in absorbance equal to 1.0 after 60 min at 275 nm (Deepak et al. 2008; Wang et al. 2009; Garg and Thorat 2014).

Experimental design for significant test of components

From many possible components, significant factors were obtained by using the one-factor-at-a-time method (OFAT method) and through a Plackett-Burrman design (PB design) (see in Fig. 1). In the OFAT method (Singh et al. 2016), soybean curd residue was the base component, and in each one experiment, only one complementary component was added to investigate its effect on MK-7 and NK production.

The PB design is a two-level design that is used to economically select significant medium components (Singh et al. 2016). Each independent variable was investigated at a high (+ 1) and a low (− 1) level. In Table 1, a PB matrix of nine variables and a 12-run design was constructed to screening significant variables using Design Expert software (version 8.0.6.1). It should be noted that the PB design could evaluate only significant factors with a large impact on the response (MK-7 or NK) (Meriem and Mahmoud 2017). The interaction

Table 1 The PB design matrix setup to the screen the primary factors for MK-7 and NK production

Run	Factors (g/L)									MK-7 production (mg/L)	Nattokinase production (U/mL)
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*		
1	100	15	15	5	20	20	10	0.8	0.8	19.62	981.10
2	100	15	5	15	20	20	20	0.3	0.3	16.83	624.63
3	100	5	5	5	10	10	10	0.3	0.3	13.49	507.07
4	150	15	15	5	20	20	10	0.3	0.3	19.45	1002.16
5	150	15	15	5	10	10	20	0.3	0.8	19.25	881.71
6	150	15	5	15	20	10	10	0.8	0.8	17.78	680.32
7	100	15	15	15	10	10	10	0.8	0.3	16.01	714.83
8	150	5	5	15	10	20	10	0.3	0.8	17.37	651.32
9	100	5	15	15	20	10	20	0.3	0.8	16.73	809.72
10	150	5	15	15	10	20	20	0.8	0.3	21.15	978.73
11	100	15	5	5	10	20	20	0.8	0.8	19.60	699.38
12	150	5	5	5	20	10	20	0.8	0.3	17.53	816.44

between different factors was investigated via response surface methodology (RSM).

Experimental design and statistical analysis by RSM

RSM was used in order to study the optimum values of and the relationships between one or more independent significant factors measured. In the central composite design, each independent variable was investigated at five coded levels, including the center point level (0), cube point level (− 1, 1), and axial point level (−α, +α), where α = 1.682. The value of the axial point (α) was $2^{k/4}$, where k was the number of variables (three in this study). Twenty sets of experiments were designed using Design Expert. Table 2 shows the three examined factors in 20 experiments. In each experiment, the medium and three significant variables were varied in their concentration according to the design produced by the software.

In RSM, the response (Y) can be predicted by the model of independent variables (X). The model was composed of an intercept term (β_0), linear coefficients (β_i), interaction coefficients (β_{ii}), and quadratic coefficients (β_{ij}), as shown in eq. 1 (Zheng et al. 2017). Coefficients were obtained using the software.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i-1}^{j-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \quad (1)$$

Results and discussion

Correlation between MK-7 production and NK activity

Before the optimization screening process, the correlation between MK-7 and NK production during the fermentation of soybean curd residue by different *Bacillus subtilis* strains was investigated. Scatter plots for the production of MK-7 and NK by 21 *Bacillus subtilis* strains are presented in Fig. 2 and in S.Table 1. Since strain BN-P15-11-1 exhibited a higher capacity for MK-7 production and NK activity, it was selected for subsequent experiments. The production of MK-7 exhibited a good linear relationship with NK, and the R^2 value of 0.72 demonstrates an acceptable correlation between MK-7 and NK. NK belongs to the serine alkaline protease family that cleaves peptide bonds between amino acids (Hedstrom 2002). NK can degrade the plasminogen activator inhibitor into smaller molecular fragments (Urano et al. 2001). In addition, NK also has the ability to directly digest fibrin. NK primarily accumulates during the preliminary stages of fermentation (Deepak et al. 2008; Cho et al. 2010), while the production of MK-7 occurs during the stationary phase (Berenjian et al. 2014). Since NK plays a crucial role in the proteolysis of culture substrate, NK may provide more favorable culture conditions for the accumulation of MK-7.

Significance test for soybean curd residue and complementary components

To optimize the production of MK-7 and NK, we first sought to identify significant factors that may have an effect on their production. Soybean curd residue was selected as the base component of the culture medium. Next, the effects of other complementary components on the concentrations of MK-7 and NK were investigated. Glucose (15 g/L, C% = 40%), glycerol (15.3 g/L, C% = 39%), sucrose (14.25 g/L, C% = 42%), starch (13.5 g/L, C% = 44%), dextrin (13.5 g/L, C% = 44%), lactose (14.25 g/L, C% = 42%), and maltose (15 g/L, C% = 40%) were tested as complementary carbon sources. Tryptone (6.4 g/L, N% = 12.5%),

Table 2 Experimental conditions for the central composite design and responses showing both the original and scaled factors

Run	Nutritional factors (g/L)			MK-7 production (mg/L)			NK activity (U/mL)		
	Soybean curd residue (*A*)	Soya peptone (*B*)	Lactose (*C*)	Experimental	Standard error	Predicted	Experimental	Standard error	Predicted
1	130	55	25	35.81	1.16	35.61	1534.13	297.76	1521.28
2	140	45	30	23.954	2.22	24.52	930.32	88.04	956.94
3	130	55	25	35.49	6.73	35.61	1516.47	283.96	1521.28
4	130	55	25	36.61	2.39	35.61	1564.83	185.67	1521.28
5	113.18	55	25	33.306	4.44	33.70	1412.21	333.41	1414.78
6	120	65	30	31.766	3.95	32.49	1315.84	78.59	1363.68
7	130	55	25	36.49	4.35	35.61	1559.74	227.07	1521.28
8	130	71.82	25	29.078	7.20	26.35	1150.34	286.61	1006.75
9	140	65	30	24.01	2.91	25.76	932.44	86.67	1004.62
10	130	55	16.59	32.004	2.74	30.41	1326.18	131.38	1227.97
11	146.82	55	25	28.406	5.83	25.87	1121.47	80.63	1003.12
12	120	45	30	31.528	1.95	30.65	1305.27	340.87	1254.55
13	120	65	20	29.26	0.83	30.21	1158.19	157.25	1213.44
14	140	45	20	25.13	3.51	25.92	980.41	54.33	1014.43
15	120	45	20	28.728	5.71	28.50	1135.23	202.89	1144.91
16	130	55	25	35.67	0.09	35.61	1523.82	211.53	1521.28
17	130	38.18	25	23.282	2.16	23.87	881.23	104.61	909.04
18	130	55	25	33.23	4.66	35.61	1408.82	157.54	1521.28
19	140	65	20	24.626	1.55	27.02	888.91	133.96	1021.50
20	130	55	33.41	31.71	2.53	31.16	1323.54	291.17	1305.97

soya peptone (10 g/L, N% = 8%), yeast extract (8 g/L, N% = 10%), $(NH_4)_2SO_4$ (3.8 g/L, N% = 21%), fish peptone (6.7 g/L, N% = 12%), $NaNO_3$ (5 g/L, N% = 16%), and beef extract (20 g/L, N% = 4%) were chosen as complementary nitrogen sources by employing an OFAT strategy. Each additional carbon (0.6%, *w*/*v*) or nitrogen (0.08%, *w*/*v*) sources was added to each medium such that the levels of these elements were consistent among the media. The inorganic salts selected included NaCl (0.5 g/L), K_2HPO_4 (0.5 g/L), $MnCl_2$ (0.5 g/L), $FeCl_3$ (0.5 g/L), $MgSO_4$ (0.5 g/L), $CaCl_2$ (0.5 g/L), and $ZnSO_4{\cdot}7H_2O$ (0.5 g/L). The effects of complementary carbon and nitrogen sources, as well as of inorganic salts, on fermentation were determined under the optimal soybean curd residue content. The optimum content of soybean curd residue for MK-7 and NK production was 120 g/L and is shown in Fig. 3a. Supplemental carbon sources, including sucrose, maltose, and lactose, exhibited significant effects on MK-7 production, but maltose showed no significant effect on NK production (Fig. 3b). The supplemental nitrogen sources soya peptone, fish peptone, and beef extract had more significant effects on MK-7 and NK production than the other tested sources (Fig. 3c). As shown in Fig. 3d, each experiment was carried out using one of these inorganic salts in single experiments. The results showed that the K_2HPO_4 had a greater influence on MK-7 and NK production than the other inorganic salts tested. Magnesium sulfate could also improve the synthesis of MK-7 with high added value.

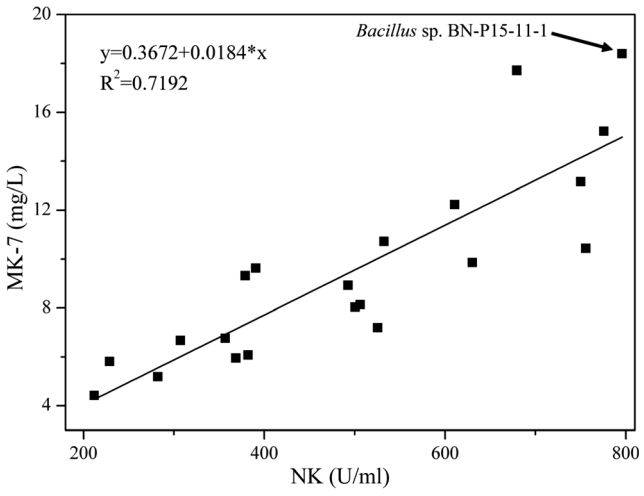

Fig. 2 Correlation between NK activity and MK-7 production in different microorganisms. The medium was composed of 10% (*w*/*v*) soybean flour and 5% (*w*/*v*) glycerol, the pH was maintained at the original value, and fermentation was carried out at 37 °C and 200 rpm in shake flasks

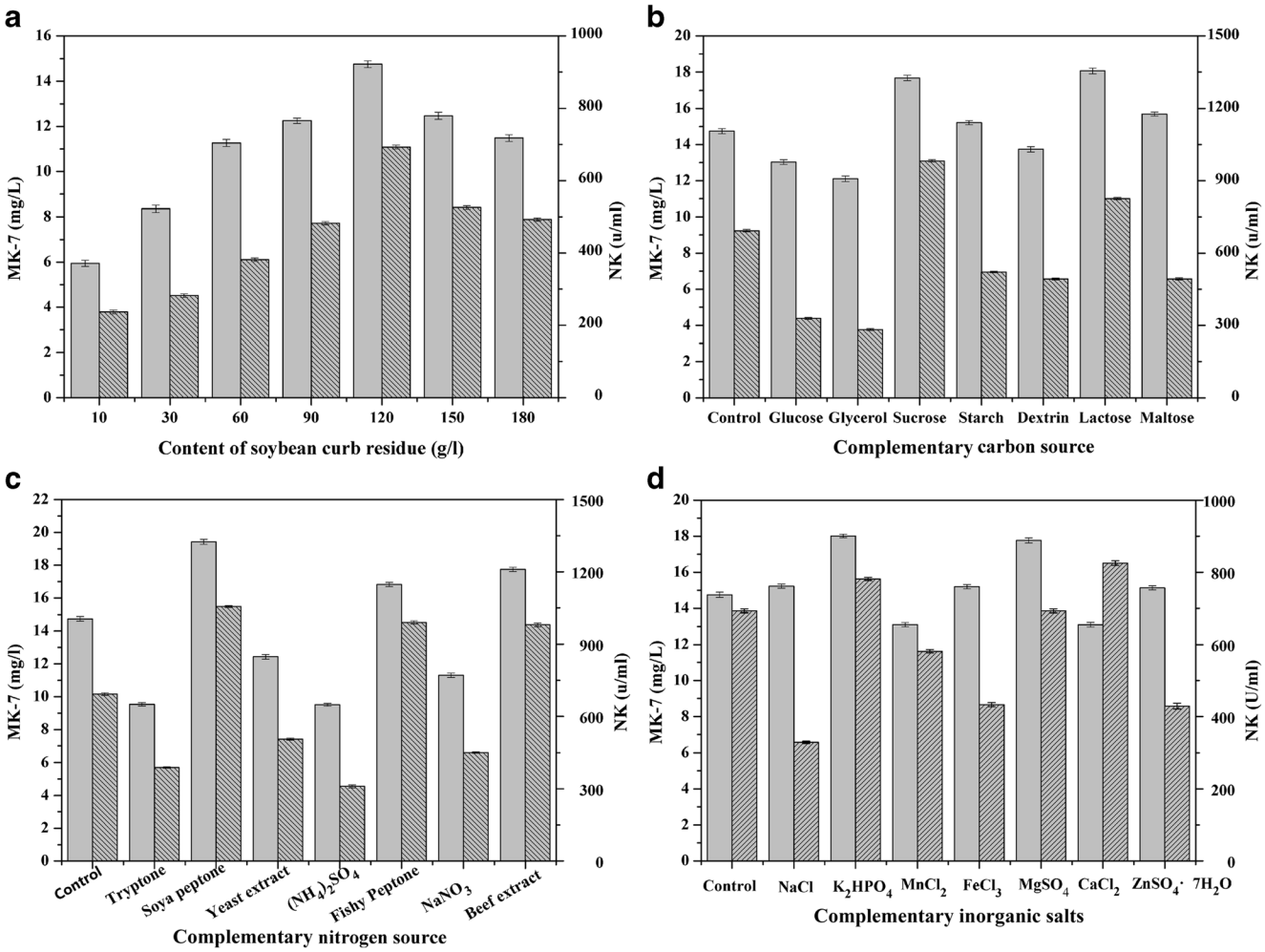

Fig. 3 MK-7 concentration and NK activity in different cultures media. **a** Different soybean curd residue contents ranging from 10 to 180 g/L. The effects of different **b** complementary carbon sources, **c** complementary nitrogen sources, and **d** inorganic salts. Fermentation was carried out at 37 °C and 200 rpm in shake flasks. Symbols: ▭, MK-7 concentration; ▨, NK activity

Identification of important variables with the PB design

The 12 runs of the PB design are shown in Table 1. Analysis of variance (ANOVA) of the model is summarized in S. Table 2. The highest MK-7 concentration (21.15 mg/L) was recorded for run 10, while the lowest production level (13.49 mg/L) was recorded for run 3. The highest NK activity (1002.16 U/mL) was recorded for run 4, while the lowest activity (507.07 U/mL) was recorded for run 3. The p value was used to indicate whether a factor was significant. When the P value was less than 0.05, the factor was significant (Thadathil et al. 2014). Figure 4 shows that maltose had no significant effect on the synthesis of MK-7 or NK. In contrast, the effects of soybean curd residue, lactose, and soya peptone on the concentrations of MK-7 and NK were the top three factors for all tested components. Thus, these three components were selected for value optimization by central composite design.

Identification of the optimal variables with the central composite design

Soybean curd residue, soya peptone, and lactose were tested at ranges from 11 to 15%, 3.5 to 7.5%, and 1.5 to 3.5% (*w*/*v*), respectively, and were selected to determine the general vicinity of the optimum values. In the steepest ascent, one condition (soybean curd residue (13%, *w*/*v*), soya peptone (5.5%, *w*/*v*), and lactose (2.5%, *w*/*v*)) was observed at the peak product concentration. Therefore, the concentrations of these factors were selected to generally represent the optimum values. The central composite design method was selected to obtain the optimum values for the three selected factors. Table 2 shows the actual values and the values predicted by the model. Runs 3 and 7 represent maximum MK-7 production values, with experimental and predicted values of 35.49 and 35.57 mg/L, respectively. Run 13 represents the maximum NK activity, with experimental and predicted values of 1600.27 and 1598.08 U/mL, respectively.

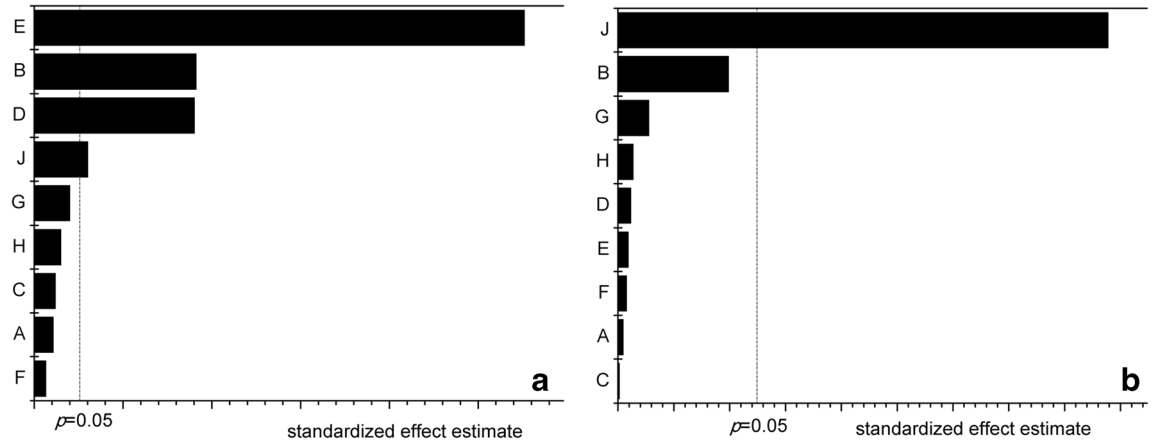

Fig. 4 Pareto chart showing the effects of different components on the biosynthesis of MK-7 (**a**) and NK (**b**). The tick labels indicate soybean curd residue (**a**), maltose (**b**), lactose (**c**), sucrose (**d**), beef extract (**e**), soya peptone (**f**), fish peptone (**g**), K_2HPO_4 (**h**), and $MgSO_4$ (**j**)

MK-7 production was designated a response factor (*Y1*), and NK activity was designated a response factor (*Y2*). *A*, *B*, and *C* represent the soybean curd residue, soya peptone, and lactose concentrations in g/L, respectively. The ANOVA and regression coefficients for the model are listed in S. Table 3. In the MK-7 production model, an *F*-value = 10.58 and *P* value < 0.05 indicated that the model was significant. Similarly, the NK production model was significant at *F*-value = 11.65 and

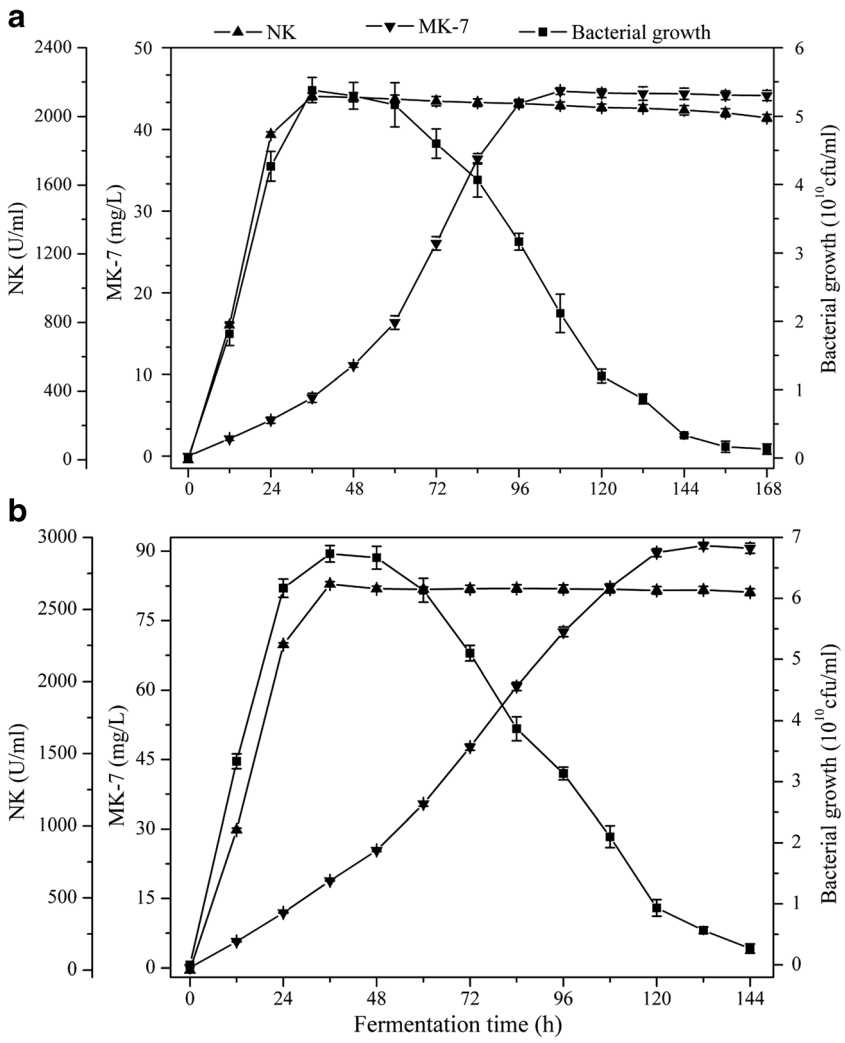

Fig. 5 Changes in CFUs and MK-7 and NK production during the course of 3.7-L bioreactor fermentation at 37 °C and 200 rpm. The medium was composed of soybean curd residue (12.2%, *w*/*v*), soya peptone (5.7%, *w*/*v*), lactose (2.6%, *w*/*v*), and K_2HPO_4 (0.6%, *w*/*v*). The aeration rate was 1.2 vvm for 168 h (**a**) and 2.0 vvm for 36 h and then was maintained at 0.8 vvm for 108 h (**b**)

P value < 0.05 (Kirrolia et al. 2014). Comparison between the predicted and observed values showed that the model had a good correlation with the experimental data. These data demonstrate that for MK-7 production, the optimum soybean curd residue concentration was between 12 and 12.5% (*w*/*v*), and the optimum lactose concentration was between 2.5 and 2.8% (*w*/*v*). Similarly, the highest value for MK-7 production was obtained by increasing the concentration of soybean peptone to 5.6% (*w*/*v*), but with any further increase in the soybean peptone concentration, the production of MK-7 decreased. For NK production, the data demonstrate that the optimum soybean curd residue concentration was between 11.5 and 12.5% (*w*/*v*), and the optimum lactose concentration was between 2.3 and 2.8% (*w*/*v*). Increasing the soya peptone concentration up to 5.7% (*w*/*v*) resulted in higher NK activity, although further increases resulted in a decrease in NK activity. There was a high level of interaction between soybean curd residue and soya peptone in MK-7 and NK production. The highest MK-7 production (36.39 mg/L) and highest NK activity (1563.53 U/mL) were predicted by the model for the following conditions: soybean curd residue (12.2%, *w*/*v*), soya peptone (5.7%, *w*/*v*), and lactose (2.6%, *w*/*v*). The statistical results for optimized values for different factors were verified in a 500-mL flask experiment. The experimental achieved MK-7 production of 36.37 mg/L and NK production of 1663.303 U/mL, which are very close to the values predicted by the model for the 500-mL flask.

Two-stage DO control strategy to produce of MK-7 and NK

Bacillus subtilis can grow vigorously in the presence of a good oxygen supply. When the cells are agitated, the desired bioproduct can be increased by ten-fold (Nikiforova et al. 2016). Typically, a scale-up step from a shake flask to a bioreactor can improve the concentration of the desired bioproduct using *Bacillus subtilis* (Dedavid e Silva et al. 2014), which was also observed in this study (see in Fig. 5a). This is because the impeller and the air distributor significantly increase the oxygen transfer coefficient in a stirred tank bioreactor relative to a shake flask (Li et al. 2013a; Xie et al. 2014). Based on the above results, the DO concentration had an important effect on the production of MK-7 and NK. It has been reported that NK biosynthesis is affected by the shaking speed in flask cultures (Kwon et al. 2011) and that MK-7 is sensitive to DO concentrations (Sato et al. 2001). However, the relationship between MK-7 production, NK activity, and DO control strategies has not been studied.

Considering the limited rotation speed of shake flasks, the effects of different oxygen supply conditions on the production of MK-7 and NK were investigated in a 3.7-L bioreactor, which was more beneficial for the industrial scale-up. A previous study showed that hydrodynamic forces affect the growth of cells. Therefore, we chose to change the aeration rates to study the effects of the oxygen

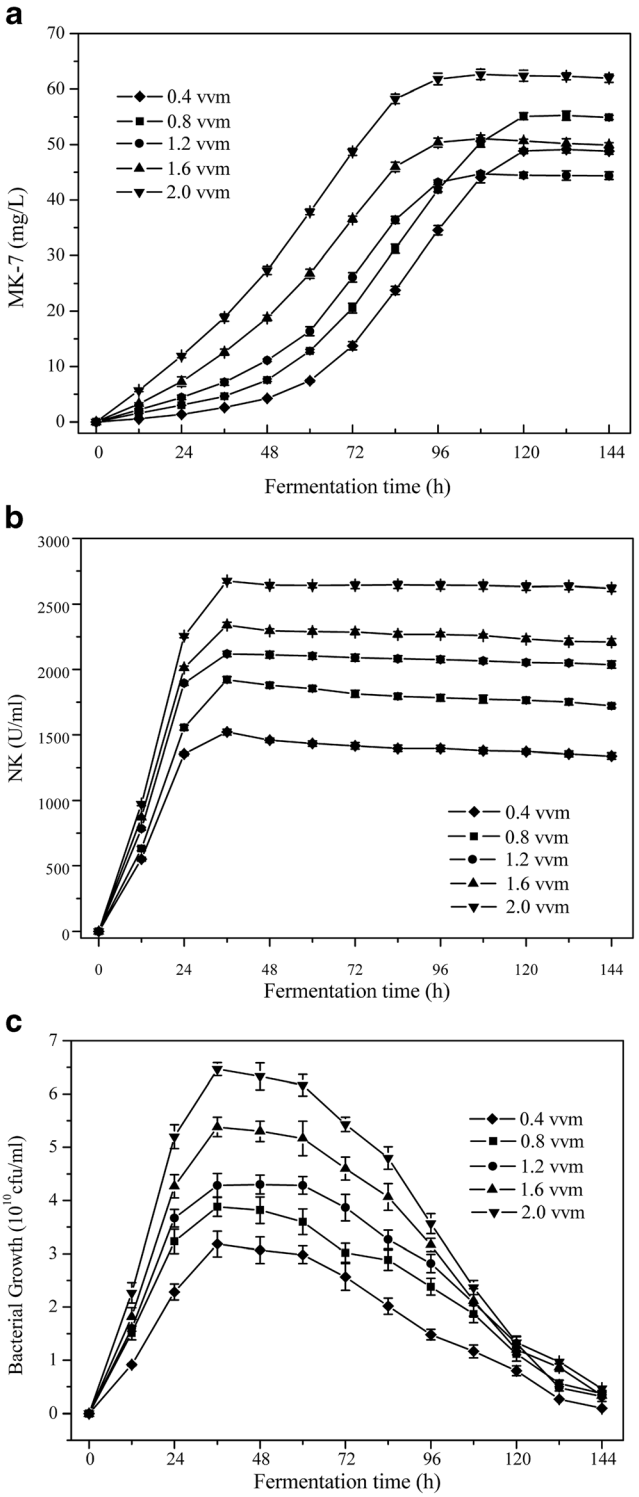

Fig. 6 Changes in **a** the MK-7 concentration, **b** NK activity, and **c** CFU levels at different aeration rates during the course of 3.7-L bioreactor fermentation. The medium was composed of soybean curd residue (12.2%, *w*/*v*), soya peptone (5.7%, *w*/*v*), lactose (2.6%, *w*/*v*), and K2HPO4 (0.6%, *w*/*v*)

supply on the fermentation process in *Bacillus subtilis* (Wang et al. 2018). Five different aeration rates, ranging from 0.4 to 2 vvm, were tested (Fig. 6). For MK-7 production, the optimum aeration rate was 2.0 vvm, which resulted in an MK-7 concentration of 62.63 mg/L. However, the MK-7 concentration quickly increased after 72 h, when the aeration rate was maintained at 0.8 vvm, and the values were even higher than those for reactions in which the aeration rate was maintained at 1.2 vvm (Fig. 6a). NK activity gradually reached maximal levels at 36 h, as shown in Fig. 6, similar to previously reported data (Kumar et al. 2011). As shown in Fig. 6b, when the aeration rate was maintained at 0.4 vvm, only NK activity at 1521.24 U/mL accumulated in the broth after 36 h of fermentation. However, NK activity reached as high as 2675.73 U/mL within 36 h when the aeration rate was maintained at 2.0 vvm. With an increased aeration rate, NK activity underwent a progressive increase, which was similar to the cell growth trend. In a previous study, NK activity was observed to be associated with bacterial growth (Kwon et al. 2011), which is in agreement with the results of this study (Fig. 5a). A highly significant correlation between the growth of *Bacillus* sp. and the DO concentration in the culture broth was observed. When the aeration rate was maintained at 0.4 vvm, bacterial levels were only 3.183×10^{10} CFU/mL. In contrast, when the aeration rate was maintained at 2.0 vvm, bacterial CFU counts increased to 6.467×10^{10} CFU/mL. Maximum bacterial growth was obtained at 36 h of fermentation and decreased gradually thereafter (Fig. 6c). NK activity, MK-7 production, and bacterial growth were all sensitive to the DO concentration in the culture broth. A high DO concentration was beneficial for bacterial growth and NK activity, but a low DO concentration was more suitable for MK-7 accumulation during the late fermentation steps. Some have studies observed that the production of MK-7 is related to the early stage of spore formation in *Bacillus subtilis* (Sato et al. 2001). Therefore, it is possible that the abundant accumulation of *Bacillus subtilis* spores at a lower aeration rate of resulted in enhanced MK-7 production. Since MK-7 is crucial for the respiration of microorganisms (Sharma et al. 1993), another explanation for the changes in MK-7 concentrations at lower DO levels may be that the concentration of MK-7 increased to allow cells to resist the negative effects of the low-DO environment.

Therefore, to enhance MK-7 production and NK activity, culture conditions should be maintained at a lower aeration rate, after cell growth and NK activity reach a maximum level. The optimum culture conditions were an aeration rate of 2.0 vvm at 37 °C for 36 h followed by an aeration rate of 0.8 vvm for 108 h for cell culture. In this case, the final values achieved were 6.73×10^{10} CFU/mL, 91.25 mg/L MK-7 and 2675.73 U/mL NK concentrations (Fig. 5b).

Conclusions

The production of MK-7 and NK was significantly improved by the optimization of medium containing soybean curd residue, soya peptone, lactose, and K_2HPO_4 using a PB design and a central composite design. The final MK-7 (36.37 mg/L) and NK (1663.303 U/mL) concentrations were two times higher than those produced under the initial conditions. A two-stage DO control strategy was used to enhance MK-7 and NK production. The concentrations of MK-7 and NK reached 91.25 mg/L and 2675.73 U/mL, respectively. The results showed the great advantage of using soybean curd residue to achieve high concentrations and yields through the simultaneous production of MK-7 and NK.

Funding This research was funded by The National High Technology Research and Development Program of China (2014AA021704) and by the Major Projects of Science and Technology in Anhui Province "Development and Demonstration of Vitamin K2 Functional Food" grant (grant no. 17030801036).

Compliance with ethical standards

Conflicts of interest We declare that we have no conflict of interest. We also thank Dr. Shen, Zhiyong for his suggestions in the writing of this manuscript.

Research involving human participants and/or animals This study does not involve human or animal experiments.

Informed consent This study does not involve human or animal experiments.

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