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A N I N T E R N A T I O N A L J O U R N A L

Optimization Medium Composition for Vitamin K_2 by *Flavobacterium* sp. using Response Surface Methodology and Addition of *Arachis hypogaea*

Mu Tan[1], Hui Liu[1], Zhemin Li[1], Xiaowen Sun[1], Zhiming Zheng[1], Genhai Zhao[*1]

[1]Key Laboratory of Ion Beam Bioengineering, Hefei Institutes of Physical Science - Chinese Academy of Sciences Hefei, Anhui, China.

ABSTRACT

The purpose of this research was to enhance the production of vitamin K_2 by fermentation optimization and Arachis hypogaea supplementation in Flavobacterium sp. mutant SP-L-01. Optimized culture condition were as follows: 6-days shake-flask culture at 37^oC with initial pH value 7.0 ± 0.2, shaking speed in 120 r/min and medium volume of 30 mL with 2% inoculums. After optimization of fermentation medium by response surface methodology (RSM), optimized medium were maltose 23.8 g/l, glucose 9.69 g/l, beef extract 15 g/l, K_2HPO_4 4.5 g/l , NaCl 3.0 g/l and $MgSO_4 \cdot 7H_2O$ 0.3 g/l. Production of vitamin K_2 after optimization reached to 10.97 mg/l, which is 79.25% higher than that before optimization (6.12 mg/l). 3 mg/mL of arachis hypogaea was added into the medium at 72 h of shake-flake cultivation, which improved the production of menaquinone-4 (MK4) up to 371% and menaquinone-6 (MK6) up to 149% higher than those of the original medium. D-(+)-catechin, one of the components of arachis hypogaea, was added alone into the medium, which also improved the vitamin K_2 synthesis.

Key words: *Arachis hypogaea*, D-(+)-catechin, response surface methodology, vitamin K_2

[1]Author for correspondence: zhgh327@126.com

INTRODUCTION

In natural environment, vitamin K exists in two different types: vitamin K_1 (VK_1, phylloquinone/ phytomenadione) and vitamin K_2 (VK_2, menaquinones) (Liu et al. 2014). VK_2 contains a series of compounds forms of which are common in 2-methyl-1, 4-napthoquinone nucleus and different in the structures of a side chain (Berenjian et al. 2011). It is mainly synthesized by microorganisms and can be divided into different types according to its length of side chains (menaquinone-n or MK-n, where n refers to the number of isoprene units on the isoprenoid tail). In bacteria, VK_2 is involved in the electron transport, oxidative phosphorylation and active transport. Previous researches have proven that VK_2 do influence blood coagulation and bone metabolism (Ishida 2008). A new discovery has revealed that VK_2 may also work on treating mitochondrial pathologies such as Parkinson's disease and amyotrophic lateral sclerosis (Vos et al. 2012). On account of those meaningful functions, ways of increasing the production of VK_2 need to be studied further. Previous studies have mostly focused on *Escherichia coli.* while seldom have been done in *Flavobacterium* sp, in which VK_2 were mainly synthesized in forms of MK4 and MK6. In *Escherichia coli*, the increased biosynthesis of MK under anaerobic conditions was due to its role as an obligatory hydrogen carrier for the oxidation of dihydroorotate coupled to fumarate reduction (Bentley and Meganathan 1982). The demand of oxygen for producing VK_2 on *Flavobacteirum* sp. still needs to be studied by exploring the fermentation condition. In 1987, Japanese researchers obtained a high amount of MK4 in cells of *Flavobacterium* sp. mutant by investigating MK production of strain K_3-15 compared with different medium components at different period (Tani et al. 1987). Also, addition of cedar wood oil increased the productivity of MK, especially MK4 (Yoshiki and Hisataka 1988). Maltose is found to be an inducer in *MAL* gene expression in Saccharomyces cells (Wang et al. 2002). In *Flavobacteria meningosepticum*, the genes for Endo (endoglycosidase) F2 and Endo F3 have been studied in 1993. These genes are fused separately to the *Mal* E gene of *E.coli* and expressed as enzymically active fusion proteins joined to the maltose-binding protein (Anthoy et al. 1993). Previous researches have focus on fermentation optimization of VK_2 in *Bacillus subtilis* mostly (Wu and Ahn 2011). According to these, maltose used as carbon source and optimized fermentation condition by using more accurate method in *Flavobacterium* sp. need to be determined in order to increase the production of VK_2.

Previous study established groundnut meal as potential low-cost substrate for alkaline protease production by *B. subtilis* SHS-04. The core component of groundnut meal is *Arachis hypogaea* (*A. hypogaea*), which is reported to provide high quality dietary protein and oil, and was rich in minerals and vitamins (E, K and B group) (Olajuyigve 2013). *A. hypogaea* is very beneficial to cells. It contains procyanidins and polyphenols, which have antioxidative, anti-carcinogenic and anti-hyperglycemic functions (Tomochika et al. 2011). Zhang et. al. had proposed that D-(+)-catechin in form of ethanol extract could be obtained by silica column chromatography from *A. hypogaea*. D-(+)-catechin can astringe excised rabbit ear vessel (Zhang et al. 1990), which is accord with the function of stypticity of VK_2. Many animal experiments showed that D-(+)-catechin decreased the permeability and fragility of capillary. This was coincidence with the hemostasis of *A. hypogaea* and VK_2. In addition, previous studies had proved the inhibition mechanism of D-(+)-catechin on decarboxylases of *Escherichia coli* (TchanGi and Shigeaki

1962). In this way, Addition of *A. Hypogaea* could be an effective way of improving the VK_2 sythesis on *Flavobacterium* sp..

In this study, we investigated the best culture condition for VK_2 production and optimized the fermentation medium by using response surface methodology (RSM). Also, we attempted to expand our understanding of the effects of *A. hypogaea* on VK_2 synthesis in *Flavobacterium* sp..

MATERIAL AND METHODS

Microoraganism

The strain was named as *Flavobacterium* sp. SP-L-01, which was obtained as a compound mutant of chemical (N-methyl-N-nitro-N-nitroso-guanidine treatment) and physical mutation (N^+ low energy ion beam) in our laboratory.

Media and fermentation composition

The seed and fermentation medium were made up of the following components (g/L): glycerol 20, peptone 10, yeast extraction 1.5, K_2HPO_4 4.5, NaCl 3, $MgSO_4 \cdot 7H_2O$ 0.3. The pH value was initially adjusted to 7.0 ± 0.2. Solid medium was in same components except for agar 1.5%. The strain was preserved in glycerin tube (glycerin: inoculum=1:1) at -80 ^oC.The seed was cultured at 37 ^oC and 200 r/min for 24 h and the fermentation broth was cultured at 37^oC and 120 r/min for 6 days.

Extraction and measurement of vitamin K_2

The fermentation broth (25 mL) was centrifuged at 15000 x g for 15min, which would be divided into cells and culture fluid. For maximum removal of glycerin, the cells were stirred with distilled water and then centrifuged at 15000 x g for 10 min. The resulting cells were freezed at -20^oC in refrigerator for 30 min and dried in vacuum cold drying machine for 12 h. After that, methyl alcohol (5 mL) was added to the dried cells and the extraction was maintained for 12 h statically. To obtain the sample in the minimum impurities, the organic phase was centrifuged at 15000 x g for 10 min and filtered through organic membranes. The concentration of VK_2 was measured by high performance liquid chromatography (HPLC). Methyl alcohol and dichloromethane were selected to be the mobile phase whose ratio was 4:1 (v/v). To measure the cell biomass, the dry cell mass was calculated by analytical balance.

Optimization experimental design of culture condition

Culture conditions were fermentation temperature (25^oC, 28^oC, 33^oC, 37^oC and 40^oC), initial pH value (6, 6.5, 7, 7.5, 8 and 8.5), shaking speed (0, 60, 120, 280, 240 and 300 r/min), inoculum size (2%, 5%, 10% and 15%) and medium volume (30 mL, 50 mL, 75 mL and 100 mL). The relative productions of VK_2 in this designed medium were all compared with original medium. We set up the control production as 100%. Each test was carried out in three sets of parallel repetition.

Experimental design

To optimize the fermentation conditions of SP-L-01, the fermentation medium that had a significant effect on VK_2 synthesis were identified by Plackett-Burman (PB) design. Experimental variables and levels in PB were shown in Table 1. In this design, eight components were chosen. Each component was set into two levels: low (-) and high (+) level. The factors with a confidence level at or above 95% were selected to be optimized later (Nanthakumar et al. 2013). Box-Behnken design was conducted to optimize the selected effective factors from PB design. The three independent variables were evaluated at three different levels (-1, 0, +1). Table 2 presented the actual factor

levels corresponding to coded factor. Response surface methodology (RSM) was used to analyze the results. Each test was carried out in three sets of parallel repetition.

Table 1-Experimental variables and levels in Plackett-Burman

Term		Value	
Variable	Component	Low(-)	High(+)
X_1	Maltose	50	70
X_2	Glucose	40	60
X_3	Glycerol	20	40
X_4	Beef Extract	20	40
X_5	Peptone	10	20
X_6	K_2HPO_4	2	5
X_7	NaCl	2	4
X_8	$MgSO_4 \cdot 7H_2O$	0.5	1

Table 2-Actual factor levels corresponding to code factor in Box-Behnken

	X_1(Maltose) (g/l)	*X2*(Glucose) (g/l)	*X3*(Beef Extract) (g/l)
-1	10	5	15
0	20	10	25
+1	30	20	40

Effect of *A. hypogaea* on vitamin K_2 production

Different concentrations (0, 0.1, 0.3, 0.5, 0.7, 1, 1.2, 1.5, 2, 2.5, 3%, 4 and 20%) and different add-time (0, 8, 12, 24 and 48) of *A. hypogaea* were designed. To further investigated the functhion of *A. Hypogaea*, D-(+)-catechin was added alone into the fermentation medium in different concentrations (0, 0.04, 0.1, and 0.2, 0.5 and 1 mg/mL). *A. hypogaea* was in pured-powder form obtained from local market. D-(+)-catechin was in crystalloid form obtained from Chengdu PureChem-Standard Co., Ltd.

RESULTS

Optimization of culture condition

Flavobacterium sp. was sensitive to temperature in this study. According to the tendency, MK4 was more likely to be influenced by temperature compared with MK6. 37^{o}C was the best for the synthesis of MK4 and MK6 (Fig. 1A). In consideration of error limit, we set the initially pH to 7 ± 0.2 (Fig. 1B). Maximum production was achieved with 120 r/min, 2% inoculum size and 30 mL medium in 250 mL shake flask (Fig. 1C, D, E).

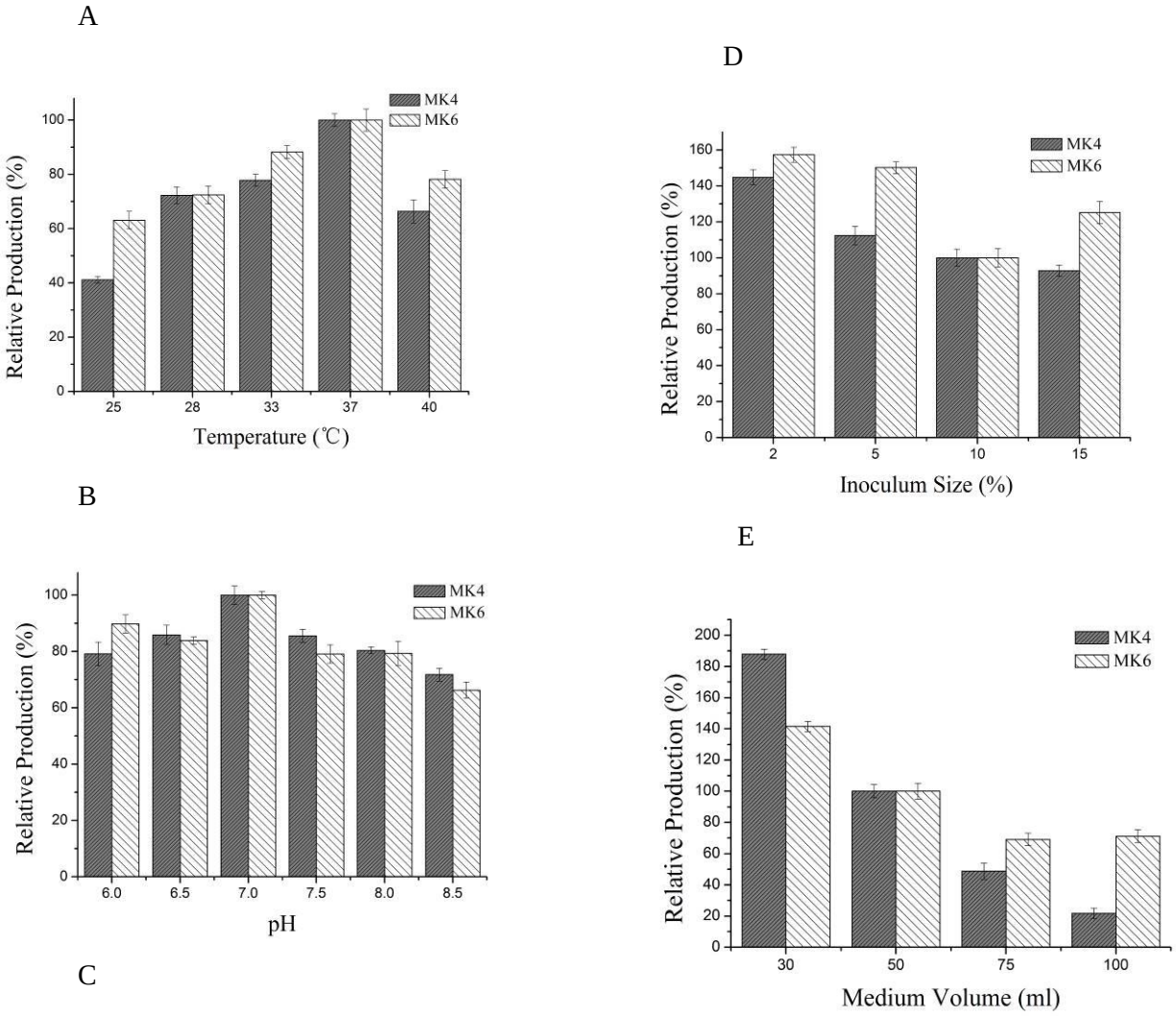

Figure 1 - Effect of fermentation conditions on production of MK4 and MK6

Plackett-Burman design

Initial selecting of the most significant components affecting vitamin K_2 production by SP-L-01 was obtained by Plackett-Burman design. Table 3 represented the Plackett-Burman for 12 trials with two levels of values for each variable and the results with respect to vitamin K_2 production. Table 4 showed the results with respect to the conefficient, mean square, F-value, *p*-value, and confidence level of each component. We screened the components for which the confidence level was at or above 95%.

Table 3-Plackett-Burman design matrix with corresponding results

Run	Variable											Production of Vitamin K2 (mg/l)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	$D1$	$D2$	$D3$	
1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	7.5
2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	10.1
3	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	4.34
4	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	4.97
5	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	3.4
6	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	3.6
7	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	3.2
8	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	3.6
9	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	7.05
10	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	4.1
11	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	6.5
12	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	6.5

*X*1-*X*8: variable; D1-D3: dummy variable; (+1): high level; (-1): low level

Table 4-Plackett-Burman regression analysis

Term		Value		Coefficient	Mean Square	F-Value	P-Value	Confidence level(%)
Variable	Component	Low(-)	High(+)					
X_1	Maltose	50	70	-1.04	12.90	42.84	0.0073	99.3
X_2	Glucose	40	60	-0.56	3.72	12.35	0.0391	96.1
X_3	Glycerol	20	40	-0.43	2.20	7.31	0.0735	92.3
X_4	Beef Extract	20	40	-1.34	21.49	71.40	0.0035	99.7
X_5	Peptone	10	20	0.32	1.23	4.08	0.1366	86.3
X_6	K_2HPO_4	2	5	0.21	2.18	3.72	0.1514	84.9
X_7	NaCl	2	4	0.19	1.24	3.13	0.1545	84.5
X_8	$MgSO_4 \cdot 7H_2O$	0.5	1	0.18	1.20	2.95	0.1655	83.5

We used the Design-Expert to analyze the results on Table 3 and obtained Table 4. The most influential variable were identified as follows: $X_4>X_1>X_2>X_3>X_5>X_6>X_7>X_8$. The confidence levels of X_4、X_1 and X_2 were above 95%. We chose the three variables as significant factors and used them for the further study. Coefficient of X_4、X_1 and X_2 were all negative, which revealed that the actual doses should be reduced subsequently.

Box-Behnken design

Maltose, glucose and beef extract were considered to be significant and optimized by the Box-Behnken design. Table 5 showed the design and results of VK_2 production in Box-Behnken experiment.

Table 5-Box-Behknen design matrix and experimental results

	X_1	X_2	X_3	Production of Vitamin K2 (mg/l)
1	1	0	1	7.8
2	0	0	0	10
3	-1	0	-1	2.125
4	0	-1	-1	10.625
5	-1	0	1	4.025
6	0	0	0	10
7	0	-1	1	3.35
8	1	0	-1	8.75
9	0	0	0	10
10	0	0	0	9.5
11	-1	1	0	3.075
12	0	1	1	5.525
13	1	1	0	3.725
14	-1	-1	0	5.2
15	0	1	-1	8.25
16	0	0	0	10.05
17	1	-1	0	6.25

A total of 17 experiments that contained 12 factorial points and 5 replicates at the center point were presented in Table 5. After being analyzed by ANOVA, the results were showed in Table 6.

Table 6 showed the analysis of variance and reliability. The model at P=0.01 is extremely significant. ANOVA for VK_2 production reveled that the F-value of the model was 27.63, and the value of "Prob>F" less than 0.0001, indicating model terms were significant. X_1, X_2, $X_1 \times X_1$, $X_1 \times X_3$ and $X_2 \times X_3$ were significant model terms in this study. The squared correlation coefficient R^2 and adjusted R^2 were 0.9260 and 0.7709, respectively, which revealed that the model can explain 93% variability in the response and showed the adequacy of the model to predict the response. Coefficient of variation (CV%=15.67) can also illustrate the precision and reliability of the model. Regression equation can describe the real relationship between each variable and response value. We can get the optimal medium from the model. Design-Expert was used to make regression fitting and led to the second-degree polynomials equation of VK_2 production with maltose, glucose and beef extract:

$$Y=15.38+1.86\times X_1+0.80\times X_2+0.11\times X_3-0.008\times X_1\times X_2-0.0057\times X_1\times X_3+0.012\times X_2\times X_3-0.036\times X_1\times X_1-0.041\times X_2\times X_2-0.0044\times X_3\times X_3$$

The three-dimensional response surface curves were made to illustrate the interaction among the three independent variables and to conclude the optimum condition (Fig. 2). Each curve represented a combined effect of two tested variables on the response with the other variable at zero level. The result predicted that the maximum VK_2 production (11.11 mg/L) was obtained with maltose concentration of 23.8 g/L, glucose concentration of 9.69 g/L and beef

extract concentration of 15 g/L. According to the optimal medium component, three sets of parallel repetition on experiments were explored and the actual production of vitamin K_2 was about 10.97 mg/l, which was similar to the predicted one and was about 79.25% higher than that of the VK_2 production before optimization (6.12 mg/L).

Table 6-Standard analysis of variance for Box-Behknen experiment results

Source	df	Sum of squares	Mean square	F-Value	P-Value(Prob>F)	Significance
X_1	1	24.85	24.85	14.07	0.0090	*
X_2	1	21.02	21.02	12.33	0.0073	*
X_3	1	10.24	10.24	4.32	0.0711	
$X_1 \times X_1$	1	53.21	53.21	17.28	0.0043	*
$X_1 \times X_2$	1	1.44	1.44	0.47	0.5161	
$X_1 \times X_3$	1	62.03	62.03	20.31	0.0036	*
$X_2 \times X_2$	1	12.13	12.13	5.18	0.0451	
$X_2 \times X_3$	1	22.18	22.18	10.68	0.0060	*
$X_3 \times X_3$	1	1.95	1.95	0.63	0.4527	
Model	9	208.20	68.24	27.63	<0.0001	
Pure Error	0.21	4	0.053			
Lack of Fit	9	6.49	0.72	3.25	0.1344	
Total	18.21	218.69				
R^2	0.9260					
Adjusted R^2	0.7709					
CV.%	15.67					

*Significant at 99% confidence level

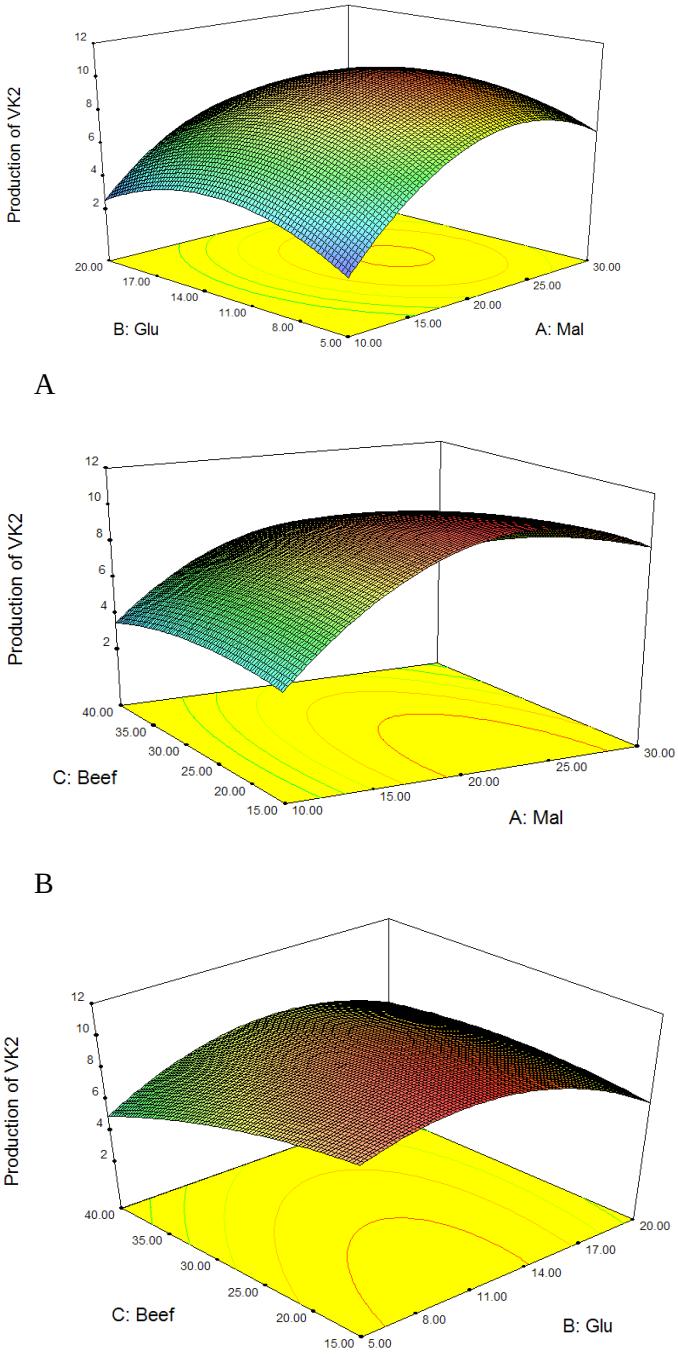

Figure 2-RSM three dimensions analysis lines

VK_2 production and cell biomass contract before and after optimization had been given in Figure 3. Sharply rising period of VK_2 production after optimization appeared 12 h earlier than that before optimization. Both two fermentation conditions reached to climax at 144 h. As to cell biomass, from 12-72 h, it remained higher after optimization than that before optimized design. However, after 72 h, the cell biomass declined deeply and was lower than that of original condition.

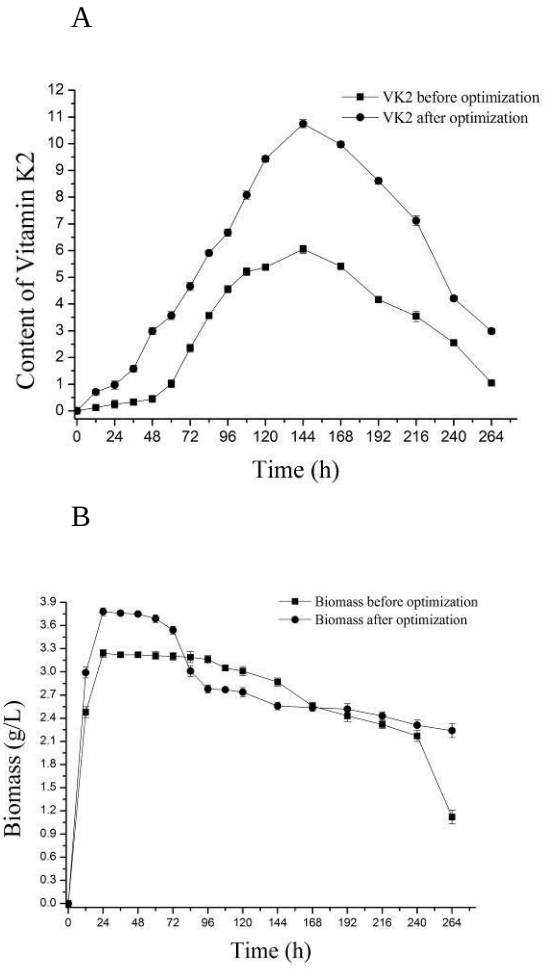

Figure 3-Fermentation process curve of mutant strain SP-L-01 before and after optimization

Involvement of *A. hypogaea* in vitamin K_2 synthesis

We set up a concentration gradient from 0.1 to 20 mg/mL of *A. hypogaea*, which would be compared with the control group (original medium without *A. hypogaea*). According to Figure 4, the tendency of VK_2 production showed a rise first followed by a decline. To be specific, when *A. hypogaea* was added with concentration (0.1-0.5 mg/mL), VK_2 output decreased, especially MK4. As the concentration rising to 2.5 mg/mL, yield of MK4 reached the

top (186%). However, the top point of MK6 appeared when A. hypogaea was added with a concentration of 3 mg/mL (137%). As mentioned above, we had preliminarily made sure that adding 3 mg/mL of *A. hypogaea* could obtain higher production than original one.

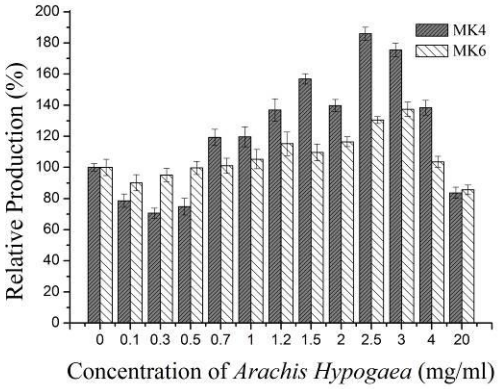

Figure 4-Effects of *arachis hypogaea* concentration on production of MK4 and MK6

Besides being a component of medium, *A. hypogaea* might be used as an inducer as well. In order to investigate the influence of add-time on VK_2 synthesis, we choose 3 mg/mL as fixed concentration firstly and added *A. hypogaea* from the beginning of fermentation to 96 h after that. Even though adding *A. hypogaea* at 8 h resulted in the worst effect, it still led to higher production than the control one. We obtained such an obvious tendency from Figure 5, which influenced MK4 yield in particular. The best effect was happened at 72 h, which enhanced production of MK4 up to 371%, as well as 149% for MK6. Add-time at 96 h had worse effect than that at 72 h, but still better than control production.

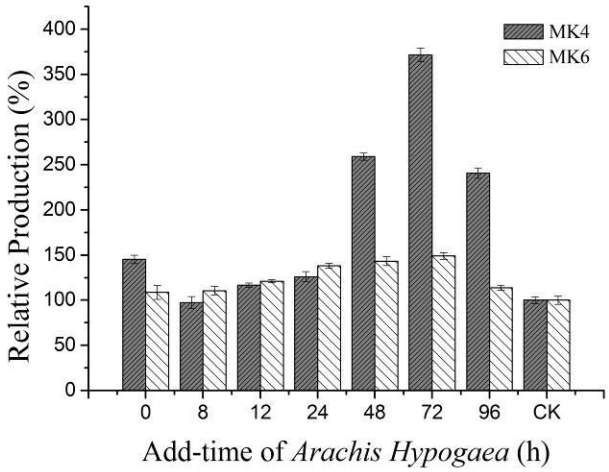

Figure 5- Effects of *arachis hypogaea* add-time on production of MK4 and MK6

At last, we made a tentative study on investigating the effective component of *A. hypogaea* medium (Fig. 6). We set the concentration of D-(+)-catechin from 0.04 to 1 mg/mL in the beginning of fermentation. The bar chart revealed that D-(+)-catechin did have an effect on enhancing VK_2 production. Both MK4 and MK6 had improved. At 0.04 mg/mL, the yield of MK4 was close to 126%. At 0.2 mg/mL, the yield of MK6 was close to 125%. It seemed as if MK4 accumulation might come to saturation state earlier than MK6. Through comparative analysis in Figure 4-6, we held opinion that D-(+)-catechin played a certain role in stimulating synthesis of VK_2, regardless of unobvious results compared with *A. hypogaea* powder. D-(+)-catechin at 0.2 mg/mL was an optimized concentration, which remained to be further investigated.

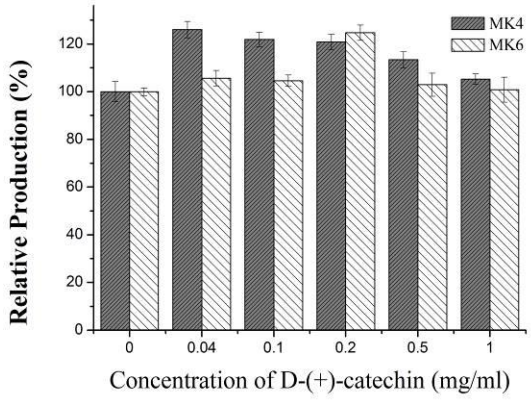

Figure 6-Effects of D-(+)-catechin concentration on production of MK4 and MK6

DISCUSSION

As to the culture condition, temperature and initial pH affect cell growth and enzymatic activity. Shaking speed, inoculum size and medium volume were all related to dissolving oxygen amount. So far, there are no literatures that are published to deal with the problem of VK_2 of the facultative anaerobe *Flavobacterium* sp. under anaerobic environment, in which VK_2 biosynthesis might be increased by anaerobiosis (Bentley and Meganathan 1982). Static cultivation with paraffin isolation was conducted here. Unfortunately, the production of VK_2 was close to zero (data not given). Shaking speed, inoculum size and medium volume curves also showed that lower oxygen amount did not enhance the VK_2 production. Our result revealed that VK_2 in *Flavobacterium* sp. did not chiefly synthesis under anaerobic environment. pH value was in the lowest level in anaerobic condition, which led to high concentration of acid and restrained the growth of *Flavobacterium* sp. (Russell and Diez-Gonzalez 1997). The interaction among fermentation medium components is complex. Response surface methodology is a common statistic method, which is very useful in the optimization of biotechnological processes. After Plackett-Burman experiment, we chose three factors and three levels. Our experimental data presented a optimized medium and culture condition for higher production of VK_2 on *Flavobacterium* sp.. Agreed with previous study, the RSM analysis in our research suggested that maltose in fermentation medium is used to an extreme and affected most in carbon sources. Comparison of fermentation curves illustrated that optimized medium and culture condition had shifted the VK_2 producing period to an early time.

A. hypogaea contained rich phenolic compounds and resveratrol, which had been known to help improve endurance ability, eliminate inflammation and prevent cardiovascular disease. Polyphenols in *A. Hypogaea* have physiologic effects, including anti-oxidative one (Tamura et al. 2013). In addition, *A. hypogaea* contains scavenging enzymes such as SOD, APX and CAT (Sankar et al. 2007). Moreover, a study found that menadione, as the superoxide generator, would arise cell toxicity (Vattanaviboon et al. 2003). *A. hypogaea* help to scavenge free radical on membrane of *Flavobacterium* sp. and slow down the cell damage to improve condition of bacterial growth. Low molecular weight catechins, especially in monomers and dimers, could significantly participate in antioxidant power of red wine (Katalinic et al. 2004). Our result presented that D-(+)-catechin at dose of 0.04-1 mg/mL improved the production of VK_2. Moreover, synthesis of Co-Q was one of the competitive ways of VK_2 metabolic and contained decarboxylase reaction. D-(+)-catechin significantly participated in antioxidant power of *A. hypogaea* and inhibited the synthesis ways of Co-Q. The confirmatory research remains to be conducted after this.

CONCLUSIONS

Optimized fermentation conditions and *A. hypogaea* supplement were investigated in *Flavobacterium* sp. mutant SP-L-01 in this study. The best culture conditions are 6-days shake-flask culture at 37°C with initial pH value 7.0 ± 0.2, shaking speed in 120 r/min and medium volume of 30 mL with 2% inoculums. By using response surface methodology, maltose 23.8 g/L, glucose 9.69 g/L, beef extract 15 g/L, K_2HPO_4 4.5 g/L ,NaCl 3.0 g/L and $MgSO_4 \cdot 7H_2O$ 0.3 g/L are determined to be the optimal fermentation medium. VK_2 production after optimization was about 10.97 mg/L, which was 75.97% higher than that before optimization (6.12 mg/L). *A. hypogaea* was found in this investigation as an useful inducer for improving the secretion of MK4 and MK6. Addition of *A. hypogaea* at 72 h of fermentation in the dose of 3 mg/mL increased the production of MK4 to 371% and MK6 to 149% compared with the original data. Moreover, D-(+)-catechin was one of the important components of *A. hypogaea* for the enhancement effect and its optimum concentration was 0.2 mg/mL.

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