Combinatorial Methylerythritol Phosphate Pathway Engineering and Process Optimization for Increased Menaquinone-7 Synthesis in *Bacillus subtilis*

Taichi Chen1,2†, Hongzhi Xia3†, Shixiu Cui1, Xueqin Lv1, Xueliang Li1, Yanfeng Liu1, Jianghua Li2, Guocheng Du1, and Long Liu1*

1Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, P.R. China
2Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, P.R. China
3Richen Bioengineering Co., Ltd., Nantong 226000, P.R. China

**Introduction**

Vitamin K2 (menaquinone) is an essential vitamin existing in the daily diet, and menaquinone-7 (MK-7) is an important form of it. In a recent work, we engineered the synthesis modules of MK-7 in *Bacillus subtilis*, and the strain BS20 could produce 360 mg/l MK-7 in shake flasks, while the methylerythritol phosphate (MEP) pathway, which provides the precursor isopentenyl diphosphate for MK-7 synthesis, was not engineered. In this study, we overexpressed five genes of the MEP pathway in BS20 and finally obtained a strain (BS20DFHG) with MK-7 titer of 415 mg/l in shake flasks. Next, we optimized the fermentation process parameters (initial pH, temperature and aeration) in an 8-unit parallel bioreactor system consisting of 300-ml glass vessels. Based on this, we scaled up the MK-7 production by the strain BS20DFHG in a 50-l bioreactor, and the highest MK-7 titer reached 242 mg/l. Here, we show that the engineered strain BS20DFHG may be used for the industrial production of MK-7 in the future.

**Keywords:** Menaquinone, methylerythritol phosphate pathway, *Bacillus subtilis*, process optimization

Vitamin K2 (menaquinone, MK) is a series of compounds that share the 2-methyl-1,4-naphthoquinone but with different lengths and saturation degrees of the polyisoprene side chain attached to the 3-position. Among these compounds is menaquinone-7 (MK-7), which is the term for 2-methyl-3-heptaprenyl-1,4-naphthoquinone, meaning the polyisoprene side chain consists of seven prenyl units [1]. MK-7 can be directly absorbed and utilized by the human body and has a longer half-life in human blood than other forms of vitamin K [2]. Addition of MK-7 in the daily diet can prevent bone fractures [2], treat vitamin K deficiency hemorrhagic disease [3], or prevent arterial calcification [4]. The biologically active MK-7 is mainly produced by microbial fermentation, including solid-state fermentation and liquid fermentation [5, 6]. The solid-state fermentation takes natto (fermented soybean) as the substrate, and the reported maximum yield is 67 mg/kg by *Bacillus subtilis natto* [7]. The parameters of solid-state fermentation are challenging to control, and a large amount of metabolic heat is generated, resulting in the slow growth of bacteria and low MK-7 production. Therefore, increasing attention is being paid to the production of MK-7 by liquid fermentation, and the strains used in this method include *Bacillus subtilis natto*, *Bacillus subtilis licheniformis*, *Flavobacterium*, etc. [8-10]. Previous studies have explored the effects of biofilm growth, dissolved oxygen changes, pH and electron generation on the production of MK-7 [2, 11-13]. In recent work, we engineered the synthesis pathway of MK-7 in *B. subtilis* using a quorum-sensing system, and the highest titer reached 360 mg/l in shake flasks and 200 mg/l in the 15-l bioreactor [14].

*B. subtilis* is Generally Recognized as Safe (GRAS) and is a typical gram-positive bacterium. The biosynthesis of MK-7 precursors in *B. subtilis* can be divided into two modules (Fig. 1). Naphthoquinone, derived from chorismic acid, is the bone structure of MK-7. The polyisoprene side chain is synthesized from glyceraldehyde-3-phosphate (G3P) and pyruvate (PYR) through the MEP pathway. Then, the polyisoprene side chain will be joined to the naphthoquinone to form demethylmenaquinone (DMK) by 1, 4-dihydroxy-2-naphthoate octaprenyltransferase (MenA). Finally, MK-7 is synthesized when the methylation of DMK is finished. In other recent work, we developed a bifunctional quorum-sensing system in *B. subtilis* 168 to engineer the synthesis modules of MK-7, and obtained a recombinant strain BS20 [14], while the MEP pathway had not been engineered. There are many studies about the MEP pathway and its final product, isopentenyl diphosphate (IPP). The accumulation of IPP can
increase the production of many natural products such as menaquinone, ubiquinone, carotenoids and taxadiene [15-19]. 1-deoxyxylulose-5-phosphate synthase (Dxs) and 1-deoxyxylulose-5-phosphate reductoisomerase (Dxr) are thought to catalyze rate-limiting steps of the MEP pathway. Up-regulating Dxs and Dxr in B. subtilis can significantly improve the production of MK-7 [5]. The titer of carotenoids in B. subtilis was increased by engineering the five enzymes (IspD - IspH) catalyzing MEP to IPP and dimethylallyl diphosphate (DMAPP) [16].

In this study, we focused on enhancing the transcription level of five isp-genes (ispD, ispE, ispF, ispH, and ispG) in the MEP pathway to promote the production of MK-7. The highest MK-7 production of 415 ± 3.2 mg/l was observed in shake culture of the engineered strain BS20DFHG. Then, we conducted fermentation optimization in an 8-unit, 300-ml bioreactor system, and the highest titer of MK-7 was 185.57 ± 6.3 mg/l at 41°C, 2 vvm and an initial pH of 7.0. Finally, we conducted fermentation in a 50-l bioreactor under optimized conditions, and 242 ± 5.5 mg/l of MK-7 was produced.

**Materials and Methods**

**Microorganisms and Reagents**

All the constructed B. subtilis strains and plasmids in this study are listed in Table 1. All microorganisms were cultivated in Luria–Bertani (LB) liquid culture or on LB agar plates at 37°C for genetic experiments. The
Shake-Flask Culture of the Engineered described [20]. Finally, the intracellular plasmid PDG148 was lost by incubation at 50°C for 12 h. promote the recombination of lox71 and lox66, which evicted the resistance marker cassette as previously

B. subtilis cassettes were combined by overlapping PCR. The purified PCR products were used to transform competent from

A wavelength of 254 nm was used for calibration and analysis. The MK-7 calibration curve was linear between

USA) at 40°C. The mobile phase consisting of methanol: dichloromethane (9:1, v/v) was used at a flow rate of 1 ml/min.

concentration of fermentation samples with a C18-ODS column (5 μm, 250 × 4.6 mm, Thermo Fisher Scientific,

MK-7 was then separated as a fermentation sample for HPLC analysis.

addition, 500 μl of fermentation broth was taken every 24 h for MK-7 concentration analysis.

MK-7 Extraction

MK-7 was extracted from the fermentation broth by extracting agent, which was a mixture of 2-propanol and n-

hexane were obtained from Sigma-Aldrich (USA).

and chloramphenicol (5 μg/ml). All antibiotics were purchased from Sangon Biotech Co., Ltd. (China). A

purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Appropriate antibiotics were added

into the medium: kanamycin (50 μg/ml), zeocin (20 μg/ml), ampicillin (100 μg/ml), spectinomycin (50 μg/ml),

and n-hexane were obtained from Sigma-Aldrich (USA).

In Situ Replacement of Promoter

The expression of isp-genes was strengthened by replacing the native promoter with the strong constitutive

promoter P43. The homogenous arms of isp-genes, target sequences and P43 promoter sequences were amplified

from B. subtilis. The modified DNA fragments and plasmids were sequenced by GENEWIZ (USA). Primers used

for genetic engineering were listed in Table S1. These three fragments and the lox71–resistance marker–lox66

cassettes were combined by overlapping PCR. The purified PCR products were used to transform competent B. subtilis cells by electroporation. The PDG148 plasmid was then transformed into antibiotic-resistant clones to promote the recombination of lox71 and lox66, which evicted the resistance marker cassette as previously described [20]. Finally, the intrachromosomal plasmid PDG148 was lost by incubation at 50°C for 12 h.

Shake-Flask Culture of the Engineered B. subtilis

A ring of engineered B. subtilis strains was picked from the plate and inoculated into 3 ml LB at 37°C with

shaking at 220 rpm for 6 h in a 15-ml tube. Each 250-ml Erlenmeyer flask contained 20 ml of fermentation

medium, which was inoculated with 1 ml of seed liquid and grown at 41°C with shaking at 220 rpm for 6 days. In

addition, 500 μl of fermentation broth was taken every 24 h for MK-7 concentration analysis.

MK-7 Extraction

MK-7 was extracted from the fermentation broth by extracting agent, which was a mixture of 2-propanol and n-

hexane (1:2, v/v) in a 4:1 ratio (organic: liquid, v/v). The mixture was vigorously shaken with a vortex mixer for

10 min and then centrifuged at 7,000 ×

For qPCRs. qPCR was performed in a 96-well plate with a total reaction volume of approximately 20 μl using SYBR Premix Ex Taq TM (Takara, China) according to the manufacturer’s specifications. The reactions were conducted with a
Statistical Analysis

All experiments were independently carried out at least three times, and the results were expressed as mean ± standard deviation (SD). All the data shown were mean values and are based on the recorded data unless otherwise indicated.

Results and Discussion

Increased MK-7 Production by MEP Pathway-Strengthened BS20DFHG Strain

To increase the flux of the MEP pathway, we overexpressed ispD genes by replacing native promoters with P₄₃ promoter stepwise. Then we conducted fermentation of the recombinant strains in 250-ml shake flasks for 6 days because the highest yield of MK-7 was reached on this day (Fig. S1). Moreover, the relative transcriptional level of ispD genes was detected on the third day of culture.

First, we replaced the promoter of the gene ispD with P₄₃ promoter in the genome of BS20, yielding the strain BS20D. Moreover, 2-C-methylerythritol 4-phosphate cytidylyltransferase (IspD) is a phosphocytidyl transferase encoded by ispD and is able to couple 2C-methyl-D-erythritol 4-phosphate (MEP) with cytidine triphosphate (CTP). The relative transcriptional level of ispD in BS20D was 1.78-times higher than the level of ispD in BS20 (Fig. 2C). The optical density (OD₆₀₀) of BS20D rose to 26 in the first two days, and then quickly dropped to 6 on the fifth day (Fig. 2A). At the same time, the increase in OD₆₀₀ value also corresponded to an increase in glucose consumption (Fig. 2B). It was shown that the fermentation titer of MK-7 by BS20D was augmented to 353.2 ± 1.2 mg/L, a 10% increase compared with the production 320.3 ± 2.5 mg/L by BS20 (Fig. 2D). Then we used P₄₃ promoter to overexpress ispF in the genome of BS20D, yielding strain BS20DF. In addition, 2-C-methylerythritol 2,4-cyclodiphosphate synthase (IspF) is a cyclodiphosphate synthase encoded by ispF. Although the relative transcriptional level of ispF in BS20DF was 1.3 times higher than that of ispF in BS20D (Fig. 2C), the fermentation titer of MK-7 by BS20DF was 332.6 ± 3 mg/L. The result showed a 3.9% MK-7 titer increase compared with BS20D but a 5.8% decrease over BS20D (Fig. 2D). This was in contrast to previous research that was more important in the flux control of MEP pathway than ispD [22]. In their study, Li focused on the systematic analysis of consecutive enzymes in the MEP pathway in B. subtilis and successfully increased the production of carotenoids. The yield of carotenoids, which also requires IPP as precursor, showed an 11.38-fold increase after additional insertion of gene ispF in ispD-overexpressed strain. The reason for the different influence degree of IspF might be that the key points of metabolic flux are usually different because of the difference in different engineered strains [22].

The strain BS20DFE was obtained by overexpressing ispE with P₄₃ promoter in BS20DFD. Although the growth curve of BS20DFE had the same trend as the other engineered strains (Fig. 2A), the highest OD₆₀₀ value of BS20DFE was only 16, and it consumed merely 12 g of glucose in 6 days (Fig. 2B). MEP was speculated to form IspF-MEP complex with IspE in E. coli although it might lead to an imbalance in the MEP pathway [23]. If the ispD, ispE, and...
ispF genes are overexpressed in BS20, the intracellular concentration of IspF may increase, and this might lead to the formation of IspF-MEP complex, resulting in an imbalance in the MEP pathway.

IspG is the term for 4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP) synthase that is encoded by ispG. The overexpression of ispG has been reported to promote the production of natural products like lycopene and isoprene that utilize IPP as a biosynthetic precursor [15, 19]. Meanwhile, it was found that activation of the downstream enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (IspH) could solve the problem of HMBPP accumulation and eliminate the negative effects of ispG overexpression [24]. Thus, we decided to overexpress ispH before ispG to avoid the potential risk of IspG toxicity.

Gene ispH was overexpressed by changing the autologous promoter to P43 promoter in the strain BS20DF, and the obtained strain was named as BS20DFH. The relative transcriptional level of ispH was 4.87-times higher than that of ispH in BS20 (Fig. 2C). The overexpression of ispH increased MK-7 titer to 370.8 ± 5.2 mg/l (Fig. 2D), showing a 15.8% increase compared with that of BS20. The growth curve of BS20DFH was similar to that of BS20, while the amount and rate of glucose consumption increased (Fig. 2B). Finally, we replaced the promoter of ispG with P43 promoter in the strain BS20DFH, yielding strain BS20DFHG. Although the strain BS20DFHG did not grow as well as BS20 in the first two days, the OD600 of BS20DFHG in the last four days declined slower than BS20 and eventually stayed at 8.8 (Fig. 2A). Thus, we obtained the highest production of MK-7 as 415 ± 3.2 mg/l (Fig. 2D), a 29% increase compared with BS20. In a previous work, the dxs, dxr, idi, and menA genes were overexpressed in BS168 to improve the production of MK-7 [5]. In this study, we overexpressed the isp genes of MEP pathway in the BS20 strain to increase the production of MK-7.

Optimizing Process Conditions with 300-ml Parallel Bioreactors

To increase the concentration of MK-7 in bioreactors, we designed a set of orthogonal experiments through the software Minitab to optimize the parameters of the fermentation process, including temperature, aeration and initial pH. The orthogonal experimental design is shown in Table 2. The experimental design of the three groups of “1”, “2”, and “3” was selected for each factor. The final fermentation titer of the strain BS20DFHG cultured in 300-ml bioreactors was used as the evaluation index. The experimental design and results are shown in Table 3. From the analysis of the extreme difference, the impact intensity of three factors on the fermentation production of MK-7 was C (initial pH) > A (temperature) > B (aeration).

The initial pH was the most important factor among these three factors. The mean of MK-7 production at pH 7.0 was 129.55 mg/l, while the mean of the MK-7 output at pH 5.5 was only 59.20 mg/l. It might be related to the fact that the strain BS20DFHG did not grow well when the initial pH was set at 5.5 (Fig. 3). When the initial pH was set at 8.5, the growth of the strain showed a delay of 10 h compared with the strains cultured at pH 7.0 (Fig. 3). The trend of growth curves in all conditions was similar to that in shake flasks. When the fermentation temperature was 41°C, the production of MK-7 was higher than that at the other temperatures, regardless of the other two
factors (Table 3). The aeration, which was expected to play an essential role in fermentation [11], however, didn't have much effect on the biosynthesis of MK-7. As a result, the optimal fermentation scheme could be determined as 41°C, 2 vvm and an initial pH of 7.0 (A3B3C2). Under these conditions, the engineered strain BS20DFHG produced the highest MK-7 titer of 185.57 ± 6.3 mg/l in a 300-ml bioreactor.

Constructing a Cell Growth Kinetic Model of BS20DFHG in 50-l Bioreactor

After optimizing the three parameters, we determined the optimal fermentation scheme for MK-7 production. According to the optimization experiments of fermentation parameters such as pH and temperature, the biomass

Table 3. Effect of three factors on the titer of MK-7.

| Run | A  | B  | C  | MK-7 production (mg/l) |
|-----|----|----|----|------------------------|
| 1   | 1  | 1  | 1  | 53.54 ± 2.4            |
| 2   | 1  | 2  | 2  | 90.37 ± 5.3            |
| 3   | 1  | 3  | 3  | 88.73 ± 6.1            |
| 4   | 2  | 1  | 2  | 112.72 ± 5.4           |
| 5   | 2  | 2  | 3  | 136.92 ± 4.9           |
| 6   | 2  | 3  | 1  | 58.75 ± 3.3            |
| 7   | 3  | 1  | 3  | 158.66 ± 6.9           |
| 8   | 3  | 2  | 1  | 65.32 ± 4.1            |
| 9   | 3  | 3  | 2  | 185.57 ± 6.3           |

Mean1: 77.54667, 108.3067, 59.20333
Mean2: 102.7967, 97.53667, 129.5533
Mean3: 112.79, 111.0167, 128.1033
Range: 35.24333, 13.48, 70.35

Fig. 3. Trends of cell growth, glucose concentration, pH and DO during fed-culture in 300-ml bioreactors. The symbols 'A-I' correspond to Run 1-9 in Table 3. (A) A1B1C1, (B) A1B2C2, (C) A1B3C3, (D) A2B1C2, (E) A2B2C3, (F) A2B3C1, (G) A3B1C3, (H) A3B2C1, and (I) A3B3C2. 'A1, A2, A3' represented three gradients of culture temperature ranging from 37 to 41°C, 'B1, B2, B3' represented three gradients of aeration ranging from 0.6 to 2.0 vvm, 'C1, C2, C3' represented three gradients of initial pH ranging from 5.5 to 8.5.
of BS20DFHG at the end of logarithmic growth would affect the final production of MK-7. So, we decided to employ the optimized scheme in 50-l bioreactor fermentation (Fig. S2). Subsequently, a cell growth kinetic model was constructed to describe and predict the cell growth of BS20DFHG. We separated the growth curve into two parts according to the increase or decrease trend of OD600. As shown in Fig. 4A, the strain BS20DFHG grew rapidly in the first 18 h, and the OD600 slowly decreased in the following 3 days. The growth of the strain BS20DFHG could be modeled by an equation in the form of Eq. (1), including the microbial biomass \( X \), the death coefficient \( K_d \) and the specific growth rate \( \mu \). In the first 18 h of the fermentation process (Fig. 4B), the growth curve indicated that there is a relationship between the specific growth rate \( \mu \) and the concentration of limiting nutrients or inhibiting substrates. The negative correlation between \( \mu \) and the concentration of carbon source \( C \) is presented in Fig. 4D. However, the concentration of glucose (10 g/l) is not considered to inhibit bacterial growth based on previous experimental results (Fig. 2A). Therefore, we turned our attention to the consumption of the nitrogen source which could be seen in Fig. 4E, because the nitrogen source usually affects the synthesis of proteins in cells and further affects the growth of strains. As can be seen in Fig. 4F, the concentration of nitrogen source \( N \) and the \( \mu \) exhibit positive correlation after linear fitting (Eq. (2)) \( A = 0.01, B = 0.0333 \). The dynamic relationship of the \( N \) and \( X \) was given in the form of Eq. (3) \( Y_{X/N} = 0.7276 \).

Due to the rich resources in the medium and the exponential growth of the strain BS20DFHG, we assumed the \( K_d = 0 \) during the first 18 h, and the growth of BS20DFHG in the 50-l bioreactor was modeled in the form of Eqs. (1), (2), and (3). In terms of the decline part, Eq. (4) was obtained after integrating Eq. (1) with \( \mu = 0 \) in the following 3 days. It could demonstrate the decline in the growth of BS20DFHG over time. \( X_0 \), which was obtained as 40.05 means the initial \( X \) of the second part and the \( K_d \) was recognized as 0.0054 (Fig. 4C). Thus, the dynamic relationship between the \( \mu \), \( N \) and \( t \) could be described by Eq. (4). So far, we have constructed a cell growth kinetic model of BS20DFHG in a 50-l bioreactor.

\[
\frac{dX}{dt} = X*(\mu - K_d) \tag{1}
\]

\[
\mu = A*N+B \tag{2}
\]

\[
\frac{dN}{dt} = \frac{dX}{dt} \cdot \frac{1}{Y_{X/N}} \tag{3}
\]

\[
X = X_0*\exp (-K_d*t) \tag{4}
\]

Fig. 4. Diagrams of cell growth kinetic model parameters. The \( \mu \) meant the biomass specific growth rate of the strain BS20DFHG, the \( C \) meant the concentration of carbon source in the bioreactor and the \( N \) meant the concentration of nitrogen source in the bioreactor. The \( \ln X \) meant logarithm of the biomass \( X \) with the constant \( e \) as the base. (A) Growth curve of the engineered strain BS20DFHG in a 50-l bioreactor. (B) The dots presented the growth curves of the engineered strain BS20DFHG in the first 18 h. The triangles meant the values of \( \ln X \). (C) The dots showed the value of OD600 from 18 h to 84 h. The red curve showed the fitted curve of OD600 and time. (D) The curve with dots showed the change of the \( \mu \) from 0 h to 16 h. The curve with hollow triangle showed the change of the \( \mu \) from 0 h to 16 h. (E) The curve with dots showed the change of the \( N \) from 0 h to 16 h. The curve with hollow triangle showed the change of \( \mu \) from 0 h to 16 h. (F) The dots showed the value of the \( \mu \) to different \( N \) and the red line was the fitted line of \( \mu \) and \( N \). (G) The red line showed the simulation curve of cell growth kinetic model and the black dots showed values of the experimental OD600 values.
In conclusion, after increasing the relative transcription level of *isp*-genes to increase the flux of the MEP pathway, we obtained the highest production of MK-7 as 415 ± 3.2 mg/l in BS20DFHG, a 29% increase compared with BS20. Then we attempted to figure out the crucial factors of the liquid fermentation process and optimized them by parallel bioreactors. Finally, a cell growth kinetic model was developed to demonstrate the growth of BS20DFHG in a 50-L bioreactor and guide the next step of optimization, and therefore, the established fermentation process paves the way for the industrial production of MK-7 in the future.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (31871784, 31870069, 21676119 and 31671845). Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX18_1786).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Walther R, Karl JP, Booth SL, Boyaval P. 2013. Menaquinones, bacteria, and the food supply: the relevance of dairy and fermented food products to vitamin K requirements. *Adv. Nutr.* 4: 463–473.
2. Mahdinia E, Demirci A, Berenjian A. 2017. Optimization of *Bacillus subtilis natto* growth parameters in glycerol-based medium for vitamin K (menaquinone-7) production in biofilm reactors. *Bioprocess Biosyst. Eng.* 41: 195–204.
3. Scheiber D, Veulemans Y, Horn P, Chatrou ML, Pottholf SA, Kelm M, et al. 2015. High-dose menaquinone-7 supplementation reduces cardiovascular calcification in a murine model of extraosseous calcification. *Nutrients* 7: 6991–7011.
4. El Asmar MS, Naoum JJ, Arbid EI. 2014. Vitamin K dependent proteins and the role of vitamin K2 in the modulation of vascular calcification: a review. *Oman. Med. J.* 29: 172–177.
5. Ma Y, McClure DD, Somerville MV, Proschogo NW, Dehghani F, Kavanagh JM, et al. 2019. Metabolic engineering of the MEP pathway in *Bacillus subtilis* for increased biosynthesis of menaquinone-7. *ACS Synth. Biol.* 8: 1620–1630.
6. Bai A, Walejko P, Kutner A, Kaczmarek L, Witkowski S. 2016. Convergent synthesis of menaquinone-7 (MK-7). *Org. Process Res. Dev.* 20: 1026–1033.
7. Mahana R, Berenjian A, Valtech P, Talbot A, Biffin R, Regtop H, et al. 2011. Enhanced production of menaquinone 7 via solid substrate fermentation from *Bacillus subtilis*. *Int. J. Food Eng.* 7: 1–23.
8. Berenjian A, Mahana R, Talbot A, Biffin R, Regtop H, Valtech P, et al. 2011. Efficient media for high menaquinone-7 production: response surface methodology approach. *N. Biotechnol.* 28: 665–672.
9. Goodman SR, Marns BL, Narconis RJ, Olson RE. 1976. Isolation and description of a menaquinone mutant from *Bacillus licheniformis*. *J. Bacteriol.* 125: 282–291.
10. Yoshi T, Hatahata T. 1989. Extracellular production of menaquinone-4 by a mutant of *Flavobacterium* sp. 238-7 with a dystergent-supplemented culture. *J. Ferment. Bioeng.* 67: 102–106.
11. Berenjian A, Mahana R, Talbot A, Regtop H, Kavanagh J, Dehghani F. 2014. Designing of an intensification process for biosynthesis and recovery of menaquinone-7. *Appl. Biochem. Biotechnol.* 172: 1347–1357.
12. Mahdinia E, Demirci A, Berenjian A. 2018. Effects of me dium components in a glycerol-based medium on vitamin K (menaquinone-7) production in biofilm reactors. *Bioprocess Biosyst. Eng.* 42: 223–232.
13. Cui S, Xia H, Chen T, Gu Y, Lv X, Liu Y, et al. 2020. Cell membrane and electron transfer engineering for improved synthesis of menaquinone-7 in *Bacillus subtilis*. *Science* 373: 109918.
14. Cui S, Lv X, Wu Y, Li J, Du G, Ledesma-Amaro R, et al. 2019. Engineering a bifunctional Phr60-Rap60-Spo0A Quorum-Sensing molecular switch for dynamic fine-tuning of menaquinone-7 synthesis in *Bacillus subtilis*. *ACS Synth. Biol.* 8: 1826–1837.
15. Heider SA, Wolf N, Hodeimeier A, Peters-Wendisch P, Wendisch VF. 2014. Optimization of the IPP precursor supply for the production of lycopene, decaprenoxanthin and astaxanthin by *Corynebacterium glutamicum*. *Front. Bioeng. Biotechnol.* 2: 28.
16. Xue D, Abdallah II, de Haan, IEM, Sibbald. MFJFB, Quax WJ. 2015. Enhanced C30 carotenoid production in *Bacillus subtilis* by systematic overexpression of MEP pathway genes. *Appl. Microbiol. Biotechnol.* 99: 5907–5915.
17. Chou HH, Keasling JD. 2013. Programming adaptive control to evolve increased metabolite production. *Nat. Commun.* 4: 2595.
18. Abdallah II, Pramastya H, van Merkerk R, Sukr asno, Quax WJ. 2019. Metabolic engineering of *Bacillus subtilis* toward taxadiene biosynthesis as the first committed step for taxol production. *Front. Microbiol.* 10: 218.
19. Ye L, Li X, Yu H. 2016. Engineered microbes for isoprene production. *Metab. Eng.* 38: 125–138.
20. Yan X, Yu HJ, Hong Q, Li SP. 2008. Cre/lox system and FCR-based genome engineering in *Bacillus subtilis*. *Appl. Environ. Microbiol.* 74: 5556–5562.
21. Cabrera-Valladares N, Martinez LM, Flores N, Hernandez-Chever G, Martinez A, Bolivar F, et al. 2012. Physiologic consequences of glucose transport and phosphoenolpyruvate node modifications in *Bacillus subtilis* 168. *J. Mol. Microbiol. Biotechnol.* 22: 177–197.
22. Li M, Hou F, Wu T, Jiang X, Li P, Liu H, et al. 2019. Recent advances of metabolic engineering strategies in natural isoprenoid production using cell factories. *Nat. Prod. Rep.* 36: 89–99.
23. Bitok JK, Meyers CF. 2012. 2C-Methyl-d-erythritol 4-phosphate enhances and sustains cyclodiphosphate synthase IspF activity. *ACS Chem. Biol.* 7: 1762–1710.
24. Li Q, Fan F, Gao X, Yang C, Bi C, Tang J, et al. 2017. Balanced activation of IspG and IspH to eliminate MEP intermediate accumulation and improve isoprenoids production in *Escherichia coli*. *Mol. Environ.* 43: 13–21.

May 2020 | Vol. 30 | No. 5