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Research Article

Enhancement of Vitamin K2 Efflux in *Bacillus subtilis* Natto via a Potential Protein Receptor for Increased Yield

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Bacillus subtilis is one of the few strains that can secrete synthetic menaquinone-7 (MK-7) to the outside of the cell, and its purpose and mechanism have not been clearly studied. As an amphiphilic protein naturally synthesized by *Bacillus subtilis*, the BslA protein may be involved in the inversion of extracellular vitamin K2 solubility. The protein structure in UniProt was used to search for the possible binding sites of MK-7, and the analysis of the higher ranking results of the genetic algorithm showed that the ASP166 residue was likely to be the binding site. They could form a stable hydrogen bond connection through ASP166, and approximately 7 proteins formed the conformation of a fixed naphthoquinone ring. We isolated and obtained the BslA protein by Ni-NTA affinity chromatography. Then, MK-7 was modified by BslA in vitro. A series of experiments, such as SEM, XPS, and WCA, showed that MK-7 and BslA proteins can realize self-assembly and transform from fat-soluble to water-soluble complexes. When the bslA protein in *Bacillus subtilis* natto was overexpressed, its MK-7 synthesis ability was further improved, especially the extracellular MK-7 content, which increased by 16%. This finding suggests that the BslA protein in *Bacillus subtilis* is likely to be involved in the extracellular secretion of MK-7 as a receptor.

1. Introduction

Vitamin K (VK) is a general term for a class of 2-methyl-1,4-naphthoquinone derivatives. Naturally, synthesized fat-soluble vitamins mainly include vitamin K1 and vitamin K2. Vitamin K1, also known as phylloquinone, is mainly found in green plants. Vitamin K2, also known as menaquinone, is mainly synthesized by microorganisms (intestinal bacteria). Although there are many types of vitamin K, the biologically active form in humans and animals is vitamin K2. There are 14 subtypes of vitamin K2 based on the number of isoprene residues on the side chain, designated menaquinone-n, or MK-n [1, 2]. Vitamin K2, as one of the indispensable vitamins for humans, plays an important role in promoting blood coagulation [3] and preventing osteoporosis [4], cardiovascular disease [5], cancer [6], and Parkinson's disease [7].

Bacillus subtilis natto has become the most important microorganism in the production of vitamin K2 due to its advantages, such as fast growth, easy cultivation, and high vitamin K2 content. The main form of vitamin K2 in *Bacillus subtilis* natto is menaquinone-7. Tsukamoto [8] identified a high-yielding (1719 μg/100 g) *Bacillus subtilis* OUV23481 strain through ultraviolet (UV) mutagenesis and N-methyl-N′-nitro-N-nitrosoguanidine mutagenesis. Sato [9] isolated a *Bacillus subtilis* natto strain from natto, and the yield of menaquinone-7 increased by 30% after NTG mutagenesis. Luo [10] improved the production of menaquinone-7 by optimizing the composition of the medium. Ebrahiminezhad [11] used magnetic nanomaterials (iron oxide nanoparticles) to immobilize *Bacillus* natto for fermentation, and the yield increased by 15%. Mahdinia [12] used a biofilm reactor to increase the mass transfer and oxygen transfer of the system, and the output of menaquinone-7 reached

35.5 mg/L. In bacteria, vitamin K2 is located on the cell membrane and participates in electron transfer and oxidative phosphorylation [13]. The excessive accumulation of intracellular vitamin K2 will cause cytotoxicity, and it will be more difficult to further increase the production of vitamin K2.

A feasible method is performed to cause time isolation. By separating the time of vitamin K2 synthesis and bacterial growth, the toxicity caused by the accumulation of vitamin K2 could be reduced. For example, Cui [14] used quorum-sensing molecular switches as a common method to allow bacteria to synthesize menaquinone-7 after reaching certain biomass, which effectively increased the final yield. Another commonly used method is performed to create spatial isolation. Fang [15] changed the permeability of cell membranes of *Flavobacterium* by adding surfactants to promote the excretion of vitamin K2 to the outside of the cell and further increased the synthesis of intracellular vitamin K2. Our group has tried adding amphiphilic surfactin derived from *Bacillus subtilis*, but the efflux of vitamin K2 is not significantly promoted, which may be due to the inability of short peptide substances to combine with extracellular vitamin K2. Some studies have shown that *Bacillus subtilis* itself can produce a protein that improves the hydrophilicity of vitamin K2 so that approximately 40% of intracellular vitamin K2 exists in the form of a soluble complex outside the cell [16, 17]. Currently, the occurrence of vitamin K2 effluxes has not been systematically and clearly studied. Existing research shows that the structure of natto-vitamin K2 has different morphologies inside and outside the cells of *Bacillus subtilis* natto [18]. Some researchers have found that extracellular vitamin K2 binds to an amphiphilic protein, which makes fat-soluble vitamin K2 water soluble [19]. The increased expression of this amphiphilic protein helps the extracellular excretion of intracellular vitamin K2, relieves cytotoxicity, releases product inhibition, and further increases the production of vitamin K2.

Computer-aided molecular structure simulations can provide weighted scores for different ligands, reducing the workload of large-scale screening. For example, in one study, the researchers achieved rapid screening of 37 quinoline derivatives through functional prediction. [20, 21] The interaction between some small molecule compounds and biological macromolecules [22] or between biological macromolecules [23] can also be predicted by in silico techniques. In addition, some researchers have tried to reveal the mechanism of protein action in cells through protein model analysis. [24] These studies demonstrate that the analysis of vitamin K2-binding factors in *Bacillus* subtilis can be achieved using computer-aided structural analysis.

This study mainly explored the effect of the naturally produced amphiphilic protein *Bacillus subtilis* natto on the synthesis of vitamin K2. According to amphiphilic characteristics, we tried to search for amphipathic proteins associated with biofilm construction in *Bacillus subtilis* in the protein database (UniProt), and AutoDock molecular docking software was used to show that this amphiphilic protein can bind to vitamin K2. Vitamin K2 modified with this amphiphilic protein was characterized by scanning electron microscopy, water contact angle, infrared spectroscopy, and X-ray photoelectron spectroscopy. Then, the expression of this protein in *Bacillus subtilis* natto was increased to detect total vitamin K2 production and extracellular vitamin K2 production.

2. Materials and Methods

2.1. Strains, Plasmids, and Primers. *Escherichia coli* DH5α cells and *Escherichia coli* Rosetta cells were purchased from Takara (Takara, China). *Bacillus subtilis* natto (BN-P5-11-1) was preserved in our laboratory [25]. The expression vectors pHYp43 and Pet-28a were purchased from YouBio (Hunan, China). All strains, plasmids, and primers used in this study are listed in Additional file 1as shown in Tables S1 and S2.

2.2. Molecular Docking Analysis of BslA. The 3-D structure of the BslA protein was downloaded from the UniProt database, and the PDB identifier code was 4BHU. ChemDraw and Chem3D were used to prepare the 3-D structure ligand molecule. According to the manual, the molecular docking procedure was carried out using AutoDock (version 4.2.6), which is considered to be the fast and precise docking between small substrates and target proteins [26]. The Lamarckian genetic algorithm (LGA) was chosen for substrate docking calculations. Finally, the docking results were visualized and analyzed by PyMOL software.

2.3. Western Blot Detection of BslA. The samples were mixed with 4 × SDS–PAGE sample loading buffer and boiled for 10 min. Protein bands were separated by SDS–PAGE with a 12% separating gel and a 5% stacking gel and transferred to nitrocellulose membranes (Beyotime, Shanghai, China). The membranes were incubated with 5% nonfat dried milk in TBS with 0.1% Tween-20 (TBST) for blocking. After blocking for 1 hour at room temperature, the membranes were incubated with 6 × His-tagged mouse monoclonal antibodies (1 : 2500 dilution, EnoGene Biotech, Nanjing, China). After washing with TBST, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated goat antimouse IgG (1 : 5000 dilution, EnoGene Biotech, Nanjing, China). Finally, immunoreactive proteins were detected with BeyoECL Star (Beyotime, Shanghai, China).

2.4. Purification of Recombinant BslA. The intracellular proteins previously extracted were concentrated and subjected to subsequent purification using Ni-NTA affinity chromatography. The crude enzyme solution was purified with a Ni-NTA Sefinose™ resin kit (BBI, UK) according to the manufacturer's specifications. Then, the eluent was passed through a desalting gravity column (BBI, UK) to remove the salts. The purified proteins of BslA were frozen and stored at −80°C in preparation for subsequent experiments.

2.5. Preparation of BslA-Modified Menaquinone-7. A 0.648 mg/mL menaquinone-7 solution was prepared by dissolving menaquinone-7 in dimethyl sulfoxide (DMSO) and using 0.22 μm syringe filters to remove possible impurities. BslA was dissolved in pure deionized water to make a solution of 19 mg/mL. The menaquinone-7 solution and the BslA solution were mixed at a molar ratio of 1 : 1. The mixtures were sonicated for 30 s and then stirred vigorously for 30 min with a magnetic stirrer, keeping the sample in an ice bath while stirring. Finally, the mixture was placed at 4°C overnight. For further analysis, the mixed solution was dried in a freeze dryer.

2.6. Characterization. The morphology of HGFI-modified and native menaquinone-7 was observed by using a scanning electron microscope (SEM) (Helios NanoLab 600i, USA). An X-ray photoelectron spectroscope (XPS) (Thermo ESCALAB 250Xi, USA) was used to evaluate functional groups and composition of BslA-modified and native menaquinone-7. The samples were analyzed under vacuum ($P < 10^{-8} mbar$) with a pass energy of 30 eV. The water contact angle (WCA) was measured by the sessile drop method at room temperature [27].

2.7. Extraction and Detection of MK-7. MK-7 was quantified at 140 h. A mixture of isopropanol and n-hexane (1 : 2, v/v) was used to extract MK-7 from the fermentation broth of B. *subtilis* natto. Five milliliters of fermentation broth were added to a 10 mL mixture and mixed at 150 rpm for 2 h. Then, 5 mL of butyl alcohol was added to this mixture and mixed at 150 rpm for 2 h. The mixture was centrifuged at $13,000 \times g$ for 10 min. The organic phase was filtered through 0.45 μm pore organic membranes and analyzed by high-performance liquid chromatography (HPLC). Methanol and dichloromethane (4 : 1, v/v) were selected as the mobile phase, and the flow rate was 1 mL/min. The concentration of MK-7 was detected at a wavelength of 248 nm, at which MK-7 exhibited strong UV absorption. A two-tailed *t*-test was used for statistical analysis.

3. Results and Discussion

3.1. Identification of Efflux Proteins in Bacillus subtilis Natto. Earlier studies have found that an amphipathic protein can promote the extracellular accumulation of vitamin K2, which is produced in *Bacillus subtilis* natto. According to the nature of the protein, a search in the protein database found that there is an amphipathic protein in *Bacillus subtilis* species, the BslA protein. The BslA protein is located on the surface of the cell membrane of *Bacillus subtilis* natto and can self-assemble into a stable elastic membrane at the interface, which gives the cell membrane hydrophobicity [28]. We reveal that the structure of BslA contains an unusual type of Ig-like fold and possesses a striking hydrophobic "cap" with physiochemical properties reminiscent of hydrophobic surfaces found in fungal hydrophobins [29]. Our previous experiment found that fungal hydrophobin HGFI can bind to MK-7, making hydrophobic MK-7 water soluble [30]. The hydrophobic amino acids on the surface of fungal hydrophobins form hydrophobic patches. The hydrophobic patches self-assemble together, giving fungal hydrophobins amphiphilic properties. The structure of BslA is composed of 1 α-helix and 13 β-sheets, and a hydrophobic cap and a main structure similar to immunoglobulin are formed as a whole. The hydrophobic cap is located above the main structure, similar to immunoglobulins, and the hydrophobic cap composed of three β-sheets makes the BslA protein amphiphilic and can bind to fat-soluble MK-7.

Table 1: Docking result information.

	Binding energy (kcal/mol)	Inhibition constant (mM)	Docking sites
1	−5.7	0.067	ASP166
2	−5.61	0.078	ASP166
3	−4.76	0.326	GLU162
4	−4.25	0.770	LEU50
5	−3.95	1.270	ASP166
6	−3.92	1.350	ASP166
7	−3.87	1.460	ASP166
8	−3.84	1.520	ALA52
9	−3.73	1.840	ASP166
10	−2.59	12.720	ALA52

The possible binding form between the BslA protein and the MK-7 molecule was predicted by a genetic algorithm using open-source software Autodock 4.2.6. The docking results provide 10 docking results containing hydrogen bonds (shown in Table.1). There are four possible docking sites, ASP166, GLU162, ALA52, and LEU50. Among them, the binding energy of the ASP166 residue is lower, and the possible docking results are significantly greater than those of other residues. It is more likely the binding site for menaquinone-7. The result with the lowest binding energy is shown in Figure 1. There is a surface structure in the β-sheet region of the BslA protein that matches the naphthoquinone ring structure in the MK-7 molecule (Figure 1), and the binding energy of the lowest matching conformation is -5.7 kcal/mol. The theoretical inhibition constant value of the docked complex is 0.067 mM. The ASP166 residue in BslA formed a hydrogen bond with hydrogen of carbon 5 of the naphthoquinone ring (Figure 1), which fixed the main ring structure of MK-7 on the surface of the protein. It can be seen from the protein surface structure model that a total of seven residues, ALA52, THR53, ILE54, MLY59, SER164, ILE165, and ASP166, form the spatial binding domain of the ligand (Figure 1), and the hydrogen bond spacing is approximately 1.956 Å.

3.2. Expression, Separation, and Purification of BslA Protein. To obtain a large amount of BslA proteins, the BslA gene was inserted into pET28a and transformed into expression strains to generate pET28a-BslA-Rosetta™. The results of sequencing using universal T7 primers demonstrated the correctness of the inserted BslA gene. After induction by IPTG, the solubility of BslA was analyzed by SDS-PAGE. The results showed that soluble *Bacillus subtilis* hydrophobin BslA with self-assembly activity was successfully expressed in Rosetta™ (Figure 2(a)).

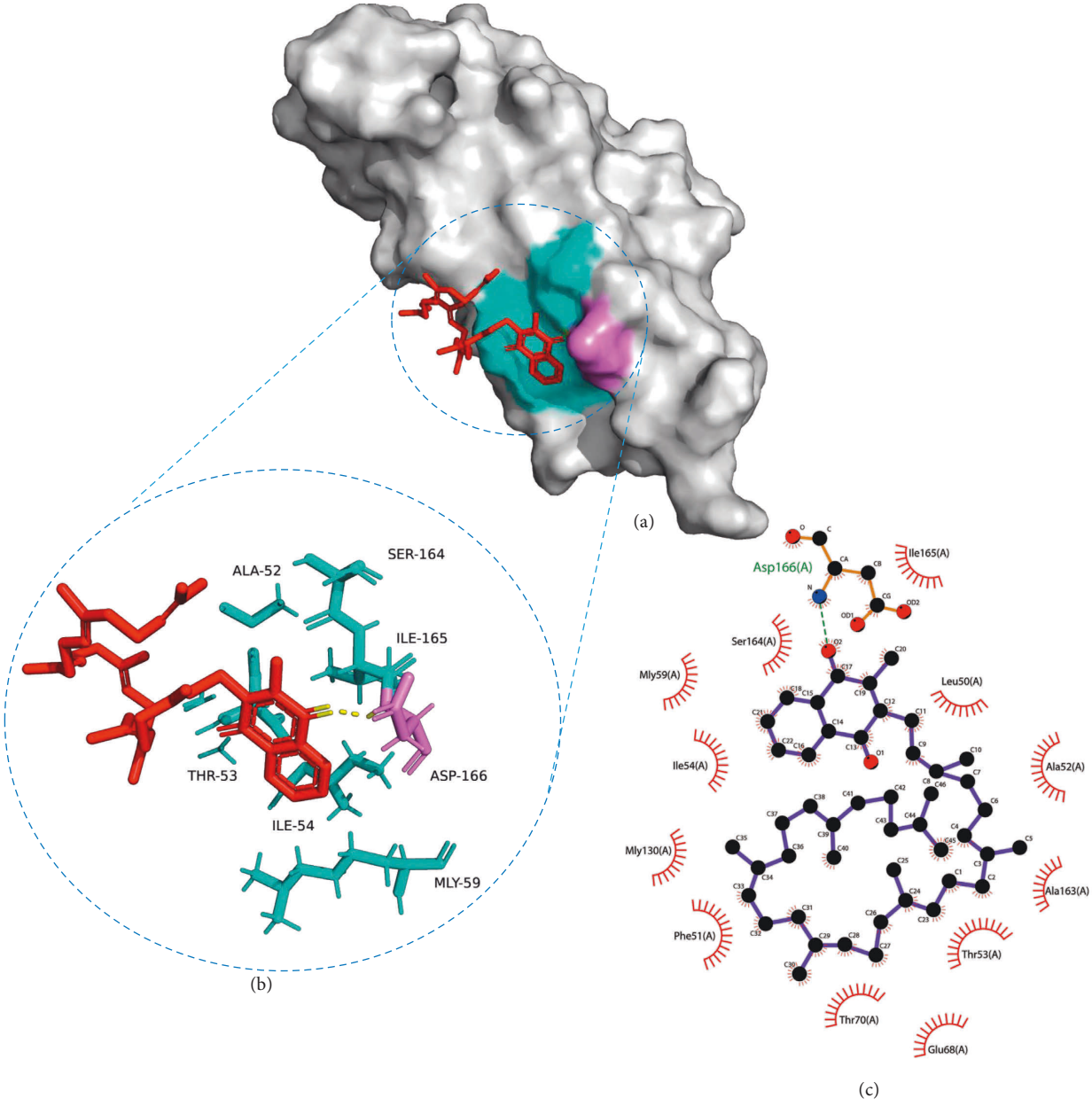

FIGURE 1: Prediction of the BslA protein binding site to menaquinone-7. (a) Surface model of menaquinone-7 binding to BslA proteins; (b) Stick model of hydrogen bonding between menaquinone-7 and BslA proteins; (c) 2D pose of protein-ligand interactions.

The Ni-NTA agarose purification resin used in this study is made of 6% cross-linked agarose as a matrix and is covalently coupled to four-coordinated nitrilotriacetic acid (NTA) to chelate nickel ions (Ni^{2+}). The target protein containing 6 × His-tag interacts with Ni^{2+} and binds to the resin. First, the binding/eluent solution containing a low concentration of imidazole was used to remove contaminated proteins, and then, the binding/eluent solution containing a high concentration of imidazole was used to elute the target protein by competitively binding Ni^{2+}. The results showed that the target protein BslA began to be eluted in the first column volume until the second and third column volumes reached the elution peak. The target protein was almost completely eluted, and the content of the target protein obtained after elution was significantly reduced (Figure 2(b)). The purified BslA protein was used for subsequent characterization in vitro.

3.3. Characterization of BslA-Modified Menaquinone-7. There is a large hydrophobic area on the surface of the BslA protein structure, which enables the BslA protein to self-assemble into a stable elastic membrane at the two-phase interface. Scanning electron microscopy images showed that the shape and dispersion of protein-modified menaquinone-7 particles changed (Figures 3(a) and 3(b)). After binding to the BslA protein, menaquinone-7 changes from the original agglomerate to a uniformly dispersed spherical shape.

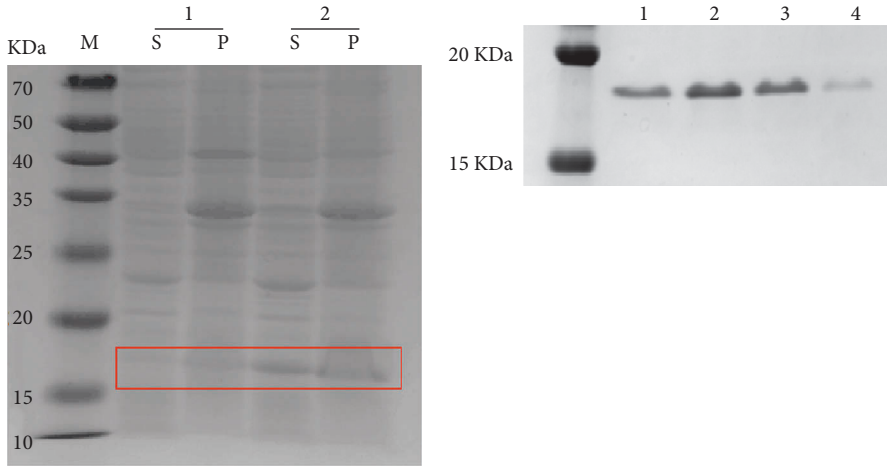

Figure 2: (a) Western blot analysis of BslA proteins. M, protein marker; S, supernatant; P, precipitate; 1, *E. coli* Rosetta with pET-28a-BslA, 30°C, 180 rpm, 4 h; 2, *E. coli* Rosetta with pET-28a-BslA, IPTG, 30°C, 180 rpm, 4 h. (b) Elution effect of BslA on Ni-NTA purification. (1–4) The eluent of the first column volume to the eluent of the fourth column volume.

In our study, the change in the wettability of the menaquinone-7 surface caused by BslA modification was investigated by WCA (Figures 3(c) and 3(d)). The mean WCA of native menaquinone-7 was 112° and dramatically reduced to 46° after modification with BslA, indicating that hydrophobins can convert hydrophobic menaquinone-7 into hydrophilic compounds. Bromley found that the BslA protein buried hydrophobic side chains inside the structure in an aqueous solution. Therefore, the reason why MK-7 binds to the BslA protein and becomes hydrophilic may be that the hydrophobic side chain inside the BslA protein structure binds to menaquinone-7, and the external hydrophilic part enables BslA-modified menaquinone-7 to stably exist in aqueous solutions.

The difference between menaquinone-7 and BslA protein is also manifested in element types, and the latter possessing some characteristic elements such as N. To determine whether BslA binds to the BslA protein, we performed an X-ray photoelectron spectroscopy (XPS) analysis. In XPS, different atoms have different characteristic absorption wavelengths. When menaquinone-7 was detected alone (Figures 3(e) and 3(f)), only C1 s and O1 s peaks were detected, indicating that the substance contained only carbon, hydrogen, and oxygen. When menaquinone-7 modified by the BslA protein was detected, there was an additional N1 s peak (Figure 4(b)). This indicated that a new nitrogen element appeared in the complex, which may be caused by the self-assembly of the BslA protein on the surface of menaquinone-7.

3.4. Overexpression of BslA in Bacillus subtilis Natto. The BslA protein was overexpressed in *Bacillus subtilis* natto, and its effect on vitamin K2 synthesis was investigated. Primer 1 and primer 2 were used to obtain the bslA gene, which was integrated into the plasmid pHYp43 containing the strong promoter P43, and then, the recombinant plasmid pHYp43-bslA was introduced into *Bacillus subtilis* natto to obtain *Bacillus subtilis* bslA. The original strain *Bacillus subtilis* natto and the recombinant strain *Bacillus subtilis* bslA were fermented in 500 mL flasks to study the effect of overexpression of BslA protein on the synthesis of vitamin K2. The results revealed that the vitamin K2 synthesis capacity of the recombinant strain *Bacillus subtilis* bslA was improved to a certain extent. As shown in Figure 5, after 140 hours of fermentation, the vitamin K2 yield was 1.2 times that of the original strain, and the extracellular vitamin K2 content also increased from 44% to 51%. The BslA protein can indeed promote the excretion of intracellular vitamin K2 to the outside of the cell and further enhance the ability of *Bacillus subtilis* to synthesize vitamin K2.

3.5. Increased Menaquinone-7 Production by Enhancing Its Efflux. To increase the expression level of the BslA protein in the recombinant bacterium *Bacillus subtilis* BslA, we optimized the initial pH and culture temperature during the fermentation process. As shown in Figure 6(a), the expression level of the BslA protein was relatively high in neutral and weakly alkaline environments. When the pH is 8.0, the protein expression level reaches the peak and the MK-7 content also reaches the maximum accumulation. At the optimal pH of 8.0, the expression level of the BslA protein increased with increasing culture temperature, and the protein expression level was highest at 42°C. However, higher temperatures affect the activity of the enzymes that synthesize MK-7, resulting in a decrease in the production of MK-7. Comprehensive considerations indicated that the optimal culture temperature for *Bacillus subtilis* bslA was 42°C (Figure 6(b)). *Bacillus subtilis* natto and *Bacillus subtilis* bslA were continuously fermented in a 20 L fermenter for 7 days, and the obtained MK-7 concentration change is shown in Figure 4. The results showed that the expression of bslA can significantly increase the production of MK-7. After 168 hours of fermentation, the yield reached the highest level of 85.1 mg/L.

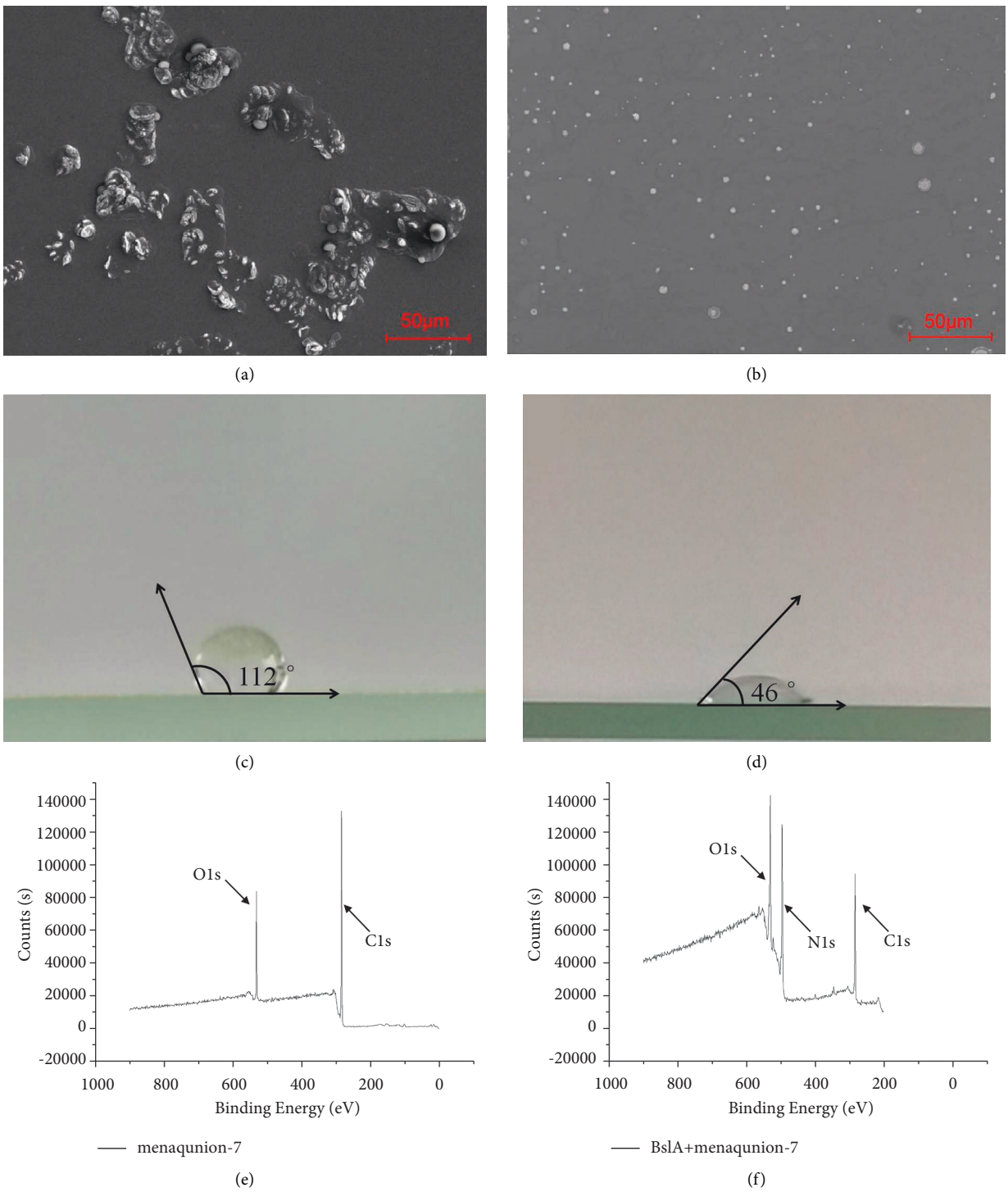

Figure 3: Physical property testing of compounds. (a) SEM of menaquinone-7; (b) SEM of BslA-modified menaquinone-7; (c) WCA of menaquinone-7; (d) WCA of BslA-modified menaquinone-7; (e) XPS of menaquinone-7; (f) XPS of BslA-modified menaquinone-7.

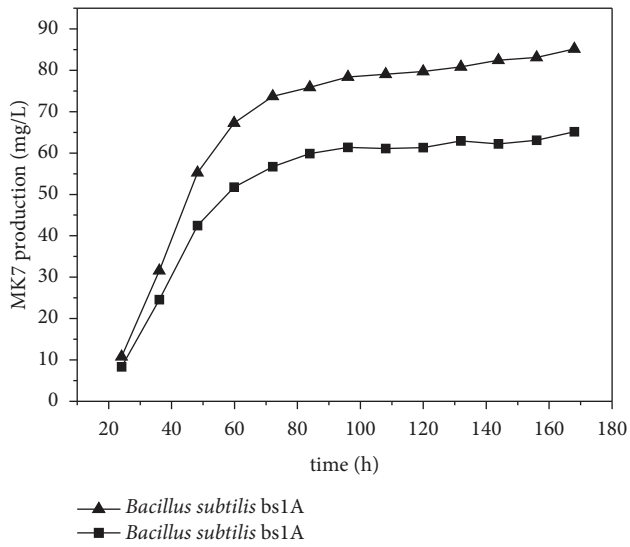

Figure 4: Changes in the concentration of MK-7 for *Bacillus subtilis* natto and *Bacillus subtilis* BslA in a 20 L biofilm reactor at 40°C, pH 8.0, 450 rpm, and 2.0 vvm.

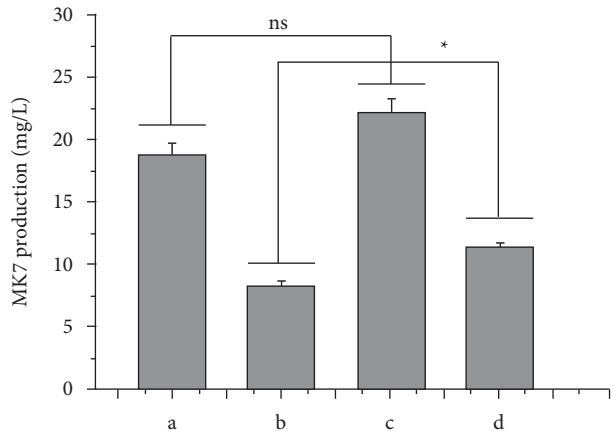

Figure 5: The MK-7 production of *Bacillus* subtilis natto. (a) Total MK-7 production of *Bacillus* subtilis natto. (b) Extracellular MK-7 production of *Bacillus* subtilis natto (BN-20644). (c) Total MK-7 production of *Bacillus* subtilis BslA. (d) Extracellular MK-7 production of *Bacillus* subtilis BslA.

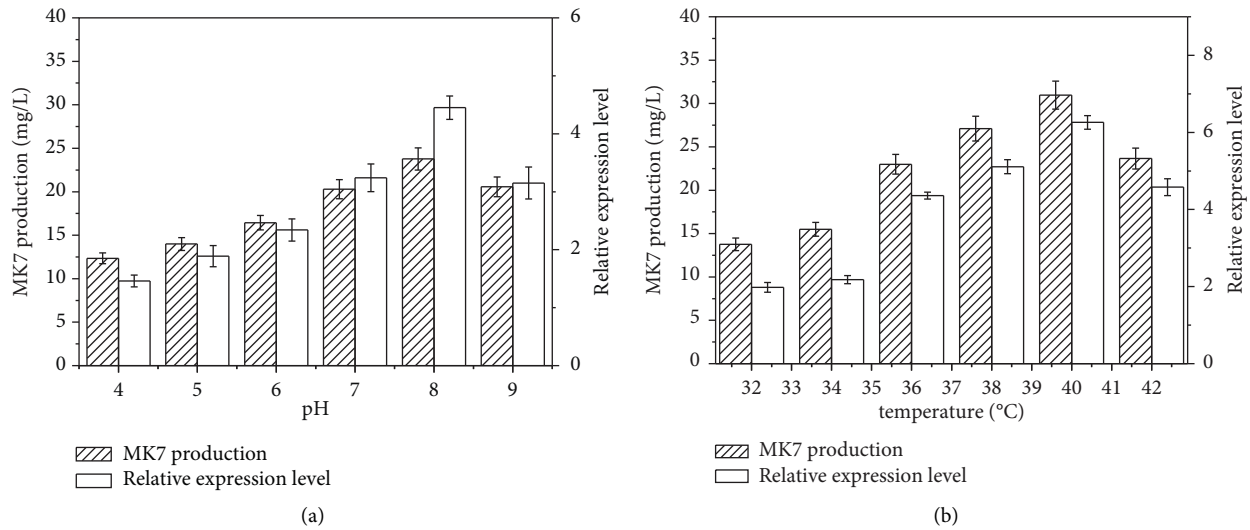

Figure 6: Effects of culture conditions on the expression level of bslA. (a) The expression of BslA at different initial pH values (at 37°C for 48 h). (b) The expression of bslA at different temperatures (at initial pH 8.0 for 48 h).

4. Conclusions

A potential amphiphilic protein was found through database search and AutoDock software, which may be the BslA protein. X-ray photoelectron spectroscopy (XPS) and water contact angle (WCA) measurements indicated that the BslA protein can self-assemble on the surface of vitamin K2. Overexpression of the BslA protein increased the total vitamin K2 content by 20% and the extracellular vitamin K2 content by 16%. The results also confirmed that the BslA protein can promote the excretion of vitamin K2 outside the cell. By optimizing the culture temperature and initial pH, the yield of *Bacillus subtilis* bslA in a 20 L fermenter reached 85.1 mg/L. Vitamin K2-producing bacteria, such as Flavobacterium and *Escherichia coli*, do not contain this amphiphilic protein themselves, and vitamin K2 cannot be secreted outside the cell. When excessive vitamin K2 accumulates excessively, it inhibits the growth of bacteria. If the BslA protein can be expressed in these bacteria, vitamin K2 will be secreted outside the cell, alleviating cytotoxicity and further improving the synthesis ability of vitamin K2. *Bacillus subtilis* extracellular vitamin K2 is involved in the formation of biofilm structures, which are so complex that only one protein can be built. This process requires multiple proteins to work together, so there may be other proteins that assist in the secretion of vitamin K2 outside the cell together with the BslA protein. This experiment needs to be further studied.

Data Availability

All analyzed data within this study can be obtained from the corresponding author upon request.

Additional Points

Vitamin K2 has received extensive attention for its compensatory role in energy transfer within mitochondria. At present, a mature large-scale Vitamin K2 production process is lacking. *Bacillus subtilis* is naturally capable of synthesizing water-soluble vitamin K2 and is considered a potential strain for its large-scale production. However, the mechanism of vitamin K2 efflux has not been fully elucidated. These studies have shown that the naturally synthesized amphiphilic protein BslA of *Bacillus subtilis* can form a hydrophilic conjugate with vitamin K2 in vitro. This finding indicates that through the in-depth study of the mechanism of vitamin K2 efflux, it is feasible to realize the spatial separation of the product from the cell, thereby increasing the yield of vitamin K2.

Ethical Approval

This research does not involve human participants, their data, or biological material.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

CL, WH, PW, and ZZ designed the study. CL and WH performed the study. CL, WH, GM, and LW analyzed the results. PW, GZ, and ZZ confirmed the results. All authors wrote and revised the manuscript and agreed to be accountable for all aspects of the presented work.

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Supplementary Materials

Supplementary Table 1. List of all strains and plasmids. Supplementary Table 2. Primers used for plasmid construction. (*Supplementary Materials*)

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