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Characteristics of *Bacillus subtilis* HNMY-13 and HNMY-15 strains in aflatoxin B_1 degradation and *Astragalus* bio-transformation

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***Astragalus* of traditional Chinese medicine (TCM) and *Bacillus subtilis* are extensively used in animal feed and for production of various fermented compounds. In order to observe the characteristics of HNMY-13 and HNMY-15 *B. subtilis* strains, this study focused on the optimization of parameters in liquid fermentation of the two strains in aflatoxin B_1 degradation, and *Astragalus* polysaccharide yield. The optimal parameters: degradation of aflatoxin B_1, the bacterial count, and the *Astragalus* polysaccharide yield analysis were examined in this study. It was shown that the percentage degradation of AFB_1 by HNMY-13 and HNMY-15 strains were 80.48 and 79.55% at 37°C, and 80.66% and 81.34 with pH 7.0. The percentage degradation by HNMY-13 was 88.07% after 48 h fermentation and by HNMY-15 was 84.81% after 60 h fermentation, respectively. The viable count of fermented sample was 8.8×10^8 and 7.6×10^8 CFU·mL^{-1} using HNMY-13 and HNMY-15 strains, respectively. The polysaccharide yielded by HNMY-13 was 4.93% at 48 h which is 1.7-fold higher than that of the control group, while polysaccharide yielded by HNMY-15 reached peak of 3.54% at 60 h. In conclusion, HNMY-13 and HNMY-15 of *B. subtilis* promote production of *Astragalus* polysaccharide in the process of liquid fermentation and can also degrade aflatoxin B_1 significantly; hence the combination could form a potential feed additive for animals.**

Key words: *Astragalus*, *Bacillus subtilis*, liquid fermentation, aflatoxin B1, polysaccharide.

INTRODUCTION

Bacillus spp. are dominant microflora in animal probiotics because they are Gram-positive, strict or facultative aerobe and endospore-forming bacteria (Chantawannakul et al., 2002). It was generally recognized that probiotics strains should be isolated from the gastrointestinal tracts (GIT) of their host. Such strains were claimed to have higher chance of survival and colonization in the intestine, which allow beneficial microbiota to thrive (Quigley, 2010). However, EU-authorized probiotics for animals were often not of intestinal origin (Nguyen et al., 2015).

According to previous studies, *Bacillus subtilis* could

biodegrade aflatoxins B_1 and ochratoxin A via biotransformation (Yu et al., 2015; Petchkongkaew et al., 2008). Thus, it could be directly applied in the feedstuffs and feeds (Gao et al., 2013). It also exhibited antimicrobial activity and high resistance to the simulated gut environment. In addition, *B. subtilis* ferments roasted soybean, wheat bran, fruit waste via solid state fermentation (SSF) for production of the novel fermented soybean, haloduracin and citric acid (Park et al., 2012; Danesh et al., 2013; Kumar et al., 2003).

Astragalus was used as supplementary forage supplement for livestock and poultry industry in China, because it contains polysaccharides, saponins, flavonoids, anthraquinones, alkaloids, amino acid, β-sitosterol and metallic elements (Ibrahim et al., 2013; Sun et al., 2013; Li et al., 2009). Recent literatures reported that crude extracts of *Astragalus* are observed to be anti-inflammatory (Kim et al., 2013), immunostimulant (Qin et al., 2012), antioxidative (Kim and Yang, 2005), and antiviral in isolated constituents (Kallon et al., 2013). *Astragalus* was fermented by lactic acid bacteria (LAB) using solid state fermentation (SSF) technology. This technology is superior to crude *Astragalus* (Sheih et al., 2011) due to its production of a wide range of antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins which could kill other microbes (Rolfe, 2000).

Even though literature has reported this based bio-transformation technology to determine the effects of *B. subtilis*, the use of *B. subtilis* on *Astragalus* effects has not been reported in literature. To the best of the authors' knowledge, this is the first investigation of *B. subtilis* from pig gastrointestinal tracts ferment *Astragalus*. It is significant to study the interaction between *Astragalus* and *B. subtilis*, producing in-feed antibiotic growth promoters (AGP) of livestock and poultry industry.

MATERIALS AND METHODS

Bacteria source and biochemistry experimentation

B. subtilis strains HNMY-13 and HNMY-15 were isolated from pigs' gastrointestinal tracts in Henan province of center of China. HNMY-13 and HNMY-15 isolates were grown on LB (Luria-Bertani) agar (Beijing Aoboxing Bio-tech company limited) at 37°C. Strains that were able to form spores on LB agar after 48 h were selected for further characterization. Further identification was achieved by performing the following tests: anaerobic test, Voges-Proskauer (VP) test, nitrate reduction test, amylohydrolysis, citrate utilization test, D-mannitol test, L-arabinose test, salt tolerance (7% NaCl in LB), growth at 37 and 42°C, and pH 5.7.

DNA extraction and 16S rRNA analysis

The bacterial isolates were grown overnight in LB agar at 37°C, then subjected to DNA extraction with bacterial genomic DNA miniprep kit (Suolaibao science and technology company, Beijing, China) according to the manufacturer's instructions. The genomic DNA is used as a template for PCR (94°C for 30 s, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 90 s and a final extension at 72°C for 10 min). The forward and reverse primers used to amplify the partial 16S rRNA sequence were 5'-CGCG GATC CCCA ATGA TATA GGTA AAAC-3' and 5'-CCGG AATT CTTA ATAG CTGT TACT TTGT-3', respectively. PCR reactions were performed in a triplicate 50 µL mixture containing 25 µL of PCR mix, 13.5 µL ultrapure water, 2 µL of primer 1 (10 µM), 2 µL of primer 2 (10 µM) and 7.5 µL of template DNA (0.2 ng/µL). Obtained sequences were compared with sequences in the GenBank non-redundant nucleotide database by BLAST analysis. Phylogeny was inferred from aligned nucleotide sequences of the 16S rRNA genes using MEGA6 software.

Optimization of fermentation parameters of *B. subtilis* HNMY-13 and HNMY-15 strains in aflatoxin B1 degradation

To determine the optimal parameters for degradation of aflatoxin B_1 by HNMY-13 and HNMY-15 strains, culture conditions for aflatoxin B_1 degradation were studied at different temperatures (25, 30, 32, 35, 37 and 40°C), pH values (5.0, 6.0, 7.0 and 8.0) and incubation periods (24, 36, 48, 60 and 72 h). Fermentation was carried out in a 500 mL Erlenmeyer flask containing 100 mL of medium inoculated with 100 µL of the spore suspension of the two strains (1.5×10^6 CFU·µL^{-1}) and incubated in an orbital shaker at 200 rpm. After the fermentation, 900 µL fermented liquid was collected from different treatment groups into a 1.5 mL sterile centrifuge tube and 100 µL AFB_1 (Aflatoxin B_1) standard substance (400 ng·mL^{-1}, Suolaibao science and technology company, Beijing, China) was added to each tube. A tube with only fermentation medium was set up as the control. Then, the AFB_1 residual content was determined using ELISA kits (Yisenbao biotechnology company, Beijing, China). The degradation rate can be described by the following formula: the percentage degradation of AFB_1 = [1-(content after processing AFB_1)/control group content]×100%.

Bacillus subtilis strains fermented *Astragalus*

Preparation of Astragalus concentrated solution

Astragalus was obtained from a central Chinese medicine market (Yuzhou, Henan, China). *Astragalus* (100 g) was taken and five-, three- and double-fold amount of water was added to boil three times, each time for 30 min. After boiling for three times, the filtrate was merged for 15 min by centrifugation (5000 ×g). The supernatant liquid was concentrated to 1 g·mL^{-1} by rotary evaporators. This suspension was later used as supplement for fermentation media.

Liquid fermentation

For the HNMY-13 and HNMY-15 strains, the LB medium was supplemented with 0.1, 0.3, 0.5, 0.7, 0.9 and 1 g·L^{-1} *Astragalus* concentrated liquid, respectively. Then they were inoculated with 10^6 CFU·g^{-1} of *B. subtilis* HNMY-13 and HNMY-15. The cultures were incubated at 37°C for 24 h under aerobic shaking condition. The samples were taken for further analysis.

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Table 1. Characterization of *Bacillus* spp. strains isolated from pig gastrointestinal tracts.

Strains	HNMY-13	HNMY-15
Sporulation (%)	100	98
anaerobic test	-	-
V-P test	+	+
nitrate reduction test	+	-
amylohydrolysis	-	+
gelatin liquefaction	-	-
citrate utilization test	-	+
D-mannitol test	-	+
L-arabinose test	-	-
LB NaCl (7.0%)	+	+
Growth pH 5.7	-	+
Growth at 37°C	+	+
Growth at 42°C	+	+

-Negative; +positive.

Microbiological analysis (total viable bacterial count)

Total viable bacterial counts were determined after the fermentation. The fermented liquid (1 mL) was mixed with 9 mL of sterilized physiological saline (0.85% NaCl). Serial dilutions were prepared in the sterilized physiological saline and 1 mL of liquid with appropriate concentration was poured in triplicate plates for the total viable bacterial count. Prepared test samples (1 mL) with the concentration of 10^{-7}, 10^{-8} and 10^{-9} dilution were transferred into sterile Petri dishes in triplicate. The warmed (45±2°C) sterile plate containing LB agar (15 mL) was mixed with the inoculums. Cultures were incubated aerobically at 37°C for 48 h. The colonies were then counted and expressed as logarithmic colony forming units per gram (log CFU·mL^{-1}) of the sample.

The *Astragalus* polysaccharides yield analysis

The fermented liquid was added to 95% ethanol (3 times volume) for 24 h, and then collected for centrifugation at 5000 ×g for 20 min. The precipitate was dried at 60°C and smashed into powder. The yield of polysaccharides in extracts was determined using the phenol-sulfuric acid method.

Statistical analysis

Data was expressed as mean±standard error of mean (S.E.M.). Statistical analysis was performed using GraphPad PRISM software version 6.0 (GraphPad Software, USA). A Two-way analysis of variance (ANOVA) with the step-down multiple-stage F post hoc test (Ryan-Einot-Gabriel-Welsch multiple F-test P=0.05) was performed to distinguish treatment mean differences.

RESULTS AND DISCUSSION

Biochemical identification of *B. subtilis* strains HNMY-13 and HNMY-15

Two spore-forming bacterial strains were isolated from GITs of pigs obtained from center of Henan province, China. Because the efficiency of sporulation is critical for industrial production of feed supplements with high yield and low cost, an initial screening was performed to assess sporulation strains and high sporulation efficiency (more than 95%). The two strains, named HNMY-13 and HNMY-15, were further characterized based on biochemical properties according to Berger's Manual of Systematic Bacteriology. Data shown in Table 1 indicated that HNMY-13 and HNMY-15 strains hydrolyze starch at a high level and metabolize glucose as determined by the Voges-Proskauer (VP) test. Under aerobic conditions, it was determined that the two strains grew at 37 and 42°C. HNMY-13 and HNMY-15 strains metabolized citrate and grew in 7.0% NaCl. However, HNMY-13 and HNMY-15 strains exhibited different results for nitrate reduction, amylohydrolysis, citrate utilization and D-mannitol test. Although biochemical identification was through traditional morphological and physiological method, this result showed that HNMY-13 and HNMY-15 strains had different growth characteristics based on the production of different metabolic products in the process of bacterial culturing.

PCR reaction and 16S rRNA analysis

The results of the PCR of the HNMY-13 and HNMY-15 strains are presented in Figure 1. The length of nucleotide sequences of the two strains was 1500 bp. The strains were assessed by 16S rRNA sequencing and BLAST analysis to determine species identity. Analysis of the phylogenetic relationship based on 16S rRNA sequences is presented in Figure 2. It also revealed that the HNMY-13 andHNMY-15 strains were closely related to *B. subtilis*. The use of 16S rRNA sequences to identify

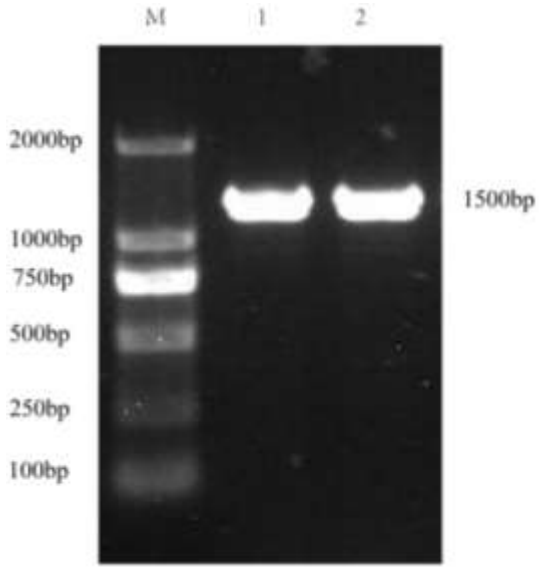

Figure 1. Agarose gel (1%) showing PCR products of HNMY-13 and HNMY-15 strains along with Marker DNA (2.0 kb). M: marker DL 2000; 1: HNMY-13 strain; 2: HNMY-15 strain.

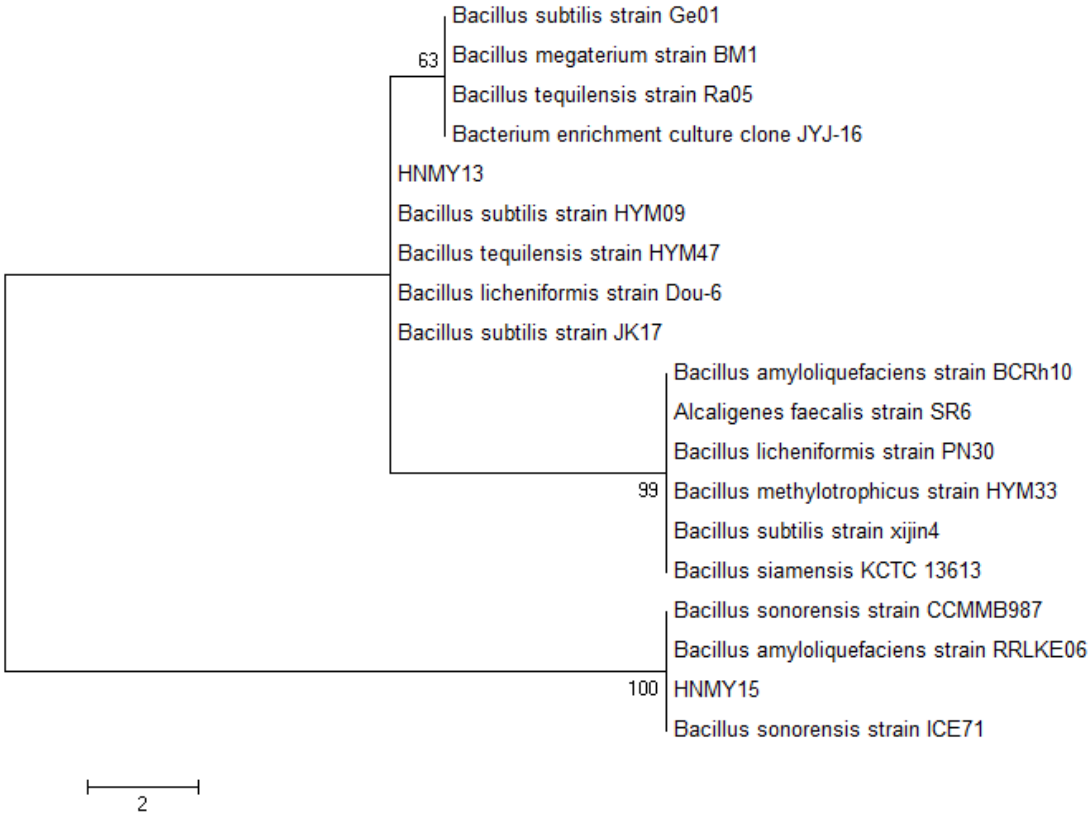

Figure 2. Phylogenetic relationship between the selected *Bacillus* strains. Dendrograms of strains based on 16s rRNA sequence alignment was obtained using MEGA6 software. Selected *Bacillus* strains are highlighted in bold and GenBank accession numbers are shown in brackets. Statistical (bootstrap) values and a scale bar representing evolutionary distance are shown. The 16s rRNA gene sequence of the *Bacillus* genus was used as the root of the phylogenetic tree.

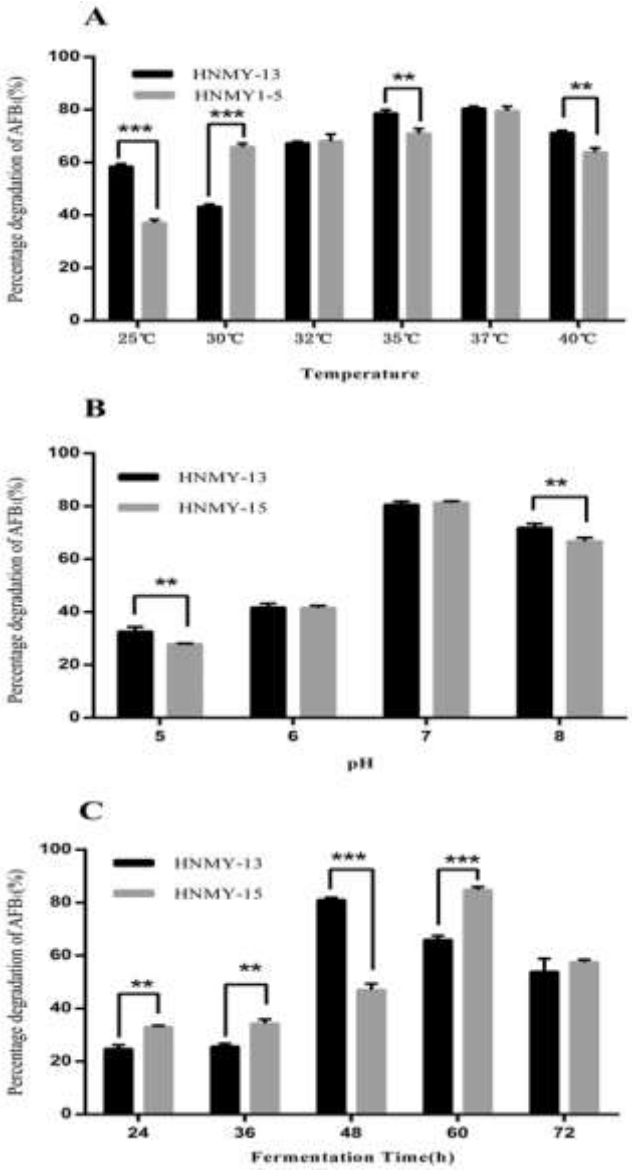

Figure 3. A: Percentage degradation of AFB_1 under different temperature (25, 30, 32, 35, 37 and 40°C) using HNMY-13 and HNMY-15 strains; B: Percentage degradation of AFB_1 at different pH values (5, 6, 7 and 8) using HNMY-13 and HNMY-15 strains; C: Percentage degradation of AFB_1 at different fermentation period (24, 36, 48, 60 and 72 h) using HNMY-13 and HNMY-15 strains. Bars with different asterisk indicates they significantly different from each other ($p<0.05$).

the species of bacteria has been successfully applied in modern microbiology, which could improve the accuracy of identification of the strain.

Optimization of HNMY-13 and HNMY-15 strains in aflatoxin B_1 degradation

The results of the optimal parameters are shown in Figure 3. The percentage degradation of AFB1 by HNMY-13 and HNMY-15 strains were respectively 80.48 and 79.55% at 37°C, and 80.66% and 81.34 with pH 7.0. The importance of temperature and pH value in the medium for aflatoxin B_1 degradation by different *B. subtilis* species has been reported by many investigators (Gao et al., 2013; Faraj et al., 1993). Percentage degradation by HNMY-13 was 88.07% after 48 h fermentation and by HNMY-15 was 84.81% after 60 h fermentation as shown

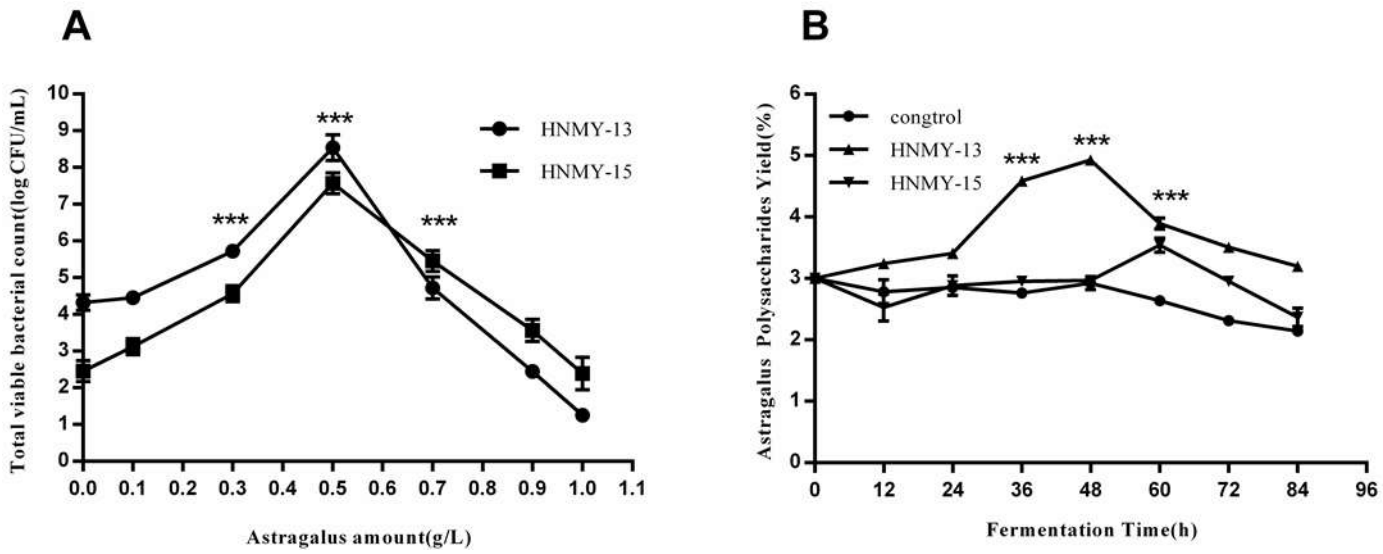

Figure 4. A: Microbial counts during fermentation with *B. subtilis* HNMY13 and HNMY15; B: The polysaccharide yield during fermentation with *B. subtilis* HNMY13 and HNMY15. Bars with different asterisk indicate they significantly differ from each other ($p<0.05$).

In Figure 3. The results revealed that different fermentation times had significant effect on HNMY-13 and HNMY-15 strains.

Microbial counts during fermentation

Microbiological monitoring of the fermented *Astragalus* co-inoculated with individual *B. subtilis* HNMY-13 and HNMY-15, respectively, was done. As shown in Figure 4A, the population of *B. subtilis* reached the peak in *Astragalus* amount at 0.5 g/L. The viable count of sample fermented was 8.8×10^8 CFU·mL^{-1} and 7.6×10^8 CFU·mL^{-1}, respectively. The result indicated that *Astragalus* might increase the population of *B. subtilis* under appropriate dosage. It was reported that *B. subtilis* natto-fermented *Astragalus* if fermented medium contained 10 g *Astragalus* (Hsu and Chiang, 2009). In this study, it was found that fermented medium containing 0.5 g could promote *B. subtilis* counts. This result revealed that *Astragalus* provided necessary polysaccharide, peptide and amino acid, such as glycine, valine, leucine, glutamic acid, tryptophan and isoleucine which were beneficial to *B. subtilis* reproduction. The result of this experiment was in line with previous studies (Gobbetti et al., 2010).

The polysaccharide yield analysis

This study presented fermented *Astragalus* by using *B. subtilis* HNMY-13 and HNMY-15 in liquid fermentation process, which was recognized as a potential bio-transformation process without causing severe environmental pollution. The *Astragalus* polysaccharide yield was higher with *B. subtilis* HNMY-13 and HNMY-15 fermentation as compared to the control group as shown in Figure 4B. Moreover, *Astragalus* polysaccharide yield by HNMY-13 was 4.93% at 48 h which was 1.7-fold higher than that of the control group, while polysaccharide yield by HNMY-15 reached peak at 3.54% after 60 h ($p<0.05$). The result showed that *Astragalus* polysaccharide yield changed significantly by *B. subtilis* fermentation. The result indicated that different bacteria had significant difference in effects on the yield of polysaccharide. In conclusion, HNMY-13 and HNMY-15 of *B. subtilis* promote production of *Astragalus* polysaccharide in the process of liquid fermentation and have the potential to be a feed additive.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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