Diversity, Enzyme Production and Antibacterial Activity of *Bacillus* Resource in Sesame-Flavored Liquor Daqu

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Short Report

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Abstract

Daqu provides enzymes and precursors for liquor fermentation, and is the core of liquor fermentation. In this study, 11 strains of *Bacillus* were isolated from sesame-flavored liquor Daqu, which can not only produce protease and amylase, but also have antagonistic effects on common pathogens *Escherichia coli* and *Staphylococcus aureus*. According to the gyrA gene phylogeny analysis, these 11 *Bacillus* strains belong to three species, B1, Y14, Y15, and YPDW9 belong to *Bacillus mojavensis*, W7, W13, YPDW6, and YPDW12 belong to *Bacillus subtilis*, W14, Y5 and YPDW1 belong to *Bacillus velezensis*. According to the results of random amplified polymorphic DNA (RAPD) typing, these 11 *Bacillus* strains are completely different. The specific primers were used to randomly amplify the biological control genes expressing lipopeptide antibiotics (bioA, bmyB, ituc, fend, srfAA, srfAB, yngG and yndJ), and they all expressed different expressions in these 11 *Bacillus* strains. This research provides new ideas for strengthening Daqu and lays a foundation for improving the quality of liquor.

Introduction

Sesame-flavored liquor is an innovative form of Chinese traditional liquor and has a unique style of "fragrant, soft and mellow". High-temperature fermentation of Daqu is the key to the special flavor of sesame-flavored liquor. Daqu is obtained through a complex fermentation process (Fan et al. 2020), made of corn, sorghum, wheat, etc. As a saccharifying agent for liquor fermentation (Tian et al. 2020), Daqu provides raw materials such as enzymes and flavor substances for liquor fermentation. The microbial community of Daqu is the key to determine the liquor flavor (Wang et al. 2017). During the fermentation process, microorganisms convert the organic macromolecules (mainly starch) in the raw materials into intermediate products such as pyruvate and amino acids, and finally produce special flavor substances of liquor (Wang et al. 2011). In this experiment, when using traditional methods to isolate bacteria from sesame-flavored liquor Daqu, it was found that *Bacillus* was dominant. Most of *Bacillus* has the ability to produce amylase and protease. In the Daqu brewing process, the main sources of microorganisms are food and airborne microorganisms, the pollution of common pathogenic bacteria such as *E. coli* is inevitable. According to reports, *Bacillus* can produce antibacterial peptides, bacteriocins, etc (Fira et al. 2018), which have inhibitory effects on common pathogens in Daqu.

In this study, a further antagonistic experiment was performed on enzyme-producing bacteria, and it was found that 11 *Bacillus* strains have antibacterial activity against *E. coli* and *Staphylococcus aureus*. These strains may also be potential probiotic resources. 11 *Bacillus* strains were classified and identified based on 16S rDNA identification, the gyrA functional gene was amplified, and then the random amplified polymorphic DNA (RAPD) was used for typing. Among the 11 strains of *Bacillus*, including 4 strains of *Bacillus mojavensis*, 3 strains of *Bacillus velezensis*, and 4 strains of *Bacillus subtilis*, they are all different. Finally, through antagonism experiment and biocontrol gene amplification, the antagonistic activity and biocontrol mechanism of 11 strains were studied. This research has made contributions to natural biocontrol resources and probiotic resources, provides new ideas for strengthening Daqu, and lays a foundation for improving the quality of sesame-flavored liquor Daqu.

Materials And Methods

Identification of Protease and Amylase Activity of Bacillus

The bacteria were inoculated in LB Agar solid medium containing 1% skimmed milk powder and cultured at 32℃ for 24 hours to observe whether there was a transparent circle around the colony. A transparent circle means that the bacteria can produce protease.

The bacteria were inoculated on LB agar solid medium containing 1% starch and cultured at 32°C for 24 hours. Drop iodine solution on the culture medium and observe after staining. If the *Bacillus* has amylase activity, a transparent circle appears around the colony, otherwise the culture medium should all turn blue.

Study on the antibacterial activity of Bacillus against E. coli and Staphylococcus aureus

E. coli was cultured in LB broth medium for 24 hours, 400 μL bacterial solution was added to 400 ml LB Agar medium cooled to 50 ℃, and the mixed medium was poured on the plate. Streak *Bacillus* on the pathogenic plate and observe whether there is a transparent inhibition zone around the colony.

Staphylococcus aureus was cultured in LB broth medium for 24 hours, 1 ml bacterial solution was added to 400 ml LB Agar medium cooled to 50 ℃, and the mixed medium was poured on the plate. Streak *Bacillus* on the pathogenic plate and observe whether there is a transparent inhibition zone around the colony.

Identification of Bacillus by gyrA

Based on the analysis of 16S rDNA, gyrA can be more effectively used for the identification and differentiation of *Bacillus* species. The DNA of *Bacillus* was extracted by freeze-thaw method, and the gyrA gene was amplified by PCR, and then sequenced. The primers used for gyrA are BS-gyrA-F (5'-CAGTCAGGAAATGCGTACGTCTT-3'), BS-gyrA-R (5'-CAAGGTAATGCTCCAGGCATTGCT-3'), and the reaction process was pre-deformation at 95 ℃ for 5 minutes, 35 cycles (95 ℃ for 1 minute, 55 ℃ for 1 minute, 72 ℃ for 1 minute), and finally 72 ℃ for 10 minutes. The reaction volume was 50 μL. The gyrA gene sequences of 11 *Bacillus* strains retrieved from GenBank were aligned using the program CLUSTAL. The neighbor-joining method of MEGA7 was used to construct a phylogenetic tree and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Kumar et al. 2016).

RAPD molecular typing of Bacillus

The RAPD molecular typing technique was used to analyze the strains of 4 *Bacillus mojavensis* namely B1, Y14, Y15 and YPDW9, 4 *Bacillus subtilis* namely W7, W13, YPDW6 and YPDW12, and 3 *Bacillus velezensis* namely W14, Y5 and YPDW1. The primers used were OPA-02 (5'-TGCCGAGCTG-3'), OPA-18 (5'-AGGTGACCGT-3'), OPL-07 (5'-AGGCGGGAAC-3'), OPL-16 (5'-AGGTTGCAGG-3'), and OPM-05 (5'-GGGAACGTGT-3') (Lefevre et al. 2016). The reaction process was pre-denaturation at 95°C for 5 minutes,

45 cycles (94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes), and finally 72°C for 10 minutes. The PCR volume was 20 μL. After amplification, 10 μL PCR products were taken and analyzed by 1% agarose gel electrophoresis, and then observed and analyzed by gel imager.

Broad-spectrum resistant Bacillus **antagonistic gene amplification.**

According to reports, bioA, bmyB, ituC, fenD, srfAA, srfAB, yngG and yndJ genes are common genes in *Bacillus* that can produce antibacterial substances (Cochrane et al. 2016). We cloned the biocontrol genes of these 11 *Bacillus* species, the primers used are shown in Table 1, and The reaction volume was 20 μL. The program is pre-denaturation 95°C for 5 minutes, 33 cycles (94°C for 1 minute, annealing temperature as shown in Supplementary Table 1, 72°C for 2 minutes), 72°C for 8 minutes. After amplification, 10 μL PCR products were taken and analyzed by 1% agarose gel electrophoresis, and then observed and analyzed by gel imager.

Table 1
Cloning biocontrol gene primers

Gene name	Primer name	Primer sequence	Primer length/bp
srfAA	srfAA-f	GAA AGA GCG GCT GCT GAA AC	273
	srfAA-r	CCC AAT ATT GCC GCA ATG AC	
srfAB	srfAB-f	GTT CTC GCA GTC CAG CAG AAG	308
	srfAB-r	GCC GAG CGT ATC CGT ACC GAG	
ituC	ituC-f	TTC ACT TTT GAT CTG GCG AT	575
	ituC-r	CGT CCG GTA CAT TTT CAC	
fenD	fenD-f	CCT GCA GAA GGA GAA GTG AAG	293
	fenD-r	TGC TCA TCG TCT TCC GTT TC	
bmyB	bmyB-f	TGA AAC AAA GGC ATA TGC TC	395
	bmyB-r	AAA AAT GCA TCT GCC GTT CC	
bioA	bioA-f	TTC CAC GGC CAT TCC TAT AC	210
	bioA-r	TTT GTC CCC TTA TCC TGC AC	
yndJ	yndJ-f	CAG AGC GAC AGC AAT CAC AT	212
	yndJ-r	TGA ATT TCG GTC CGC TTA TC	
yndG	yndG-f	GAA CTG TCC GAA ACA TGT CCG	372
	yndG-r	CTG AGC TCT TGA ACG GTC CGG	

Results

Identification results of enzyme production and bacteriostatic activity

The main substances in Daqu are protein and starch. Enzyme-producing bacteria can accelerate the fermentation process of Daqu, and can convert protein and starch into small molecular substances. According to whether there were transparent circles on the LB medium containing 1% skimmed milk powder or starch, it was determined that 25 strains of *Bacillus* can produce protease, 22 strains of *Bacillus* can produce amylase, and 22 *Bacillus* strains can produce both protease and amylase. The results are shown in Supplementary Table 2 and Supplementary Fig. 1.

According to whether the enzyme-producing bacteria had a transparent circle on the LB medium inoculated with *E. coli* and *Staphylococcus aureus*, 11 strains can antagonize *E. coli* and 12 strains can antagonize *Staphylococcus aureus*. 11 strains of *Bacillus* that can antagonize two pathogenic bacteria at the same time, namely B1, W7, W13, W14, Y5, Y14, Y15, YPDW1, YPDW6, YPDW9, YPDW12. The results are shown in Supplementary Fig. 2.

Identification of Bacillus **with high enzyme activity and antibacterial activity**

Based on the results of previous experiments, the accession numbers of 16S rDNA of 11 *Bacillus* strains with high enzyme production and antibacterial activity are shown in Table 2, The phylogenetic tree is shown in Fig. 1.

Table 2
Identification of *Bacillus* by 16S rDNA

Original number	The bacteria with the highest similarity in NCBI database	Similarity(%)	Accession number
B1	*Bacillus subtilis* NCIB 3610^T (ABQL01000001)	99.58	MW512270
W7	*Bacillus velezensis* CR-502^T (AY603658)	99.23	MW512271
W13	*Bacillus velezensis* CR-502^T (AY603658)	99.85	MW512272
W14	*Bacillus nakamurai* NRRL B-41091^T (LSAZ01000028)	99.72	MW512273
Y5	*Bacillus subtilis* NCIB 3610^T (ABQL01000001)	99.58	MW512274
Y14	*Bacillus velezensis* CR-502^T (AY603658)	99.54	MW512275
Y15	*Bacillus cereus* ATCC 14579^T (AE016877)	99.44	MW512276
YPDW1	*Bacillus subtilis* NCIB 3610^T (ABQL01000001)	99.72	MW512277
YPDW6	*Bacillus velezensis* CR-502^T (AY603658)	99.69	MW512278
YPDW9	*Bacillus nakamurai* NRRL B-41091^T (LSAZ01000028)	99.72	MW512279
YPDW12	*Bacillus velezensis* CR-502^T (AY603658)	99.39	MW512280

The identification based on 16S rDNA and gyrA gene showed that 11 strains of *Bacillus* with high enzyme activity and antibacterial activity belong to three species respectively. Among them, B1, Y14, Y15, and YPDW9 belong to *Bacillus mojavensis*, W7, W13, YPDW6, and YPDW12 belong to *Bacillus subtilis*, and W14, Y5, and YPDW1 belong to *Bacillus velezensis*. The phylogenetic tree of gyrA is shown in Fig. 2.

RAPD typing of 11 bacteria with high enzyme activity and antibacterial activity

The RAPD molecular typing was used to further analyze 4 strains of *Bacillus mojavensis*, 4 strains of *Bacillus subtilis*, and 3 strains of *Bacillus velezensis*, and found that these 11 strains were of different types. The results are shown in Fig. 3.

Biocontrol Gene Amplification of Bacillus

The lipopeptide antibiotic is an important metabolite of *Bacillus*, which is encoded by 8 biocontrol genes, namely bioA (273bp), bmyB (308bp), ituC (575bp), fenD (293bp), srfAA (395bp), srfAB (210bp), yngG (212bp) and yndJ (372bp), and has broad-spectrum antibacterial activity. 4 strains of *Bacillus mojavensis*, 4 strains of *Bacillus subtilis*, and 3 strains of *Bacillus velezensis* isolated from Daqu of

sesame-flavored liquor were used to amplify lipopeptide antibiotic genes with biological control potential and they all had different expressions. As shown in Fig. 4.

Discussion

The dominant bacteria in Daqu of sesame-flavored liquor mainly include *Kroppenstedtia*, *Lactobacillus*, *Saccharopolyspora*, *Bacillus*, *Thermoactinomyces*, and *Weissella* (Xie et al. 2020). *Bacillus* is a kind of heat-resistant microorganism, commonly found in mature Daqu, which can secrete a variety of hydrolytic enzymes, including amylase, protease, lipase, cellulase, glucanase, etc., used for the hydrolysis of macromolecules, and produce flavor compounds in the brewing process (He et al. 2019). As early as 2010, Anissa Haddar et al. reported that *Bacillus mojavensis* A21 can produce at least 6 extracellular proteases (Haddar et al. 2010); Later in 2018, A Bacillus strain capable of simultaneously producing high alkaline protease and heat-resistant amylase was identified as *Bacillus mojavensis* (Hammami et al. 2018). In 2016, an author isolated a strain of *Bacillus mojavensis* BmB4 with broad-spectrum antibacterial properties from plant endophytic bacteria, which has antibacterial activity against *E. coli.*, *Salmonella typhi*, and *Staphylococcus aureus*. After identification by LC-MS/MS, the bacterium can synthesize lipopeptides, surface proteins, rheumatic acid, fengmycin, and other antibacterial substances. Fengmycin is synthesized in non-ribosomal form by the polypeptide synthetase encoded by fenC, fenD, fenE, fenA and fenB genes (Jasim et al. 2016).

Bacillus subtilis is a dominant microorganism not only in liquor Daqu (He et al. 2019), but also in vinegar Daqu (Li et al. 2014) and yellow Jiuqu (Wang et al. 2020). The high-temperature tolerance and high enzyme activity made *Bacillus subtilis* are important in liquor Daqu. *Bacillus Subtilis* can produce flavor substances such as ethyl acetate and its derivative ligustrazine (Xu et al. 2018). Shen et al. added *Bacillus subtilis* LBM 10019 and *Bacillus vallismortis* LBM 10020 to the sorghum extract to increase the content of 2-furanthiol in sesame-flavored liquor to ultimately produce l-cysteine to improve the flavor of sesame-flavored liquor quality (Shen et al. 2020). It is well known that *Bacillus subtilis* has good biocontrol effects, but there is little about liquor. The *Bacillus subtilis* and *Bacillus amyloliquefaciens* isolated from Maotai Daqu have natural antagonistic effects on *Streptomyces*. *Streptomyces spp.* producing geosmin is the most common and most serious source of pollution in liquor (Zhi et al. 2016). Some researches have proposed that lipopeptide antibiotics are *Bacillus* metabolites with broad-spectrum antibacterial activity encoded by fenD, bmyB, ituC, yndJ, bioA, srfAA, srfAB, and yngG (Cochrane et al. 2016; Joshi et al. 2006). As a carrier for high-protease production, *Bacillus* has the antibacterial activity of the spectrum, but also the function of probiotics. Marie Lefevre and others mentioned that *Bacillus subtilis* CU1, as a newly discovered probiotic for the immune health of the elderly, is clinically safe and well tolerated, and has no adverse effects on liver and kidney function, vital signs, etc (Lefevre et al. 2016).

There have been few reports about *Bacillus velezensis* in Daqu. Wang et al(2018) discovered that *Bacillus velezensis* is a thermostable *Bacillus* during Daqu fermentation. He et al.(2019) strengthened Daqu by adding *Bacillus velezensis* and *Bacillus subtilis* and found that the liquefaction power, saccharification

power, and esterification power of Daqu increased significantly, and also the content of volatile compounds such as alcohols, the esters, and pyrazines. *Bacillus velezensis* exist widely in nature, its metabolites are abundant and have spectral antibacterial activity. Fan et al.(2018) reported that *Bacillus velezensis* FZB42 had 13 gene clusters that can produce secondary metabolites with potential antibacterial effects, including the cyclic lipopeptides surfactin, bacillomycin, fengycin (Chen et al. 2009). These substances can not only inhibit the growth of plant pathogens and fungi, but also Can induce plant systemic resistance. Yang et al.(2020) obtained 7 *Bacillus velezensis* strains from corn seeds that have good antagonistic effects against pathogenic maize strains and successfully cloned the biocontrol genes of *Bacillus velezensis*. In recent years, with the indepth research on the genetic, biological and physiological characteristics of *Bacillus velezensis*, more and more researchers have sequenced the whole genome sequence of this strain to obtain its biological control mechanism.

In a conclusion, this study explored the *Bacillus* in sesame-flavored liquor Daqu in both enzyme production and antibacterial directions. This not only provides a direction for speeding up the fermentation process of Daqu, but also provides a theoretical basis for exploring the enzymes and precursors in liquor fermentation. Besides, this study also explored the reasons why 11 *Bacillus* strains can inhibit *E. coli* and *Staphylococcus aureus* from a genetic point of view, which is of great significance to deeply explore the function of Daqu microorganisms and to strengthen Daqu and liquor quality control.

Declarations

Acknowledgments

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This study does not contain any study with human participants or animals performed by any of the authors.

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Figures

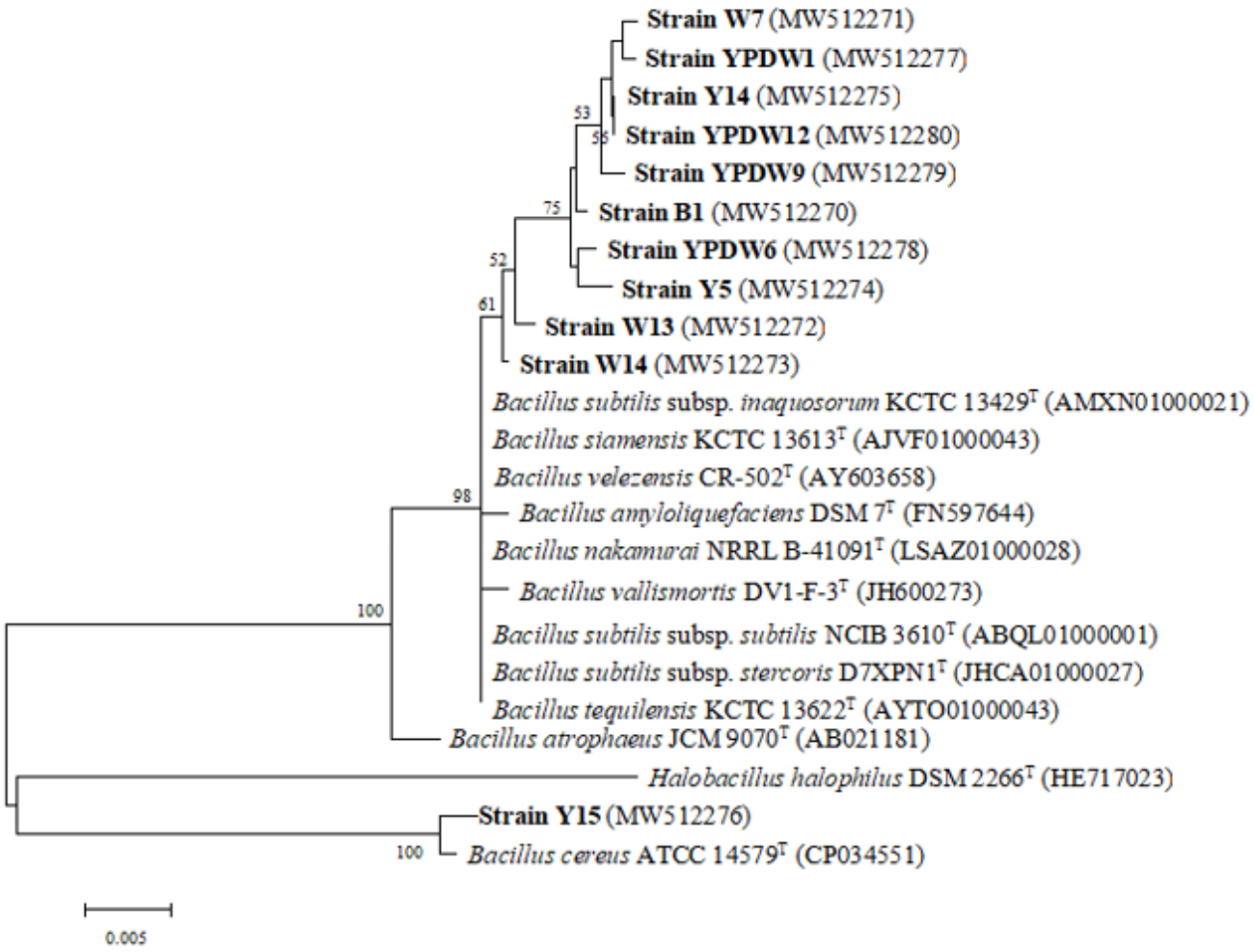


Figure 1

Phylogenetic analysis of the 16S rRNA gene showing the relationships of 11 strains to closer species within the genus Bacillus and Halobacillus halophilus as outgroup. Note: The scale bar indicated 0.005 substitutions per nucleotide position.

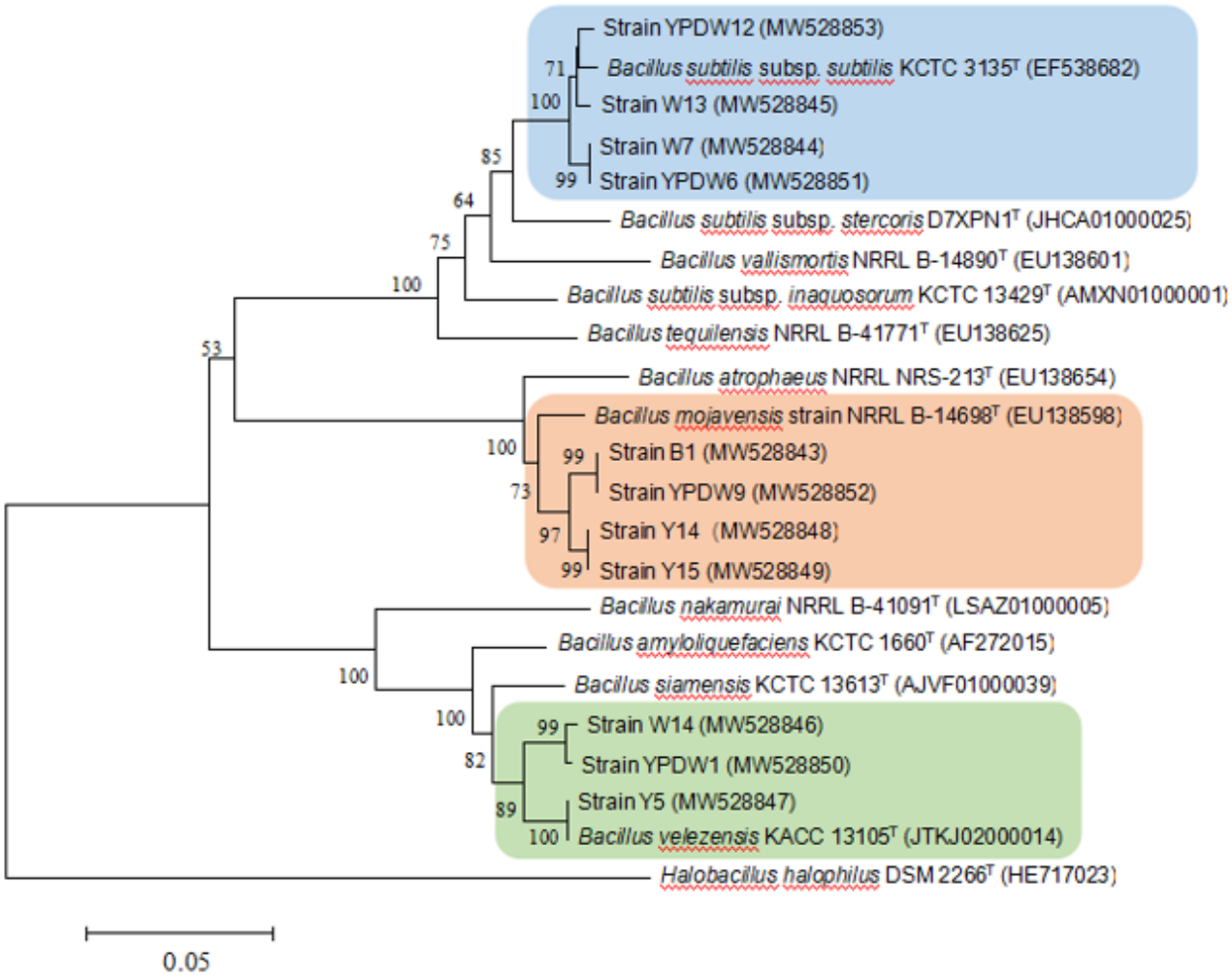
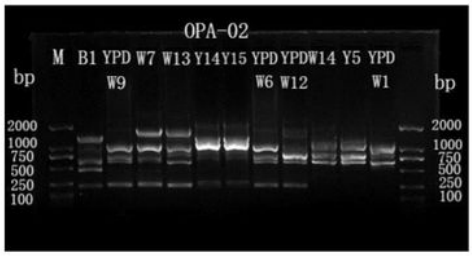
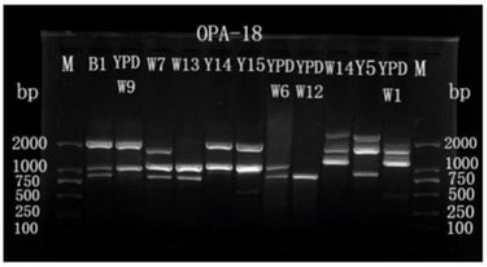


Figure 2

Phylogenetic analysis of gyrA gene showed that 11 strains of Bacillus with antagonistic activity were mainly divided into three species, namely Bacillus mojavensis, Bacillus subtilis and Bacillus velezensis.The scale bar indicated 0.05 substitutions per nucleotide position. Each species is represented by a different color in the picture.



(A)



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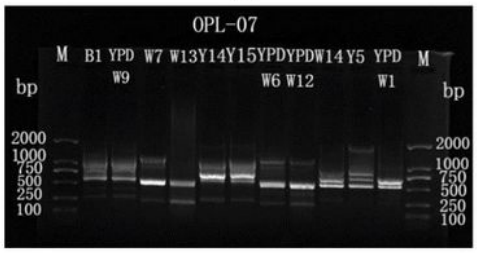

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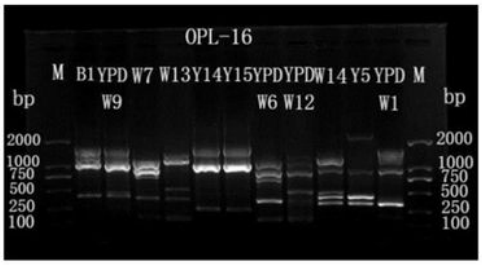

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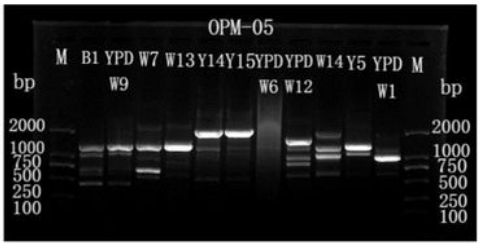

(E)

Figure 3

Schematic diagram of Bacillus RAPD molecular typing. A, B, C, D, and E represent 5 different random sequences. According to the amplification of 5 random sequences, B1, Y14, Y15, and YPDW9 were identified as different strains, W7, W13, YPDW6, and YPDW12 were different strains, and W14, Y5, and YPDW1 were different strains.

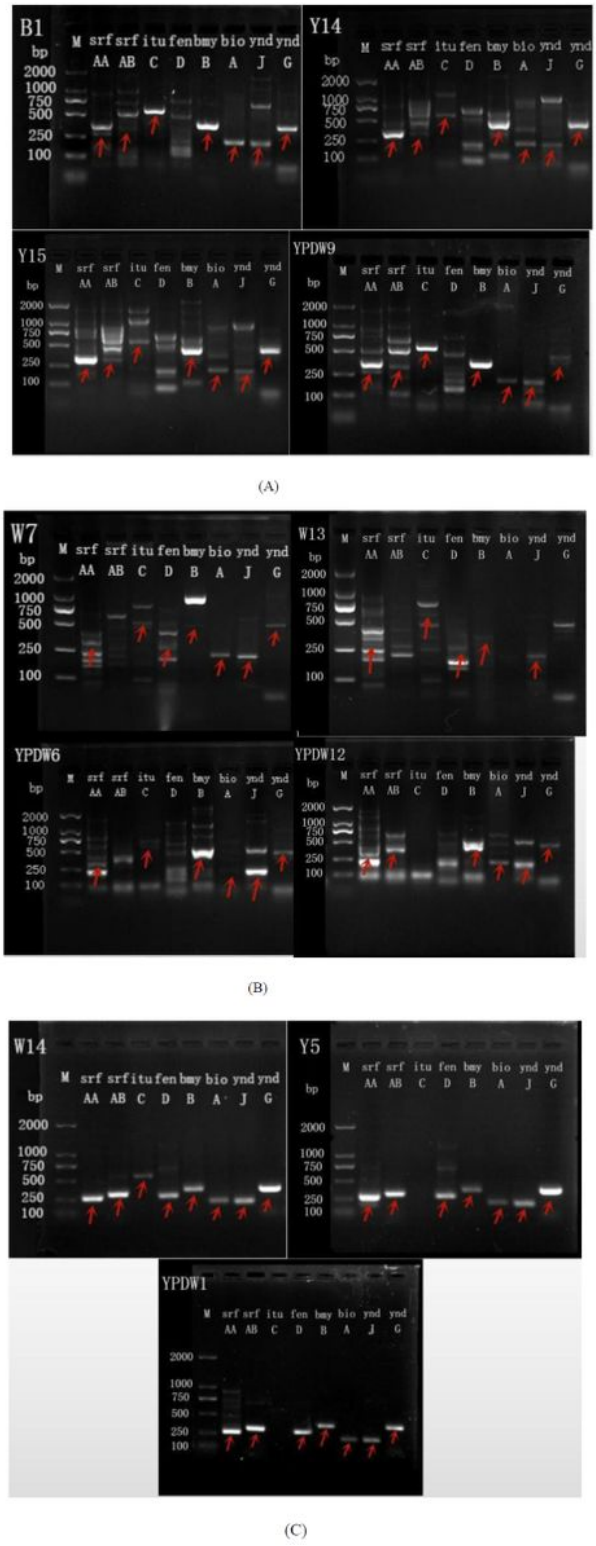

Figure 4

Cloning results of 8 biocontrol genes bioA (273bp), bmyB (308bp), ituC (575bp), fenD (293bp), srfAA (395bp), srfAB (210bp), yngG (212bp) and yndJ (372bp) from Bacillus. A represents 4 Bacillus mojavensis strains; B represent 4 Bacillus subtilis strains, and C represents 3 Bacillus velezensis strains. In each electrophoresis result, the position of the biocontrol gene is marked with a red arrow.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementrayFigure.docx
- SupplemrntaryTable.docx