Simultaneous Production of B-glucanase and Protease From Bacillus Velezensis Strain Identified in the Manure of Piglets.

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Research

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Abstract

Background: β-glucanase and protease, are the representative of most worldwide enzyme market. In this study, a strain producing β-glucanase and protease, identified as one type of Bacillus velezensis and named Y1, was isolated from the manure of piglet.

Results: Its optimal β-glucanase-producing conditions were as: fermentation temperature of 37 °C, fermentation time of 60 h, initial pH of 6.0, outfit fluid amount of 50 mL/250 mL and carbon to nitrogen ratio was 1:1. The properties of the β-glucanasewere that the most suitable reaction temperature was 65°C, the optimum pH was pH 6.0. The optimal protease-producing conditions were fermentation temperature of 37 °C, fermentation time of 60 h, initial pH of 7.0, outfit fluid amount of 75/250 mL and carbon to nitrogen ratio of 2:3.The properties of the protease showed that the most suitable reaction temperature was 50°C, the optimum pH was 6.0. And the enzyme had the best stability at 45°Cand pH 7.0. The effects of each factor on producing β-glucanase and protease by *B. velezensis* Y1 were as follows: temperature > time > pH > loaded liquid volume. The amplified PCR fragments of β-glucanase and protease were 1434 bp containing an open reading frame of 1413 bp encoding a protein with 444 amino acids and 1752 bp containing an open reading frame of 1521 bp encoding a protein with 506 amino acids, respectively.

Conclusion: This study identified the enzymes production, optimized the fermentation conditions of β-glucanase and protease by *B. velezensis*. As well as the enzyme properties were investigated and genes were cloned.

1. Introduction

Enzymes are most efficient biocatalysts, providing, numerous benefits as compared to the traditional chemical catalysts (1). They have a large potential to catalyze the biochemical reactions, and being considered as environment friendly and cost effective alternatives to the chemical catalysts used in the industry (2). *Bacillus velezensis* can inhibit the growth of harmful fungus by secreting enzymes to degrade fungus cell wall (3, 4) There have been some researches on cellulose and protease produced by *B. velezensis* (5, 6). Some series of the known β-glucanase and protease produced by microbial fermentation is still considered as the most important production method.

The enzymes from *B. velezensis* have been verified to be the key substance to inhibit the parasitic harmful fungus and bacteria in plant and gut of animals respectively. Most of the fungal cell wall is mainly composed of chitin and an amorphous filler Laminaria polysaccharide as a structural skeleton, and also contain a small amount of protein and fat (7). Extracellular cell wall degrading enzymes such as chitinase, glucanase, cellulase and protease are responsible for degrading the cell wall of target fungus and utilizing its nutrients, beside this enzymes can inhibit the mycelial growth and spore germination of plant pathogenic fungi, thereby inhibiting pathogenic fungi (8, 9).

As a novel biocontrol bacterium, *B. velezensis* has been widely used as a biological control agent in agricultural fields due to its strong ability to suppress other pathogenic bacteria (10, 11). Therefore, the study of the β-glucanase and protease production from *B. velezensis* has extremely important value. In order to industrialize these two enzymes from *B. velezensis*, the present study aimed to optimize the fermentation conditions of β-glucanase and protease by *B. velezensis*, isolated from the manure of piglets. Furthermore, the enzyme properties were investigated and its genes were also cloned. The results of this study provided the base of using *B.velezensis* Y1 strain as a potential animal probiotic.

2. Material And Methods

2.1. Medium

LB culture medium (1.5% of agar was added for solid medium).

Fermentation medium for β-glucanase consists of bran 10%, soybean meal 15%, CMC-Na 1.5%, and NaCl 2.5%. Fermentation medium for protease consists of bran 10%, soybean meal 15%, and NaCl 2.5%. 1% CMC-Na solution was used as a substrate for β-glucanase activity assay and 2% Casein solution was used as a substrate for protease activity assay.

2.2. Methods

2.2.1 The strain isolation

Sterile equipment and water was used to collect piglet manure. Sampling was carried out at WENS in Hefei, Anhui. Five 35-day-old piglets were randomly selected and their fresh manure (about 5 g) was mixed by glass rod in the beaker. 1 g of the mixed fecal sample was placed in a test tube containing 10 mL of sterile water, and quickly sealed and brought back to the laboratory. All experimental steps were performed quickly to prevent contamination. The mixture was serially diluted to 10^{-2}, 10^{-4}, 10^{-6}, 10^{-7}, and 10^{-8}, respectively. Additionally, 0.1 mL of each concentration was culture on LB medium at 28 ˚C for 36 hours until a single colony was acquired by the streak plate method. A single colony was picked and streaked on a fresh agar plate in order to ensure pure culture, streaking was performed for 3 times.

2.2.2. Morphological characterization

The isolated colony was selected for morphological analysis followed by smear preparation, Gram staining and microscopic examination. The morphology of the bacteria was observed under the microscope.

2.2.3. Molecular identification of strains based on 16S rDNA

Genomic DNA was extracted using a bacterial DNA extraction kit (CWBiotech, Beijing, China) according to the manufacturer's instructions. Amplification of the 16S rDNA gene was done by using primers: 27f: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492r: 5'-TACGGYACCTTGTTAi9CGACTT-3'in a PCR reaction. The 25 μL PCR reaction mixture contained 2 μL primers (10 pmol/ul), 1μL DNA, 9.5μL Taq DNA MasterMix (5U/

μL), and 12.5μL ddH_2O. PCR amplification conditions were: 94˚C for 10 minutes (preheating), and 35 cycles of 94˚C for 35 seconds, 54˚C for 40 seconds, and 72˚C for 90 seconds, followed by a final extension at 72˚C for 10 minutes. The PCR product size was confirmed by electrophoresis on a 1.2% agarose gel and then purified using a DNA purification kit (CWBiotech, Beijing, China).The purified PCR products were checked in the agarose gel and then ligated into *pEASY-T3* cloning vector (TransGen Biotech, Beijing, China). The recombinant system was used to transform into the *Trans-T1* strain. The positive colonies were identified. The target 16S rDNA was isolated and sequenced by General Biosystems (Hefei, China).

The sequence of 16S rDNA was checked for homology in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Strains with high similarity to the 16S rDNA sequences of this strain were selected from GeneBank, and the phylogenetic tree was constructed by using Neighbor-joining method with MEGA 5.4 software.

2.2.4. Preparation of crude enzymes

Bacterial culture in the fermentation medium was continuously centrifuged at 37 ˚C for 48 hours at 200 rpm then centrifuged at 4 ˚C for 15 minutes at at 6000 rmp by a high-speed freezing centrifuge to obtain a crude enzyme and the liquid was maintained at 4 ˚C. The activity of β-glucanase was determined by DNS method (12). And the activity of protease was determined by Folin phenol method (13).

2.2.5. Optimal fermentation conditions for β-glucanase and protease production

Definition of enzyme activity unit: at certain conditions, the amount of enzyme needed to produce 1 μ mol of reducing sugar from the substrate hydrolyzed by the enzyme per minute is defined as a unit of activity (U).

Calculation of enzyme activity: U = X × n/t/v

X—The quality of reducing sugar produced by enzyme-substrate reaction (μ mol)

n—Dilution multiple of enzyme × solution

t—reaction time (min)

v—Volume of Enzyme Solution (mL)

All the data was analyzed by Excel.

Optimization of fermentation temperature: Five Erlenmeyer flasks were filled with 100 mL fermentation medium and inoculated with the bacterium and incbated at 21, 29, 37, 45, and 53 °C for 48 hours at 200 rpm in a shaking incubator to test the effect of temperature on the fermentation production of this strain.

Optimization of fermentation time: For optimization of fermentation time, the 250 mL Erlenmeyer flask was filled with 100 mL fermentation medium and the fermentation was carried out from 24 to 108 h at the optimum temperature, the production was measured at 12-h intervals.

Optimization of initial pH: To test the most suitable pH on β-glucanase and protease production by the strain, the fermentation conditions were based on the above optimization conditions, and the pH was adjusted from 5.0 to 9.0.

Optimization of liquid load: The fermentation based on the above optimization conditions, in 250 mL fermentation flasks, six volumes of 25, 50, 75, 100, 125, and 150 mL medium were compared for β-glucanase and protease production.

Optimization of carbon to nitrogen ratio: In order to determine the optimal proportion of carbon and nitrogen sources, the total quality of carbon and nitrogen sources was unchanged and the added proportions were designed to 1:9, 1:4, 2:3, 1:1, 3:2, 4:1, and 9:1, respectively.

2.2.6. Orthogonal test for optimization of fermentation conditions

On the basis of the single factor test, four factors that had a great influence on the enzyme production of the strain were selected. An L9 (3^4) orthogonal table was chosen using the β-glucanase and protease activity value in the fermentation supernatant fluid as the inspection index, and fermentation temperature (A), fermentation time (B), pH value (C) and liquid load (D) were used as the experimental factors. Each factor was designed with three experiment levels, the factors and levels of orthogonal tests for β-glucanase and protease producing were shown in Table 3 and Table 4, respectively. The treatment of the enzyme solution and the de termination of the enzyme activity were still carried out as the method as of the above single factor test.

Table 3
Factors and levels of orthogonal tests forβ-glucanase fermentation.

Level	A fermentation temperature (˚C)	B fermentation time (h)	C pH value	D liquid load (mL)
1	29	60	5	25
2	37	72	6	50
3	45	84	7	75

Table 4
Factors and levels of orthogonal tests for protease fermentation.

Level	A fermentation temperature (˚C)	B fermentation time (h)	C pH value	D liquid load (mL)
1	29	48	6	50
2	37	60	7	75
3	45	72	8	100

2.2.7. Enzyme properties of β-glucanase

Optimal temperature for β-glucanase reaction: The optimum temperature for enzymatic activity was examined by incubating at different temperatures (30–75 °C) with an interval of 5 °C. The relative activity was calculated contrasting to maximum activity as.

Optimal pH for β-glucanase reaction: The optimum pH of enzyme activity was measured within the pH ranging from 3.0 to 9.0 at suitable temperature.

Thermal stability of β-glucanase: Thermal stability of enzyme was assayed by incubating it in water bath at 55, 60, 65, and 70 °C for 10, 20, 30, 60, 120, and 240 minutes, respectively. A certain amount of enzyme was periodically withdrawn for activity assay. The residual activity was measured.

pH stability of β-glucanase: To determine the pH stability, the enzyme was incubated at different pH values for 17 hours at 30 °C. The residual activity of each sample was recorded.

Metal ions: Under the optimum assay conditions, the effect of various metal ions on the enzyme activity was determined by adding Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, Al^{3+}, Mn^{2+}, and K^{+} (1 mmol/L) to the substrates. The relative activity was calculated contrasting to the control group where the reaction was carried out in the absence of above metal ions.

Enzyme activity to substrates: CMC-Na (control group), cassava dregs, absorbent cotton, soybean meal, filter paper, and microcrystalline cellulose were chosen as the substrate, respectively. The β-glucanase activity was tested under the optimum assay conditions.

2.2.8. Enzyme properties of protease

Optimal temperature for protease reaction: The optimum temperature for enzyme activity was measured at different temperatures (30–60 °C) with an interval of 5 °C. The relative activity was calculated contrasting to maximum activity as 1.

Optimal pH for protease reaction: The pH of the enzyme reaction solution was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, respectively, and the enzyme activity was measured at the optimal temperature.

Thermal stability of protease: The same amount of enzyme solution was taken and placed in water bath at 45 50, 55, and 60 °C for 10, 20, 30, 60, 120, and 240 minutes, respectively. A certain amount of enzyme was periodically taken for activity assay. The residual activity was measured.

pH stability of protease: To determine the pH stability, the enzyme was incubated at different pH values for 17 hours at 30 °C. The residual activity of each sample was recorded.

Metal ions: Under the optimum assay conditions, the effect of various metal ions on the enzyme activity was determined by adding Na^{+}, K^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} (1 mmol/L).. The relative activity was calculated contrasting to the control group where the reaction was carried out in the absence of above metal ions.

2.2.9. Gene Cloning

According to the gene information of *B. velezensis* in GeneBank, one pair of primers was designed by the Primer premier 5.4 software as follows: cel-F: 5’-AGAGGATCCATGAAACGGTCAATCTCTATTTT-3’; cel-R: 5’-AAACTCGAGTAACTAATTTGGTTCTGTTCCCC-3’. The PCR was followed using thermal cycle of 94 °C for 4 minutes, followed by 35 cycles each of 94 °C for 30 seconds, 60 °C for 40 seconds, and 72 °C for 40 seconds, and a final extension of 10 minutes at 72 °C.

The primers for protease gene were designed as follows:

pro-F: 5’-CGCGGATCCGTGGGTTTAGGTAAGAAATTG-3’;

pro-R: 5’-GCGTCGACTTACAATCCGACTGCATTCC-3’. The PCR was followed using thermal cycle of 94 °C for 5 minutes, followed by 30 cycles each of 94 °C for 40 seconds, 50 °C for 40 seconds, and 72 °C for 90 seconds, and a final extension of 10 minutes at 72 °C.

The above PCR products were observed in the agarose gel and then purified using an EasyPure Quick Gel Extraction kit. The purified PCR products were checked in the agarose gel and then ligated into *pEASY-T3* cloning vector. The recombinant system was used to transform into the *Trans-5a* strain. The positive colonies were identified. The target genes were sequenced by Gneral Biosystems (Hefei, China).

The signal peptide of β-glucanase gene and protease gene was predicted by Signal P (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight and the theoretical isoelectric point was predicted by DNAMAN 7.0. The structure protein was predicted by SWISS-MODEL (https://www.swissmodel.expasy.org/interactive).

2.3. Ethics approval

This article does not contain any studies involving human participants or animals.

3. Results

3.1. Morphological characterization

After incubation on LB medium plate at 28 °C for 36 hours, the selected strain grew slowly and the bacterial colony was white, moist, flat, opaque, and anomalistic. The bacteria were rod-shaped and Gram-positive after Gram stain (Fig. 1).

3.2. Molecular identification

The 16S rDNA was extracted from the strain Y1, amplified through PCR and detected by 1.2% agarose gel electrophoresis. The PCR products showed a band at about 1500 bp that conformed to the theoretical length of bacterial 16S rDNA. The 16S rDNA gene sequence was used for constructing a phylogenetic tree with the neighbor-joining method. The sequence showed 99% homology with *B. velezensis* GQJK49 (CP021495.1), *B. velezensis* NBIF-001 (CP020893.1), and *B. velezensis* JTYP2 (CP020375.1). The phylogenetic tree was constructed by MEGA 5.4 software (Fig. 2). As a result, the strain was identified as *B. velezensis* and named as *B. velezensis* Y1.

3.3. Optimization of fermentation conditions for β-glucanase

In order to determine the suitable fermentation temperature, the strain Y1 was cultured in 100 mL of fermentation medium for 48 h at 21, 29, 37, 45, and 53 °C. The result obtained revealed the strain Y1 had the highest activity to produce β-glucanase under the fermentation temperature at 37 °C (Fig. 3A). The ability of Y1 strain to produce β-glucanase was increased with increase in temperature from 30 °C to 37 °C, At temperature above 37 °C, a sharp decline in β-glucanase activity was observed. Optimization of fermentation time for enzymes production was done by performing the fermentation from 24 to 108 h with 12-h intervals (Fig. 3B). It was apparent that the enzyme activity increased with the prolongation of fermentation time within 72 h and reached the peak at 72 h. After 84 h, the enzyme activity decreased significantly. The effect of initial pH (5.0–9.0) on the enzymes production in our investigation as shown in Fig. 3C revealed an increase in β-glucanase production with increasing the initial pH from 5.0 to 6.0 with the maximum at 6.0. Thereafter, it decreased slowly with the minimum at 9.0. In order to acquire the optimized liquid load of medium, the fermentation was carried at six different volumes. The ideal liquid load was 50 mL/250 mL (Fig. 3D). The enzyme activity in the fermentation medium increased by increasing carbon to nitrogen ratio and was found to be maximum at 1:1. (Fig. 3E)

3.4. The result of orthogonal test

According to the results of single factor experiment, four factors were selected for orthogonal test. As can be seen from the R value in Table 1, β-glucanase production was most affected by temperature, followed by time, pH and loading volume. The R value is the range of the test factor, which reflects the variation of the test index when the test factor level fluctuates. The larger the R value, the greater the influence of this factor on the test index. According to the size of the R value, the order of the factors can be judged. The most ideal conditions for bacterial fermentation were A2B1C2D2 according to their effect respectively,

which stood for fermentation temperature of 37 °C, fermentation time of 60 h, initial pH of 6.0, and medium volume of 50/250 mL.

Table 1
Results of L_9 (3^4) orthogonal test for β-glucanase fermentation

Treatment	A	B	C	D	Enzyme activity (U/mL)
1	A1	B1	C1	D1	0.99
2	A1	B2	C2	D2	1.03
3	A1	B3	C3	D3	0.96
4	A2	B1	C2	D3	1.30
5	A2	B2	C3	D1	1.23
6	A2	B3	C1	D2	1.14
7	A3	B1	C3	D2	0.97
8	A3	B2	C1	D3	0.87
9	A3	B3	C2	D1	0.85
K1	2.98	3.26	3.01	3.07	
K2	3.66	3.13	3.17	3.14	
K3	2.69	2.94	3.15	3.13	
k1	0.99	1.09	1.00	1.02	
k2	1.22	1.04	1.06	1.05	
k3	0.90	0.98	1.05	1.04	
R	0.32	0.11	0.06	0.03	
Note: A: Fermentation temperature; B: fermentation time; C: pH value; D: liquid load.					

3.5. Optimization of culture conditions for protease

It can be seen from Fig. 4A that *B. velezensis Y1* strain had the strongest ability to produce protease under the temperature 37 °C. The protease production started increasing with the increase in temperature from 30 °C and reach to maximum at 37 °C. Further increase in temperature showed a dramatic decrease in the protease production. At the optimum temperature, the protease activity was measured every 12 hours (Fig. 4B). Protease activity reached highest at 60 h of fermentation. After 72 h, the enzyme activity of protease decreased gradually. Protease production increased from pH 5.0 to 7.0 and reached to the maximum at 7.0 (Fig. 4C), indicating that the neutral medium was beneficial for the production of the enzyme. Thereafter, it decreased slowly and reach to the minimum at 9.0. The ideal liquid load for

protease production was 75/250 mL (Fig. 4D). The strain grew better and had a higher enzyme activity in the fermentation medium with carbon to nitrogen ratio of 2:3 (Fig. 4E). The enzyme-producing activity was increased by increasing carbon to nitrogen ratio and found to be most stable at 2:3.

3.6. The result of orthogonal test

As can be seen from the R value in Table 2, the protease production was most affected by temperature, followed by time, pH, and medium volume. The most ideal conditions for bacterial fermentation were A2B2C3D1 according to their effect respectively, which stood for fermentation temperature of 37 °C, fermentation time of 60 h, initial pH of 8.0, and medium volume of 50 /250 mL.

Table 2

Results of L_9 (3^4) orthogonal test for protease fermentation.

Treatment	A	B	C	D	Enzyme activity (U/mL)
1	A1	B1	C1	D1	38.81
2	A1	B2	C2	D2	40.67
3	A1	B3	C3	D3	37.15
4	A2	B1	C2	D3	47.49
5	A2	B2	C3	D1	51.56
6	A2	B3	C1	D2	42.05
7	A3	B1	C3	D2	25.58
8	A3	B2	C1	D3	26.49
9	A3	B3	C2	D1	24.00
K1	116.63	111.87	107.35	114.37	
K2	141.09	118.72	112.16	108.29	
K3	76.07	103.20	114.28	111.13	
k1	38.88	37.29	35.78	38.12	
k2	47.03	39.57	37.39	36.10	
k3	25.36	34.40	38.09	37.04	
R	21.67	5.17	2.31	1.08	
Note: A: fermentation temperature; B: fermentation time; C: pH value; D: liquid load.					

3.7. Enzyme properties of β-glucanase

β-glucanase activity was measured using Carboxymethylcellulose Salt (CMC) as a substrate. The enzymatic reactions were assayed at various temperatures ranging from 30 to 75 °C with a 5 °C interval. As shown in Fig. 5A, the appropriate temperatures of β-glucanase reaction ranged from 60 °C to 70 °C, but the activity reached the peak at 65 °C, and the enzyme activity decreased by 26.97% and 44.86% at 60 °C and 70 °C, respectively. The stability of β-glucanase was better at 55 °C, and more than 91.39% of β-glucanase activity retained until 4 hours. The activity of β-glucanase retained 69.17% at 60 °C and 30.04% at 65 °C after 4 hours. The activity decreased drastically at 70 °C after 30 minutes (Fig. 5B). The effect of different pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) was tested at the optimum temperature. The optimum pH of enzymatic reaction was pH 6.0. β-glucanase activity was reduced 18.0% at pH 5.0 and 26.3% at pH 7.0 (Fig. 5C). As for the pH stability, the enzyme activity was stable at the range 5.0–8.0. But the enzyme activity was not high may be affected by pH, but it was most stable at pH 6.0. At pH 5.0, 7.0, and 8.0, the β-glucanase exhibited 96.5%, 86.8% and 82.2% residual activity (Fig. 5D). The effect of various known metal ions on β-glucanase activity such as Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, Al^{3+}, Mn^{2+}, and K^{+} was measured. The presence of K^{+} and Ca^{2+} would enhance the activity of β-glucanase by 26.57%, and 16.29%. But the Zn^{2+} and Mg^{2+} had a slight inhibitory effect on enzyme activity while Al^{3+} and Cu^{2+} had a strong inhibitory effect on enzyme activity (Fig. 5E). The decomposition capacities of β-glucanase to the substrates were different. The β-glucanase showed a greater activity with microcrystalline cellulose (98.98%), filter paper (79.92%), and absorbent cotton (76.05%) compared with that of the control. However, the activities with cassava dregs (47.26%) and soybean meal (37.7%) were weak (Fig. 5F).

3.8. Enzyme properties of protease

Casein was used as a substrate for protease activity assay. The enzymatic reactions were assayed at different temperatures with every 5 °C interval, ranging from 30–60 °C. Figure 6A indicated that the appropriate temperatures of protease reaction ranged from 45 °C to 55 °C and reaches the peak at 50 °C, and the enzyme activity decreased by 33.51% and 20.07% at 45 °C and 55 °C. But the activity was only 22.23% at 60 °C. The stability of protease was better at 50 °C, and more than 91.11% of protease activity retained until 4 hours. The activity of protease retained 73.70% at 45 °C after 2 hours and 55.21% after 4 hours. The enzyme activity was only 13.12% after incubation at 55 °C for 4 hours. The activity decreased drastically at 60 °C after only 10 minutes (Fig. 6B). The effect of different pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) was tested at the optimum temperature. The optimal pH of enzymatic reaction was pH 6.0. The protease activity was 83.23% at pH 7.0, and still 71.24% at pH 10.0, while only 24.54% at pH 5.0 (Fig. 6C). As for the pH stability, the enzyme activity was stable at the range 5.0 to 10.0. The enzyme activity reached the highest at pH 7.0. At pH 6.0, the protease activity was 56.82%, while the enzyme still exhibited 98.51%, 93.87% and 86.25% residual activity at pH 8.0, 9.0, and 10.0 respectively (Fig. 6D). The effect of various known metal ions on protease activity, such as Na^{+}, K^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} was detected. The presence of Mn^{2+} would enhance the activity of protease by 50.60%. But the Na^{+}, K^{+}, and Ca^{2+} had a slight inhibitory effect on enzyme activity while Cu^{2+}, Zn^{2+}, and Mg^{2+} had a strong inhibitory effect on enzyme activity (Fig. 6E). Activity of proteases from several bacterial strains has been reported to be influenced by metal ions. In our study, the presence of Mn^{2+} would enhance the activity of

Bacillus velezensis protease. But the Na^+, K^+, and Ca^{2+} had a slight inhibitory effect on enzyme activity while Cu^{2+}, Zn^{2+}, and Mg^{2+} had a strong inhibitory effect on enzyme activity. It can be seen that the action of this enzyme depends on the activation of metal ions.

3.9. Gene cloning

The amplified PCR fragment of β-glucanase was 1434 bp containing an open reading frame of 1413 bp encoding a protein with 444 amino acids. N-terminal contains a signal peptide consisting of 12 amino acids (Fig. 7A). The molecular weight is predicted to be 52.31 kDa, and the theoretical isoelectric point is 11.20. The nucleotide sequence of β-glucanase was 100% similar to *B. subtilis* S6 endo-1,4-β-glucanase gene (HQ650233.1) and 99% similar to *B. amyloliquefaciens* MN-13 endo-1,4-β-glucanase gene (KY849862.1), *B. subtilis* C-36 endo-1,4-β-glucanase gene (DQ782954.1), and *B. oceanisediminis* IARI-SP-10 endo-β-1,4-glucanase gene (KF240856.1) (Fig. 8 and Fig. 9A). The structure of the β-glucanase gene is showed in Fig. 10A.

The amplified PCR fragment of protease was 1752 bp containing an open reading frame of 1521 bp encoding a protein with 506 amino acids. N-terminal contains a signal peptide consisting of 25 amino acids (Fig. 7B). The molecular weight is predicted to be 55.3 kDa, and the theoretical isoelectric point is 8.05. The nucleotide sequence of β-glucanase was 99% similar to *B. subtilis* A7 neutral protease precursor gene (KJ958383.1), 98% similar to *B. amyloliquefaciens* K11 neutral protease gene (KM603515.1), *B. subtilis* DB104 neutral protease precursor gene (EU035553.1), and *Bacillus* sp. B16 neutral protease base 16 precursor gene (AY708654.1) (Fig. 11 and Fig. 9B). The structure of the protease gene is showed in Fig. 10B.

4. Discussion

4.1. The stain isolation

A strain producing β-glucanase and protease was isolated from the manure of the piglets, and identified as one type of *Bacillus velezensis. Bacillus velezensis* was firstly isolated from the the mouth of the river Vélez in Málaga (Southern Spain), has originally described by Ruiz-García et al (14). Many currently known *Bacillus velezensis* were isolated from plant roots and soil (15, 16). To the best of our knowledge, this is the first report that *Bacillus velezensis* has been isolated from the manure of piglets. There were many reports about β-glucanase and protease produced by *B. subtilis* (17, 18). *B. velezensis* had 99% similarity with *B. subtilis*, therefore, we speculated that *B. velezensis* can also produceβ-glucanase and protease.

4.2. Optimization of fermentation conditions for β-glucanase

The result obtained revealed the strain had the highest activity to produce β-glucanase under the fermentation temperature at 37 °C. A similar temperature optimum was reported form *Bacillus* sp. (19,

20). There was a sharp decrease after 37 °C was probably due to enzyme denaturation and conformation change, as enzymes are proteins. The enzyme activity increased with the prolongation of fermentation time within 72 h and reached the peak at 72 h which is identical with some other *Bacillus* sp.(21, 22), and much shorter than some reported *Bacillus* sp.(23). However, there were a few reports that the optimal enzyme fermentation time for *Bacillus* sp. was 36 h and 48 h (24, 25). In the post-fermentation stage, nutrients were continuously consumed, the microorganisms were gradually fading, resulting in a decrease in enzyme production. Figure 3C revealed an increase in β-glucanase production with increasing the initial pH form 5.0 to 6.0 with the maximum at 6.0. Thereafter, it decreased slowly with the minimum at 9.0. Nagar et al reported similar optimum pH optimum for enzyme production (26). However, enzyme production by *B. circulans* AB 16(27), *B. pumilus* ASH (28), *B. subtilis* ASH (29), and *B. qingshengii* sp. nov (30) was highest at pH 7.0. An initial pH of the fermentation medium affects the metabolic progress of organic matter by affecting changes in microbial community structure. In general, the higher the biodiversity, the more complex metabolic relationships and the more stable structure. The microorganisms are in the net growth phase at the pH 5.0–8.0 which is more conducive to the anabolism of microorganisms. The amount of liquid loading mainly affects the oxygen capacity of the fermentation medium, which will affect the enzyme production and the growth of bacteria. If the volume of liquid is too high, the oxygen capacity will be insufficient. If the volume of liquid is too low, it becomes difficult for the bacteria to acquire sufficient nutrients, which will lead to the reduction of enzyme production. The ideal liquid load of the strain to produce enzyme was 50/250 mL. There was a higher enzyme activity in the fermentation medium with carbon to nitrogen ratio of 1:1, it was consistent with the theory that carbon source is one of the essential constituents of the microbial fermentation medium, which affects the overall cellular growth and metabolism.

4.3. Optimization of culture conditions for protease

B. velezensis Y1 strain has the strongest ability to produce protease by fermentation under the temperature 37 °C. Many researchers reported 37 °C as an optimum temperature for protease production by *Bacillus* (31, 32). The protease production was increased with the increase in temperature and found to be maximum at 37 °C, further increase in temperature And cause a dramatic decrease in production, as the fermentation temperature can affect the activity and stability of enzyme. When the temperature was too high, the decrease of enzyme production is probably due to the thermotropy of enzyme and the growth of the bacteria is slow at low temperature. At the optimum temperature, protease activity reached highest at 60 h of fermentation, which was identical with some other researches (33, 34), and much shorter as reported by (31, 32). However, the optimal fermentation time of protease for *Bacillus pumilus* D3 was 48 h (35). After 72 h, the enzyme activity of protease decreased gradually. The enzyme production went down with any prolongation in fermentation period. The pH mainly affects the charged state and the redox potential of microbial cells, which affects the absorption of nutrients by microorganisms and the secretion of enzymes. Protease production increased from 5.0 to 7.0 and reached to the maximum at 7.0, indicating that the neutral medium was beneficial for the production of the enzyme. Thereafter, it decreased slowly to the minimum at 9.0. Another study reported similar optimum pH for protease production (36). Furthermore, there were also reports that the best pH for

protease production was 8.0(37), 9.0 (38), 10.0 (39), and 11.0 (38, 40). The pH value affects the absorption of nutrients by affecting the degree of ionization of the nutrients, cell membrane charge and membrane permeability. For most species, it generally has the ability to maintain its cytoplasmic acidity under near-neutral conditions, which is beneficial to metabolism and various enzyme reactions. The pH of the substrate has an indirect effect on cell metabolism and enzyme production. There are some differences in the growth reproduction and neutral protease-producing ability of the strain under different initial pH conditions. The ideal liquid load for protease production was 75 /250 mL. Liquid volume mainly affects the oxygen content of the fermentation medium. Volume of fermentation medium is very important for air supply, nutrient supply, and growth of microorganism and production of enzyme. Too much or too little amount of the liquid can inhibit the protease production. The production of protease was stable at optimum ratio of carbon and nitrogen sources which were essential for cellular growth and metabolism. The strain grew better and had a higher enzyme activity in the fermentation medium with carbon and nitrogen ratio of 2:3.

4.4. Enzyme properties of β-glucanase

In order to utilize β-glucanase effectively, the enzyme properties of β-glucanase were studied in this experiment. The appropriate temperatures of β-glucanase reaction ranged from 60 °C to 70 °C, and reached the peak at 65 °C which was much higher than some other *Bacillus* sp. (41–43).The stability of β-glucanase was better at 55 °C, and more than 91.39% of β-glucanase activity retained until 4 hours. Which showed that the enzyme had a good stability. The optimum pH of enzymatic reaction was pH 6.0. β-glucanase activity was reduced 18.0% at pH 5.0 and 26.3% at pH 7.0. As for the pH stability, the enzyme activity was stable at the range 5.0 to 8.0, But the enzyme activity was not high, might be because of the influence of pH. The enzyme activity was most stable at pH 6.0. At pH 5.0, 7.0, and 8.0, the enzyme exhibited 96.5%, 86.8% and 82.2% residual activity. These results showed that the enzyme has the characteristic of alkali-resistance and acid-resistance, which means the strain, was suitable for animal intestinal environment (44). Therefore, it is one possible application of β-glucanase from *B. velezensis* Y1 in animal breeding as an exogenous. The presence of K^+ and Ca^{2+} would enhance the activity of β-glucanase, but the Zn^{2+} and Mg^{2+} have a slight inhibitory effect on enzyme activity while Al^{3+} and Cu^{2+} have a strong inhibitory effect on enzyme activity, which showed that role of the enzyme depends on the activation of metal ions. Gaur et al. reported that the enzyme activity of *B. vallismortis* increased significantly in the presence of Na+, Mg2+, and Ca2+ (45). Some enzymes are not effective under normal conditions, and can only function with the participation of certain metal ions and cofactors. There are many mechanisms of metal ions acting on enzymes. Some metal ions participate in the catalytic reactions as active centers of enzymes, some connect enzymes and substrates as bridges, some neutralize charges and promote the binding of substrates to enzymes, and some stabilize the spatial conformation of enzymes after binding with enzymes (46, 47). The decomposition capacities of β-glucanase to the substrates were different. The β-glucanase had the highest activity with CMC-Na, the enzyme activity was slightly decreased with microcrystalline cellulose, filter paper and compared with the control, and the activities with cassava dregs, absorbent cotton and soybean meal were weak. These results indicated that the enzyme selectivity was towards cellulose substrate.

4.5. Enzyme properties of protease

Figure 6A indicated that the appropriate temperatures of protease reaction ranged from 45 °C to 55 °C and reached the peak at 50 °C. Protease from *B. pumilus* (48), *B. safensis* S406 (49) *B. nealsonii* PN-11(50), and *B. licheniformis* A10 (51) have been reported to have an optimum temperature of 50 °C, 60 °C, 65 °C, 70 °C respectively. And the stability of protease was better at 50 °C. As for the pH stability, the enzyme activity reached the highest at pH 7.0. At pH 6.0, the protease activity was 56.82%, while the enzyme still exhibited 98.51%, 93.87% and 86.25% residual activity at pH 8.0, 9.0, and 10.0 respectively. The wide pH stability of *B. veleznesis* Y1 suggested that this could be a potential candidate to be used in the tannery and detergent industries. Activity of proteases from several bacterial strains has been reported to be influenced by metal ions. In our study, the presence of Mn^{2+} enhanced the activity of *Bacillus velezensis* protease. But the Na^{+}, K^{+}, and Ca^{2+} have a slight inhibitory effect on enzyme activity while Cu^{2+}, Zn^{2+}, and Mg^{2+} have a strong inhibitory effect on enzyme activity. It can be seen that the action of this enzyme dependent on the activation of metal ions. *Bacillus megaterium* protease activity was increased by cations Ca^{2+} and Mg^{2+} and was strongly inhibited by Cu^{2+} and Zn^{2+} (52). *Bacillus stearothermophilus* protease was significantly stimulated by Ca^{2+} and Mg^{2+} whereas Hg^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+} inhibited the enzyme activity (53).

5. Conclusion

A strain producing β-glucanase and protease, identified as one type of *Bacillus velezensis*, named Y1, was isolated from the manure of piglets. The optimal fermentation conditions of β-glucanase were temperature of 37 °C, time of 60 h, initial pH of 6.0, outfit fluid amount of 50/250 mL and carbon to nitrogen ratio was 1:1. However the optimal fermentation conditions of protease were temperature of 37 °C, time of 60 h, initial pH of 7.0, outfit fluid amount of 75/250 mL and carbon to nitrogen ratio is 2:3 High yield of β-glucanase and protease can be obtained by optimizing the fermentation conditions. The enzymes were weak acid β-glucanase and protease that possesses strong resistance to heat, acidity and alkalinity. The presence of some metal ions could enhance the activity of β-glucanase and protease, which showed that the enhancement of enzyme activity was dependent on the activation of metal ions. Besides, the β-glucanase is specific to the celloluse substrate.*Bacillus velezensis* is widely distributed in nature and is capable of inhibiting a variety of fungi and bacteria. In addition, it is also rich in metabolites. Because of these characteristics, *B. velezensis* was widely used in plants. To our knowledge, there is little research on this strain as probiotic applied to animal feed as a replacement of antibiotics. In the near future, research on the replacement of antibiotics by *B. velezensis* in animal feed will be reported by our laboratory.

Declarations

Availability of data and materials

Not applicable

Competing Interests

The authors declare no competing interests.

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Author contribution statement

ZGW designed, performed and wrote the experiments, and helped draft the manuscript. AK, MYcarried out the laboratory work, participated in data analysis, participated in the design of the study and drafted the manuscript. CJW, BHD and RY collected field data. All authors gave final approval for publication.

Consent for Publication:

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Figures

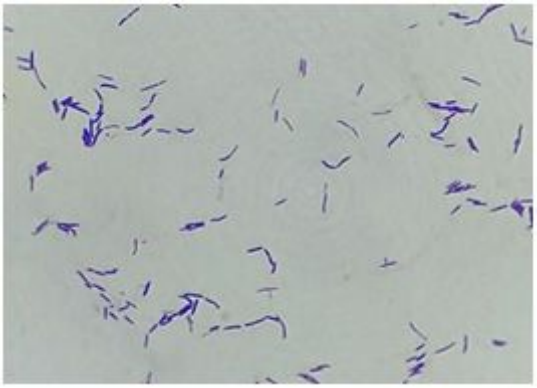

Figure 1

Results of bacteria (Y1) after Gram stain examination.

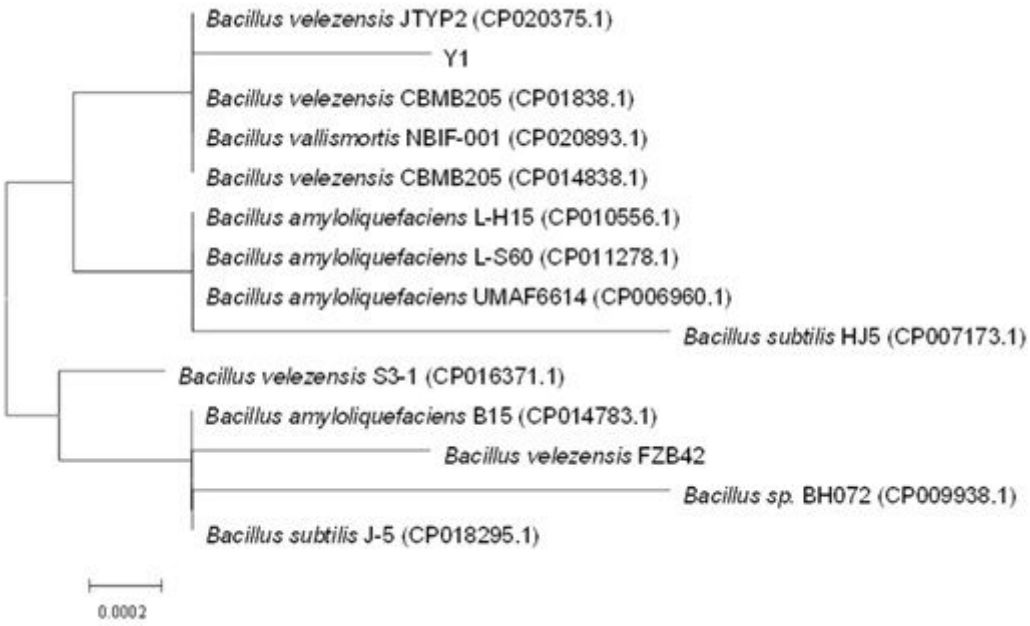

Figure 2

Phylogenetic tree based on 16S rDNA gene sequences of strain B. velezensis, which indicated the position of the isolate among the sequences of closest phylogenetic neighbors obtained from NCBI BLAST analysis.

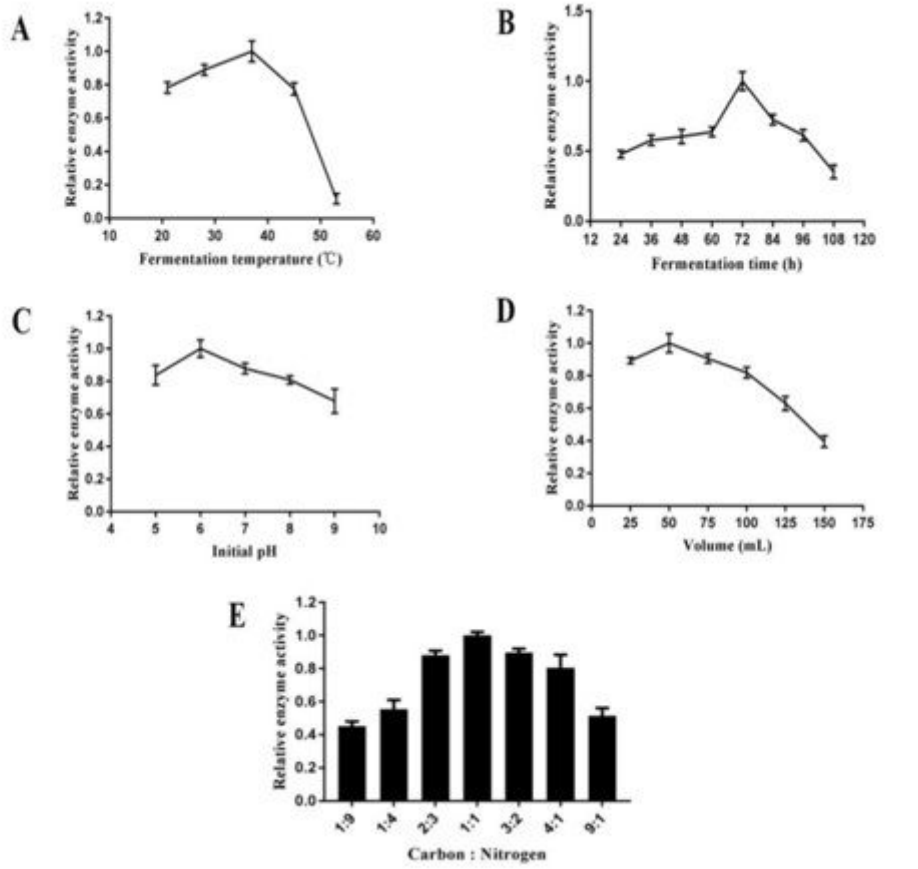

Figure 3

Optimization of fermentation conditions of β-glucanase secreted by B. velezensis Y1. A: Effect of fermentation temperature on enzyme production; B: Effect of fermentation time on enzyme production; C: Effect of initial pH of medium on enzyme production; D: Effect of medium volume aeration on enzyme production; E: Effect of carbon and nitrogen ratio of medium on enzyme production.

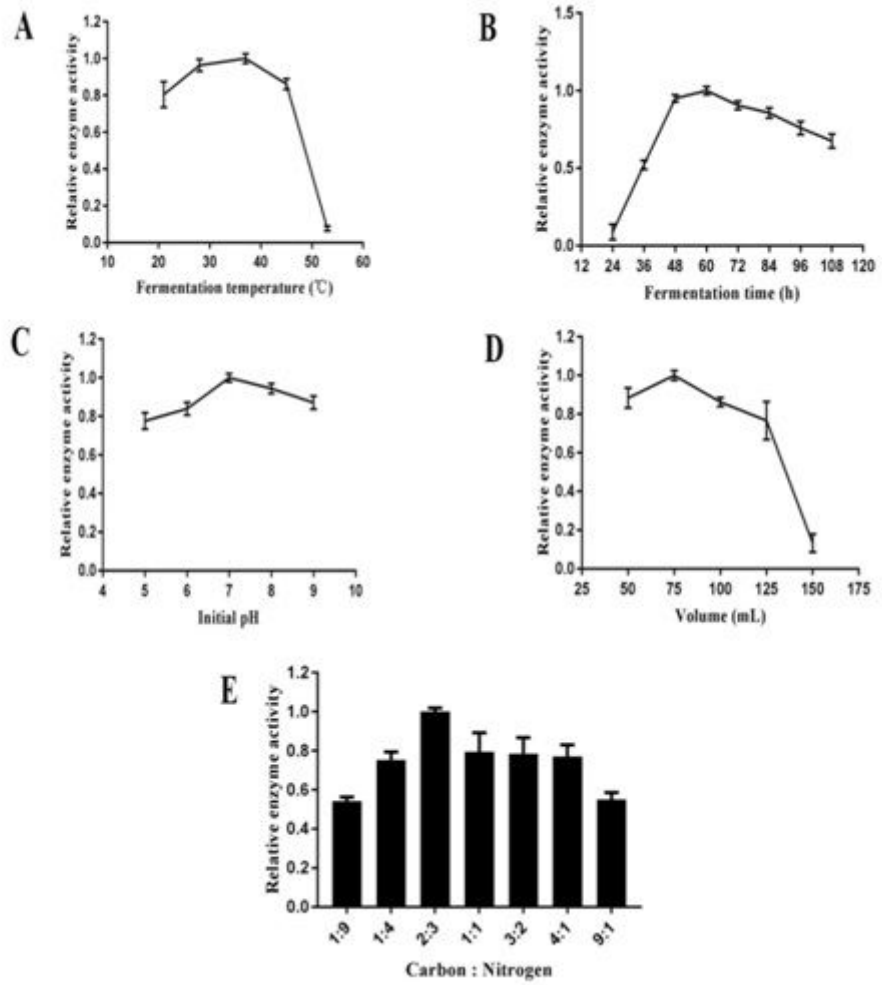

Figure 4

Optimization of fermentation conditions of protease secreted by B. velezensis Y1. A: Effect of fermentation temperature on enzyme production; B: Effect of fermentation time on enzyme production; C: Effect of initial pH of medium on enzyme production; D: Effect of medium volume aeration on enzyme production; E: Effect of carbon and nitrogen ratio of medium on enzyme production.

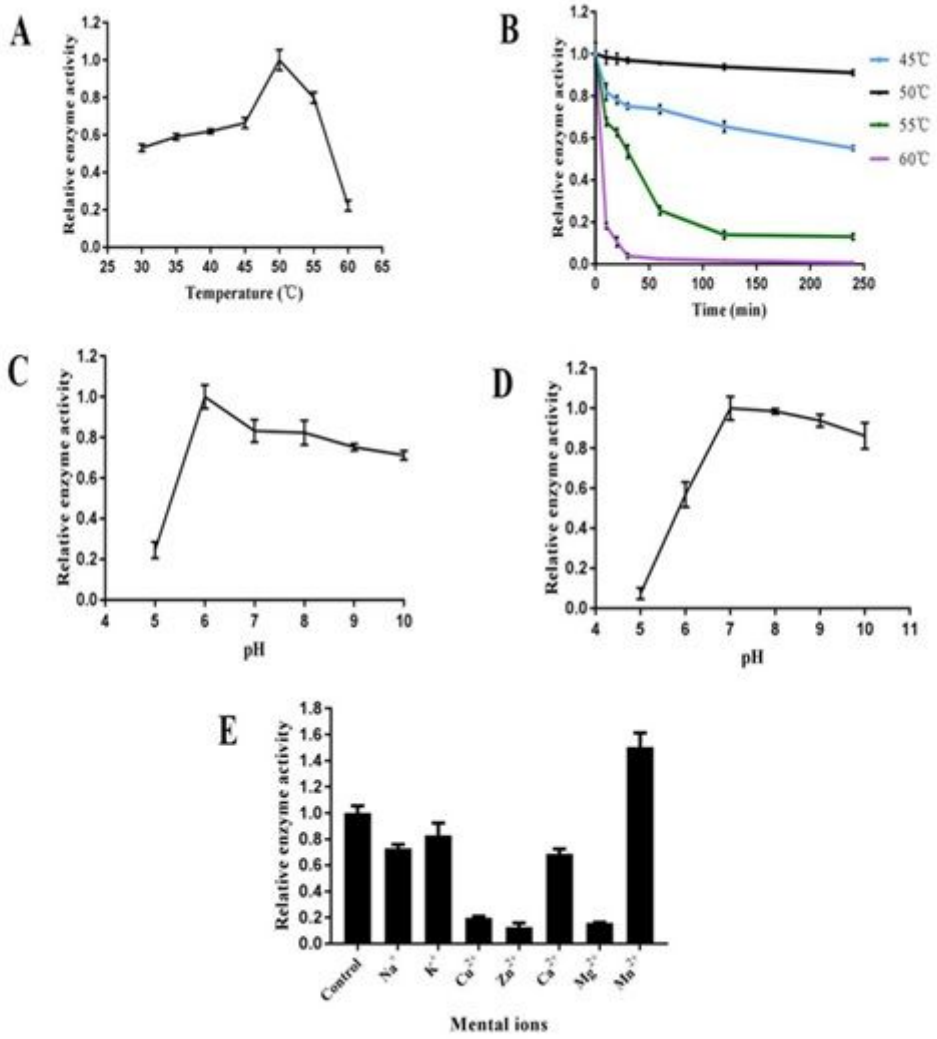

Figure 5

Properties of protease secreted by B. velezensis Y1. A: Effect of temperature on protease activity; B: Thermal stability; C: Effect of pH on protease activity; D: pH stability; E: Effect of metal ions on protease activity.

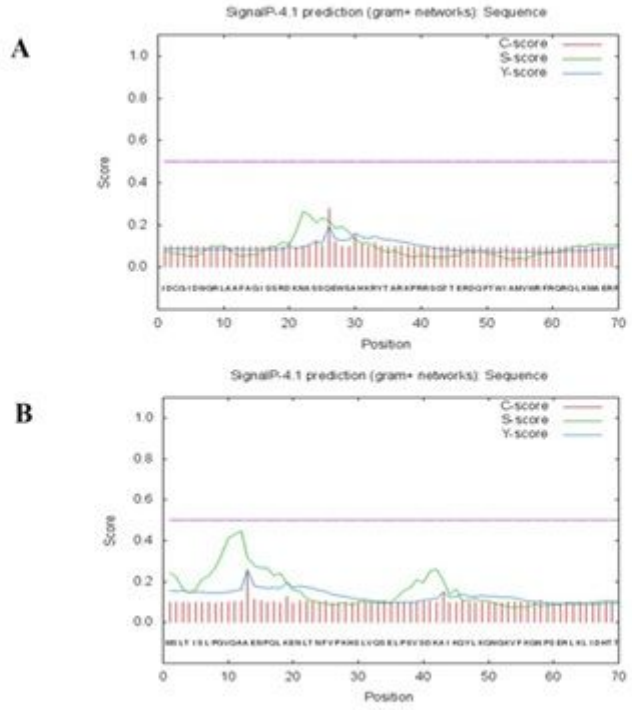

Figure 6

Signal peptide prediction of the β-glucanase gene and protease gene. A: Signal peptide prediction of the β-glucanase gene; B: Signal peptide prediction of protease gene.

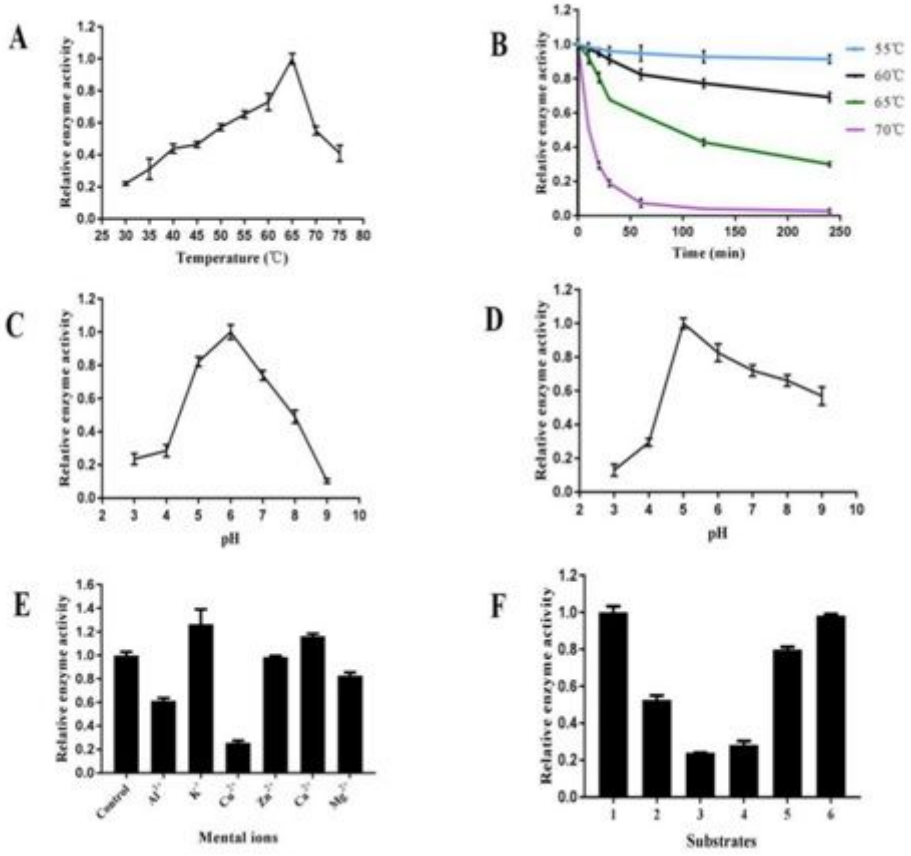

Figure 7

Properties of β-glucanase secreted by B. velezensis Y1. A: Effect of temperature onβ-glucanase activity; B: Thermal stability; C: Effect of pH onβ-glucanase activity; D: pH stability; E: Effect of metal ions onβ-glucanase activity; F: The selectivity of β-glucanase to substrates. 1: CMC-Na; 2: Cassava dregs; 3: Absorbent cotton; 4: Soybean meal; 5: Filter paper; 6: Microcrystalline cellulose.

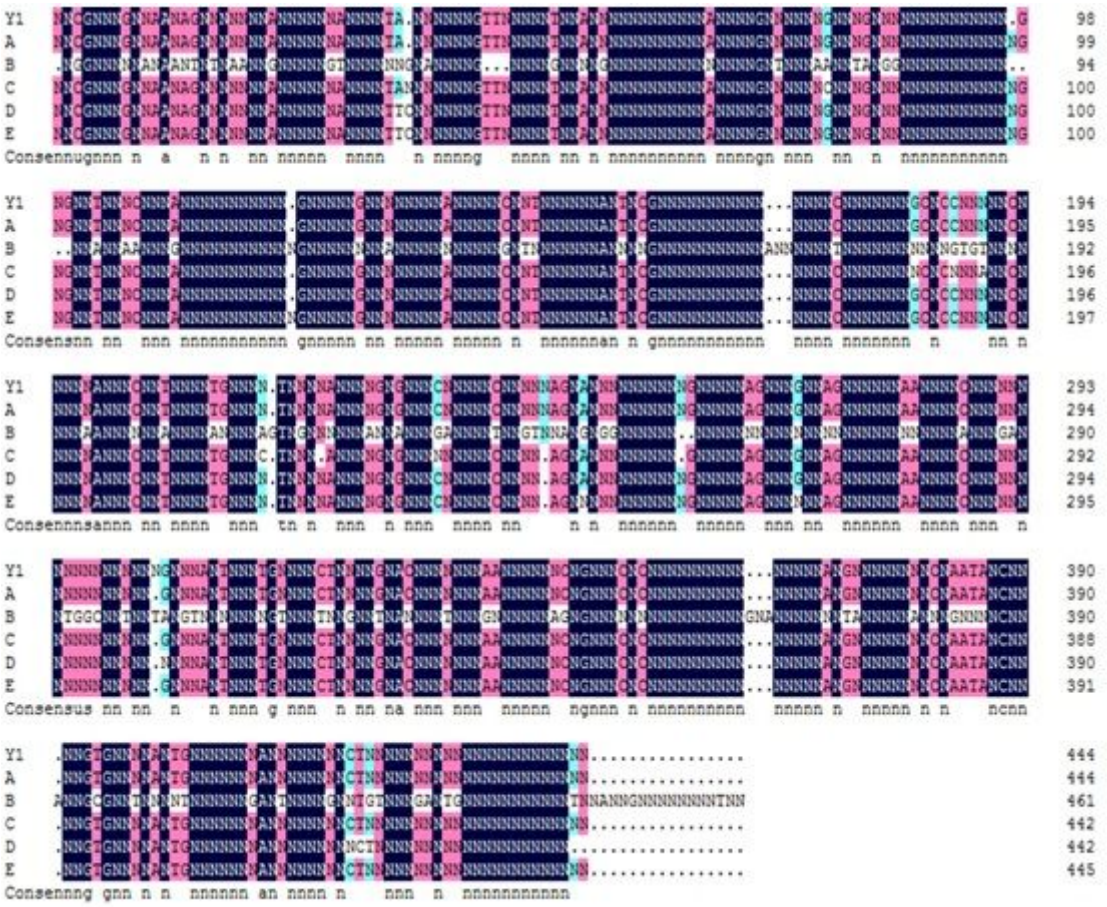

Figure 8

Multiple alignment of the β-glucanase amino acid sequences with homologous proteins. A: Bacillus amyloliquefaciens MN-13 endo-1,4-β-glucanase precursor gene; B: Bacillus oceanisediminis IARI-SP-10 endo-β-1,4-glucanase gene; C: Bacillus subtilis DLG endo-β-1,4-glucanase gene; D: Bacillus sp. TZT-02 β-1,4-endo-glucanase gene; E: Uncultured bacterium clone endos19 endo-1,4-β-glucanase gene.

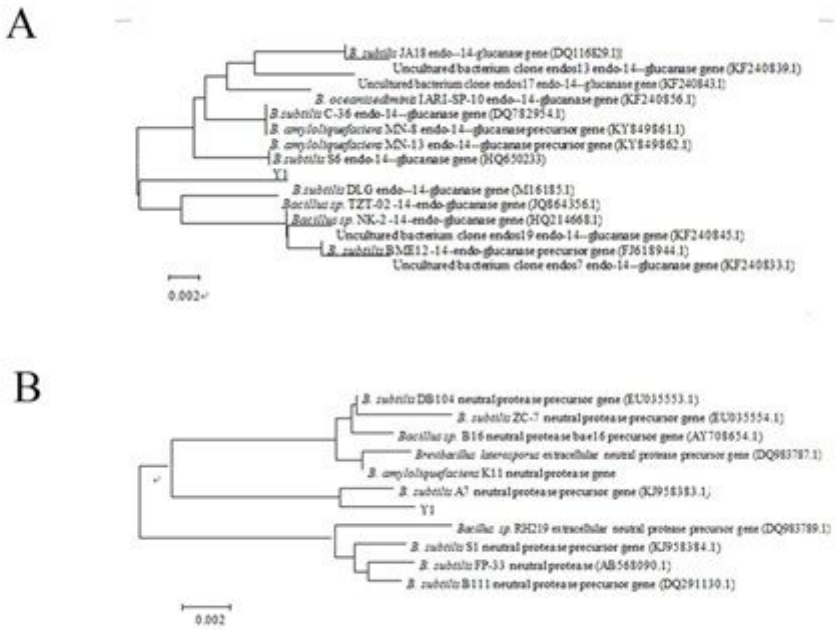

Figure 9

Phylogenetic position of the β-glucanase gene and protease gene sequences of B. velezensis and other related taxa. A: Phylogenetic position of the β-glucanase gene; B: Phylogenetic position of the protease gene.Fig.

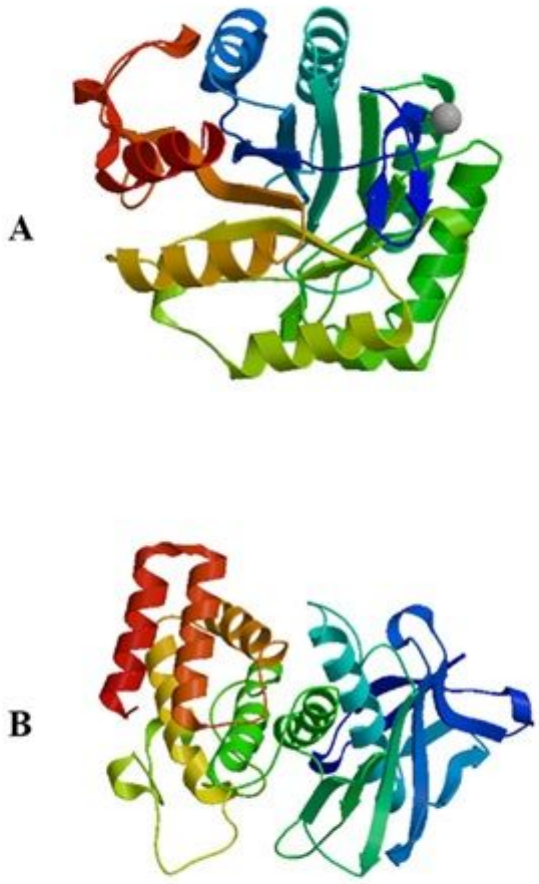

Figure 10

The structure of the β-glucanase and protease sequences of B. velezensis. Note: A: The structure of the β-glucanase; B: The structure of the protease.

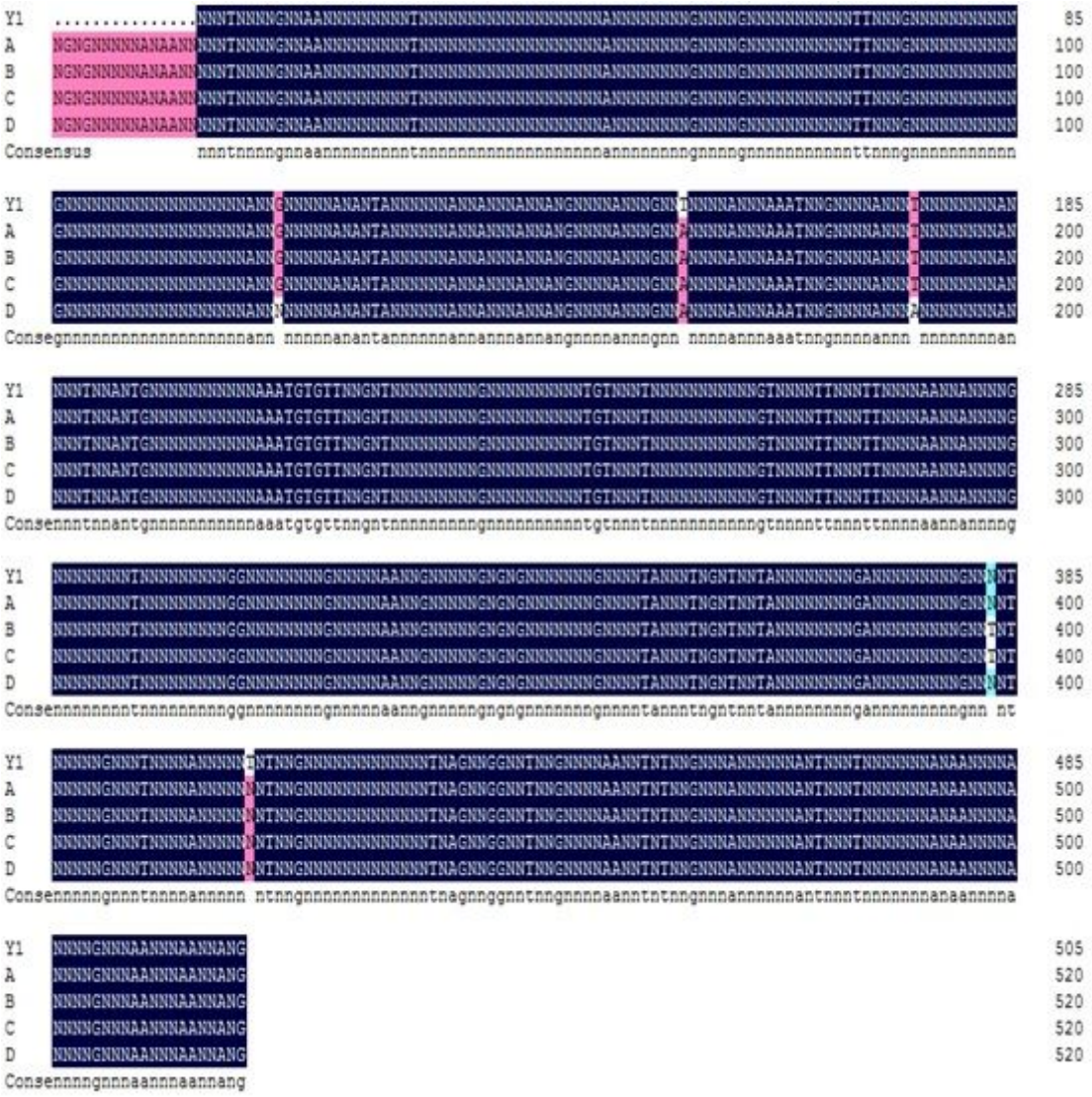

Figure 11

Multiple alignment of the protease amino acid sequences with homologous proteins. A: Bacillus amyloliquefaciens K11 neutral protease gene; B: Bacillus subtilis S1 neutral protease precursor gene; C: Bacillus sp. RH219 extracellular neutral protease precursor gene; D: Brevibacilluslaterosporus extracellular neutral protease precursor gene.