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Screening of protease-producing microorganisms and optimization of their fermentation processes for the efficient preparation of Broussonetia papyrifera feed

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Abstract

With the rapid development of China's livestock industry, the demand for protein feed has been surged, underscoring the importance of developing and utilizing new types of feed. As a novel and unconventional protein source, fermented *Broussonetia papyrifera* feed has become a promising alternative to traditional feeds due to its high nutritional value, biologically active ingredients and wide range of applications. However, the efficient utilization of crude proteins in *B. papyrifera* has been hindered by currently used microorganisms and fermentation processes. In the present study, nine proteolytic bacterial strains and eight yeast and fungal strains were isolated from the root zone soil of *B. papyrifera* by qualitative and quantitative methods. Among them, the protease activities of strains *Paenibacillus* sp. AY and *Rhodotorula mucilaginosa* FG were determined to be 21.95 and 55.16 U/mL, respectively. As compared with the control group, the contents of acid-soluble proteins and ammonia nitrogen in the fermented feed were significantly increased by 26.7% and 12.2% ($P < 0.05$) by *Paenibacillus* sp. AY, respectively, while they were respectively enhanced by 43.3% and 24.5% by *R. mucilaginosa* FG, and their effects were not lower than those of type strains. Finally, to increase the quality of fermented *B. papyrifera* feed, the cultivation conditions were further optimized by single-factor experiments and orthogonal design. Under optimum conditions, the content of acid-soluble proteins reached 7.63, which was 27.2% higher than that of the control. Our results lay a solid foundation for the establishment of a novel process for the efficient utilization of crude proteins in *B. papyrifera* feed and can accelerate the application of *B. papyrifera* feed in animal production.

Introduction

Over the past two decades, China has undergone remarkable social and economic development to become the world's second largest economy. Rapid economic growth and urbanization have promoted a shift in residents' dietary structures and nutritional preferences, leading to a large increase in demand for livestock products, especially for protein-based foods and dairy products[1,2], which requires more grains that are used as feed to meet the requirement of meat production[3]. The competition for grains used as human food or livestock feed has therefore emerged as a new concern for China's food security[4]. It is predicted that the supply-demand gap of feed grains will reach 109.20 million tons and 179.40 million tons in 2030[5], resulting in significant import dependence and a rapid expansion of feed raw material imports. Consequently, to realize the sustainable development of animal husbandry, there is an urgent need to explore novel unconventional feed resources to strike a balance between feed grain security and food security simultaneously.

Paper mulberry (*Broussonetia papyrifera* (L.)) is an ecologically, economically and medicinally important plant in the Asia-Pacific region with features like ease of breeding, fast growth, strong tillering ability, etc[6]. It has a long history of utilization in papermaking, barkcloth making, medicine, and livestock breeding. As a nonfood and unconventional protein feed source approved by Chinese Ministry of Agriculture, *B. papyrifera* exhibits many advantages: (1) containing abundant crude proteins, crude fat,

crude fiber, polysaccharides, amino acids, vitamins, microelements, and essential trace elements, thus displaying high nutritional value[6–8]. Among them, the crude protein content of its stem and leaves is up to 18%-22%, which is similar to that of alfalfa (≈20%)[6]; (2) producing a great number of bioactive compounds, like flavonoids, flavanes, polypenols, alkaloids, and broussopapyrin A, which confers protective effects on livestock and poultry, including antiviral, antimicrobial, anti-inflammatory, anticancer, antioxidant, antinociceptive and anti-tyrosinase activity[9–11]; (3) using paper mulberry as an alternative to traditional protein feed can not only improve the digestibility of feed, but also modulate the composition of growth performance, gut microbiota, and immunity of monogastric animals, ruminants and aquatic animals[12–14]. Being a feedstuff with wide environmental adaptability, abundant nutrients, high bioactive compound content and wide range of applications, paper mulberry has thus become a promising substitute for traditional protein feeds[15].

Although the crude protein content of *B. papyrifera* is high, their amino acid composition is not balanced, the materials can't be easily digested by animals when directly fed, and palatability is low[16]. Therefore, microbial fermentation technology has been employed to prepare *B. papyrifera* feed, which can convert these crude proteins into acid-soluble proteins that can be easily absorbed by animals. During this process, microorganisms and the corresponding cultivation conditions play key roles. Until now, various commercially-available type strains and fermentation processes have been adopted to increase the digestibility of crude proteins[17]. However, their utilization efficiency in *B. papyrifera* feed remains low. Hence, it is highly warranted to select appropriate microbial strains and establish an optimal fermentation process for the preparation of *B. papyrifera* feed.

In the present study, the root zone soil of *B. papyrifera* collected from our university was used as the starting material, and qualitative and quantitative techniques will be utilized to screen microbial strains with high protease activity. Then, the effects of the strains obtained on the content of acid-soluble proteins will also be investigated to choose appropriate candidates. After these strains are identified by 16S rRNA sequencing method, their cultivation conditions will be optimized by single-factor experiment and orthogonal design methods to increase the utilization efficiency of crude proteins in the fermented *B. papyrifera* feed. Our results will not only establish an optimal process for the efficient preparation of *B. papyrifera* feed, but also help alleviate the protein feed supply crisis.

Materials and methods

Chemical reagents, microbial strains and culture media

Folin-Ciocalteu's phenol reagent and casein were bought from Sangon Biotech Co., Ltd. (Shanghai, P.R. China). Rose Bengal medium, nutrient broth medium, malt extract broth and skim milk powder were the products of Solarbio Life Sciences (Beijing, P.R. China). Paper mulberry *(B. papyrifera)* leaf powder was supplied by Sheqi Jingou Agriculture and Animal Husbandry Co. Ltd. (Nanyang, P.R. China). Bacterial and yeast genomic DNA extraction kits were purchased from TransGen Biotech Co., Ltd. (Beijing, P. R. China). All other chemicals used in this study were commercially available in analytical grade.

Bacillus subtilis AS 1.398 and *Saccharomyces cerevisiae* were stocked in our laboratory, while *Aspergillus oryzae* RIB40 was kindly provided by associate professor Bin He from Jiangxi Science and Technology Normal University. Enrichment medium consisted of 20 g paper mulberry leaf powder and 1 L distilled water. Medium containing 15 g skim milk powder, 15 g agar and 1 L distilled water was used for screening protease-producing bacteria, while Rose Bengal medium was employed to isolate yeasts and filamentous fungi. *Paenibacillus* sp. strains were grown in TYC medium consisting of 15 g tryptone, 5 g yeast extract, 50 g sucrose, 20 g sodium acetate, 1 g NaCl, 2 g $NaHCO_3$, 0.1 g Na_2SO_4, 0.2 g L-cysteine, 2 g $Na_2HPO_4{\cdot}12H_2O$ and 1 L distilled water. All media were autoclaved at 121^{o}C for 15 min.

Primary and secondary screening of protease-producing microorganisms

Samples were collected from the root zone soil of *B. papyrifera* in the vicinity of the College of Physical Education, Nanyang Normal University, Nanyang, Henan Province, P. R. China (32.993422^{o} N and 112.536167^{o} E). To isolate protease-active bacteria, all the samples were weighed (5 g), mixed into 500 mL conical flasks containing 100 mL sterile saline, and shaken vigorously for 30 min. Afterward, 5 milliliter samples were withdrawn, inoculated into enrichment medium and incubated at 37^{o}C for 24 h. Traditional 10-fold dilution technique has been adopted to isolate protease-harboring microorganisms through agar plate about 10^{-1} to 10^{-6} dilution factor. From the test tubes labeled 10^{-4}, 10^{-5} and 10^{-6}, 0.2 mL was evenly spread onto plates supplemented with the screening medium and subsequently cultivated in an incubator. During the incubation period, the morphological characteristics of the growing microorganisms were monitored. In addition, the ratio of clearance zone diameter (D) to colony diameter (d) was also determined[18], and strains with a large ratio were selected and then isolated to obtain pure colonies by the streak plate method. For the primary screening of yeasts and fungi, all the samples were also cultured in the enrichment medium. Thereafter, they were inoculated into Rose Bengal medium, and incubated at 30^{o}C for 3 days.

Selected bacterial isolates were grown in 3 mL nutrient broth medium, and incubated at 37^{o}C and 200 r/min for 24 h. Subsequently, 1% (v/v) inoculums of the growth cultures were again inoculated into 20 mL freshly prepared nutrient broth medium and subjected to 24 h of shaking cultivation under the same condition. For yeast and fungal strains, malt extract broth medium was used, and the strains were incubated at 30^{o}C and 200 r/min for 3 d. Post-incubation, all the cultures were centrifuged at 12,000 rpm and 4^{o}C for 10 min, and the supernatants collected were assayed using Chinese national standard GB/T 23527–2009[19] to determine their protease activities.

Re-screening of isolates suitable for the preparation of B. papyrifera feed

After selected isolates and type strains were individually cultivated under their corresponding conditions, 0.6% (v/v) inoculums were inoculated into the *B. papyrifera* powder medium containing 0.1 g brown sugar, 50 g *B. papyrifera* powder and 50 mL water. They were then thoroughly mixed, transferred into plastic bags equipped with a one-way valve and incubated for 6 d. The fermented feed obtained were

heated at 105^{o}C for 15 min, and incubated at 60^{o}C until dried. *B. papyrifera* powder medium without any inoculums was used as a control. The content of acid-soluble proteins in dried samples were determined by Pony Testing International Group (Zhengzhou, P.R. China), while free ammonia nitrogen content was measured according to Chinese national standard GB/T 12143–2008[20].

Molecular identification of isolated strains

Molecular identification was performed for the taxonomic characterization of isolated strains[21]. Total DNA of isolated strains was extracted using bacterial and yeast genomic DNA extraction kits according to manufacturer's instructions, whereas total RNA sample of fungal isolates was extracted with the High Pure RNA Isolation Kit (Roche Diagnostics) and cDNA was synthesized using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's protocol, respectively. With the genomic DNA of cDNA as a template, 16S rDNA sequences of bacterial strains and the ITS gene regions of fungal isolates were amplified by PCR using universal primers, respectively, and sequenced by Sangon Biotech Co., Ltd. The sequencing results obtained was analyzed by nucleotide BLAST on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the phylogenetic trees were constructed by MEGA 11.0 using neighbor-joining method[22]. The confidence values of branches of the phylogenetic trees were determined by bootstrap analyses based on 1000 resamplings.

Single-factor experiments to improve the quality of fermented B. papyrifera feed

Effect of cultivation temperature on the content of acid-soluble proteins

After *Paenibacillus* sp. AY and *R. mucilaginosa* FG were cultivated in TYC medium and malt extract broth to late logarithmic growth phase, respectively, they were mixed at a ratio of viable cells of 1:1, and 0.6% (v/v) inoculums were inoculated into the *B. papyrifera* powder medium as previously described. The experimental setup involved incubation at five distinct temperatures of 28^{o}C, 31^{o}C, 34^{o}C, 37^{o}C, and 40^{o}C. After 5-day fermentation, samples were withdrawn to determine the content of acid-soluble proteins.

Influence of fermentation time on the content of acid-soluble proteins

After 0.6% (v/v) inoculums were inoculated into the *B. papyrifera* powder medium, they were cultured for 4, 5, 6, 7 and 8 days, respectively, while keeping other conditions akin to above section. After 5-day incubation, samples were withdrawn to determine the content of acid-soluble proteins.

Influence of loading amount on the content of acid-soluble proteins

B. papyrifera powder media composed of 40, 45, 50, 55 and 60 g *B. papyrifera* powder were individually adopted to prepare the feed, while keeping all other conditions at a fixed level. After 5-day cultivation, the

content of acid-soluble proteins was assessed.

Effect of water content on the content of acid-soluble proteins

The volume of water was changed to 40, 45, 50, 55 and 60 mL, respectively, to investigate its influence on the content of acid-soluble proteins. All other components were maintained at a fixed level.

Orthogonal experimental design of fermentation conditions

Based on the results of single-factor experiments, the three process parameters were determined to be influencing factors: fermentation temperature (A), fermentation time (B) and water content (C). Three values were set for each influencing factor, and the values of each level for each factor were shown in Table 1. Considering the number of experiments and the reliability of the experimental results, a 3-factor 3-level experimental plan was adopted in this study, using an L9 (3^3) orthogonal table.

Table 1
Factors and levels of the orthogonal experimental design

Levels	Factors		
	A	B	C
1	28	4	40
2	31	5	45
3	34	6	50

Statistical analyses

All experiments were performed in triplicate, with results expressed as mean ± standard deviation (SD). The figures were drawn by Origin (version 2022; OriginLab Corporation, Northampton, MA, USA) and statistical analysis of data was conducted using SPSS software (version 2019b; SPSS Inc., Chicago, IL, USA). Results were considered significantly different when *P*-values were less than 0.05. The least significant difference (LSD) test was used to examine the differences between the parameters. For Tables 1 to 4, data with different superscripts in the same column are significantly different from each other ($P < 0.05$), while those having the same superscripts are not significantly different from each other ($P > 0.05$).

Table 2
Screening of protease-producing bacteria by plate assay and enzymatic activity assay methods

Isolates	Diameter of transparent zone (D, mm)	Diameter of colony (d, mm)	Ratio (D/d)	Activity of proteases (U/mL)
AH	7.5	1.1	6.82	20.05 ± 2.84^{d}
AK	12.2	1.5	8.13	44.70 ± 3.52^{b}
AL	10.3	1.0	10.30	62.82 ± 3.24^{a}
AM	11.6	1.2	9.67	65.21 ± 1.27^{a}
AS	12.7	2.0	6.35	67.01 ± 2.96^{a}
AT	11.5	1.5	7.67	30.03 ± 1.01^{c}
AV	11.6	1.6	7.25	48.49 ± 2.11^{b}
AW	13.4	1.6	8.38	62.03 ± 0.99^{a}
AY	10.1	1.5	6.73	21.95 ± 0.14^{d}

Table 3
Effects of different bacterial strains on the quality of fermented *B. papyrifera* feed

Isolates	Content of acid-soluble proteins (%)	Content of ammonia nitrogen (mg/100g)
Control	5.96 ± 0.09^{i}	95.2 ± 0.3^{i}
AH	8.41 ± 0.01^{c}	115.6 ± 0.4^{e}
AK	7.61 ± 0.13^{d}	108.5 ± 0.7^{f}
AL	7.00 ± 0.06^{g}	129.5 ± 1.3^{b}
AM	7.24 ± 0.13^{g}	115.5 ± 1.1^{e}
AS	8.78 ± 0.10^{b}	125.8 ± 0.9^{c}
AT	7.83 ± 0.13^{de}	119.0 ± 1.2^{d}
AV	7.23 ± 0.06^{g}	133.0 ± 0.7^{a}
AW	9.06 ± 0.06^{a}	105.4 ± 1.4^{fh}
AY	7.55 ± 0.06^{df}	106.8 ± 1.1^{f}
B. subtilis AS 1.398	6.42 ± 0.07^{h}	109.4 ± 1.5^{fg}

Table 4
Effects of different yeast and fungal strains on the quality of fermented *B. papyrifera* feed

Isolates	Content of acid-soluble proteins (%)	Content of ammonia nitrogen (mg/100g)
Control	4.76 ± 0.01^{e}	166.6 ± 1.3^{f}
FA	5.89 ± 0.04^{d}	179.3 ± 1.5^{e}
FB	6.26 ± 0.21^{c}	181.5 ± 1.7^{d}
FE	6.61 ± 0.09^{abc}	197.2 ± 0.9^{c}
FG	6.82 ± 0.04^{a}	207.4 ± 1.1^{ab}
FK	6.48 ± 0.07^{bc}	210.8 ± 0.7^{a}
S. cerevisiae	6.79 ± 0.03^{a}	204.0 ± 1.2^{b}
A. oryzae RIB40	6.39 ± 0.01^{c}	200.6 ± 1.1^{bc}

Table 5
Results of orthogonal experimental design

number	A (°C)	B (d)	C (mL)	Content of acid-soluble protein (%)
1	28	4	40	6.99 ± 0.03^{a}
2	28	5	45	7.08 ± 0.02^{ab}
3	28	6	50	7.13 ± 0.03^{c}
4	31	4	45	7.54 ± 0.03^{e}
5	31	5	50	7.63 ± 0.02^{a}
6	31	6	40	7.58 ± 0.01^{b}
7	34	4	50	6.84 ± 0.02^{d}
8	34	5	40	6.88 ± 0.03^{e}
9	34	6	45	6.79 ± 0.03^{f}
*k*1	7.07	7.12	7.15	
*k*2	7.58	7.2	7.14	
*k*3	6.84	7.17	7.2	
R	0.71	0.06	0.05	

Results

Primary and secondary screening of protease-producing microorganisms

To isolate protease-active bacteria, all the samples were cultured in the enrichment medium and then spread onto screening medium containing skim milk powder, and the size of colonies and transparent zones formed by each bacterial strain was measured after one-day cultivation at 37°C. As shown in Table 2, isolates AH, AK, AL, AM, AS, AT, AV, AW and AY exhibited ratios of transparent zones diameter to colony diameter (D/d) greater than 6.0, indicating their proficiency in protein degradation. Conversely, isolates that failed to display transparent zones were deemed incapable of hydrolyzing proteins and consequently excluded from further utilization. The initially screened isolates with transparent zones were cultivated in nutrient broth medium. Subsequent determination of protease activity demonstrated enhanced activities of isolates of AL (62.82 U/mL), AM (65.21 U/mL), AS (67.01 U/mL), AW (62.03 U/mL) compared to other isolates (Table 2).

For screening of protease-harboring yeasts and fungi, all samples were cultivated in enrichment medium, and subsequently spread onto Rose Bengal medium. After cultivation at 30°C for 3 days, single yeast

colonies or mycelium were individually inoculated into freshly prepared malt extract broth. Post-incubation, samples were withdrawn to investigate their protease activities. As shown in Fig. 1, strains FB and FG demonstrated significantly higher ($P < 0.05$) proteolytic activities compared to other isolates, which were 45.90 and 55.16 U/mL, respectively, indicating that they possess potentially robust protease productivity with efficient protease secretion activity. Nevertheless, no protease activity was detected in isolates FH, FJ, FL and FP.

Re-screening of appropriate strains for the efficient preparation of B. papyrifera feed

To further investigate the effects of bacterial strains on the quality of fermented *B. papyrifera* feed, all selected isolates and type strain *B. subtilis* AS 1.398 were individually inoculated into *B. papyrifera* powder medium. The contents of acid-soluble proteins and ammonia nitrogen were determined after 6-day fermentation. As compared with the control, all the strains tested could significantly enhance the contents of acid-soluble proteins and ammonia nitrogen ($P < 0.05$, Table 3). Besides, all the strains showed higher content of acid-soluble proteins in the fermented feed than type strain *B. subtilis* AS 1.398, while isolates AK, AW and AY exerted similar effects on the content of ammonia nitrogen compared to the type strain.

Similarly, effects of yeast and fungal strains on the quality of fermented *B. papyrifera* feed were also studied. As shown in Table 4, all the isolates could significantly ($P < 0.05$) improve the contents of acid-soluble proteins and ammonia nitrogen in the fermented feed compared to the control. Among them, both contents of acid-soluble proteins and ammonia nitrogen of *B. papyrifera* feed fermented by FG were higher than those of *B. papyrifera* feed prepared by type strains *S. cerevisiae* and *A. oryzae* RIB40.

Molecular identification

After comparing of the DNA sequencing results with the sequences deposited in NCBI database, it has been shown that bacterial isolates AH, AL and AV exhibit close homology with *Stenotrophomonas maltophilia* (GenBank accession NO. KT748642.1), strains AM, AS and AW share high similarity with *Aeromonas hydrophia* (accession NO. OL774807.1), while AT and AK display high homology with *B. anthracis* (accession NO. OQ293911.1). Therefore, all of these eight bacterial strains are opportunistic pathogens. Besides, isolate AY displays high close homology (96.51%) with *Paenibacillus tuaregi* Marseille-P2472 (accession NO. NR_179515.1), as depicted in Fig. 2 by the results of phylogenetic analysis. Genome sequencing and annotation revealed that this strain doesn't encode toxic genes (data not shown). Based on all these findings, it can be reasonably inferred that strain AY belongs to genus *Paenibacillus* and has been designated as *Paenibacillus* sp. AY with an assigned accession number of PX112460 in the GenBank database.

Sequencing results of yeast and fungal isolates were also analyzed by comparing them to the NCBI BLAST database. It has been found that isolates FA, FB and FE have a close homology with *Plectosphaerella cucumerina* W1-6 (accession NO. MK773577.1), *P. cucumerina* W1-6 (accession NO. MK773577.1) and *Cryptococcus laurentii* R134 (accession NO. HG532080.1), respectively. Isolates FG

and FK exhibit a high similarity with *Rhodotorula mucilaginosa* AUMC 10727 (accession NO. KX385847.1), which was further confirmed by phylogenetic analysis (Fig. 3). Therefore, isolate FG was designated as *R. mucilaginosa* FG, and its ITS gene sequence has been assigned a GenBank accession number of PX112467.

Based on the results of primary and secondary screening, as well as re-screening, it can be found that isolates *Paenibacillus* sp. AY and *R. mucilaginosa* FG not only exhibit relatively higher proteolytic activities, but also increase the fermentation quality of *B. papyrifera* feed. Therefore, they were selected for subsequent studies.

Single-factor experiments for optimizing B. papyrifera feed preparation process

The “change one factor at a time” method was employed to investigate the impact of culture temperature, fermentation time, loading amount and water content on the quality of fermented *B. papyrifera* feed. Figure 4A illustrates the effects of culture temperature on the quality of *B. papyrifera* feed. The content of acid-soluble proteins reached a maximum value of 9.63% at 31^{o}C after 5-day incubation, but decreased sharply when the fermentation temperature was below or above this temperature. Therefore, 31^{o}C was observed as the optimal culture temperature for *B. papyrifera* feed preparation by the present isolates.

Impact of fermentation time on the quality of fermented *B. papyrifera* feed was also investigated in a similar way. As shown in Fig. 4B, the content of acid-soluble proteins reached a maximum value of 7.75% after 5 days of incubation, but decreased when the fermentation time was shorter or longer than this time. Accordingly, 5-day incubation could be recommended for the preparation of *B. papyrifera* feed by these isolates.

Effects of loading amount of *B. papyrifera* leaf powder on the quality of *B. papyrifera* feed were then studied. The content of acid-soluble proteins in the fermented feed exhibited a similar trend of initial increase followed by a subsequent decrease, with a maximum of 7.16% when 45 g *B. papyrifera* leaf powder was added (Fig. 4C). Consequently, this amount emerged as the effective experimental condition for optimal fermented *B. papyrifera* feed preparation.

Finally, the influence of water content on the quality of *B. papyrifera* feed was also investigated. As shown in Fig. 4D, the optimum water content was 45 mL, while the content of acid-soluble proteins decreased when water content was lower or higher than this value. Thus, 45 mL water could be considered optimal for enhancing the content of acid-soluble proteins in fermented *B. papyrifera* feed.

Orthogonal experimental design for optimizing B. papyrifera feed preparation process

According to the results of single-factor experiments, culture temperature, fermentation time and water content were considered as influencing factors. Thus, 3-factor 3-level orthogonal experimental design (Table 1) was adopted to investigate the interactions between these dominant factors and optimize the

fermentation process. The range analysis demonstrated that the hierarchical order of factors influencing content of acid-soluble proteins is A > B > C, corresponding to culture temperature > fermentation time > water content (Table 4). Based on mean value (*K*) analysis, the optimal combination was determined to be $A_2B_2C_3$, representing the following conditions: 31^{o}C cultivation temperature, 5-d fermentation and 50 mL water. Under the optimum conditions, triplicate experiments yielded a content of acid-soluble proteins of 7.63 ± 0.02%, which was 27.2% higher than the initial result.

Discussion

Over the past few decades, *B. papyrifera* has received significant attention from researchers due to its wide distribution, high yield, strong environmental adaptability, comprehensive nutrition and high protein content. To make full use of this plant in feed industry, especially its crude proteins, microbial fermentation represents a promising technique[23]. Currently, the majority of research concerning preparation of *B. papyrifera* feed has been centered on commercial microorganisms, which may be not suitable for the fermentation of *B. papyrifera* and hinder the efficient utilization of crude proteins. Thus, isolating a proper microbial strain and establishing an optimal fermentation process has become one of the most important issues for the efficient preparation of *B. papyrifera* feed. In the present study, using the root zone soil of *B. papyrifera* as the starting material, nine bacterial and eight yeast and fungal strains with relatively high protease activities were screened and isolated by qualitative and quantitative methods (Table 2 and Fig. 1). Further analysis showed that bacterial isolate AY and fungal isolate FG could significantly ($P < 0.05$) increase the contents of acid-soluble proteins and ammonia nitrogen in fermented *B. papyrifera* feed compared to the control, which were also higher than or similar to those of type strains (Table 3 and Table 4). Finally, these two strains were identified and designated as *Paenibacillus* sp. AY and *R. mucilaginosa* FG (Fig. 2 and Fig. 3), respectively. To the our knowledge, this is the first report of screening appropriate microbial strains from the root zone soil of *B. papyrifera* for the efficient utilization of its crude proteins.

Previous evidence has shown that *Paenibacillus* sp. strains produce various antimicrobials, exopolysaccharides, and hydrolytic enzymes, which offer protection against many pathogens for livestock and poultry[24]. Among these strains, many have already been adopted as feed additives to enhance the growth performance of animals, including *P. polymyxa* AM20[25], *P. pabuli* E1[26], and *P. konkukensis* SK 3146[27], etc. Furthermore, Yue et al. demonstrated that *P. polysaccharolyticus* XY5 promotes the degradation of fiber component in a co-fermentation system of mulberry leaves and distillers' grains[28]. *R. mucilaginosa* strains can also produce cartenoids, beta-carotene, astaxanthin, single-cell proteins, lipids, enzymes and other functional bioproducts from low-cost agricultural waste materials[29,30]. In addition, dietary supplementation with *R. mucilaginosa* strains could enhance the growth performance, antioxidant capacity, immune function of animals, and modulate the composition of their gut microbiota[31,32]. It has been found that solid-state fermentation with *R. mucilaginosa* TG 529 could remove free gossypol and enhance the nutritional value of cottonseed meal[33]. Therefore,

Paenibacillus sp. and *R. mucilaginosa* strains exhibit huge developmental and application potential, and can be used for the fermentation of *B. papyrifera* feed.

Fermentation parameters have significant effects on the quality of fermented feed, and various methods can be adopted to optimize the process. A previous report by Zhang et al.[34] used orthogonal experimental design to optimize fermentation conditions to increase the quality of fermented *B. papyrifera* feed, whereas Lv et al. simultaneously employed single-factor experiments and response surface method to optimize fermentation conditions of *B. papyrifera* feed[35]. In this study, the co-culture conditions of the isolates *Paenibacillus* sp. AY and *R. mucilaginosa* FG were optimized by single-factor experiments and orthogonal design through changes in culture temperature, fermentation time, loading amount and water content. The highest acid-soluble protein content was attained under the cultivation temperature of 31^{o}C, fermentation time of 5 d, water content of 50 mL, loading amount of 45 g. Under these conditions, the content of acid-soluble proteins reached 7.63%, which is higher than the result obtained by Lv et al. (6.84%)[35], but is much lower than that reported by Zhang et al. (12.56%)[34]. This may be attributed to the fact that the initial contents of acid-soluble proteins in *B. papyrifera* leaf powder used in these studies are different. Anyway, future research should focus on adopting surface response method, genetic algorithm, and neural network model to further optimize the fermentation process of *B. papyrifera* feed. In addition, proteases involved in the degradation of crude proteins in *B. papyrifera* leaf powder should also be identified by omic technologies, such as transcripomics, proteomics, etc.

Conclusion

This study has demonstrated that the two selected isolates, designed *Paenibacillus* sp. AY and *R. mucilaginosa* FG, can effectively degrade crude proteins in *B. papyrifera* leaf powder and increase its fermentation quality. Single-factor optimization provided favorable culture conditions, including 31^{o}C cultivation temperature, fermentation time of 5 d, 45 g *B. papyrifera* leaf powder, and 45 mL water to maintain maximum content of acid-soluble proteins by the isolates. Furthermore, orthogonal experimental design yielded a substantial improvement in the quality of fermented *B. papyrifera* feed. Under the optimal conditions, the content of acid-soluble proteins reached a maximum of 7.63 ± 0.02%. Hence, this study not only screens promising strains for *B. papyrifera* feed preparation which greatly improved the utilization efficiency of crude proteins, but also provides a valuable reference for the screening and cultivation of similar strains in future research endeavors.

Declarations

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have prepared to influence the work reported in this paper.

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Author Contribution

Z. D. and L. Y. conceptualized the scientific ideas. X. L., K. Z. and S. Y. were the major contributors to the experiments and data analysis. Z. D. wrote the draft manuscript and provided financial support. C. T., D. L. and Y. K. reviewed and edited the manuscript. All authors have read and have agreed to the published version of the manuscript.

Data Availability

The datasets generated during the current study are available in the NCBI repository under the accession numbers of PX112460 and PX112467.

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Figures

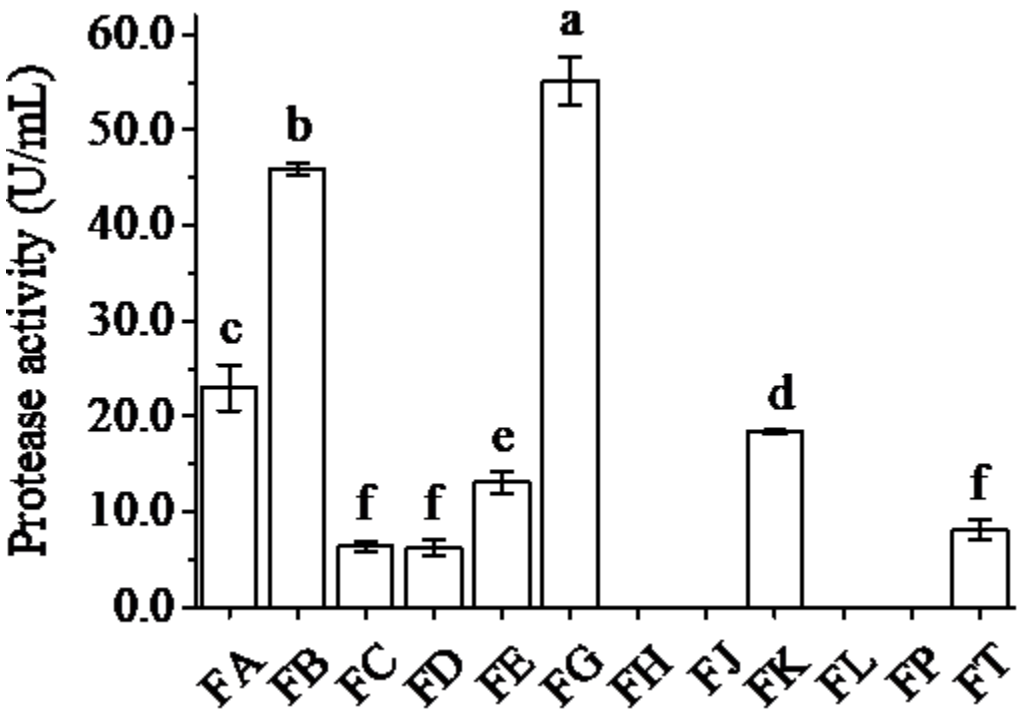


Figure 1

Protease activities of various yeast and fungal strains. Different superscript lowercase letters in bar charts represent significant difference (P<0.05)

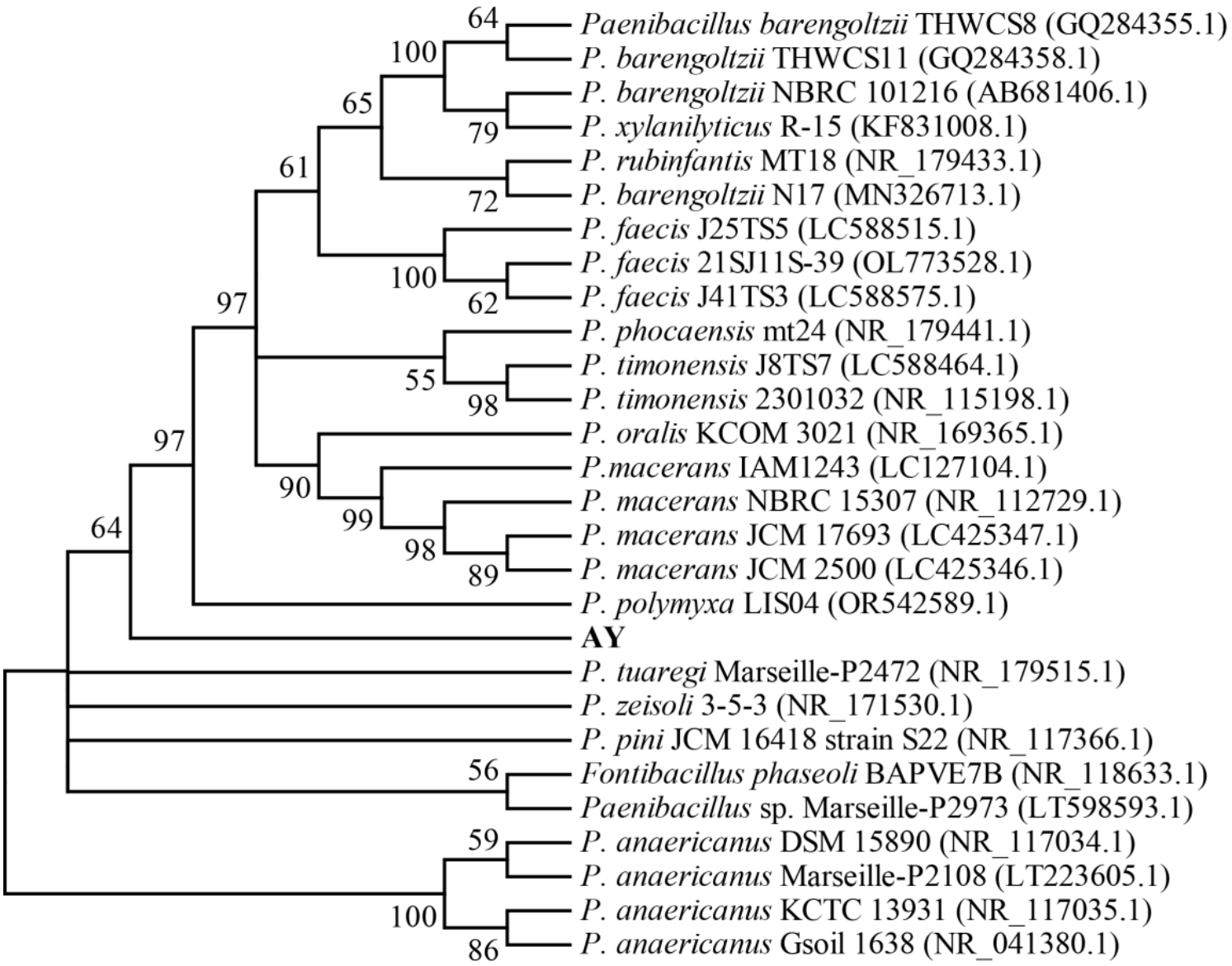


Figure 2

Phylogenetic analysis of bacterial strain AY. Bootstrap resampling with 1000 pseudoreplicates was conducted to assess support for each individual branch, and values lower than 50% were not shown.

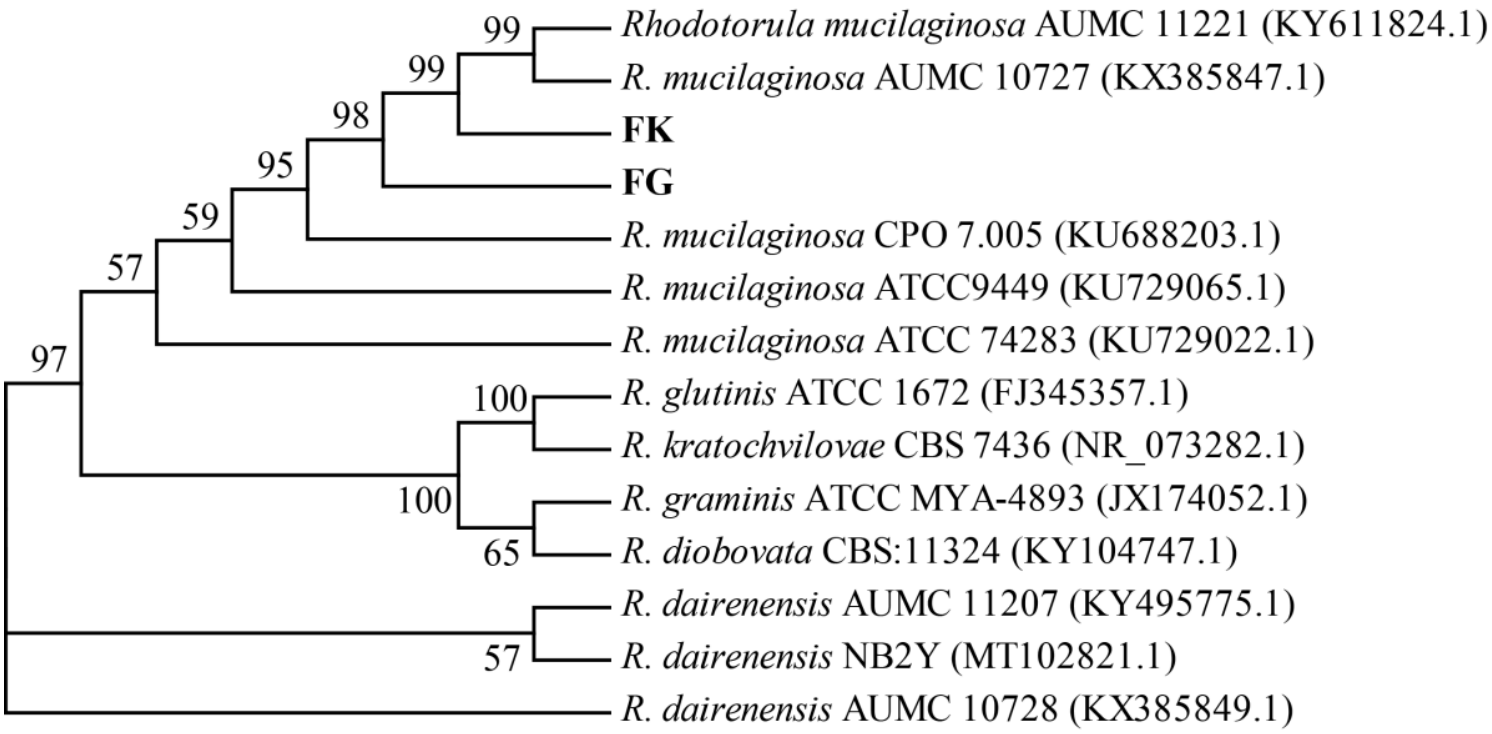


Figure 3

Phylogenetic analysis of fungal strains FG and FK. Bootstrap resampling with 1000 pseudoreplicates was conducted to assess support for each individual branch, and values lower than 50% were not shown.

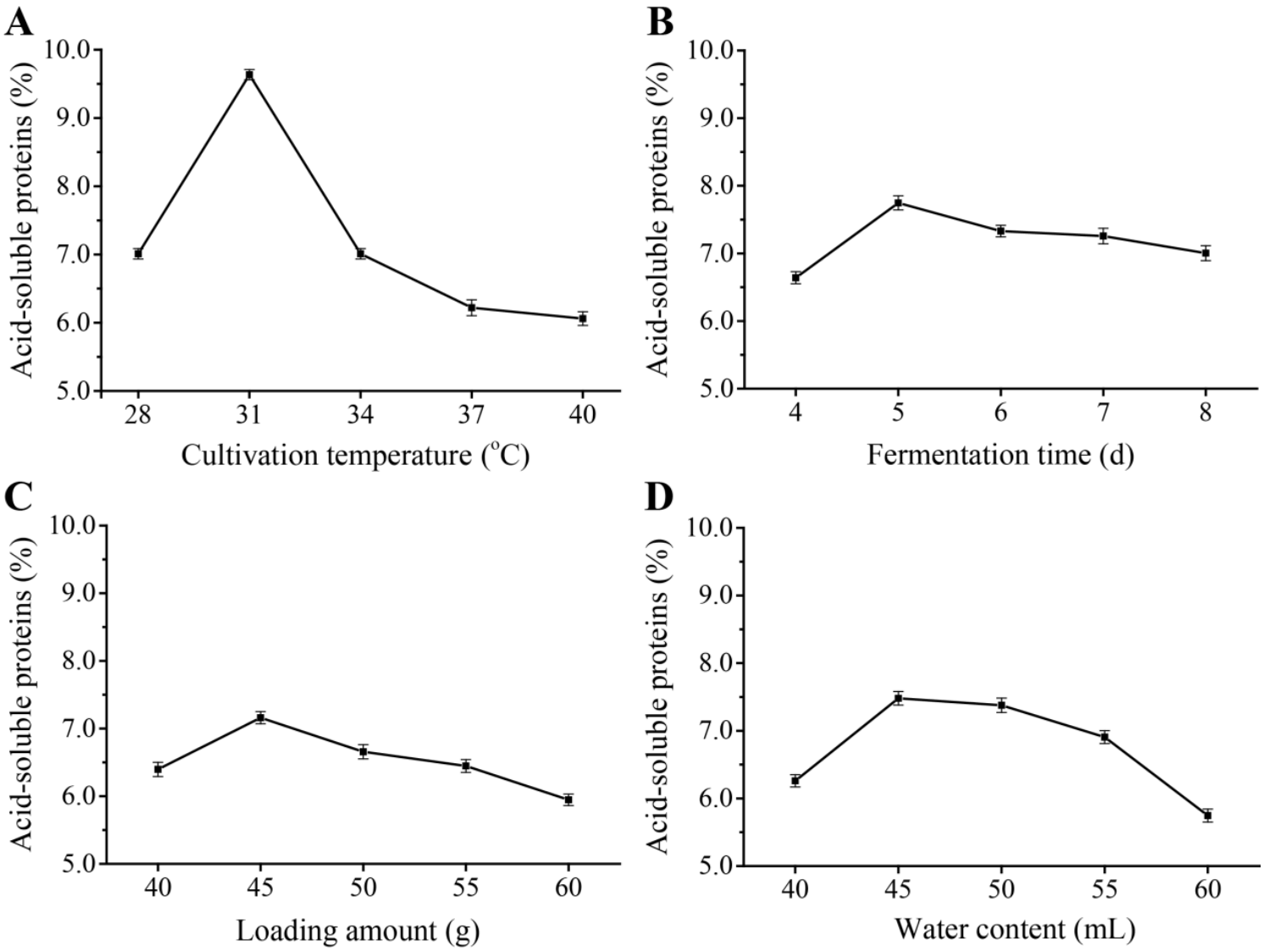


Figure 4

Effects of cultivation temperature (A), fermentation time (B), loading amount (g) and water content (g) on the contents of acid-soluble proteins in the fermented feed