RESEARCH ARTICLE

Isolation, identification, optimization of medium composition, and enhancement of the fermentation process for probiotic *Bacillus* strains isolated from chicken intestines

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Received: April 24, 2025; **accepted:** August 20, 2025.

The global ban on the use of antibiotic growth promoters in livestock has necessitated the need for effective alternatives. *Bacillus* probiotics hold significant promise. However, their application is often constrained by limited functional diversity, environmental adaptability, germination efficiency, and safety concerns. This research isolated and characterized novel *Bacillus* probiotic strains from the intestines of free-range chickens to address these limitations. Eighty-four isolates were subjected to rigorous screening for acid (pH 3.0) and bile salt (0.3% porcine bile) tolerance. Sixteen strains exhibiting > 40% survival under acidic conditions and > 70% survival in the presence of bile salts were selected for further analysis. These strains also demonstrated > 50% survival in simulated gastric juice (0.3% pepsin, pH 1.2, 3 h) and produced amylase, cellulase, and protease. Strains S5 and L10 identified as *Bacillus subtilis via* 16S rDNA sequencing showed superior enzyme production profiles. Medium optimization for strain S5 was conducted using an orthogonal design, resulting in the highest biomass yield at OD_{600} with 8 g/L glucose, 40 g/L peptone, and 1 g/L sodium chloride (NaCl). Response surface methodology (RSM) refined the fermentation parameters to an initial pH of 7.0, an inoculation volume of 1%, and a fermentation duration of 23.84 h. Model validation demonstrated high reliability as predicted OD_{600} value of 0.708 comparing to the actual value of 0.695 with a deviation of 0.13 ± 1.80%. The findings demonstrated the probiotic potential of *B. subtilis* strains S5 and L10 characterized by robust gastrointestinal tolerance, multi-enzyme activity, and scalable fermentation performance. This study provided a foundation for the development of effective, antibiotic-free feed additives to support sustainable livestock production.

Keywords: probiotic; *Bacillus*; enzyme production; medium composition; fermentation process; response surface methodology (RSM).

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Introduction

With the development of the social economy and rising living standards, the demand for poultry and livestock by-products has increased. Meanwhile, public concern regarding the quality and safety of these products has grown. Antibiotics have long played a crucial role in poultry and livestock production. However, their misuse has led to drug residues, weakened immune systems in poultry, and the emergence of endogenous and secondary infections [1-3]. More critically, the long-term misuse of antibiotics has led to the development of drug-resistant pathogens, disrupting microbial balance and posing threats to human health [4, 5]. These issues have attracted global attention and prompted many countries to impose restrictions on the use of antibiotics in animal husbandry. The European Union banned the use of antibiotics in animal husbandry in 2006. In China, the use of antibiotics in feed has been prohibited since January 1, 2020, with all growth-promoting feed additives, except those derived from traditional Chinese medicine, being withdrawn. Thus, the development and implementation of antibiotic alternatives have become essential.

Microbial preparations are widely regarded as ideal substitutes for antibiotic feed additives owing to their safety, efficiency, lack of residues, and cost-effectiveness [6, 7]. Studies show that probiotics primarily exert their effects in the gastrointestinal tract of animals by promoting microbial proliferation, supplying nutrients, regulating pH, modulating immune responses, enhancing digestion and nutrient adsorption, adsorbing toxins, inhibiting pathogens, and improving feed utilization and metabolism [1, 8]. Commonly used probiotics include *Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Enterococcus faecium*, *Aspergillus niger*, and *Bacillus licheniformis* [6]. *Bacillus* has attracted significant research interest owing to its unique physiological characteristics such as heat resistance, tolerance to acidic and alkaline environments, enzyme production along with its broad application potential. *Bacillus* species as gram-positive spore-forming bacteria can endure extreme conditions like high temperature, acid and alkaline environments, and desiccation, which ensures high survival rates during feed processing, storage, and passage through the animal digestive system. Moreover, *Bacillus* can competitively exclude pathogens and secrete digestive enzymes including protease, amylase, and lipase to promote the digestion and absorption of feed. Additionally, *Bacillus* can regulate the intestinal microbiota balance and enhance animal immune function, ultimately improving animal health and production performance [9]. Thus, incorporating *Bacillus* into animal feed can effectively improve gastrointestinal function and promote animal growth, positioning it as a promising alternative to antibiotics. However, several limitations persist in the current application of *Bacillus* preparations. Existing *Bacillus* formulations primarily focus on the secretion of digestive enzymes and the inhibition of pathogens. However, their functional diversity, particularly in regulating intestinal microbiota balance and enhancing host immune responses, remains limited. For example, although *Bacillus coagulans* effectively inhibits pathogens and promotes nutrient digestion and absorption, research on its role in regulating the intestinal microbiota and immune function is limited [10]. Moreover, the health benefits of *Bacillus* preparations vary across different host species, underscoring the need for *Bacillus* preparations with broader functional capabilities [11]. Most current *Bacillus* preparations are derived from laboratory-preserved strains or a limited number of well-characterized isolates, which may not be fully adaptable to diverse host environments. Some *Bacillus* strains exhibit good probiotic characteristics under laboratory conditions but may lose viability due to environmental stresses within the animal gut [12]. Additionally, the significant differences in gut environments among different hosts necessitate that *Bacillus* preparations possess enhanced adaptability to exert optimal effects, however, deficiencies still remain [11]. Although *Bacillus* spores are highly tolerant to environmental stresses, their

germination rate and recovery of metabolic activity within the host remain critical challenges. Studies have shown that certain *Bacillus* spores exhibit low germination rates in the gastrointestinal tract, resulting in insufficient gut activity, which not only affects the probiotic function of *Bacillus* but also hinders its effectiveness in practical applications. Some studies have found that certain *Bacillus* strains demonstrate poor germination rates and metabolic reactivation in the gut, preventing them from delivering their full probiotic potential [12]. Further, the safety of *Bacillus* preparations is also a concern. Some *Bacillus* strains may harbor pathogenic genes such as those encoding enterotoxins, which pose potential health risks to the host. Additionally, the issue of antibiotic resistance in *Bacillus* strains must be addressed to avoid the dissemination of resistance genes through probiotic applications [12]. Certain commercially available *Bacillus* strains have been found to carry enterotoxin genes, raising safety concerns. Therefore, considering these limitations, the widespread application of *Bacillus* preparations remains constrained. Continued efforts are needed to screen and develop more efficient and safer *Bacillus* strains from natural sources to develop higher-quality probiotic preparations [13].

The gastrointestinal tract of livestock and poultry is a source of probiotic strains. This study aimed to isolate probiotic *Bacillus* strains from the intestinal contents of local free-range chickens in Zhumadian City, Henan Province, China, determine their taxonomic classification, investigate their probiotic properties, and optimize both the fermentation medium components and the fermentation process. These efforts would lay the foundation for the development of highly resistant and specific *Bacillus*-based probiotic preparations.

Materials and methods

Experimental animal resource

Six male Zhengyang Three-yellow chickens (*Gallus gallus domesticus*), a native Chinese, yellow-feathered breed, aged 12 - 18 months, from local free-range farmers in Zhumadian City, Henan Province, China were involved in this study. All subjects were traditionally reared in non-intensive backyard systems with natural foraging. This study was approved by the Animal Ethics Committee of Huanghuai University (Zhumadian, Henan, China), and all experimental procedures strictly adhered to the ethical guidelines and regulations for the use of animals in research.

Isolation and screening of *Bacillus* strains

Healthy adult free-range chickens were selected and euthanized immediately *via* cervical bleeding upon arrival after less than 3-hour transport. The large intestines, small intestines, and ceca were immediately collected aseptically. The intestines were opened using sterile scissors, and the intestinal walls were rinsed with sterile physiological saline to remove any contents. The mucosal surfaces were subsequently washed with 1 mL of sterile physiological saline to dislodge adherent probiotics. A 100 µL aliquot of the wash solution was incubated at 80°C for 20 min followed by serial dilution to 10^{-6} using a 10-fold gradient. The 80°C for 20 min treatment selectively eliminated non-spore-forming contaminants while permitting heat-resistant *Bacillus* spores to survive and subsequently germinate, ensuring cultured colonies being the predominantly target probiotics [14]. Aliquots of 100 µL from dilutions ranging from 10^{-2} to 10^{-6} were evenly spread on Luria-Bertani (LB) solid medium and incubated at 37°C for 16 - 20 h. Single colonies exhibiting abundant and distinct morphological characteristics were selected and purified by streak plating. The pure cultures were inoculated into 2.5 mL of LB liquid medium and incubated at 37°C with shaking at 180 rpm for 16 - 20 h. The resulting cultures were mixed with glycerol at a final concentration of 40% (v/v) and stored at -80°C for future use.

Acid and bile salt tolerance tests

For assessing acid tolerance, the strains were inoculated into LB liquid medium (pH 3.0) using an inoculation volume of 3% (v/v). Non-acidified LB medium served as the control. The experiment was repeated three times. For bile salt tolerance testing, the strains were inoculated at a volume of 3% (v/v) into LB liquid medium supplemented with 0.3% (w/v) porcine bile salt (Chongqing Saipunasi Technology Co., Ltd, Chongqing, China) with LB medium lacking bile salt as the control. Following incubation at 37°C for 24 h, the OD_{600} was measured, and strains demonstrating high survival rates were selected.

Tolerance to simulated gastric juice

The bacteria were harvested by centrifugation at 8,000 rpm for 15 min. The pellets were resuspended in physiological saline to a final concentration of 0.1 g/mL. A 0.5 mL aliquot of the bacterial suspension was mixed with 4.5 mL of simulated gastric juice containing 0.3% pepsin and 0.5% NaCl (pH 1.2) to form the experimental group, while another 0.5 mL of the bacterial suspension was mixed with 4.5 mL of 0.1 mol/L phosphate buffered saline (PBS) (pH 7.2) as the control group. Both groups were incubated at 37°C for 3 h followed by dilution plating and incubation at 37°C for 24 h. The bacterial concentration was calculated as below.

$$Y_1 = L_1/L_0 \times 100\%$$

where Y_1 was the bacterial concentration (%). L_1 was the concentration of *Bacillus* in the experimental group. L_0 was the concentration of *Bacillus* in the control group. The experiment was repeated three times.

Physiological and biochemical characterization of strains

(1) Staining microscopy

The purified isolated strains were streaked onto LB solid medium and incubated at 37°C for 16 - 24 h before colony morphology observation. Subsequently, Gram staining was performed, and the bacterial cell morphology was examined under a microscope.

(2) 16S rDNA sequence analysis

Genomic DNA was extracted using a genomic DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China) following manufacturer's instructions. The 16S rDNA of the tested strains was amplified using polymerase chain reaction (PCR) with the forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG -3') and the reverse primer 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') and the TSE101 Gold Mix (GenScript, Wuhan, Hubei, China) in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, California, USA). Each PCR reaction was performed in a 50 µL reaction volume comprising 45 µL of 1× TSE101 Gold Mix, 1 µL of each primer (20 µmol/L), and 1 µL of template DNA. The PCR reaction was performed at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 90 s with a final extension at 72°C for 10 min. PCR products were visualized using 1% agarose gel electrophoresis and subsequently sequenced by Wuhan GenScript Biotechnology Co., Ltd (Wuhan, Hubei, China). The sequencing results were submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and analyzed for homology using the Basic Local Alignment Search Tool (BLAST) to determine the species.

Screening of enzyme-producing *Bacillus* strains

Enzyme-producing *Bacillus* strains were initially screened using previous reported methods [15-19]. The strains were cultured in LB liquid medium at 37°C with shaking at 180 rpm for 16 - 24 h. The bacteria were harvested by centrifugation at 8,000 rpm for 10 min and then resuspended in 5 mL of physiological saline. Sterile filter paper was immersed in the bacterial suspension, and excess liquid was removed before placing the paper onto the medium. The plates were incubated at 37°C for 24 h. Amylase production was assessed using an amylase-inducing medium with 1% peptone, 0.5% yeast extract, 0.5% NaCl, 1% soluble starch, 2% agar (pH 7.2). After incubation, plates were stained with iodine solution. A clear halo around the bacterial colony indicated amylase production. Cellulase production was evaluated on a

cellulase-inducing medium with 1% peptone, 1% yeast extract, 0.5% NaCl, 1% carboxymethyl cellulose sodium, 0.1% dipotassium phosphate, 1% sodium carbonate, 0.02% magnesium sulfate heptahydrate, 2% agar (pH 7.2). After incubation, plates were stained with 0.2% Congo red for 30 min followed by washing with distilled water, rinsing with 1 mol/L sodium chloride, and fixing with 5% acetic acid. A clear halo around the colonies indicated cellulase production. Protease production was assessed using a protease-inducing medium with 1% peptone, 0.5% yeast extract, 0.5% NaCl, 3% skim milk, 2% agar (pH 7.2). Proteolytic activity was indicated by a hydrolysis halo around the bacterial colonies, and enzyme activity was quantified by measuring the halo diameter using a vernier caliper. The enzyme production capacity was calculated as follows. Each group included three parallel controls.

Enzyme production capacity = Diameter of hydrolysis halo - Diameter of bacterial strain

Optimization of medium components

Medium optimization was conducted through sequential screening protocols. Tested strains were inoculated into LB liquid medium and incubated at 37°C with 180 rpm shaking for 12 h. Carbon source was screened using soluble starch, glucose, sucrose, lactose, maltose, and malt extract at 2% concentrations (g/mL). Nitrogen sources including tryptone, peptone, ammonium sulfate, urea and salt ions including NaCl, KCl, $MgSO_4$, $MnSO_4$ were similarly tested at 2% (g/mL) and 0.1 g/100 mL concentrations, respectively, with OD_{600} determining optimal components. Subsequent concentration optimization employed identified optimal components of glucose at 1, 2, 8, 10, 20, 30 g/L, peptone at 5, 10, 15, 20, 30, 40, 80 g/L, and NaCl at 1, 3, 5, 8, 10, 15, 20 g/L in LB medium under identical culture conditions. A three-factor three-level $L_9(3^3)$ orthogonal design was implemented using glucose (A), peptone (B), and NaCl (C) with OD_{600} as the response variable. The factor-level combinations were shown in Table 1.

Table 1. Factors and levels of the orthogonal experiment.

Level	Glucose (A)	Peptone (B)	Sodium chloride (C)
1	2 g/L	3 g/L	1 g/L
2	5 g/L	4 g/L	4 g/L
3	8 g/L	5 g/L	7 g/L

Optimization of the fermentation process

Following strain activation on LB agar, single colonies were sequentially cultured in 5 mL LB broth at 37°C, 180 rpm, for 12 h before transferring to 100 mL LB medium (3% v/v inoculum) under identical conditions to prepare seed cultures. For single factor optimization, seed cultures were inoculated (baseline 3% v/v) into fermentation media and incubated at 37°C with 180 rpm shaking. All experiments were conducted in triplicate, and OD_{600} was measured post-incubation. The single factor experiments for fermentation optimization were conducted as follows. The pH optimization was performed with the initial pH being adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, respectively. The fermentation duration optimization was conducted by testing fermentation durations of 16, 18, 20, 22, 24 h using the optimized medium. For inoculation volume optimization, 1.0, 3.0, 5.0, 10.0, 15.0% inoculation volumes were examined. Further, the defoamer dosages of 0.1, 0.3, 0.5, 0.7, 1.0% were tested. All experiments were performed in triplicate, and the OD_{600} values were measured after incubation for each condition. Subsequently, significant parameters identified through single factor screening were subjected to response surface optimization using a Box-Behnken design with OD_{600} as the response variable. Factor-level matrices were generated by using Design-Expert® v8.06 (Stat-Ease, Inc., Minneapolis, MN, USA) with model-derived theoretical optima validated experimentally.

Statistical analysis

GraphPad Prism 8.0.2 (GraphPad Software, Boston, MA, USA) was employed for the statistical analysis of this research. Intergroup differences were assessed using Student's t-test and visualized using the “Aa” labeling system,

where distinct uppercase letters (A, B) designated highly significant differences ($P < 0.01$), while distinct lowercase letters (a, b) indicated significant differences ($P < 0.05$), and groups sharing identical letter combinations such as Aa indicated no statistical significance.

Results

Isolation and screening of *Bacillus* strains

In this study, 84 *Bacillus* strains exhibiting diverse colony morphologies were initially screened and designated as L1 – L20 for those isolated from the large intestine, C1 – C26 for those isolated from the cecum, and S1 – S37 for those isolated from the small intestine. These strains were then subjected to acid and bile salt tolerance assays. The results showed that 25 strains including L1, L2, L3, L10, L11, L12, C4, C7, C12, C24, S5, S6, S11, S12, S13, S15, S17, S18, S19, S21, S23, S29, S33, S34, S36 exhibited acid tolerance with survival rates exceeding 40% after 24 h of acid exposure, indicating strong acid resistance. Following 24 h of bile salt treatment, 42 strains including L1, L2, L3, L4, L5, L7, L8, L9, L10, L11, L13, L16, L17, C4, C6, C9, C12, C13, C14, C15, C16, C19, C23, C24, S5, S6, S12, S15, S18, S19, S22, S23, S26, S27, S28, S31, S32, S34, S35, S36, S37, S38 exhibited survival rates above 70%, indicating strong bile salt tolerance. Based on their combined acid and bile salt resistance, 16 strains of L1, L2, L3, L10, L11, C4, C12, S5, S6, S12, S15, S18, S19, S23, S34, S36 demonstrating the highest tolerance were selected for further experimentation. The simulated gastric juice tolerance test revealed that the survival rates of the 16 strains of L1, L2, L3, L10, L11, C4, C12, S5, S6, S12, S15, S18, S19, S23, S34, S36 all exceeded 50% (Figure 1), indicating their tolerance to simulated gastric conditions with no significant differences observed among them. *Bacillus* as a gram-positive bacterium possessed a thick cell wall primarily composed of peptidoglycan and teichoic acid. The peptidoglycan layer formed a dense, robust, and multilayered mesh-like structure. During Gram staining, the crystal violet–iodine complex was strongly retained by this cell wall, causing the cells to remain purple even after ethanol decolorization. This classical bacterial classification method distinguished gram-positive and gram-negative bacteria. The 16 strains exhibited typical gram-positive characteristics, appearing as rod-shaped cells, either singly or in chains, and forming spores with features consistent with those of *Bacillus*.

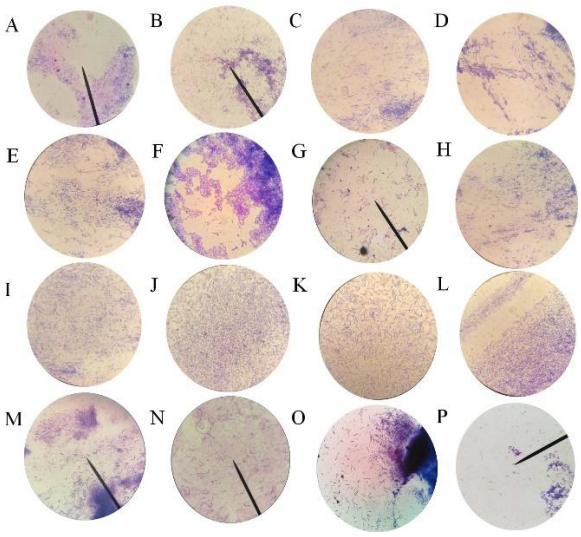

Figure 1. Gram staining results of the 16 selected strains (200×). **A.** L1. **B.** L2. **C.** L3. **D.** L10. **E.** L11. **F.** C4. **G.** C12. **H.** S5. **I.** S6. **J.** S12. **K.** S15. **L.** S18. **M.** S19. **N.** S23. **O.** S34. **P.** S36.

The enzyme-producing capabilities of the selected *Bacillus* strains were evaluated by measuring the diameters of the transparent (hydrolysis) zones and the bacterial colonies, as well as the differences between these diameters. The amylase produced by *Bacillus* hydrolyzed soluble starch in the medium into smaller molecules of dextrin, maltose, and glucose. This enzymatic activity depleted the starch surrounding the colonies, resulting in transparent zones observable under a microscope. All 16 selected strains formed transparent zones, confirming their ability to produce amylases. Congo red dye binds to cellulose to form a red complex but does not bind to the hydrolysis products of cellulase such as glucose and oligosaccharides. When *Bacillus* was cultured on a medium containing carboxymethyl

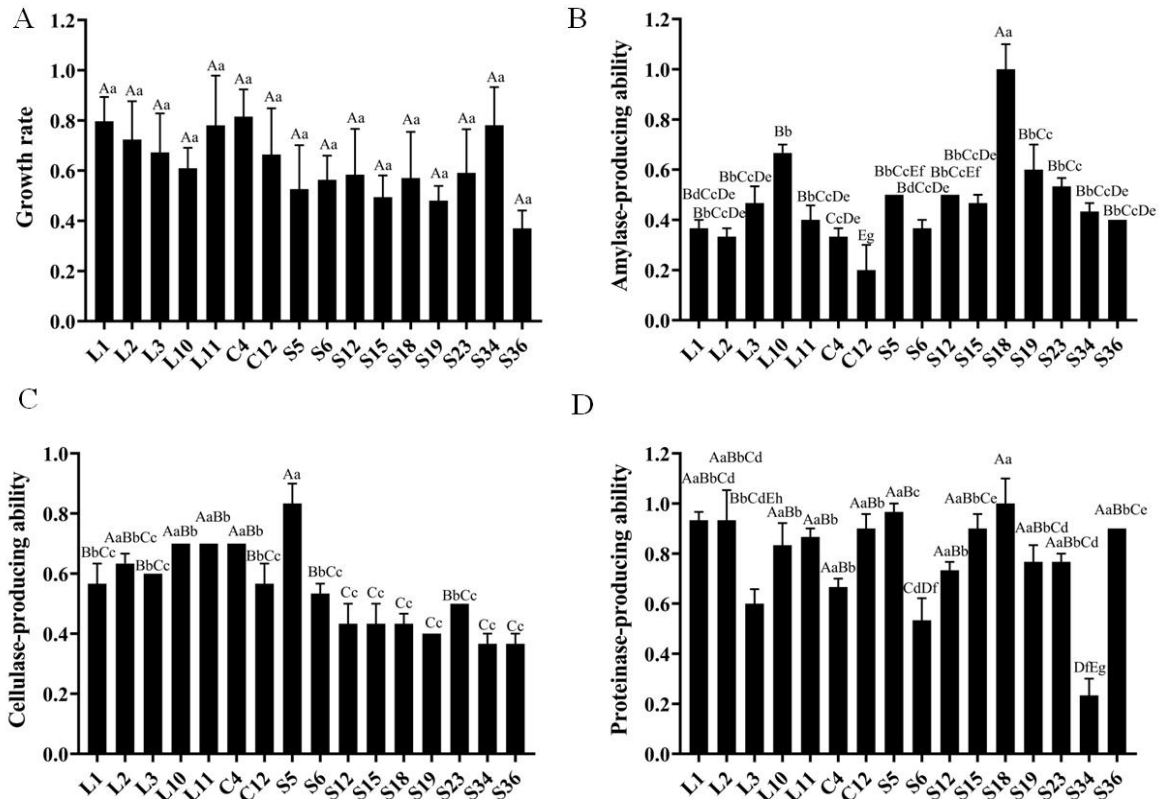

Figure 2. Screening of tested strains, survival rates in simulated gastric juice (**A**), amylase production (**B**), cellulase production (**C**), and protease production (**D**).

cellulose sodium and secreted cellulase, the cellulase hydrolyzed carboxymethyl cellulose sodium, reducing its ability to bind Congo red. After Congo red staining, the red coloration disappeared in the hydrolyzed regions, resulting in the formation of transparent zones. All 16 selected strains formed transparent zones, indicating their ability to produce cellulase. Similarly, the protease produced by *Bacillus* hydrolyzed proteins in the medium such as casein in skim milk into smaller peptides and amino acids. On a skim milk-containing medium, this proteolytic activity resulted in the formation of transparent zones around the colonies. All 16 selected strains exhibited transparent zones, confirming their ability to produce protease. The enzyme production capabilities of the 16 selected strains were assessed by calculating the ratio of the diameters of the stained (or transparent) zones to the colony diameters. The results demonstrated that strains S18, L10, and S19 exhibited the largest differences between the stained zone and colony diameters with values of 1.00 ± 0.14, 0.67 ± 0.05, and 0.60 ± 0.14, respectively (Figure 2B). Strains S5, L10, L11, and C4 demonstrated large differences between the diameters of the stained zones and the colony diameters with values of 0.83 ± 0.09, 0.70 ± 0.00, 0.70 ± 0.00, and 0.70 ± 0.00, respectively (Figure 2C), while strains S18, S5, L1, and L2 also exhibited large differences between the diameters of the stained zones and the colony diameters with values of 1.00 ± 0.14, 0.97 ± 0.05, 0.93 ± 0.05, and 0.93 ± 0.17, respectively (Figure 2D). Despite the influence of various factors on enzyme production capabilities such as solid and liquid culture conditions, strain-specific growth and enzyme production rates, and differences in colony size and stacking on plates, the enzyme production capabilities of the selected strains under standardized enzyme production conditions were compared. The size of the

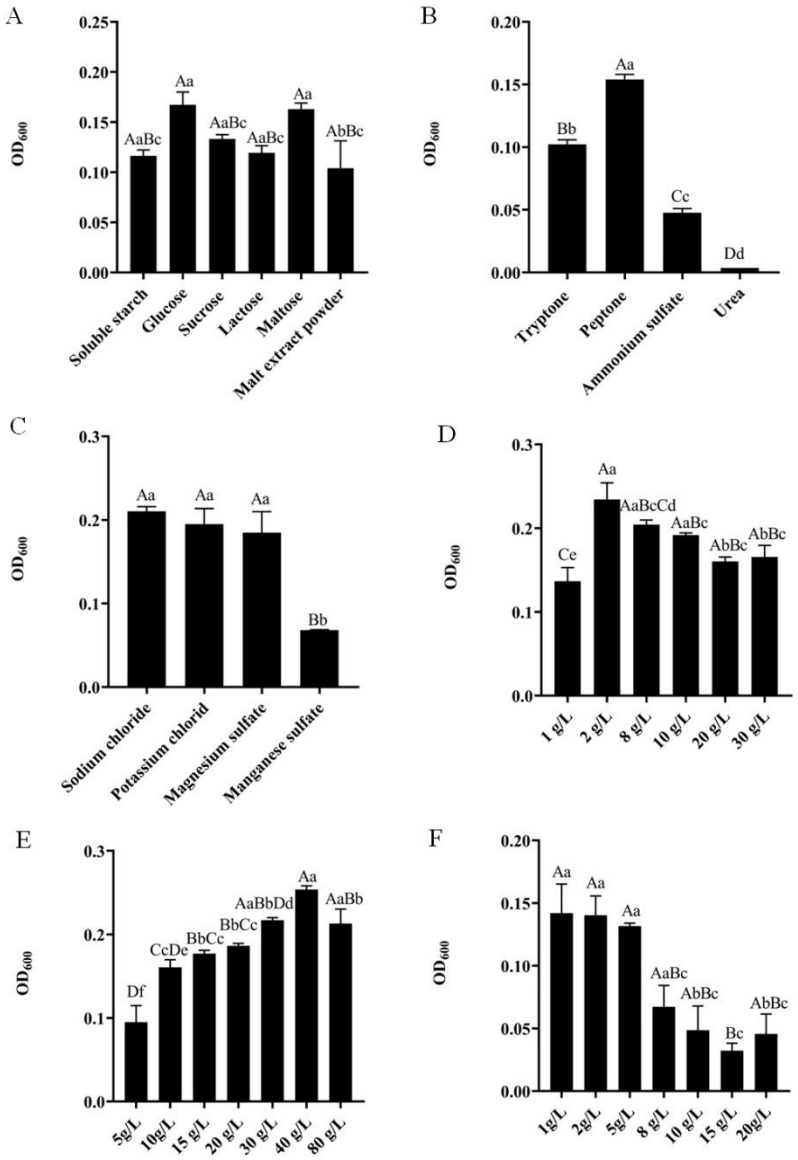

Figure 3. Effects of carbon source type (**A**), nitrogen source type (**B**), salt ion type (**C**), carbon source concentration (**D**), nitrogen source concentration (**E**), NaCl concentration (**F**) on the fermentation of strain S5.

transparent zone directly reflected the enzyme concentration and represented the enzyme production capability of the strains. The results showed that the enzyme production capabilities of different strains were not consistent. Based on the production levels of amylase, cellulase, and protease, strains S5 and L10 were selected for further experiments and subjected to molecular biological identification. Homology analysis using the GenBank nucleotide sequence database revealed that both strains were *Bacillus subtilis*.

Optimization of medium components

In this study, the medium components for the tested strains were optimized using strain S5. Various carbon sources including soluble starch, glucose, sucrose, lactose, maltose, and malt extract were evaluated in LB liquid medium to identify the most suitable carbon source for bacterial growth. The results showed that strain S5 could ferment all six carbon sources. The OD_{600} value for strain S5 was significantly higher in glucose than it in malt extract ($P < 0.05$).

However, although the OD_{600} value with glucose was higher than those obtained with soluble starch, sucrose, lactose, and maltose, the differences were not statistically significant ($P > 0.05$). Therefore, glucose along with soluble starch, sucrose, lactose, and maltose was identified as an optimal carbon source for strain S5 (Figure 3A). Glucose was selected for further experiments with an optimal concentration determined to be 2 g/L (Figure 3D). Various nitrogen sources including tryptone, peptone, ammonium sulfate, and urea were tested in LB liquid medium to identify the optimal nitrogen source for bacterial growth. The results showed that strain S5 could ferment tryptone, peptone, and ammonium sulfate, but not urea (Figure 3B). Among these, peptone yielded the highest OD_{600} value, which was significantly greater than those observed with tryptone, ammonium sulfate, and urea ($P < 0.01$). Therefore, peptone was identified as the optimal nitrogen source for strain S5 and was subsequently used in further experiments at an optimal concentration of 40 g/L (Figure 3E). The optimal ionic compounds for strain S5 fermentation were identified as NaCl, KCl, and MgSO4 (Figure 3C). NaCl was selected for further experiments with an optimal concentration range of 1 – 5 g/L (Figure 3F). Based on the effects of various carbon sources, nitrogen sources, and salt ions on the fermentation performance of the tested strains, this study further optimized the concentrations of glucose, peptone, and NaCl, as well as their optimal combinations. The OD_{600} value of the fermentation broth of strain S5 served as the primary metric to identify the ideal ratio of glucose, peptone, and sodium chloride. The results of orthogonal experiments with different factors and levels were shown in Table 2, and the corresponding analysis of variance was provided in Table 3. The results of the orthogonal experiment indicated that, among the selected factors, sodium chloride (C) exhibited the largest range, suggesting that it had the greatest influence on the biomass of strain S5. This was followed by glucose (A), while peptone (B) had the least impact. Variance analysis further confirmed that both sodium chloride and glucose significantly affected biomass. Based on these findings, the optimal combination was determined to be $A_3B_2C_1$, corresponding to a final medium composition of 8 g/L glucose, 40 g/L peptone, and 1 g/L sodium chloride.

Table 2. Results of the orthogonal experiment for optimization of medium components for strain S5.

Experiment	Factors			$OD_{600\ nm}$
	A	B	C	
1	1	1	1	0.287
2	1	2	2	0.237
3	1	3	3	0.248
4	2	1	2	0.265
5	2	2	3	0.291
6	2	3	1	0.308
7	3	1	3	0.266
8	3	2	1	0.327
9	3	3	2	0.294
K_1	0.257	0.273	0.307	
K_2	0.288	0.285	0.265	
K_3	0.296	0.283	0.268	
R	0.039	0.012	0.042	
Factor importance order	C > A > B			
Optimal combination	$A_3B_2C_1$			

Fermentation process optimization of strain S5

To optimize the fermentation conditions for strain S5, this study varied culture duration, pH, inoculation volume, and defoamer dosage. The initial pH levels tested were 6.0, 6.5, 7.0, 7.5, and 8.0. Inoculation volumes were 1.0%, 3.0%, 5.0%, 10.0%, and 15.0%. Culture durations were 16, 18, 20, 22, and 24 h. The defoamer concentrations were 0.1%, 0.3%, 0.5%, 0.7%, and 1.0%. The results showed that the optimal conditions were a pH of 6.5 (Figure 4A), an inoculation volume of 3% (Figure 4B), a culture duration of 22 h (Figure 4C), and a defoamer dosage in the range of 0.1% to 1.0% (Figure 4D). Since defoamer dosages ranging from 0.1% to 1.0% had no significant impact on the fermentation performance of strain S5, response surface experiments were conducted to determine the optimal pH, inoculation volume, and agitation speed for the fermentation of strain S5 (Table 4). The OD_{600} value was used as the response variable, and medium components were optimized using RSM. A quadratic regression equation with the OD_{600} value of the fermentation broth of strain S5 as

Table 3. Variance analysis of the orthogonal experiment for optimization of medium components for strain S5.

Factor concentration	Sum of squares	Degrees of freedom	F ratio	F value	*P* value
Glucose	0.002	2	5.578	5.140	< 0.05
Peptone	0.001	2	0.006	5.140	
Sodium chloride	0.003	2	5.863	5.140	< 0.05
Error	0.01	6			

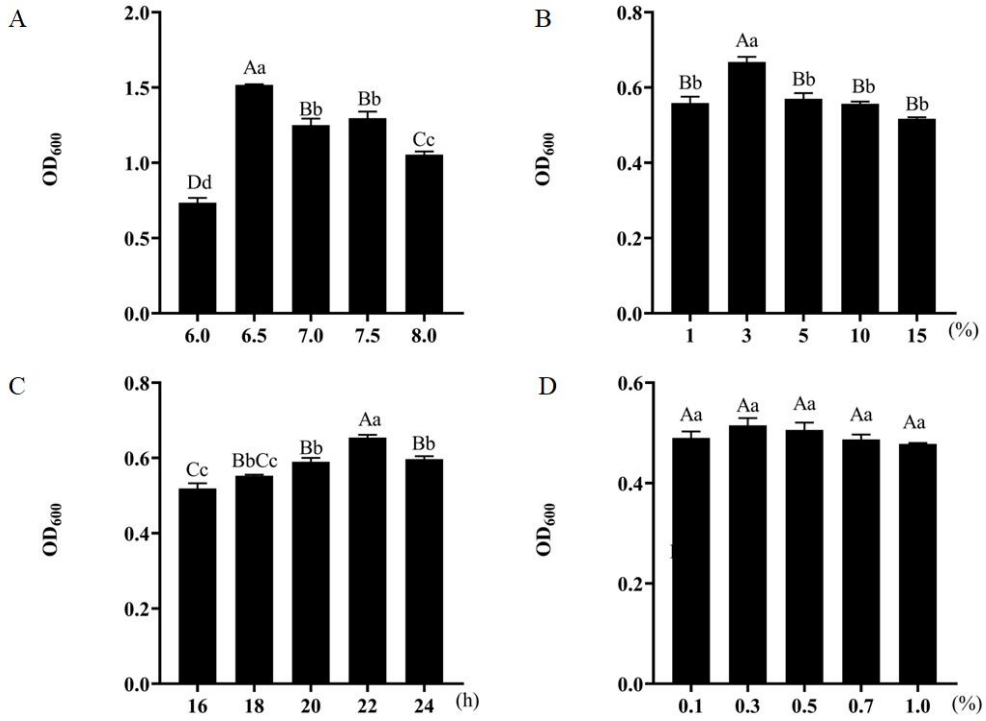

Figure 4. Effects of pH (**A**), inoculation volume (**B**), culture time (**C**), defoamer dosage (**D**) on fermentation of strain S5.

the objective function was as follows.

$$Y = -12.70358 + 1.78515A + 0.56614B + 0.85111C + 0.054750AB - 0.062500AC - 0.018500BC - 0.21230A^2 - 0.019519B^2 - 0.00545625C^2$$

Table 4. Response Surface Experimental Design for Optimization of Fermentation Conditions for Strain S5.

Code	pH (A)	Culture time (B) (h)	Inoculation volume (C) (%)
-1	6.0	20	1
0	6.5	22	3
1	7.0	24	5

The variance analysis of the regression model yielded an F-value of 6.92 ($P < 0.01$) and an R^2 of 0.90, indicating that the model accounted for 90% of the response values (Table 5). The adjusted R^2 was 0.77, suggesting a good model fit. The lack-of-fit test produced an F-value of 1.03 and a *P* value of 0.4690, which were not significant ($P > 0.05$). These results indicated that the regression equation provided a suitable model for the central composite experiment. The interactions AB, AC, BC, A^2, and B^2 had P values less than 0.05, indicating significant effects on the model. These factors correspond to A (pH), B (culture time in hours), and C (inoculation volume). The main effects of A, B, and C, along with the quadratic term of inoculation volume

Table 5. Variance analysis results for optimization of fermentation conditions for strain S5.

Variations	Sum of squares	Degrees of freedom	Mean square	F value	*P* value
Regression model	0.096	9	0.011	6.92	0.0092
A	0.0004651	1	0.0004651	0.30	0.5994
B	0.001891	1	0.001891	1.23	0.3040
C	0.0008405	1	0.0008405	0.55	0.4837
AB	0.012	1	0.012	7.80	0.0268
AC	0.016	1	0.016	10.16	0.0153
BC	0.022	1	0.022	14.25	0.0069
A^2	0.012	1	0.012	7.71	0.0274
B^2	0.026	1	0.026	16.69	0.0047
C^2	0.002006	1	0.002006	1.30	0.2909
Residual	0.011	7	0.001537		
Lack of fit	0.004689	3	0.001563	1.03	0.4690
Pure error	0.006073	4	0.001518		
Cor total	0.11	16			

Note: R^2 = 0.90.

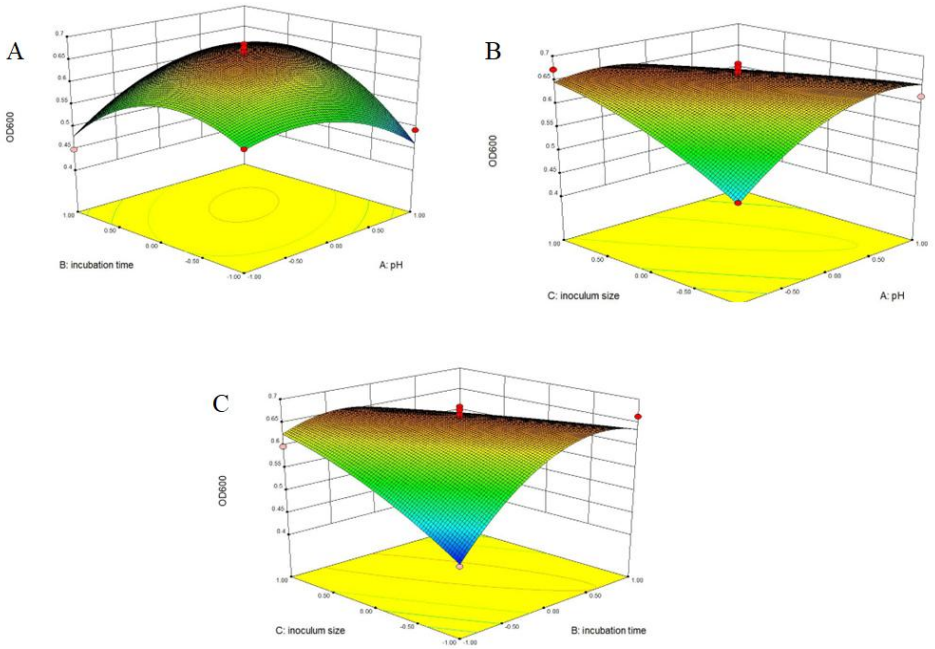

Figure 5. Response surface experimental results of interactions between pH and inoculation volume (**A**), pH and culture time (**B**), inoculation volume and culture time (**C**).

(C^2) were nonsignificant (P > 0.05), indicating these parameters did not substantially influence the model response. The order of influence on the model was as follows: among the single factors, B > C > A, while, among the interaction terms, BC > AC > AB. All quadratic terms, except C^2, exhibited highly significant effects. The response surface plots illustrating the effects of

fermentation process conditions on the OD_{600} value of the fermentation broth of strain S5 showed that factors A, B, and C exhibited a clear quadratic parabolic relationship with the OD_{600} value of the fermentation broth of strain S5 (Figure 5). Based on this model, in conjunction with the results of single factor experiments, the predicted optimal fermentation conditions were pH 7.0, a culture duration of 23.84 h, and an inoculation volume of 1%. Under these conditions, the predicted OD_{600} value was 0.708, while the actual measured value was 0.695, resulting in a deviation of 0.13% ± 1.80%. This small deviation indicated that the optimized region identified by the quadratic polynomial mathematical model was consistent with the experimental objectives.

Discussion

Bacillus strains can form spores, allowing them to survive under extreme conditions such as high temperature, acidic and alkaline conditions, and desiccation. This ability is particularly advantageous for withstanding the acidic environment and bile salts present in the gastrointestinal tract. These bacteria secrete various digestive enzymes that promote feed digestion and nutrient adsorption in the host, regulate the intestinal microbiota, and enhance host immune function, which are considered safe and non-toxic with immune-boosting properties, making them an ideal alternative to antibiotics [20]. The use of such bacteria can help alleviate issues associated with excessive antibiotic use and improve both animal health and production performance. In this study, the S5 strain was isolated from the intestines of free-range chickens raised by local farmers in Zhumadian City, Henan Province, China. Following Gram staining, physiological and biochemical tests, and 16S rDNA sequencing, the isolate was identified as *B. subtilis*. Studies have shown that *B. subtilis* is rich in digestible proteins, vitamins such as vitamin B6, thiamine, biotin, riboflavin, niacin, pantothenic acid, and minerals such as magnesium and zinc, making it a commonly used feed additive in poultry and livestock industries [21]. A key characteristic of effective probiotics is their ability to survive in acidic conditions, in the presence of bile salts, and within simulated gastric juice. *B. subtilis* forms spores in extreme environments, exhibiting acid and bile salt tolerance, as well as stable survival within the gastrointestinal tract, which confers a natural advantage in the development of probiotic preparations. Several *B. subtilis* strains that are capable of surviving low pH and high bile salt concentrations have been reported, and these strains produce beneficial compounds such as organic acids and exhibit probiotic potential [22]. The two *B. subtilis* strains (S5 and L10) isolated in this study exhibited high tolerance to bile salts, low pH, and simulated gastric juice, indicating strong probiotic potential. Additionally, both strains demonstrated enzymatic activities including the production of amylase, cellulase, and protease, further confirming their probiotic potential. In fermentation processes, bacterial cell concentration is positively correlated with final yield, thus, increasing cell density is a core objective in fermentation research. Higher cell density improves production efficiency, reduces production costs and equipment requirements, ultimately achieving higher economic returns. Previous studies have focused on optimizing fermentation medium components and process parameters to increase cell concentration [23, 24]. Oh *et al*. employed RSM to optimize carbon and nitrogen sources in the medium, developing a cost-effective medium for high-density fermentation of lactic acid bacteria [25]. Our research group previously optimized the medium composition for probiotic lactic acid bacteria isolated from chicken intestines using RSM [3]. Additionally, Zhang *et al*. used orthogonal design, single-factor experiments, and response surface experiments to optimize both medium components and fermentation conditions for *Bacillus velezensis* XY40-1, achieving a 92.97% increase in cell yield [11]. This research optimized the shake flask fermentation medium and process conditions for strain S5 using a combination of single-factor experiments, orthogonal experiments, and RSM. The final

optimized medium composition consisted of 8 g/L glucose, 40 g/L peptone, and 1 g/L sodium chloride. The optimized fermentation conditions were determined as initial pH of 6.5, culture duration of 22 h, and inoculation volume of 3%. Under these conditions, *Bacillus* growth was optimal. The results showed that sodium chloride and glucose concentrations significantly influenced the growth of strain S5, highlighting the key roles of salt ions and carbon sources. The nitrogen source was also essential for the strain's growth. Previous studies have shown that the yield of strain S5 does not vary significantly within the temperature range of 30 – 42°C, thus, temperature was excluded as an optimization parameter in this study. Additionally, since pH can fluctuate during fermentation, initial pH was considered a process condition rather than a medium component.

Conclusions

The present study focused on the isolation, identification, and optimization of fermentation conditions for probiotic *Bacillus* strains derived from the chicken intestinal tracts and resulted in two promising strains, S5 and L10. They were both identified as *B. subtilis*, exhibiting excellent probiotic potential. The fermentation conditions for the selected strain S5 were systematically optimized, while RSM was applied to further refine the fermentation parameters, identifying the optimal conditions as an initial pH of 7.0, a cultivation duration of 23.84 h, and an inoculum volume of 1%. Under these optimized conditions, the biomass of strain S5 reached 0.695, which closely matched the predicted value of 0.708. The findings highlighted the potential of chicken intestinal *B. subtilis* strains as effective probiotics for use in livestock and poultry. The optimized fermentation conditions provided a robust foundation for large-scale production and further *in vivo* studies. Future research should aim to evaluate the probiotic effects of these strains in animal models and examine whether the optimized conditions can be applied to other related *Bacillus* strains. Additionally, a comprehensive evaluation of the safety and efficacy of these strains as feed additives is essential to support their practical application in the livestock industry.

Acknowledgements

This research was funded by the Scientific and Technological Research Project Foundation of Henan Provincial Scientific and Technological Department (Grant No. 242102111020), the Major Science and Technology Project of Zhumadian City (Grant No. 44, ZMDSZDZX2024007), the Horizontal project of Huanghuai University (Henan Dade Guang Animal Pharmaceutical Co., LTD.) (Grant No. 2018411717000020), the Young Backbone Teachers Foundation of Huanghuai University, the Scientific Research Foundation for Advanced Talents of Huanghuai University (Grant No. 1000.12.01.1678).

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