CONSERVATION OF TOBACCO FIELD SOIL BY MUTANT STRAIN *BACILLUS PUMILUS* YYFP-3 AND OPTIMIZATION OF ITS FERMENTATION CONDITIONS

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Abstract. This study aimed to enhance soil quality by screening strains with conservation effects in tobacco-planted soil. *Bacillus pumilus YYFP* was used as the starting strain, and ARTP-DES technology was employed for composite mutagenesis.The impact of the mutant strain on tobacco planting soil was evaluated, and optimal fermentation conditions were determined using response surface methodology. The results indicated that the amylase-producing activity of the high-quality mutant strain YYFP-3 was 278.77 U/mL. After applying a high concentration of the YYFP-3 mutant strain in combination with organic fertilizer to tobacco-growing soil, the soil pH approached neutrality. Compared to the control group (CK), the contents of soil organic carbon, total nitrogen, available phosphorus, and available potassium increased by 20.33%, 22.06%, 74.34%, and 12.36%, respectively. The optimal fermentation conditions of the Box-Behnken design were 12.94 g/L soluble starch, 4.22 g/L calcium chloride, and 3.66% inoculum, yielding an enzyme activity of 535.50 ± 4.26 U/mL. This study demonstrated that the mutant strain YYFP-3, when used as a microbial fertilizer in combination with organic fertilizer, has the potential to enhance soil quality, reduce the need for chemical fertilizers, support the conservation of tobacco plant soils, and present promising application prospects.
Keywords: *continuous tobacco planting soil, microbial agents, amylase, mutagenesis, fermentation conditions optimization*

Introduction

Tobacco is one of the primary economic crops in China, with both its planting area and yield ranking first worldwide. However, improper fertilizer application can lead to insufficient soil carbon (C) storage, decreased enzyme activity, and imbalanced microbial communities, all of which negatively affect soil quality (Madear et al., 2020). This, in turn, influences the growth, quality, and yield of tobacco plants (Uo et al., 2012). Maintaining good soil quality is essential for ensuring the quality of tobacco leaf (Wang et al., 2025). Effectively enhancing the physicochemical properties and biological activity of tobacco planting soil is crucial for promoting the sustainable development of the tobacco industry, which is a pressing issue in current tobacco production. Microbial fertilizers not only increase soil enzyme activity in the rhizosphere but also improve soil physicochemical properties and the structure of microbial communities. In addition, they facilitate nutrient uptake by plant roots, thereby increasing crop yields (Uo et al., 2012). Furthermore, microbial fertilizers play a significant role in enhancing the agricultural ecological environment and contribute to a reduction in chemical fertilizer usage. The effectiveness of microbial fertilizers in improving soil conditions is well documented. For instance, Shrivastava et al. (2023), demonstrated that the simultaneous application of

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biochar and biofertilizers effectively enhanced soil quality and rice productivity. Pérez-Álvarez et al. (2024), found that rhizobial fungal fertilizers exhibited the highest nitrate reductase activity in maize leaves, which, in turn, improved maize photosynthesis to a certain extent, resulting in increased biomass and yield.

Wild strains selected from nature often exhibit disadvantages, such as low production performance and poor tolerance. Therefore, it is essential to use various mutagenesis methods to mutate and select wild strains. Atmospheric pressure room temperature plasma (ARTP) mutagenesis induces microbial mutations by emitting plasma-containing active particles that cause deoxyribonucleic acid (DNA) damage (Yu et al., 2024). Diethyl sulfate (DES) contains one or more active alkyl groups that alkylate DNA, leading to mutations due to base mispairing during DNA replication (Shafique et al., 2020). Current research on the application of microbial fertilizers in flue-cured tobacco has primarily focused on screening microbial fertilizer varieties and their effects on plant growth and development, with less attention given to mutagenized microbial strains and their application methods. Previous studies have demonstrated that microorganisms, such as arbuscular mycorrhizal fungi, *Bacillus* spp., and nitrogen (N)-fixing bacteria, can effectively regulate plant growth and enhance soil nutrients (Matos et al., 2022). In this study, amylase, a key enzyme in the C cycle, was used as an indicator, and a combination of ARTP and DES mutagenesis technologies was used to screen for superior strains, thereby enhancing the efficacy of microbial fertilizers. In addition, this study explored the impact of these strains on soil physicochemical properties and optimized the fermentation conditions for high-quality mutant strains. The goal was to provide a theoretical foundation for the industrial application of the mutant strain *Bacillus pumilus* YYFP-3 to promote the sustainable and healthy development of the tobacco industry.

Materials and methods

Materials

The *B. pumilus* YYFP strain (SUB15755396 YYFP PX495925) was isolated from tobacco planting soil in Zhugou Town, Queshan County, Zhumadian City, Henan Province, and temporarily stored in the Microbiology Laboratory at the College of Agriculture, Henan University of Science and Technology, it will be preserved at the China Culture Collection Center of Wuhan University.

The seed medium (g/L) consisted of 10.0 g NaCl, 10.0 g peptone, and 5.0 g yeast extract, which were autoclaved at 121℃ for 20 minutes. The initial fermentation medium (g/L) consisted of 5.0 g NaCl, 10.0 g peptone, and 5.0 g soluble starch, which were autoclaved at 121℃ for 20 minutes. The amylase screening medium (g/L) included 18.0 g agar, 10.0 g peptone, 10.0 g soluble starch, and 5.0 g NaCl, and was also autoclaved at 121℃ for 20 minutes, the plates were then inverted for incubation.

Experimental Site Overview: The experiment was conducted in Zhugou Town, Queshan County, Zhumadian City, Henan Province, China. The physicochemical properties of the experimental soil are as follows: pH of 5.50, organic matter content of 10.87 g/kg, total nitrogen (TN) concentration of 0.68 g/kg, total phosphorus (TP) concentration of 0.32 g/kg, total potassium (TK) concentration of 8.74 g/kg, available phosphorus (AP) level of 35.42 mg/kg, available potassium (AK) level of 138.16 mg/kg, and alkaline dissolved N concentration of 76.53 mg/kg.

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Methodology

Mutagenic treatment of B. pumilus YYFP

ARTP-DES Mutagenesis: A 10 μL aliquot of a bacterial suspension with a concentration of 1×10^7 to 1×10^8 CFU/mL was applied to the surface of a metal carrier. Helium was used as the working gas with a power setting of 110 W, an airflow rate of 10 L/min, and an irradiation distance of 2 mm (Ji et al., 2025). Mutagenesis was conducted for 0, 15, 30, 45, 60, 75, 90, 105, and 120 seconds. Subsequently, the bacterial solution was serially diluted and spread onto agar plates, followed by incubation at 28°C for 48 hours to generate a lethal curve. The target strains screened by ARTP mutagenesis were prepared as a bacterial suspension at a concentration of 1×10^7–1×10^8 CFU/mL. Two milliliters of this bacterial suspension were added to 8 mL of phosphate buffer at pH 7.0. Subsequently, 0.1 mL of the DES stock solution was added and mixed thoroughly. Reactions were conducted at 30°C for 0, 15, 30, 45, and 60 minutes. To terminate the reaction, 0.5 mL of 25% sodium thiosulfate was added to 1 mL of treatment solution to terminate the reaction. The treatment solution was diluted, coated, and incubated at 28°C for 48 hours to generate the lethality curve (*Eq. 1*).

$$\text{Lethality} = \frac{\text{Number of blank control colonies-Number of colonies after mutagenesis}}{\text{Number of blank control colonies}} x\ 100 \quad \text{(Eq.1)}$$

Screening for positive mutant strains

Initial Screening: The 16 positive mutant strains obtained through mutagenesis were purified and inoculated onto amylase screening medium. After incubation at 28°C for 24 hours, iodine solution was added to cover the entire medium surface. Following several minutes of standing, the ratio of the diameter of the clear zone (D) to the diameter of the colony (d) (D/d) was measured. Using this method, the 16 positive mutant strains were serially passaged for 8 generations on the amylase screening medium to assess D/d ratio stability for genetic stability evaluation. Finally, 5 positive mutant strains that maintained a D/d value consistently greater than 50% of the parental strain after 8 generations were selected for amylase activity determination (rescreening).

Secondary Screening: The strains selected from the initial screening were inoculated into the initial fermentation medium and cultured at 28°C with a shaking speed of 180 rpm for three days. After centrifuging the fermentation broth at 8000 rpm for ten minutes, the supernatant was collected as a crude enzyme solution. Amylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method described by Ousmane et al. (2022).

Determination of soil physical and chemical properties

Preparation of bacterial suspension

The mutant strain *B. pumilus* YYFP-3, obtained through mutagenesis, was inoculated into seed medium and incubated at 28°C with shaking at 180 rpm for 18 hours. The culture was then centrifuged at 4000 rpm for 10 minutes to collect bacterial cells. After washing the cells three times with sterile water, the concentration of the bacterial suspension was adjusted to 10^6, 10^7, and 10^8 CFU/mL using sterile water.

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Experimental design

Soil samples for the experiment were collected in early May 2025 from tobacco fields in Zhugou Town, Queshan County, Zhumadian City, Henan Province. The soil was sieved through a 0.50 cm×1 cm mesh screen. The experiment was conducted in the solar greenhouse at the Henan University of Science and Technology farm, where the temperature was maintained at approximately 25°C and relative humidity ranged from 50% to 70%. A pot experiment was conducted with five treatments, each replicated six times. All pots were arranged in a completely randomized block design within the greenhouse: ① Conventional chemical fertilization (CK); ② 20% reduction in chemical fertilizer combined with organic fertilizer (T1); ③ 20% reduction in chemical fertilizer combined with organic fertilizer and a low-concentration bacterial suspension (T2); ④ 20% reduction in chemical fertilizer with a medium-concentration bacterial suspension (T3); ⑤ 20% reduction in chemical fertilizer combined with organic fertilizer and a high-concentration bacterial suspension (T4). The chemical fertilizers applied to the CK group included: compound fertilizer (N-P_2O_5-K_2O = 13.5-0-44.5, 7.88 g), potassium carbonate (K_2CO_3, 2.67 g), superphosphate ($Ca(H_2PO_4)_2 \cdot CaSO_4 \cdot H_2O$, 14.75 g). Groups T1, T2, T3, and T4 incorporated organic fertilizer (fermented sheep manure, 20 grams) into the CK group's soil with a 20% reduction in chemical fertilizer. For all treatments, soil and fertilizer were thoroughly mixed before potting. Each pot contained 8 kg of soil and received 1,000 mL of water daily. Apply 400 mL of the microbial solution via root drenching every 15 days; apply an equal volume of clean water to the control plants.

Determination of soil physicochemical properties

Thirty days after transplanting the tobacco seedlings, the rhizosphere soil was collected using the root-shaking method. The soil samples were thoroughly mixed and passed through a 2 mm sieve. The physicochemical properties of the soil were determined according to the method outlined by Shidan (Bao, 2000). Soil pH was measured using the potentiometric method; TN was assessed using the Kjeldahl method; soil organic carbon (SOC) was determined through potassium dichromate titration; AP was measured using the 0.5 mol/L $NaHCO_3$ method; and AK was evaluated using the flame photometric method.

Optimization of fermentation conditions for amylase-producing mutant strains

Single-Factor Experiment: Seed culture medium: 100 mL liquid volume in a 250 mL conical flask. Initial pH 7, incubated at 28 ℃ with shaking at 180 rpm. The effects of various factors on amylase production in the YYFP-3 mutant strain were investigated. These factors included carbon sources (lactose, maltose, glucose, sucrose, and soluble starch), nitrogen sources (ammonium sulfate, peptone, tryptone, sodium nitrate, and casein), inorganic salts (sodium chloride, calcium chloride, manganese chloride, zinc chloride, and magnesium chloride), pH (4, 5, 6, 7, 8, 9, 10), fermentation temperature (20℃, 25℃, 30℃, 35℃, 40℃, 45℃), shaker rotation speed (160 rpm, 180 rpm, 200 rpm, 220 rpm, 240 rpm), and inoculum size (1 %, 2%, 3%, 4%, 5%) (*Eq. 2*). The inoculum volume refers to the process of inoculating a seed culture suspension into the initial fermentation medium at a specific ratio.

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$$V_{\text{Inoculum size}}=V_{\text{Volume of Seed Culture Medium}}\times A\% \quad \text{(Eq.2)}$$

Plackett-Burman (PB) design experiment, steepest ascent test, and Box-Behnken tests were conducted using Design-Expert 13 software (Kannapally et al., 2025). The PB design was used to evaluate the effects of the seven independent variables on amylase production and to identify the significant influencing factors. The steepest ascent method was used to determine the optimal direction and step size for significant factors identified in the PB experiment. Based on the significant factors selected from the PB test and the concentrations determined from the steepest ascent, the Box-Behnken design was applied to analyze the interactions between the factors and to optimize the formulation of the fermentation medium. Finally, to validate the predicted values obtained using the response surface methodology, fermentation experiments were conducted under theoretically optimal fermentation conditions. Each experiment was repeated three times, and the average values were calculated. By comparing the experimental results with the predicted values, the reliability, accuracy, and practicality of the fermentation conditions optimized using the response surface methodology were confirmed.

Data processing

Experimental data were processed using Microsoft Excel 2010 software, and data analysis was performed with SPSS 22.0 software. Statistical tests were conducted using one-way analysis of variance (ANOVA). Different lowercase letters indicate significant differences between treatment groups, with a significance level set at 5% ($P<0.05$). For the fermentation condition optimization section, Design-Expert 13 software was used to analyze experimental data. Analysis of variance and regression equations were performed using F-tests. Data are expressed as mean ± standard error (SE).

Results

Determination of mutagenic lethality of ARTP-DES

The ARTP-DES mutagenic lethality curve for *B. pumilus* YYFP is shown in *Figure 1*. Mutagenic lethality increased with increasing mutagenic exposure times. Specifically, at an ARTP mutagenic time of 105 seconds, lethality reached 97.44%. The target strain, after being subjected to ARTP mutation, will undergo further DES mutation，at a DES mutagenic time of 60 minutes, lethality was 95.58%. Positive mutation rates are generally considered to be higher when lethality is 90% or higher (Hu et al., 2020). Therefore, we selected an ARTP mutation time of 105 seconds and a DES mutation time of 60 minutes.

Screening of positive mutant strains and measurement of enzyme activity

Sixteen positive mutant strains were screened after the application of the mutagenic treatment solution. The ratio of the diameter D of the transparent zone to the diameter d of the colony in the starch screening medium indicates that the higher the ratio, the stronger the enzyme-producing capacity of the strain; a larger D/d ratio correlates with enhanced enzyme production. After six consecutive passages, five of the 16 positive mutant strains exhibited stable D/d values and were designated YYFP-1, YYFP-2, YYFP-3, YYFP-4, and YYFP-5. The D/d values and amylase activity measurements for the

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starting strain, YYFP, and the five positive mutants are presented in *Figure 2*. Among these, mutant strain YYFP-3 demonstrated the highest D/d value. Preliminary analysis suggested that YYFP-3 was the most effective enzyme-producing strain among the five positive mutants. Amylase activity assay results indicated that the mutant strain YYFP-3 achieved the highest amylase production, with an enzyme activity of 278.77 U/mL, followed by the mutant strain YYFP-1. Amylase production by the mutant strain YYFP-3 was approximately double that of the starting strain YYFP. Based on the D/d value observed during the initial screening, mutant strain YYFP-3 was selected for further experiments. A comparison of the amylase activity plates of the mutant strain YYFP-3 and the starting strain YYFP is shown in *Figure 3*.

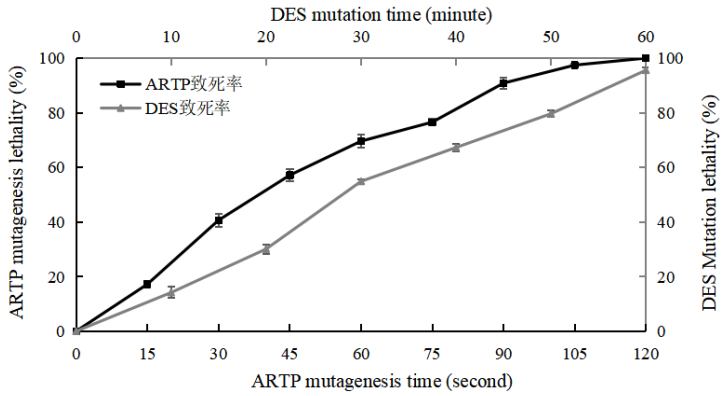

Figure 1. *Lethality curves of B. pumilus YYFP at different ARTP-DES mutagenesis times (data expressed as mean ± standard error [SE])*

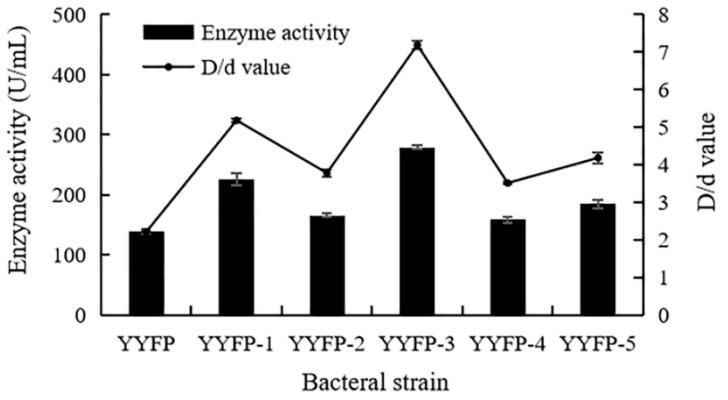

Figure 2. *Comparison of amylase production capacity between the starting and mutant strains (data are expressed as mean ± SE)*

Effects of mutant strain YYFP-3 on soil physicochemical properties

The effects of various concentrations of mutant strain YYFP-3 on soil physicochemical properties are presented in *Table 1*. Compared with the control group, the application of organic fertilizers enhanced soil quality to varying degrees, with a more pronounced effect observed after the addition of YYFP-3 liquid. Application of different concentrations of the mutant strain YYFP-3 resulted in increased soil pH, SOC, TN, AP, and AK content. As the concentration of *B. pumilus* YYFP-3 increased, soil pH exhibited

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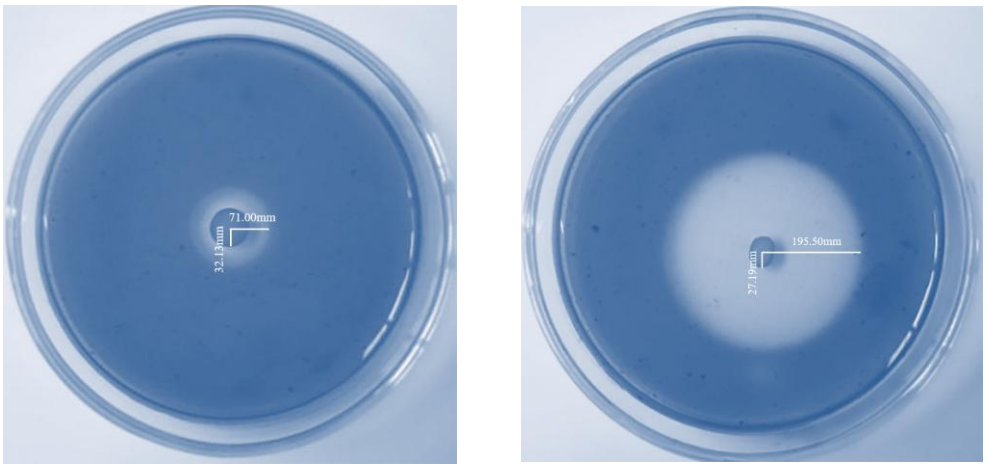
an upward trend; however, after reaching a certain concentration, pH levels stabilized, and further increases in bacterial liquid concentration had no significant effect on pH. SOC content demonstrated a positive correlation with the inoculated bacterial liquid, increasing as the inoculation concentration increased. The T2, T3, and T4 treatment groups showed significant differences compared with the CK control group, with SOC increasing by 16.65%, 19.14%, and 20.33%, respectively. The TN content did not show significant differences before and after the application of low to medium concentrations of bacterial liquid; however, after applying high concentrations, the soil TN content increased by 22.06% compared with the CK group. The soil AP content significantly increased after the application of the bacterial liquid, with increases of 32.33%, 56.18%, and 74.34%, respectively. Compared with the CK group, the soil AK content also increased with the application of low to medium concentrations of bacterial liquid, with the most substantial increase (12.36%) observed at high concentrations. The overall treatment effect was ranked in the order T4 > T3 > T2 > T1 > CK, with the highest effect observed at the highest concentration of the bacterial liquid.



(a) (b)

Figure 3. *Comparison of YYFP strains before and after mutagenesis. (a) Starting strain YYFP (D=142.00 mm, d=64.25 mm, D/d=2.21). (b) Positive mutant YYFP-3 (D=391.00 mm, d=54.38mm, D/d=7.19)*

Table 1. *Effect of starting strain YYFP and mutant strain YYFP-3 on physicochemical properties of soil*

Treatment	pH value	SOC (g/kg)	TN (g/kg)	AP (mg/kg)	AK (mg/kg)
CK	5.50 ± 0.03 ^{a}	10.87 ± 0.26 ^{a}	0.68 ± 0.04 ^{a}	35.42 ± 0.57 ^{a}	138.16 ± 1.65 ^{a}
T1	5.70 ± 0.03 ^{b}	11.55 ± 0.32 ^{b}	0.73 ± 0.03 ^{a}	40.87 ± 1.96 ^{b}	142.60 ± 1.26 ^{ab}
T2	5.75 ± 0.08 ^{b}	12.68 ± 0.16 ^{c}	0.75 ± 0.02 ^{ab}	46.87 ± 0.89 ^{c}	145.55 ± 3.31 ^{b}
T3	5.98 ± 0.03 ^{c}	12.95 ± 0.13 ^{c}	0.77 ± 0.05 ^{ab}	55.32 ± 1.49 ^{d}	146.81 ± 3.73 ^{b}
T4	6.07 ± 0.15 ^{c}	13.08 ± 0.15 ^{c}	0.83 ± 0.03 ^{b}	61.75 ± 1.10 ^{e}	155.23 ± 3.90 ^{c}

Note: CK represents the conventional chemical fertilizer treatment group. T1:20% reduction in chemical fertilizer combined with organic fertilizer. T2:20% reduction in chemical fertilizer combined with organic fertilizer and a low-concentration bacterial suspension. T3:20% reduction in chemical fertilizer with a medium-concentration bacterial suspension. T4:20% reduction in chemical fertilizer combined with organic fertilizer and a high-concentration bacterial suspension. Different lowercase letters in the same column indicate significant differences between the treatments ($P < 0.05$). Data are expressed as the mean ± SE

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Optimization of fermentation conditions for mutant strain B. pumilus YYFP-3

Single-factor optimization experiment

The effects of various carbon sources, nitrogen sources, and inorganic salts on amylase production by mutant strain YYFP-3 are illustrated in *Figure 4*. When soluble starch was used as the carbon source, amylase activity was significantly higher than when sucrose, glucose, lactose, or maltose was used. The highest amylase activity was observed when pancreatic peptone was used as the nitrogen source, followed by peptone and casein. The amylase activity of the mutant strain YYFP-3 peaked when calcium chloride was added as an inorganic salt.

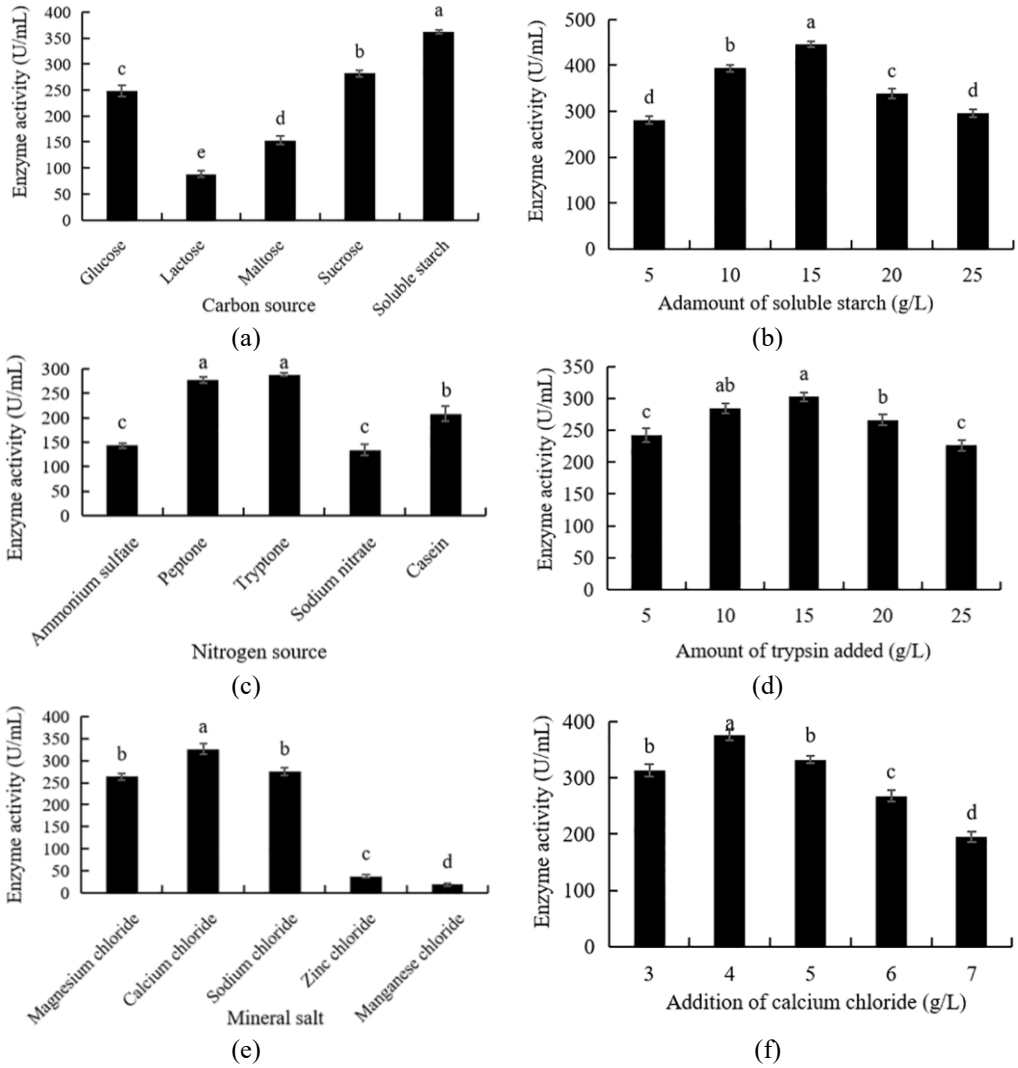

Figure 4. *Effects of various carbon sources, nitrogen sources, and inorganic salts on amylase production by the mutant strain YYFP-3. (a) Effect of various types of carbon sources on amylase production by mutant strain YYFP-3. (b) Effect of sucrose addition on amylase production in mutant strain YYFP-3. (c) Effect of various nitrogen sources on amylase production by mutant strain YYFP-3. (d) Effect of tryptone addition on amylase production by mutant strain YYFP-3; (e) Effect of various types of inorganic salts on amylase production by mutant strain YYFP-3. (f) Effect of calcium chloride addition on amylase production by mutant strain YYFP-3 (data are expressed as mean ± SE; significant differences are considered at $P < 0.05$)*

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Other inorganic salts, such as sodium chloride and magnesium chloride, also promoted amylase production, although their effects were slightly less pronounced than those of calcium chloride. In contrast, inorganic salts such as zinc chloride and manganese chloride inhibited amylase synthesis. As the levels of soluble starch, tryptone, and calcium chloride increased, the amylase activity initially increased and then decreased. Amylase activity peaked when the concentration of soluble starch is 15 g/L, the concentration of pancreatic peptone is 15 g/L, and the concentration of calcium chloride is 4 g/L. Therefore, 15 g/L soluble starch, 15 g/L tryptone, and 4 g/L calcium chloride were selected as the optimal concentrations

The effects of various incubation temperatures, pH values, inoculum amounts, and shaker speeds on amylase production by mutant strain YYFP-3 are illustrated in *Figure 5*. The amylase activity of the mutant strain YYFP-3 initially increased and then decreased with increasing incubation temperature, pH, inoculum amount, and shaker speed. At an incubation temperature of 35℃, mutant strain YYFP-3 exhibited the highest amylase activity. In addition, this strain demonstrated broader pH tolerance under alkaline conditions, with higher and more stable enzymatic activity. However, excessive acidity inhibited amylase synthesis, leading to a decline in activity when the pH exceeded 9. The optimal inoculum size was found to be 3%, beyond which amylase activity began to decrease. This decline may be attributed to overinoculation, which can cause significant bacterial proliferation in the early stages, resulting in the production of excessive metabolic waste and nutrient depletion. This ultimately inhibits bacterial growth and amylase synthesis during the later stages of growth. The highest amylase activity was observed at a rotational speed of 220 rpm. Therefore, the optimal conditions for amylase production were determined to be an incubation temperature of 35℃, an initial pH of 9.0, an inoculum size of 3%, and a rotational speed of 220 rpm.

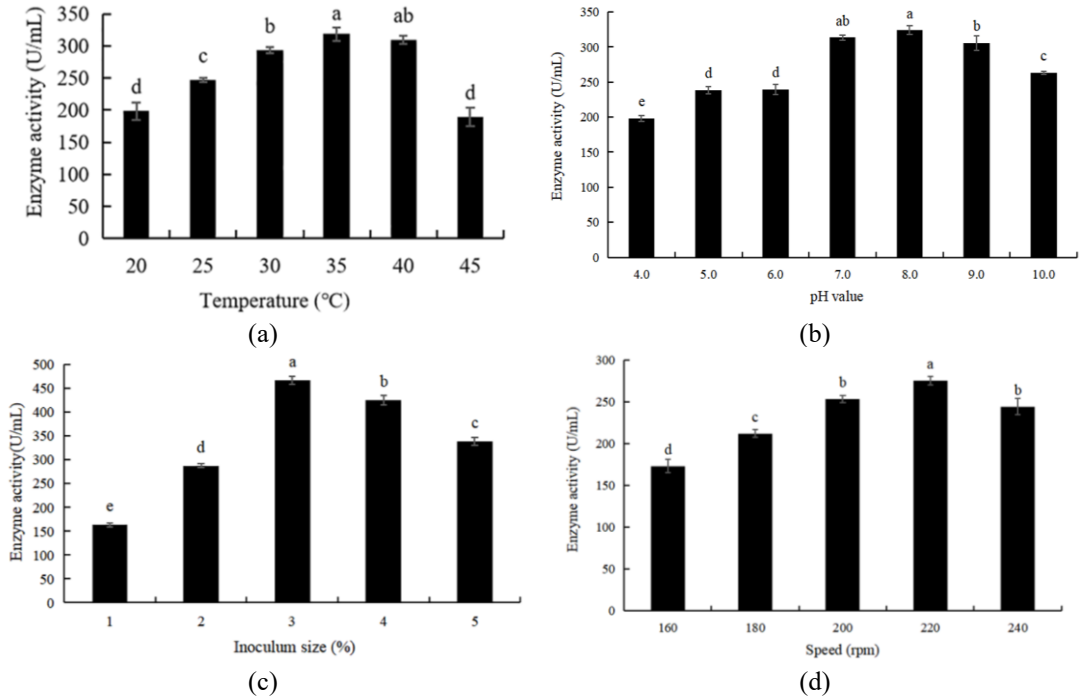

Figure 5. *Effects of various cultivation temperatures (a), initial fermentation pH (b), inoculation amount (c), and rotational speed (d) on amylase production by mutant strain YYFP-3. Data are expressed as mean ± SE, with significant differences considered at $P < 0.05$*

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PB design experiment

The seven factors examined in this study were: soluble starch addition (A, g/L), tryptone addition (B, g/L), calcium chloride addition (C, g/L), temperature (D, ℃), pH (E), inoculum amount (F, %), and rotational speed (G, rpm). For each factor, two levels were selected: high (+1) and low (-1). The PB experimental design and results are presented in *Table 2* along with an investigation of their effects on enzyme activity. The results of the significance analysis for these factors are presented in *Table 3*. Based on the findings of the PB test, the *P*-value of the model was <0.01, indicating that it was highly significant. The *P*-values for soluble starch addition (A) and fermentation inoculum (F) were both <0.01, demonstrating that these two factors had a highly significant effect on amylase production by the mutant strain YYFP-3. In addition, the *P*-value for calcium chloride addition (C) was <0.05, suggesting that calcium chloride also had a significant effect on amylase production by the mutant strain YYFP-3. *P*-values for all other factors were > 0.05. Consequently, three factors—soluble starch addition, inoculum size, and calcium chloride addition—were identified as the primary influencing factors in the subsequent hill-climbing test.

Table 2. *PB test design and results*

Assay	Variable levels							Enzyme activity (U/mL)
	A	B	C	D	E	F	G	
1	-1	1	-1	1	1	-1	1	374.32 ± 8.01
2	1	-1	1	1	1	-1	-1	357.69 ± 2.22
3	-1	1	1	1	-1	-1	-1	379.11 ± 6.96
4	-1	-1	1	-1	1	1	-1	433.16 ± 7.63
5	1	1	-1	-1	-1	1	-1	383.38 ± 6.11
6	1	1	1	-1	-1	-1	1	353.14 ± 8.92
7	1	-1	-1	-1	1	-1	1	345.21 ± 5.70
8	-1	-1	-1	-1	-1	-1	-1	375.31 ± 9.45
9	-1	-1	-1	1	-1	1	1	426.17 ± 6.18
10	-1	1	1	-1	1	1	1	430.92 ± 9.49
11	1	-1	1	1	-1	1	1	392.30 ± 8.03
12	1	1	-1	1	1	1	-1	386.37 ± 11.34

Table 3. *Significance analysis of PB test*

Sources	Sum of squares	DF	Mean square	*F*	*P*
Model	9661.55	7	1380.22	67.29	0.0006**
A-Soluble starch	3363.29	1	3363.29	163.97	0.0002**
B- Tryptone	42.55	1	42.55	2.07	0.2232
C-Calcium chloride	257.21	1	257.21	12.54	0.0240*
D-Temperature	2.22	1	2.22	0.1080	0.7589
E-pH	27.80	1	27.80	1.36	0.3091
F-Inoculum size	5964.36	1	5964.36	290.78	<0.0001**
G-Shaker speed	4.13	1	4.13	0.2012	0.6770
Error	82.05	4	20.51		
Cor total	9743.60	11			

Note: * indicates significant difference, $P < 0.05$; ** indicates extremely significant difference, $P < 0.01$

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Steepest ascent test

The steepest ascent test design was based on experimental results obtained from the PB test. The experimental design and results are presented in *Table 4*. The highest enzyme activity recorded was 501.48 ± 4.14 U/mL, achieved with a soluble addition of 11.5 g/L, a calcium chloride addition of 4.2 g/L, and an inoculum amount of 4%. Consequently, this condition was selected as the central point for the testing.

Table 4. *Climbing test design and results*

Assay	A-Soluble starch (g/L)	C-Calcium chloride (g/L)	F-Inoculum size (%)	Enzyme activity (U/mL)
1	15.0	4.0	3	496.28 ± 4.64
2	11.5	4.2	4	501.48 ± 4.14
3	8.0	4.4	5	431.57 ± 7.98
4	4.5	4.6	6	354.54 ± 5.54
5	1.0	4.8	7	286.02 ± 8.70

Box-Behnken test

To investigate the interactions between soluble starch, calcium chloride, and inoculum, a three-factor, three-level response surface experiment was conducted. Based on the results of the steepest slope test described above, the soluble starch addition rate was determined to be 11.5 g/L, the calcium chloride addition rate 4.2 g/L, and the inoculum rate 4% as the center point of the response surface experiment. The factors and their respective levels are presented in *Table 5*, and the experimental design and results are listed in *Table 6*. The experiment was conducted using a 17-group experimental design, with each experimental group performing 3 replicates.

Table 5. *Box-Behnken test factors and levels*

Fermentation conditions	Level		
	-1	0	+1
A-Soluble starch (g/L)	8.0	11.5	15.0
C-Calcium chloride (g/L)	4.0	4.2	4.4
F-Inoculum size (%)	3.0	4.0	5.0

Regression analysis of the data in *Table 6* yielded a quadratic polynomial regression equation for enzyme activity versus soluble starch addition (A), calcium chloride addition (C), and inoculum size (F).

$$R = 527.48 + 19.94A + 3.86C - 31.87F + 0.1967AC - 1.85AF - 0.9658CF - 24.96A^2 - 23.80C^2 - 48.08F^2$$

The results of the quadratic multinomial model and the analysis of variance (ANOVA) are presented in *Table 7*. The *P*-value of the model was <0.01, indicating that the model was highly significant. However, the *P*-value of the out-of-fit term was 0.1547 >0.05, suggesting that the errors in the test were relatively small. The correlation coefficient (R^2) was 0.9993, and the adjusted R^2 was 0.9983, indicating that the equation was significant. C.V.% was 0.3524, which was <10, suggesting that the accuracy of the test was high. The ANOVA results indicated that the *P*-values for the first-order terms of soluble starch (A),

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calcium chloride (C), and inoculum (F), as well as their second-order terms, were all <0.01, demonstrating a highly significant effect on enzyme activity. However, the interaction between soluble starch, calcium chloride, and the inoculum size was not significant, indicating that the relationship between these factors and amylase activity is not linear (Kenza et al., 2024).

Table 6. *Box-Behnken design trials and results*

Assay	Variable levels			Enzyme activity (U/mL)
	A-Soluble starch (g/L)	C-Calcium chloride (g/L)	F-Inoculum size (%)	
1	-1	0	-1	466.12 ± 7.51
2	0	-1	-1	482.13 ± 7.61
3	0	0	0	526.58 ± 7.50
4	1	-1	0	496.05 ± 7.66
5	0	0	0	527.56 ± 7.05
6	-1	0	1	405.42 ± 4.46
7	1	0	1	439.05 ± 8.12
8	0	1	-1	492.13 ± 8.01
9	0	0	0	528.67 ± 7.49
10	-1	-1	0	454.03 ± 5.31
11	0	1	1	427.13 ± 5.98
12	1	1	0	503.81 ± 8.79
13	0	0	0	525.91 ± 3.04
14	1	0	-1	507.16 ± 3.52
15	0	-1	1	421.00 ± 5.59
16	0	0	0	528.68 ± 10.30
17	-1	1	0	461.00 ± 4.97

Table 7. *Response surface ANOVA*

Source	Sum of squares	Df	Mean square	*F*	*P*	Significant
model	27653.11	9	3072.57	1065.63	< 0.0001**	significant
A-Soluble starch (g/L)	3180.03	1	3180.03	1102.90	< 0.0001**	
C-Calcium chloride (g/L)	119.04	1	119.04	41.29	0.0004**	
F-Inoculum size (%)	8124.30	1	8124.30	2817.67	< 0.0001**	
AC	0.1560	1	0.1560	0.0541	0.8227	
AF	13.73	1	13.73	4.76	0.0655	
CF	3.74	1	3.74	1.30	0.2920	
A^2	2622.90	1	2622.90	909.67	< 0.0001**	
C^2	2384.76	1	2384.76	827.08	< 0.0001**	
F^2	9735.93	1	9735.93	3376.27	< 0.0001**	
Residua	20.18	7	2.88			
Lack of Fit	14.05	3	4.68	3.05	0.1547	Not significant
Pure Error	6.14	4	1.53			
Cor Total	27673.29	16				

Note: * indicates significant difference, $P < 0.05$; ** indicates extremely significant difference, $P < 0.01$; $R^2 = 0.9993$; $AdjR^2 = 0.9983$; $PredR^2 = 0.9915$; CV% = 0.3524

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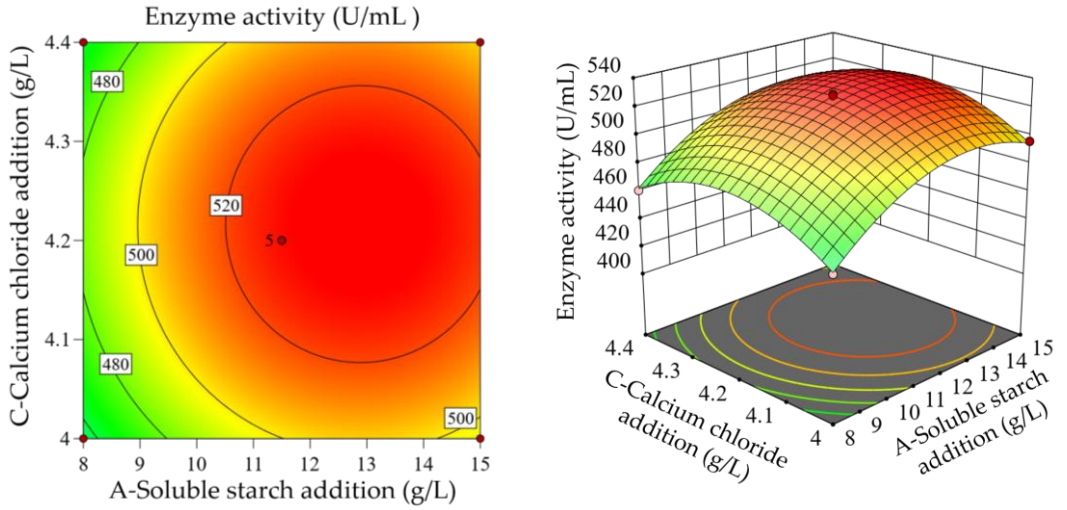
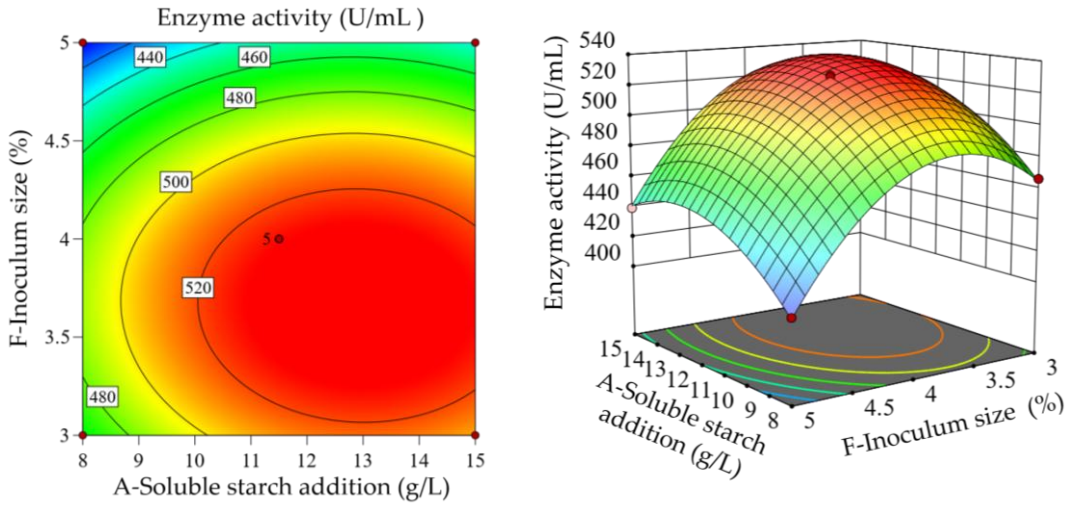
Contour plots and three-dimensional surface plots illustrating the interaction of these factors with amylase-producing enzyme activity are presented in *Figures 6, 7, and 8*. Each response surface analysis plot demonstrated the interrelationships between two independent variables, with the third variable maintained at its middle level. As shown in the plots, increasing the values of the two factors initially led to an increase in the enzyme activity, followed by a decrease. This pattern indicated that there was a specific combination of two interacting factors, soluble starch (A) and calcium chloride (C), along with the inoculum size (F), which maximized the Y value.



(a) (b)

Figure 6. *Interaction between soluble starch and calcium chloride addition. (a) High-line graph of the interaction between soluble starch addition and calcium chloride addition; (b) Response surface diagram of the interaction between soluble starch addition and calcium chloride addition*



(a) (b)

Figure 7. *Interaction between soluble starch and inoculum size. (a) High-line graph of the interaction between soluble starch addition and inoculum size; (b) Response surface diagram of the interaction between soluble starch addition and inoculum size*

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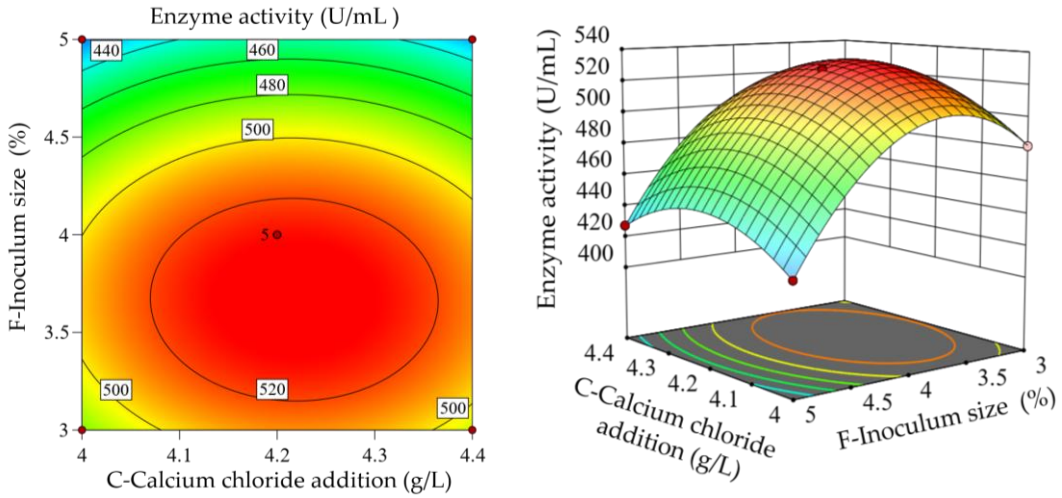

Figure 8. *Interaction between calcium chloride addition and inoculum size. (a) High-line graph of the interaction between calcium chloride addition and inoculum size; (b) Response surface diagram of the interaction between calcium chloride addition and inoculum size*

The optimal combination of significant factors was determined using Design Expert 13 software: soluble starch at 12.94 g/L, calcium chloride at 4.22 g/L, and an inoculum size of 3.66%. The model predicted enzyme activity of 537.185 U/mL under the specified test conditions. The test was repeated three times to validate the accuracy of the model, yielding an amylase activity measurement of 535.50 ± 4.26 U/mL. This result represents 99.69% of the predicted value, indicating that the model is highly reliable and consistent with expected test outcomes.

Discussion

Microbial mutagenesis is a practical, efficient, and relatively simple method of microbial breeding. Compared with natural processes, mutagenesis can induce high-frequency mutations in organisms, facilitating the acquisition of mutant strains with desirable traits (Gupta et al., 2022; Behnam et al., 2025). Common physical mutagenesis methods include ultraviolet (UV) radiation, X-rays, gamma rays, and ARTP technology (Mukhammadiev et al., 2024). Commonly used chemical mutagens include DES, methyl nitrosoguanidine, and ethyl methanesulfonate (Huang et al., 2021). In recent years, an increasing number of researchers have adopted a combination of multiple mutagenesis techniques to demonstrate the synergistic effects of combined mutagenesis. The strategic application of two or more mutagens and mutagenesis methods significantly enhances the mutagenesis outcome compared with single methods (Xia et al., 2022). For instance,Ishaq et al. (2025), mutagenized a thermophilic strain of *Aspergillus* fumigatus using UV radiation alone, EMS alone, or a UV-EMS combination. They found that the highest exoglucanase activity was produced by *Aspergillus* after UV-EMS compound mutagenesis. In this study, ARTP-DES combined with mutagenesis was used to induce mutations in *B. pumilus* YYFP, resulting in an exceptional amylase-producing mutant strain, *B. pumilus* YYFP-3, which produced approximately 2.01 times the amount of amylase than the original strain, demonstrating significant mutagenesis effects.

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The results of this study indicate that the application of organic fertilizer combined with *B. pumilus* YYFP-3 can effectively regulate the soil pH. As the concentration of *B. pumilus* increased, soil pH exhibited an upward trend. However, once a certain concentration was reached, the pH stabilized and further increased in the bacterial liquid concentration no longer significantly affected the pH levels. Ewunetu et al. (2024), found that the application of organic fertilizers could improve the pH of acidic soils, making them more neutral. Suhani et al. (2024), indicated that increasing the application of microbial agents accelerates the decomposition of soil organic matter and enhances nutrient absorption by plant roots. However, excessive application of microbial agents can lead to nutrient imbalances or fluctuations in the soil pH. This observation aligns with the trend noted in our experiment, where soil pH initially increased and then stabilized as *B. pumilus* YYFP-3 concentration increased. Compared with the control group, the application of organic fertilizer combined with *B. pumilus* YYFP-3 significantly increased the levels of SOC, TN, AP, and AK in tobacco-growing soils. Treatment with high concentrations of *B. pumilus* YYFP-3 was more effective than treatment with medium or low concentrations. This enhancement may be attributed to the ability of *B. pumilus* to promote the decomposition of organic matter, thereby releasing nutrients completely. In addition, *B. pumilus* possesses functions such as nitrogen fixation, P solubilization, and K release (Ewunetu et al., 2024; Suhani et al., 2024; da Costa Neto et al., 2024; Ishaq et al., 2025; Dushku et al., 2025). Consequently, the combination of organic fertilizers and microbial agents can enhance organic matter decomposition, address nutrient deficiencies resulting from reduced chemical fertilizer use, and potentially provide more effective nutrients than chemical fertilizers. This finding aligns with those of Prashun et al. (2025).

The growth of the strain and yield of secondary metabolites are closely related to fermentation conditions (García et al., 2021; Liu et al., 2025). Compared with traditional linear regression and orthogonal experiments, the Box-Behnken design response surface methodology offers several advantages, including shorter cycles, fewer trials, and greater precision (Zhang et al., 2025). Based on these mutations, the fermentation conditions for the *B. pumilus* YYFP-3 mutant strain were optimized using response surface methodology, resulting in enhanced amylase activity in the YYFP-3 mutant strain. The initial pH of the culture medium can regulate the bacterial absorption of external nutrients and secretion of enzyme proteins by altering the permeability and stability of the cell membrane. In addition, it can affect the ionization degree of the components in the fermentation medium, thereby influencing the enzyme activity in the fermentation broth (Han et al., 2024). At fermentation temperatures <30°C, the growth of the YYFP-3 mutant strain was inhibited, adversely affecting enzyme production. Elevated temperatures significantly disrupted the metabolic processes of the YYFP-3 mutant strain, compromising the stability of amylase in the fermentation broth and leading to a marked decrease in enzyme activity (Chen et al., 2025). Furthermore, when the inoculum size of the YYFP-3 mutant strain was set at 1%, enzyme activity was the lowest. This may be attributed to the small inoculum size, which results in slow bacterial growth and subsequently hampers enzyme production. Conversely, a larger inoculum size leads to a high concentration of bacteria in the fermentation broth, which quickly depletes nutrients in the culture medium and causes premature bacterial aging and death, thereby affecting the extracellular accumulation of amylase (Mabrouk et al., 2024). Enhancing soil amylase activity aids crops in better nutrient absorption from the soil, promotes the soil C conversion process, and facilitates the exchange of materials between crops and the soil.

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The exogenous introduction of the mutant strain YYFP-3 increased soil amylase concentration, which may have indirectly influenced soil physicochemical properties by promoting the conversion of organic matter in the soil.

In summary, the mutated strain Bacillus pumilus YYFP-3 obtained through mutagenesis exhibits strong amylase production capacity and high genetic stability. When applied in combination with organic fertilizer, it reduces chemical fertilizer usage and improves soil physicochemical properties. Response surface experiments identified the optimal fermentation conditions for mutant strain YYFP-3, providing a theoretical basis for its industrial application. The strain demonstrated excellent soil improvement effects in tobacco-growing fields, making it suitable for promotion in tobacco cultivation with promising application prospects. However, as the core objective of this study was to evaluate the synergistic potential of the mutant strain under conditions of reduced chemical fertilizer application combined with organic fertilizer, the experimental design did not include a "mutant strain alone without organic fertilizer" group or a "control strain group." Future research will incorporate these treatments to investigate the synergistic effects of YYFP-3 with other growth-promoting bacteria, determine optimal application ratios for each microorganism, and establish the most effective application rates when combined with organic fertilizers. This will maximize the synergistic enhancement effects between microbial fertilizers and organic fertilizers.

Conclusions

The amylase production activity of the high-yield amylase mutant strain YYFP-3 obtained through mutagenesis reached 278.77 U/mL, representing a 3.21-fold increase compared to the parental strain YYFP. Following fermentation condition optimization, its amylase production activity increased to 1.92 times that of the unoptimized strain. Under conditions of 20% reduced chemical fertilizer application, the combined use of organic fertilizer and high-concentration B. pumilus YYFP-3 bacterial suspension effectively regulated soil pH. This treatment significantly increased the content of organic carbon, total nitrogen, available phosphorus, and available potassium in tobacco-growing soil by 20.33%, 22.06%, 74.34%, and 12.36%, respectively. The combination of mutant strain YYFP-3 with organic fertilizer reduces reliance on chemical fertilizers and demonstrates excellent soil improvement effects for tobacco cultivation.

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Safety statement. The bacterial strain used in this study is classified as a Level 2 safety strain according to the Chinese agricultural industry standard "NY 1109-2006 General Technical Guidelines for Biosafety of Microbial Fertilizers."It has previously passed acute toxicity tests, with no significant impact observed on the growth and activity of test mice even at high doses. Therefore, its use in the field is considered relatively safe. The strain employed in this study holds value for further research.

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