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BREEDING, GROWTH CHARACTERISTICS AND FUNGAL INHIBITORY EFFECT OF THERMOTOLERANT *BACILLUS VELEZENSIS* DERIVED FROM DISTILLER'S GRAINS

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Abstract

This study used a starter strain, *Bacillus velezensis* TS01, isolated from distiller's grains. To directionally breed thermotolerant strains at 42-46°C for 532 h and 50 generations, EVOL (cell adaptive evolution technology) was used. Strain 46-1 has a growth of 250% higher than that of the original strain at 46°C, with stable entry into the logarithmic phase and a prolonged stationary phase. It grew optimally at 2% ethanol and 4% NaCl, grew well at pH 3, and produced amylase, protease, and cellulase. When it was tested against six pathogenic fungi, it exhibited the highest inhibition rate against *Eutypella microtheca* (72.60±0.99%). Then, it was followed by 58.60±4.24% and 56.53±0.11% against *Mortierella gemmifera* and *Talaromyces fuscoviridis*, respectively. 39.49±0.61% against *Mortierella elongata*; 38.78±0.44% against *Fusarium oxysporum* and 20.68±1.45% against *Trichoderma harzianum*. This research study provides a basis for greater biocontrol efficacy of *B. velezensis* in a high-temperature environment and for developing microbial fungicides.

Key words: *Bacillus velezensis*; EVOL cell adaptive evolution technology; Thermotolerance; Growth curve; Bacteriostatic activity; Biological characteristics

Introduction

Bacillus velezensis, a gram-positive bacterium, inhibits plant pathogenic fungi and bacteria by secreting antibacterial peptides, lipopeptides and hydrolases, showing great biocontrol potential against crop diseases (Johny *et al*., 2024; Wang *et al*., 2025; Petermann *et al*., 2025; Khan *et al*., 2025; Park *et al*., 2025; Bhimana *et al*., 2025; Esawy *et al*., 2025). Its strong environmental adaptability and biosafety to humans and animals make it a promising microbial alternative to chemical pesticides for green agricultural production (De la Cruz-Rodríguez *et al*., 2023; Xu *et al*., 2024; Petermann *et al*., 2025; Park *et al*., 2025).

In practical applications, high-temperature environments, including summer field soil and greenhouses, often reduce the metabolic activity and proliferation capacity of *B. velezensis*, greatly weakening its field control effect (Hurtado-Bautista *et al*., 2024). Most existing studies on *B. velezensis* focus on its antibacterial activity under standard temperatures, while research on directional domestication of thermotolerant strains is scarce. The lack of high-temperature-adapted biocontrol strains restricts its use in hot agricultural environments (Moreno-Velandia *et al*., 2021). Meanwhile, plant diseases (such as Fusarium wilt and root rot) caused by pathogenic fungi like *Fusarium oxysporum* occur frequently. These not only result in crop yield reduction but also disrupt the soil microecological balance, bringing significant losses to agricultural production (De la Cruz-Rodríguez *et al*., 2023; Le, 2025). In addition, fungi of the genera *Talaromyces* and *Eutypella* are also widely present in agricultural ecosystems, posing potential threats to crop health (Mao *et al*., 2021).

Distillers' grains, brewing byproducts rich in cellulose and protein, form an acidic, organic-rich microenvironment that selects stress-tolerant microorganisms (Zhang *et al*., 2024; Yang *et al*., 2025). *B. velezensis* TS01 isolated from this habitat owns natural stress resistance, laying a solid foundation for subsequent thermotolerant domestication. EVOL cell adaptive evolution technology is an automated high-throughput microbial domestication tool that creates continuous selection pressure to accumulate directional mutations and outperforms traditional domestication methods in efficiency.

This study uses *B. velezensis* TS01 isolated from distiller's grains as the starting strain and adopts EVOL cell adaptive evolution technology to breed thermotolerant strains. It explores its high-temperature growth traits and multiple stress tolerances (ethanol, acid, bile salt, sodium chloride), analyzes its amylase, protease and cellulase production capacity, and systematically tests its inhibitory activity against *F. oxysporum* and five other common pathogenic fungi. The results can provide theoretical support for improving the biocontrol efficiency of *B. velezensis* under high temperature and guide the development of efficient, stable microbial fungicides.

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Materials and Methods

Experimental materials

Strains: The test strains (TS01, six pathogenic fungi, etc.) were all provided by the Tangshan Key Laboratory of Applied Technology, Tangshan Normal University.

Culture media: LB Medium (g/L): Tryptone 10.0, Yeast Extract 5.0, NaCl 5.0 (2% agar for solid form); Protease Medium (g/L): Tryptone 5.0, NaCl 5.0, Skim Milk Powder 10.0 (pH 7.2, 1.5% agar for solid form); Amylase-Producing Medium (g/L): Peptone 10.0, Beef Extract 3.0, NaCl 5.0, soluble starch 10 (1.5% agar for solid form); PDA Medium (g/L): Potato infusion 200, Glucose 20.0 (1.5% agar for solid form). Basal Powder Medium (BPM) (Qingdao Haibo Biotechnology Co., Ltd., Qingdao, China) contained (g/L): peptone 10.0, yeast extract 5.0, NaCl 10.0, glucose 20.0, pH 7.0 ± 0.2 (15.0 g/L agar added for the solid form). Congo Red Medium was purchased from Qingdao Haibo Biotechnology Co., Ltd.

Experimental methods

Screening and breeding of high-temperature-resistant strain TS01

Preliminary experiment (determination of strain domestication conditions): TS01 was inoculated into the seed medium and cultured for 16 h before being transferred to the fermentation medium at a 2% inoculum. It was then cultured at various temperatures, and the OD_{600} absorbance was measured after 24 h.

EVOL cell domestication experiment: After the original strain was inoculated and cultured for 16 h, 2% of the bacterial solution was transferred to a new fermentation medium and subsequently introduced into the automatic microbial adaptive evolution instrument (EVOL cell). Following the configuration of the experimental parameters, the experiment commenced. Throughout the process, parameters such as domestication temperature and passage time were adjusted as necessary based on the strain's growth. After domestication, the bacterial solution was extracted and spread onto LB plates for culture (Guo *et al*., 2021).

Verification of the high-temperature-resistant strain TS01: The TS01 strain (CK) and the thermotolerant strain of TS01 were inoculated into LB liquid medium and cultured at 46°C for 18 h. Subsequently, they were transferred to fermentation medium at an OD_{600} of 0.12 and cultured at 46°C for an additional 24 h, after which the OD_{600} was measured.

Growth curve of thermotolerant strains of TS01: The screened thermotolerant strain TS01 was inoculated in LB liquid medium and cultured until the value of OD_{600} nm was 0.6-0.8. At a ratio of 1:100, the bacterial solution above has been inoculated into fresh LB liquid media. It was then cultured at 46°C with 200 rpm, and the OD_{600} nm value was measured at 12-h intervals. GraphPad 5.0 software was used to plot the growth curve. Each group was set with 3 replicates.

Multiple stress tolerance experiments and analysis of enzyme-producing characteristics of TS01 mutant strain

Multiple stress tolerance experiments of the TS01 mutant strain: Prepare BPM liquid media containing stress factors at different concentrations respectively: ethanol (0% (CK), 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, and 10%), pH gradients (3.0, 4.0, 5.0, 6.0, and 7.0 (CK), adjusted with 1mol/L HCl/NaOH), bile salts (0% (CK), 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%), and sodium chloride (0% (CK), 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, and 10%), with 3 replicates set for each group. Culture the TS01 mutant strain overnight until OD_{600}=0.8. Inoculate at a 1:100 ratio into the above culture media. Incubate the bacterium at 37°C for 20 h. Using software like GraphPad 5.0, a line graph was sketched based on information collected in three trials of the experiment.

Analysis of enzyme-producing characteristics of TS01 mutant strain: The BPM liquid medium with the TS01 mutant strain, an OD_{600} of 0.8, overnight culture at 37°C and 180 rpm. A suitable volume of the bacterial suspension was taken up and inoculated on the digestive enzyme medium at different spots and incubated at 37°C for 24 h. A hydrolysis zone was observed on the medium. The diameters of the hydrolysis zone (D) and the colony (d) were measured and recorded using the cross method, with a precision of 0.1 mm. The ability of the TS01 mutant strain to produce digestive enzymes was calculated based on the D/d ratio.

Inhibition experiment of thermotolerant strains of *B. velezensis* TS01 on different fungi: Preparation of the bacterial solution involved inoculating the screened thermotolerant strain TS01 into LB liquid medium. The culture was incubated with shaking overnight at 37°C and 180 rpm until reaching the logarithmic growth phase (OD_{600} = 0.8), which resulted in the production of the bacterial fermentation broth.

In the plate confrontation experiment, *T. fuscoviridis*, *F. oxysporum*, *T. harzianum*, *M. gemmifera*, *M. elongata*, and *E. microtheca* were cultured on PDA plates. Once the colonies exhibited robust growth, a sterile puncher was employed to extract 6 mm-diameter mycelial discs from the edges of the colonies. The mycelial disc was then inoculated at the center of the PDA plate, followed by the application of 2.5 µL of bacterial solution, which was spotted 2 cm away on both sides of the mycelial disc. Plates without bacterial inoculation served as controls (CK). The plates were incubated at 30°C, and the growth of the fungal colonies was observed and recorded (Zhou *et al*., 2022; Xia *et al*., 2024).

Determination of inhibition rate: Cultivation time was set based on fungal growth rate: 3 days for *E. microtheca*, 5 d for *M. gemmifera*, 7 d for *T. fuscoviridis*, 4 d for *F. oxysporum*, 2 d for *T. harzianum*, and 3 d for *M. elongata* (until control colonies reached 60% of the plate diameter). The inhibition rate was calculated according to the formula:

$$\text{Inhibition rate (\%)} = \frac{\text{Diameter of control colony} - \text{Diameter of treated colony}}{\text{Diameter of control colony}} \times 100\ \%$$

Data statistics: Data analysis and processing were conducted using Excel 2010. Statistical analyses were performed using GraphPad Prism 8.0 software. Comparisons between the two groups were analyzed by unpaired t-test. For comparisons among three or more groups, one-way ANOVA followed by Tukey's post-hoc multiple comparison test was used, with the significance threshold set at $\alpha = 0.05$. Tukey-adjusted P-values were used to judge differences between treatments, and $p<0.05$ was considered statistically significant. Mean values, standard deviations, and significance letter markers for all inhibition-rate comparisons are presented in Table 2.

Table 1. Hydrolysis circle characteristics of strain 46-1 for three extracellular enzymes.

Enzyme type	Hydrolysis zone diameter (D, mm)	Colony diameter (d, mm)	D/d
Amylase	1.85 ± 0.08	1.40 ± 0.07	1.32 ± 0.01
Protease	2.32 ± 0.02	1.28 ± 0.03	1.81 ± 0.04
Cellulase	1.71 ± 0.06	1.03 ± 0.07	1.66 ± 0.13

Table 2. Antifungal inhibition rates of *B. velezensis* 46-1 against different test fungi.

Fungal pathogens	Inhibition rate (%)
T. fuscoviridi	56.53 ± 0.11b
F. oxysporum	38.78 ± 0.44c
T. harzianum	20.68 ± 1.45d
M. gemmifera	58.60 ± 4.24b
M. elongata	39.49 ± 0.61c
E. microtheca	72.60 ± 0.99a

Results

Breeding of thermotolerant strains of *B. velezensis* TS01: To directionally breed thermotolerant strains, a preliminary experiment was conducted to establish the optimal domestication temperature range. The original strain TS01 was inoculated into the fermentation medium (initial OD_{600}=0.1) and cultured at various temperatures for 24 h, after which the OD_{600} values were measured. The results indicated that as the temperature increased, the inhibitory effect on strain growth intensified, with a significant enhancement of this effect observed at 44°C. Consequently, a core domestication temperature range of 42-46°C was determined to mitigate strain mortality due to abrupt temperature increases and to promote the directional expression of heat resistance-related genes through gradient pressure (Fig. 1A).

In this study, EVOL cell adaptive evolution technology was employed to domesticate TS01. The original strain, cultured for 16 h, was transferred to a new fermentation medium with an inoculation amount of 2%. Subsequently, it was placed in an automatic microbial adaptive evolution instrument. The total duration of domestication was 532 h, during which 50 generations of strain passage were completed (Fig. 1B). After the domestication process, the bacterial solution was extracted for coating cultures on LB plates. The target thermotolerant strain, designated as *B. velezensis* 46-1, was obtained through single-colony streaking isolation.

To verify the high-temperature growth ability of strain 46-1, both the original strain TS01 (control, CK) and strain 46-1 were inoculated into LB liquid medium and cultured at 46°C for 24 h. The OD_{600} values were subsequently measured. The results indicated that the growth of strain 46-1 was 250% significantly higher than that of the original strain ($p<0.0001$) (data derived from the high-throughput screening report of the EVOL adaptive evolution platform). This finding demonstrates that, following directional domestication, the growth capacity of strain 46-1 in high-temperature environments has been significantly enhanced, thereby successfully achieving the objective of breeding thermotolerant strains (Fig. 1C).

Growth curve analysis: The growth curve of the *B. velezensis* 46-1 strain was plotted using GraphPad software (Fig. 2). The results showed that 46-1 could grow well in LB medium. The OD_{600} nm gradually increases with increasing culture time until the 56th hour, and after this point, there are no significant changes in the OD. This suggests that the strain has a good growth stability at 46°C.

Multiple stress tolerance and enzyme-producing characteristics of mutant strain 46-1

Multiple stress tolerance of mutant strain 46-1: Mutant strain 46-1 was subjected to tolerance tests for ethanol, acid, bile salt, and sodium chloride. All the strains were cultured in BPM liquid medium for 20 h. The findings are as follows: In the media containing 0%-10% ethanol, the growth of the strain improved with the increasing of ethanol concentration. When the ethanol concentration is 0%-2%, the OD_{600} value peaks (the optimal growth) at a concentration of 2% ethanol. After exceeding 2%, the higher the concentration of ethanol, the stronger the inhibitory effect on the growth of the strain, and when the concentration of ethanol reached 10%, the growth of the strain was strongly inhibited (Fig. 3A). In the medium with pH 3.0-7.0 (with pH 7 as the control), the OD_{600} value of the strain was 0.430±0.006 (good growth) at pH=3; as the pH increased (acidity weakened), the OD_{600} value decreased gradually, and it was only 0.086±0.007 at pH=7, with the strain's growth significantly inhibited, indicating that an acidic environment is more conducive to its growth (Fig. 3B). In the medium containing 0–0.5% bile salts (with no bile salts as the control), the OD600 value decreased in a dose-dependent manner with increasing bile salt concentration, reaching its highest value (0.332 ± 0.006) at 0% bile salt concentration. This indicates that bile salts inhibit the strain's growth, with inhibition becoming more pronounced at higher concentrations (Fig. 3C). In the medium containing 0%-10% sodium chloride (with no sodium chloride as the control), the growth of the strain showed a "first increase then decrease" trend with the change of sodium chloride concentration; the OD_{600} value increased gradually when the sodium chloride concentration was 0%-4%, and reached the maximum value of 0.749±0.005 (optimal growth) at 4%; after exceeding 4%, the higher the concentration, the stronger the inhibitory effect (Fig. 3D).

Experimental results on enzyme-producing characteristics of strain 46-1: Strain 46-1's digestive enzyme production was evaluated via hydrolysis zones on protease/amylase/Congo red media: it produced amylase (D=1.85±0.08 mm, d=1.40±0.07 mm, D/d=1.32±0.01), protease (D=2.32±0.02 mm, d=1.28±0.03 mm,

D/d=1.81±0.04), and cellulase (D=1.71±0.06 mm, d=1.03±0.07 mm, D/d=1.66±0.13) (Fig. 4, Table 1).

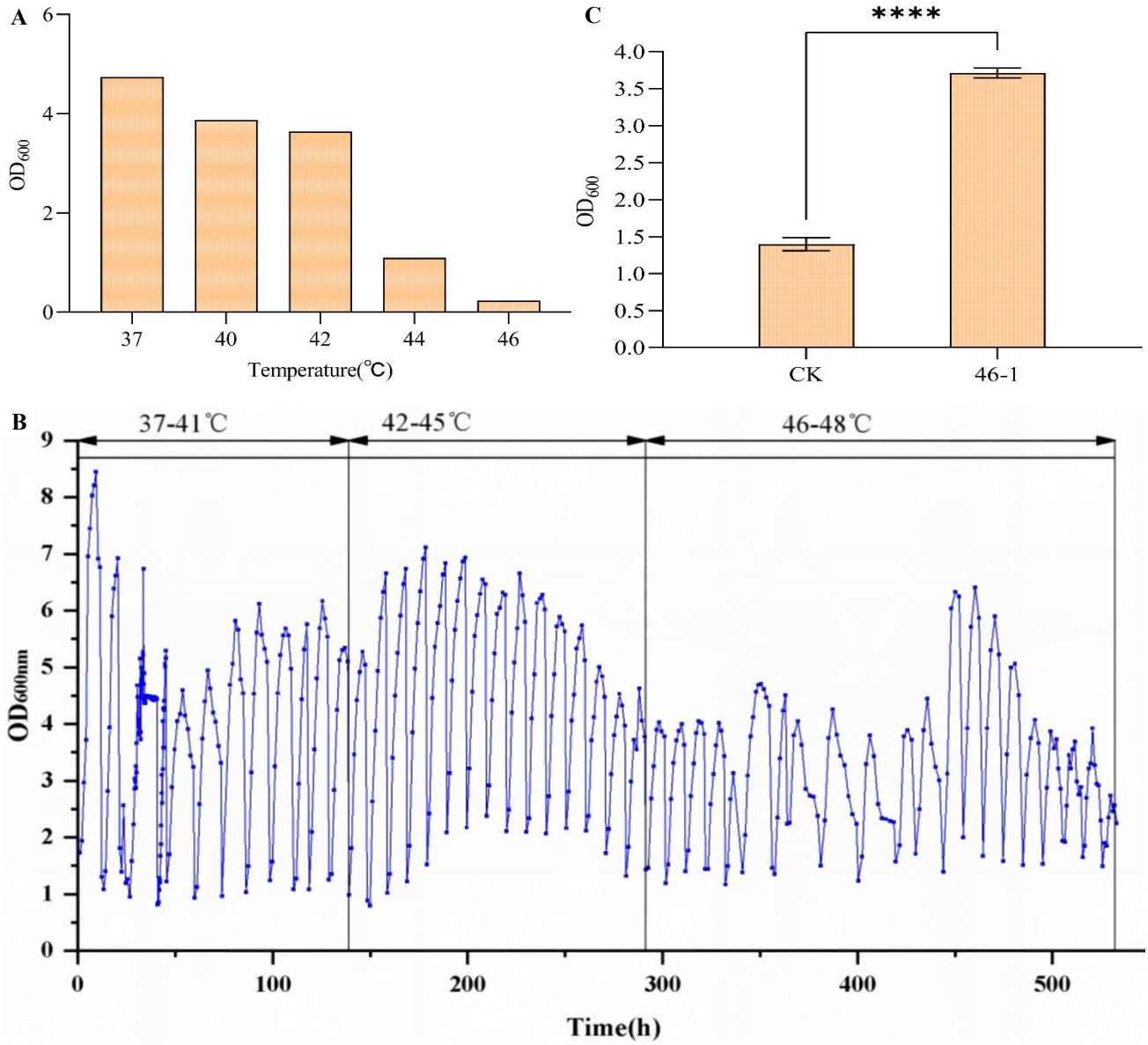

Fig. 1. Directional breeding results of thermotolerant *B. velezensis* TS01. (A) Effect of temperature on the growth of original strain TS01 (initial OD_{600}=0.1, 24 h culture); (B) Cumulative time curve of EVOL cell domestication (total 532 h, 50 generations); (C) Growth comparison between original strain TS01 (CK) and thermotolerant strain 46-1 at 46°C (24 h culture).

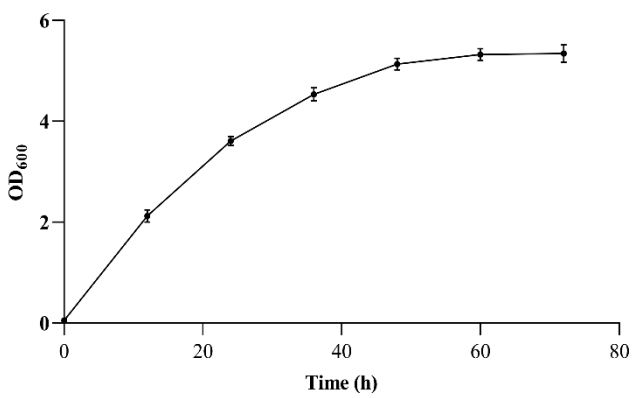

Fig. 2. Growth curve of *B. velezensis* 46-1.

Inhibitory effect of *B. velezensis* 46-1 on fungi: Plate confrontation experiments showed *B. velezensis* 46-1 inhibited *F. oxysporum* (38.78±0.44%) and 5 other fungi (Fig. 5, Table 2): highest rate on *E. microtheca* (72.60±0.99%), 58.60±4.24%/56.53±0.11% on *M. gemmifera*/*T. fuscoviridis*, 39.49±0.61% on *M. elongata*, and 20.68±1.45% on *T. harzianum*.

Discussion

In this study, thermotolerant *B. velezensis* cell adaptive evolution (42-46°C, 532 h/50 generations) of TS01 originally derived from distiller's grain gave rise to 46-1. At 46°C, its growth was 250% greater than that of the wild strain, confirming that directional domestication improves high-temperature adaptability (Liu *et al*., 2021). The temperature range of 42-46°C prevented death caused by temperature shock, promoted the expression of heat-resistance genes, and the length of application, 532 h,

permitted the accumulation of mutations in the genome. The 46°C growth curve showed that 46-1 stably entered the logarithmic phase and maintained a long stationary phase, indicating its high-temperature stability.

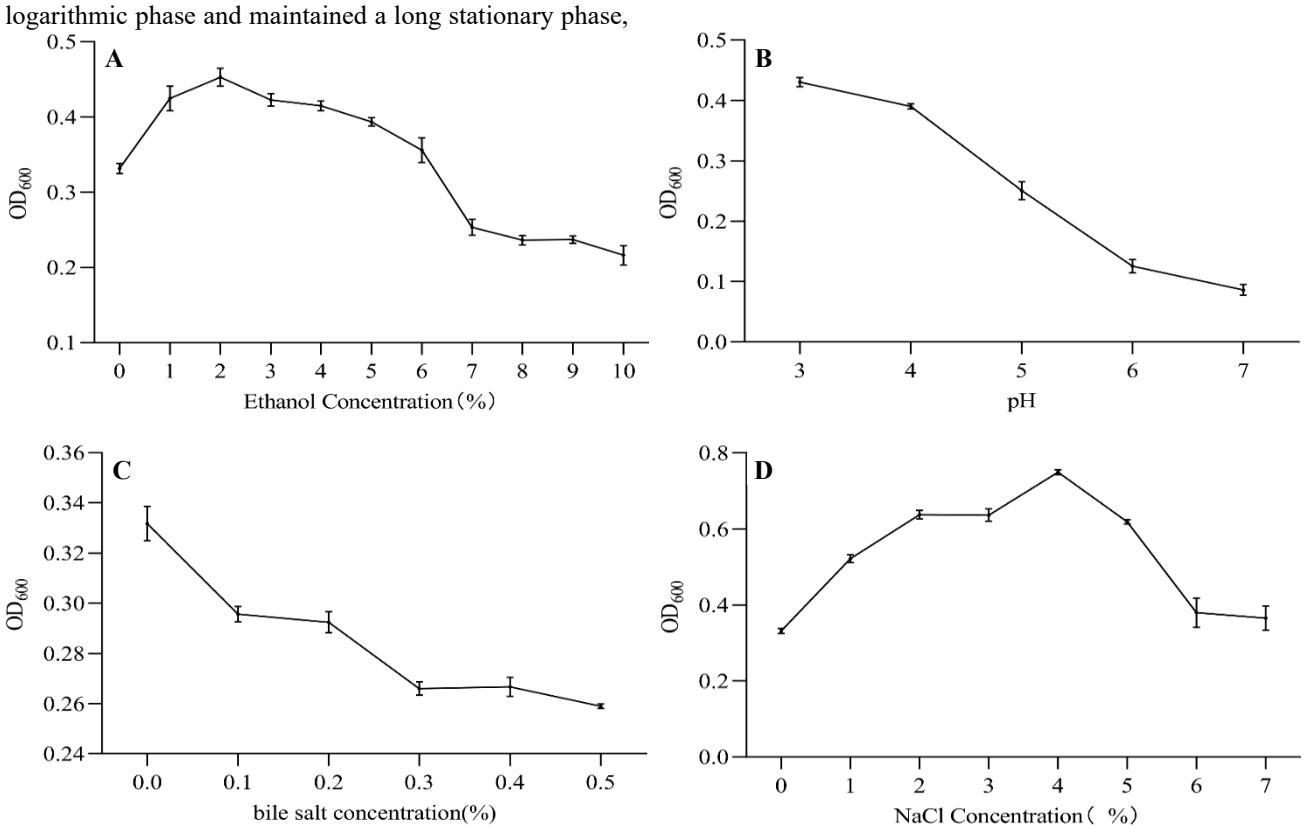

Fig. 3. Growth of mutant strain *B. velezensis* 46-1 under different stress factors.

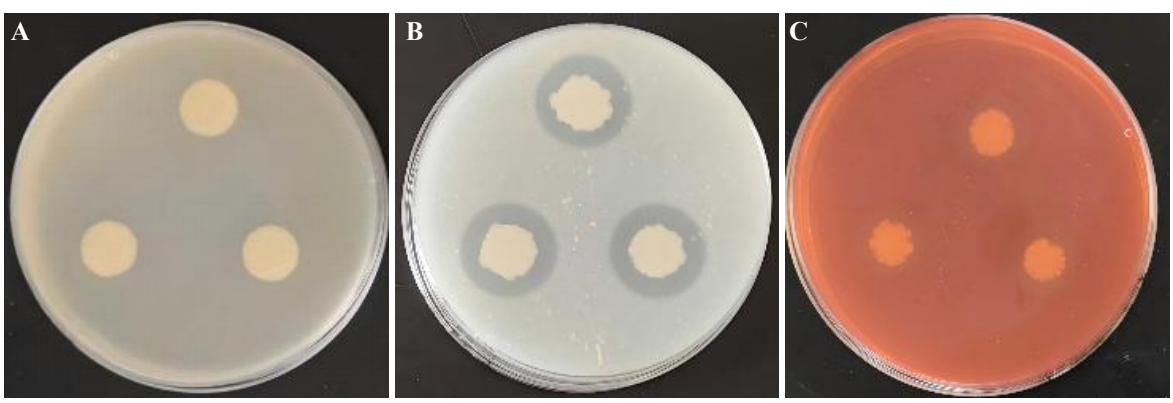

Fig. 4. Hydrolysis zones of strain 46-1 on digestive enzyme-producing media.
(A) Amylase-producing medium; (B) Protease-producing medium; (C) Congo red medium

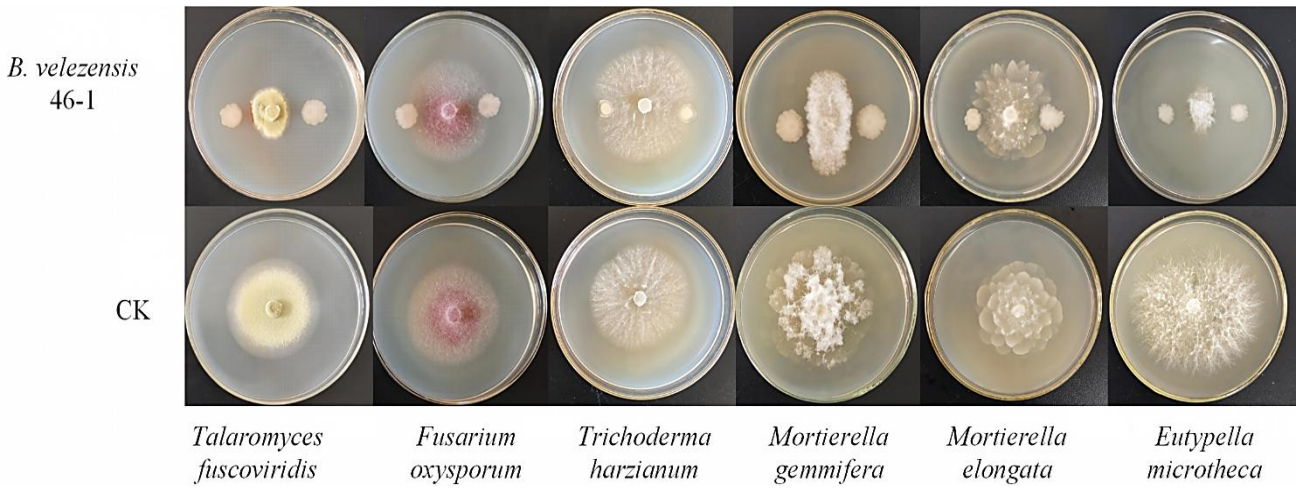

Fig. 5. Inhibition of *B. velezensis* 46-1 on different fungi.

Rising soil temperatures driven by global climate change have severely restricted the field application efficiency of conventional *B. velezensis* biocontrol agents in summer greenhouses and open farmland, while the limitation of chemical fungicides pushes agricultural production to seek eco-friendly sustainable biocontrol alternatives, which highlights the practical value of thermotolerant strains like 46-1 developed in this work (Hurtado-Bautista *et al*., 2024; Moreno-Velandia *et al*., 2021). Strain 46-1 has good resistance to various stresses as well as high-temperature resistance. The strain has a maximum growth at an ethanol concentration of 2% (absorbance value peak) and a high absorbance of 0.749±0.005 at a sodium chloride concentration of 4%. Moreover, the highest optical density across the pH gradient was recorded at pH 3.0 (absorbance 0.430±0.006). This apparent preference for strongly acidic conditions is unusual for *B. velezensis*, which is generally regarded as a neutrophile, and it should be interpreted with caution. Optical density reports turbidity rather than viability, and colony-forming unit counts were not performed at pH 3.0; the present data therefore cannot distinguish active replication from an increase in turbidity, and no precipitation of BPM medium components was observed that would obviously account for the reading. The acid tolerance of strain 46-1 is nevertheless consistent with the acidic, organic-rich distiller's grains from which the parent strain TS01 was isolated. Confirming that growth at pH 3.0 reflects true proliferation will require viable-count verification, and the pH optimum reported here should be regarded as provisional until that verification is available. These results demonstrate that strain 46-1 possesses multi-stress tolerance, likely shaped by long-term adaptation to the acidic and organic-rich environment of distiller's grains (Liu *et al*., 2021). Such traits suggest an ecological advantage for survival in complex agro-environments (Kuang *et al*., 2024). Notably, strain 46-1 exhibited gradually weakened growth with rising bile salt concentrations, reflecting its poor bile salt tolerance. Bile salts themselves are slightly alkaline substances, and livestock manure and mature manure compost generally maintain a weakly alkaline environment enriched with residual bile salts from animal intestinal secretions, forming dual stress of alkaline pH and bile salt toxicity for microorganisms (Nie *et al*., 2020). The intended application niches of this strain are high-temperature greenhouses and field soil with negligible bile salt content and moderate pH, so this limitation barely interferes with its biocontrol performance against soil-borne fungal diseases in farmland. Nevertheless, this trait would restrict its direct utilization in animal waste fermentation and manure composting, and further adaptive domestication targeting bile salt resistance is required if extending its application to compost fermentation.

In terms of antibacterial activity, strain 46-1 showed inhibitory effects on all 6 tested fungi. Among them, the inhibition rate against *E. microtheca* was the highest (72.60±0.99%), the inhibition rates against *M. gemmifera* and *T. fuscoviridis* also exceeded 50%, while the inhibition rate against *F. oxysporum* was 38.78±0.44%, and the inhibition rate against *T. harzianum* was the lowest (20.68±1.45%). This difference may relate to fungal cell wall structures, metabolic pathways, and sensitivity to antibacterial substances (Takahashi *et al*., 2012; Garcia-Rubio *et al*., 2019). For example, *B. velezensis*-derived chitinase may specifically degrade *E. microtheca* cell walls (Hong *et al*., 2022), while *T. harzianum* (a biocontrol fungus) may reduce inhibition via antagonistic secretion or nutrient competition (Topçu *et al*., 2025; Ishfaq *et al*., 2025). Furthermore, strain 46-1 can produce amylase, protease, and cellulase (with D/d ratios of 1.32±0.01, 1.81±0.04, and 1.66±0.13, respectively). The digestive enzymes not only assist in the decomposition of organic matter in the environment to procure nutrients, but they can also synergistically degrade the cell wall of pathogenic fungi (e.g., cellulase decomposes fungal cell wall cellulose, protease decomposes surface proteins), which enhances the antibacterial effect. This "dual antibacterial mechanism" of "active substance secretion + enzyme degradation" is formed (Chen *et al*., 2023; Zhou *et al*., 2024; Schönbichler *et al*., 2020).

Strain 46-1 is still active against fungal pathogens at high temperatures. This quality makes it suitable for applications in field-dried farmland or facility greenhouses during summer with high temperatures. The antibacterial mechanism is probably through multiple pathways. First, degradation of the integrity of the fungal cell membranes by lipopeptide compounds (these are iturin and surfactin) (Zhao *et al*., 2017; Zhang *et al*., 2025); second, secreting proteases, cellulases, etc., to degrade fungal cell walls (Meng *et al*.,

2024); third, inhibiting the proliferation of pathogenic bacteria through nutrient competition (Hibbing *et al*., 2010). Nevertheless, this study only confirmed the antibacterial effect via plate confrontation experiments, and the absence of pot experiments and field trial data could not fully reflect the actual biocontrol effect of the strain in nature. Furthermore, the major antibacterial substances (e.g., specific lipopeptides or enzymes) of strain 46-1 have still not been identified, and how its thermotolerance genes and antibacterial genes are correlated remains unknown (Younis *et al*., 2024). Various studies in the future can identify antibacterial substances using metabolomics, explore heat-resistance genes using transcriptomics, and verify field efficacy using potfield trials.

Distillers' grains, a by-product of brewing, are a rich source of nutrients like cellulose and protein. Therefore, distillers' grains can enhance the by-products of the screening of functional microorganisms (Zhang *et al*., 2024). The TS01 strain was isolated from distillers' grains, and it showed excellent performance after domestication. Its good performance not only indicates a new direction for resource utilization of agricultural waste. Furthermore, it suggests that the micro-ecology of distillers' grains may contain rich functional microbial resources, worthy of further exploration.

While this study provides foundational data supporting the thermotolerance and biocontrol potential of strain 46-1, three principal limitations warrant attention. First, the antifungal efficacy was evaluated exclusively via plate confrontation assays; consequently, the in vivo performance against soil-borne diseases remains to be validated through future pot and field trials. Second, although we characterized phenotypic traits such as enzyme production and inhibition rates, the specific bioactive compounds and the molecular basis of thermotolerance remain unclear. Third, growth under stress was quantified solely by optical density; without viable-count confirmation, the reported optima — in particular the apparent optimum at pH 3.0 — describe turbidity rather than demonstrated proliferation.

Addressing these gaps will require integrating non-targeted metabolomics with transcriptomic profiling to identify key secondary metabolites and differentially expressed genes under thermal stress. Collectively, these follow-up studies will bridge the gap between laboratory-based phenotypic characterization and the practical deployment of *B. velezensis* 46-1 as a robust biocontrol agent in warming agricultural ecosystems.

Conclusion

In this study, a thermotolerant strain 46-1 was successfully bred from *B. velezensis* TS01 (isolated from distiller's grains) via EVOL cell adaptive evolution technology (domesticated at 42-46°C for 532 h, 50 generations). At 46°C, strain 46-1 grew 250% better than the original strain (stable logarithmic phase, prolonged stationary phase), with excellent multi-stress tolerance (optimal at 2% ethanol/4% NaCl, good at pH=3) and production of amylase, protease (strongest, D/d=1.81±0.04), and cellulase. It exhibited inhibitory effects on 6 tested pathogenic fungi, with the highest inhibition rate (72.60±0.99%) against *E. microtheca*, over 50% against *M. gemmifera* and *T. fuscoviridis*, 39.49±0.61% against *M. elongata*, 38.78±0.44% against *F. oxysporum*, and the lowest (20.68±1.45%) against *T. harzianum*. This strain shows potential for biocontrol in high-temperature agricultural environments, but its actual efficacy needs to be further verified via pot experiments (e.g., wheat Fusarium wilt control at 40–45°C) and field trials (e.g., summer greenhouse tomato root rot control). Considering that multiple target pathogenic fungi (e.g., *Fusarium* spp.) also exist in industrial wastewater, the stress tolerance and antifungal traits of strain 46-1 suggest additional potential for water pollution control applications. To translate this lab-bred strain into practical application, future work will prioritize three directions: first, optimizing the large-scale fermentation process to improve spore yield and metabolite production efficiency; second, evaluating the synergistic biocontrol effects of strain 46-1 when combined with other stress-tolerant beneficial microbes to build composite biocontrol consortia; third, refining downstream formulation techniques to extend shelf life under ambient storage conditions. These efforts will lay a solid foundation for deploying strain 46-1 as a stable, cost-effective biocontrol agent for warming agricultural ecosystems.

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Author's Contributions: Yazi Li and Aamir Rasool: Screening and isolation of thermotolerant strains (after EVOL domestication), methodology design, manuscript review and revision; Jinxiao Yang: Growth curve determination and data analysis; Zhixin Zhang: Plate confrontation assay and inhibition rate calculation; Other authors: Operation of EVOL cell domestication, preparation of culture media, and project coordination; Ke Xu: Project design and funding acquisition.

Declaration of Competing Interest: We declare that we have no financial or personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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