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Isolation and Identification of Wheat-Associated Fungi, Screening of Antagonistic Bacteria, and Medium Optimization

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ABSTRACT

Wheat is highly susceptible to postharvest mold contamination, posing significant threats to food safety and economic stability. This study isolated and identified 40 fungal strains from stored wheat, with *Alternaria* as the predominant genus. *Bacillus velezensis* MY1 exhibited broad-spectrum antagonism against key pathogens including *Alternaria alstroemeriae*, *Chaetomium globosum*, and *Aspergillus aflatoxiformans*, with an average inhibition rate of 67.56%. Orthogonal optimization defined a fermentation medium comprising sucrose (30 g/L), beef extract (20 g/L), and K_2HPO_4 (5 g/L), which enhanced biomass yield 2.4-fold compared to conventional LB medium.

KEYWORDS

Bacillus velezensis; biocontrol; Antifungal activity; Fermentation optimization; Orthogonal array design

Addressing the critical issue of postharvest mold growth in wheat, this study developed *Bacillus velezensis* MY1 as a highly effective biocontrol agent, which exhibits significant inhibitory activity against a broad spectrum of fungi. Leveraging an optimized fermentation process, we present a solution enabling efficient cultivation and environmental sustainability, aiming to substantially reduce grain losses during storage. This work holds considerable value for safeguarding food security, mitigating postharvest economic losses, and decreasing reliance on chemical fungicides.

1 Introduction

Wheat, a cornerstone of global food security, is highly susceptible to spoilage by toxigenic molds during storage due to its hygroscopic nature. In regions like China, prolonged storage under fluctuating temperature and humidity promotes localized moisture accumulation, creating ideal conditions for fungal proliferation. This spoilage leads to significant nutritional degradation—reducing starch, protein, and lipid content—and, more critically, the accumulation of hazardous mycotoxins. Key spoilage fungi include Alternaria, Aspergillus, Fusarium, and Penicillium species, which produce metabolites such as aflatoxins, ochratoxins, deoxynivalenol, and fumonisins. These toxins pose severe, long-term risks to human health, including carcinogenicity and organ damage, and are challenging to eliminate once formed.

Current control strategies face notable limitations. Physical methods are energy-intensive and costly, while chemical fungicides raise concerns over residues and pathogen resistance. Biological control presents a sustainable alternative, with Bacillus species emerging as promising agents due to their environmental compatibility, stress tolerance, and multiple antimicrobial mechanisms. However, research has largely focused on crop seedling diseases or postharvest produce, leaving a gap in effective biocontrol solutions for stored wheat.

To address this, we isolated and identified six prevalent fungal strains from wheat grains and conducted antagonistic screening. A strain of Bacillus velezensis (MY1) demonstrated significant inhibitory activity. We systematically optimized the fermentation medium for MY1 using single-factor and orthogonal experiments to maximize biomass production. This work identifies a potent biocontrol agent and establishes an efficient fermentation process, providing a practical foundation for developing microbial formulations to reduce mold contamination and economic losses in wheat storage.

2 Materials and methods

2.1 Wheat variety and microbial strains

The wheat variety used in this study was Jimai 22. The antagonistic bacterial strains were isolated from tobacco and sorghum field soils and are preserved at the Sichuan Province Engineering Technology Research Centerof Liquor-Making Grains, Sichuan University of Science & Engineering. A total of 30 strains were tested, including *Bacillus velezensis* MY1, *Bacillus amyloliquefaciens* Ycx12, *Bacillus velezensis* T3, *Bacillus sp.* BZ1, and *Bacillus sp.* MY2.

2.2 Culture media

LB medium, NA medium, and PDA medium were prepared according to the formulations described by Swarnalee

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2.3 Isolation and purification of wheat endophytic fungi

Wheat seeds were randomly selected and surface-sterilized by immersion in 75% ethanol for 10–20 s, followed by treatment with 5% sodium hypochlorite for 1 min. The seeds were then rinsed three times with sterile water and dried with sterile filter paper. Subsequently, the seeds were evenly placed on PDA plates (5 seeds per plate) and incubated at 28 °C for 7 days. Representative fungal hyphae from the edge of individual colonies were repeatedly isolated and purified through subculturing。

2.4 Isolation and purification of wheat surface fungi

Wheat samples were mixed with sterile water at a mass-to-volume ratio of 1:2 and shaken for 12 h to obtain a microbial suspension. After thorough mixing, the suspension was serially diluted (10^{-1} to 10^{-6}), and 100 μL of each dilution was spread onto PDA plates. The plates were incubated at 28 °C for 7 days. Pure cultures were obtained through repeated streak plating.

2.5 Identification of wheat fungi

Morphological identification: Colonial characteristics—including form, colour, texture, and margin features—were examined. Hyphal and conidial morphologies were observed under a light microscope to facilitate preliminary taxonomic classification.

Molecular Identification: Genomic DNA was extracted from fungal isolates using a commercial kit. The fungal universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for PCR amplification. The amplified products were sequenced by Shanghai JieLi Biology Co., Ltd. The resulting sequences were compared against the NCBI database using BLAST analysis, and a phylogenetic tree was constructed with MEGA 11 software.

2.6 Screening of antagonistic bacteria for inhibiting wheat fungi

A 5-mm mycelial plug of each fungal isolate was placed at the center of a PDA plate. Then, 5 μL of bacterial fermentation broth was symmetrically inoculated 2.5 cm away from the fungal plug. A control group was set up without bacterial inoculation. Each treatment was performed in triplicate. After incubation at 28 °C for 7 days, the fungal colony diameter was measured using the cross method. The inhibition rate was calculated according to the following formula:

Inhibition rate (%) = [(Control colony diameter − Treated colony diameter) / Control colony diameter] × 100.

2.7 Optimization of fermentation medium for antagonistic strain

Strain MY1, demonstrating the most potent antifungal activity, was selected for further study. A single colony was inoculated into 100 mL of LB liquid medium and cultured at 28 °C with agitation at 180 r/min for 18 h to prepare a seed culture.

A single-factor experiment was conducted to evaluate the effects of carbon sources (peptone, glucose, sucrose, lactose, mannitol, and soluble starch), nitrogen sources (yeast extract, glutamic acid, urea, beef extract, ammonium sulfate, and sodium nitrate), and inorganic salts (KH_2PO_4, K_2HPO_4, NaCl, KCl, $FeSO_4$, and $MgSO_4$) on bacterial growth. Each component was tested at varying concentrations (0.5%, 1%, 2%, 3%, 4%, and 5%) to determine its optimal supplementation level. A 100 μL aliquot of the seed culture was inoculated into 100 mL of each test medium in Erlenmeyer flasks and cultured at 28 °C with shaking at 180 r/min for 24 h. The fermentation broth was diluted 10-fold, and the OD_{600} was measured.

Based on the above results, sucrose (carbon source), beef extract (nitrogen source), and K_2HPO_4 (inorganic salt) were selected as key factors, each tested at three concentration levels. An $L_9(3^3)$ orthogonal array design was employed to determine the optimal composition of the fermentation medium(Table 1)。

Table 1 Factors and levels of L9 (3^3) orthogonal test

Level	Factor		
	Sucrose (A)	Beef extract (B)	K_2HPO_4 (C)
1	1.5 %	2 %	0.5 %
2	2 %	3 %	1 %
3	3 %	4 %	1.5 %

3 Results and analysis

3.1 Isolation of fungi from wheat samples

A total of 40 fungal strains were isolated from the wheat samples and classified into seven genera,including *Alternaria*, *Acremonium*, *Trichoderma*, *Chaetomium*, *Talaromyces*, *Aspergillus*, and *Candida*. *Alternaria* was the dominant genus, representing 27 of the isolated strains. Six commonly occurring strains—MN3, MN17, MB41, MB42, MB44, and MB45—were selected as representative isolates for subsequent experiments.

3.2 Identification of wheat fungi

3.2.1 Morphological identification

Isolate MN3 initially exhibited light yellow colonies that gradually deepened to an olive-green hue, with a velvety texture and well-defined margins. Conidia were obclavate, characterized by both transverse and longitudinal septa, forming a distinct brick-wall pattern. Hyphae were brown, robust, and regularly septate .Isolate MN17 produced white, cottony colonies with a red-pigmented substrate. Perithecia were subglobose and densely covered with curled, hair-like appendages. Hyphae were light brown, septate, and highly branched. Ovoid, hyaline ascospores were sparsely produced. Isolate MB41 initially formed white colonies that developed pale green margins over time, exhibiting a velvety texture. Hyphae were hyaline and extensively branched, displaying a dendritic morphology. Conidia were spherical and colorless. Isolate MB42 displayed colonies with white to light pink surface pigmentation and pale yellow reverse coloration, both centrally and peripherally. The colonies were thick, velvety, and well-circumscribed. Hyphae were hyaline, predominantly erect, and sparingly branched. Conidia were ovoid, aseptate, and transparent. Isolate MB44 transitioned from white to grey-green, with a rough surface and circular morphology. The reverse side showed yellow central pigmentation. Conidia were spherical and light green.Isolate MB45 exhibited yellow-green colonies, often with a colorless to pale yellow reverse. Conidia were spherical, yellow-green, and borne in loosely arranged columns that fragmented easily.

3.2.2 Molecular identification

Based on integrated morphological characteristics and phylogenetic analysis (Fig 1), the isolates were conclusively identified as follows: MN3 as *Alternaria alstroemeriae*, MN17 as *Chaetomium globosum*, MB41 as *Trichoderma nordicum*, MB42 as *Acremonium sclerotigenum*, MB44 as *Talaromyces pseudofuniculosus*, and MB45 as *Aspergillus aflatoxiformans*.

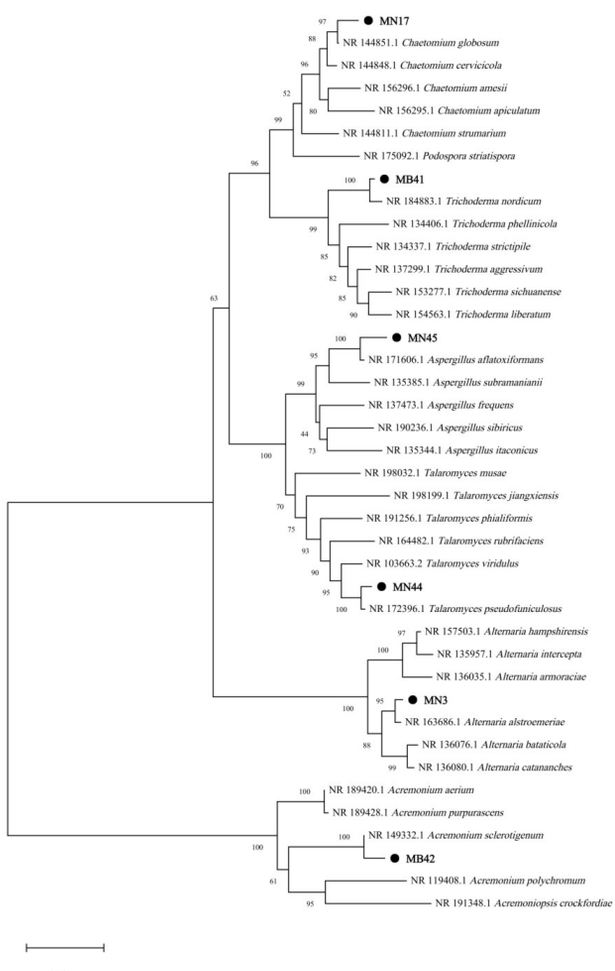

Figure 1 Phylogeny of selected wheat fungal strains

3.3 Screening of antagonistic bacteria for inhibiting wheat fungi

Plate confrontation assays revealed that five bacterial strains exhibited significant inhibitory effects against the test fungi (Fig 2). As shown in Figs. 2 and 3, *Bacillus velezensis* MY1 demonstrated particularly strong antagonistic activity. The hyphal growth of isolates MN3, MN17, and MB42 was markedly restricted on treatment plates, forming only limited, block-like expansions around the inoculation point without normal radial spread. Pigment production in MN3 was also inhibited. The inhibition rates for these fungi were 75.07%, 71.79%, and 58.19%, respectively. A clear inhibition line formed between strain MY1 and the margin of MB41 mycelia, corresponding to an inhibition rate of 59.61%. Furthermore, MY1 effectively suppressed the sporulation and dispersal of MB44 and MB45, forming distinct inhibition zones with inhibition rates of 72.22% and 68.50%, respectively.Compared to the other four bacterial strains, *B. velezensis* MY1 not only exhibited superior and broader-spectrum antifungal efficacy but also maintained stable antagonistic activity after repeated subculturing, indicating high genetic stability. Based on these results, strain MY1 was selected for further investigation.

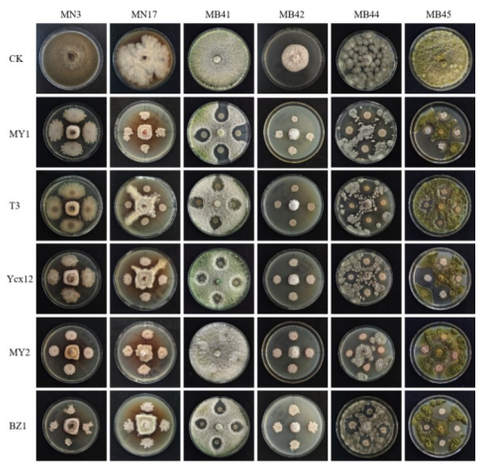

Figure 2 Bacterial antagonistic effects on test fungi

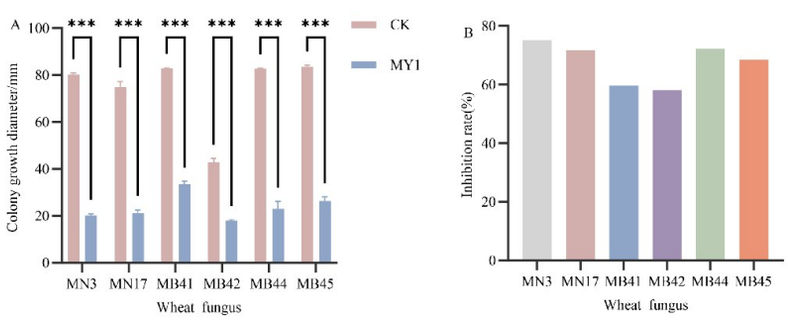

Figure3 Fungal colony diameter and inhibition rate by MY1

3.4 Optimization of fermentation medium for strain MY1

3.4.1 Medium composition optimization

Assessment of OD_{600} values after 24 hour cultivation revealed significant differences in the growth of strain MY1 across six carbon sources (Fig 4-A). Sucrose yielded the highest biomass, with an OD_{600} of 0.53, followed by glucose and peptone. Consequently, sucrose was selected as the optimal carbon source.Among nitrogen sources tested (Fig 4-B), all except urea supported bacterial growth, with beef extract producing the highest OD_{600} value of 0.64, establishing it as the preferred nitrogen source.During inorganic salt screening, K_2HPO_4 proved markedly superior to other salts, resulting in an OD_{600} of 0.57 after 24 hours(Fig 4-C).

3.4.2 Optimization of carbon, nitrogen, and inorganic salt concentrations

Following the identification of optimal nutrient sources—sucrose (carbon), beef extract (nitrogen), and K_2HPO_4 (inorganic salt)—their concentrations were further optimized through gradient assays.The results indicated that sucrose at a concentration of 2% yielded the highest OD_{600} value of 0.60, establishing an optimal supplementation level of 20 g/L (Fig 4-D). Beef extract performed best at 3%, achieving an OD_{600} of 0.53, corresponding to an optimal concentration of 30 g/L (Fig 4-E). K_2HPO_4 most significantly enhanced bacterial growth at 0.5%, with an OD_{600} reaching 0.58, confirming 5 g/L as the optimal dosage (Fig 4-F).

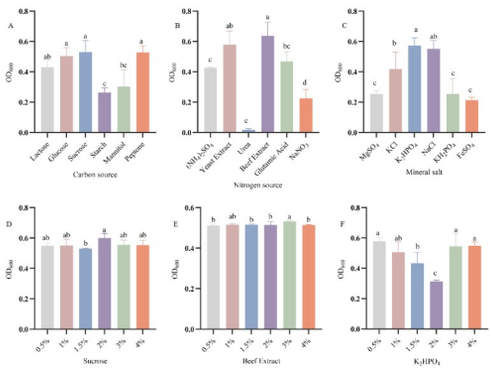

Figure 4 Orthogonal experimental results

3.4.3 Orthogonal experimental optimization

Orthogonal array testing revealed that sucrose (Factor A) exerted the most significant influence on bacterial growth, whereas beef extract (Factor B) had the least impact. The optimal level combination was identified as $A_3B_1C_1$, corresponding to sucrose 30 g/L, beef extract 20 g/L, and K_2HPO_4 5 g/L. This combination yielded the highest OD_{600} value among all tested conditions (Table 2). When strain MY1 was cultured for 24 hours in the optimized medium versus standard LB medium, the biomass obtained under the optimized condition was approximately 2.4-fold higher, confirming this formulation as the superior fermentation medium for MY1.

Table 2 Orthogonal test results of MY1 culture conditions

Number	Factor			OD_{600}
	Sucrose (A)	Beef Extract (B)	K_2HPO_4 (C)	
1	1	1	1	0.79
2	1	2	2	0.56
3	1	3	3	0.62
4	2	1	3	0.65
5	2	2	1	0.81
6	2	3	2	0.98
7	3	1	2	1.14
8	3	2	3	1.17
9	3	3	1	0.75
K1	0.657	0.860	0.980	
K2	0.813	0.847	0.653	
K3	1.020	0.783	0.857	
R	0.363	0.077	0.327	

4 Discussion

Forty fungal strains were isolated from wheat grains, dominated by *Alternaria alstroemeriae*, along with *Chaetomium globosum*, *Trichoderma nordicum*, *Acremonium sclerotigenum*, *Talaromyces pseudofuniculosus*, and *Aspergillus aflatoxiformans*. Alternaria species are major pathogens causing significant losses in cereals, vegetables, and fruits. For instance, *Alternaria alternata*—the predominant pathogen in diseased wheat—produces alternaria toxins and alters starch composition, degrading pasting properties and grain quality. Other isolates are also known pathogens: *A. sclerotigenum* causes soft rot in lilies and necrosis in apples, Talaromyces species induce postharvest decay in fruits, and *A. aflatoxiformans* produces the highly stable and carcinogenic aflatoxin B_1, posing serious food safety risks.

Biocontrol agents such as *Bacillus velezensis* act through multiple mechanisms. Studies report that *B. velezensis* inhibits fungal pathogens via nutrient competition, secretion of antimicrobial lipopeptides, chitinases, and glucanases, and induction of host resistance. In this study, *B. velezensis* MY1 exhibited broad-spectrum inhibition against the isolated fungi, with inhibition rates of 75.07% (*A. alstroemeriae*), 71.79% (*C. globosum*), 59.61% (*T. nordicum*), 58.19% (*A. sclerotigenum*), 72.22% (*T. pseudofuniculosus*), and 68.50% (*A. aflatoxiformans*), demonstrating its potential as a biocontrol resource.

Medium composition critically influences microbial growth and metabolite yield. Prior optimizations have significantly enhanced biomass and sporulation in related strains. Here, single-factor and orthogonal tests optimized the medium for MY1, yielding a formulation of sucrose 30 g/L, beef extract 20 g/L, and K_2HPO_4 5 g/L. This optimized medium supports efficient biomass production, facilitating the development of MY1 as a biocontrol agent.

5 Conclusion

Forty fungal strains were isolated from wheat samples and identified through morphological and molecular methods as belonging to the genera *Alternaria*, *Chaetomium*, *Trichoderma*, *Acremonium*, *Talaromyces*, and *Aspergillus*. Antagonistic assays revealed that *Bacillus velezensis* MY1 exhibited significant inhibitory activity against representative strains from all six genera, with an average inhibition rate of 67.56%. The strongest suppression was observed against *Alternaria spp.*, achieving an inhibition rate of 75.07%. Further optimization of the fermentation medium—comprising sucrose (30 g/L), beef extract (20 g/L), and K_2HPO_4 (5 g/L)—increased the biomass yield of strain MY1 by 2.4-fold compared to the pre-optimized conditions.

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