# Screening and Identification of Chitinase-Producing Strains and Their Inhibitory Effect on Dominant Molds in Corn Straw Postprint

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## Abstract

This study aimed to investigate the inhibition of dominant mold growth in corn straw by chitinase-producing strains, providing a theoretical basis for improving straw utilization. A chitinase-producing strain BS-1 was screened from corn straw samples using colloidal chitin medium, while dominant molds were isolated and purified. Strain BS-1 and the dominant molds were identified through morphological observation and 16S rDNA or 18S rDNA sequencing. The chitinase activity of strain BS-1 at 24, 48, 72, 96, 120, 144, and 168 h of fermentation was determined using the 3,5-dinitrosalicylic acid colorimetric method, and the inhibitory effect of BS-1 fermentation broth on dominant molds in corn straw was detected using the Oxford cup method. The results showed that strain BS-1 isolated from corn straw samples was identified as Bacillus subtilis, and the four dominant molds were Mucor circinelloides, Fusarium oxysporum, Aspergillus oryzae, and Aspergillus niger, respectively. The chitinase activity of strain BS-1 reached its maximum value of 3.23 U/mL after 120 h of cultivation at 37 °C. The fermentation broth of strain BS-1 exhibited significant inhibitory effects on the four dominant molds in corn straw, with inhibition zone diameters of 18.13, 18.48, 17.55, and 15.68 mm, respectively. These findings demonstrate that the screened chitinase-producing Bacillus subtilis BS-1 can effectively inhibit the growth of the four dominant molds, namely Mucor circinelloides, Fusarium oxysporum, Aspergillus oryzae, and Aspergillus niger, in corn straw.

## Full Text

### Screening and Identification of Chitinase-Producing Strain and Its Inhibitory Effect on Dominant Molds in Corn Stalk

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**Abstract:** This study aimed to investigate the inhibition of dominant mold growth in corn stalk using a chitinase-producing strain, providing a theoretical basis for improving straw utilization efficiency. A chitinase-producing strain designated BS-1 was screened from corn stalk samples using colloidal chitin medium, while dominant molds were simultaneously isolated and purified. Strain BS-1 and the dominant molds were identified through morphological observation and 16S rDNA or 18S rDNA sequence analysis. The chitinase activity of strain BS-1 was measured at 24, 48, 72, 96, 120, 144, and 168 h of fermentation using the 3,5-dinitrosalicylic acid colorimetric method, and the inhibitory effect of BS-1 fermentation broth on dominant molds in corn stalk was evaluated by the Oxford cup method. The results showed that strain BS-1 isolated from corn stalk was identified as *Bacillus subtilis*, and the four dominant molds were *Mucor circinelloides*, *Fusarium oxysporum*, *Aspergillus oryzae*, and *Aspergillus niger*, respectively. The highest chitinase activity of strain BS-1 reached 3.23 U/mL after 120 h of cultivation at 37 °C. The fermentation broth of strain BS-1 exhibited significant inhibition against all four dominant molds from corn stalk, with inhibition zone diameters of 18.13, 18.48, 17.55, and 15.68 mm, respectively. In conclusion, the screened chitinase-producing *Bacillus subtilis* BS-1 can effectively inhibit the growth of the four dominant molds (*Mucor circinelloides*, *Fusarium oxysporum*, *Aspergillus oryzae*, and *Aspergillus niger*) in corn stalk.

**Keywords:** corn stalk; dominant molds; antifungal; chitinase; *Bacillus subtilis*

## Introduction

China is a major agricultural country with the world' s largest straw production, particularly corn stalk, which serves as an important roughage source for ruminants. According to a Ministry of Agriculture survey report, corn stalk production reached 265 million tons in 2009, and National Bureau of Statistics data indicate that corn planting areas continue to rise, with corn stalk production increasing correspondingly. Improper straw disposal in recent years has caused severe air pollution, especially PM2.5 and PM10, which pose significant risks to human respiratory health. The National Development and Reform Commission, Ministry of Agriculture, and Ministry of Finance have implemented preferential policies including subsidies, electricity pricing, and tax incentives to vigorously promote research on straw feed utilization. However, due to variable weather conditions during mechanical harvesting, bundling, and processing of corn stalk, as well as soil contamination, stored straw is highly susceptible to mold and spoilage.

To address this problem, various chemical preservatives such as propionic acid and propionates have been developed, but their application to corn stalk—a low-cost resource—has been limited due to their lack of biological activity, high

cost, and strong corrosive effects on processing machinery and operators' skin and respiratory systems. Therefore, developing a biologically active microbial preparation to replace traditional chemical preservatives, reduce storage costs, and solve straw mold problems is imperative.

Chitin, also known as chitosan, is the second most abundant renewable resource in nature after cellulose and constitutes the main component of fungal cell walls. Chitinase enzymes act on $\beta$-1,4 glycosidic bonds to degrade chitin into N-acetylglucosamine (NAG). Kishore et al. reported that certain bacteria can secrete chitinase to inhibit fungi by destroying their cell walls. Various chitinase-producing strains have been documented, such as *Serratia proteamaculans*, *Penicillium ochrochloron*, and *Brevibacillus laterosporus*, which exhibit strong inhibitory effects against fungi including *Fusarium oxysporum*, *Aspergillus niger*, and wheat scab fungi. Current research on chitinase applications primarily focuses on antifungal pest control in rice, cotton, tobacco, and other plants, as well as biodegradation of chitin industrial waste, with no reports on its application for mold inhibition in feed resources such as straw. Microbial treatment of straw has mainly concentrated on degradation, such as the composite microbial system screened by Liu et al., which achieved a 38.5% degradation rate of corn stalk after 16 days at 22 °C, and the low-temperature composite system developed by Sarula et al., which reached a 32.21% degradation rate in 15 days. This study screened dominant molds and high-yield chitinase-producing bacteria from corn stalk, measured the chitinase activity of the high-yield strain and its inhibitory effect on dominant molds, aiming to identify mold species causing corn stalk spoilage and verify the antifungal capacity of chitinase-producing strains, thereby providing theoretical support and practical guidance for corn stalk storage using microbial treatment to replace traditional chemical preservatives.

## Materials and Methods

### 1.1 Corn Stalk and Strains

Corn stalk was obtained from Qixingpao Farm, Nongken Jiusan Administration, Heilongjiang Province. The chitinase-producing strain and four mold strains were all isolated and purified from corn stalk bales.

### 1.2 Main Culture Media and Reagent Preparation

Potato dextrose agar (PDA) and high-salt Czapek-Dox medium were used for mold isolation, purification, and cultivation. Fermentation medium was used for activation and cultivation of chitinase-producing strains, with the following formula: LB broth supplemented with 25% (V/V) of 1% colloidal chitin. Colloidal chitin medium was used for screening chitinase-producing strains, with the formula: 1% colloidal chitin 250 mL, $K_2HPO_4 \cdot 3H_2O$ 0.7 g, $KH_2PO_4$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, distilled water to 1,000 mL. All media were sterilized at 121 °C for 20 min before use, with 2% agar added for

solid media.

The preparation of 1% colloidal chitin followed the method of Roberts et al. with minor modifications: 5 g of chitin powder (purchased from Sigma) was dissolved in 200 mL of pre-cooled concentrated hydrochloric acid (4 °C) with rapid stirring for 2 h, then added to 1,000 mL of pre-cooled 50% ethanol (4 °C) with rapid stirring for another 2 h. The mixture was stored overnight at 4 °C, centrifuged to collect the precipitate, which was repeatedly washed and centrifuged with deionized water until neutral pH, then dispersed in a small amount of deionized water. A portion of the colloidal chitin was dried at 105 °C to determine its concentration, which was adjusted to a final concentration of 1% (m/V) and sterilized at 121 °C for 20 min.

3,5-dinitrosalicylic acid (DNS) solution: 3.15 g of $C_7H_4N_2O_7$ was dissolved in 600 mL deionized water with stirring and heated in a water bath to 45 °C. A 0.2 g/mL NaOH solution (100 mL) was gradually added with stirring until clear and transparent. Then 91.0 g of $C_4H_4KNaO_6 \cdot 4H_2O$, 2.5 g of $C_6H_5OH$, and 2.5 g of $Na_2SO_3$ were added with 300 mL deionized water, stirred at 45 °C until completely dissolved, and brought to 1,000 mL, then stored in the dark.

### 1.3 Isolation and Purification of Antagonistic Strains and Dominant Molds

Five grams of collected corn stalk was placed in a glass-stoppered flask containing 250 mL sterile physiological saline and shaken at 28 °C for 30 min. One milliliter of the suspension was serially diluted 10-fold to prepare $10^{-3}$, $10^{-4}$, and $10^{-5}$ dilutions. One hundred microliters of each dilution was spread on PDA plates and incubated inverted at 28 °C for 3 days. Molds with large colonies and vigorous growth, as well as strains showing antagonistic effects against molds, were observed. Single colonies of dominant molds were picked with an inoculation loop and transferred to high-salt Czapek-Dox medium, while antagonistic strains were transferred to LB medium. Pure cultures were obtained through streak plate method until complete purification was achieved.

### 1.4 Screening of Chitinase-Producing Strains

Mold antagonistic strains purified from corn stalk were spot-inoculated on colloidal chitin medium plates with three replicates per strain. After 3 days of inverted incubation at 37 °C, plates were examined for clear zones of chitin degradation around colonies.

### 1.5 Morphological Observation of Molds

Sterile PDA medium was cut into 1 cm × 1 cm slices and placed on sterile slides positioned on U-shaped glass rods in sterile petri dishes. A small amount of mold spores was inoculated at the edge of the agar block and covered with a sterile coverslip. The preparations were incubated right-side up at 28 °C for 3-5 days. Slides were then observed under an AMG EVOS inverted microscope

at low magnification, with high magnification used when necessary. Preliminary identification was performed based on colony characteristics and hyphal structures with reference to *Standard Atlas of Food Hygiene Microbiology Identification* and *Manual of Fungal Identification*.

### 1.6 Molecular Biological Identification of Dominant Molds and Chitinase-Producing Strain

Molds were subjected to 18S rDNA amplification using primers ITS1 (5' -TCC GTA GGT GAA CCT GCG G-3' ) and ITS4 (5' -TCC TCC GCT TAT TGA TAT GC-3' ). The chitinase-producing strain was subjected to 16S rDNA amplification using primers 27F (5' -AGA GTT TGA TCC TGG CTC AG-3' ) and 1492R (5' -TAC GGC TAC CTT GTT ACG ACT T-3' ). The PCR program consisted of: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR products were gel-purified and sequenced by Genewiz Biotechnology Co., Ltd. (Beijing). Sequence homology analysis was performed using BLAST software against GenBank, and a phylogenetic tree of the chitinase-producing strain' s 16S rDNA was constructed using MEGA4.1 software.

#### 1.7.1 Establishment of NAG Standard Curve

Using NAG as a standard, 100 mg was accurately weighed, dissolved in deionized water, and brought to volume in a 50 mL volumetric flask. Aliquots of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL were transferred to glass test tubes, diluted with deionized water to 2 mL, mixed with 3 mL of DNS solution, and heated in a boiling water bath for 10 min. After cooling to room temperature, the mixture was brought to 25 mL in a volumetric flask. Absorbance at 540 nm ($OD_{540}$) was measured using a UV spectrophotometer. A standard curve was plotted with $OD_{540}$ as the x-axis and NAG concentration as the y-axis.

#### 1.7.2 Chitinase Activity Assay

Five hundred microliters of centrifuged diluted enzyme solution was mixed with 2 mL phosphate-buffered saline (PBS) and 1 mL of 1% colloidal chitin solution. The mixture was immediately incubated in a water bath at 37 °C for 1 h, then rapidly centrifuged at 10,000 × g for 10 min at 4 °C. Two milliliters of the supernatant was transferred to a test tube, mixed with 3 mL of DNS reagent, and boiled for 10 min. After immediate cooling to room temperature with cold water, the mixture was brought to 25 mL in a volumetric flask. Inactivated crude chitinase solution served as the control, with three replicates per treatment. $OD_{540}$ values were measured and compared with the standard curve to calculate chitinase activity. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1  mol of NAG per minute under these conditions.

#### 1.8.1 Preparation of Chitinase-Producing Strain Fermentation Supernatant and Mold Spore Suspension

The chitinase-producing strain was inoculated at 2% into fermentation medium and cultivated at 37 °C for 120 h. The culture was then centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was collected and stored at 4 °C for later use. The four mold strains were inoculated on PDA slants and cultivated at 28 °C for 5 days. Spores were harvested from the slants using sterile water containing 0.05% (V/V) Tween-80, filtered through sterile gauze, and spore concentration was determined using a hemocytometer and adjusted to $10^6$ spores/mL.

#### 1.8.2 Antifungal Test

In a sterile workstation, 5 mL of sterile water agar medium was poured into petri plates and allowed to solidify horizontally. One hundred microliters of mold spore suspension was mixed with 7–10 mL of sterile, cooled (approximately 50 °C) glucose potato semi-solid medium (0.75% agar) to achieve a final spore concentration of $10^4$ spores/mL. The mixture was immediately poured onto the water agar plates. After solidification, three Oxford cups were placed evenly on the surface, and 200  L of fermentation supernatant was added to each cup, with sterile water as the control. Plates were incubated horizontally at 28 °C for approximately 48 h, after which inhibition zones were observed and their diameters measured.

### 1.9 Statistical Analysis

Experimental data were analyzed using one-way ANOVA in SAS 9.2 statistical software. Results are expressed as means ± standard deviation (means±SD), with $P < 0.05$ considered statistically significant.

## Results

### 2.1 Screening and Identification of High-Yield Chitinase-Producing Strain

After purification, mold antagonistic strains were inoculated on colloidal chitin medium plates. After 3 days of cultivation, obvious chitin degradation zones appeared around chitinase-producing strains.

**2.1.1 PCR Amplification Results** Following PCR amplification of the chitinase-producing strain' s 16S rDNA, agarose gel electrophoresis verification showed a bright characteristic band at approximately 1,500 bp [Figure 1: see original paper].

M: marker (D2000); 1: BS-1 amplification product; 2: negative control.

Fig. 1 PCR amplification of chitinase-producing strain 16S rDNA

**2.1.2 Sequencing Results and Homology Analysis** After sequencing and assembly, a full-length gene sequence of 1,408 bp was obtained, consistent with the electrophoresis results. BLAST sequence comparison in the GenBank database revealed that the 16S rDNA shared 99% homology with most *Bacillus subtilis* sequences. A phylogenetic tree constructed using MEGA4.1 software [Figure 2: see original paper] showed that the chitinase-producing strain clustered with *Bacillus subtilis*, indicating the closest phylogenetic relationship and confirming its identification as *Bacillus subtilis*, designated BS-1.

Fig. 2 Phylogenetic tree of strain BS-1 based on 16S rDNA sequence homology

### 2.2 Identification of Dominant Molds

**2.2.1 Isolation, Purification, and Morphological Observation of Dominant Molds** Dominant mold strains were isolated, purified, and observed morphologically under an AMG EVOS inverted microscope [Figure 3: see original paper]:

Mold A: Non-septate, branched hyphae with spherical sporangia at branch tips containing numerous smooth, spherical to elliptical sporangiospores. Initially white hyphae later turned gray-white to black, with rapid mycelial spread and no rhizoids or stolons. Preliminary identification: *Mucor* genus.

Mold B: Raised, floccose colonies with white, septate, dense hyphae and rapid growth. Macroconidia were sickle-shaped and slightly curved. Preliminary identification: *Fusarium* genus.

Mold C: Loose colony texture with relatively fast growth, initially white, gradually turning yellow, and finally light green-brown, with light brown reverse. Vesicles were nearly spherical with a single layer of phialides, and conidial heads were radiate. Preliminary identification: *Aspergillus* genus.

Mold D: Loose colony texture with rapid spread, initially white, later turning yellowish, and finally black. Spherical vesicles at the apex were covered with a layer of metulae and phialides, with radiate conidia and variable-length conidiophores and well-developed hyphae. Preliminary identification: *Aspergillus* genus.

Fig. 3 Colony shape and hyphal characteristics of dominant molds

**2.2.2 18S rDNA Sequence Determination of Dominant Molds** PCR amplification of 18S rDNA from the four purified mold strains showed bright characteristic bands at approximately 600 bp [Figure 4: see original paper].

A–D: amplification products of molds A to D; Y: negative control; M: marker (D2000).

Fig. 4 PCR amplification of dominant molds 18S rDNA

**2.2.3 Sequencing Results and Homology Analysis** After sequencing and assembly, gene sequences of approximately 600 bp were obtained for all strains, consistent with electrophoresis results. BLAST sequence comparison in GenBank, combined with colony and hyphal characteristics, identified the four dominant molds from corn stalk as *Mucor circinelloides*, *Fusarium oxysporum*, *Aspergillus oryzae*, and *Aspergillus niger*.

### 2.3.1 Establishment of NAG Standard Curve

Using $OD_{540}$ as the x-axis and NAG concentration as the y-axis, a standard curve was plotted [Figure 5: see original paper].

Fig. 5 Standard curve of NAG

### 2.3.2 Chitinase Activity at Different Culture Times

The color intensity of reaction mixtures at different culture times was measured by DNS colorimetry, and the NAG concentration was calculated using the formula: NAG ( g/mL) = (OD_{sample} - OD_{blank}) / 0.0141. Chitinase activities at different culture times are shown in Table 1 . Chitinase activity began to increase rapidly when strain BS-1 was cultured for 72 h, reaching a maximum of 3.23 U/mL at 120 h, which was significantly higher than activities at 24, 48, 72, 96, and 168 h ($P < 0.05$).

Table 1 Chitinase activity at different culture time (U/mL)

| Time (h) | Chitinase activity |
|---|---|
| 24 | 0.37 ± 0.10 |
| 48 | 0.60 ± 0.07 |
| 72 | 1.29 ± 0.14 |
| 96 | 2.87 ± 0.08 |
| 120 | 3.23 ± 0.15 |
| 144 | 3.08 ± 0.12 |
| 168 | 2.71 ± 0.14 |

Values in the same row with different letter superscripts indicate significant difference ($P < 0.05$), while the same letter superscripts indicate no significant difference ($P > 0.05$). The same as below.

## 2.4 Determination of Antifungal Effect of High-Yield Chitinase-Producing Strain

As shown in Fig. 6 [Figure 6: see original paper], BS-1 fermentation broth exhibited inhibitory effects against all four mold strains, with inhibition zones of varying sizes appearing around the Oxford cups, while no inhibition zones were observed in control wells. BS-1 fermentation broth showed the strongest

inhibition against *Fusarium oxysporum*, with an inhibition zone diameter of 18.48 mm (Table 2 ). The inhibition zone diameters in descending order were: *Fusarium oxysporum* > *Mucor circinelloides* > *Aspergillus oryzae* > *Aspergillus niger*. No significant differences were observed among the first three strains ($P > 0.05$), but all were significantly higher than *Aspergillus niger* ($P < 0.05$).

Table 2 Diameter of inhibition zone of BS-1 fermentation fluid towards four molds

| Mold | Inhibition zone diameter (mm) |
|---|---|
| *Mucor circinelloides* | 18.13 ± 0.95 |
| *Fusarium oxysporum* | 18.48 ± 1.10 |
| *Aspergillus oryzae* | 17.55 ± 1.45 |
| *Aspergillus niger* | 15.68 ± 0.83 |

1: control hole; 2, 3: test holes.

Fig. 6 Inhibition of BS-1 fermentation fluid towards four molds

## Discussion

### 3.1 Screening and Identification of Chitinase-Producing Strain BS-1

Currently, chemical treatment is the primary method for utilizing corn stalk as ruminant roughage, aiming to improve nutritional value while extending storage time. This study, however, aimed to inhibit mold growth in corn stalk using biologically active strains to enhance storage stability. Numerous microorganisms can produce chitinase, including many bacteria, fungi, and actinomycetes, with *Bacillus* being an important enzyme-producing genus. The *Bacillus subtilis* BS-1 strain screened from corn stalk samples showed obvious chitin degradation zones on colloidal chitin medium plates, confirming its chitinase production capability. Its application for mold inhibition in corn stalk offers dual advantages: the produced chitinase can degrade fungal cell walls to inhibit mold growth, and because the strain was directly isolated from corn stalk bales, its survival adaptability and activity in straw are superior to strains obtained through other sources. Moreover, its biological activity and low application dosage make it a cost-effective option for inhibiting mold in corn stalk bales, warranting further investigation of its production and utilization value.

### 3.2 Determination of Chitinase Activity of BS-1

Recent research on microbial chitinase production has made progress, but most studies have focused on cultivation conditions, enzyme activity improvement, and enzymatic properties. The gap between laboratory findings and practical application remains a bottleneck for chitinase promotion, primarily due to low enzyme yield and poor activity of screened strains. This study expands the resource pool of chitinase-producing and biocontrol *Bacillus* strains, preliminarily

determined the chitinase activity of BS-1, and explored its inhibitory effects on certain fungi. Based on these findings, further strain modification and optimization of enzyme production conditions could promote the development of biocontrol applications.

Chitinases represent a diverse group of hydrolases with varying enzymatic properties depending on their source materials. Due to differences in activity assay methods, substrates, unit definitions, and expression formats, reported chitinase activities vary considerably among strains, such as *Aeromonas hydrophila* (0.39 U/mL), *Brevibacillus* sp. (1.25 U/mL), and *Aspergillus niger* (82 U/mL). In this study, *Bacillus subtilis* BS-1 exhibited low chitinase activity during the first 2 days of cultivation, with activity increasing progressively over time and reaching a maximum of 3.23 U/mL at 120 h, followed by a gradual decline as the strain entered the decline phase. These results are similar to those reported by Dong et al. regarding chitinase activity from four pathogenic fungi cultured on scale insect cuticle medium. The maximum chitinase activity of BS-1 exceeded that of the original industrial chitinase-producing strain *Serratia marcescens*, indicating high potential industrial application value. Further research is needed on the thermal stability, pH stability, effects of metal ions on enzyme activity, as well as enzyme purification, gene cloning, and sequence analysis of *Bacillus subtilis* BS-1.

### 3.3 Inhibitory Effect of BS-1 Fermentation Broth on Dominant Molds in Corn Stalk

Research suggests that chitinase primarily acts on fungal cell walls. Chitin is the main component of most fungal cell walls, and its degradation leads to cell wall thinning, spherical protrusion formation, and eventual rupture of the protoplast membrane, resulting in protoplasm leakage and inhibition of hyphal growth. The Oxford cup plate diffusion assay in this study demonstrated that BS-1 fermentation broth showed significant inhibitory effects against all four dominant mold strains from corn stalk samples. Variations in inhibition zone diameters may be attributed to differences in mold growth rates and chitin content or structure in cell walls. Additionally, because these molds belong to different genera, BS-1 strain exhibits a broad antifungal spectrum, potentially inhibiting other spoilage-causing fungi while suppressing dominant molds. Although further experiments are needed to determine whether BS-1 chitinase can inhibit molds in other roughages such as alfalfa and pelleted feeds, its potential for application is substantial based on its effects on corn stalk molds.

## Conclusion

The dominant molds in corn stalk samples were identified as *Mucor circinelloides*, *Fusarium oxysporum*, *Aspergillus oryzae*, and *Aspergillus niger*. The high-yield chitinase-producing strain BS-1 isolated from these samples was identified as *Bacillus subtilis*, which achieved maximum chitinase activity of 3.23 U/mL after 120 h of fermentation at 37 °C and 200 r/min. The chitinase produced by

BS-1 strain demonstrated significant inhibitory effects against the four dominant molds in corn stalk.

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*Note: Figure translations are in progress. See original paper for figures.*