

# Cellulase Production by a Newly Isolated Soil Fungus *Penicillium Griseofulvum* A2

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## Research Article

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# Abstract

Rice straw is one of the most abundant resources in the world. Returning straw to field is an effective method facilitating sustainable agricultural production in many countries. However, the crop residues cannot be degraded easily due to recalcitrant structure and composition of lignocellulosic material. A straw-degrading fungus *Penicillium griseofulvum* A2 was isolated from rice straw culture medium and identified by morphology and ITS rRNA gene sequencing. Enzyme-producing condition of A2 was optimized, including nitrogen source ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>Cl, urea, and peptone), pH value (5.0 to 7.5), and incubation time (1 to 20 d). Results showed that *P. griseofulvum* A2 produced the highest CMCase (33.97 ± 0.33 U/mL) utilizing urea as nitrogen source and growing at pH 7.0-7.5 after 15 d cultivation. While, NH<sub>4</sub>Cl was more suitable for β-glucosidase (15.40 ± 1.77 U/mL) production under pH 7.0 condition after 10 d cultivation. The isolated fungus *P. griseofulvum* A2 is a good potential strains to decomposed rice straw.

## 1 Introduction

Rice straw (RS) is the byproducts of paddy crop as well as an abundant resource of lignocellulosic biomass. Its worldwide production is 800 to 1000 million tonnes. More than 75% of RS is produced by Asia countries like China and India (20). Crop straw returning to field is a widely used method for facilitating sustainable agricultural production in many countries (29). However, straw is hard to degrade due to its recalcitrant structure and composition of lignocellulosic material including lignin, hemicellulose and cellulose (20). Furthermore, the presence of silica (7.5–13.9%) in RS make it more recalcitrant than other straw residues including wheat straw and corn straw (20).

Straw-degrading microorganisms playing an important role in straw decomposition process, which could greatly speed up the straw ripening process, increase farmland fertility, and play important role in keeping sustainable development of agriculture (8). Microbial degradation of lignocellulose depends on the secretion of cellulase including exoglucanase (EC3.2.1.91), endoglucanase is also called CMCCase (EC3.2.1.4), and β-glucosidase (EC3.2.1.21)(22). Cellulase activity is an important indicator of lignocellulose-degradation ability of the microbial strains. Digestion of straw lignocellulose to saccharides requires the synergy of these three enzymes (9). The high cost of cellulase is one of the main bottlenecks for the lignocellulose decomposition (10). Thus, it is quite important to screening microorganisms with effective cellulase activity and applying on-site plants for enzyme production.

Many straw-degrading microorganisms are well explored for straw degradation, including *Trichoderma*, *Chaetomium*, *Penicillium*, *Aspergillus*, *Acremonium*, *Rhizopus*, etc (19, 24, 25, 28, 4). The most widely used cellulase producing strain is *Trichoderma reesei*. However, *T. reesei* often has insufficient β-glucosidase activity, resulting in cellobiose accumulation and reduced enzymatic hydrolysis efficiency (27). By increasing β-glucosidase activity, the competitive product inhibition of cellobiose can be overcome to a certain extent (9). *P. purpureus* and *P. roquei* have been shown to exert higher activity on lignocellulose compared with *T. reesei*, because they produce more β-glucosidase and hemicellulas (3,

11). *P. griseofulvum* can produce more  $\beta$ -glucosidase to supplement traditional cellulase products. However, their straw decomposition efficiencies still need increasing. It is necessary to screen different cellulolytic strains. And, natural habitat is a key factor influencing cellulase characteristics. Hence it is important to explore new habitats to search for novel strains with high cellulase producing potential.

As culture condition is important for converting lignocellulosic biomass to saccharides. For filamentous fungi, cellulase gene expression was mainly regulated by transcription activation factors, carbon source metabolism inhibitors, pH and nitrogen source (7). Carbon sources are key to enzyme production because they induce the expression of cellulase and allow microorganisms to secrete various types of enzymes (7). Studies have shown that pH of media strongly influence many enzymatic reactions by affecting the transport of various chemical products and enzymes on cell membranes (12). Naturally, nitrogen stimulates fungal cell growth, which in turn enhances biomass formation and cellulase enzyme expression(2). Thus, culture condition optimization is quite important for RS decomposition.

In this study, RS-degrading strains was isolated from alpine regions to screen microorganisms with better characters. Culture conditions were optimized for cellulase production by the newly isolated fungus *P. griseofulvum* A2, including nitrogen, pH and incubation time. The main aim is screen new strain with increased cellulase, in order to overcome the limitations that prevent a RS decomposition.

## 2 Materials And Method

### 2.1 Fungal isolation and identification

The sample was collected from residues of herbage from the high-altitude area of Setira Mountain in Linzhi County, Tibet, China. The strains were activated and enriched on Carboxymethyl cellulose (CMC) medium (26) at 30°C. The fungal colonies were screened on CMC agar medium.

The isolated strain is characterized on the its colony morphology, microscopic features (light microscope H550S Nikon, Japan), and ITS sequence. Colonies were grown on the plate, and the colony characteristics were studied by observing their size, and color that they produce. Microscopic evaluation by Lactophenol cotton blue staining was done to understand spore size, shape, and arrangement (6). The genome of the fungus was extracted by the method of CTAB (23) and then used ITS common primer (ITS1 5'- TCCGTAGGTGAACCTGCGG-3', ITS4 5'-TCCTCCGCTTATTGATATGC-3') PCR amplification. The PCR mixture was carried out in a volume of 50  $\mu$ L, contained 25  $\mu$ L of Permixon Taq (TaKaRa), 1  $\mu$ L of each forward and reverse primer, 2  $\mu$ L of template and 21 $\mu$ L distilled water. The PCR amplification conditions were 4 min of preheating at 95°C, 30 s denaturation at 95°C, 30 s of primer annealing at 55°C, 45 s extension step at 72°C for 35 cycles, and postcycling extension of 10 min at 72 °C. The reactions were carried out in a thermal cycler (K960 Thermal Cycler, Hangzhou, China). The PCR amplification products were sent to Beijing Augct Company (China) for sequencing. The resulting sequences are compared with the ITS sequences in NCBI. Then MEGA7.0 software is used to construct phylogenetic tree by Neighbor-Joining method (6).

## 2.2 Cellulases production through liquid state fermentation

For cellulase production, the isolated strain was cultured in medium containing (g/L), RS 10;  $(\text{NH}_4)_2\text{SO}_4$  1.4;  $\text{KH}_2\text{PO}_4$  0.2;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.4;  $\text{MgSO}_4$  0.3; Peptone 1.0; NaCl 0.3;  $\text{FeSO}_4$  0.005;  $\text{MnSO}_4$  0.0016;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.0014;  $\text{CoCl}_2$  0.002 (13). Sterilized medium in the flask was homogeneously mixed with active inoculum 0.1% (v/v) and incubated at 30 °C. Nitrogen source, pH, and culture time were optimized in liquid enzyme production medium by control variable method. Each experiment was carried out in triplicate.

## 2.3 Enzyme assay

5 mL of fermentation liquid was centrifuged at 4 °C for 15 minutes at 5000 r/min. After centrifugation, the supernatant was taken as the crude enzyme solution. CMCase activity was measured using 0.5 mL of 1% (w/v) carboxymethyl cellulose (CMC) as substrate with 0.5 mL of enzyme solution at 50°C, for 30 min, followed by the addition of 3.0 mL of dinitrosalicylic acid (DNS) reagent (15) according to Ghose' method (5). One unit (U) of CMCase was defined as the amount of enzyme that released 1 µg of glucose per minute under experimental conditions.

$\beta$ -Glucosidase activity was determined by incubating 0.5 mL of enzyme solution with 0.5 mL of 1% cellobiose in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C for 30 min and the liberated glucose was estimated by the glucose oxidase–peroxidase (GOD-POD) method (5). One unit (U) of  $\beta$ -glucosidase was defined as the amount of enzyme that released 1 µg of glucose per minute under experimental conditions.

## 2.4 Statistic analysis

Significance of difference was assessed by one-way ANOVA, using IBM SPSS Statistics 24. Data represented as mean  $\pm$  SE.

## 3 Results And Discussion

### 3.1 Identification of *P. griseofulvum* A2

Colonies of *P. griseofulvum* A2 was cultured for 48 h at 30 °C on the CMC agar medium. The individual colonies of A2 are regularly round, milky gray-green, with neat edges, raised mosses, and flocculated surface hyphae in the air (Fig. 1(a)). The conidia were oval in shape, smooth in surface, blue-green in color and 3–5 µm in length. The conidial stalk also has a septum, and the surface is rough (Fig. 1(d)). The conidial stalk has been branched many times to produce small stalks, which are shaped like a broom and stained with blue by lactophenol cotton blue staining, as shown in Fig. 1(b-c).

The ITS sequence was deposited in NCBI Genbank with the accession number MW874977. The homology comparison found that A2 has a sequence similarity of 100% compared with *P. griseofulvum* (KR296884). The phylogenetic tree constructed by the ITS rDNA sequence of the strain and its close

strain is shown in the Fig. 2. Based on the results of the strain, it was identified as *Penicillium* Genus and named *Penicillium griseofulvum* A2.

## 3.2 Effect of nitrogen source on cellulase production

Nitrogen source is major regulatory factor on cellulase production and precursor of the enzyme protein. Therefore, the type of nitrogen source also affects the activity and yield of cellulase. In this study, peptone, urea,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NH}_4\text{Cl}$  were used as nitrogen sources and cultured in pH 6.5 medium for 5 days (Fig. 3). The optimum nitrogen source of CMCCase is urea. But for  $\beta$ -glucosidase, the optimum nitrogen source is  $\text{NH}_4\text{Cl}$ . However, peptone is optimum nitrogen source for *Aspergillus fumigatus* (14), which is an organic nitrogen. Inorganic nitrogen sources such as ammonium sulfate promoted growth, and organic nitrogen sources such as yeast extract had a greater impact on enzyme production (18, 16, 14). The nitrogen sources used by different strains were also very different. The maximal activities were  $12.57 \pm 0.51$  U/mL for CMCCase, and  $4.06 \pm 0.41$  U/mL for  $\beta$ -glucosidase (Fig. 3(a-b)). In this study, the optimal nitrogen sources are inorganic nitrogen, which is low cost than most organic nitrogen, which could greatly decrease the production cost.

## 3.3 Effect of initial pH on cellulase produced

The pH of the medium affects the dissociation of charged groups on the cell membrane surface and its microstructure, which in turn affects the absorption of nutrients and the secretion of metabolites, resulting in changes in the growth and metabolism of microorganisms (12). Additionally, the reason for the effect of pH on cellulase is the hydrogen ion effect. Hydrogen ion concentration strongly affects many enzymatic processes and the transport of compounds on cell membranes (1). Studies have shown that growth media pH strongly influence many enzymatic reactions by affecting the transport of various chemical products and enzymes on cell membranes.

In this study, the optimal pH values of these cellulolytic enzymes were also determined on the 5th day with ammonium sulfate as the nitrogen source (Fig. 4). The maximal activity of CMCCase was at pH values of 7.0–7.5. However,  $\beta$ -glucosidase showed the highest activity at pH 7.0. The maximal activities were  $18.13 \pm 1.1$  U/mL for CMCCase, and  $4.56 \pm 0.21$  U/mL for  $\beta$ -glucosidase (Fig. 4(a-b)). A2 produced more cellulase under neutral condition, which was consistent with the result of Ines BH' study (7). However,  $\beta$ -glucosidase also has high enzyme activity when the acid condition is pH 5.5. Cellulase production from the different bacteria and fungi is mainly based on pH, and acidic pH (4.0–6.0) supports the production of fungal cellulases(21). In addition, acid cellulase has potential applications in the animal feed industry, clarifying fruit juices, and newspaper acid inks.

## 3.4 Effect of culture time on cellulase production

With the growth of microorganisms, the nutrient is consuming in the fermentation broth. As the cell tends to senescence and autolysis, the production capacity of the product slows down or stops correspondingly. It is necessary to determine the fermentation time. The production of cellulase by *Penicillium* was studied with 1% rice straw as substrate and  $(\text{NH}_4)_2\text{SO}_4$  as nitrogen source at pH 6.5. The

activities of CMCase and  $\beta$ -glucosidase were maximal within 15 days, then decreased dramatically. *Penicillium sp.* FSDE15 (17) reached their maximum enzyme production after 10 days. A2 reached its maximum enzyme production in 10–15 days too. The maximal activities were  $33.97 \pm 0.33$  U/mL for CMCase, and  $15.40 \pm 1.77$  U/mL for  $\beta$ -glucosidase, as shown in Fig. 5(a-b).

## 4 Conclusion

In this study, a soil fungal, *P. griseofulvum* A2, was isolated from alpine regions, which could utilize RS as substrate to produce cellulase including CMCase, and  $\beta$ -glucosidase. Culture condition was optimized for cellulase production. These results indicate that *P. griseofulvum* A2 can effectively produce cellulase, which has potential to speed up straw degradation.

## Declarations

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### Authors Contributions

In this paper, Fachun Guan and Ligang Hou conceived and designed research. Fachun Guan and Chao Wang prepared the fungal isolation and identification, under the supervision of Yanru Cui, Xie Jiao and Zhonghe Li. Jie Liu and Jinling Cai prepared the cellulase production, Chunyan Qi and Ligang Hou prepared the enzyme assay. Fachun Guan and Yanru Cui wrote the manuscript. All authors contributed to the writing of the paper.

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### Ethical Approval

Not applicable.

### Consent to Participate

The authors declare that they consent to participate.

### Consent for Publication

The authors declare that they consent for publication.

### Competing Interests

The authors declare that they have no competing interests.

## Availability of data and materials

Available upon request

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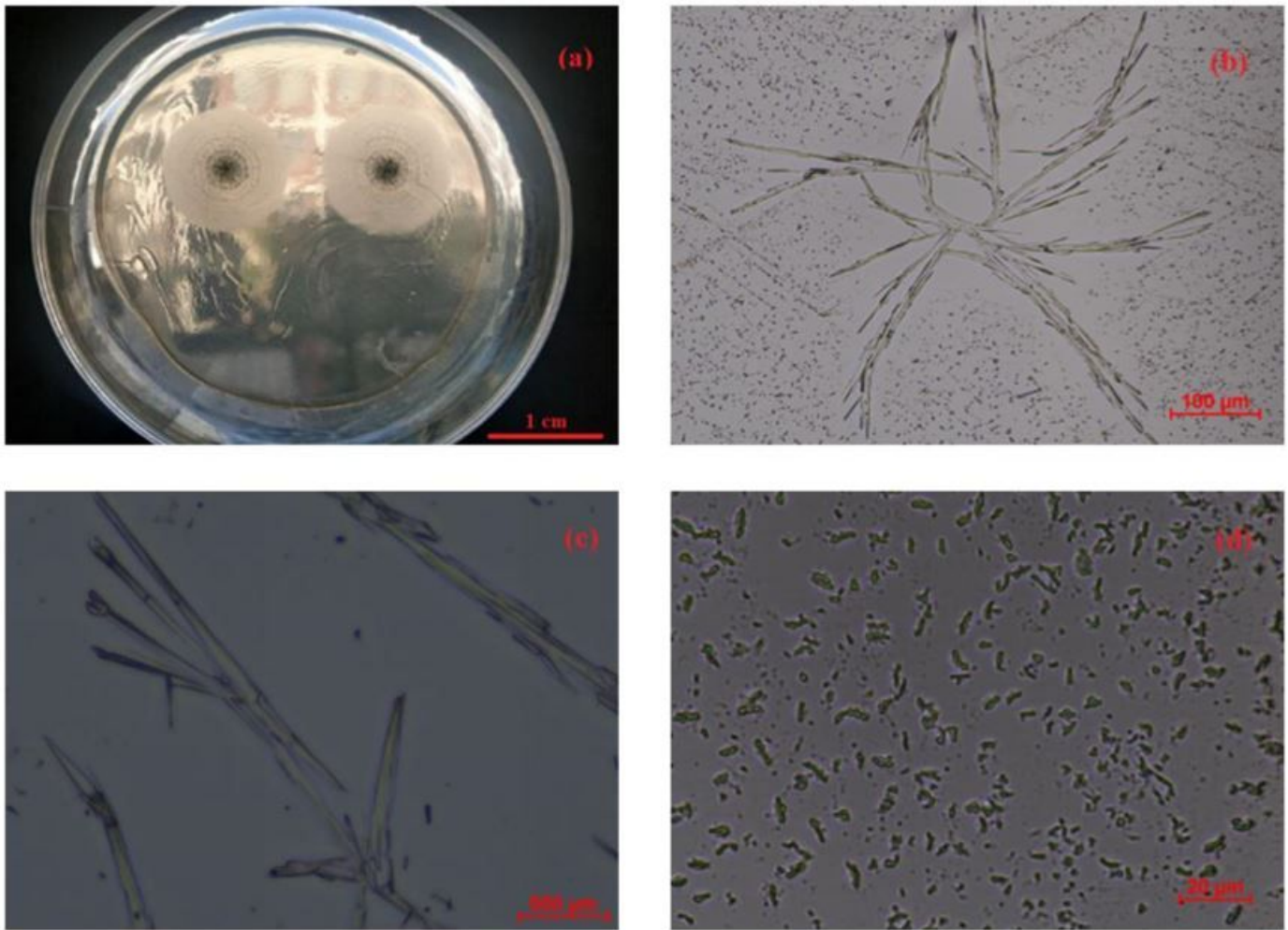
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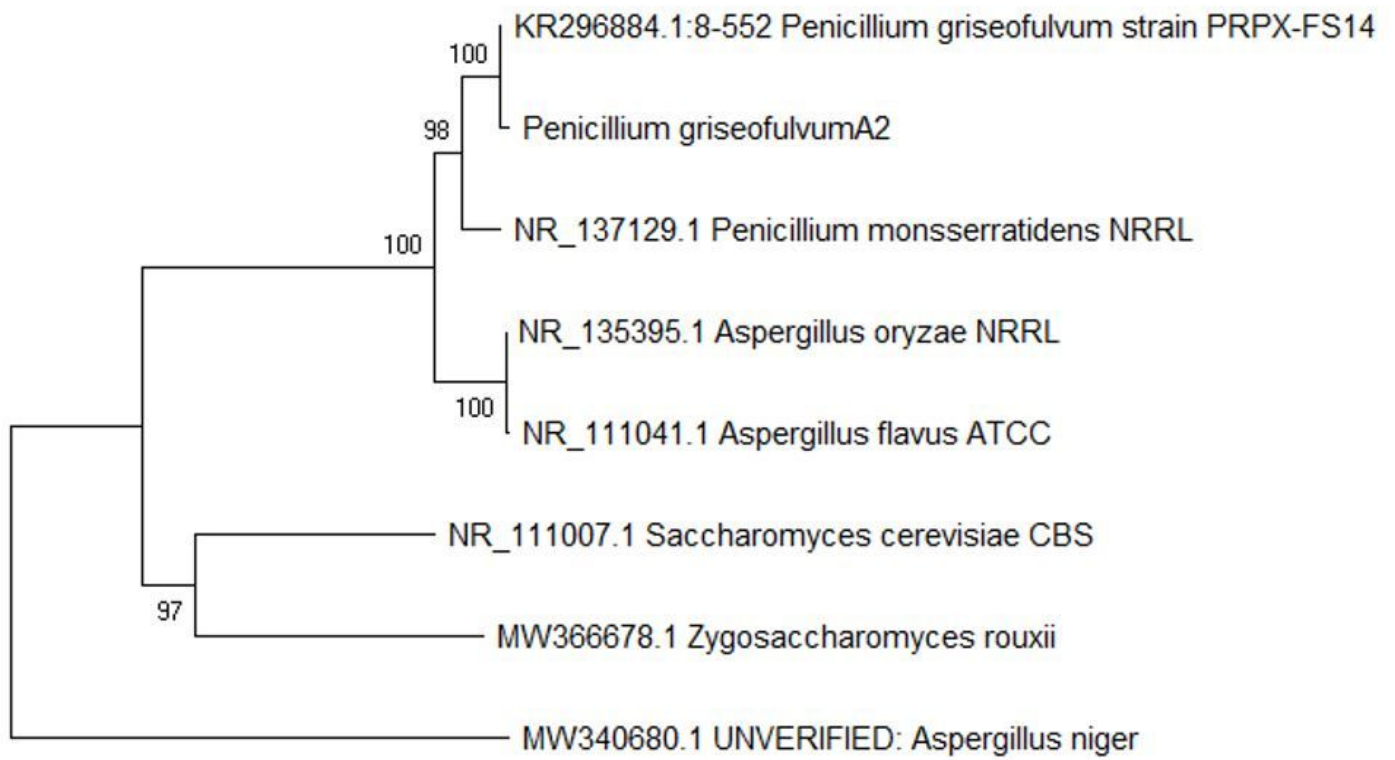
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## Figures



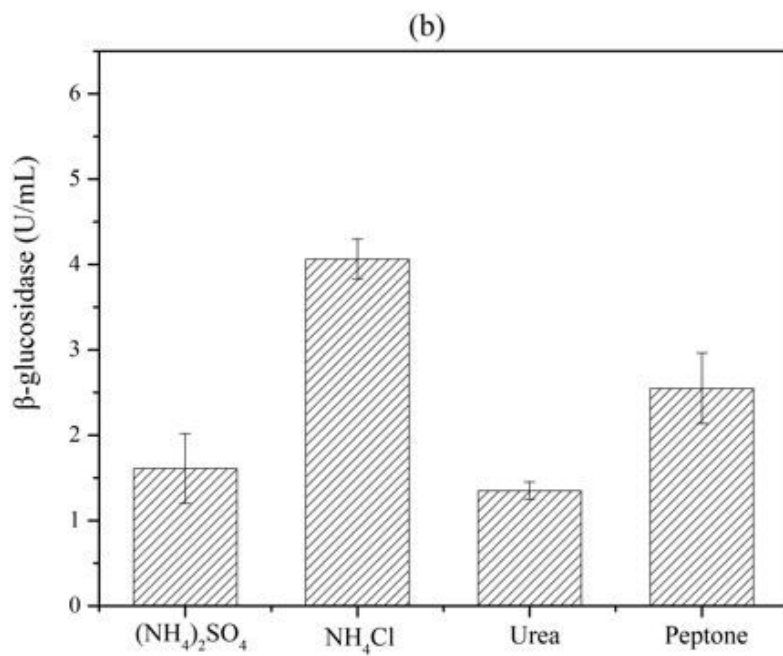
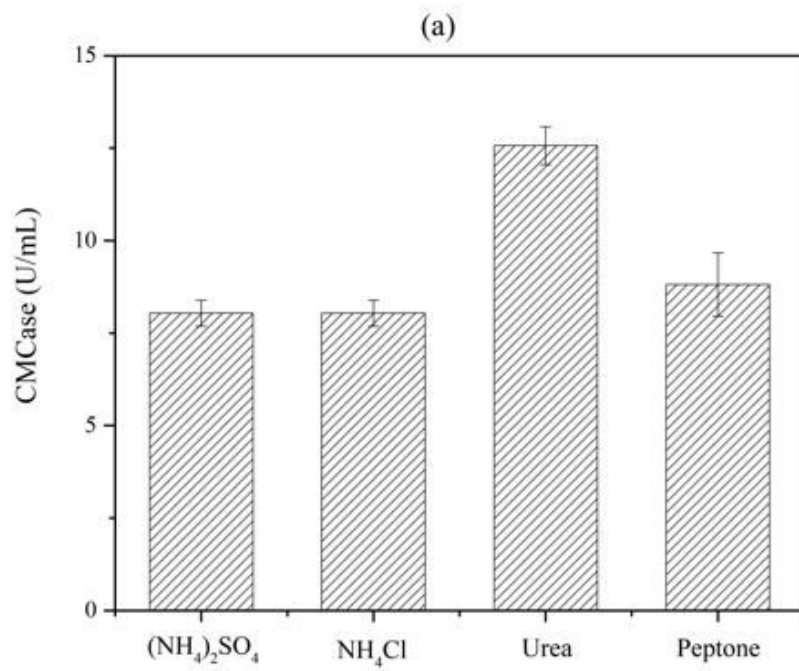
**Figure 1**

Morphological characteristics of *P. griseofulvum* A2. (a) Colony morphology; (b) Unstained hyphae morphology; (c) Stained hyphae morphology (d) Spore morphology.



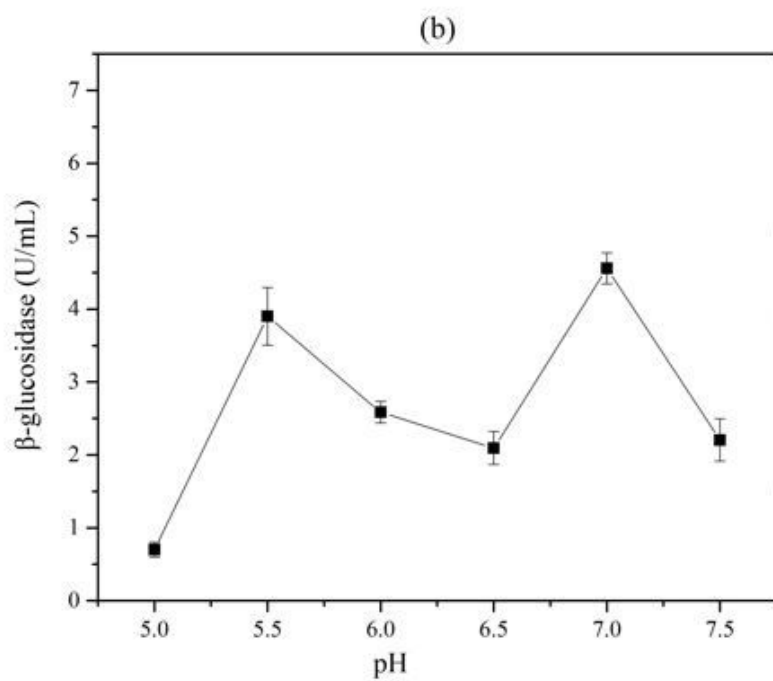
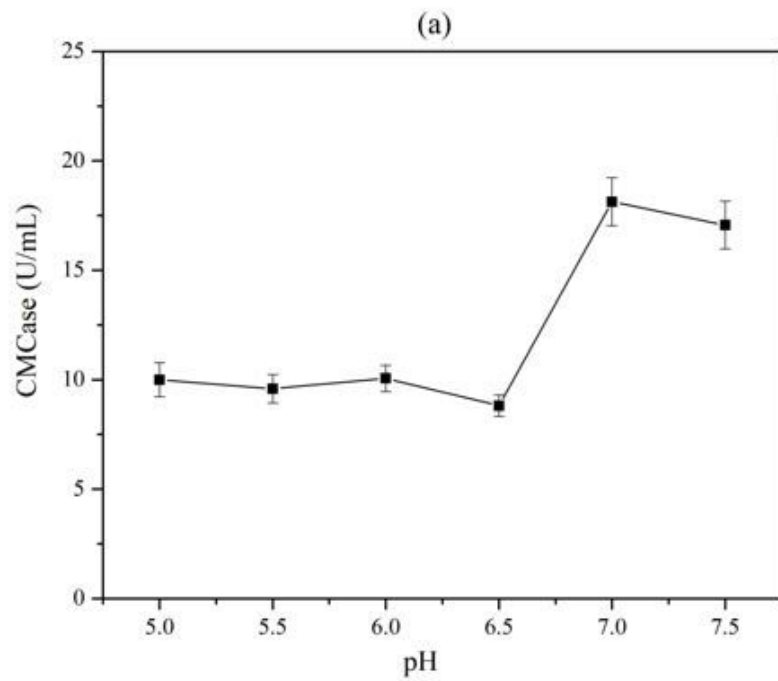
**Figure 2**

Phylogenetic tree of the ITS sequence of *P. griseofulvum* A2. Numbers in parentheses represent accession numbers in GenBank. Numbers at each branch point represent the bootstrap values on Neighbor-joining analysis of 1000 replication data sets. Bar 0.050 is the sequence divergence.



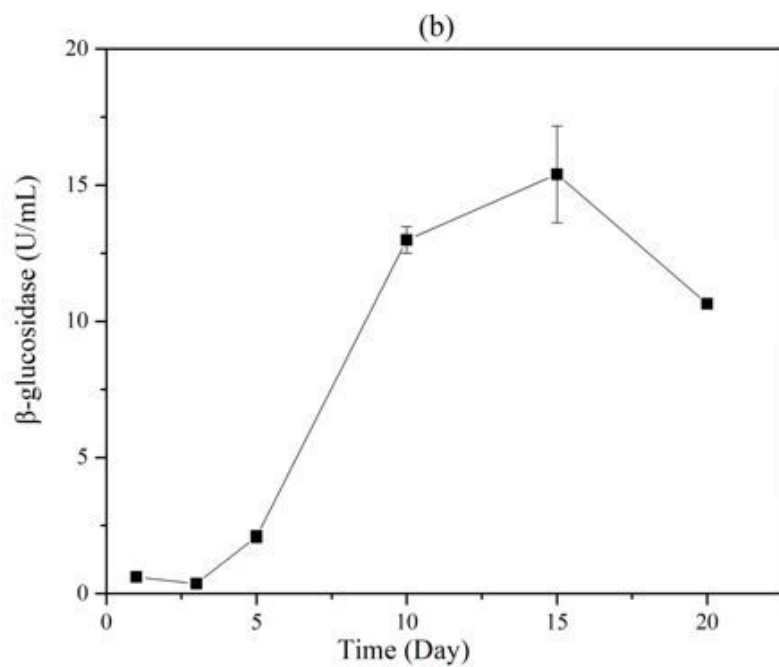
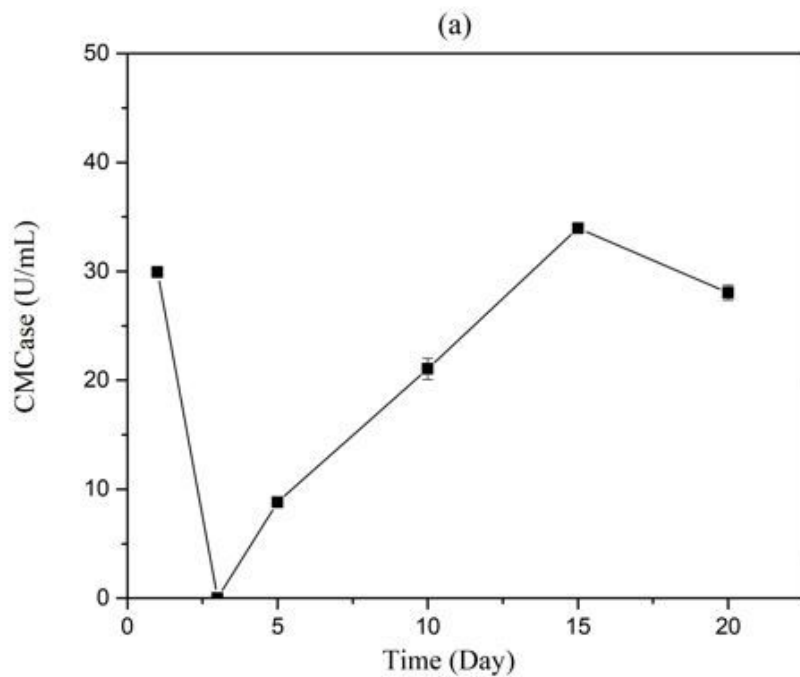
**Figure 3**

Effect of nitrogen source on cellulase production of *P.griseofulvum* A2. (a) CMCase; (b)  $\beta$ -glucosidase.



**Figure 4**

Effect of pH on cellulase production of strain *P. griseofulvum* A2. (a) CMCCase; (b)  $\beta$ -glucosidase.



**Figure 5**

Effect of pH on cellulase production of *P. griseofulvum* A2. (a) CMCase; (b)  $\beta$ -glucosidase.

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