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Full Length Research Paper

Biodegradation of free-gossypol by a new fungus isolated from cotton planted soil

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A new fungal strain isolated from a soil microcosm was investigated due to the finding that it could biodegrade free-gossypol on agar plates. Benzyl chloride was used for extracting fungal genomic DNA which was isolated from mycelium grown in liquid culture, then 18S rDNA was specifically polymerase chain reaction (PCR) amplified using a pair of primers EF4/EF3 (1.4 kb). The strain was further identified as a member of the genus *Aspergillus* through the morphological and molecular biological methods. The isolated strain had a high tolerance of free-gossypol and 2 g/L was chosen as the best addition amount. Furthermore, the dry weight method was employed to determine the growth curve and optimum temperature of the gossypol-degrading fungus in different carbon sources. The results showed that it was best to be researched after incubation for 72 h at 30°C. To the extent of our knowledge, this is the first report on biodegradation of gossypol on solid plates without other carbon sources and this might help in decomposing some anti-nutritional factors in feed resources.

Key words: Free-gossypol, fungus, biodegradation, isolation, growth characteristics.

INTRODUCTION

Cottonseed meal (CSM) as a protein resource is limited to use in feed industry because of the presence of gossypol ($C_{30}H_{30}O_8$) which is a toxic polyphenolic binaphthyl compound found primarily in the pigment glands of cotton plant (*Gossypium* sp.). In recent years, the effects of gossypol on growth and reproduction have been extensively studied and reported (Blom et al., 2001; Robinson et al., 2001; Lee et al., 2002; Santos et al., 2003). Feeding gossypol to animals resulted in irregular estrous cycles and growth restriction. Many studies showed that the toxic effects of free-gossypol were much greater in non-ruminants (Gamboa et al., 2001; Henry et al., 2001) including fish (Cheng and Hardy, 2002; Lee et al., 2006) than ruminants (Nagalakshmi et al., 2001). Ruminants can tolerate greater levels of gossypol because free-gossypol is transformed into bound-gossypol in rumen fluid. However, concentrations of total gossypol did not change during fermentation of cottonseed by rumen microorganism (Schneider et al., 2002). For this reason, a maximum limitation the Food and Drug Administrations (FDA) sets for free-gossypol in human food products and ingredients is 0.45 g/L (Lusas and Jividen, 1987).

In order to improve the use of cottonseed products in animal feeds, a number of methods have been used to remove free-gossypol from cottonseed, especially microbial fermentation (Weng and Sun, 2006a, b; Zhang et al., 2006a, b; 2007). Some research found that a few microorganisms were capable of degrading gossypol, including *Candida tropicalis*, *Torulopsis candida*, *Aspergillus flavus* and *Aspergillus niger* (Wu and Chen, 1989; Shi et al., 1998; Weng and Sun, 2006a). These previous research can only transform free-gossypol into bound-gossypol instead of removing it completely, and therefore can not block the absorption of gossypol. Thus, it is necessary to provide an approach for biodegradation of gossypol and prevention of its absorption into animal body. It is hoped that the question will be resolved with our proposed method.

The purpose of this paper is to confirm whether hydrolytic

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enzyme of gossypol is induced during fungi survival using gossypol as a sole carbon source. In the present study, a new fungal strain named HQ-1 was isolated from a cotton-planted site, which was expected to have a greater tolerance to gossypol and degrade it on solid medium. And also, the biodegradation characteristics of gossypol by this strain were simply investigated through the tolerance test, growth curves and optimum temperature. Further studies on gossypol-degrading enzymes will be summarized in our next study.

MATERIALS AND METHODS

Isolation of gossypol-degrading strain

Soil samples were collected from cotton planted soil in Zhejiang University, Hangzhou, China. Small quantities of the polluted soil about 1 g were put on plates together with Basal Medium (5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 1 g/L NaCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2$, 0.2 g/L yeast extract, 15 g/L agar) contained 2 g/L gossypol and 0.01 g/L chloramphenicol antibiotic, to prevent the growth of bacteria. After 2 weeks, the fungal strains that grew were inoculated on Czapek's medium (3 g/L $NaNO_3$, 0.5 g/L KCl, 1 g/L K_2HPO_4, 0.01 g/L $Fe_2(SO_4)_3$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 30 g/L sucrose, 15 g/L agar) and incubated at 30°C. To obtain pure cultures, mycelium was repeatedly transferred onto new plates. The strains were maintained at 4°C on Potato Dextrose Agar (PDA) medium (200 g/L peeled potatoes, 20 g/L dextrose, 15 g/L agar). The chemical reagents were all obtained locally and free-gossypol was from JiangNan University of China.

Observation of morphology and structure

The filamentous morphology of the isolated strain growing in different carbon source (glucose and gossypol) was studied using scanning electron microscope (SEM) Philips XL30ESEM and transmission electron microscope (TEM) JEOL JEM-1230.

Genomic DNA extraction, PCR amplification and sequencing

The mycelia were inoculated into Czapek's medium for 3 days at 30°C under static conditions and harvested from the liquid medium by filtration. Genomic DNA was extracted using benzyl chloride (Moller et al., 1992) and purified DNA samples were stored at -20°C. The 18S rDNA as a template was amplified using the universal primer pairs of EF4 (5'-GGAAGGG[G/A]TGTATTTATTAG-3') / EF3 (5'-TCCTCTAAATGACCAAGTTTG-3') (Smit et al., 1999). The polymerase chain reaction (PCR) reaction conditions: denaturation at 94°C for 2 min, then 30 cycles at 94°C for 50 s, primer annealing at 50°C for 50 s, extension at 72°C for 2 min, followed by 10 min at 72°C to complete the elongation. After checking the PCR products by electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide and visualized under ultraviolet (UV) light, the 18S rDNA were sequenced by Shanghai Shengon, China. The nucleotide sequence analysis of the sequences was done at BLAST site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST).

Tolerance test

The isolated strain was tested for its growth situation on solid media containing gossypol. The medium used was Basal Medium and five different doses of gossypol (0.5, 1, 2, 4 and 8 g/L) were added to the medium (Dritsa et al., 2007). Fungal spores were harvested from well sporulating colonies and suspended in sterile water. The concentrations of the spore suspensions were determined using a hemacytometer and adjusted to approximately 1.0×10^6 spores/ml. A 5 µl aliquot of the final suspension was then spotted on each agar plate containing a different dose of gossypol. All plates were incubated at 30°C and three replicates were performed for each treatment at each time point. The diameters of individual colonies were measured every 24 h and used as the measurement of the mycelial growth. The optimum inoculum level of gossypol achieved by this step was fixed for subsequent experiment.

Growth curves in different carbon source

The growth curves generation was based on dry weight method (Qian and Huang, 2008). A 10 µl aliquot of spore suspension was inoculated into 250 ml Erlenmeyer flasks, with 50 ml Basal Medium (20 g/L glucose or 2 g/L gossypol as carbon source) in each flask. Dry weights of mycelial biomass at the end of incubation periods served as growth rate of the strain. Three replicates were performed for different carbon source. All the flasks were incubated at 30°C on a gyratory shaker at 150 rpm. The mycelia were collected every 12 h, washed with the distilled water, filtered to remove extraneous water, wrapped with filter paper, and dried at 80°C in a desiccator to constant weight. The optimum growth time achieved by this step was fixed for subsequent experiment.

Incubation temperature in different carbon source

A 10 µl aliquot of spore suspension was inoculated into 50 ml Basal Medium at various temperature (20, 25, 30, 35, 40 and 45°C) to study their influence in different carbon source (glucose and gossypol). Three replicates were performed for each temperature. The mycelia were collected after 72 h and dried to weight, respectively.

Statistical analysis

The data were treated with the SPSS statistical package for one-way ANOVA followed by LSD post-hoc test was used to determine significant differences among treatment groups. For all analysis, differences were considered to be significant at $p < 0.05$ (Steel and Torrie, 1980).

RESULTS

Morphological identification

The isolated strain named HQ-1 grew well on Basal Medium plates containing 2 g/L gossypol as the sole carbon source. The average diameter of colony with white mycelia and black spores on glucose plates was larger than gossypol plates after incubating 3 days at 30°C. SEM and TEM observations confirmed visible fungal elements including conidiophores and hyphae dichotomously branched (Figures 1 and 2). The micrograph of the strain growing in gossypol medium showed that the mycelium was thinner and the mycelial surface was smoother than that growing in glucose medium (Figure 1). This could be due to adhering to the surface of mycelia by gossypol.

The straight-cut sections of the mycelium showed that

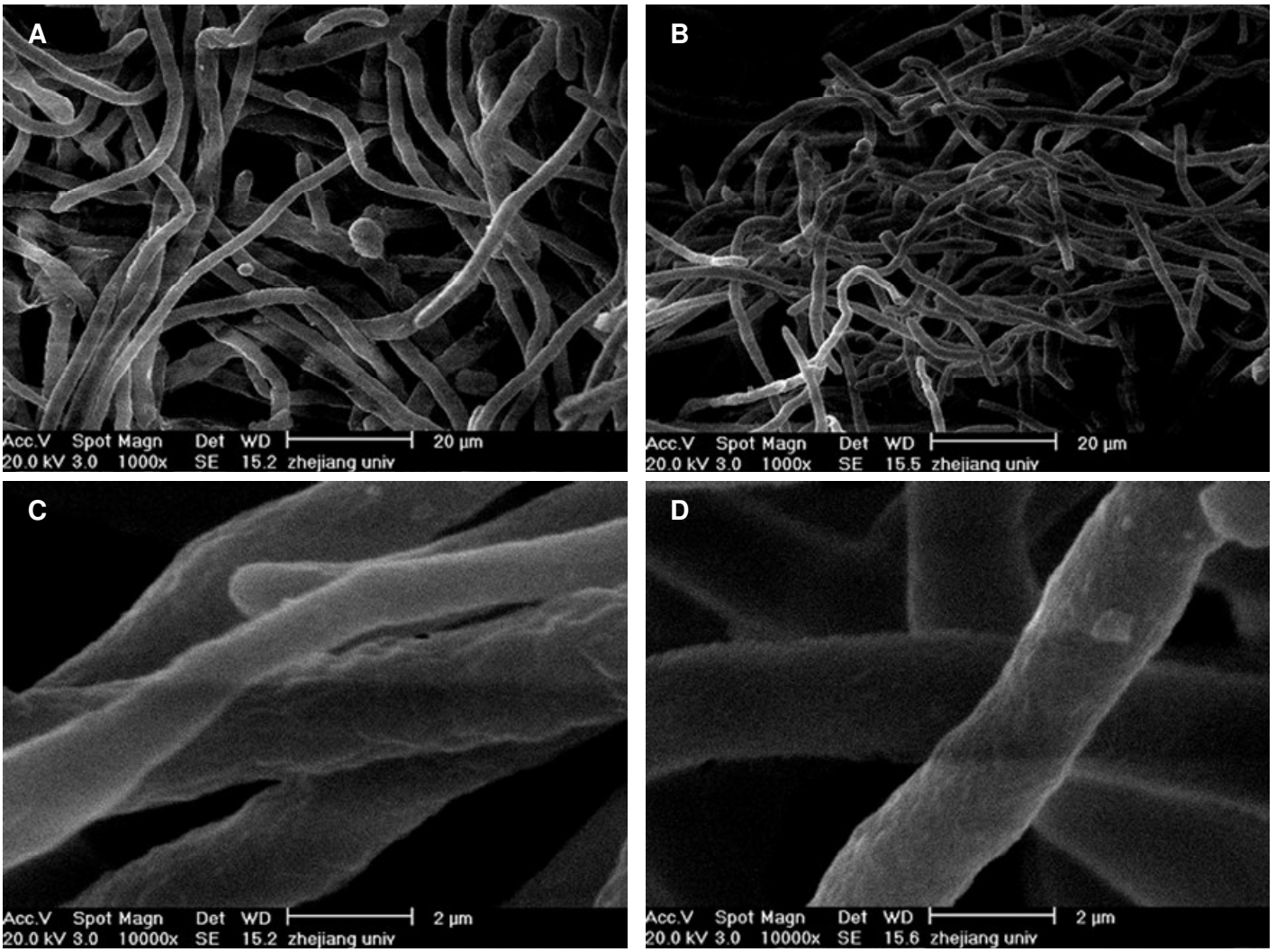

Figure 1. Scanning electron microscope. A, The growth of HQ-1 in glucose medium, 1000×; B, The growth of HQ-1 in gossypol medium, 1000×; C, The growth of HQ-1 in glucose medium, 10000×; D, The growth of HQ-1 in gossypol medium, 10000×.

the cytoarchitecture of the strain growing in glucose medium could be seen clearly and the diaphragm of the mycelium was also distinct (Figure 2A). In contrast, the cytoarchitecture of that growing in gossypol medium was faint and could not see the diaphragm (Figure 2B). As shown in Figure 2C and D, the results of the crosscut sections of the mycelium were analogous. The reason may be that the toxicity of free-gossypol destroyed the structure of the cell. The morphology of the strain seems to be very similar to *Aspergillus*, which is a type of common fungi often isolated from soil, with a worldwide distribution (Domsch et al., 1980).

Molecular identification

Genomic DNA was extracted successfully by the mycelium using the benzyl chloride method. The primer pair EF4/EF3 was fungal-specific primer, which generated amplicons of about 1.4 kb, was used for testing of a range of fungal strains (Smit et al., 1999). By means of PCR and using purified DNA from the isolate of HQ-1 as a template, amplicons of around 1.5 kb was obtained (Figure 3). The 18S rDNA sequences of the fungus HQ-1 were compared in NCBI-BLAST Database, and showed 99% identity with sequences of *A. niger* (EU853156.1) (Figure 4).

Gossypol tolerance

The gossypol-degrading strain HQ-1 showed very sensitivity towards gossypol. The growth rate obviously decreased with increasing concentration of gossypol. The colony diameters of 0.5 and 1 g/L were 2.70 ± 0.26 cm and

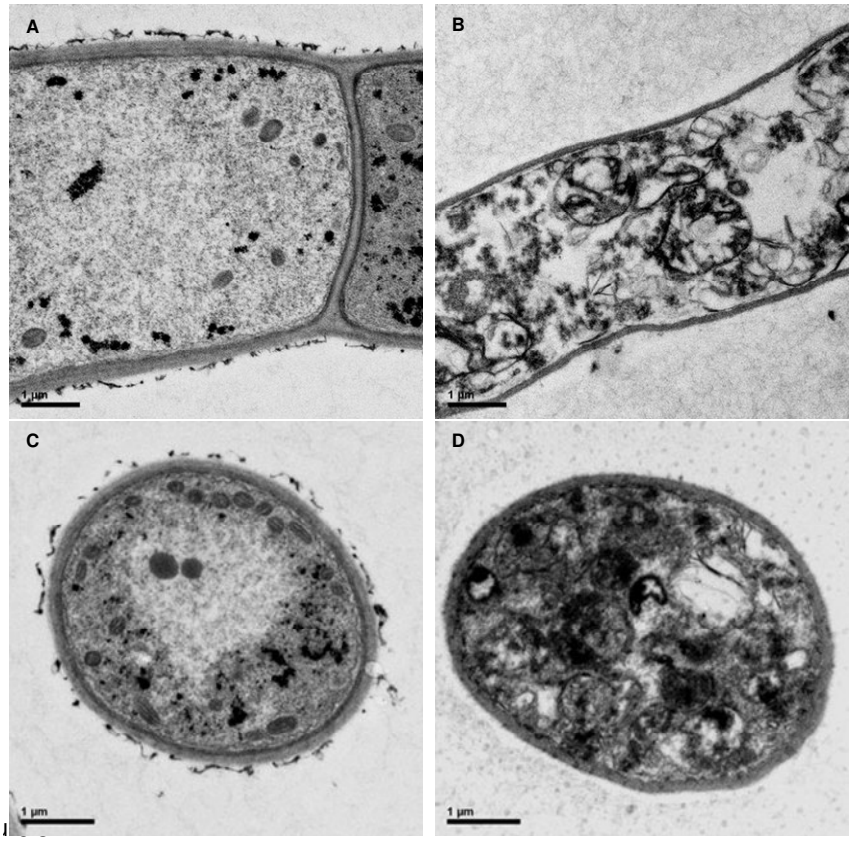

Figure 2. Transmission electron microscope. A, The growth situation of HQ-1 in glucose medium, 20000×; B, The growth situation of HQ-1 in gossypol medium, 20000×; C, The growth situation of HQ-1 in glucose medium, 25000×; D, The growth situation of HQ-1 in gossypol medium, 25000×.

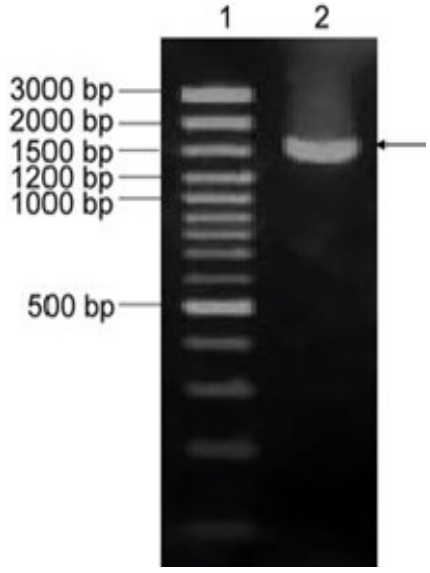

Figure 3. Electrophoresis of PCR products of genomic DNA from the strain HQ-1. Lane 1, DNA marker, the size is listed on the left; Lane 2, PCR product of HQ-1 genome, the predicted size was about 1.4 kb.

1.80 ± 0.26 cm for 3 days, respectively. Then, a visible concentration was a turning point. Even at the highest decrease was observed at 2 g/L 0.63 ± 0.15 cm and this concentration (8 g/L) the strain HQ-1 was still able to grow from the 4th day (0.50 ± 0.14 cm) and this concentration was far higher than the maximum limitation (≤ 0.45 g/L) (Figure 5). This high tolerance of gossypol probably occurred because the strain was isolated from a cotton planted soil, thus developing a remarkable tolerance to gossypol.

Growth curves

Because of testing the number of total mycelia, the dry weight method was not able to display the downtrend in the decline phase. The results are presented in Figure 6. It could be concluded that the strain was in the logarithmic phase at 48 to 84 h period which was the best time for research. In some studies, the incubation time employed was 48 h (Zhang et al., 2006b) or 60 h (Weng and Sun, 2006a) for filamentous fungi or yeast. However, gossypol as the carbon source was difficult to be applied because of its complex structure, hence incubation for 72 h would be

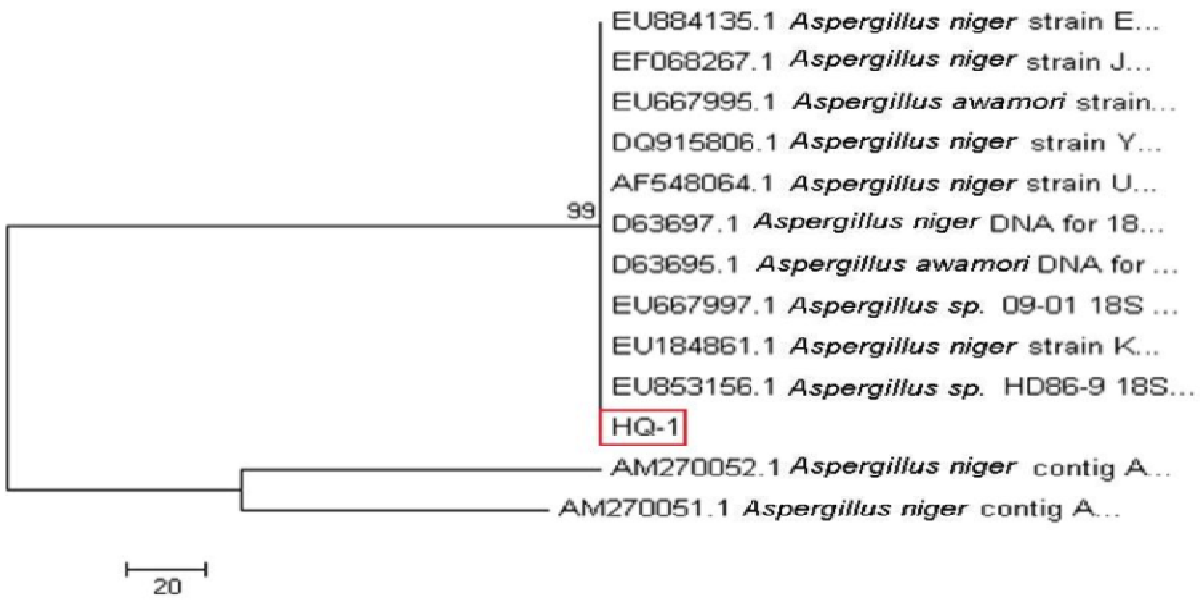

Figure 4. Phylogenetic tree based on 18S rDNA sequences of gossypol-degrading strain HQ-1. The scale bar indicates the number of base substitution per site.

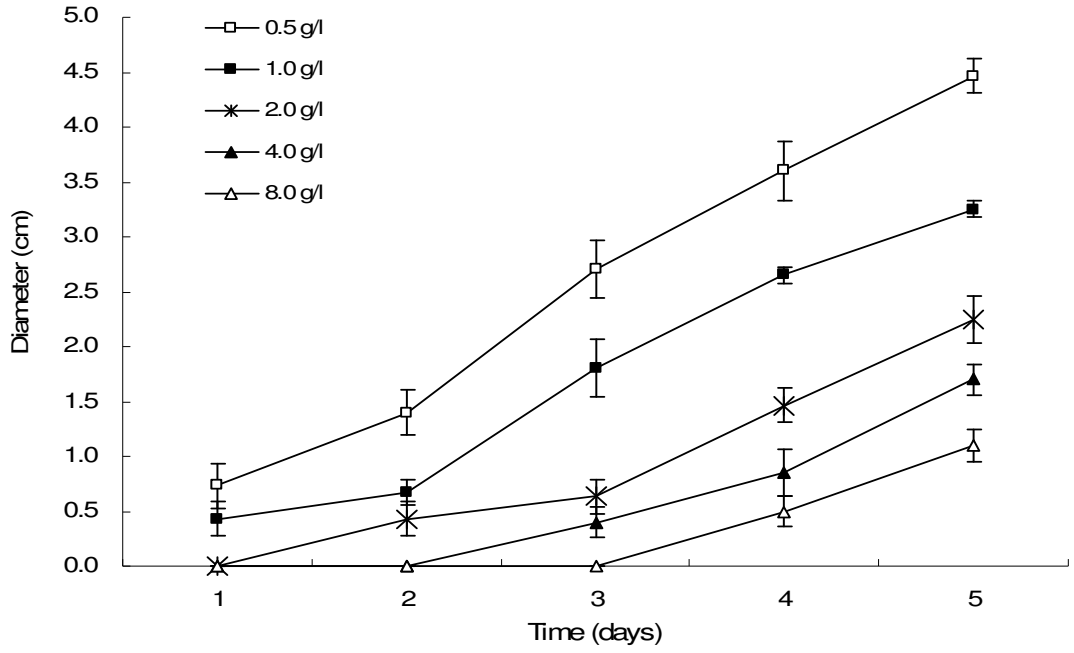

Figure 5. Growth rate of the strain HQ-1 in solid medium containing free-gossypol with five different doses of 0.5, 1, 2, 4 and 8 g/L (diameter vs. time).

utilized in present and after studies. And it was also observed that the growth rate of strain HQ-1 in the glucose was significantly higher than in the gossypol ($p < 0.05$). The reason may be that gossypol is the two naphthalene ring linked through the C-C so that its degradation process is more complicated than the glucose.

Optimum temperature

The maximum biomass (0.0766 g for glucose and 0.0738 g for gossypol) was both attained at 30°C (Figure 7). In the range of 25 to 35°C the microbial biomass was almost the same high but it obviously decreased at 20, 40 and 45°C.

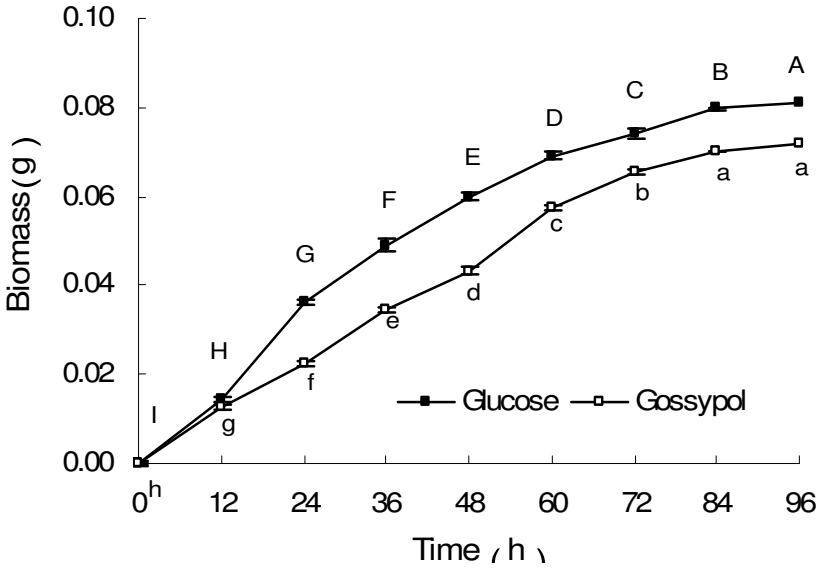

Figure 6. Growth curves of the strain HQ-1 in different carbon source. Dots of the same pattern with various letters were significant differences ($p < 0.05$). Dots of the different pattern with capital or small letters were significant differences ($p < 0.05$).

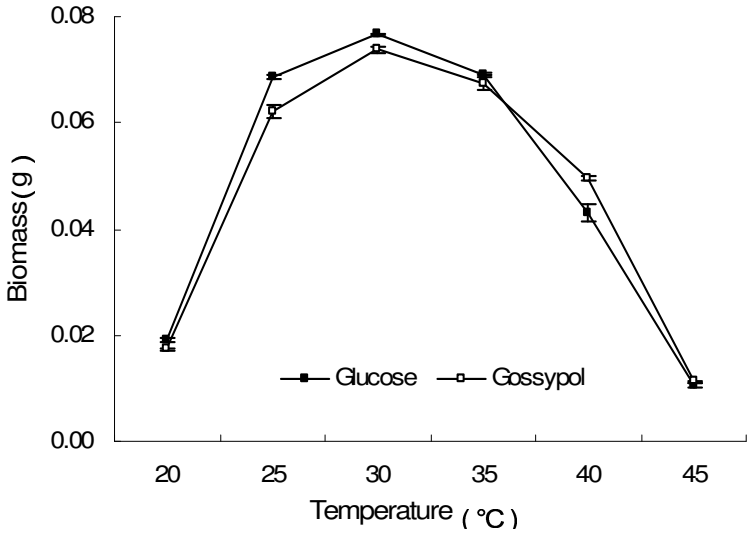

Figure 7. Effect of various temperatures on growth of the strain HQ-1.

The biomass in gossypol substrate was lower than in glucose at 20 to 35℃ but it was conversed at 35 to 45℃. And after 35℃ the microbial biomass rapidly declined. These results showed that this strain was not high temperature resistant, however the more resistant in the gossypol.

DISCUSSION

In this study, a gossypol-degrading strain HQ-1 was isolated from soil by sugar-free Basal Medium plates containing free-gossypol. Through the morphological and molecular biological identification, the 18S rDNA sequence of HQ-1 was confirmed homologous with the *A. niger* as high as 99%. This strain has a high tolerance of free-gossypol and 2 g/L was chosen as the best addition amount. Furthermore, the dry weight method was employed to determine the growth curve of strain HQ-1, and the result showed that the strain grew better in medium

with glucose than with free-gossypol.

The presence of gossypol in CSM, which is an important

source of feed raw materials, has an adverse effect on animals, especially on monogastric ones. Some previous studies have showed that microbial fermentation was capable of greatly decreasing free-gossypol levels in CSM (Weng and Sun, 2006a; Zhang et al., 2006a), and the reason is not clear up to now. In this work, a new strain from gossypol-polluted soil was isolated and identified using morphological and molecular methods. The approach of isolating fungi from contaminated sites offers a promising prospect for screening strains which are tolerant of high levels of pollutants. Gossypol is one of polyphenol compounds, which have attracted serious concern worldwide. Numerous studies have shown that these compounds can be degraded by fungi isolated from soil (Potin et al., 2004; Dritsa et al., 2007). This approach may help in efficiently biodegrading other anti-nutritional factors in feed resources.

As to our knowledge, no work of biodegrading gossypol by microorganisms has been published and this was the first-ever such research with gossypol-containing medium. Shi et al. (1998) reported that *A. niger* could degrade gossypol but this strain was isolated from moldy CSM. CSM is rich in a variety of nutrients like amino acids, proteins, crude fibers, calcium, phosphorus etc., which may affect the degradation of gossypol. However, the strain HQ-1 could be cultivated under Basal Medium using gossypol as the only carbon source. This may be due to the effects of microbial secreted enzymes or other biological compounds that are able to degrade gossypol, the proper reason or mechanism has not been identified and such research is under way.

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