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Identification of antagonistic bacteria for *Amorphorallus konjac* soft rot disease and optimization of its fermentation condition

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Soft rot disease of *Amorphorallus konjac*, an important and potentially destructive corms disease, is caused by *Pectobacterium* species. Now, the conventional methods of controlling the disease include the breeding variety and the chemical control, but the effects are uncomfortable. The aim of this study was to screen antagonistic bacteria for soft rot disease and optimize its fermentation conditions. The antagonistic bacterium (strain C12) confirmed *Bacillus subtilis* by the identification of Biolog system and analysis of 16S rDNA gene sequence. Antimicrobial spectrum of the strain C12 was determined by growth rate method, which could restrain the growth of 12 pathogens. The fermentation conditions of the strain C12 were studied by using the single-factor method. The optimal fermentation conditions for antagonistic bacteria were as follow: Medium initial pH 7.0, the fermentation temperature 31°C, the quantity of medium 50 mL in a 250 mL flask, the inoculation volume 2.5%, the incubation time 22 h and the rotation speed 180 rpm, the glucose as carbon source and yeast as nitrogen source. The fermentation liquor of the strain C12 was twice than the streptomycin in control effect of pot experiment. The research provides reference for controlling soft rot disease of *A. konjac*. The findings suggested that the strain C12 could be exploited as a biocontrol agent for soft rot pathogens.

Key words: Soft rot disease, antagonistic bacteria, characterization, fermentation condition, optimization.

INTRODUCTION

Soft rot disease is one of the destructive diseases of vegetables. It causes a greater total loss of produce than any other bacterial disease. The disease can be found on crops in the field, in transit, in storage and during marketing, and results in great economic losses (Bhat et al., 2010). The disease is conventionally controlled by cultivation measures (such as crop rotation, intercropping) and chemical control (Ronald et al., 2004). Chemical control is usually inappropriate because pathogens can develop resistance and also pesticides can pollute the environment. Therefore, people pay much attention to the biological control. It was reported that *Pseudomonas fluorescens* (Hendawy et al., 1998), *Lysobacter enzymogenes* (Folman et al., 2003), *Erwinia carotovora* subsp. *Betavasculorum* Ecb168 (Costa and Joyce, 1994), *E. carotovora* subsp. *carotovora* Ecc 32 (Seo et al., 2004) and *Streptomyces* (Zamanian et al., 2005) and so on could restrain the *Pectobacterium carotovorasub* sp. *carotovora. Amorphophallus konjac* K. Koch ex N.E.Br. (Araceae) originates in South East Asia (Hetterscheid

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Abbreviations: **P.c.c**, Pectobacterium carotovorasub sp. Carotovora; **PCR**, polymerase chain reaction; **BLAST**, Basic Alignment Search Tool; **DO**, dissolved oxygen.

andIttenbach,1996)andisnowmainlydistributedthroughout Southern and South Eastern China and Vietnam (Brown and Aroids, 2000). China is the main producer of konjac with an area under cultivation of ~200 million acres (Xu et al., 2001) and has approximately 400 factories devoted to the production of konjac flour and related goods (En, 2008). Due to the increasing demand for konjac flour, konjac is now regarded by the Chinese government as an agronomically important crop which has great potential in both domestic and international markets (WFS, 2003). Projects involving planting konjac in mountainous regions of Southern China have been implemented by provincial governments to help combat rural poverty (WFS, 2003). But the soft rot disease of konjac could cause losses between 30 and 50% in the total production, some as high as 80% and even the complete destruction (Xiu et al., 2006). This disease is becoming the bottleneck of konjak industry. No konjac cultivars resistant to the soft rot disease have been reported so far. Recent researches have focused on biological control. Zhou et al. (2004) found that the extracts from *Orostachys fimbriatus* had the antibacterial activities against *Pectobacterium* spp.. Sheng et al. (2007) used the antibiotic extracted from microbial product to prevent and treat soft rot in konjac. *Bacillus subtilis* BSn5 from calli tissue of konjac and *Serratia marcescens* strain 21-2 from rotten corms of konjac showed antibacterial activity against *Pectobacterium carotovorasub* sp. *Carotovora* (*P.c.c*) (Zhou et al., 2007; Wu et al., 2012). But until now effective ways to control this pathogen in the field have not been available. The primary objective of this study is to screen and indentify the antagonistic bacteria against from *P.c.c* the soil of the rhizosphere of konjac, and grope the optimal fermentation conditions, so that the antagonistic bacteria could be quickly developed as a biological control agent of soft rot disease of konjac.

MATERIALS AND METHODS

Strains and culture medium

The strain of *P.c.c* (Registry number: FJ463871) and other pathogens were provided by the laboratory. PSA medium (200 g·L^{-1}potato, 18 g·L^{-1} sucrose) and KMB medium (20 g·L^{-1} peptone, 15 mL glycerol, 2.5 g·L^{-1} K_2HPO_4, 0.73 g·L^{-1} $MgSO_4$) were reference of the microbiology experiment (Fang, 1998).

Isolation of bacterial antagonist

One gram of soil sample, collected from rhizosphere soil of konjac, was suspended in 10 ml of sterile water and vortexed for 45 s. The sample was serially diluted and 100 µL of each dilution was added to molten PSA agar maintained at 55°C along with 10^8 cfu·mL^{-1} of *P.c.c* and poured in sterile petri dishes. After incubation at 28°C for 2 days, bacterial colonies showing zones of inhibition were selected. Further, the antagonistic bacteria were inoculated onto PSA at 180 rpm, 28°C for 24 h. The cell-free supernatants were obtained by centrifugation at 5000×g for 15 min and filtered on a 0.45 µm Mill`ipore filter (Millipore Co., USA). Culture supernatants were added in the wells, made in PSA using sterile metal cylinders, and the plates were incubated at 28°C for 24 h. Bacterial colonies showing zones of inhibition were reselected (Fang, 1998). The streptomycin (0.24 mg·mL^{-1}) (ZHONGNONG, Co., China) was controlled to compare the effect of the antagonistic bacteria. The experiment was repeated three times.

Inhibition of the different fungi

Inhibition of the different fungi for antagonistic bacteria was determined by growth rate method. The bacteriostasis rate was calculated by the antibacterial circle diameter.

The bacteriostasis rate (%) = (Control bacteria colony diameter-Treatment bacteria colony diameter)/(Control bacteria colony diameter-The colony diameter)

Identification of the strain based on 16S rRNA and Biolog plates

The sequence of the 16S rDNA was obtained from the total DNA of the antagonistic bacteria by polymerase chain reaction (PCR) amplification with sense primer 5'-AGAGTTTGATC CTGGCTCA G-3' and antisense primer 5'-CGGCTACCTTGTTACGACTTC-3' (Weisburg et al., 1991). The PCR amplification conditions were as follows: one denaturation step (3 min at 95°C), 35 cycles of amplification (30 s at 95°C, 30 s at 50°C, 1.5 min at 72°C), and a final elongation step of 10 min at 72°C. PCR products were inserted into the pGEM T-easy vector for sequencing. The Biolog system was used to support the 16S rDNA genus identification. All protocols for preparation and identification of microorganisms are outlined in the Biolog™.

Fermentation conditions of the antagonistic bacteria

The antagonistic bacteria grew in KMB medium for these experiments. The fermentation conditions of the antagonistic bacteria were studied by using the single-factor method. Different factors were regulated by the requirement of experiment. The antagonistic bacteria was cultured at 31°C, pH 7.0, broth content 50 mL/250 mL, inoculum concentration 2.5%, the fermentation cycle 22 h and the shaker revolution 180 rpm. Different factors: pH (2-11), fermentation temperature (22, 25, 28, 31, 34, 37 and 40°C), liquid volume (30, 50, 70, 100, 130 and 150 mL medium in 250 mL triangular flask), inoculation amount (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) (volume fraction), culture time (12, 18, 24, 30, 36, 42 and 48 h), rotation speed (150, 180, 210, 240 and 270 rpm), carbon source (glucose, fructose, lactose, mannitol, maltose, sucrose and glycerin) and nitrogen source ($NaNO_3$, $C_2H_5NO_2$, Yeast, $(NH_4)_2SO_4$, KNO_3, Tryptone, $(NH_4)H_2PO4$, NH_4NO_3, L-Glu). The optical density (OD) was surveyed by spectrophotometer (INESA, Co., China). Each treatment was repeated for three times.

Pot experiments

A. konjac plants were grown in a greenhouse until the 3-month-old, where the temperature ranged between 25 and 30°C, and the humidity was more than 80%. The healthy plants were drenched with the 24-h-old-grown culture (approximately 1×10^7 cfu·mL^{-1}, 50 ml/plant) of the antagonistic bacterial. At the same time, the sterile distilled water and streptomycin (0.24 mg·mL^{-1}) were as the negative control. After one week, the plants were challenge-inoculated with 50 ml of *P.c.c* strain (approximately 1×10^7 cfu·mL^{-1}) by pouring the bacterial suspension around the root zone. The incidence of soft rot was recorded periodically over a period of time up to 30 days.

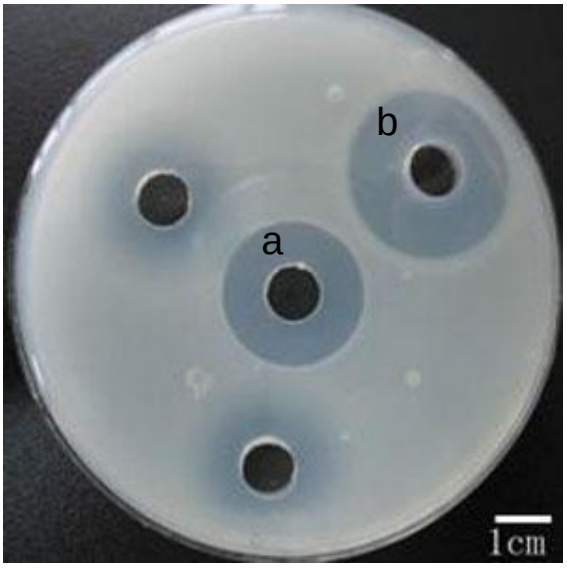


Figure 1. The antimicrobial activity in fermentation supernatants of strain C12 to *P.cc* on PDA medium in 9-cm-diameter Petri dish. The antimicrobial activity was determined by measuring the translucent inhibition zones after incubating the dish 24 h at 30°C. A. streptomycin; B, fermentation liquor of strain C12.

Each treatment was repeated three times. The data were analysed statistically.

RESULTS

Screening of antagonistic bacteria

Twenty-four antagonistic bacteria from the soil of the rhizosphere of konjac against *P.c.c* were obtained by zones of inhibition. The antagonistic effect was further studied by using sterile metal cylinders. The strain C12 had the largest inhibition zone diameter (about 2.6 cm) among the twenty-four antagonistic bacteria. The inhibition zone diameter of the streptomycin was about 2.1cm. The antibacterial circle diameter of the strain C12 was about 0.5 cm wider than the streptomycin (Figure 1).

Determination of the antifungal spectrum

The antifungal spectrum of the antagonistic bacteria strains C12 was determined by growth rate method (Table 1). The strains C12 could inhibit the 12 kinds of pathogens, and the inhibition rate of the 11 pathogens was above 50%. The inhibition rate was greater than 90% of the strawberry root rot (*Rhizoctonia solani*) and Fusarium Wilt of watermelon (*Fusarium oxysporum* f.sp. *niveum*). The results showed that strain C12 had a wide antifungal spectrum.

Identification the antagonistic bacteria

Similar degree of strain C12 with *B. subtilis* was 0.562 by the BIOLOG system (Gin III 5.2 Microstation). The whole 1,420 bp nucleotides 16S rDNA of strain C12 (Registry number: JX960647) was aligned with all related sequences in the NCBI database by the Basic Alignment Search Tool (BLAST) program. The Max ident was 99% with *B. subtilis* in Genbank. So the strain C12 was proved to be *B. subtilis.*

Fermentation conditions of the antagonistic bacteria

The effect of pH value on cell density indicated that the strain C12 could grow under $5 < pH < 10$ (Figure 2a). The strain C12 could grow in a wide pH range. The fermentation temperature has little influence on the growth of strain C12. The strain C12 grew best at 31°C (Figure 2b). Soft rot disease of konjac is easily occurred at 25-30°C. Therefore, the optimum growth temperature of the strain C12 was consistent with the outbreak temperature of soft rot disease.

Liquid volume had great influence in the growth of strain C12. The strains C12 grew best when liquid volume was 50 mL, and then liquid volume was 30 and 70 mL. So the ventilation volume was too large or small, which are not beneficial to the growth of strain C12 (Figure 2c). The strain C12 grew best under the inoculation amount 2.5%, then the 2.0and 3.0% (Figure 2d). Antagonistic bacteria strain C12 reached a maximum growth value when culture time in 22 h, then the strain C12 started aging period (Figure 2e). The antagonistic bacteria strains grew best at 180 rpm. When the shaking speed was more than 180 rpm, the growth of the strain C12 was gradually reduced (Figure 2f).

We could see that the carbon source utilization rate was glucose > sucrose > fructose > maltose > mannitol > lactose > starch > glycerin for antagonistic bacteria (Figure 2g). The highest utilization rate of antagonistic bacteria was yeast, then tryptone, and the other nitrogen source utilization rate was very low (Figure 2h).

Effects of biological bacteria against soft rot disease in pot experiments

30 trees konjac were experimented in each treatment. The plants were all deaths after 30 d by the water treatment, the survival rate of the plant was 16.7% by streptomycin, and the survival rate of the plant was 33.3% by the fermentation liquor of strain C12 (Table 2). The results of pot experiments illuminated that the strain C12 could effectively control soft rot disease of konjac.

DISCUSSION

The strain C12 was identified to be *B. subtilis* by the BIOLOG system and 16S rDNA. Because the bacterium *B. subtilis* produces a variety of antibacterial and antifungal antibiotics such as Zwittermicin-A, kanosamine and lipopeptides from iturin, surfactin and fenzycin fami-

Table 1. The antibacterial spectrum of strain C12.

Pathogenic bacteria	The inhibition rate (%)	Pathogenic bacteria	The inhibition rate (%)
Alternaria alternata	86.7	*Fusarium graminearum*	72.9
Colletotrichum acutatum	55.0	*Rhizoctonia bataticola*	80.0
Colletotrichum gloeosporioides	78.0	*Rhizoctonia solani*	98.7
Myrothecium inundatum	45.8	*Sclerotinia sclerotiorum*	86.7
Fusarium oxysporum f.sp. *batatas*	68.3	*Sphaeronaemella fragariae*	51.2
Fusarium oxysporum f.sp. *niveum*	94.9	*Sclerotium rolfsii Sacc*	71.8

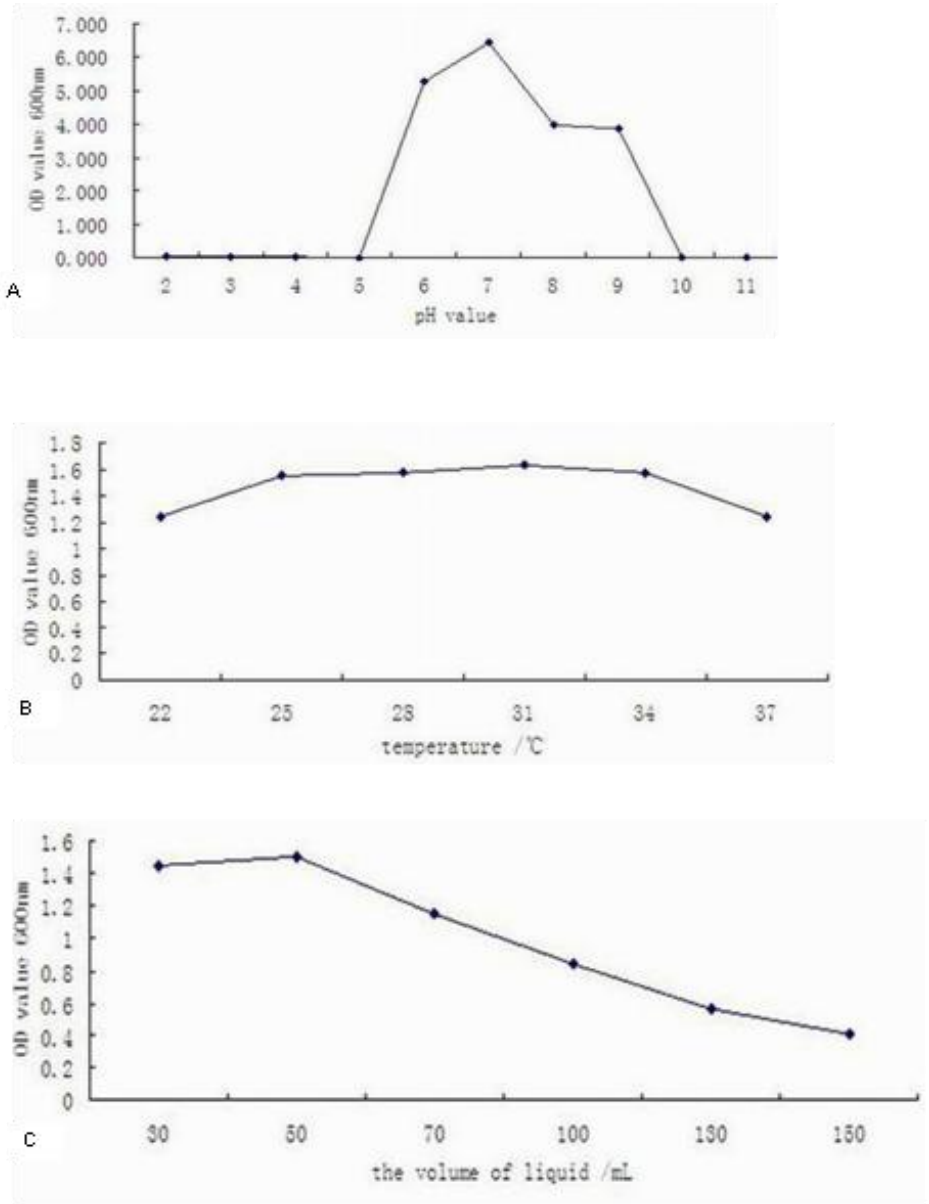
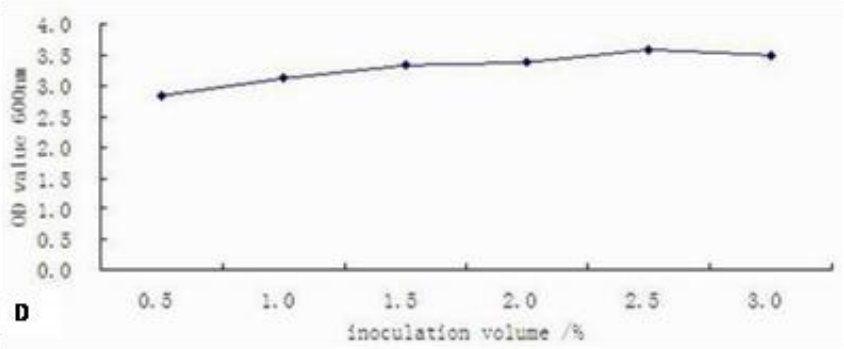
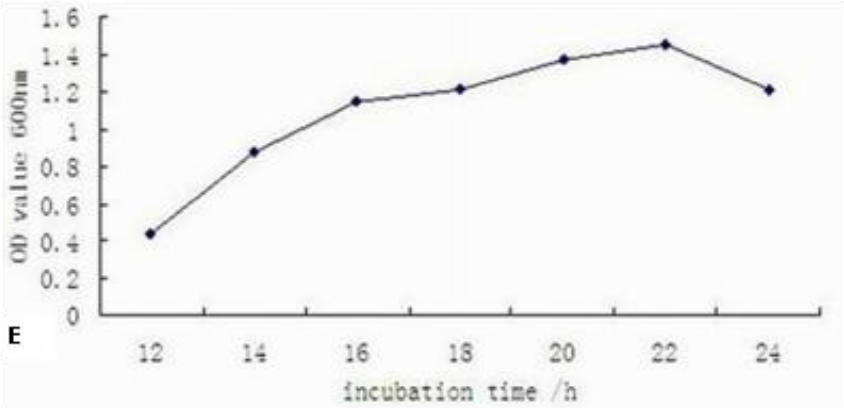


Figure 2. Effect of different factor on cell density for the strain C12. a, Effect of pH value on cell density; B, effect of temperature on cell density; C, effect of the volume of liquid on cell density; D, effect of inoculation volume on cell density; E, effect of incubation time on cell density; F, effect of rotation speed on cell density; G, effect of different C-source on cell density; H, effect of different N-source on cell density.





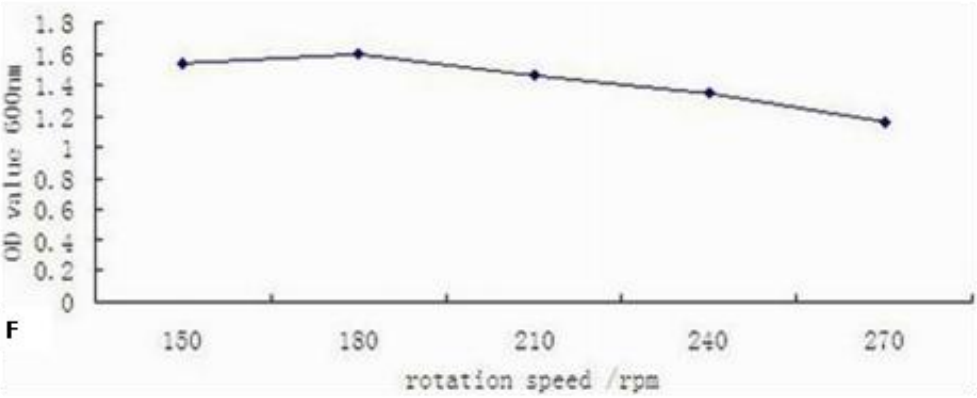


Figure 2. Contd.

families, so the strain C12 not only inhibited the soft rot pathogen of konjac but also exhibit strong inhibitory activity against various plant pathogenic fungi, such as *R. solani*, *Alternaria alternate*, *Fusarium graminearum* among others.

Many bacteria could be used as biological control agents. Among them, *B. subtilis* is now widely recognized as a powerful tool in bio-control as a prevalent soil inhabitant. As a soil-dwelling rhizobacterium, naturally present in the immediate vicinity of plant roots, *B. subtilis* is able to maintain stable contact with higher plants and promote their growth. In addition, *B. subtilis* had broad host range, formed endospores and produced different antibiotics with a broad spectrum activity. Soft rot disease of konjac was a soil-borne disease, and almost no chemical measure was effective in the control of this disease

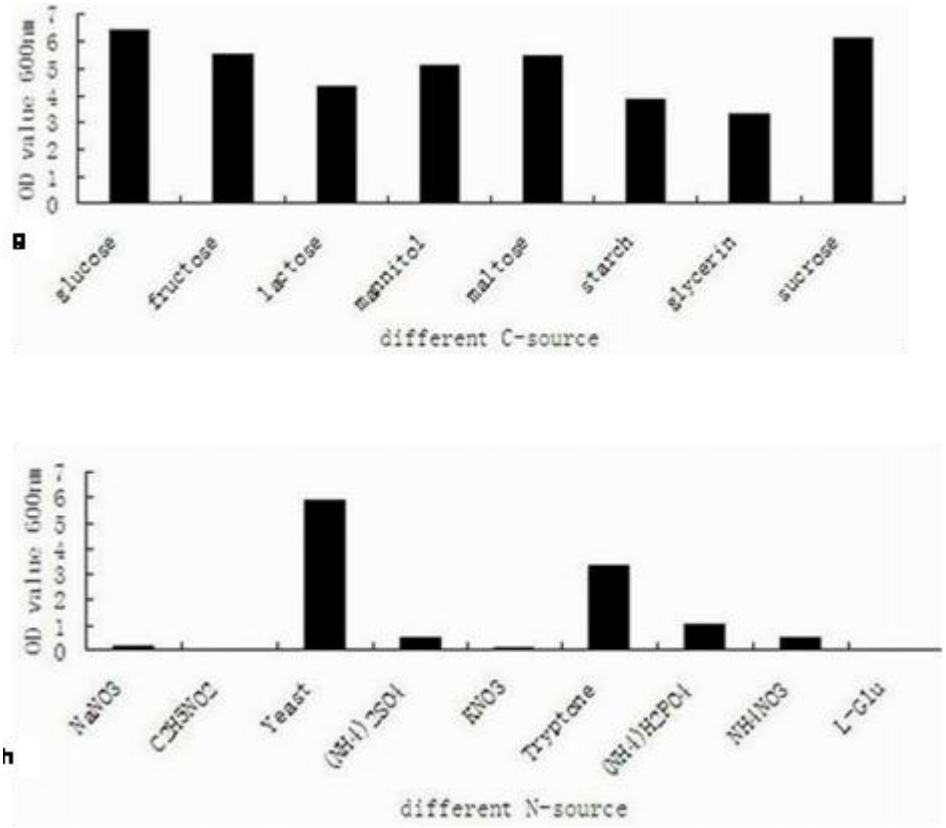


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Table 2. Control efficiency of antagonistic bacteria against soft rot of konjac in the greenhouse.

Treatment	Concentration	Inoculate time (d)	The number of inoculated plants	Survival rate (%)
C12	1×10^{7}cfu·mL^{-1}	30	30	33.3
Streptomycin	0.24 mg·mL^{-1}	30	30	16.7
CK	—	30	30	0

(Krzysztofa et al., 2007). Therefore, the strain C12 was expected to be a bio-control bacterium for soft rot disease of konjac.

The optimal medium volume in the flask and the rotary speed were around 50 mL and 180 rpm, respectively. Our study revealed that rotary speeds, medium volume in the flask are related to the dissolved oxygen (DO) in shaken flasks (Yan et al 2012). A proper DO level was beneficial to the growth of antagonistic bacteria. Lai et al. (2005) found that the shear effect on cell morphology such as mycelium growth or pellet formation was closely influenced by the DO level.

Carbon sources and nitrogen sources played an important role in the growth of the strain C12, not only because of limiting the supply of an essential nutrient is an effective means of restricting growth but also because of the choice of limiting nutrients can have specific metabolic and regulatory effects (Doull and Vining, 1990; Elibol, 2004). The initial pH, the fermentation temperature, the inoculation volume, the incubation time and so on were disadvantage to the growth of the strain C12 when they were too large or small. The information obtained is considered fundamental and useful for developing a cultivation process for efficient production of antibiotics on a large scale for the strain C12.

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