Bioresource Technology 114 (2012) 536–541

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Study of the anti-sapstain fungus activity of *Bacillus amyloliquefaciens* CGMCC 5569 associated with *Ginkgo biloba* and identification of its active components

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ARTICLE INFO

Article history:
Received 28 January 2012
Received in revised form 17 March 2012
Accepted 19 March 2012
Available online 28 March 2012

Keywords:
Bacillus amyloliquefaciens CGMCC 5569
Sapstain fungi
Anti-sapstain fungi activity
Lipopeptides

ABSTRACT

An endophytic bacterium, designated strain *Bacillus amyloliquefaciens* CGMCC 5569 was isolated from Chinese medicinal *Ginkgo biloba* collected from Xuzhou, China. Both the filtrate and the ethyl acetate extract of strain CGMCC 5569 showed growth inhibition activity against the sapstain fungi *Lasiodiplodia rubropurpurea*, *L. crassispora*, and *L. theobromae* obviously (>65%) based on the comparison of the length of zones on the petri dish. From the ethyl acetate extract of the filtrate, the antifungal compounds were obtained as a series of lipopeptides, which including series of fengycin, surfactin and bacillomycin. It showed strong growth inhibition activity *in vitro* against the *L. rubropurpurea*, *L. crassispora* and *L. theobromae* by about 70.22%, 69.53% and 78.76%, respectively. The strong anti-sapstain fungus activity indicated that the endophytic *B. amyloliquefaciens* CGMCC 5569 and its bioactive components might provide an alternative bio-resource for the bio-control of sapstain.

1. Introduction

The concept of biological control as a method for protecting crops and other perishable commodities has received increased attention from the scientific research community in recent years. This is a consequence, in part, of the increasing awareness of both industry and the general public of the environmental impact of chemicals used for crop protection and preservation purposes. Wood discoloration is a complex biological process that can involve a wide variety of microorganisms, often interacting with one another, and influenced by the changing environmental conditions under which the wood is placed. Sapstain is a major problem for timber producers as well as pulp and paper manufacturers since fungi colonization and disfigurement of freshly fell materials prior to drying can result in significant economic losses (Bruce et al., 2003). Although the sapstain fungi cause little or no significant damage to the structure elements of the timber, they have a detrimental effect on the aesthetic value of the wood due to the colonization by their pigmented mycelia. It causes significant economic losses to the China lumber industry recently. Control of sapstain is normally achieved through rapid air or kiln drying of the lumber or through the use of diffusible chemical preservatives. At present, chemical control remains the main measure to reduce the wood discoloration (Yang, 2005); however, it may pose significant risks to environment and public health.

Significant advances in control the disease and stain fungi have been achieved both in research and application by the use of bio-control microorganisms, like bacteria and actinomycetes. Bacteria have been commercialized and used in controlling crop diseases, such as *Bacillus subtilis*, *B. polymyxa*, *B. pumilus*, *B. amyloliquefaciens*, *B. cereus*, and *B. licheniformis*. Recently, *Bacillus* species have been used widely as bio-control agents (Chen et al., 2009; Zhao et al., 2010). *Bacillus* spp. can produce structurally diverse secondary metabolites with a wide spectrum of antifungal activity. Several strains of *B. subtilis* and *B. amyloliquefaciens* have been found to produce lipopeptides, and these bioactive lipopeptides showed a great potential for biotechnological, biopharmaceutical and agricultural applications (Schallmey et al., 2004).

Many of these antifungal substances have been identified, including mycobacillin, iturin, bacillomycin, surfactin, mycosubtillin, fungi-stain, subsporins and rhizocticins (Fiddaman and Rossall, 1993; Kunst et al., 1997; Touré et al., 2004; Stein, 2005; Liu et al., 2010). These compounds, made of amino acids and a fatty acid, are easily biodegradable in the soils (Cho et al., 2003). Considerable interest lies in using *Bacillus* producing lipopetide antibiotics, such as iturin A and surfactin as biocontrol agents due to their antagonistic and repressive activities against plant pathogens. These amphiphilic cyclic biosurfactants have many advantages over other fungicides: low toxicity, high biodegradability and environmentally friendly characteristics (Kim et al., 2004). The endophytic

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http://dx.doi.org/10.1016/j.biortech.2012.03.062

B. amyloliquefaciens CGMCC 5569 used in this study was isolated in our laboratory from *Gingko biloba* and showed high levels of anti-sapstain fungus properties. We showed that CGMCC 5569 inhibited anti-sapstain fungus suggesting that the strain and antibiotic production were involved in sapstain-suppression. Identification of the antibiotics produced may improve our understanding of the mechanism involved in this and other biocontrol systems. The goal of this study was to purify and identify certain antibiotics produced by CGMCC 5569, which is responsible for the inhibition of sapstain fungi. Using chromatography, HSCCC and mass spectrometer, series of lipopeptides that inhibit sapstain fungi were isolated and identified in this study. The filtrate, ethyl acetate extract, and components showed a strong growth inhibition activity against the sasptain. And the results were significantly better than chemical fungicide nystafungi. The strong anti-sapstain fungus activity indicated that the endophytic *B. amyloliquefaciens* CGMCC 5569 and its bioactive components might provide an alternative bio-resource for the bio-control of sapstain.

2. Methods

2.1. Microorganism

Strain *B. amyloliquefaciens* CGMCC 5569 was isolated from ginkgo (*G. biloba*) collected from Xuzhou, China. The strain was deposited in the China General Microbiological Culture Collection Center (CGMCC) and maintained on LB medium (Lysogeny Broth Medium).

The sapstain strain of *Lasiodiplodia theobromae* was provided by the Forest Microbial Resources of China (CFCC). The stain of *L. rubropurpurea* and *L. crassispora* were kindly provided by Professor Gui-hua Zhao and maintained on PDA (Medium, Potato Dextrose Agar Medium).

2.2. Identification of the strain CGMCC 5569

Morphological observations were made with scanning electron microscope (SEM) using the method of Vendan et al. (2010). Utilization of carbon and nitrogen sources was carried out according to standard methods. Strain CGMCC 5569 was identified by biochemical and physiological methods by a kit (French, bio-Merieux, sa). The ability to produce enzymes was also studied and the morphological description was characterized by SEM.

Strain CGMCC 5569 was grown on LB medium. Genomic DNA extraction, amplification and 16S rRNA genes sequencing was analyzed according to described procedures (Liu et al., 2007). The 16S rRNA gene sequence data of the strain in this study were deposited in GenBank (JQ756988) and also compared with those of some type strains within the genus bacterium (retrieved from the GenBank/EMBL/DDBJ database). Phylogenetic analysis was performed using mega version 4.0 after multiple alignment of data by Clustal-X.

2.3. Fermentation and extraction

The fermentation was performed in LB medium at 30 °C, 100 rpm for 72 h. The obtained culture broth (12 L) was centrifuged at 4000 rpm at room temperature for 5 min. The cell-free supernatant was extracted exhaustively five times with ethyl acetate (filtrate:ethyl acetate = 1:2 vol/vol). The solvent was removed by using a rotary vacuum evaporator R 206 D (SENCO, Shanghai, China) under reduced pressure to yield a brown viscous tarry residue (5.2 g) and stored at 4 °C.

2.4. Anti-sapstain fungus assays

Petri dishes containing PDA were used for anti-sapstain fungus activity assay procedure by Duru et al. (2003). After removal of the cells by centrifugation of the culture broth at 4000 rpm for 5 min and filtration through 0.22 μm membrane filter. Culture filtrate (1 mL) mixed with 10 mL of the PDA medium was poured into a Petri dish. Discs of the target fungi, from the fresh margin of the mycelia, were spaced equally on the Petri dish. The dish was incubated at 28 ± 0.5 °C for 72 h. The inhibitory activity of the filtrate against fungal growth was recorded as the percentage reduction of mycelia growth in comparison with that of the control plates (Liu et al., 2007). Make up different concentrations from 0.1 to 1.0 mL/mL and used similar procedure above to determine the anti-sapstain fungus activity of culture filtrate. The tests were conducted in triplicate for each treatment.

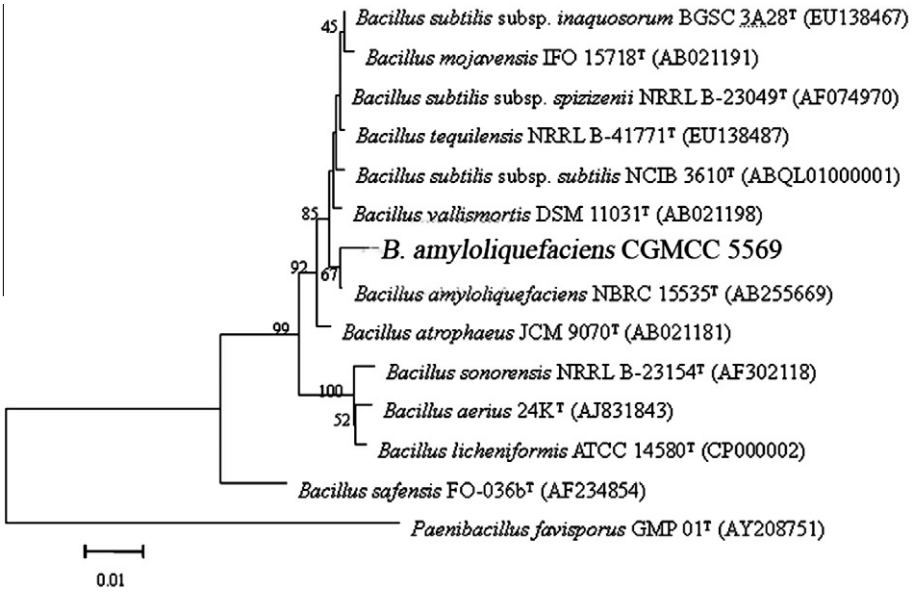

Fig. 1. A neighbor-joining phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain CGMCC 5569 among members of the genus *Bacillus* species. Numbers on branch nodes are percentage bootstrap values (1000 resamplings). The "T" in the figure means "type strain".