African Journal of Microbiology Research Vol. 6(9), pp. 2156-2161, 9 March, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1610
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Isolation and identification a tannin-tolerant fungus producing protopectinase

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Acceptance 24 January, 2012

A strain SHPP01, which produces a protopectinase and has the ability of tannin tolerance, was screened from Shanghai University campus with protopectin as the solo carbon source. It was identified as *Aspergillus terreus*, and designated as *A. terreus* SHPP01 based on its morphology and internal transcriptional spacer sequence. Tannin tolerance assay was carried out later to explore the effect on the characters of SHPP01; the result showed the dry weight of *A. terreus* SHPP01 increased about 1.39 times, when tannin concentration increased from 2 to 10 g/L. With tannin concentration increasing protopectinase production decreased firstly, and increased later, the result showed that tannin concentration below 10 g/L has not significantly affected protopectinase production, and *A. terreus* SHPP01 has the good ability of tannin tolerance than others in previous literatures. This study provides the foundation to utilize *A. terreus* SHPP01 to extract pectin from persimmon peel in microbial fermentation.

Key words Molecular identification, tannin tolerance, *Aspergillus terreus*, protopectinase.

INTRODUCTION

Pectin is a water-soluble, heteroploysaccharide that contains galacturonic acid and methoxylgalacturonic acid as their main components (Sakai, 1993). In the plant Kingdom, pectin exists in the middle lamella, linking primary cell walls (containing cellulose and hemicelluloses such as xylan and mannan as the main components) and from water-insoluble protopectin, which is solubilized by protopectinase with restricted hydrolysis. Pectin is widely used as a functional ingredient in the food industry, pharmacy and cosmetic manufacture (Canteri-Schemin et al., 2005) because of its ability to form aqueous gels, dispersion stabilizer.

The most commercial pectin is still been produced by chemical extraction from plants in China. The process of chemical extraction of pectin consumes energy and produces many industrial wastes (Shi et al., 1996; Kim et al., 2004). Microbial and enzymatic extract pectin, however, can reduce such drawbacks and produce pectin with higher quality. In this way, Sakai and Okushima (1980) isolated different microorganisms able to produce protopectinase to extract pectin from citrus peel. In 1999, acidic protopectinase from *Aspergillus kawachii* IFO 4308 were used to extract pectin from lemon peel (Contreras Esquivel et al., 1999), and the pectin yield was up to 17.4% (on dry peel).

All kinds of plant tissues are used as the substrate to extract pectin, such as apple pomace (Zheng et al., 2007), pumpkin, citrus peel (Kim et al., 2004), sunflower heads (Wiesenborn et al., 1999), peach pomace (Pagan et al., 2001). China is the place of origin and the production country of persimmon. There are many persimmon peel wastes in persimmon processing every year. This investigation is motivated by the increasing economic importance of an efficient utilization of plenty of persimmon peel wastes. The objective of the study is to isolate a novel fungus capacity of using protopectin as the sole carbon source, to identify it as *Aspergillus*

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terreus through morphological and molecular method, and study tannin tolerance of this strain subsequently.

MATERIALS AND METHODS

Enrichment of tannin-tolerant microorganism

A few soil samples were collected from natural sources at various sites in Shanghai University campus, Shanghai, China. The samples were pretreated as follows. The 100 g of soil samples were mixed uniformly with 5 g of dry persimmon peel and kept in the room temperature for 5 to 7 days.

Isolation of tannin-tolerant microorganism degrading protopecin

10 g of the pretreated soil sample were suspended in 100 ml sterilized water, and the supernatant was serially diluted and spread on selective agar medium and incubated at 28°C for 96 h. The selective medium (g/L) contained protopectin 5, sodium nitrate 2, monopotassium phosphate 1, potassium chloride 0.5, magnesium sulphata heptahydrate 0.5, ferrous sulphate heptahydrate 0.01, agar 20 and distilled water 970. The plates were observed constantly for appearance of fungal colonies, stained with 0.015% Congo red solution for 5 min, and de-stained with sterilized water. The microorganisms degrading protopectin were selected by viewing hydrolytic zones around the colonies against the background; the one having a biggest hydrolytic zone was denominated SHPP01.

Morphological identification of the fungal isolate

For viewing the colonial morphology, the strain SHPP01 cultivated on PDA plates at 28 °C for about 5 days. Macroscopic characteristics such as shape, color and surface texture were described in details. The microscopic features (for example, the shape of hyphae, conidia and conidiophores) were measured from strains grown on PDA plates 2 to 3 days. The method of morphological identification was established by Wei (1979), Raper and Thom (1949).

DNA extraction, PCR amplification, cloning and DNA sequencing of fungal ITS1-5.8S-ITS2 region

The strain SHPP01 were grown in 250 ml Erlenmeyer flasks containing 50 ml of potato dextrose broth with 140 rpm at 28°C for 48 h. The mycelia were harvested by filtration and grinded with quartz sands thoroughly. The genomic DNA was extracted Paavanen-Huhtala et al. (1999). DNA extracts were stored at -20°C prior to use. The internal transcribed spacer (ITS) region, the small subunit (ITS1-5.8S-ITS2) of the rDNA genes were amplified by using the primer set ITS1 (5'-TCCGTAGGT GAACCTGCGG-3') and ITS4 (5' -TCCTCCGCTTATTGATATGC-3') (Zhao et al., 2010). PCR reactions were performed in an Eppendorf Mastercycler gradient (Eppendorf, Holstein City, Germany). Thermal cycling was carried out using an initial denaturation step at 95°C for 5 min, followed by 30 cycles of de-naturation at 95°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 60 s. Cycling was completed by a final elongation step at 72°C for 10 min.

For sequencing of the ITS1-5.8S-ITS2 region obtained from the isolated strain, a PCR product of the previous isolates was purified by DNA purification kit, cloned into pGM-T kit (Tiangen Biotechnology Co.Ltd, Beijing, China) and transformed into *Escherichia coli* DH5α competent cells by using ampicillin. The recombinant plasmid was purified and sequenced with M13 Universal primers in Applied Biosystems 3730xl DNA Analyzer (Shanghai Majorbio Biotechnoloyg Co.Ltd, Shanghai, China). Subsequently, the sequence had been submitted to Genbank database.

Phylogenetic analysis

Sequences of ITS region of the strain SHPP01 was used as query sequence to search for similar sequences from GenBank using BLAST program. The most similar reference sequence with query sequences were obtained and used for the following phylogenetic analyses. These sequences were aligned using ClustalX program (Jeanmougin et al., 1997). To construct the relevant phylogenetic tree, MEGA 4.1 software was employed in this study (Kumar et al., 2007). Ambiguous positions, which may not be homologous, were eliminated and gap positions were deleted manually. The alignment data were subsequently analyzed by the neighbor-joining (NJ) method (Kimura two-parameter distance calculation)(Saitou and Nei, 1987). For each search, 1,000 replicates of random stepwise sequence addition were performed. Statistical support for the internal branches was estimated by bootstrap analysis with 1,000 replications.

Effects of tannin on the growth and protopectinase production of the strain

The strain was transferred into the concentration of 2, 4, 6, 8 and 10 g/L of tannin in the fermentation media (g/L) containing glucose 20, peptone 4, yeast extract 2 and protopectin 5, respectively, and cultured at 28°C for 2 days. After fermentation, the dry weight of organisms was determined (Lu et al., 2005), and protopectinase activity was measured by sulphate-carbazole method (McComb and McCready, 1954).

RESULTS AND DISCUSSION

Enrichment of tannin-tolerant microorganism

In order to enrich the tannin-tolerant microorganism for an extreme facility in obtaining targeting strains later in this study, about 3.75% tannin mixed in the soil sample as a selective pressure to inhibit tannin-sensitive microorganisms; after the soil sample had kept in the room temperature for 5 to 7 days, only tannin-tolerant organisms had grown and enriched in the environment.

Isolation of tannin-tolerant microorganism degrading protopectin

Some strains appeared in the selective agar medium with protopectin as the solo carbon source, and 5 isolates were selected according to the transparent hydrolytic zone formation on agar plates stained Congo red solution, which pointed out those had the capacity of degrading protopectin. The isolated fungus forming a biggest hydrolytic zone was designated as SHPP01 in the subsequent investigation.

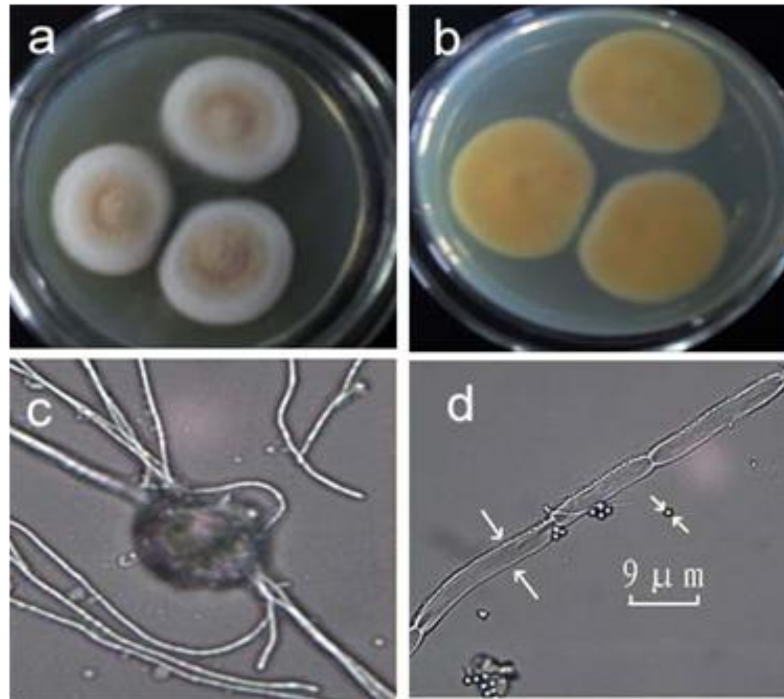

Figure 1. Morphological examination of strain SHPP01. a and b showed the obverse and reverse of colonies of SHPP01, respectively. c–d showed light micrographs of conidiophore and branching hyphae of strain SHPP01. c showed the conidiophores. d showed the hyphae.

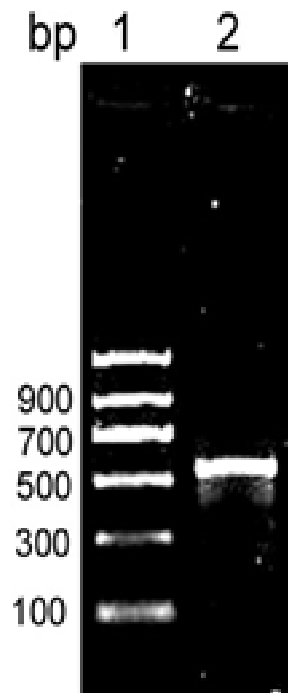

Figure 2. Electrophoresis of the PCR amplification of ITS region. Lane1: MarkerII; lane 2: PCR product

Fungal morphological observation

Reaching 4 to 5 cm diameter on PDA after 5 days, colony's forms were circular, radially raised and their textures were softy and villous. Colony's color was beige to buff to whitish grey with fuzzy edges and turned into a different color from the center outwards (Figure 1a), while the reverse was pale yellow (Figure 1b). Conidiophore was globose to ellipsoidal, hyaline to slightly yellow-grey (Figure 1c) and its diameter was about 2.7 μm. Hyphae were septate and hyaline and their diameter were about 6.3 to 8.1 μm (Figure 1d). According to these characteristics, it was primarily identified as *A. terreus*.

Phylogenetic analysis

For the further identification of earlier result, the ITS1-5.8S-ITS2 region of SHPP01 was examined by electrophoresis (Figure 2), purified and sequenced. The result showed the PCR product was about 600 bp, which was consistent with the expected length of PCR amplification.

The ITS sequence of SHPP01 was submitted to GenBank and its accession number JF738047 was gained. The ITS sequence of SHPP01 was compare to that of different strains obtained from the NCBI database using the neighbor-joining method (Saitou and

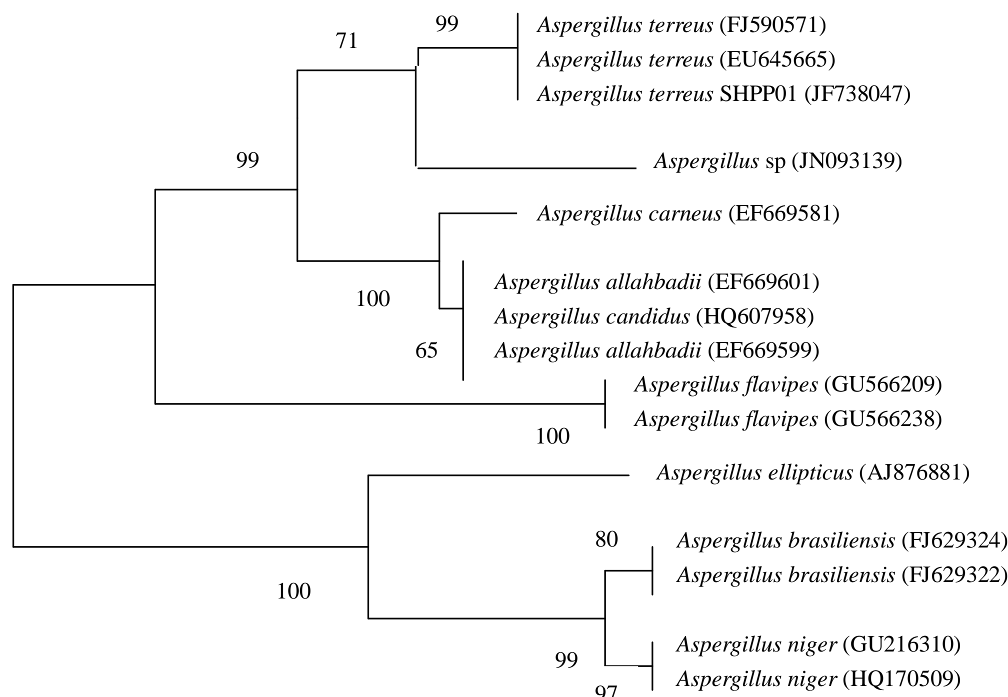

Figure 3. Neighbor joining phylogenetic tree on aligned ITS1-5.8S-ITS2 rDNA sequences.

Nei, 1987). The approximate phylogenetic position of the strain was shown in Figure 3.

Confidence values above 50% obtained from a 1,000-replicate bootstrap analysis were shown at the branch nodes. Bootstrap values from neighbor-joining method were determined. *Aspergillus ellipticus* (FJ629324), *Aspergillus brasiliensis* (FJ629324), *A. brasiliensis* (FJ629322), *Aspergillus niger* (GU216310) and *A. niger* (HQ170509) were used as the out group

A search for similar ITS region sequences from GenBank showed that strain SHPP01 had higher sequence similarities with the species *A. terreus* than any other reference taxa. All 14 sequences of reference taxa were obtained for constructing a phylogeny. In the neighbor-joining (NJ) tree (Figure 3), strain SHPP01 and other two reference taxa, *A. terreus* (EU645665) and *A. terreus* (FJ590571) formed a clade with 99% bootstrap support. In this clade, the reference taxa, *Aspergillus* sp (JN093139), also clustered together with them. The results of similarity comparisons of the ITS1- 5.8S-ITS2 region sequence revealed that strain SHPP01 had the highest nucleotide similarities with *A. terreus*. According to the results of the morphological and molecular identification, it could be confirmed that SHPP01 was *A. terreus* and was designated as *A. terreus* SHPP01 in our study.

Molecular approaches have been successfully applied to resolve many problems in identification of fungi, which cannot be done effectively using morphological or cytological methods. It has often been reported that the ITS regions of the rDNA gene are often variable with nucleotide composition and that this characteristic can be used to distinguish both morphologically distinct fungal species and strains of the same fungal species (Boysen et al., 1996; Shao et al., 2004). Thus, the ITS region of the rDNA gene is believed to represent a convenient target for the molecular identification of specific species and subspecies level of fungi. This technique is one of the most commonly used today for identifying species, as it is a quick, reliable, and reproducible method (Ocon et al., 2010). In this research, the ITS region sequence was amplified and sequenced and compared to others and identified the novel isolation strain SHPP01 as *A. terreus*, which showed that molecular approach and morphological method revealed the similar results.

Effects of tannin on the growth and protopectinase production of *A. terreus* SHPP01

The effects of tannin on the growth and protopectinase production of *A. terreus* SHPP01 was investigated (Figure 4). The results showed that the dry weight of *A. terreus* SHPP01 increased with the increase of tannin concentration. The dry weight of *A. terreus* SHPP01 increased about 1.39 times, when tannin concentration increased from 2 to 10 g/L, which indicated that there was the not significant effect of tannin on the growth of *A. terreus* SHPP01. However, with an increase in tannin concentration protopectinase production decreased firstly

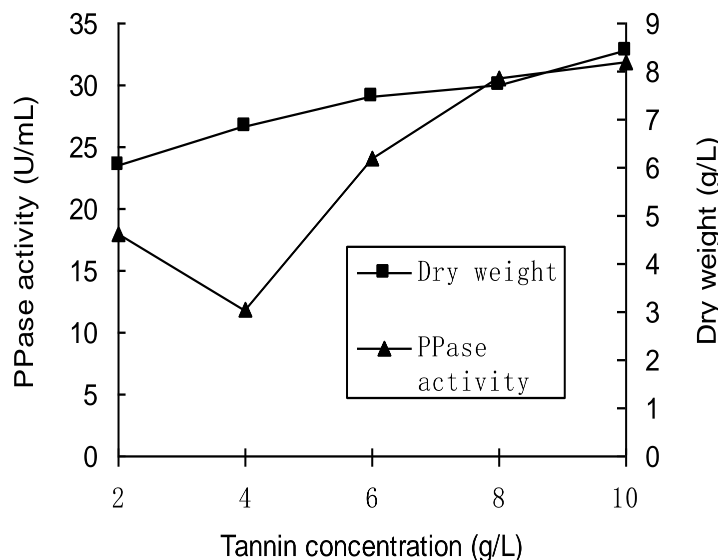

Figure 4. Effect of tannin on the characterization of *Aspergillus terreus* SHPP01.

at tannin concentration of 2 to 4 g/L and increased later at the higher concentration.

The results also inferred that protopectinase production was not affected by tannin at the low concentration of 2 g/L. when tannin concentration increased from 4 to 10 g/L there may be a certain change in the metabolism of *A. terreus* SHPP01 and some biomass could be produced to eliminate tannin effect. As can be seen from Figure 4, tannin had the inhibitory effect on protopectinase production, which is coordinated with some reports (Jamroz et al., 2009; Amarowicz et al., 2008). At low concentration of tannin, tannin not only inhibited protopectinase production, but also induced *A. terreus* SHPP01 to excrete tannase, which could liberate tannin to eliminate its inhibit to cell enlargement and protopectinase production.

Currently, protopectinase has attracted many researchers' attentions. Sakai et al. (2000a) and (2000b) had purified and characterized of acid-stable protopectinase produced by *Aspergillus awamori* and thermostable protopectinase from thermophilic *Bacillus* sp TS 47, respectively. *A. niger* CD-01 had been identified to produce protopectinase and its fermentation conditon had been optimized (Xia et al., 2009). Mignone and Cavalitto (2007) ulitilize statistic method to optimize protopectinase production by a *Geotrichum klebahnii*. protopectinase gene from *Trichosporon penicillatum* had been cloned and expressed in *Saccharomyces cerevisiae* (Iguchi et al., 1997). In our previous study, we had cloned and expressed protopectinase-N gene from *Bacillus subtilis* in *Pichia pastoris*, and optimized medium for protopectinase production by *A. terreus* in submerge culture (Liu et al., 2005; Fan et al., 2011). However, few attentions have paid to the effect of tannin on protopectinase production and characteristic of strains producing protopectinase.

In order to accomplish microbial and enzymatic extract pectin from persimmon peel, the strain producing protopectinase must have a good capacity of tannin tolerance because that tannin existing in persimmon peel has the inhibitory effect on microbial growth and metabolism. In this study, *A. terreus* not only excretes protopecitnase and but also has a good tannin tolerance; therefore, *A. terreus* has a potential utilization of persimmon peel waste to make pectin.

In the present study, we isolated and screened a strain SHPP01 from Shanghai University campus, which produces protopectinase and has the good capacity of tannin tolerance. This strain was identified as *A. terreus* SHPP01 by morphologic examination and internal transcriptional spacer sequence analysis.

ACKNOWLEDGMENTS

This research is financially supported by the Shanghai Science and Technology Committee (11DZ2272100) and Shanghai Key Laboratory of Bio-Energy Crops.

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