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Enhanced production and antioxidant activity of endo-polysaccharides from *Phellinus igniarius* mutants screened by low power He-Ne laser and ultraviolet induction

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ABSTRACT

In this study, a *Phellinus igniarius* mutant was screened through low power He-Ne laser and ultraviolet (UV) induction. The mutant was then used to improve the production of endo-polysaccharides. In the shake flasks, the dry weight of the mycelial biomass and the production of endo-polysaccharides derived from the screened mutant (JZx) fermentation were 20.715 g/L and 1.428 g/L, respectively, which were 40.31% and 56.58% higher than those of the control strain (CK). In addition, isozyme electropheresis spectrogram analysis results indicated that the genetic materials of the screened mutants were altered. The endo-polysaccharides obtained from the JZx fermentation were mainly composed of D-glucose, L-rhamnose, and D-mannose in a molar ratio of 2.0:16.0:1.0 and with low-molecular weights (MWs) (1.5 kDa, 61%). These endo-polysaccharides exhibited stronger antioxidant activities in vitro, contained stronger hydroxyl radical scavenging capacity, and higher Trolox equivalent antioxidant capacity (TEAC) (195.43 μmol Trolox/g sample) and ferric reducing ability of plasma (FRAP) (20.57 μmol Fe^{2+}/g sample) values compared with those of the CK. Therefore, the mutant screening through low power He-Ne laser and UV-induction could be an efficient and practical method for the development of the *Phellinus* strains and thus could improve the production and antioxidant activities of their endo-polysaccharides.

1. Introduction

Phellinus igniarius is a basidiomycete fungus under the genus *Phellinus* of the Polyporaceae family. It is well-known for its significant biological activities and medicinal properties (Mizuno, 1999). The fruiting bodies of *P. igniarius* have been traditionally used as folk medicine for many years in China, Japan and Korea because of their prominent anti-inflammatory and hemostasis functions. In addition, they also invigorate the liver, promote blood circulation, and reinforce the spleen (Dai, Zhou, Cui, Chen, & Decock, 2010). As a major class of bioactive components of *P. igniarius*, polysaccharides have been important and beneficial because they demonstrate antioxidative, antibacterial, antiviral, antitumor, antimutagenic, and immunomodulatory activities (Li, Yang, Ma, Yan, & Guo, 2015; Song, Lin, Yang, & Hu, 2008; Suabjakyong, Nishimura, Toida, & Van Griensven, 2015). Therefore, these compounds have increasingly attracted attention for their health benefits. They have been used in the food, medical, and cosmetic industries as therapeutic agents because they exhibit low toxicity and have minimal side effects.

In recent years, wild or natural *P. igniarius* has become increasingly rare, and its field-cultivation cycle is time consuming. Thus, the development and use of *P. igniarius* are limited (Chen, Xiao, Li, & Zhang, 2007). Generally, cultivation of a fruiting body of *P. igniarius* requires six months, and the product quality is difficult to control when it is cultivated in a solid substrate. The product composition also varies among batches (Liu et al., 2009). Notably, liquid or submerged fermentation has some advantages. It increases mycelia and polysaccharide production even at compact space over a short incubation time and availability of convenient control with less chance of contamination (Hwang, Kim, Choi, & Yun, 2003; Suabjakyong et al., 2015). Therefore, submerged fermentation has become a promising alternative that generates highly efficient production of mycelial biomass and polysaccharides.

Although natural breeding can maintain the stability of the strains and produce pure strains, the probability of natural variation is extremely low, and the output frequently occurs in the fluctuation

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range of biological production. As modern techniques, genetic engineering and metabolic engineering are increasingly considered as effective methods for microorganism breeding. However, these techniques encounter serious impediments because of limited insights into the genetics, physiology, and biochemistry of organisms (Olano, Lombó, Méndez, & Salas, 2008). Until now, traditional mutation is still the most effective strategy for improving the productive capacity of the strains (Khaliq et al., 2009). In this regard, UV is widely used in mutagenesis-selection protocol. The mutagenic and lethal mechanisms of UV radiation have been elucidated in several microorganisms (Alifano, Lorusso, Nassisi, Tala, & Tredici, 2008; Clark, 1996; Ikehata & Ono, 2011; Ravanat, Douki, & Cadet, 2001). However, because of the lower rate of UV on positive variation, researchers have focused on mutation screening through compound mutation in various ways (Yu et al., 2011). A low-power laser irradiation technology has generated considerable interest with regard to microorganism mutation breeding. Based on a homemade XeCl 308 nm excimer laser, an innovative and effective mutagenesis protocol has been developed for antibiotic-producing industrial strains (Alifano et al., 2008). Laser at wavelength of 620 nm has significant growth-stimulating effect, especially He-Ne laser at wavelength of 632.8 nm (Van Breugel & Bär, 1992). However, to the best of our knowledge, reports on the use of low-power He-Ne laser and UV irradiation to induce microorganism mutation are few.

Many research findings demonstrated that the antioxidant activity of the polysaccharides and their derivatives mainly rely on separation procedures, physicochemical properties, water solubility, and even their primary structures. Among the processes regarding these polysaccharides, chromatographic purification of actives anionic polysaccharides, oligoglucuronans, and their derivatives are increasingly attracting interest (Petera et al., 2015; Wang et al., 2016). Some studies focused on the relationship between the structure and antioxidant activity of polysaccharides, elucidation of their antioxidant mechanism at the molecular level, and improvement of their various biological activities through molecular modifications, such as sulfation, carboxymethylation, and regioselective oxidation (Delattre et al., 2015; Elboutachfaiti et al., 2011; Wang et al., 2016). For example, Delattre et al. (2015) reported that a xanthouronic acid sodium salt called xanthouronan, which was produced from xanthan through TEMPO-mediated oxidation, exhibits stronger antioxidant activities than native xanthan. In addition, physical irradiation treatments, such as ultrasound, microwave, and UV radiation, have been used to modify polysaccharides in order to enhance their physicochemical properties and biological activities (Delattre & Vijayalaksmi, 2009; Drímalová, Velebný, Sasinková, Hromádková, & Ebringerová, 2005).

Thus, a novel microbial mutation method combining low power He-Ne laser and UV radiation was used to screen *P. igniarius* strains to increase the production of endo-polysaccharides. Meanwhile, the physicochemical properties, primary structures, and antioxidant activities of endo-polysaccharides of wild and mutant strains were investigated.

2. Materials and methods

2.1. Strain

The wild strain of *P. igniarius* NO.5.95 was purchased from China General Microbiological Culture Collection Center, CGMCC (Beijing, China) and grown in solid slant medium, potato dextrose agar (PDA) slant.

2.2. Media and Cultural Conditions

The stock culture was maintained on potato dextrose agar (PDA) slants. The slants were incubated at 26 °C for 7 days and then stored at 26 °C. The mycelium culture prepared for the protoplast was statically grown in a 250 mL flask containing 100 mL of basal potato medium (PDB) at 26 °C. The screened *P. igniarius* strain was initially grown on a PDA medium in a petri dish, and then transferred into the seed medium by punching out 5 mm of the agar plate culture using a cutter. The seed culture was grown in a 250 mL flask, which contains 100 mL of PDB basal medium, at 26 °C placed on a rotary shaker incubator (130 rev min^{-1}) for 8 days. The flask-culture experiments were performed in a 250 mL flask containing 100 mL of medium inoculated with 10% (v/v) of the seed culture. The fermentation medium consisted of the following components: maize flour (50 g/L), bran (15 g/L), mulberry shoot powder (10 g/L), KH_2PO_4 (2 g/L), $MgSO_4$ (1 g/L).

2.3. Protoplast generation

P. igniarius mycelia were cultivated and statically grown in a flask for 10 days. The mycelia were then harvested by centrifugation at 10,000*g* for 10 min before they were triturated and homogenized by a sterilized Eppendorf tube. The mycelia were then incubated in an incubator shaker (100 rev min^{-1}) and allowed to undergoenzymatichydrolysis in 1% lywallzyme mixed with 0.25% driselase (Sigma) containing 0.6 mol/L of mannitol. The enzyme solution was filtered through a sterile 0.22-μm millipore filter before use. After incubation, the protoplasts were obtained by centrifuging at 5000*g* for 10 min and then were washed twice with sterile osmotic stabilizer 0.6 mol/L mannitol to remove the enzymes. Finally, pure protoplasts were obtained by using sterile G3 sand core funnel.

2.4. Mutagenesis and rational screening

Each 1 mL of appropriately diluted suspension was placed into a 1.5 mL Eppendorf tube. The fiber of the laser (LJL40-HA, Shanghai Institute of Laser Technology, China) was vertically irradiated through the tube top, which had an output power of 40 mW and transmission efficiency of 80%. The distance between the suspension liquid level and fiber optic terminus was 10 cm, and the spot radius was 10 mm. The exposure time was 30 min. The suspension was then spread over a plate, which had a diameter of 70 mm. The plate was placed 20 cm from the 15-W UV lamp. The UV directly radiated suspension in the plate. To avoid any photo recombination influence, the irradiation was performed in the dark. After the exposure, the irradiated suspension was diluted, and 100 μL of sample from each diluted suspension was plated on screening agar plates, which contained PDA media supplemented with 0.6 mol/L of mannitol. Although all the operations were performed in the dark to prevent light repairs, red light was allowed. The samples wrapped with black paper were placed in an incubator, and the temperature was set to 26 °C. Black paper was removed after 24 h. The protoplast suspension without any physical radiation was used as control.

The performances of all the colonies on each screening plate were investigated after 7 days of cultivation at 26 °C. After each run, the positive mutants, which exhibited excellent and stable genetic traits, were successively cultured five times on separate agar plate PDA media. The strain that exhibited faster mycelium growth rate than that of the CK (average growth rate plus the coefficient of variation) was regarded as a positive mutant strain. The fermentation experiment on the selected mutants was used to determine the biomass yield capacity. The mycelia in submerged culture were washed with distilled water and then freeze dried in the vacuum before they were weighed.

2.5. Extraction of endo-polysaccharides from P. igniarius

Dried mycelia (100*g*) ground in a disintegrator and mixed with 95% ethanol statically for 10 times overnight. The mixtures were centrifuged at 10,000*g* for 10 min, and the precipitate was extracted with 1000 mL of distilled water at 100 °C thrice for 2 h. The supernatant was collected through centrifugation (5000 rpm, 10 min) and then concentrated