Original paper

Nitrous Acid and Diethyl Sulfate, as Chemical Mutagenic Agents, to Improve the Biomass, Metabolites, Enzyme Activity, and Antioxidant Activity of Wild *Phellinus igniarius*

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Various secondary metabolites with antioxidative effects have been reported to exist in *Phellinus igniarius*. However, the trace quantity of these metabolites in normal fermentation conditions deeply limits its medical values and potent clinical application. Some elicitors such as bioinducer and chemical inducer can improve the productivity of secondary metabolites which have certain biological activities in plants and fungus. In this study, nitrous acid (NA) and diethyl sulfate (DES), as potent killing and mutagenic chemical agents, to treat *P. igniarius* and enhance the metabolites contents. The variation of biomass, metabolite production, enzyme activity, and antioxidant efficacy of two mutant strains were investigated. The mutant strains exhibited better viability and produced more secondary metabolites. Altogether, these results indicate that chemical mutagenesis is an effective strategy to obtain high-activity mutant strains of *P. igniarius* with high yields of total flavonoids and total polysaccharides, the main constituents with antitumor and antioxidant activities.

Keywords: chemical mutagens, *Phellinus igniarius*, antioxidant activity, secondary metabolites

Introduction

Phellinus igniarius (L.ex Fr.) Quél belongs to the family *Hymenochaetaceae*. As a perennial edible fungus, the effective anti-cancer capabilities of *Phellinus igniarius* (*P. igniarius*) have been a popular topic of international studies (Hsin *et al.*, 2017; Li *et al.*, 2015a; Zhou *et al.*, 2014). *P. igniarius* contains many bioactive compounds that have been reported to possess antibacterial, antioxidative, antitumor, and antimutagenic activities and has been widely used in China, Japan, and Korea for many years (Konno *et al.*, 2015; Wang *et al.*, 2007; Wang *et al.*, 2005; Zhang *et al.*, 2014). The secondary metabolites of *P. igniarius* feature biological activities and include polysaccharides, polyphenols, flavonoids, and other organic compounds (Gao *et al.*, 2017; Lee *et al.*, 2015; Lee *et al.*, 2013). Given the limited and unstable supply of wild *P. igniarius*, efforts to achieve artificial fermentation have caused extensive

concern in recent years (Hur, 2008). In previous studies, we obtained various structurally unusual secondary metabolites possessing novel biological activities by submerged fermentation (Wu *et al.*, 2010). However, the content of these metabolites in normal fermentation conditions is extremely low, thereby limiting the use of the species in further medical applications.

In this work, *P. igniarius* was induced by chemical mutagens, including diethyl sulfate (DES) and nitrous acid (NA), to accelerate its pace of growth and increase the production of secondary metabolites. DES and NA are reproducible, simple, and efficient mutagenic compounds that could damage DNA, often resulting in gene mutations (Hoffmann, 1980). Many studies have shown that the chemical mutation method is an effective strategy for breeding high-producing strains (Li *et al.*, 2015b; Zhang *et al.*, 2013). The present study aims to investigate and analyze the effects of inducing treatments on *P. igniarius* on

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promoting changes in enzyme activity, metabolite production, and antioxidant activity.

Materials and Methods

P. igniarius (CGMCC 5.95) was purchased from China General Microbiological Culture Collection Center (CGMCC, Beijing, China) and kindly provided by Professor Dai Jungui (Biosynthesis Lab, Institute of Medicine of Chinese Academy of Medical Sciences, Beijing). It was stored in slants of modified Martin medium (MMM) (including tryptone, 5 g; yeast extract powder, 2 g; glucose, 20 g; K_2HPO_4 , 1 g; $MgSO_4$, 0.5 g; agar, 20 g; distilled water, 1 L; pH 6.2–6.5) at 4 °C at Ningxia Engineering and Technology Research Center of Modern Hui Medicine, Ningxia Medical University, China. The medium was autoclaved at 121 °C for 30 min prior to culture.

NA and DES mutagenesis *P. igniarius* was activated by incubating at 28 °C on an MMM agar plate for 1 week. Then, the mycelia were transferred to a 250 mL flask containing glass beads and normal saline. The flask was shaken for 20 min to prepare a mycelium suspension. About 2 mL of mycelium suspension was added to 0.005 μ M sodium nitrite solution ($NaNO_2$) containing acetate buffer (2 mL, pH 4.4), and the mixture was incubated at 28 °C for 5, 15, 30, 45, 60, or 90 min. At each time interval, 0.5 mL of the above solution was mixed with dibasic sodium phosphate solution (Na_2HPO_4 , 1 mL, pH 8.6) to end NA treatment. Finally, 0.2 mL each of mycelium suspension was spread on MMM agar plates. After incubation at 28 °C for 5–7 d, the surviving colonies were counted, and the dose-survival curve was plotted in terms of time of NA exposure as a function of percentage of survival. Plates that showed less than 5 % survival were further screened for dominant strains. According to our previous research, we applied 0.5 % DES to the mycelium suspension for 4 min (Wang *et al.*, 2015). After establishing the optimal breeding method through screening of mutation agents and conditions, the dominant strains were selected based on the biomass and laccase activity, as well as hyphal density. The mutant strains which named PINA-1 and PIDES-2 were selected through screening testing. These strains were subcultured in an MMM agar plate in the dark until they showed active proliferative capacity and then stored at 4 °C before use.

Biological assay After *P. igniarius*, PINA-1, and PIDES-2 were inoculated on MMM agar plates, the variation in colony diameter of the three strains was determined as the growth index. To assess the change of biomass, mycelia of the same age and volume taken from the MMM agar plate and placed in modified Martin liquid medium. Mycelia grown in shake flasks with baffles at 28 °C and 180 rpm. At 30 days of cultivation, mycelia were removed by filtration from the fungal culture, washed three times with sterile distilled water, dried in a hot air oven at 65 °C until a constant weight was reached, and weighed for growth analysis. Laccase activity was determined using

guaiacol as a substrate (MMM contains 0.04 % guaiacol). Guaiacol could produce a bright and even red-brown oxidative circle, and the diameter and color of this circle were used as a testing index for the active strains.

Observation of morphology Mycelium pellet was obtained from liquid medium and placed in a glass slide for morphological examination under a light microscope. The morphology of hyphae was characterized by scanning electron microscope (SEM, Hitachi S-3400N, Japan). The hyphae samples were fixed on a specimen holder with an aluminium tape and sputtered with a thin layer of gold prior to micrograph examination. The SEM was run under high vacuum condition at an accelerating voltage of 10 kV.

Sample preparation The three strains were cultivated via the method described above, and each strain was organized into three sets of five. Then, 5 mL of fungal culture supernatant (FCS) from each flask was harvested every third day of the 30 days culture period. The collected samples were transferred into centrifuge tubes and stored at -20 °C before use.

Quantification of secondary metabolites

Total phenolics (TP) The polyphenols of the culture samples (5 mL) were extracted three times with ethyl acetate (EtOAc, v/v, 1:1). The EtOAc extract was then evaporated under reduced pressure and dissolved in 1 mL of 60 % ethanol. TP content was determined according to the Folin–Ciocalteu method with some modifications (Fujita *et al.*, 2017). The extracts were mixed with 0.6 mL of 2 N Folin–Ciocalteu's reagent. After standing for 8 min in the dark, 1.2 mL of 10 % Na_2CO_3 was added to the mixture, and the resulting solution was diluted with 60 % ethanol to a volume of 10 mL. The solution was kept in the dark for 30 min at room temperature, after which its absorbance was assessed at 760 nm. Gallic acid was used to establish the calibration curve, and TP content was reported in terms of milligrams per milliliter of culture broth of gallic acid equivalents (GAE).

Total flavonoids (TF) TF content was determined using the method described by the previous literature (Zhang *et al.*, 2016). Briefly, the fermentation liquor was centrifuged for 10 min at 8000 rpm. Then, the supernatant was combined with 1 mL of 70 % ethanol solution and 1 mL of 5 % (M/V) $NaNO_2$ solution in 60 % ethanol up to 6 mL and kept for 6 min at room temperature. Addition of 1 mL of 10 % (M/V) $Al(NO_3)_3$ solution that had been incubated for 6 min was followed by addition of 10 mL of 4 % (M/V) NaOH solution and 70 % ethanol to achieve a total volume of 25 mL. After incubating for 15 min at room temperature for color development, the spectrum of the solution was determined at 510 nm. The spectrum of 70 % ethanol was considered the contrast. The results were expressed as milligrams per milliliter of culture broth of rutin equivalents (RE).

Total polysaccharides (TPS) The phenol-sulfuric acid method was employed to assess polysaccharide content (Kushwaha and Kates, 1981). The fermented liquid sample was

diluted 100 times with sterilized distilled water before detection. Exactly 1 mL of the diluted sample was treated with 1 mL of 5 % phenol solution and 5 mL of sulfuric acid. The absorbance at 490 nm was determined after the mixture was incubated for 30 min at room temperature. The TPS concentration in the fermented liquid was expressed as milligrams per milliliter of culture broth of glucose equivalent (GE).

Ferric reducing antioxidant power (FRAP) assay The FRAP values of the fermentation samples were determined by the method described by the previous literature (Yan *et al.*, 2016). The FRAP reagent contained 0.3 M acetate buffer (pH 3.6), 0.1 M 2,4,6-tris(2-pyridyl)-1,3,5-triazine in 0.4 M HCl, and 0.2 M ferric chloride hexahydrate at a volume ratio of 10:1:1. The culture broth (0.1 mL) of different periods was mixed with 2.9 mL of the reagent for 10 min at room temperature. The absorbance of the solution was assessed at 593 nm. Ferric sulfate solutions (2 mM) were used for calibration, and the results were expressed in $\mu\text{g}/\text{mL}$ (as FeSO_4 equivalents $\mu\text{g}/\mu\text{g}$).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay The ability of the culture broth to scavenge DPPH radicals was assessed by the reported method with minor modifications (Luo *et al.*, 2016). The reaction system was mixed with 1 mL of culture broth and 2 mL of 400 μM DPPH–ethanol solution and incubated for 30 min at room temperature in the dark. The absorbance was measured at 517 nm using a UV spectrophotometer. The initial medium was used as a blank control at the same dosage. The ability to scavenge DPPH radicals was calculated using the following equation:

$$\text{DPPH-scavenging activity (\%)} = [1 - A_s/A_0] \times 100\% \quad \cdots \cdots \text{Eq. 1}$$

where A_0 is the absorbance of the blank and A_s is the absorbance of the samples.

Enzyme activities The polyphenol oxidase (PPO), peroxidase (POD), and phenylalanine ammonia-lyase (PAL) activities of all strains were examined using commercial chemical assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol.

Statistical analysis All experiments were performed in triplicate. Means were expressed with their standard error (SE) and compared by one-way ANOVA to evaluate the impacts of chemical induction on *P. igniarius*. A P value of <0.05 was considered to indicate statistical significance.

Results and Discussion

Relationship between NA mutagenesis time and death rate NA can remove the amino group of bases to cause gene mutation, which is beneficial in screening dominant strains during microbial genetic breeding. In this work, different concentrations of NA were chosen as mutagenic agents. The

Fig. 1. The relationship of NA mutagenesis time with death rate

series of working solutions inflicted damage on *P. igniarius*, resulting in mycelium death at higher concentrations of NA. Therefore, the concentration of NA was reduced and the mutagenesis time was extended accordingly to optimize the conditions of NA mutagenesis. Fig. 1 shows the relationship between fatality rate and mutagenesis time at dose of 5 mM NA treatment. The fatality rate increased gradually with increasing mutagenesis time. After 60 min of treatment, only seven clones were regrown on the screening solid plate, and a fatality rate of 97.2 % was observed. The clones were picked up and inoculated on solid slant medium to culture three stable generations from which a dominant strain was obtained and named PINA-1.

The change of growth characteristics The influence of two induction factors on the cell viability and biomass production of *P. igniarius* was evaluated (Fig. 2A). The results showed that the mycelium dry weight of PIDES-2 was notably higher than that of *P. igniarius* by approximately 20.9%; by comparison, the mycelium dry weights of PINA-1 and the original strain showed no significant difference. In former research, the mycelial biomass of PIDES-1 was lower than *P. igniarius*. (Wang *et al.*, 2015) The final colony diameters of PINA-1 and PIDES-2 were greater than of that of the original strain in solid medium by approximately 8 %. Laccase activity was also observed on 0.04 % guaiacol MMM agar plates. While the colony diameter of wild type *P. igniarius* was wider compared to that of the mutant strains, the oxidative circle of the wild type was obviously smaller and lighter in color than those of the mutants (Fig. 3).

Structural change of mutant strains The mycelial pellet of PIDES-2 showed a loose form under microscopic observation; by contrast, the original *P. igniarius* hyphae branches were intimately entwined (Figs. 3). The heightened integration of hyphae branches could stunt the growth and decay of the pellets. The branching hyphae of PIDES-2 were closely packed and radiated outward, resulting in an increase in its dry weight. The SEM was employed to analyze the morphology and

Fig. 2. Biomass productions for original *P. igniarius*, PINA-1, and PIDES-2 after 30-day fermentation. (a) Mycelium dry weight. (b) Growth of colony diameter

*indicated significant differences as compared to *P. igniarius* at $p < 0.05$.

microstructure of the fungal hyphae. As seen in Fig. 4, the diameter of hyphae of wild *P. igniarius* was around 3.85 μm , while the hyphae of mutant strains were with the diameter of around 2 μm . The hyphae of *P. igniarius* was straight and had less bifurcation, it also had a smooth surface with some protuberance. After chemical induction, the hyphae became finer and had more branches. The surface of PINI-1 was rough and the surface of PIDES-2 was wrinkled. Thus, chemical mutagenesis can cause a change of mycelium morphology, which affected biomass production and metabolism probably. This work showed that DES and NA mutagenesis caused variability in the apparent structure of a fungal mycelium and affected the viability of the fungus itself.

Analysis of chemical constituents The effects of chemical mutagens on the TP, TF, and TPS contents of the strains are presented in Figs. 5a, 5b, and 5c, respectively. The TP contents of the untreated fungus remained markedly higher than those of the treated strains over nearly the entire duration of cultivation and peaked at 21 d. In the initial stages of fermentation, no

significant difference of TP contents was found among three strains. The TF amounts in the induced strains exhibited an increasing trend. The TF contents of PINA-1 and PIDES-2 were greater than that of *P. igniarius* by 62.4% and 73.9%, respectively, by day 24 of cultivation (Fig. 5b). The TF contents of PIDES-2 reached maximum yields at day 27, and dipped slightly like other strains. As shown in Fig. 5c, the TPS production of the three strains first increased from 20 g/L (primary culture contains 20 g/L glucose) to the maximum amount about 6–9 d into cultivation and then declined rapidly thereafter. The TPS contents of PINA-1 and PIDES-2 approached peak levels on day 9 and exceeded that of *P. igniarius* by 25.65% and 26.13%, respectively. The TPS contents of all strains started to decline on day 12, especially PINA-1 and PIDES-2. The PINA-1 produced more polysaccharides in later period of culture, though a similar tendency of TPS contents existed in all. It was reported that total flavenes and exopolysaccharides production of *P. igniarius* was enhanced 1.78- and 1.33- fold via chromosomal integration with the *Viteoscilla* hemoglobin gene. (Hu Zhu *et al.*, 2011) The result showed that chemical mutagen is an alternate way to get the same effect, it could also efficiently enhance total flavonoids and polysaccharides production in *P. igniarius*.

Analysis of enzyme activity POD, PPO, and PAL activities were assessed from the FCS of the strains, as shown in Fig. 5. PPO and POD are extracellular enzymes produced by many fungi and higher plants with functions in lignin and aromatic hydrocarbon degradation. The production of these enzymes is essential to the normal growth of microorganisms. The PPO activity of all the three strains peaked twice during the incubation period. The PPO activities of PINA-1 and PIDES-2 increased rapidly on the third day of incubation, while that of *P. igniarius* rose sharply after the ninth day, remaining 38% higher than those of the others. These results show that the oxidative circle (Fig. 3) of PINA-1 is correlated with its increase in PPO activity. The PPO activity of PINA-1 peaked on day 21 and then decreased thereafter, similar to those of *P. igniarius* and PIDES-2. The POD activities of *P. igniarius* and PIDES-2 peaked on day 9 of inoculation and then declined steadily over time. In fact, at day 9 of incubation, PIDES-2 demonstrated over twice the POD activity of other strains. The POD activities of PINA-1 and PIDES-2 were greater than that of *P. igniarius* throughout the incubation process. PAL is an endoenzyme with a key role in the phenylpropanoid pathway. The PAL activities of PINA-1 were markedly higher than those of *P. igniarius* and PIDES-2 at the end of the incubation period, likely because of autolysis of microorganisms in its culture. The maximum POD and PAL activities of the mutant strains were higher than that of the untreated fungus at different time points. In general, chemical mutagenesis exerts an important impact on the accumulation of metabolites.

Analysis of antioxidant activity The antioxidant capacity

Fig. 3. The morphology and activity change of strains after mutagenesis.

of fungus can be attributed to synergistic reactions between different compounds, such as polyphenols, flavonoids, and other chemical substances. In this study, the antioxidant properties of the FCS obtained from the three strains were determined by testing for scavenging activity against DPPH and FRAP. The DPPH free-radical scavenging activity of *P. igniarius* and mutant fungus first increased and then decreased, while that of PINA-1 increased again at 24 day. Fig. 6a clearly indicates that the DPPH scavenging activity of PIDES-2 is consistently lower than that of *P. igniarius*, and the difference between strains was significant ($P < 0.05$). These results are consistent with the total phenolic contents of the three strains. The polyphenols of *P. igniarius* are effective antioxidants (Shou *et al.*, 2016), and the FRAP method could be used to evaluate ferric ion reducing antioxidant power instead of direct free-radical scavenging activity. In contrast to the result of DPPH scavenging activity, the FRAP activity of PINA-1 remained higher than that of the untreated fungus for approximately 15-day into the 30-day incubation period. However, the DPPH scavenging activity of PINA-1 was near or below that of *P. igniarius* until 21 day. After that, the scavenging activity of PINA-1 raised and higher than *P. igniarius*. The average level of FRAP activity in PIDES-2 exceed the level of untreated

strain from 21-day cultivation. This results is different to the results from DPPH experiment. A significant difference was observed between the FRAP of *P. igniarius* and those of the other strains after day 18 ($P < 0.05$). The total phenolic content in the fermentation cultures of the mutant strains, particularly in PIDES-2, decreased.

This paper presents a novel method for producing valuable secondary metabolites and extracting new compounds by the fermentation culture of *P. igniarius* or other precious fungi for food, it has the significant value in science and application.

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Conflict of interest

The authors declare no conflict of interest.

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Fig. 4. SEM images of the hyphae with two different magnifications. (A, B) *P. igniarius*. (C,D) PINA-1. (D,E) PIDES-2.

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Fig. 5. Enzyme activities of three strains. (a) Total phenolic (b) Total flavonoids (c) Total polysaccharides (d) PPO activity (e) POD activity (f) PAL activity. *indicated significant differences as compared to *P. igniarius* at $p < 0.05$.

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Fig. 6. Antioxidant activity of three strains. (a) DPPH free radical scavenging activity. (b) Ferric reducing antioxidant power. *indicated significant differences as compared to *P. igniarius* at $p < 0.05$.

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