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Quantitative and morphologic analysis on exopolysaccharide and biomass production from a truffle endophytic fungus *Hypocreales* sp. NCHU01☆

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ABSTRACT

In this study, exopolysaccharides (EPS) produced from *Hypocreales* sp. NCHU01 was carried out in the submerged cultivation. To find the optimal conditions, factors such as carbon and nitrogen source concentrations, pH, temperature, magnesium ion, and potassium ion levels were considered. The results showed that the optimal conditions were as follows: sucrose, yeast extract, magnesium sulfate and potassium dihydrogen phosphate were 10.6 g/L, 10.92 g/L, 1.0 g/L and 1.01 g/L, respectively. The optimization conditions were set at 25 °C with an initial pH 6.5. The maximum EPS production was about 1329 ± 23 mg/L, which was 3.34-fold to that of the original condition. The sucrose consumption rate was calculated to be 8.89 (g/L/day). Biomass production and EPS production rates were 3.92 and 0.18 (g/L/day), respectively. The yields for EPS and biomass were calculated to be 0.02 and 0.44 (g/g), respectively. The study displayed an increasing molecular weight distribution in the harvested EPS over a longer cultivation time. The quantitative relationship between mycelial biomass and EPS production was also built.

1. Introduction

Plants and underground mushrooms commonly act as hosts to a multitude of microorganisms including parasites, symbionts, endophytes, epiphytes and mycorrhizal fungi. Some of them may be able to produce the secondary metabolites closely related to their hosts [1]. Nowadays, the endophytic fungi residing in the intercellular spaces of plants and mushrooms have become important sources for discovery of the novel and bioactive secondary metabolites [2].

Truffles are renowned for environmental and forestry applications owing to the advantage that mycorrhizae provide for host plants, and for their unique and characteristic aroma, making them the highest economically valued edible fungi in the world [3,4]. They are also regarded as complex microhabitats hosting bacteria and yeasts. It has been demonstrated that they contain a great number of bacteria and several yeasts in their gleba. Three kinds of endophytic fungus, *i.e.*, *Paecilomyces fumosoroseus*, *Verticillium insectorum* and *Verticillium leptobactrum*, belonging to *Hypocreales* order have been isolated from *Tuber magnatum* [5]. An endophytic fungal strain isolated from the fruiting body of *T. magnatum Pico* was identified as *Hypocreales* sp. according to its internal transcribed spacer (ITS) sequence. Based on the rDNA-ITS sequence (GenBank accession number HQ608125) analysis together with the morphologic traits, this strain was grouped into the order of *Hypocreales*. Nowadays, there is no paper concerned about endophytic fungus of truffle to the *secondary metabolites production*.

In a pre-study, the endophytic fungus *Hypocreales* sp. NCHU01 isolated from *T. magnatum* fruiting body was proved to be capable of biomass, triterpenoids and exopolysaccharides (EPS) productions. Owing to its specificity as a truffle accompanying strain, a process for the simultaneous production of mycelial biomass and EPS by the submerged fermentation was developed. The study concerning about EPS production *via* an endophytic strain of truffle was the first time mentioned.

In the development of submerged culture, carbon and nitrogen sources were the critical sources in the medium for obtaining high EPS yield. Metal ions, such Mg^{2+} and K^{+}, acting as the enzyme

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activators in the metabolic pathway, were also considered as the key factors [6,7]. Besides, the temperature and the initial pH of the fermentation are also essential for achieving a promising cultivation. The response surface methodology (RSM) was applied to statistically analyze and optimize the conditions governing the *Hypocreales* sp. NCHU01 fermentation process [8–10]. The morphologic and the quantitative analysis on the mycelial growth were carried out. The relationship between EPS molecular weight distribution and the mycelial biomass was also built in the cultivation process.

2. Materials and methods

2.1. Microorganism

The strain used in this study was isolated from the fruiting body of *T. magnatum Pico* and was identified as *Hypocreales* sp. NCHU01 according to its internal transcribed spacer (ITS) sequence based on the rDNA-ITS sequence (GenBank accession number HQ608125). It was maintained on a potato-dextrose-agar slant. The slant was inoculated with mycelia and incubated at 25 °C for 3 days. Seed medium consisted of the following components (g/L): glucose, 5; peptone, 1; yeast extract, 1; KH_2PO_4, 0.2; $MgSO_4{\cdot}7H_2O$, 0.2. For the seed culture, 90-mL of the seed medium with an initial pH of 6.0 was prepared in a 250-mL flask, and then 10-mL mycelium suspension from a slant culture was used as inoculums, followed by a 3-day incubation at 25 °C in a rotary shaker (100 rpm).

2.2. Flask cultivation

A shake-flask culture was performed in a 250 mL Erlenmeyer flask containing 100 mL of the main medium. The main medium consisted of the following components (g/L): sucrose, 5; yeast extract, 2; KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; vitamin B1 hydrochloride, 0.15. A volume of 90 mL medium in a 250 mL shake flask was inoculated with 10 mL seed broth (with *ca.* 350–450 mg cell dry weight per liter, mg CDW/L). The pH was adjusted to the desired value by the addition of either 0.1 N HCl or 2.5 M NaOH. Media were sterilized at 121 °C for 20 min and the carbon source was autoclaved separately. The flasks were incubated on a rotary shaker at 100 rpm and 25 °C for 57 days. Time courses of the biomass, sucrose consumption, and the EPS production were monitored during the whole cultivation. Mycelial morphology was photographed using an optical microscope (BX40,Olympus, Japan) equipped with a CCD camera (Micrometrics® 318CU, Laite tech. Co., Taipei, Taiwan). The cell mycelia area (A_m) and perimeter (P) were obtained directly from the CCD pictures using the analysis software (Micrometrics SE Premium Version 3.0). The pellet hydraulic radius (D_m) was calculated based on the equation $D_m = A_m/P$ [11].

2.3. Experimental design

A two-level fractional factorial design (FFD) was employed to determine the path of steepest ascent (PSA) leading toward the neighborhood of the optimum process response. Central composite design was then employed to fully elucidate the response surface near the optimum, and provide an approximation to the true response. The RSM was used for the optimization of six factors (carbon source, nitrogen source, magnesium ion, potassium ion, temperature and initial pH) to yield the maximum EPS production from *Hypocreales* sp. NCHU01.

Concerning the interaction between the experimental factors, a 2^{6-2} partially FFD was applied. According to the experimental data, a mathematical model related EPS concentration to carbon source and nitrogen source; culture temperature and initial pH value were established *via* multiple regression: furthermore the optimal value was obtained. All of the experiments were designed and analyzed by the software Design-Expert (version 6.0.2).

2.4. Assays

2.4.1. Cell biomass

Three flasks were taken each time. Dry cell weight (DCW) was obtained by filtering the broth through a mesh with 30 mm pore size, followed by washing the cells three times with distilled water and drying at 60 °C to a constant weight.

2.4.2. Residual sucrose analysis

The assay was performed with a PU-980 HPLC pump (Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-980, Jasco, Tokyo, Japan) and an ASP-2-Hypersil column (4.6 mm i.d. × 250 mm, 5 μm, Thermo Fisher Scientific Co., USA) kept at a 30 °C oven (Analab, Taipei, Taiwan). The mobile phase consisted of acetonitrile and de-ionized water (70/30, v/v). The flow rate was set at 1 mL/min and the injection volume was 20 μL. The data was analyzed using integration software (SISC 3.2, Scientific Information Service Co., Taipei, Taiwan).

2.4.3. Exopolysaccharide analysis

For the determination of EPS amount, the mycelial biomass in the broth was removed by filtration, followed by the addition of 95% (v/v) ethanol at four times the volume to precipitate the crude EPS. Then the EPS was collected by centrifugation at 10,000 g. The crude EPS was re-suspended in 1 M NaOH at 60 °C for 1 h, and the total sugar concentration was measured using the phenol–sulfuric acid method [12].

2.4.4. Molecular weight analysis

Molecular weight of EPS was estimated based on a calibration curve built by the pullulan standard (5.9–1600 kDa) (Showa Denko K.K, Tokyo, Japan). A gel permeation chromatography (Analytical Scientific Instruments, Richmond, CA) coupled with a RI-2000 detector (Showa Denko K.K, Tokyo, Japan) was used. Three columns, *i.e.*, KB-802.5, KB-804 and KB-805 (0.8 cm × 30 cm, Showa Denko K.K, Tokyo, Japan) were connected in series. DI-water was used as the mobile phase. The column temperature was set at 35 °C. The flow rate was set at 1.0 mL/min and the injection volume was 20 μL [13]. In addition, the percentage of high molecular weight EPS was defined as (Area of EPS with MW higher than 300 kDa)/(Area of total EPS).

2.4.5. Statistical assay

Multiple flasks were run at the same time, and three flasks were used each time for daily sampling. Each data point was expressed as a mean with a standard deviation. The optimal study was designed and analyzed with the Design-Expert program (version 6.0.2). The comparison between the results was carried out by performing the Tukey test ($P \leqq 0.05$).

3. Results and discussion

3.1. Effect of carbon and nitrogen sources

In order to seek the best carbon source for cell growth and the EPS production, different carbon sources, *i.e.*, glucose, sucrose, fructose, lactose, and maltose, were tested in the submerged fermentation of *Hypocreales* sp. NCHU01 [14]. The results are displayed in Fig. 1A. Sucrose was found to be the most effective carbon source for EPS production. The highest EPS production was 397 ± 20 mg/L. Therefore, sucrose was chosen as the carbon source for subsequent studies.