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Glucose fed-batch integrated dissolved oxygen control strategy enhanced polysaccharide, total triterpenoids and inotodiol production in fermentation of a newly isolated *Inonotus obliquus* strain

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ABSTRACT

Submerged fermentation is promising for producing secondary metabolites of *Inonotus obliquus,* a folk medicine to treat cancer and other diseases. However, there limited progress work in strain selection or process optimization in order to enhance production of its bioactive metabolites. In this work, a new *Inonotus obliquus* strain was isolated from the sclerotia collected in Belarus, which was identified by ITS sequencing and phylogenetic analysis. The dissolved oxygen (DO) condition was optimized, it showed that 50% DO level facilitated secondary metabolites production. Finally, a glucose fed-batch integrated DO control strategy was developed by feeding 10 g/L glucose when residual sugar decreased to 10 g/L, while controlling DO level at 50%. The biomass, polysaccharide, triterpenoids and inotodiol production enhanced by 52%, 31%, 54% and 65%, respectively. This study demonstrated that the new strain shown good fermentation property. Glucose fed-batch integrated DO control strategy effectively enhanced secondary metabolites production in fermentation of *Inonotus obliquus*.

1. Introduction

The medicinal mushroom *Inonotus obliquus* (*I. obliquus*), commonly known as chaga, is a white rot fungus that belongs to the family Hymenochaetaceae of Basidiomycetes. It has been used as a folk remedy to treat gastrointestinal cancer, cardiovascular disease and diabetes for a long time in Russia and East-European countries [1,2].

I. obliquus has many therapeutic effects, such as anti-tumor [3–5], anti-hyperglycemic [6] and immuno-modulatory [7,8]. The phytochemical research revealed that triterpenoids and polysaccharide were two important active constituents of *I. obliquus*. Phytochemical investigations of *I. obliquus* mostly described lanostane triterpenoids, most of which were exclusive to *I. obliquus* in nature, which showed antitumor activities. Zhao et al. reported that four triterpenoids exhibited strong cytotoxicity against A549 tumor cell lines, and seven triterpenoids exhibited moderate cytotoxicity against A549, HT29, Hela or L1210 tumor cell lines [3]. Inotodiol, a lanostane triterpenoid, exhibited an anti-tumor promoting activity in vivo carcinogenesis test [9]. Nomura et al. investigated the antitumor activity of the triterpenoids form *I. obliquus* sclerotia. Among the triterpenoids examined, only inotodiol inhibited mouse leukemia P388 cell proliferation [10]. It was also reported that triterpenoids from submerged culture of *I. obliquus* had anti-hyperglycemic activity by inhibiting alpha-glucosidase activity [6].

Due to host specificity, rarity in nature, and slow growth rate, the sclerotia of *I. obliquus* are not a reliable source for an industrialized production of these valuable bioactive metabolites [11]. Submerged cultivation of medicinal mushrooms is a promising alternative for the efficient production of secondary metabolites. But much work should be done to enhance *I. obliquus* polysaccharide and triterpenoids production in submerged fermentation process.

Higher fungi fermentation is an aerobic process. The viscosity of fermentation broth increased in higher fungi fermentation process, because of the increase of cell concentration, changes in microbial

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morphology, and the accumulation of extracellular products [12]. Dissolved oxygen (DO) is an important factor affecting the higher fungi fermentation yield. Different DO control strategy was developed in higher fungi fermentation process [13–15]. Tang et al. reported a pH-shift and DOT-shift integrated fed-batch fermentation process for production of ganoderic acid and *Ganoderma* polysaccharide [13]. A two-stage DO control strategy was developed in fermentation of *Cordyceps militaris* [14]. DO was controlled at 60% from the beginning of cultivation and then shifted to a lower control level of 30% when specific cordycepin formation rate started to decrease, and the cordycepin production enhanced by 15% [14]. But the effect of DO control strategy was not studied in *I. obliquus* fermentation process.

Fed-batch was a useful strategy in fermentation process [16–20]. A sucrose fed-batch fermentation process of *Ganoderma lucidum* (*G. lucidum*) was developed for production of polysaccharide and triterpenoids by feeding of sucrose when the residual sugar below to 15 g/L [19], and the biomass and polysaccharide production enhanced 62% and 50%, respectively. A pH, DO-shift integrated fed-batch fermentation strategy enhanced ganoderic acid and polysaccharide production by medicinal mushroom *G. lucidum* [18]. The fed-batch fermentation by glucose feeding was performed in *Grifola frondosa* fermentation process when the glucose concentration was lower than 5 g/L, which greatly enhanced the production of biomass and extracellular polysaccharide [21]. However the fed-batch fermentation was not studied in *I. obliquus* fermentation process, which might be a useful strategy to enhance *I. obliquus* polysaccharide and triterpenoids production.

There are limited reports studied the effect of DO or fed-batch strategy on simultaneous production of polysaccharide and triterpenoids in fermentation of *I. obliquus* until now. In this work, a new *I. obliquus* strain was isolated and identified from *I. obliquus* sclerotia, which were collected in Belarus. Then, the impact of DO on fermentation of the new *I. obliquus* strain was investigated in order to enhance polysaccharide, total triterpenoids and inotodiol production. A glucose fed-batch integrated DO control strategy was developed by feeding glucose when the residual sugar was lower than 10 g/L, while controlling the DO at 50%. The polysaccharide, total triterpenoids and inotodiol production were enhanced greatly.

2. Methods

2.1. Strain isolation and identification

I. obliquus sclerotia were sampled in Belarus. The surface of *I. obliquus* sclerotia were disinfected with 75% ethanol, and the disinfected sclerotia were cut into small pieces. The *I. obliquus* pieces were inoculated on PDA plates with penicillin-streptomycin. It was cultured for 5 days at 30 °C until the mycelia formed. The mycelia were picked up with a disinfected inoculating shovel to a new PDA plate, and cultured for 7 days at 30 °C until the mycelia covered the plate. The mycelia was harvested and ground in liquid nitrogen. The DNA was extracted according to the protocol of fungus genome extraction kit (Sangon Biotech Company, China).

The new strain was identified by ITS sequencing. Two primers, ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3), were used for amplification of ITS domain in the polymerase chain reaction (PCR). The PCR mixture volume was 50 μL containing 25 μL PCR buffer, 400 μM dNTP, 200 μM each of primers, 2.5 U Taq polymerase, 150 ng template DNA. The PCR was accomplished using PCR machine (Eppendorf Company, Germany) with an initial step of 94 °C for 5 min, followed by 35 cycles of 94 °C, 56 °C and 72 °C for 45 s, 45 s and 60 s, and final step at 72 °C for 10 min to ensure that the PCR product extension is completed. The sequencing of amplified ITS fragments were performed in Sangon Biotech (Shanghai, China).

2.2. Cultivation condition

The *I. obliquus* was inoculated on potato dextrose agar slants and cultured at 30 °C for 7 days. The mycelia were scraped off and suspended in the sterilized water, and inoculated to the seed medium. Seed medium consisted of the following components (g/L): glucose 30, oat flour 10, yeast extract 10, KH_2PO_4 1, $MgSO_4$ 0.5. For the first-stage preculture, 90 mL seed medium with initial pH of 5.5 was prepared in a 500 mL flask, and then 10 mL mycelium suspension from a slant culture was inoculated, and cultured for 4 days at 30 °C on a rotary shaker (150 rpm). For the second-stage preculture, 90 mL seed medium was prepared in a 500 mL flask, and inoculated with 10 mL first seed, then cultured for 3 days at 30 °C on a rotary shaker (150 rpm). For fermentation process, 10 mL second seed was inoculated to the 500 mL shake flask which containing 90 mL fermentation medium. The fermentation medium consisted of the following components (g/L): glucose 35, yeast extract 10, KH_2PO_4 1, $MgSO_4$ 0.5, $CaCl_2$ 0.4. It was cultivated for 10 days at 30 °C, 150 rpm.

2.3. Optimization of DO level in 7-L bioreactor

The bioreactor used was a 7-L agitated bioreactor (Baoxing Company, China) with two six-bladed Rushton impellers (6 cm i.d.). The bioreactor was equipped with the probes of pH (Mettler-Toledo, Switzerland), DO (Mettler-Toledo, Switzerland), temperature and antifoam. The DO probes were calibrated before inoculation. The DO was set to 10%, 30% and 50%, separately, by controlling the agitation (from 50 to 500 rpm) and aeration level (from 0.5 to 2 VVM) automatically. Agitation was first considered to control DO level. The agitation was changed automatically from 50 rpm to 500 rpm to control the DO level. If the DO level cannot reach the set point, aeration rate was changed from 0.5 to 2 VVM. For fermentation process, 500 mL second seed was inoculated to the 7 L bioreactor which containing 4.5 L fermentation medium. It was cultivated for 10 days at 30 °C.

2.4. Fed-batch fermentation in shake flask

Ten milliliter glucose with the concentration of 110 g/L was feeding to the 500 mL shake flask, with 100 mL fermentation broth. The glucose was feeding when the residual sugar level decreased to about 15, 10 and 5 g/L, respectively.

2.5. DO control integrated glucose fed-batch fermentation

Five hundred milliliter glucose with the concentration of 110 g/L was feeding to the 7 L bioreactor, with 5 L fermentation medium. The glucose was feeding when the residual sugar level decreased to about 10 g/L. The OD of one bioreactor was set to be 50% by controlling the agitation speed and aeration rate automatically. In another bioreactor, the agitation speed and aeration rate were set to be 300 rpm and 1 VVM, without controlling DO level.

2.6. Analysis of biomass, intracellular polysaccharide, total triterpenoids and inotodiol

Biomass was assayed by dry cell weight mehods [19]. Mycelia from bioreactor were isolated by centrifugation (10 min, 10000*g*), and washed three times with sterile water. It was dried to constant weight at 60 °C.

Intracellular polysaccharide was assayed according to the literature [19]. Twenty five milligram mycelia powder was suspended in 1 m L NaOH (1 mol/L) at 60 °C for 1 h. The precipitation was resuspended in the same condition after centrifugation (12000*g*, 10 min). The supernatant was combined, and the polysaccharide level was measured by phenol-sulfuric acid method.

Total triterpenoids were analyzed by spectrophotometry, using