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The morphology of *Ganoderma lucidum* mycelium in a repeated-batch fermentation for exopolysaccharide production

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ABSTRACT

The morphology of *Ganoderma lucidum* BCCM 31549 mycelium in a repeated-batch fermentation (RBF) was studied for exopolysaccharide (EPS) production. RBF was optimised for time to replace and volume to replace. *G. lucidum* mycelium showed the ability to self-immobilise and exhibited high stability for repeated use in RBF with engulfed pellets. Furthermore, the ovoid and starburst-like pellet morphology was disposed to EPS production in the shake flask and bioreactor, respectively. Seven RBF could be carried out in 500 mL flasks, and five repeated batches were performed in a 2 L bioreactor. Under RBF conditions, autolysis of pellet core in the shake flask and shaving off of the outer hairy region in the bioreactor were observed at the later stages of RBF (R4 for the shake flask and R6 for the bioreactor). The proposed strategy showed that the morphology of *G. lucidum* mycelium can withstand extended fermentation cycles.

1. Introduction

Medicinal mushrooms have been shown to be the most popular choice of traditional medicine, especially those from the phylum Basidiomycota such as *Ganoderma lucidum*,*Ganoderma atrum*, *Ganoderma tsugae* and *Ganoderma applanatum* [1]. Although all have been used in traditional Chinese medicine for the prevention and treatment of human diseases [2], *Ganoderma lucidum* (Fr.) Karst (Polyporaceae) has been the organism of choice for creating useful bioproducts [2,3], with a significant number of reports in the literature attesting to its benefits [4–6].

To date, the effects of inoculation concentration [7], dissolved oxygen [8], pH [9,10], environmental conditions (e.g. shear rate and catabolite repression) [11], medium composition [12], two-stage culture process [13], pH-shift and dissolved-oxygen transfer (DOT)-shift integrated fed-batch fermentation [14], and oxygen supply [15] have been studied for *G. lucidum* fermentation strategy. Most existing research has focused on improving the fermentation process to increase metabolite activity [16]. However, only one study on increasing metabolite productivity by reducing the fermentation time, cost for seed culture and inoculation between each fermentation cycle has been reported [17]. Similarly, this work addresses how morphological characteristics play an important role in the extended fermentation cycle.

Repeated-batch fermentation (RBF) is an adaptation of an existing technique or alternative strategy in which the medium, or a portion of the medium, is removed and fresh medium introduced periodically or repeatedly without changing the existing culture [17,18]. Based on this, RBF differs from fed-batch techniques as it has the advantage of reducing the fermentation time, cost for seed culture, medium usage, and inoculation requirements between fermentation cycles [19]. RBF has been shown to enhance the productivity of microbial fermentations [18,20], as it requires less time for washing and sterilising the bioreactor, omits the seed preparation time, results in high growth rates, and shortens the initial inoculation procedure between batches [19]. These advantages have the potential to lead to significant savings in terms of both time and labour, as yield remains constant with reduced fermentation time [21]. Consequently, this process enables the reuse and storage of pellets while also maintaining their long-term cell activity [22], especially for the production of bioactive metabolites [18,22,23].

G. lucidum morphology is an important factor that affects the rheological properties of the fermentation broth, and its control is highly desired from an industrial perspective [24]. Generally, two growth forms, the filamentous and the pelleted form, can be

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observed in most fungal fermentations and the pelleted form is usually less viscous than the filamentous form [22,25]. Pellets are characterised by mycelia developing into stable, spherical aggregates consisting of a relatively dense, branched and partially intertwined network of hyphae [26]. A number of reports have addressed the factors influencing *G. lucidum* morphology, rheology and production of fungal exopolysaccharide (EPS) [8,25,27]. Amongst many aspects of morphology, the effect of long continuous culture (RBF) has not been studied to date.

In order to investigate this effect, this work focused on morphological relationship and the optimisation of RBF to reduce fermentation time while enhancing EPS productivity. In addition, the optimum morphology for EPS production during RBF cycles was determined.

2. Materials and methods

2.1. Microorganism and medium

G. lucidum BCCM 31549 stock culture was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/MUCL), [Agro] Industrial Fungi and Yeast Collection (Leuven, Belgium). The fungus was transferred onto potato dextrose agar (PDA, Oxoid Limited, Hampshire, UK) to avoid any contamination and ensure sustainability, as shown in previous research [3,17]. PDA plates were inoculated and incubated at 30 °C for 7 days, and stored at 4 °C. The compositions of media was, in g/L: Glucose 50, Yeast Extract (YE) 1, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgS0_4$ 0.5 and NH_4Cl 4, unless otherwise stated.

2.2. Fermentation conditions in shake flask and bioreactor

The method for inoculum preparation of Wan et al. [17] for *G. lucidum* BCCM 31549 was used and involved two seed culture stages, both cultivated for 10 days at 30 °C, initial pH 4 and 100 rpm. Four mycelial agar squares (5 mm × 5 mm) from a 10-day-old plate were inoculated into a 500 mL Erlenmeyer flask containing 100 mL of medium (first seed culture). To produce additional growing hyphae tips, mycelium from the first seed culture were then homogenised using a sterile Warring blender for 20 s. This material was used as the inoculum for the second seed culture (500 mL Erlenmeyer flask containing 200 mL medium) and then transferred to the bioreactor; during inoculum production, it was inoculated into new fresh medium during the late exponential phase (from day 9 to day 11), meaning that cells were biochemically most active and in the optimum physiological state. EPS fermentation was performed in a 500 mL (20 mL working volume) shake flask and a 2.5 L stirred-tank (STR) bioreactor (New Brunswick Bioflow 3000, Edison L.N, USA) [2 L working volume]. 20% (v/v) of the seed culture was used to inoculate the fermenter, unless otherwise stated. The cultivation was carried out at 30 °C with pH maintained at 4.0, dissolved oxygen (DO) was controlled, aeration rate was at 2.0 vvm, and agitation speed was controlled at 100 rpm. The compositions of media were, in g/L: Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1 and NH_4Cl 4.

2.3. Repeated-batch fermentation

RBF was carried out for the maximum possible number of cycles. To determine a suitable broth replacement ratio, the existing fermentation broth was removed at a pre-determined broth replacement ratio [50% to 90% (v/v)] and replaced with fresh medium to permit continuous growth of *G. lucidum* mycelium (Table 1). To determine the appropriate broth replacement time point, three batch fermentation growth phases were obtained from the shake flask. These were designated as increasing EPS concentration (at the end of logarithmic growth phase), highest EPS concentration (transition phase) and stabilizing EPS concentration (stationary phase). During RBF, the fermentation cycle was started until it achieved the highest growing point (i.e. the organism became too viscous, or autolysis had occurred). At this point, the existing medium fermentation mixture was replenished and the process was repeated for the subsequent cycles that affected morphology.

2.4. Analytical methods

2.4.1. Exopolysaccharide (EPS)

EPS was obtained from the harvested fermentation broth. From this broth, supernatant was collected through centrifugation at 8000 rpm for 15 min. Crude EPS was precipitated from the supernatant by the addition of four volumes of 95% (v/v) ethanol and left overnight at 4 °C to form one volume of cell-free filtrate. The precipitate was then separated by centrifugation at 10,000 rpm for 15 min, a process that was repeated twice. The precipitate was then filtered through a pre-dried and weighted GF/C filter paper and washed twice with 5 mL of 95% (v/v) ethanol, as described by Wan et al. [17]. Precipitate was then placed in a desiccator and dried for 24 h at room temperature to a constant weight before the weight of EPS was estimated. All assays were carried out in triplicate.

2.4.2. Image analysis

A light microscope (Nikon OPIPHOT-2, Japan) with a coupled camera (JVC, TK-C1381 Colour Video Camera) was used to assess the morphology details of the collected samples [17]. Five mL of culture samples were re-suspended in 5 mL of a fixative solution, according to the technique defined by Packer and Thomas [28], and maintained at 4 °C until assessment. Observation of mycelium in the samples required fixative solution which was prepared by mixing 13 mL of 40% (v/v) formaldehyde, 5 mL of glacial acetic acid and 200 mL of 50% (v/v) ethanol. A 0.1 mL aliquot of each fixed sample was transferred to a slide, air dried and stained with methylene blue [9]. The microscopic images for samples from increasing RBF cycles were compared.

2.5. Statistical analysis

All analyses were carried out in triplicate and the respective mean ± S.D determined using GraphPad Prism 5 (Version 5.01) software and shown as error bars as described in the earlier work [17]. Where error bars were not visible, it was assumed that they

Table 1
Mode of operation (broth replacement ratio) of repeated-batch processes by *G. lucidum* BCCM 31549 in the shake flask.

Experiments	Working culture (v/v)	Harvesting percentage (v/v)	Fresh broth replacement (v/v)
A	10%	90%	90%
B	20%	80%	80%
C	30%	70%	70%
D	50%	50%	50%

*Total working volume: 200 mL in a 500 mL Erlenmeyer flask.