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Exopolysaccharide production by *Ganoderma lucidum* immobilised on polyurethane foam in a repeated-batch fermentation

Wan Abd Al Qadr Imad Wan-Mohtar [a,*], Roslinda Abd Malek [b], Linda M. Harvey [a], Brian McNeil [a]

[a] *Fermentation Centre, SIPBS, HW429, John Arburthnott Building (Hamnett Wing), 161 Cathedral Street, University of Strathclyde, Glasgow G4 0RE, Scotland, UK*

[b] *Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor Bahru, Johor, Malaysia*

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ABSTRACT

Exopolysaccharide (EPS) production by *Ganoderma lucidum* BCCM 31549 immobilised on polyurethane foam (PUF) cubes exhibited high ability for continuous EPS production in a repeated-batch fermentation (RBF). RBF by immobilised mycelium was optimised for broth replacement time point and broth replacement ratio. Interestingly, seven batches could be fermented consecutively in the shake flask for 55 days using 80% broth ratio and at day 13 time point, where this smart immobilised approach was deployed. Fermentation time was reduced from 13 days to 6 days, and the medium usage was also reduced due to the broth replacement volume procedure. Critically, immobilised *G. lucidum* mycelium grown on PUF cubes could be reused at least for seven successive cycles without any loss of EPS efficiencies with the PUF cubes retained the original structure, pointing out to be an ideal immobilisation matrix. The EPS productivity in immobilised culture at 0.045 g/L day^{-1} was higher than the value in a freely suspended culture (0.029 g/L day^{-1}). The proposed immobilisation strategy proved that PUF as a physical carrier would provide an economical solution to enhance EPS production for extended liquid fermentation of mushrooms.

1. Introduction

In recent times, a vast range of valuable polysaccharide has been extensively exploited from fermented medicinal mushrooms, mainly *Ganoderma lucidum* (Reishi in Japan and Lingzhi in China) (Ji et al., 2015; Li et al., 2016; Wan-Mohtar et al., 2016; Xu et al., 2015). This is because *G. lucidum* hold various important biological activities, health-preserving and therapeutic agent, and medicinal uses (Paterson, 2006) which can be utilised for creating useful products (Ferreira et al., 2015). These pharmacological benefits came from one of the important active constituents named polysaccharides, which have the essential bioactivities for battling infections: antioxidant, antitumour, anti-inflammatory, antiviral, antibacterial, and immunomodulatory properties (Ferreira et al., 2015; Maftoun et al., 2013). Cultivation of this polysaccharide in the most economically way can be achieved from extensive studies, development, and improvements implemented for *G. lucidum* (Ferreira et al., 2015; Li et al., 2016; Wan et al., 2016b).

Cultivating the wild-type *G. lucidum* in submerged liquid fermentation (SLF) is considered as a promising alternative for an effective polysaccharide production because several months are needed to cultivate the fruiting body, and to control both environment and bioprocess (Tang et al., 2011). However, SLF is costly and time-consuming to satisfy the current market demand (Wan et al., 2016b). Therefore, extensive improvements have been studied to overcome this problem, especially during their liquid production stage (Wan et al., 2016b) and in understanding the extracted polysaccharide (Wan-Mohtar et al., 2016). Recent improvement technique for polysaccharide production of *G. lucidum* has been done via gene expression (Ji et al., 2015; Li et al., 2016) which generated higher exopolysaccharide (EPS) level (highest at 1.76 g/L)(Xu et al., 2015) than those of the wild-type strain. However, wild-type *G. lucidum* cultivation in a bioreactor has been shown to improve EPS production using extended fermentation technique (Wan et al., 2016b) compared to unreported bioengineered strain. Both approaches, wild-type and bioengineered *G. lucidum*, are proven to be significant in EPS production although bioengineered *G. lucidum* has not been tested to survive in an extended fermentation culture as their behaviour, morphology, and resistance level towards catabolite repression might differ.

In the previous study on extended fermentation cultivation of

* Corresponding author.
E-mail addresses: wan.bin-wan-mohtar@strath.ac.uk, wanqadir1987@gmail.com (W.A.A.Q.I. Wan-Mohtar).

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G. lucidum by Wan et al. (2016b), there is a drawback caused by the problematic mycelium overgrowth that reduces the effectiveness of the implemented strategy, repeated-batch fermentation (RBF) both in the shake flask and bioreactor. In liquid culture, *G. lucidum* is susceptible to any available supports of the vessel used by clinging to the bioreactor walls (shake flask only), agitators, and baffles (Wan et al., 2016b), thus creating inferior oxygen supply and mixing which are major operational problems (Espinosa-Ortiz et al., 2016). This overgrowth disturbance is generated from mycelial filaments that naturally tend to aggregate because of hyphal entanglement, which due to continued growth, can produce a dense form around the core called a pellet (Wan et al., 2016b). Pellet growth has some advantages, for example, reduced viscosity in the liquid phase. However, the main downside with pellet culture is lack of oxygen diffusion caused by internal mass transport resistance that may reduce biosynthetic activity (Espinosa-Ortiz et al., 2016; Fang and Zhong, 2002; Wagner et al., 2003). There are no reasonable means by which the structure of the pellets can be controlled to reduce the diffusion resistance within the structure. In this regard, the weakness of fungal pellets fermentation has led to the searching for alternative culture methods such as fungal immobilisation strategy (Prasad et al., 2005) which can possibly be combined with RBF system.

Fungal immobilisation strategy refers to the techniques in which "there is a physical captivity or localisation of microorganisms that permits their cost-effective re-use"(Wang et al., 2005). Immobilised fungal cells have established an increasing attention since the 1970s (Anderson et al., 1983) for potential metabolites production (Wang et al., 2005). To date, this has generated various immobilisation tools include entrapment, adsorption, covalent bonding, and cross-linking (Peart et al., 2016; Rogalski et al., 2006; Yang et al., 2005; Zawawi et al., 2016). Amongst all, entrapment and adsorption via physical carriers are the most frequently utilised, especially for a self-immobilisation system. Compared to the freely suspended culture system, immobilised cell systems make it easy to separate cells from the liquid medium which aids RBF system (Wan et al., 2016b). This would help the operation of both extended fermentation culture and succeeding downstream processes (Wang et al., 2005).

An immobilised mycelium system might be an efficient way to improve EPS productivity for *G. lucidum*. This is because it has the potential to increase the mycelium amount per bioreactor volume by the mycelia being deposited on, in, and even adherent to the chosen support matrices. As stated above, the adsorption by passive adhesion to surfaces would be preferable because it would provide a direct contact between the nutrients and the adhered cells thus reducing any diffusion problems (Loyarkat et al., 2015). A variety of matrices such as agar carrageenan, calcium alginate gels, polyacrylamide, and polyurethane foam (PUF) have been used. However, the nature of the mycelia to be immobilised, substrates, and bioproducts produced, and the culture conditions are vital considerations for the choice of the immobilisation matrix and procedures. Adsorption to surfaces or porous materials (a particular type of physical attachment) has been one of the most widely studied methods for the immobilisation of microorganism (De Ory et al., 2004). It represents a special form of cellular adhesion based on the ability of certain fungi to fix themselves to solid surfaces (Moonmangmee et al., 2002; Prasad et al., 2005) with improved physiological conditions (Rogalski et al., 2006).

Many literature reports focused on the enhancement of bioproduct productions using immobilisation approach (Behera et al., 2010; De Ory et al., 2004; Nakamura et al., 1999) including the use of adsorption to surfaces, and encapsulation within PUF during the immobilisation (De Ory et al., 2004; Wagner et al., 2003) as mycelial entrapment into the carrier avoids mass transfer restrictions (Moonmangmee et al., 2002). Eventually, this would improve the reliability and perhaps allows a continuous immobilised fermentation strategy for *G. lucidum*. It is strongly believed that by attaching the hyphae of *G. lucidum* onto the solid surfaces (carriers), the culture time may be extended. In addition, immobilisation allows for easy separation of mycelial mass from the bulk liquid, minimises contamination, improves operational stability and mycelial viability, and reduces production costs compared to freely suspended fermentation processes (Ariyajaroenwong et al., 2012; Tripathi et al., 2010). Also, most immobilisers are low-cost and free from toxicity problems, reusable, decent mechanical strength and robust (Loyarkat et al., 2015).

Immobilisation of *G. lucidum* CCRC 36123 by Yang et al. (2000) in a shake flask using PUF indicated that the mycelium adopted on a solid support was favourable for both fungal growth and EPS formation though this strategy has not yet been applied in RBF strategy. Thus, the aim of this research was to study the ability of PUF as a low-cost carrier for the immobilisation of *G. lucidum* for EPS production and to investigate the carrier and culture stability in RBF to improve EPS productivity. The effects of the carrier, broth replacement ratio, and broth replacement time were also investigated. To the best of our knowledge, the use of a physical carrier for repeated-batch immobilisation strategy on *G. lucidum* BCCM 31549 remains unreported. This technique offers a great potential for industrial applications (Wang et al., 2005), particularly for continuous fungal pellets which are useful in airlift bioreactor (Espinosa-Ortiz et al., 2016).

2. Materials and methods

2.1. Fungi

G. lucidum BCCM 31549 was described according to the previous study by Wan et al. (2016b) which was obtained from the Belgian Coordinated Collections of Microorganisms, (BCCM/MUCL), Leuven, Belgium. The fungus was subcultured onto potato dextrose agar (PDA, Oxoid Limited, Hampshire, UK) to maintain the culture viability. PDA plates were inoculated and incubated at 30 °C for seven days and stored at 4 °C.The strain was preserved on PDA slant.

2.2. Fermentation strategy

In the present investigations, *G. lucidum* BCCM 31549 was studied to produce EPS in submerged agitation culture using cotton-plugged 500 mL Erlenmeyer flask containing 200 mL of production medium according to the RBF strategy in the previous work (Wan et al., 2016b). A Rotary Shaker Incubator (New Brunswick, Edison, L.N., USA) was used for incubation. The inoculum groundwork involved two seed culture stages, both were cultivated for 10-days at 30 °C and 100 rpm. Four mycelial agar squares (5 mm × 5 mm) from a 10-day-old plate was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of medium (1st seed culture). Later, the mycelium from the 1st seed culture was homogenised using a sterile Warring blender for 20 s (Wan et al., 2016b). This was used as the inoculum for the main culture (500 mL Erlenmeyer flask containing 200 mL medium and a carrier). The medium compositions for seed culture medium, batch fermentation medium, and RBF were (g/L): glucose, 50; yeast extract, 1; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $MgSO_4$, 0.5; NH_4Cl, 4 – unless otherwise stated (Wan et al., 2016b). The cultivation was carried out using 10% (v/v) initial inoculum incubated at 30 °C with the initial pH 4, and 100 rpm agitation speed.

2.2.1. Immobilisation

Polyurethane foam (PUF) (Plastifoam, Manchester, UK) was used as a carrier. Prior to use, the PUF was cut into cube-shaped