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Pellet diameter and morphology of European *Ganoderma pfeifferi* in a repeated-batch fermentation for exopolysaccharide production

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ABSTRACT

The pellet diameter and morphology of an underutilized European *Ganoderma pfeifferi* (EGP) mycelium was studied in a repeated-batch fermentation (RBF) for exopolysaccharide (EPS) production. In preliminary fermentation, growth screening was performed for initial pH, glucose concentration, and agitation speed. At 30 °C, the favourable conditions for EPS production (0.58 g/L) and EPS productivity (0.058 g/L day^{-1}) were 15 g/L glucose, initial pH 6, and 100 rpm, while the highest dry cell weight (DCW; 3.63 g/L) was achieved at 9 g/L glucose, initial pH 4, and 120 rpm. Morphologically verified, large, compact pellets (diameter: 40 μm < d < 40.67 μm) were associated with high DCW while small dispersed pellets (diameter: 11.67 μm < d < 14.67 μm) were associated with high EPS production. In RBF, EGP mycelium exhibited the ability to self-immobilize and high stability for repeated use with increasing smooth pellet diameter from RBF1 (7.33 μm < d < 7.67 μm) to RBF4 (16.67 μm < d < 18.33 μm). The fermentation period was subsequently shortened from 48 (batch) to 24 days in four consecutive cycles of RBF, and the productivity of EPS increased from 0.053 g/L day^{-1} to 0.108 g/L day^{-1}. In an FTIR analysis, crude EPS of EGP showed the presence of β-glucan characteristics at 1075 cm^{-1} and 891 cm^{-1} wavelengths, similar with European *G. lucidum* BCCM 31549. In addition, ^{1}H NMR showed similar β-glycosidic linkages when compared with laminarin standard. The proposed strategy demonstrated that *G. pfeifferi* pellet morphology can withstand extended fermentation cycles for efficient EPS production.

1. Introduction

Among the numerous species within the *Ganoderma* genus, the most investigated are *G. lucidum*, *G. aplanatum* and *G. tsugae* (Paterson, 2006) while *G. pfeifferi* has been reported only nine times since 2000 (Fraga et al., 2014; Paterson, 2006). The published literature has focused on phytochemicals such as triterpenoids and sesquiterpenoids and potential medical effects such as anti-aging, antimicrobial and anti-inflammatory activities (Hoang-Minh et al., 2011). According to Lindequist et al. (2015), *G. pfeifferi* contains farnesylhydroquinones, a type of sesquiterpene that is rarely found in mushrooms and has not been identified in any other species from the family *Ganodermataceae*. The efficient identification of other potentially bioactive compounds from *G. pfeifferi* is therefore of significant interest.

With the success in cultivating *G. lucidum* by submerged-liquid fermentation (SLF) (Lee et al., 2007; Mahapatra and Banerjee, 2013; Wan-Mohtar et al., 2016b), the potential for cultivating *G. pfeifferi* in SLF cannot be dismissed. This method gives a high biomass yield and reduces the time needed to produce bioproducts thus reducing the risk of contamination (Papagianni, 2004). Although cultivation in SLF is associated with high mycelial stability and product quality (Zhang and Hu, 2012), this process is unable to sustain market demand due to high production costs and lengthy duration (Reddy et al., 2002). Several attempts have therefore been made to improve the efficiency of the process, including the modification of *G. lucidum* genes to increase polysaccharide production (Wan-Mohtar et al., 2016a). However, efforts to improve the production rate are not described for *G. pfeifferi* and unlike *G. lucidum*, the cultivation of *G. pfeifferi* has not been evaluated

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using extended fermentation techniques or investigated with respect to morphological characteristics in SLF.

Polysaccharides from *Ganoderma*, especially β-glucans have been extensively studied because of their significant medicinal properties (Paterson, 2006) and anticancer activities including their ability to inhibit carcinogenesis (Fraga et al., 2014). Exopolysaccharides (EPS) production from fungi depends primarily on the type of fungal strain, physical conditions maintained during fermentation, and type of medium components used in EPS production (Mahapatra and Banerjee, 2013). The production of EPS from fruiting bodies is more complex compared with that of mycelia, but this limitation has been overcome by the use of SLF (Lee et al., 2007). For example, EPS production from *G. pfeifferi* in the cosmetics and pharmaceutical industries has been achieved by implementing SLF (Mahapatra and Banerjee, 2013).

Results from morphological studies on *G. lucidum* have been reported by Wan-Mohtar et al. (2016b) but no similar studies on *G. pfeifferi* have been described to date. When culturing fungi in a liquid medium, the most common growth forms will be hyphal and pellet, with the hyphal form made up of a dense intertwined network comprising the mycelium while pellets are spherical or ellipsoidal hyphal masses with structures including loosely packed, "fluffy"-to-compact, dense pellets (Papagianni, 2004). The pelletisation of mycelium depends on culture conditions and is thought to be strain-specific (Zhang and Hu, 2012). According to Wan-Mohtar et al. (2016b), *G. lucidum*, which belongs to the same genera as *G. pfeifferi*, exhibits specific pellet structures both in the shake flask and the bioreactor. Thus, it is imperative to control any changes in morphology in *G. pfeifferi* mycelium that can affect the properties of the fermentation broth (Reddy et al., 2002).

Repeated batch fermentation (RBF) represents a promising liquid cultivation technique according to previous research (Wan-Mohtar et al., 2016a) as it produces a greater biomass concentration in a shorter time compared with batch fermentation. RBF involves the removal of broth fermentation medium at specific time intervals and its replacement with an equal volume of fresh broth fermentation medium (Hoang-Minh et al., 2011). Furthermore, the use of a single seeding process results in reduced duration and sterilization costs (Moreira et al., 2017; Postemsky et al., 2017). In summary, RBF gives a high EPS yield (Wan-Mohtar et al., 2016a) with a single inoculation for each fermentation cycle.

To date, no studies have addressed the factors influencing *G. pfeifferi* pellet morphology and EPS production. Among the many morphological parameters, the effect of RBF on pellet diameter has not yet been studied. To investigate this effect, changes in the morphology of *G. pfeifferi* throughout the EPS production process were observed. First, various culture parameters (pH, glucose concentration and agitation speed) were adjusted for conducive EPS and biomass production prior to the generation of a batch fermentation growth curve. Second, the RBF strategy was applied to reduce fermentation time while improving EPS productivity. Throughout the batch and RBF, the effects of pellet morphology and diameter on EPS production were analysed. This strategy enables pellet morphology to be favourable during EPS production in liquid fermentation and constitutes the first report of the repeated batch culture of EPS-producing *G. pfeifferi*.

2. Materials and methods

2.1. Microorganism and medium

A stock culture of *G. pfeifferi* IMI 379841 originating from European beech tree (*Fagus sylvatica*) was obtained from Dr. Helen Steward (CABI UK Centre [Egham], Bakeham Lane, Egham Surrey, TW20 9TY, United Kingdom). The stock culture was subcultured onto potato dextrose agar media (PDA, Oxoid Limited, Hampshire, UK) and incubated at 30 °C until the fungus was fully grown on the plate. A slant culture was prepared using the same media to maintain the strain at 4 °C to ensure strain longevity. The medium composition for seed culture and RBF was as follows (g/L): yeast extract, 1; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $MgSO_4$, 0.5; and NH_4Cl, 4, unless otherwise stated (Wan-Mohtar et al., 2016a).

2.2. Screening of culture conditions

Screening of *G. pfeifferi* growth involved two stages of seed culture with various cultivation parameters of initial pH (4, 5, or 6), agitation speed (80, 100, or 120 rpm), and glucose concentration (3, 9, or 15 g/L), maintained at 30 °C for 10 days according to Lin and Yang (2006). For initial seed culture, four mycelial agar squares (5 mm × 5 mm) were incised from an 8-day old plate using a sterile scalpel and inoculated into a 250-mL Erlenmeyer flask containing 100 mL of medium. Mycelium from the first seed culture was homogenized for 20 s using a sterile blender to produce additional growing hyphae tips. Next, 20% (v/v) of the homogenate was used to inoculate the second seed culture, which contained 80 mL of medium (100 mL of working culture) in a 250-mL Erlenmeyer flask. The total cultivation time was approximately 20 days. The parameters for growth, EPS, and biomass production after the second seed culture were recorded for further use in generating a growth pattern.

2.3. Batch fermentation

Batch fermentation was performed in a 250-mL (100 mL of working medium) Erlenmeyer flask inoculated with 20% (v/v) second seed culture according to the screened conditions for EPS production only. Growth curve and time profiles were established to determine the highest production time point representing the broth replacement time point in RBF. The two conditions (Section 2.2) were condition A (initial pH 6, 15 g/L glucose, 100 rpm) and condition B (initial pH 4, 9 g/L glucose, 120 rpm)

2.4. RBF in shake flask

The selected broth replacement time point condition from the batch fermentation was used in RBF. The entire broth culture was removed according to the total volume of culture (80%) as described by Wan-Mohtar et al. (2016b) and immediately replaced with the same volume of sterile medium to initiate a new cycle. When the EPS production rate was considered sufficiently high and stable, the next cycle was started. Four cycles of RBF were performed, and each broth fermentation cycle was assayed for EPS production and morphologically observed.

2.5. Analytical methodology

2.5.1. EPS

The fermented mycelial broth was transferred to a centrifuge tube and centrifuged at 11000 × *g* for 15 min. Supernatant was collected and crude EPS was precipitated by mixing with four volumes of 95% ethanol and overnight incubation at 4 °C. Next, the precipitates were centrifuged at 17000 × *g* for 15 min, and the process was repeated twice. The precipitate was filtered using pre-dried and weighed GF/C filter paper (Whatman Ltd., Kent, UK) and washed twice using 5 mL of 95% (v/v) ethanol. The precipitate was placed in a desiccator and dried to a constant weight prior to estimation of EPS weight (Wan-Mohtar et al., 2018, 2016c; 2016a).

2.5.2. Dry cell weight (DCW)

For estimation of *G. pfeifferi* DCW, 10 mL of sample was filtered through a weighed GF/C filter using a Buchner funnel filter set attached to a water pump. The resulting mycelial biomass was washed three times with distilled water, dried at 50 °C in a food dehydrator (650 W) for 4 h, and cooled in a desiccator for 24 h before weighing. The pre-weighed filter mass was subtracted from the mass with the filtrate and multiplied by the dilution factor to obtain DCW in g/L using equation

Table 1
Screening of culture conditions in a controlled shake-flask fermentation of *G. pfeifferi* IMI: 379841 for EPS production in 10 days.

Glucose concentration (g/L)	Agitation speed (rpm)	Initial pH 4		Initial pH 5		Initial pH 6	
		EPS concentration (g/L)	EPS productivity (g/L day^{-1})	EPS concentration (g/L)	EPS productivity (g/L day^{-1})	EPS concentration (g/L)	EPS productivity (g/L day^{-1})
3	80	0.030 ± 0.002^{a}	0.003 ± 0.0002	0.030 ± 0.002^{bc}	0.003 ± 0.0002	0.056 ± 0.003^{b}	0.006 ± 0.0003
	100	0.027 ± 0.004^{a}	0.003 ± 0.0004	0.027 ± 0.004^{a}	0.003 ± 0.0004	0.073 ± 0.010^{a}	0.007 ± 0.001
	120	0.133 ± 0.002^{a}	0.013 ± 0.0002	0.133 ± 0.002^{cd}	0.013 ± 0.0002	0.104 ± 0.045^{d}	0.01 ± 0.004
9	80	0.370 ± 0.046^{bc}	0.037 ± 0.005	0.370 ± 0.046^{cd}	0.037 ± 0.005	0.347 ± 0.031^{b}	0.035 ± 0.003
	100	0.310 ± 0.010^{b}	0.031 ± 0.001	0.310 ± 0.010^{a}	0.031 ± 0.001	0.363 ± 0.040^{a}	0.036 ± 0.004
	120	0.370 ± 0.046^{bc}	0.037 ± 0.005	0.370 ± 0.046^{d}	0.037 ± 0.005	0.403 ± 0.012^{cd}	0.04 ± 0.001
15	80	0.483 ± 0.061^{d}	0.048 ± 0.006	0.483 ± 0.061^{b}	0.048 ± 0.006	0.487 ± 0.015^{bc}	0.049 ± 0.002
	100	0.493 ± 0.032^{d}	0.049 ± 0.003	0.493 ± 0.032^{a}	0.049 ± 0.003	0.580 ± 0.056^{a}	0.058 ± 0.006
	120	0.460 ± 0.062^{cd}	0.046 ± 0.006	0.460 ± 0.062^{d}	0.046 ± 0.006	0.567 ± 0.045^{d}	0.057 ± 0.005

All the results are expressed as mean ± SD from three experiment (n = 3). One-way ANOVA has been conducted for each row with ($_P$ < 0.05). Since analysis is significant, a post-hoc analysis (Tukey HSD Test) was carried out to identify which pair(s) are statistically different. Same letter denote means are not significantly different ($_P$ > 0.05).

(1).

$$Concentration\ (g/L) = \frac{Dryweight\ (g)}{Volume\ used\ (mL)} \times 100 \quad (1)$$

2.5.3. Pellet morphology

An inverted microscope (LEICA DFC295, Wetzlar, Germany) with a coupled camera (JVC, TK-C1381 Colour Video Camera) was used to assess the morphology of collected samples. A 20-mL aliquot of culture sample was transferred from the shake flask to a Petri dish. Ten pellets from each culture were selected at random from the Petri dish and placed onto a slide for observation under a LEICA DM1000 microscope at 4 × magnification. The Petri dish was subsequently placed under a LEICA DFC295 inverted microscope for further morphological observation and analysis. The pellets were selected at random in triplicate and each diameter was measured using a Microscopic Objective Stage Micrometre Calibration Slide 10 mm/100 0.1 mm (OMAX, A36CALM7), which was placed onto a LEICA DM1000 microscope at 4 × magnification for measurement of the pellet diameter ranges.

2.5.4. Statistical analysis

All analyses were carried out in triplicate and the respective mean ± S.D was determined using IBM SPSS Version 24 and GraphPad Prism 5 (Version 5.01) software and shown as error bars. Where error bars are not apparent, it can be assumed that they are smaller than the symbol. A *t*-test was used to plot the fermentation graphs, and kinetic parameters were compared using a *t*-test and post-hoc test (Tukey Multiples Comparison Test).

2.5.5. Kinetic calculation

Kinetic parameters for *G. pfeifferi* fermentation were calculated as described by (Wan-Mohtar et al., 2016a) using equation (2) and equation (3).

3. Batch culture

X max – Xo = Maximum EPS production – Initial EPS production.

tii-ti = The time of maximum EPS production – Initial time

$$EPS\ productivity,\ P_{eps}(g/L\ day^{-1}),\ \frac{\text{X max} - \text{X o}}{\text{tii} - \text{ti}} \quad (2)$$

4. Repeated-batch culture

- Cycle 1 (R1) = Maximum EPS production at initial R2 – EPS production at maximum EPS from batch culture/Total time of RBF (5 days)
- Cycle 2 (R2) = Maximum EPS production at initial R3 – EPS production at R1/Total time of RBF (5 days)
- Cycle 3 (R3) = Maximum EPS production at initial R4 – EPS production at R2/Total time of RBF (5 days)
- Cycle 4 (R4) = Maximum EPS production at initial R5 – EPS production at R3/Total time of RBF (5 days)

$$\text{EPS productivity, } P_{EPS} = (g/L\ day^{-1}), \frac{\text{X max} - \text{X o}}{\text{the time for product recovery at certain cycle in repeated batch culture (day)}} \quad (3)$$

4.1. Fourier-transform infrared spectroscopy (FTIR)

The crude exopolysaccharides were characterized using FTIR analysis, and the frequency range are measured as wave numbers in the range of 4000–650 cm^{-1}. EPS sample (0.5 g) was placed on clean window of Agilent Cary 630 equipped with diamond ATR (Attenuated Total Reflectance). Then, the pressure clamp was closed until a click was heard and analysed using a real-time Micro-Lab software.

4.2. Proton nuclear magnetic resonance (^{1}H-NMR)

Using 600 Mhz Agilent, USA, the NMR analysis was performed. Crude EPS (10 mg) was mixed with 500 µl of deuterium oxide ($D_2$0-*d*) at room temperature. The mixture was then vortexed and sonicated for 15 min. Once completely dissolved, the mixture was centrifuged at 10000 × *g* for 10 min. A clear supernatant was transferred to 5 mm NMR tube (Norell, Sigma Aldrich, Canada) for analysis.

5. Results and discussion

5.1. Screenings of culture conditions for EPS production

EPS are produced by all fungal species and are in high demand for their medicinal properties. In the present study, EPS was extracted using ethanol. EPS production and productivity are shown in Table 1. The highest EPS concentration (0.580 g/L) was recorded at culture conditions of initial pH 6, 15 g/L of glucose and 100 rpm (Condition A), while the lowest EPS concentration (0.027 g/L) was reported at pH 4 and pH 5 with similar glucose concentration (3 g/L) and agitation (100 rpm). Fifteen g/L of glucose resulted in the highest EPS production and had a positive effect on *G. pfeifferi* EPS production as reported by Hsu et al. (2017) in a study involving *G. lucidum*. This outcome was attributable to the role of glucose in the glycolysis pathway to produce energy and act as a precursor for sugar nucleotides in polysaccharide synthesis (Xu et al., 2015). This result showed that glucose

concentration had a direct correlation with EPS production: as glucose concentration increases, EPS production increases also, and this finding was in accordance with studies by Ding Zhang and Hu (2012) and Yuan et al. (2012).

In the present study, however, *G. pfeifferi* was shown to tolerate mild acidity within the range of pH 4–6 while producing a significant amount of EPS, as shown by the presence of pellets deposited in the clear liquid culture. However, the highest EPS production occurred at pH 6, indicating that *G. pfeifferi* preferred less acidic culture conditions, unlike *G. lucidum* which preferred the more acidic condition of pH 4 (Wan-Mohtar et al., 2016b). Evidently, the three pH levels (4, 5 and 6) did not significantly affect EPS production, as shown in Table 1, with only small differences in concentration observed among the levels, indicating that EPS produced by *G. pfeifferi* was unaffected by acid conditions, contrary to previous reports (Mohammadi et al., 2011).

EPS production was also affected by agitation speed as shown in Table 1. *G. pfeifferi* favoured an agitation speed of 100 rpm over 80 rpm or 120 rpm, as this produced the highest EPS concentration (0.580 g/L). A similar result was obtained for the counterpart *G. lucidum* mycelial growth in a submerged fermentation, whereby the ideal agitation speed for EPS production was 100 rpm (Yang and Liau, 1998).

5.2. Screenings of culture conditions for biomass production

Compared with EPS production, biomass production for *G. pfeifferi* favoured at 120 rpm and moderate glucose concentration (9 g/L) in the pH range of 4–6, given that the largest amount of biomass achieved was 3.63 g/L at pH 4, followed by 3.30 g/L at pH 5 and 3.27 g/L at pH 6 (Fig. 1). This result indicated that 120 rpm was the preferred agitation speed for propagation of the mycelial biomass to ensure a sufficient supply of oxygen (Fazenda et al., 2010). Furthermore, an agitation speed of 120 rpm allowed for improved glucose utilization compared with the lower speeds of 100 rpm or 80 rpm. This follows a pattern reported by Nair et al. (2016), who observed reduced glucose utilization at lower agitation rates. In the present study, the favourable agitation speed for *G. pfeifferi* to produce the maximum biomass differed from that of *G. lucidum*, which previously determined as 100 rpm for maximum mycelial concentration. Higher agitation speeds (> 100 rpm) resulted in lower biomass yields for *G. lucidum*, suggesting that shear stress on the mycelium was the contributing factor (Yang and Liau, 1998). Therefore, the present study findings indicate that *G. pfeifferi* can tolerate agitation speeds of 120 rpm without succumbing to the effects of shear stress observed with *G. lucidum.*

5.3. Pellet and diameter analysis of G pfeifferi for screened of culture conditions

5.3.1. Initial pH 4

According to Wajid Khan et al. (2013), pH has an effect on mycelial growth. Therefore, the initial pH will affect pellet morphology as the pellets are composed of entangled mycelium. As shown in Fig. 2A, pellets exhibited a relatively irregular shape, with a compacted mycelial mass and smooth outer layer. Remarkably, culture conditions at pH 4 favoured the formation of a large, disproportionate ball of mycelial mass, which dispersed at the bottom of the shake flask. A similar structure was observed under all culture conditions yielding a high biomass production. The mycelial mass gradually transformed to small, clumped and irregular pellets with minimal diameter at 3 g/L and 80 rpm (Fig. 2A).

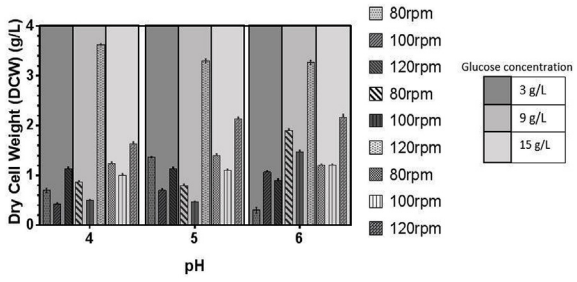


Fig. 1. Biomass concentration of *G. pfeifferi* IMI:379841 in a 10-day controlled shake-flask fermentation using condition: Agitation speed (80, 100 or 120 rpm), glucose concentration (3, 9 and 15 g/L) and initial pH value (4, 5 or 6). All other fermentation conditions were all the same [(g/L): KH_2PO_4 1, K_2HPO_4 1, $MgSO_4$ 1, YE 2, NH_4Cl 8], 20% (v/v) of inoculum and temperature 30 °C.

As shown in Fig. 2B, initial pH 4 gave the highest biomass production compared with other pH levels using condition B (9 g/L of glucose, 120 rpm) with diameters of 40.00 μm < d < 40.67 μm, respectively. The increase in pellet diameter with increased agitation speed differed from the pellet diameter trends observed in other studies, which showed that the higher the agitation speed, the smaller the pellet diameter (Cui et al., 1998a, 1998b; Purwanto et al., 2009). Taken together, these data show that *G. pfeifferi* cells have a greater capacity than *G. lucidum* to withstand high shear stress at 120 rpm (Wan-Mohtar et al., 2016b).

5.3.2. Initial pH 5

As shown in Fig. 3B, pellets formed the largest diameter (31.67 μm < d < 38.67 μm) at initial pH 5 when agitated at 80 rpm with 9 g/L of glucose, while the smallest diameter (10.00 μm < d < 10.67 μm) was found at 120 rpm and 3 g/L of glucose. Of note, neither biomass nor EPS production were uniform at initial pH 5 (Fig. 3A). As shown in Fig. 3A, pellets were observed to co-exist in two forms in the culture: a large round-shaped form and a small irregular-shaped form.

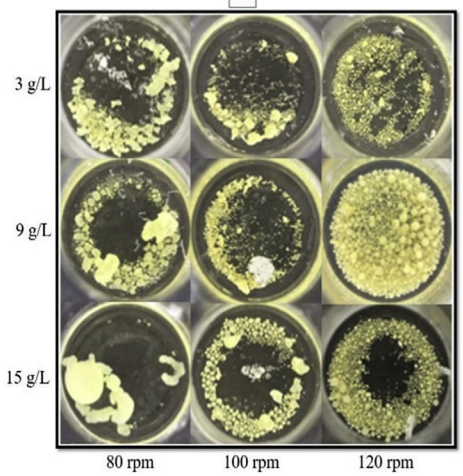
5.3.3. Initial pH 6

As demonstrated in Fig. 4B, the largest and smallest diameter of pellets produced under the initial pH 6 condition was 37.33 μm < d < 41.0 μm (9 g/L of glucose at 80 rpm) and 11.67 μm < d < 14.67 μm (15 g/L of glucose at 100 rpm), respectively. The smallest pellet diameter correlated with the highest EPS production (0.58 g/L) while increasing pellet size correlated with low EPS production, as the largest diameter in initial pH 6 produced 0.056 g/L of EPS. This observation supported the finding of Wan-Mohtar et al. (2016a) that a reduction in EPS concentration was attributable to large pellets in *G. lucidum*.

In appearance (Fig. 4A), pellets produced at pH 6 reduced in size as the agitation rate increased. At 80 rpm, large globular pellets were predominant in the culture while smaller pellets formed compared with those at 100 rpm. However, at 120 rpm, a distinct morphology was observed with 9 g/L of glucose, with smooth, round pellets prevalent compared with the small, dispersed pellets observed at other glucose concentrations. Meanwhile, condition A (initial pH 6, 15 g/L of glucose, and 100 rpm) produced a mix of small and medium-sized pellets with a smooth edge. The majority of the small mycelial mass yielded a higher EPS concentration at pH 6 compared with larger pellets, resulting from a sizeable surface area and increased oxygen diffusion (Xu et al., 2015).

5.4. Effect of G. pfeifferi pellet morphology on EPS production in batch fermentation

An EPS production time profile was constructed for *G. pfeifferi* based on condition A (initial pH 6, 15 g/L of glucose, and 100 rpm) to determine the transition point, which was the favourable broth replacement time point for RBF. As shown in Fig. 5B, *G. pfeifferi* successfully followed a normal *Ganoderma* sp. growth pattern, and EPS production reached a peak value at day 12 (0.65 g/L). Meanwhile, the pellet diameter grew steadily from day 3 (10.33 μm < d < 11.67 μm) until day 9 (17.00 μm < d < 19.00 μm) and decreased slightly at day 12 (15.33 μm < d < 16.00 μm). The decreasing trend in EPS production from day 3 to day 6 may be attributable to initial stress following the



B

Glucose (g/L)	Agitation speed (rpm)	Initial pH 4 Pellet diameter (μm)
3	80	10.33 ± 2.51 < d < 14.00 ± 1.00
	100	8.00 ± 1.00 < d < 8.00 ± 1.73
	120	10.00 ± 1.00 < d < 10.67 ± 0.58
9	80	29.67 ± 2.51 < d < 33.00 ± 3.46
	100	30.67 ± 1.15 < d < 35.67 ± 1.15
	120	40.00 ± 2.00 < d < 40.67 ± 1.15
15	80	26.67 ± 1.53 < d < 33.33 ± 1.53
	100	23.00 ± 1.00 < d < 26.67 ± 1.52
	120	18.00 ± 1.73 < d < 19.67 ± 0.58

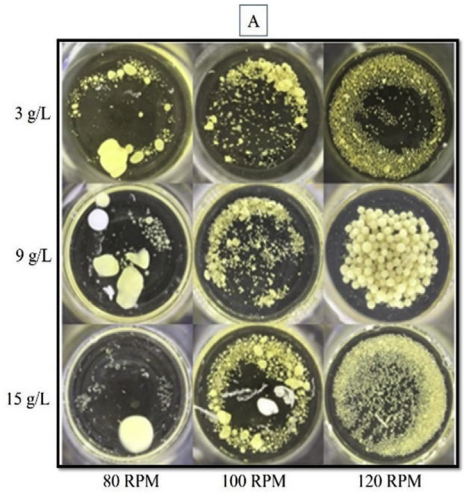
Fig. 2. Pellet morphology (A) and diameters (B) of *G. pfeifferi* IMI:379841 for EPS production in a controlled shake-flask fermentation using a fixed initial pH 4 media, different glucose concentration (g/L) and agitation speed (rpm). The conditions and medium compositions were at [(g/L): Glucose (3, 9, and 15 respectively), KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], temperature at 28°C.

introduction of *G. pfeifferi* liquid media and adaptation to the culture conditions. This behaviour was previously demonstrated by Yang and Liau (1998), who showed that polysaccharides attached to the mycelial mass instead of accumulating in the media.

Stress appeared to affect pellet morphology (Fig. 5A) on day 3 as the mycelium clumped together into a pellet form, possibly due to self-immobilization, giving the fungus protection in the liquid medium, especially against shear effects (Wan Mohtar et al., 2016b). The increasing pellet diameter from day 3 to day 6, as shown in Fig. 5A, confirmed this observation as the fungus was actively self-immobilized into larger pellet forms for maximal protection. The fungal pellets observed on day 6 exhibited very short hyphal tips protruding from surface of the growing pellet. These protuberances increased in length and detached from the pellet surface by day 12, resulting in dispersed pellets. These protuberances are newly formed pellets, liberated from the parent pellet to form second-generation pellets. This event is therefore associated with maximum EPS production for *G. pfeifferi*, similar to the findings of studies by Wan-Mohtar et al. (2016b) in *G. lucidum*.

5.5. Effect of G. pfeifferi pellet morphology on EPS production in repeated-batch fermentation

Pellet morphology plays a major role in EPS production. As shown in Fig. 6B, for RBF, pellet diameters were gradually increased from R1, R2, and R3, culminating with the largest pellet in R4 (diameter: 16.67 μm < d < 18.33 μm). The fermentation period was successfully shortened from 48 days (batch) to 24 days in four consecutive (6 days × 4) cycles of RBF. The morphological changes of *G. pfeifferi* during RBF for EPS production are shown in Fig. 6A, indicating that dispersed second-generation pellets from R0 (initial cycle) transformed to small hairy pellets at R1. The hairy surface increased up to R2, and may be attributed to rapid growth of the fungal pellets (Sinha et al., 2001). This hairy surface then decreased at R3, having most likely been sheared off due to prolonged shear stress (Xu et al., 2006). The rapid growth observed was consistent with the principle of RBF, whereas each cycle involves the removal of homogenous old media and its substitution with new media, thus providing an enriched environment for fungal culture. However, the addition of media leads to stress, which plays a role in the increasing production of EPS as a form of response by cells to protect themselves in stressful environments (Ordax et al., 2010). Since the highest EPS production and productivity were both present in R4 (0.65 g/L), the morphology of pellets in this cycle could be the key to distinguish the level of EPS in *G. pfeifferi* cultures. As shown in Fig. 6B, the presence of large, smooth pellets at R4 indicates that there were second-generation pellets growing in the culture. Cultivation of *G. pfeifferi* using the RBF method successfully revived and expanded the mycelium mass in each cycle without a detrimental lag phase. The presence of hairy pellets signified the presence of regions of active tips (Wan-Mohtar et al., 2016b), which were vital in ensuring the continual growth of the fungus.

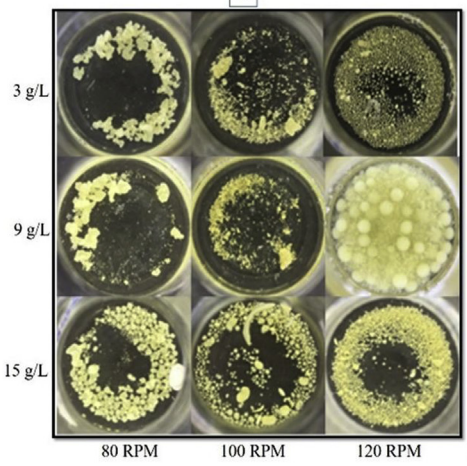


B

Glucose (g/L)	Agitation speed (rpm)	Initial pH 5 Pellet diameter (μm)
3	80	29.33 ± 1.15 < d < 36.67 ± 1.15
	100	13.33 ± 1.53 < d < 14.67 ± 1.53
	120	10.00 ± 1.00 < d < 10.67 ± 0.58
9	80	31.67 ± 1.53 < d < 38.67 ± 1.53
	100	27.33 ± 2.52 < d < 32.00 ± 2.52
	120	16.00 ± 1.73 < d < 18.33 ± 1.53
15	80	16.00 ± 1.73 < d < 35.67 ± 1.15
	100	30.33 ± 0.58 < d < 32.33 ± 2.52
	120	20.67 ± 1.15 < d < 23.67 ± 1.15

Fig. 3. Pellet morphology (A) and diameters (B) of *G. pfeifferi* IMI:379841 for EPS production in a controlled shake-flask fermentation using a fixed initial pH 5 media, different glucose concentration (g/L) and agitation speed (rpm). The conditions and medium compositions were at [(g/L): Glucose (3, 9, and 15 respectively), KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], temperature at 28°C.

A

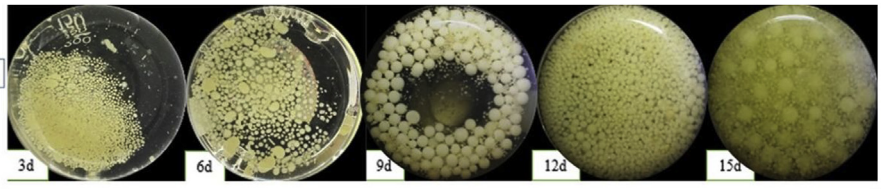


B

Glucose (g/L)	Agitation speed (rpm)	Initial pH 6 Pellet diameter (μm)
3	80	24.00 ± 1.73 < d < 27.67 ± 0.58
	100	16.33 ± 1.53 < d < 19.67 ± 1.53
	120	12.33 ± 0.58 < d < 13.33 ± 1.15
9	80	37.33 ± 2.08 < d < 41.00 ± 1.00
	100	20.00 ± 1.73 < d < 21.67 ± 1.53
	120	30.33 ± 0.58 < d < 31.33 ± 0.58
15	80	16.00 ± 1.73 < d < 20.00 ± 1.00
	100	11.67 ± 0.58 < d < 14.67 ± 0.58
	120	12.67 ± 1.15 < d < 19.00 ± 1.00

Fig. 4. Pellet morphology (A) and diameters (B) of *G. pfeifferi* IMI:379841 for EPS production in a controlled shake-flask fermentation using a fixed initial pH 6 media, different glucose concentration (g/L) and agitation speed (rpm). The conditions and medium compositions were at [(g/L): Glucose (3, 9, and 15 respectively), KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], temperature at 28°C.

A

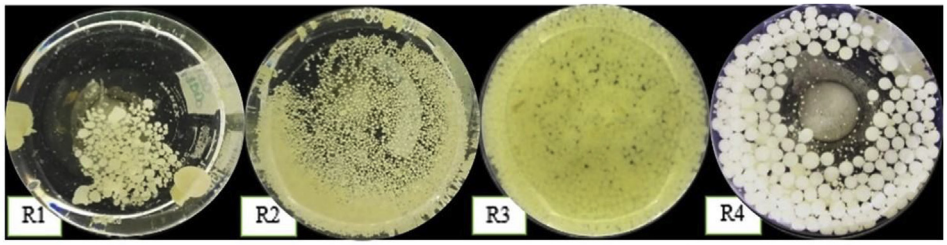


B

Day	EPS production (g/L)	EPS productivity (g/L day^{-1})	Pellet Diameter (μm)
0	0.12 ± 0.02	-	-
3	0.32 ± 0.01	0.07	11.67 ± 1.53 < d < 10.33 ± 0.58
6	0.23 ± 0.01	0.02	11.33 ± 1.15 < d < 13.00 ± 1.00
9	0.37 ± 0.02	0.03	17.00 ± 1.00 < d < 19.00 ± 1.00
12	0.65 ± 0.02	0.04	15.33 ± 1.53 < d < 16.00 ± 1.00
15	0.48 ± 0.02	0.02	12.00 ± 1.00 < d < 13.67 ± 1.53

Fig. 5. Pellet morphology (A) and diameters (B) of *G. pfeifferi* IMI:379841 for EPS production in a batch fermentation using condition A: [15 g/L of Glucose, 20% (v/v) inoculum, initial media pH6, and 100 rpm]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], and temperature 30°C. 1–15 d means fermentation period in days.

A



B

Fermentation period (days) Batch	RBF	Cycle	EPS production (g/L)	EPS productivity (g/L day^{-1})	Pellet diameter (μm)
48	6	R1	0.32 ± 0.06	0.053 ± 0.03	7.33 ± 0.58 < d < 7.67 ± 1.15
		R2	0.23 ± 0.01	0.038 ± 0.02	13.11 ± 1.15 < d < 13.67 ± 0.58
		R3	0.37 ± 0.02	0.062 ± 0.01	15.33 ± 0.58 < d < 16.00 ± 1.00
		R4	0.65 ± 0.02	0.108 ± 0.02	16.67 ± 2.08 < d < 18.33 ± 1.53

Fig. 6. Pellet morphology (A) and diameters (B) of *G. pfeifferi* IMI:379841 for EPS production in a repeated-batch fermentation using condition A: [15 g/L of Glucose, 20% (v/v) inoculum, initial media pH6, and 100 rpm]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], and temperature 30 °C. R1 (cycle 1), R2 (cycle 2), R3 (cycle 3), and R4 (cycle 4).

5.6. Characterisation of exopolysaccharide from the mycelium of G. pfeifferi using Fourier -transform infrared spectroscopy (FTIR)

Spectral characteristics of crude exopolysaccharide from the mycelium *G. pfeifferi* was identified using FTIR spectroscopy. In Fig. 7, the IR spectra result of mycelial glucan from European *G. lucidum* BCCM 31549 (A) and glucan from European *G. pfeifferi* IMI 379841 (B) is compared. The stretching vibration of O-H group on the sugar residue was indicated in broad-stretched peak from 3500 cm^{-1} to 3000 cm^{-1}. The stretching vibration of C-H in sugar ring was associated with weak peak at 2923 cm^{-1}. At absorbance 1637 cm^{-1}, the presence of C=O was observed. The presence of C-O-C and -OH in pyran structure (β – glucan) was associated with strong peak at 1075 cm^{-1} and weak peak at 891 cm^{-1} indicating the α - linked glycosyl. The obtained result was compared to IR result of European *G. lucidum* BCCM 31549 obtained from Wan Mohtar et al. (2016d) and found out to have similar spectral arrangements in the same ranges. Thus, these structural confirmations indicated the exopolysaccharide from European *G. pfeifferi* IMI 379841

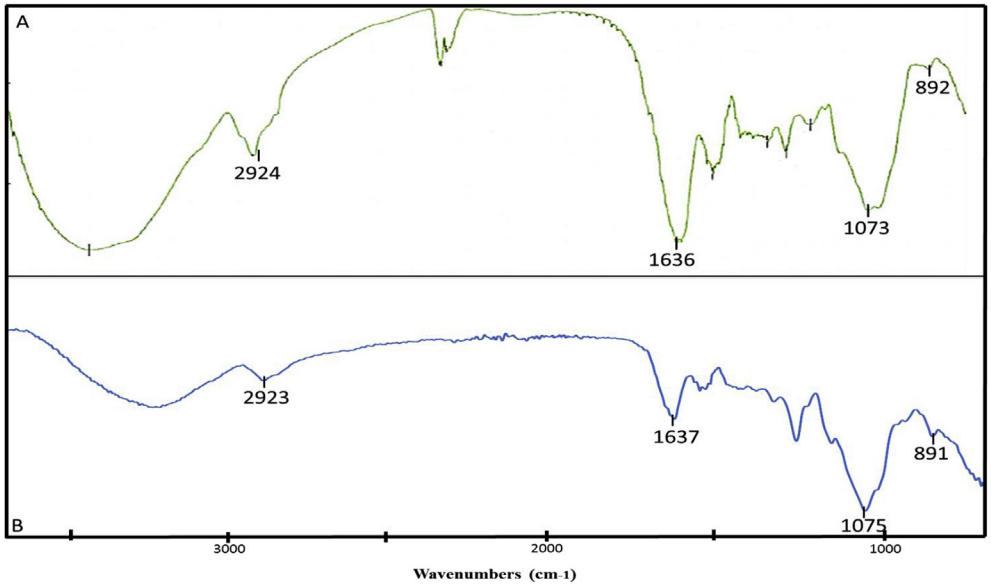


Fig. 7. Comparison of β-glucan IR spectra. A: glucan derived from the mycelium European *G. lucidum* BCCM 31549 (Wan Mohtar et al., 2016d) and B: glucan from European *G. pfeifferi* IMI 379841.

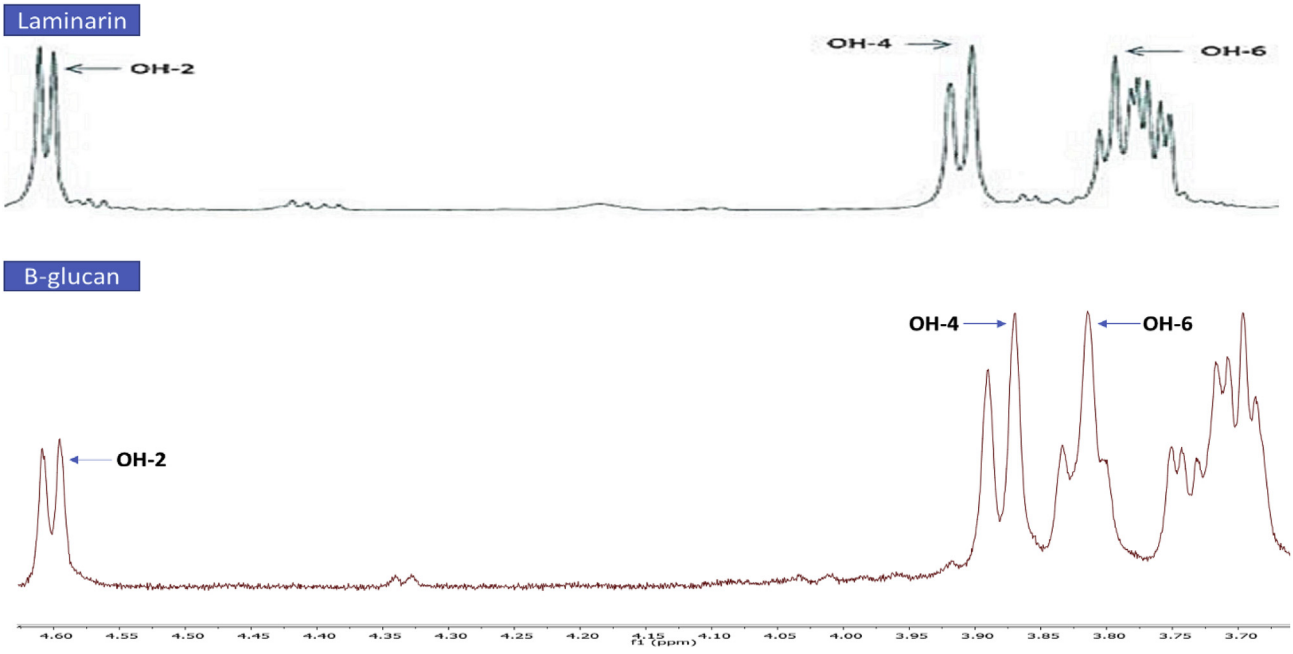


Fig. 8. Comparison of β-glucan ^{1}H-NMR spectra derived from the mycelium European *G. pfeifferi* IMI 379841with Laminarin (standard β-1,3-D-glucan) from *L. digitata*.

contains β-glucan and α-glycosyl linkages.

5.7. Characterisation of exopolysaccharide from the mycelium of G. pfeifferi using proton nuclear magnetic resonance (^{1}H-NMR)

To further confirm the presence and the structure of mycelial β-glucan of *G. pfeifferi*, proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic analysis was performed at 80 °C using D_20-*d* as solvent. The result was compared to laminarin, a standard of β-1,3-D-glucan. The signals at δ 4.58 (OH-2), 3.87 (OH-4) and 3.81 (OH-6) were similar to the standard. Therefore, the FTIR (Fig. 7) and ^{1}H-NMR results (Fig. 8) warranted that BG was composed of (1–3)-β-D-linkages (a typical structure of β-glucan), which was in agreement with Wan Mohtar et al. (2016d).

5.8. Comparison of the current work with studies of other Ganoderma sp. on EPS production and pellet morphology

Table 2 details seven reported studies on improvements in EPS production or descriptions of pellet morphology for any *Ganoderma* species using a batch or fed-batch fermentation technique. Five out of the seven reported studies (Ding et al., 2012; Fazenda et al., 2010; Kim et al., 2006; Lee et al., 1999a, 1999b; Papinutti, 2010; Wagner et al., 2004) demonstrated the relationship between pellet morphology and high EPS production in a fermentation process. These studies indicate that there is a correlation between pellet morphology and the concentration of EPS produced by *G. lucidum* (Ding et al., 2012; Fazenda et al., 2010; Kim et al., 2006; Lee et al., 1999a, 1999b; Papinutti, 2010; Wagner et al., 2004), whereas the current study highlighted this

Table 2
Comparison of the current work on *Ganoderma* sp. fermentation regarding EPS production and its morphology.

Ganoderma species	Technique	EPS productivity, (g/L day^{-1})	Pellet morphology	Pellet diameter (μm)	Reference
G. pfeifferi	Batch	0.05	Small-dispersed pellets	14.33 < d < 16.00	*Current work*
IMI 379841	Repeated-batch	0.1	Smooth large pellets	16.67 < d < 18.33	
G. lucidum CMB 0246	Fed-batch	0.44	Small pellet size	NA	Wagner et al. (2004)
G. resinaceum DG-6556	Fed-batch	0.38	NA	NA	Kim et al. (2006)
G. lucidum	Batch	0.7	NA	NA	Papinutti (2010)
G. lucidum	Batch	0.4	Slight roughness around the edge pellets	800 ≤ d < 2500	Ding et al. (2012)
G. lucidum CCRC36123	Fed-batch	0.9	Freely dispersed and clumped pellets	NA	Fazenda et al. (2010)
G. lucidum AS1 7004	Batch	3.35	Large pellet size with low fractal dimension	2000 ≤ d < 2100	Lee et al. (1999a)
G. lucidum AS1 7004	Batch	0.91	Filamentous pellet	NA	Lee et al. (1999b)

correlation for *G. pfeifferi*. Only two studies (Ding et al., 2012; Lee et al., 1999a) described the morphology and diameter of *G. lucidum* pellets. One study (Ding et al., 2012) merely classified the pellet diameter as medium sized, whereas the current study presents diameter ranges together with macroscopic and microscopic images. To the best of our knowledge, the current data represent the first reported study on pellet morphology in *G. pfeifferi* in a controlled extended liquid fermentation. Although EPS production of *G. pfeifferi* was the lowest compared with other reports, other unexplored factors may have affected EPS production for this underutilized species. Therefore, further research is required to optimize the EPS yield from *G. pfeifferi*.

6. Conclusion

Large *G. pfeifferi* pellets were observed at ideal biomass production conditions, while relatively small and smooth-edged pellets were prevalent at high EPS production. Batch fermentation at condition A (9 g/L glucose, initial pH 4, and 120 rpm) generated medium-sized pellets with protuberances on day 12, with the highest EPS production (0.65 g/L). RBF generated optimal EPS production at cycle 4 (R4), with large second-generation pellets and reduced fermentation time. Throughout this study, *G. pfeifferi* showed resilience to shear stress and marked adaptability to the RBF strategy with high survivability for sustainable EPS production in an extended fermentation strategy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101118.

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