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# Production of exopolysaccharide by *Ganoderma lucidum* in a repeated-batch fermentation

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ABSTRACT

Exopolysaccharide (EPS) productivity of the slow-growing *Ganoderma lucidum* BCCM 31549 mushroom was enhanced in repeated batch fermentation (RBF) process by reducing the fermentation time. The results showed that by using 2.5-L STR unbaffled bioreactor, the fermentation time could be reduced from 10 days per batch to 5 days, due to lag-phase elimination by adopting a RBF strategy. RBF approach was also shown to achieve a semi-continuous mode of production by eliminating the inoculation time between each cycle. The fermentation period was shortened from 50 to 25 days in five consecutive cycles of RBF, with the productivity of EPS increased from 0.81 g/L day$^{-1}$ to 1.32 g/L day$^{-1}$ by using N-limiting medium. The presence of clamp connections in RBF ensures the reproducibility of this strategy. The proposed strategy proved to provide easy operation and thus can be adapted for EPS production.



## 1. Introduction

Exopolysaccharides (EPS) are essential components produced by higher fungi mainly *Ganoderma lucidum*. The EPS created by this therapeutic filamentous fungus have a number of significant functionalities, possess multiple biological activities and exhibit potential medicinal uses (Yang et al., 2013). This mushroom was utilized by traditional Chinese medication for the treatment and prevention of human infections, namely gastric cancer, hypertension, hepatitis, chronic bronchitis, and hypercholesterolemia (Paterson, 2006). Also, it is the chosen organism for creating useful products (supplements, antimicrobial products, and biofilms) (Paterson, 2006), with significant interest in the literature due to its bioactive properties (Li et al., 2013; Liu et al., 2012; Ruan and Popovich, 2012). However, isolating EPS from the fungus is costly and the production rate is insufficient to meet the current market demand. Such phenomena occur because the traditional liquid fermentation requiring long cultivation time, of approximately six months and so is time-consuming, economically unviable and potentially the process is prone to contamination (Leskosek-Cukalovic et al., 2010).

Fermentation for EPS production by *G. lucidum* has been extensively studied and developed in the previous times (Chang et al., 2006; Fazenda et al., 2010; Tang and Zhong, 2002). The filamentous fungus, *G. lucidum*, normally has a low growth rate, resulting in an extended fermentation duration, and low EPS productivity that hinders the industrial scale production. The separate preparation for fungal seed culture from fermentation processes further prolonged the process time.

Previous literature reporting the repeated-batch fermentation strategy (RBF) has been shown to be effective in improving microbial cultures productivity. This approach involves repeatedly substituting a portion of culture with a fresh sample (Birhanli and Yesilada, 2010; Mirończuk et al., 2014; Qu et al., 2013; Wenyan et al., 2014; Zhang et al., 2014) repeatedly. This would not only evade catabolite repression arising from high substrate concentrations but also reduce the fermentation time significantly. This reduction was due to the introduction of the seed culture as well as the fermentation processes.

Previous studies using *G. lucidum* have mainly concentrated on EPS production using batch (Kim et al., 2006), fed-batch (Zhu et al., 2010) and two-stage strategy fermentation (Xu et al., 2010), but there are limited studies on EPS using RBF, which is a different cultivation condition from the batch system (Birhanli and Yesilada, 2006). The RBF system has shown numerous advantages in the long-term fermentation process, such as uncomplicated operating conditions (Birhanli and Yesilada, 2006), self-immobilisation pellet system (Wang et al., 2005), long-term activity maintenance of sustained secretion of bioactive metabolites (Birhanli and Yesilada, 2006), increased economic efficiency (Birhanli and Yesilada, 2006),

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and metabolite mass production (Paterson, 2006; Smith et al., 2002). Birhanli and Yesilada (2006) have shown that the maximum metabolite production (laccase enzyme) obtained by RBF of *F. trogii* was about nine times greater than agitated batch culture, and 13 times higher than static batch culture.

In the present study, the RBF strategy was developed and established as development of this culture method was expected to improve the EPS productivity. Firstly, different batches and RBF experiments were piloted and compared to optimize the parameters of the repeated batch culture, comprising the broth replacement time point and broth replacement ratio precisely for slow-growing *G. lucidum* mycelia. Secondly, the repeated batch culture was further improved to shorten the fermentation time. As of now, to our knowledge, this approach constitutes the first report on the repeated batch culture of EPS-producing *G. lucidum*.

## 2. Materials and methods

### 2.1. Microorganism and medium

A stock culture of *G. lucidum* BCCM 31549 was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/MUCL), Agro Industrial Fungi and Yeast Collection (Leuven, Belgium) as a well-preserved culture slant tube. The fungus was subcultured onto potato dextrose agar (PDA, Oxoid Limited, Hampshire, UK) upon receipt from the supplier to avoid any contamination and to ensure its viability. This approach is also suggested in the previous research (Fazenda et al., 2010). Plates were inoculated and incubated at 30 °C for seven days and stored at 4 °C. The strain was preserved on PDA slant. The compositions of seed culture medium, batch fermentation medium, and repeated batch fermentation medium were all of the same metrics at (g/L): Glucose 30, Yeast Extract 1, $KH_2PO_4$, 0.5, $K_2HPO_4$, 0.5, $MgSO_4$ 0.5, $NH_4Cl$ 4, unless otherwise stated.

### 2.2. Culture conditions

The inoculum preparation of *G. lucidum* BCCM 31549 involved two seed culture stages, both cultivated for ten days at 30 °C and 100 rpm. Four mycelial agar squares (5 mm × 5 mm: extracted using a sterile scalpel) from a ten day old plate was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of medium (first seed culture). To produce more growing hyphae tips, the mycelium from the first seed culture was homogenized using a sterile Warring blender for 20 s. This was used as the inoculum for the second seed culture (500 mL Erlenmeyer flask containing 200 mL medium) and subsequently transferred to the bioreactor. During the inoculum production, it is inoculated into the new fresh medium while they were in late exponential phase (from day 9 to day 11), which means the cells are biochemically at their most active and desired physiological state. EPS fermentation was executed in 500-mL (200-mL working volume) shake flask and 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 is used to inoculate the fermentor, unless otherwise stated. The cultivation was carried out at 30 °C with the pH value maintained at 4.0, dissolved oxygen (DO) was controlled, aeration rate at 2.0 vvm, and agitation speed was controlled at 100 rpm.

### 2.3. Repeated-batch fermentation

The RBF was performed for multiple cycles until the working culture became adequately viscous for the growth or analysis of cells. To determine an appropriate feeding ratio, the old fermentation broth was drained using a peristaltic pump at a predetermined broth replacement ratio [50%, 70%, 80% or 90% (v/v)] and replaced with a fresh preparation of media to permit the *G. lucidum* fungi cells to continue growth. To determine the appropriate feeding time point, three phases were obtained from the shake flask. These were designated with increasing EPS concentration (at the end of logarithmic growth phase), highest EPS concentration (transition phase) and stationary EPS concentration (stationary phase). The next cycle started once a stable, high cell density and high production rate of the EPS is accomplished. Something along the lines of the desired volume of the culture was retained in the reactor and uses as the inoculum for the subsequent fermentation. To this volume an appropriate volume of media was added. Condition A and Condition B (in the results) were used either in shake flask or the bioreactor according to the situations.

### 2.4. Analytical methods

#### 2.4.1. Residual glucose

An HPLC-RI determined the residual sugar in the fermentation broth using an ionic exchange column (Rezex ROA organic acid, phenomenex). The mycelial cells were recovered by centrifugation (HERMLE, model Z160M) at 10,000 rpm for 10 min, after which the supernatant was collected. The supernatant was filtered through a 0.45 μm sterile Corning syringe filter (Sigma-Aldrich, Gillingham, Dorset, U.K). The 0.008 $NH_2SO_4$ was used as the mobile phase at a flow rate of 0.6 mL/min at 55 °C, and the effluent was monitored by an RI detector (RI-1530, Jasco, Japan) (Hsieh et al., 2006). The residual glucose in the in the fermentation broth was also determined by 2900D Biochemistry Analyzer with a glucose biosensor (YSI, Xylem's Tunbridge Wells, U.K).

#### 2.4.2. Dry cell weight (DCW)

The DCW from the harvested *G. lucidum* fermentation process was estimated by filtering a 10–15 mL sample through a pre-dried and weighed GF/C filter (Whatman Ltd., U.K.) using a Buchner funnel filter set attached to a water pump, followed by repeated washing (three times) of the mycelial biomass with distilled water, Mili-Q® Advantage A10 (Millipore, Bedford, MA, USA). The mycelial or pellets filter cake was dried for 2 min in a microwave oven (650 W) or an overnight dryer and cooled in a desiccator for 24 h before weighing. Calculation of the DCW was done by subtraction of pre-weighed filter mass from the mass with the filtrate and multiplied by the dilution factor to get DCW in g/L. All values are taken based on averages of at least three independent trials.

#### 2.4.3. Exopolysaccharide (EPS)

EPS was obtained from the centrifuged (8000 rpm for 15 min) supernatants of the harvested fermentation broth. Then, the crude EPS was precipitated by the addition of four volumes of 95% (v/v) ethanol and left overnight at 4 °C to 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. It was then reassigned to desiccators, left to dry to constant weight, and the weight of EPS was then estimated. All assays were carried out in triplicates.

#### 2.4.4. Residual nitrogen (N)

The residual nitrogen in the fermentation broth was determined using a High-Performance Ammonia Electrode model IS 570-NH3 (Thermo Scientific, UK) coupled with Corning ISE pH/mV meter 240 with a BNC connector. Later, a standard ammonium chloride dilution was prepared to obtain an ammonia standard curve ($y=mx+c$, $R^2=0.99$). A 12.5 ml aliquot of the sample was

mixed with 250 μL of pH-adjusting ISA (Thermo-fisher, UK) and stirred at a moderate, uniform rate. The ammonia electrode was rinsed in distilled water, blotted and placed into the mixture. When a stable reading was obtained the values were recorded and used to calculate the ammonia concentration. The mV values were recorded when a stable reading was displayed. The unknown concentrations of the samples were determined using the prepared calibration curve.

*2.4.5. Image analysis*

The morphology details of the samples collected were assessed using a light microscope (Nikon OPIPHOT-2, Japan) through a coupled camera (JVC, TK-C1381 Color Video Camera). 5 mL of culture sample were re-suspended in 5 mL of a fixative solution according to the method described by Packer and Thomas (1990) and kept at 4 °C until measured. The fixative solution 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. An aliquot (0.1 mL) of each fixed sample was transferred to a slide, air dried, and then stained with methylene blue (Kim et al., 2006). In contrast to human observation, which may be inconsistent, unreliable, and biased, digital image examination is a quick, accurate, and reproducible for evaluating microscopic images (Treskatis et al., 2000).

*2.4.6. Kinetic calculations*

The *G. lucidum* fermentation kinetic parameters were calculated as follows (Stanbury et al., 2013):

$dx/dt = \mu$ max
$R_{batch}$ = biomass concentration per hour (g/L day $^{-1}$)
X max = maximum cell concentration achieved at stationary phase
X o = initial cell concentration at inoculation
$t^{i}$ = time during which the organism grows at $\mu$ max
$t^{ii}$ = time during which the organisms IS not growing at $\mu$ max

**Batch culture**

- Biomass productivity (g/L day$^{-1}$), $PX = \dfrac{Xmax - X\,o}{ti + tii}$ (1)
- $Yield_{(DCW)} = \dfrac{\text{biomass produced}}{\text{glucose consumed}}$ (2)
- EPS productivity, $P_{EPS}$(g/L day$^{-1}$); $\dfrac{Xmax - X\,o}{ti + tii}$ (3)
- Yield (EPS) $= \dfrac{\text{EPS produced}}{\text{glucose consumed}}$ (4)
- Specific production rate of EPS, $Q_{EPS/X}$, [(g/g) day$^{-1}$]; $\dfrac{PEPS}{DCWconcentration}$ (5)

**Repeated-batch culture**

- Biomass productivity (g/L day$^{-1}$), $PX = \dfrac{Xmax - X\,o}{\text{the time for product recovery atcertaincycle in repeated batch culture(day)}}$ (6)
- $Yield_{(DCW)} = \dfrac{Xmax - Xinitial}{(\text{Initial glucose} + \text{glucose added}) - R\ \text{glucose end level}}$, [cycle time (day)] (7)
- EPS productivity, $P_{EPS}$ = (g/L day$^{-1}$); $\dfrac{Xmax - Xo}{\text{the time for product recovery atcertaincycle in repeated batch culture(day)}}$ (8)
- $Yield_{(EPS)} = \dfrac{EPSmax - EPSinitial}{(\text{Initial glucose} + \text{glucose added}) - R\ \text{glucose end level}}$, [cycle time (day)] (9)
- Specific production rate of EPS, $Q_{EPS/X}$ = [(g/g) day$^{-1}$]; $\dfrac{PEPS}{DCWconcentration}$ (10)

*The time for product recovery or cycle time was based on the actual time spent in this study.*

*2.5. Statistical analysis*

All analysis were carried out in triplicate and the respective mean ± S.D determined using the software, GraphPad Prism 5 (Version 5.01) and shown as error bars. If the error bars do not appear, it is assumed that they are smaller than the size of the symbol. A *t*-test was used for the plotting of fermentation graphs. One-way ANOVA and ad-hoc post-test (Bonferroni's Multiples Comparison Test) were used for kinetic parameters comparison.

## 3. Results and discussion

*3.1. Characteristics of RBF in the shake flask*

As shown in Table 1, the different broth replacement ratios were determined in the shake flask for up to five cycles of RBF. The

**Table 1**
Kinetic parameters of different broth replacement ratios by *G. lucidum* BCCM 31549 using repeated-batch fermentation[a].

| Broth replacement ratio[b](R3) | Biomass concentration (max), $x$ (g/L) ($X_2 - X_1$)[c] | Biomass productivity $P_x$(g/L day$^{-1}$) | EPS concentration (max), $_{EPS}$ (g/L) ($EPS_2$–$EPS_1$)[d] | EPS productivity, $P_{EPS}$(g/L day$^{-1}$) | Specific production of EPS, $Q_{EPS/x}$[(g/g) day$^{-1}$] | $Yield_{EPS}$(EPS yield on glucose), ($g_{EPS}$/$g_{GLU}$) | $Yield_{DCW}$(DCW yield on glucose), ($g_{DCW}$/$g_{GLU}$) |
|---|---|---|---|---|---|---|---|
| 50% | 1.04 ± 0.04 | 0.173 ± 0.04 | 0.13 ± 0.03 | 0.022 ± 0.01 | 0.021 ± 0.006 | 0.037 ± 0.01 | 0.29 ± 0.1 |
| 70% | 1.17 ± 0.07 | 0.195 ± 0.03 | 0.19 ± 0.08 | 0.031 ± 0.002 | 0.027 ± 0.007 | 0.022 ± 0.01 | 0.14 ± 0.02 |
| 80% | 1.94 ± 0.10 | 0.324 ± 0.02 | 0.21 ± 0.01 | 0.035 ± 0.004 | 0.018 ± 0.006 | 0.042 ± 0.01 | 0.38 ± 0.05 |
| 90% | 1.39 ± 0.09 | 0.231 ± 0.03 | 0.21 ± 0.01 | 0.035 ± 0.003 | 0.025 ± 0.004 | 0.015 ± 0.01 | 0.10 ± 0.02 |

*One way ANOVA has been carried out for each row with the P value of $< 0.0001$. Bonferroni's post-test shows the Biomass concentration was significantly different ($P < 0.05$) against Biomass concentration, EPS concentration, EPS productivity, Specific production of EPS, Yield $_{EPS}$ and Yield $_{DCW}$, respectively.

[a] Fermentations were carried out in shake flasks with the conditions and medium compositions of [(g/L): Glucose 50, $KH_2PO_4$ 0.5, $K_2HPO_4$ 0.5, $MgSO_47H_2O$ 0.5, yeast extract 1, $NH_4Cl$ 4], 100 rpm, initial pH 4, 10% (v/v) initial inoculum and temperature at 30 °C

[b] All values were at the third cycle (R3) of RBF in 500-mL shake flask.

[c] (X2 − X1) means the value of end biomass concentration minus initial biomass concentration for the each of the cycles.

[d] (EPS2–EPS1) means the value of end EPS concentration minus initial EPS concentration for the each of the cycles.

biomass concentration of (g/L): 1.04, 1.17, 1.94 and 1.39, were obtained on 50%, 70%, 80% and 90% of broth replacement ratios respectively. The third cycle (R3) was the highest for all strategies using RBF. The corresponding biomass productivity for 50%, 70%, 80% and 90% of broth replacement ratios were (g/L day$^{-1}$): 0.173, 0.195, 0.324, and 0.231, respectively. To date, there have been no reports on the use of RBF on the mushroom *G. lucidum*.

Our work indicates that 80% broth replacement ratio was the most optimal biomass producer (1.94 g/L) and at the same time the highest biomass productivity (0.324 g/L day$^{-1}$) compared with other tested ratios. The EPS concentration for (%): 50, 70, 80 and 90 of broth replacement ratios (v/v) were (g/L): 0.13, 0.19, 0.21, and 0.21, respectively. The corresponding broth replacement ratios on EPS productivity were (g/L day$^{-1}$): 0.022, 0.031, 0.035, and 0.035. Observations have shown that 80% broth replacement ratio returned to the highest EPS concentration and EPS productivity compared to other broth replacement ratios. The yield of EPS and DCW that have been produced were calculated as Eqs. (7) and (9) at the end of the process. The EPS yield on glucose for 50%, 70%, 80% and 90% broth replacement ratios were ($g_{EPS}/g_{GLU}$): 0.037, 0.022, 0.042 and 0.015 while DCW yield on glucose were ($g_{DCW}$/$g_{GLU}$): 0.29, 0.14, 0.38 and 0.10, respectively. As expected, 80% broth replacement ratio gave both the highest Yield $_{EPS}$ (0.042 $g_{EPS}/g_{GLU}$) and $Yield_{DCW}$ (0.38 $g_{DCW}/g_{GLU}$) compared to other broth replacement ratios.

Overall, 80% broth replacement ratio kinetics showed the most sustainable and highest concentration and yield for both EPS and DCW. With this strategy, 80% (v/v) of the total fermented culture were harvested and the residue has indirectly formed 20% (v/v) of an active inoculum for the subsequent cycles. Hence, the

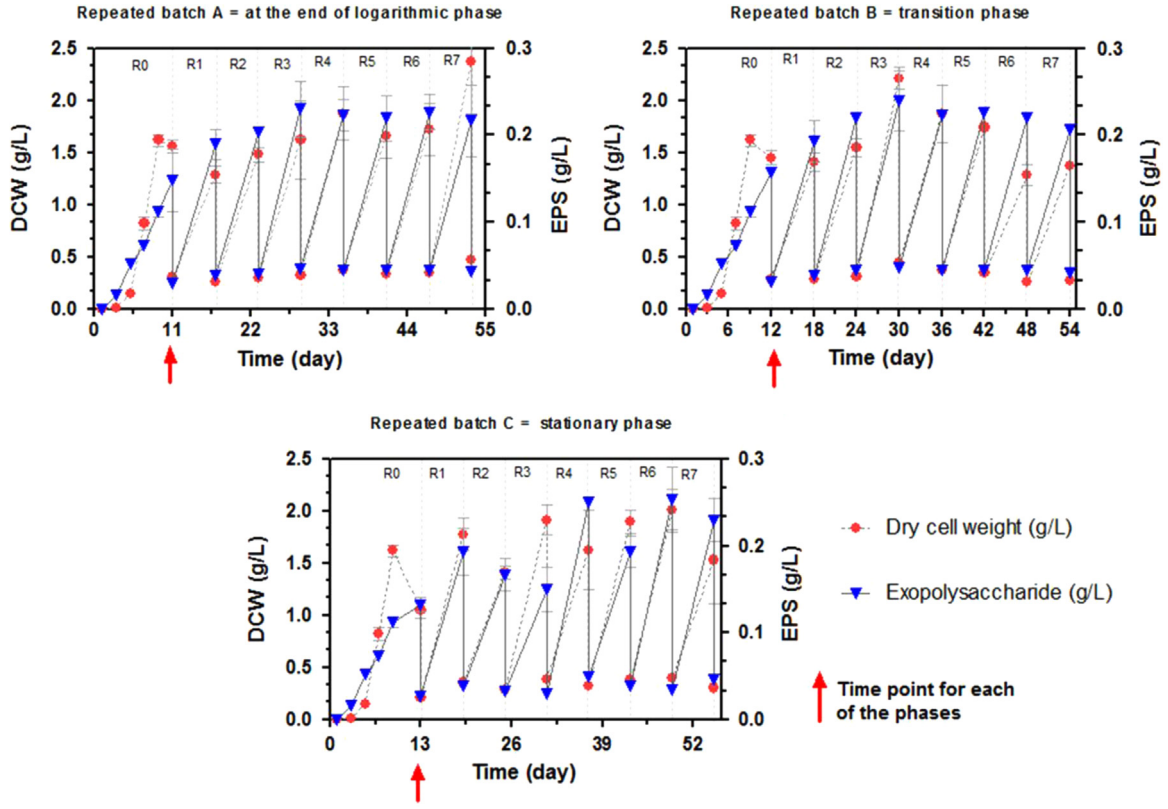


**Fig. 1.** Effect of broth replacement time point on dry cell weight (DCW) and exopolysaccharide (EPS) production during repeated-batch fermentation of *G. lucidum* BCCM 31549 at A = at the end of logarithmic phase, B = transition phase, and C = stationary phase in the 500 mL shake flask. All other fermentation conditions were all the same [(g/L): Glucose 50, $KH_2PO_4$ 0.5, $K_2HPO_4$ 0.5, $MgSO_47H_2O$ 0.5, Yeast Extract 1, $NH_4Cl$ 4], 100 rpm, 10% (v/v) inoculum, initial pH 4, and temperature 30 °C. R1-R7 means fermentation repetition in cycles.

fermentation results corroborate that 80% broth replacement ratio was the ideal strategy both for EPS and DCW production in repeated batch cycles. This conclusion is in agreement with the previous work by Zhang et al. (2014), which used 80% (v/v) of replaced volume during the RBF process of *Baccillus coagulans*.

#### 3.1.1. Broth replacement time points

To determine the effects of different broth replacement time points, the EPS formations from typical batch fermentation in shake flasks were observed with different time points (Fig. 1). The experiments were carried out at the chosen broth replacement ratio of 80% (v/v) and the replacement time points were set at A=end of logarithmic growth phase (day 11), B=transition phase (day 12), and C=stationary phase (day 13). Samples from the RBF experiments were taken at six day intervals from each cycle. Based on Fig. 1, it is observed that a sustainable continuous fungal growth among the three experiments (repeated batch A, B and C) occurs on the production of EPS and DCW. The process of repeated-batch cells have shown its ability with repeated use and a total of seven batches (approximately 42 days) could be fermented consecutively using 500 mL Erlenmeyer flasks which mean the *G. lucidum* cells have shown robustness to repeated fermentation cycles prior upscaling. Between the first cycle (R1) to the seventh (R7), the lag phase reduced and all of these were able to produce EPS and DCW continuously. The approximate amount of EPS obtained was the same, or higher, than the batch fermentation (R0). These results were comparable to prior research by Naritomi et al. (2002) which indicates that repeated batch culture methods lead to higher productivity than with standard batch culture.

For the maximum biomass productivity ($P_X$), time point A reached the highest value of 0.318 g/L day$^{-1}$ at the seventh cycle (R7) and 0.318 g/L day$^{-1}$ for time point B at third cycle (R3). Meanwhile, time point B and C generated the highest EPS productivity ($P_{EPS}$) of 0.033 g/L day$^{-1}$ at third cycle (R3) and 0.036 g/L day$^{-1}$ at the fourth cycle (R4), respectively. It seems that there were no significant differences ($P > 0.05$) on EPS productivity between time points B and C. However, time point B was chosen as the ideal broth replacement time point for RBF as it showed the most ideal growth curve (Fig. 1) compared to time points A and C, and reached the highest total value of $Q_{EPS/x}$ at 0.164 (g/g) day$^{-1}$. The reasons for higher values are because the time points were different morphologically. In the last two cycles, the EPS production rate did not change despite the viscous broth of *G. lucidum* pellets which adhered to the inside wall of the flask.

*G. lucidum* RBF assumed into a variety of pellet shapes throughout the processes, but only some of these progressed in EPS production. According to the shake flask work by Wagner et al. (2004), EPS production was the highest once the ovoid pellets have taken its shape. Time point B has produced ovoid pellets at the third cycle (R3), while time point C managed to produce ovoid pellets at the seventh cycle (R7). Time point A was unable to produce ovoid pellets throughout the cycles (results not shown). The formation of ovoid pellets at R3 of time point B corresponds to its kinetics showing the ideal value for both biomass and EPS productivity. Thus, repeated batch B was chosen for future work in the bioreactor.

During the process, the same fungal cells in the culture were used in the RBF, therefore it is critical to keep the biomass and EPS concentrations below the highly toxic level by changing the media (optimising time point and replaced volume) at an appropriate fermentation time, as mentioned by Wenyan et al. (2014). From the results, the RBF using shake flask has to be stopped at the seventh cycle due to the color changes, toxic metabolites built up and led to possible autolysis. Experiments in the bioreactor was then carried out to determine the highest cycle that RBF can endure.

### 3.2. Upscaling

#### 3.2.1. Characteristics of EPS batch fermentation process

During the bioreactor batch fermentation using condition A (Fig. 2(1)), biomass concentration (DCW) started to increase at day 3 (1.69 g/L) and remained unchanged until day 10. Afterwards, the growth was accelerated reaching its highest value at day 11 (2.82 g/L) and later the biomass slowly reduced and subsequently remained nearly constant. On the other hand, the EPS was low at an early stage but also accelerated at day 10 (0.27 g/L) reaching 0.58 g/L by day 11, and then slowly tapering off. Day 11 was the critical point for important events such as the onset of EPS acceleration and biomass growth. At this time, the dissolved oxygen (DO) was at 10% (Fig. 2(1) at day 11), thus creating a limited environmental conditions, which were favourable for EPS production (Hsieh et al., 2006). The time profile in Fig. 2(1) for condition A was related to its fungal morphology (Fig. 2(2)). This is because, the morphology changes as the process progresses. At day 3, Fig. 2 (2) of condition A, the pellets were in solid sphere shape with some filaments surrounding the surface. As the day continues, the pellets broke into small feathers on day 8, and they clumped together to form large fluffy pellets at day 9. Here, the onset of EPS production occurred on day 10 which coincided with pellet break-up. The protuberances arising from the pellet surface detached from its parent (1st generation pellet), forming the new progeny, and this may have caused the increase in the EPS production.

For batch fermentation using condition B in Fig. 2(3), both biomass and EPS production started to increase/change at day 2. Later, they proceeded slowly until day 10; however the biomass growth began to be more active from day 8 onwards. The highest biomass was obtained at day 11 with the value of 5.66 g/L as well as EPS (0.87 g/L). At this time, the DO percentage was at 10%, which is similar with condition A, thus confirming the limiting environmental conditions spark EPS and biomass growth. Later, both of them slowly disintegrated into an early death phase (day 12).

In morphological terms, these changes occurred in the process under condition B are shown in Fig. 2(4), as the pellet shape refers to EPS productions. As seen in Fig. 2(4), at day 3, the biomass started form sphere-shaped pellets. Those pellets were then engulfing each other to create a larger pellet at day 5 and producing short filaments at the surfaces. At this time around (from day 5 to day 9), the EPS values were constant as the shape of the pellets were not ready for metabolites secretion (Wagner et al., 2004). As day 10 arrived, the hairy pellets broken into starburst shapes consistent with the onset of EPS production as mentioned by Wagner et al. (2004). Here, this dense fungal mycelium grew as a network of filaments that extended apically, branching away from each other across, to form a cluster-pellet structure. These pellets degraded into small hyphal trees at day 11 and these clumped together to reshape the pellet structure. Here, the EPS production was the highest as the second generation pellets were being formed. By day 15, the reshaping of pellet structure was complete, and the cycle of producing the third generation pellets were taking place, however due to nutrient exhaustion and waste build-up, the process was slowed down.

Overall, the EPS and biomass concentrations were higher for batch fermentation by using condition B compared to condition A. Both processes produced two different sets of pellets from first generation followed by second and subsequently to third generation pellets. These two formation patterns were possibly formed due to different osmotic pressure of conditions A (high glucose) and B (low glucose), thus affecting the stress level of the fungus in producing the second generation and third generation pellets, respectively. Furthermore, the kinetic parameters of these conditions were calculated and compared to understand the scale of productivity.

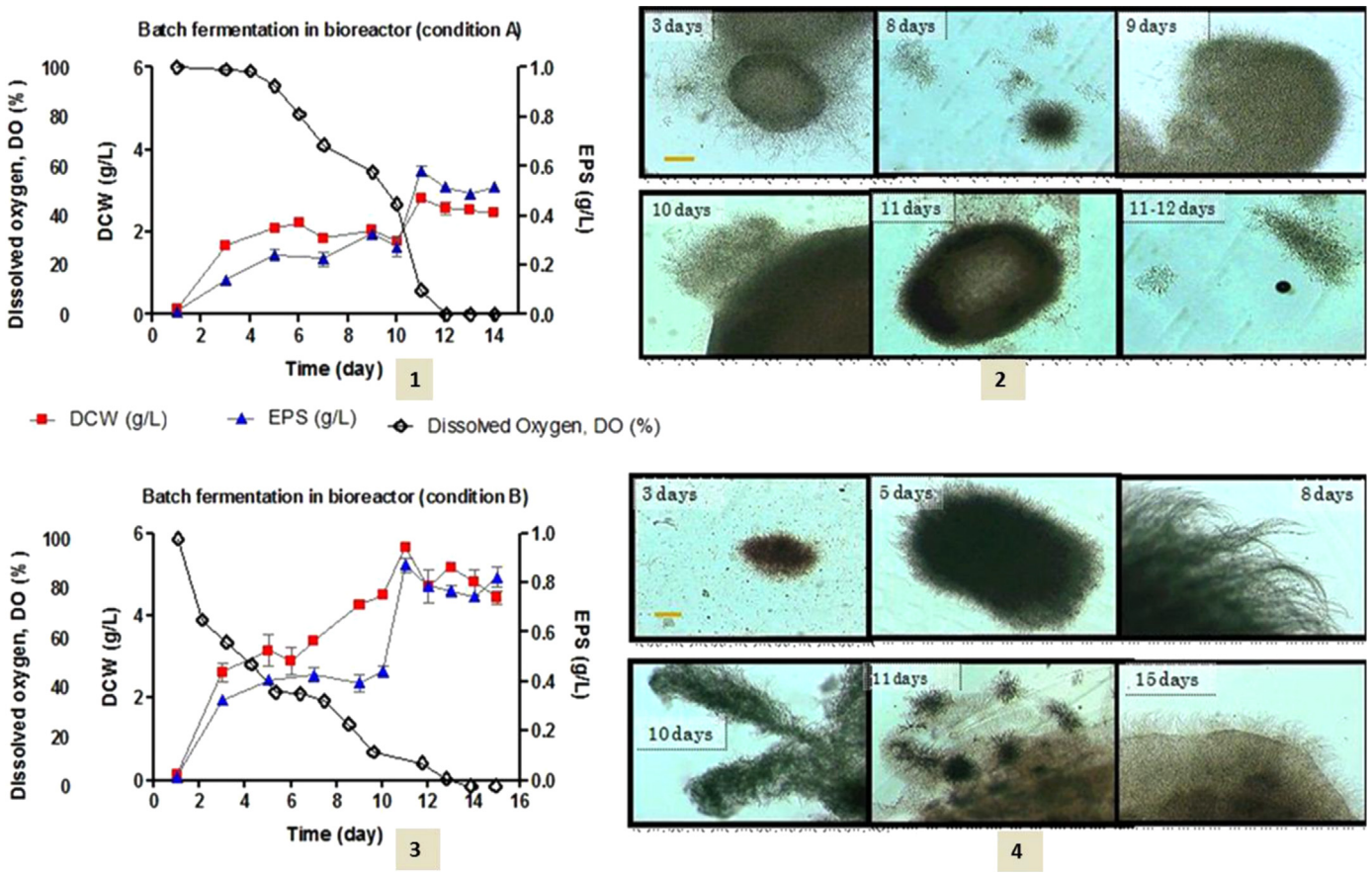


**Fig. 2.** Time course and morphological changes during bioreactor batch fermentation of *G. lucidum* BCCM 31549 using Condition A: [50 g/L of Glucose, 10% (v/v) inoculum] and Condition B: [30 g/L of Glucose, 20% (v/v) inoculum] in the 2-L baffled bioreactor. All other fermentation conditions were all the same [(g/L): $KH_2PO_4$ 0.5, $K_2HPO_4$ 0.5, $MgSO_47H_2O$ 0.5, Yeast Extract 1, $NH_4Cl$ 4], 100 rpm, pH 4, 2% vvm of air and temperature 30 °C. (2) Images (3 and 4) were taken at 4-fold magnification. Bar = 150 μm.

The biomass productivity of condition B (0.515 g/L $day^{-1}$) was 2 times higher than condition A (0.256 g/L $day^{-1}$). In the case of EPS concentration, condition A (0.58 g/L) was lower than condition B (0.87 g/L), while the EPS productivity of condition B (0.079 g/L $day^{-1}$) was 1.5 times higher than condition A (0.053 g/L $day^{-1}$). In summary, the high osmotic pressure environment (due to 50 g/L of Glucose in the system) that is associated with condition A may have affected the EPS secretion, while a higher inoculum percentage of condition B had possibly led to improved the biomass concentration compared to condition A, thus resulted in a small pellet size and higher EPS concentration. Observations on the yield of both conditions have shown that the yield of biomass on the glucose consumed for condition A was 0.19 $g_X/g_{GLU}$, while the rating was 0.33 $g_X/g_{GLU}$ for condition B. The yield of EPS on the sugar consumed for condition B (0.05 $g_{EPS}/g_{GLU}$) was 2.5 times higher than condition A (0.02 $g_{EPS}/g_{GLU}$). Therefore, condition B was the right candidate for the following investigation.

The residual N concentration for condition B was 3.6 g/L of $NH_4Cl$ compared to condition A resulted in 3.5 g/L $NH_4Cl$. Hence, approximately less than 1 g/L of N was consumed by the fungus for both conditions. Following these processes, RBF of *G. lucidum* under the optimal conditions was carried out in the same bioreactor to compare the effectiveness of the two different fermentation techniques (repeated batch and batch), which reportedly can enhance both EPS and biomass productivities.

*3.2.1.1. Bioreactor environment for RBF.* During the RBF with a baffled-bioreactor, the fungal cells were unable to grow (cells were able to grow prior RBF). Thus, the baffle had to be removed and a new set-up was implemented (Fig. 3). Following that, the fungus managed to adapt to the RBF system in an unbaffled bioreactor. As a result, the mode of operation for RBF in the bioreactor was amended and compared in Table 2. Based on the table, day 10 was chosen in the current work as the broth replacement time point. The fermentation results of the repeated cycle's experiments were taken at five day of intervals from each cycle.

Once the baffle was removed, the EPS concentration improved remarkably from 0.87 g/L (baffled) to 8.10 g/L (unbaffled), while the EPS productivity experienced a 10-fold increase from 0.08 g/L $day^{-1}$ (baffled) to 0.81 g/L $day^{-1}$ (unbaffled). In addition, both specific productions of EPS and Yield $_{EPS}$ were also raised from the baffled conditions [0.01 (g/g) $day^{-1}$ and 0.05 $g_{EPS}/g_{GLU}$] compared with the unbaffled conditions [0.81 (g/g) $day^{-1}$ and 0.50 $g_{EPS}/g_{GLU}$]. This EPS enhancement under unbaffled condition was associated with higher N source consumption than the baffled condition. Implementing unbaffled condition led to a significant reduction in biomass concentration of 5.66 g/L, biomass productivity of 0.52 g/L $day^{-1}$ and $Yield_{DCW}$ of 0.33 $g_{DCW}/g_{GLU}$ compared to the baffled conditions of 1.60 g/L, 0.16 g/L $day^{-1}$ and 0.18 $g_{DCW}/g_{GLU}$, respectively. Therefore, it can be concluded that manipulation of the bioreactor environment by removing the baffle reduces the shear stress of the working culture (reduced pellet size), hence by changing one condition (unbaffled) resulted in the increased EPS with a reduced fungal growth, and vice versa (Seviour et al., 2011; Znidarsic and Pavko, 2001).

*3.2.2. Characteristics of EPS on RBF*

Compared to the batch fermentation, the current RBF in the

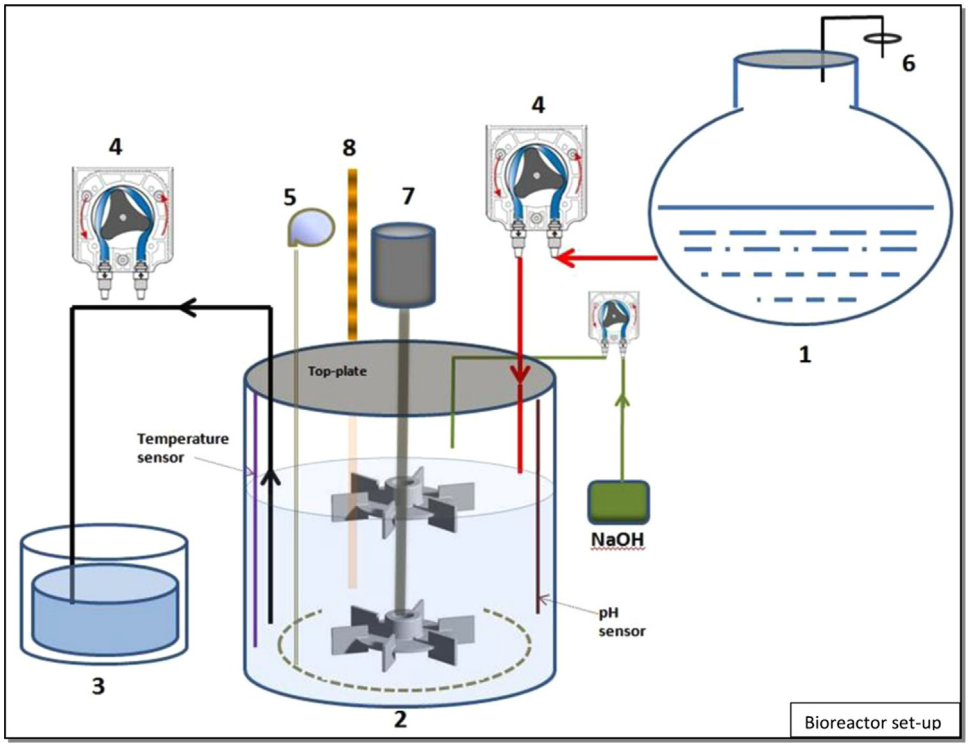


**Fig. 3.** Diagram of experimental equipment: (1) medium reservoir; (2) fermentor; (3) broth receiver; (4) peristaltic pump; (5) air distributing implement; (6) filter; (7) impeller motor; (8) dissolves oxygen sensor. [Figure is by the author].

**Table 2**
Comparison of *G. lucidum* BCCM 31549 batch fermentation in the 2.5-L bioreactor.

| Batch fermentation technique [1] | Biomass concentration, (g/L) ($X_2-X_1$)[2] | Biomass productivity, $P_x$(g/L $day^{-1}$) | EPS concentration, (g/L)($EPS_2$–$EPS_1$)[3] | EPS productivity, $P_{EPS}$(g/L $day^{-1}$) | Specific production of EPS, $Q_{EPS/X}$[(g/g) $day^{-1}$] | $Yield_{EPS}$(EPS yield on glucose), ($g_{EPS}/g_{GLU}$) | $Yield_{DCW}$(DCW yield on glucose), ($g_{DCW}/g_{GLU}$) | End N(ppm) |
|---|---|---|---|---|---|---|---|---|
| Baffled[4](at day 11) | 5.66 ± 0.9 | 0.52 ± 0.02 | 0.87 ± 0.1 | 0.08 ± 0.01 | 0.01 ± 0.01 | 0.05 ± 0.01 | 0.33 ± 0.03 | 878.0 ± 2 |
| Unbaffled[4](at day 10) | 1.60 ± 0.1 | 0.16 ± 0.02 | 8.10 ± 0.3 | 0.81 ± 0.03 | 0.50 ± 0.03 | 0.89 ± 0.08 | 0.18 ± 0.02 | 662.4 ± 2 |

*One way ANOVA has been carried out for each row with the P value of $< 0.0001$. Bonferroni's post-test shows the Biomass concentration was significantly different ($P < 0.05$) vs End N, Biomass productivity vs End N, EPS concentration vs End N, Specific production of EPS vs End N, Yield $_{EPS}$ vs End N, Yield $_{DCW}$ vs End N, End N vs End $NH_4Cl$ concentration, respectively.

[1] Fermentations were executed with the conditions and medium compositions of [(g/L): Glucose 30, $KH_2PO_4$ 0.5, $K_2HPO_4$ 0.5, $MgSO_47H_2O$ 0.5, yeast extract 1, $NH_4Cl$ 4], 2% vvm of air,100 rpm, controlled pH 4, 20% (v/v) inoculum and temperature at 30 °C.

[2] (X2 − X1) means the value of end biomass concentration minus initial biomass concentration.

[3] (EPS2–EPS1) means the value of end EPS concentration minus initial EPS concentration.

[4] The values were taken at day 11 (baffled) and day 10 (unbaffled) as which the EPS was the highest for each condition.

shake flask has shortened the fermentation period. However, seed cultivation for *G. lucidum* was critical for EPS fermentation, especially in a bioreactor. This is because the seed culture morphology would affect the fermentation features (Ji et al., 2014). Typically, it would take 20 days for seed cultivation (10 days for PDA slant, 10 days for the inoculum preparation) in the shake flask and 30 days for seed cultivation in the 2-L bioreactor (10 days for PDA slant, 10 days for the inoculum preparation, 10 days for the seed). Therefore, extensive stretching (high stress environment) costed for the long period of seed cultivation. This drawback can be avoided by implementing RBF strategy, which would save the time for sterilization, cleaning, seed culture preparation, and inoculation process between batches (Xue et al., 2010). In general, this culture method only required as a one-off seed culture, which would shorten the fermentation period significantly.

As a result, the RBF of *G. lucidum* for EPS production was established (Fig. 4), based on the optimized RBF condition that suits the fungal mycelia. That was, 80% (v/v) of the fermentation broth at the end of R0 was removed as the seed for subsequent cycle, R1 and onwards. When the first cycle (R1) was completed after 120 h (5 days), the fermentation broth was discharged, and 400-mL of cell culture was left to serve as the subsequent inoculum. Second cycle (R2) started by supplementing 1600 mL of freshly-prepared fermentation medium via a self-developed peristaltic pump (Fig. 3 (4)). Similarly, the third cycle (R3) was initiated using the same strategy. Overall, the repeated-batch experiment lasted for approximately 840 h (35 days) until the fifth cycle (R5).

### 3.2.3. *N-limiting RBF strategy*

Nitrogen (N) limitation is an efficient and economical strategy for increasing EPS production for some *Ganoderma* species (Zhao et al., 2011). Ammonium from ammonium chloride utilized in this work is the primary N source for *G. lucidum* (Zhao et al., 2011), which are also the key compounds involved in N regulation of EPS

metabolism (Marzluf, 1997). By using either N-limiting or N-free medium on RBF process, the EPS growth and productivity could be enhanced. Therefore, the accumulations of EPS in response to N-limiting and N-free media were measured in the RBF system.

In this method, the N-normal, N-limiting, and N-free media were used during the first cycle (R1) and its subsequent cycles. The N-limiting medium contained 0.4 g/L of $NH_4Cl$ (source of N) which is 10% (w/v) of the original 4 g/L of $NH_4Cl$ (N-normal), which was mentioned in the process. Meanwhile, the N-free medium contained no $NH_4Cl$ during RBF. Both of these media were prepared and implemented during the broth replacement events for each RBF cycle. The aim of this procedure was to determine the effect of N limitation on *G. lucidum* EPS production during the RBF process.

From Fig. 4(X), N-normal medium on RBF favours biomass production, while N-limiting medium (Fig. 4(Z)) on RBF clearly supports the EPS production. The N-free medium on RBF (Fig. 4(Y)) has shown the importance of N source during the repeated batch processes. The repeated batch processes ended at the fifth cycle (R5) due to the decrement in EPS percentages.

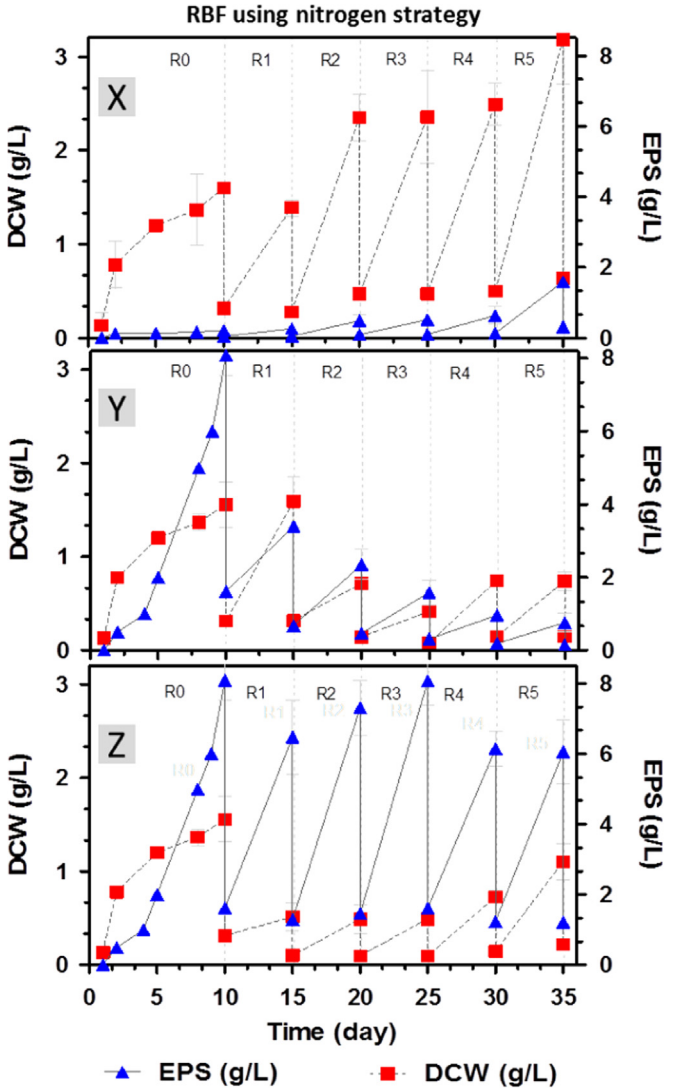


**Fig. 4.** Time course of a repeated-batch fermentation of *G. lucidum* BCCM 31549 using condition B: [30 g/L of Glucose, 20% (v/v) inoculum] in the 2.5-L unbaffled bioreactor. All other fermentation conditions were all the same [(g/L): $KH_2PO_4$ 0.5, $K_2HPO_4$ 0.5, $MgSO_47H_20$ 0.5, Yeast Extract 1, $NH_4Cl$ 4], 100 rpm, pH 4, 2% vvm of air and temperature 30 °C. Nitrogen concentration were at (X) 4 g/L, (Y) 0 g/L, and (Z) 0.4 g/L, respectively during each cycles.

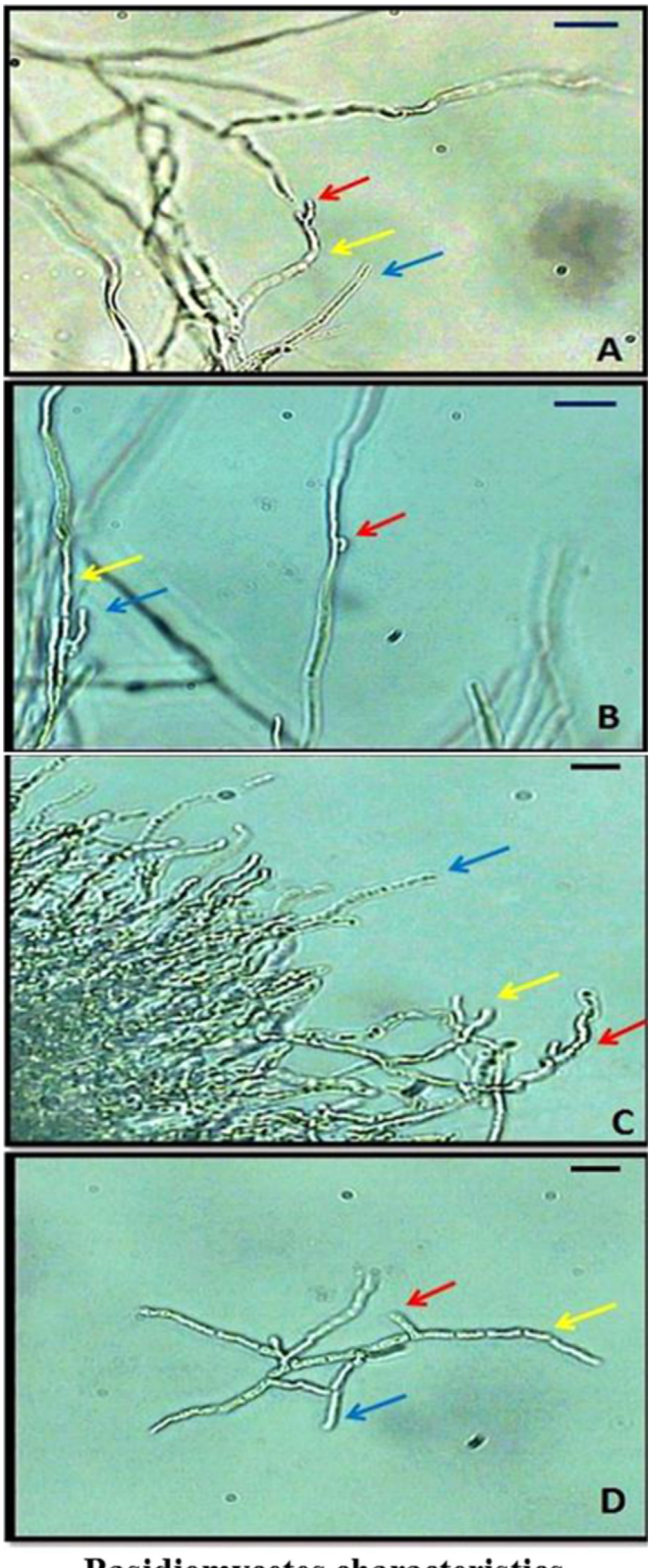


**Fig. 5.** Details of the presence of clamp connections/crozier (red arrow), septum (yellow arrow) and hyphal tip (blue arrow) during repeated-batch fermentation of *G. lucidum* BCCM 31549. Image A and B showed hyphal morphology during the RBF in the 2.5-L unbaffled bioreactor at R5 and R2, respectively. Image A and B were taken at 25-fold magnification and bar = 150 μm. Image C and D showed hyphal morphology during RBF in the 500-mL shake flask at day 10 and day 48, respectively. Image C and D were taken at 20-fold magnification and bar 150 = μm.

Morphologically, the compact exterior of the growing mycelia may cause restriction of oxygen and mass transfers, and thus affecting the EPS fermentation (Ji et al., 2014) in RBF process. Also, different mycelial-pellet sizes eventually affected the EPS concentrations that are high in smaller pellets. If the pellets are smaller than the critical size of oxygen diffusion, less oxygen limitation could occur (good oxygenation), as a consequence the fermentation conditions can be manipulated to favour the EPS over biomass, which is desired.

### 3.3. RBF interactions on the life cycle of G. lucidum

The clamp connections are intended to function in ensuring that each Basidiomycetes "cell" or compartment has a compatible pair of nuclei, and so the dikaryotic state is maintained (Fazenda et al., 2008). In the present work, the increment of the shear rate in the bioreactor effects the morphology of *G. lucidum*, consequently breaking the pellets into more fragmented hyphae, and henceforth encouraging the development of the filamentous form (Fazenda et al., 2010).

Based on these events, the means of transferring nuclei from one hyphal fragment to another or so-called clamp connections were found in Fig. 5 (Image A, B, C, D), which illustrate the details of *G. lucidum* hyphae during the RBF that are both in the bioreactor (image A and B) and shake flask (image C and D). The red arrow indicates the clamp connections, the yellow arrow indicates the septum and blue arrow indicates the hyphal tip. Overall, the findings of the clamp connections in RBF cultures were both astonishing and inspiring, since in liquid cultures submerged hyphae may fail to form clamp connections (Carlisle et al., 1994), which attributed to the use of severe agitation in the current stirred-tank bioreactor (Fazenda et al., 2010). Without the clamp connections, the primary fungal mycelium would not divide synchronously (Deacon, 2013) and the dikaryotic stage of the cells will get disrupted, thus halting the growth and failing RBF.

Therefore, maintaining the dikaryotic stage during the RBF process even with the presence of shear stress is crucial for fungal rejuvenation and EPS production. As part of comparative analysis, liquid culture fermentation of ectomycorrhizal fungi (*Laccaria proxima*) by Carlisle et al. (1994) and revealed that clamp connections were always lost and hence reduced the ability to infect the plant symbiont, ultimately resulted in halting the potential commercialization opportunity which is also supported by Fazenda et al. (2010). The presence of key elements in the present RBF work proved that the investigation herein will widen the applicability of Basidiomycetes in extending batch cultures by using high-scale bioreactor.

### 3.4. Comparison of batch and RBF

RBF has successfully generated 5 days of cycles for every five successful batches, resulting in a total of 55 days fermentation (25+30 days of seed culture). If the conventional batch strategy was applied, 10 days with five successful batches would generate 80 days of total fermentation time (50+30 days of seed culture). These reductions have supported the practicality of RBF strategy for improving the extended batch cultures. As shown in Table 3, compared to the batch, the EPS productivity, specific production of EPS, and Yield $_{EPS}$ of the N-limiting medium on RBF strategy were higher. Therefore, the RBF could positively improve the productivity of fungal EPS fermentation. When compared with the previous RBF work on *Mortierella alpina* (Zygomycota) by Ji et al. (2014), the extended batch strategy has proven to shorten the fermentation time similarly to the current work on *G. lucidum* (Basidiomycota).

It is known that N supply plays a vital role in fungal EPS production, and may also affect fungal morphology (Fazenda et al., 2008), especially when RBF technique is applied with different N conditions. Based on the Table 3, the N-limiting medium (0.4 g/L $NH_4Cl$) used in this study clearly promotes EPS production and was shown to be higher than N-normal condition (4 g/L $NH_4Cl$), while N-free medium (0 g/L $NH_4Cl$) was the lowest. These phenomena were similar to the work of Zhao et al. (2011), where the secondary metabolites production was improved for *G. lucidum* under N-limiting environment.

Overall, the biomass concentration and biomass productivity were descending in the order of N-normal > N-free > N-limiting, while the EPS concentration, specific production of EPS and EPS productivity were ascending in the order of N-free < N-normal < N-limiting. In the end, Yield $_{EPS}$ work well with N-limiting compared to N-normal and N-free. If compared with *G. lucidum* that is grown in the wild, they generally live on substrates that are low in most nutrients except carbon, which is due to the low N content in wood. It is found that the *G. lucidum* behaviour in

**Table 3**
Comparison of batch and repeated-batch strategy in the 2.5-L unbaffled bioreactor using *G. lucidum* BCCM 31549.

| Culture strategy[a] | Time (day) | Biomass concentration, $x$ (g/L) ($X_2-X_1$)[b] | Biomass productivity, $P_x$(g/L day$^{-1}$) | EPS concentration, $_{EPS}$ (g/L)($EPS_2$–$EPS_1$)[c] | EPS productivity, $P_{EPS}$(g/L day$^{-1}$) | Specific production of EPS, $Q_{EPS}/x$((g/g) day$^{-1}$) | $Yield_{EPS}$(EPS yield on glucose), ($g_{EPS}/g_{GLU}$) | $Yield_{DCW}$(DCW yield on glucose), ($g_{DCW}/g_{GLU}$) | End N(ppm) |
|---|---|---|---|---|---|---|---|---|---|
| Batch Repeated-batch | 10 | 1.56 ± 0.35 | 0.16 ± 0.04 | 8.08 ± 1.2 | 0.81 ± 0.04 | 0.52 ± 0.04 | 0.89 ± 0.09 | 0.17 ± 0.04 | 878 ± 2.00 |
| N-normal (third cycle) | 5 | 1.89 ± 0.20 | 0.38 ± 0.08 | 4.64 ± 0.6 | 0.93 ± 0.02 | 0.49 ± 0.08 | 0.73 ± 0.02 | 0.30 ± 0.02 | 991 ± 2.00 |
| N-free (first cycle) | 5 | 1.28 ± 0.05 | 0.26 ± 0.02 | 1.78 ± 0.5 | 0.36 ± 0.04 | 0.28 ± 0.03 | 0.74 ± 0.04 | 0.53 ± 0.07 | 149 ± 2.00 |
| N-limiting (third cycle) | 5 | 0.40 ± 0.25 | 0.08 ± 0.05 | 6.61 ± 0.5 | 1.32 ± 0.04 | 3.34 ± 0.09 | 1.03 ± 0.08 | 0.06 ± 0.05 | 46.3 ± 3.00 |

*The values were taken at the day as which the EPS was the highest for each RBF strategy [N-normal at the third cycle, N-free at the first cycle and N-limiting at the third cycle]

[a] Fermentations were executed with the conditions and medium compositions of [(g/L): Glucose 30, $KH_2PO_4$ 0.5, $K_2HPO_4$ 0.5, $MgSO_47H_2O$ 0.5, yeast extract 1], 100 rpm, controlled pH 4, 20% (v/v) inoculum, 2% vvm of air and temperature at 30 °C. repeated-batch fermentation was done at 80% (v/v) broth replacement ratio and during the transition phase of broth replacement time point.

[b] ($X_2-X_1$) means the value of end biomass concentration minus initial biomass concentration

[c] ($EPS_2$–$EPS_1$) means the value of end EPS concentration minus initial EPS concentration

**Table 4**
Comparison of the current work on *G. lucidum* EPS fermentation using bioreactor.

| Technique | Bioreactor | EPS production, $(_{EPS})$ (g/L) | EPS productivity, $P_{EPS}$ (g/L day$^{-1}$) | Fermentation period (days) | Yield $_{EPS}$ ($g_{EPS}$/$g_{GLU}$) | Reference |
|---|---|---|---|---|---|---|
| RBF | 2.5-L jacketed bioreactor vessel (new Brunswick bio-flow 3000, Edison LN, USA) with Rushton turbine impeller (without baffle) | 6.61 | 1.32 | 5 | 1.03 | *Current work* |
| Batch | 10-L stirred-tank bioreactor (7-L working volume, Baoxing company) 300-L stirred-tank bioreactor (Baoxing Co.) | 1 | 0.08 | 12 | NA | (Wei et al., 2014) |
| | | 1.2 | 0.24 | 5 | NA | (Wei et al., 2014) |
| Fed-batch | 7.5-L stirred-tank bioreactor biotech JS bioreactor system by Baoxing bioengineering (shanghai, China) | 2.59 | 0.14 | 17 | NA | (Tang et al., 2011) |
| Fed-batch | 15-L stainless steel bioreactor (bio-stat C. DCU; B. Braun biotech international, Switzerland) | 4.55 | 0.65 | 5 | NA | (Fazenda et al., 2010) |
| Fed-batch | 10-L stirred tank bioreactor (bioengineering AG, Switzerland) | 9.6 | NA | NA | 1.37 | (Berovic et al., 2003) |
| Fed-batch | 2-L agitated bioreactor | 0.53 | 0.05 | 10 | NA | (Tang and Zhong, 2002) |
| Batch | 2-L bioreactor (Bioflow model C32, new Brunswick scientific) | 1.5 | 0.5 | 3 | NA | (Yang and Liau, 1998) |

the current RBF strategy was the same with *G. lucidum* in the wild, as most of these cultures are naturally N-limited (Fazenda et al., 2008).

With respect to biomass growth in a high concentrated N medium, it was observed that biomass concentrations were higher as the N increased, which is the case with the RBF system (Table 3) and this is also notified by Hsieh et al. (2006). Under these conditions, EPS synthesis demonstrated an opposite pattern to that of observed for growth. This is because EPS concentration was higher at lower N concentration. Thus, maximum EPS levels were reached in an N-limiting medium compared to N-free medium in RBF strategy.

### 3.5. Comparison of the current work with the literature

Table 4 shows the comparison of the current *G. lucidum* fermentation applications with previously reported research either using batch, fed-batch or RBF for the purpose of improving EPS production with the utilisation of a bioreactor. There were only six previous studies involving *G. lucidum* in bioreactors which produced EPS, including four fed-batches and two batches (Berovic et al., 2003; Fazenda et al., 2010; Tang and Zhong, 2002; Tang et al., 2011; Wei et al., 2014; Yang and Liau, 1998). To the best of our knowledge, the RBF study reported here is the most recent application of *G. lucidum* EPS production.

The current work was most effective in producing EPS (6.61 g/L for 5 days of RBF) compared to the closest counterpart (4.55 g/L for 5 days of fed-batch) (Wei et al., 2014) in Table 4. Also, even though the current RBF applied 2.5-L bioreactor as the growing vessel, they had managed to produce EPS consistently for at least three consecutive cycles compared to other higher vessel volumes. Besides, the removal of the baffle for the current RBF work might favour EPS production as compared to the other work as homogeneity in the bioreactor improves significantly.

## 4. Conclusions

The optimized repeated-batch strategy shortened the fermentation period and enhanced the EPS productivity significantly, which generated strong potential for the large-scale EPS production. The presence of clamp connections in RBF strategy ensures the reproducibility of the fungus growth in the liquid environment for both in the shake flask and bioreactor.

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