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Enhanced exopolysaccharide production by *Cordyceps militaris* using repeated batch cultivation

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***Cordyceps militaris* exo-polysaccharides (EPS) have been reported to possess many benefits, such as anti-tumor, anti-inflammatory and antioxidant activities. In this study, the production of EPS via cultivation in a bioreactor was investigated. Glucose and yeast extract were determined to be the most suitable carbon and nitrogen sources for EPS production. The appropriate levels of glucose and yeast extract were 40 g/L and 10 g/L, respectively, resulting in EPS production of 1.686 g/L in a submerged culture. In the stirred-tank fermentor, an agitation rate of 150 rpm and aeration rate of 1.5 vvm were the most effective for EPS production. Due to the anchoring of mycelial cells on the wall of fermentor, a repeated batch approach was used. EPS production of *C. militaris* could be enhanced to a maximum of 5.713 g/L, with a productivity of 476 mg/L/day in the second run. The repeated batch approach was expected to generate higher EPS production, increase EPS yield and productivity and further simplify cultivation operations for bio-industrial application.**

[**Key words:** *Cordyceps militaris*; Fermentation; Agitation; Aeration; Repeated batch; Submerged culture]

Mushroom polysaccharides have supposed health benefits due to their biological and pharmacological activities, including anti-inflammatory, anti-oxidative, anti-tumor and anti-diabetic (1–6). Although polysaccharides can be found in various foods, such as cucurbits, cereal, chicory root, citrus and seaweed (1,2,7–10), edible mushrooms, which are noteworthy of being both food and medicine, provide an abundant supply of polysaccharides whose safety has been well established (5,11). *Cordyceps*, a genus of mushroom, is an entomopathogenic fungus belonging to the class Ascomycetes, which forms bodies in insect hosts. This particular genus has been widely used in traditional medicine in China, Japan, Korea and other Asian countries (12–14). Among the species, *Cordyceps militaris* is popular for its rich amount of bioactive metabolites, like adenosine, cordycepin and polysaccharides (15–19), and has generally been used for medicinal or health food purposes.

Polysaccharides produced by *C. militaris* can be found inside and outside cells as intra- and exo-polysaccharides (EPS), respectively (11), which are reputed to possess various bioactivities, such as anti-tumor (20), antiinflammatory (21), antioxidant (22,23), anti-hyperlipidemic and hepatoprotective activities (17,24). Due to their health and medical benefits, it is worth developing a process to produce *C. militaris* EPS for use in food and medicine (5,11,15).

In the literature, some reports mention the enhancement of EPS production from *C. militaris*. Kim et al. (25) reported that the most suitable carbon and nitrogen sources for EPS production were sucrose and corn steep powder. Shih et al. (18) reported that yeast extract was the best nitrogen source for EPS production by *C. militaris* in a submerged culture. Cui and Zhang (26) proposed a two-stage cultivation method to enhance EPS production, while Kim et al. (27) obtained a higher concentration of EPS under optimized culture conditions using sucrose, peptone and K_2HPO_4 with a pH of 6. Cui and Zhang (28) found that the addition of metal ions and surfactant were beneficial to EPS production. Due to difficulties with mycelial growth in scaled-up bioreactors, most studies focused on the nutrients, additives or environmental tests in the shake flasks. Few studies investigated the cultivation and operation conditions of the bioreactor during *C. militaris* EPS production (25,29).

Due to the attachment property of the mycelial cells, *C. militaris* cells would grow and accumulate along the inside wall of the reactor during the cultivation, which could serve as the self-immobilized cells for repeated batch operation. This study tried to apply the immobilized cells to enhance the EPS production by *C. militaris*. To determine the medium suited for submerged culture of *C. militaris*, various carbon and nitrogen sources and concentrations were tested in a flask culture. The selected medium was then used in a 5 L bioreactor and the effects of aeration and agitation on the biomass and EPS productions were examined. In addition, a repeated batch operation was developed to immobilize the mycelial cells for repeated EPS production by *C. militaris*. Finally, reports in the literature concerning EPS production were listed and compared.

MATERIALS AND METHODS

Chemicals and reagents The potato dextrose agar (PDA) was obtained from BD Difco, Franklin Lakes, NJ, USA. Glucose and yeast extract were purchased from

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Sigma–Aldrich, St. Louis, MO, USA. All other solvents and chemicals used were of analytical grade and obtained from the local dealer.

Strain and cultivation conditions *C. militaris* (BCRC 33735) was obtained from the Culture Collection Research Center at the Food Industry Research and Development Institute (Hsinchu, Taiwan). The strain was cultured on a PDA plate at 25°C for 7 days. One square unit (1 cm × 1 cm) of mycelium was inoculated on to a fresh PDA plate for a 7-day growth to activate the cells. The seed medium (100 ml) in a 500 ml flask containing 4% (w/v) glucose (Glu), 1% yeast extract (YE), 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% $FeSO_4 \cdot 7H_2O$ (initial pH adjusted to 6.0) was sterilized at 121°C for 25 min, followed by inoculation with two square units (2 cm × 2 cm) of mycelia. Cultivation was carried out on a rotary shaker at 150 rpm and 25°C for 7 days and the harvested broth was used as the inoculum to main culture. The main flask medium (100 ml, the same composition as the seed medium) was inoculated with 5% (v/v) of the seed culture and incubated on the rotary shaker at 150 rpm and 25°C for 14 days (30).

Cultivation in the fermentor A 5 L fermentor (BTF-A5L, Biotop Industrial Co., Taiwan) containing 3 L of the main medium was used for the submerged cultivation of *C. militaris*. A volume of 10% (v/v) of the seed culture was used as the inoculum. Incubation took place at 25°C, 100 rpm and 1 vvm (i.e., volume of air to volume of broth per min) without pH control for 14 days. The foaming was controlled via the addition of antifoam (Akasil antifoam 30PS, Amochem, Malaysia).

Repeated batch (RB) cultivation was carried out by harvesting cultivation broth and adding fresh medium to the bioreactor at the end of each cultivation. The first repeated batch culture had the same conditions as the batch cultivation. The second cycle harvested the broth out of the fermentor then topped up the volume to 3 L with fresh medium, followed by the same batch process. Subsequent batch cycles were conducted in a similar manner as the second run. The samples collected during the RB operation were subjected to glucose, biomass and EPS analysis.

Assays Mycelial biomass in the broth was obtained by centrifugation at 7000 ×*g* for 10 min. The resulting cell pellet was washed three times with distilled water and dried at 60°C to a constant weight. The cell concentration is represented as the grams of dry cell weight (g DCW) per liter. For the biomass assay in fermentor, the measurement is based on the broth sampled in each time interval.

For the quantitative determination of glucose in the culture broth, 10 µl of diluted cell-free broth was mixed with 1 ml glucose liquid reagent (Tonyar Biotech, Inc.) and incubated at 100 rpm for 10 min at 37°C. After incubation, the absorbance of the reaction mixture was measured at 500 nm. Glucose was used as the standard to build the calibration curve. All procedures were performed according to the manufacturer's instructions (Pointe Scientific, Inc.)

To determine EPS, the collected broth was centrifuged at 7000 ×*g*. Two mL of the supernatant was mixed with 8 ml of 95% (v/v) ethanol and left to stand overnight at 4°C to precipitate the crude EPS. The EPS was then collected by centrifugation at 16,000 ×*g* for 30 min. The resulting precipitate was rinsed 2 times with 8 ml of 75% (v/v) ethanol, followed by drying at 60°C to remove residual ethanol. The dried precipitate was re-suspended in water and subjected to phenol-sulfuric acid assay with glucose as the standard for the calibration curve (31).

Statistical analysis To obtain the statistical results, all the data were analyzed by Origin software (version 7.0). Experiments were performed in triplicate and expressed in mean ± SD; the significant difference between means was identified via Tukey analysis (ANOVA).

RESULTS AND DISCUSSION

Effect of medium composition on biomass and EPS production The optimal temperature and initial pH conditions for cultivating *C. militaris* were conducted in flask tests. It was found that the best conditions were 25°C with the initial pH set at 6.0. In contrast, it was found that the pH value in the cultivation process (fermentor) was in range of 5.0–6.0 (data not shown). Due to the robust pH in cultivation, the process was conducted without pH control. To find the best medium for cultivating *C. militaris,* various carbon and nitrogen sources were tested. In the literature, Park et al. (32) found that a medium with sucrose and corn steep powder is best for exopolymer production from *C. militaris*. Cui and Jia (33) proposed a medium containing glucose, yeast extract and peptone for EPS production in *C. militaris* cultivation. Mao et al. (34) suggested the combination of glucose and peptone was the best medium for EPS production from *C. militaris*. In this study, glucose (GL) and yeast extract (YE) were found to be the most effective for cultivation in pre-tests (data not shown). A medium with 40 g/L of glucose and 10 g/L of yeast extract was therefore applied as the based medium for EPS production.

The concentrations of GL and YE were varied to evaluate the effect of composition on mycelial growth and metabolite production. The time courses of GL consumption, mycelial biomass and EPS production are shown in Fig. 1. It was found that biomass and EPS production increased when GL and YE levels in the medium also increased. A maximum mycelial biomass of 44.0 g/L and EPS level of 2.256 g/L were obtained on day 14, when the concentration of YE and GL were both 80 g/L. In contrast, the lowest biomass of 15.5 g/L and EPS level of 1.129 g/L were found on days 12 and 6, respectively, when GL and YE were both set at 20 g/L.

In addition, cells would only grow until day 12 when GL was 40 g/L and YE was 10–20 g/L. If the concentrations of GL and YE were both in the range of 40–80 g/L, the cells would grow an additional two days. If GL supplementation was as low as 20 g/L, the biomass immediately decreased once glucose was exhausted on day 6. These results indicate that cell growth timing is closely related to GL concentration. With insufficient Glu, the biomass will stop growing earlier. Meanwhile EPS production stopped on day 12

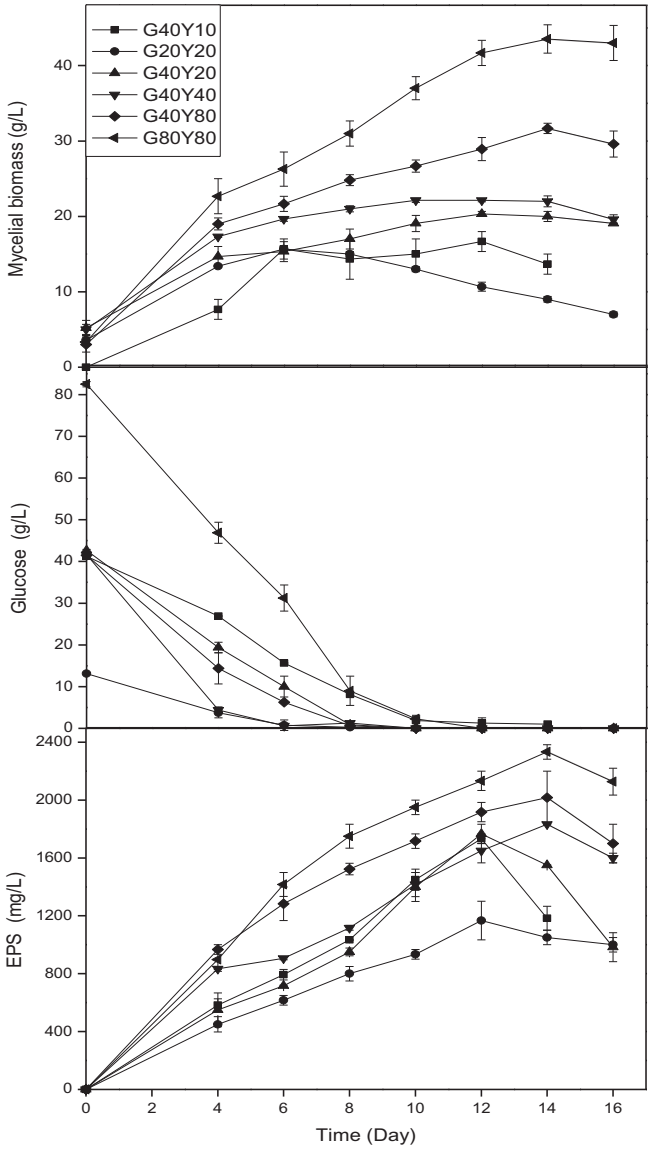

FIG. 1. Time courses of mycelial biomass, glucose consumption and EPS production of *C. militaris* with various combinations of glucose and yeast extract supplementation. Symbols G and Y represent glucose and yeast extract levels, respectively. Values correspond to concentration (g/L).

when the concentration of YE was set below 40 g/L. With an increased YE supplementation of 40–80 g/L, EPS production would last until day 14. This indicates that the length of EPS production is related to YE concentration.

To analyze the effects of YE and GL in the medium, the maximum mycelial biomass and EPS production were recorded; these are summarized in Table 1. To ease the comparison, the experimental data were arranged into four sets (some of the data would appear twice). As seen in set 1, where GL concentration was set at 40 g/L, the biomass was found to increase greatly with increasing YE concentration. Likewise, when GL concentration was increased while YE stayed constant, there was a remarkable increase in EPS production, as observed in set 2 and set 3.

When the GL is set at 40 g/L, the increase of biomass and EPS following the increase of YE was calculated via the linear regression. The results are listed in Table 1. The average biomass and EPS yields per YE were estimated as 0.21 g CDW/gYE and 3.85 mg EPS/gYE. On the other hand, the biomass yields per GL were calculated to be 0.26 gCDW/gGL and 0.3 gCDW/gGL when YE was set at 20 g/L and 80 g/L respectively (set 2 and set 3). The EPS yields per GL were calculated to be 29.6 mg EPS/gGL and 7.75 mg EPS/gGL when YE was set at 20 g/L and 80 g/L, respectively (set 2 and set 3). These results indicate that GL contributes more to EPS production than YE, while its yield to biomass is only a little higher than YE.

In addition, the specific EPS production (Table 1) decreased with increased YE concentration in set 1. This is consistent with the findings of Mian et al. (35), where the specific EPS production increased with a lower nitrogen concentration. The metabolic pathway of *C. militaris* favors cell growth under high nitrogen source levels, which results in a low specific yield of EPS. It was found that higher GL and YE levels were beneficial to both biomass and EPS production. However, the specific EPS production decreased dramatically with increased GL and YE concentrations.

As seen from the data of the specific growth rate (Table 1), it was found that while increased YE with constant GL, an increased specific growth rate was observed (except for GL40YE10 case). In addition, when the C: N ratio is set to 1, the increase in C and N levels would result in a significant increase in specific cell growth rate. The ratio of C and N sources was reported to influence the metabolic functions of the cells. Park et al. (32) indicated that the best mycelial biomass produced by *C. militaris* was obtained when the C:N ratio of the medium was set at 20:1. Masuda et al. (16) and Mao et al. (34) also found that EPS produced from *C. militaris* was best when the C:N ratio was set at 2:1. In our observations, when C:N ratio was set at 1:1 (Table 1, set 4), a medium with higher substrate levels produced a higher biomass and EPS levels but a lower specific yield of EPS. These results indicate that the concentrations of C and N sources, rather than the C:N ratio, guide biomass and EPS production.

Owing to the fact that the specific EPS yield is directly related to EPS production by the mycelial cells, the medium with 40 g/L GL and 10 g/L YE was adopted for running the 5 L fermentor.

Effects of agitation on biomass and EPS production Agitation is required to mix all the components and break the air bubbles for better oxygen mass transfer in the fermentor. A low agitation rate may reduce oxygen transfer rate and slow down cell growth, while high agitation can cause higher shear stress and lead to cell damage (36–39). To investigate the effect of agitation on EPS production from *C. militaris*, several agitation rates (100, 150, 200 and 250 rpm) were investigated. The results are shown in Fig. 2.

It was found that the lowest biomass of 18.4 g/L was harvested on day 8 when the agitation rate was set at 100 rpm. When the highest agitation rate of 250 rpm was applied, the mycelial biomass could reach its maximum of 20.3 g/L on day 6. In comparison, a mycelial biomass of 20.9 g/L could be obtained on day 8 with an agitation rate of 200 rpm. The rate of GL consumption was found to increase with agitation rate. In general, GL would be exhausted by day 8 with a high agitation rate of 150–250 rpm. Only with a low agitation rate of 100 rpm would GL still exist on day 10.

EPS production of 0.89 g/L was found at agitation rates of both 200 and 100 rpm after 10 and 12 days, respectively. In contrast, the highest EPS production of 1.18 g/L was obtained on day 12 with an agitation rate of 150 rpm. At the lower agitation rate, the cells grew slowly and limited EPS formation due to the low mass transfer rate. Conversely, at a higher agitation rate, the shear force may hinder EPS formation from *C. militaris* due to cell damage. Park et al. (40) reported that mycelial biomass of *C. militaris* increased with higher agitation, but the greatest exobiopolymer yield was achieved at a moderate rate. As such, an agitation rate of 150 rpm was chosen for *C. militaris* cultivation in the fermentor.

Effects of aeration on biomass and EPS production Air supply is an important factor for cell growth and metabolite production by fungi (18,41–43). For EPS production in the submerged *C. militaris* culture, adequate oxygen must be supplied continuously via aeration. Three aeration rates (1, 1.5 and 2 vvm) were tested during *C. militaris* cultivation at 150 rpm and 25°C; the results are shown in Fig. 3. According to the time course of mycelial growth, the initial growth rate was a little higher with the higher aeration rate. The greatest mycelial biomass of 20.63 g/L was obtained on day 8 with the highest aeration rate of 2 vvm. The consumption rate of glucose was also found to increase with aeration rate. This indicates that under higher aeration, cells would grow faster and

TABLE 1. The maximum mycelial biomass and EPS production from *C. militaris* submerged culture with various combinations.

Item	GL (g/L)	YE (g/L)	Biomass[a] (CDW g/L)	EPS[b] (g/L)	Specific EPS yield (mg/g CDW)	Calculated productivity (mg/L/day)	Specific growth rate (day^{-1})	Calculated biomass yield	Calculated EPS yield
Set 1	40	10	16.7 ± 1.3	1.69 ± 0.13	101	141	0.260	0.21[c] (gCDW/gYE)	3.85[c] (mgEPS/gYE)
	40	20	20.7 ± 1.5	1.72 ± 0.17	83	143	0.191		
	40	40	22.4 ± 1.8	1.79 ± 0.17	80	128	0.240		
	40	80	31.9 ± 2.1	1.96 ± 0.12	61	140	0.348		
Set 2	20	20	15.5 ± 0.6	1.13 ± 0.10	103	94	0.257	0.26[d] (gCDW/gGL)	29.6[d] (mgEPS/gGL)
	40	20	20.7 ± 1.2	1.72 ± 0.16	83	143	0.191		
Set 3	40	80	31.9 ± 2.1	1.96 ± 0.12	61	140	0.348	0.30[d] (gCDW/gGL)	7.75[d] (mgEPS/gGL)
	80	80	44.0 ± 2.8	2.27 ± 0.21	51	162	0.363		
Set 4	20	20	15.5 ± 0.6	1.13 ± 0.10	103	94	0.257	-[e]	-[e]
	40	40	22.4 ± 1.8	1.79 ± 0.15	80	128	0.240		
	80	80	44.0 ± 2.8	2.27 ± 0.21	51	162	0.363		

[a] The data express the highest mean biomass level in the cultivation; CDW, cell dry weight. Data are expressed in mean ± standard deviation.
[b] The data express the highest EPS level in the cultivation. Data are expressed in mean ± standard deviation.
[c] The values are obtained by linearly fitting the relationship of biomass vs YE, and EPS vs YE.
[d] The values are obtained by directly calculating the line slopes of biomass vs GL, and EPS vs YE.
[e] The negative sign indicate no calculated data.

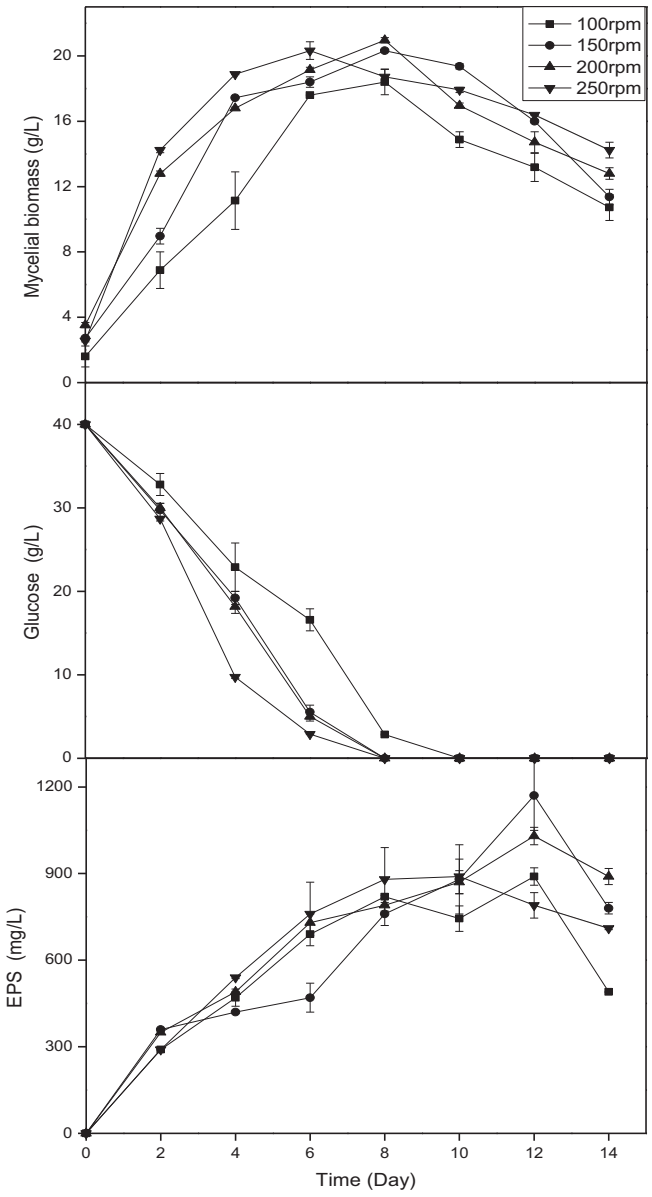

FIG. 2. Time courses of mycelial biomass, glucose consumption and EPS production of *C. militaris* with various agitation rates and an aeration rate of 1 vvm.

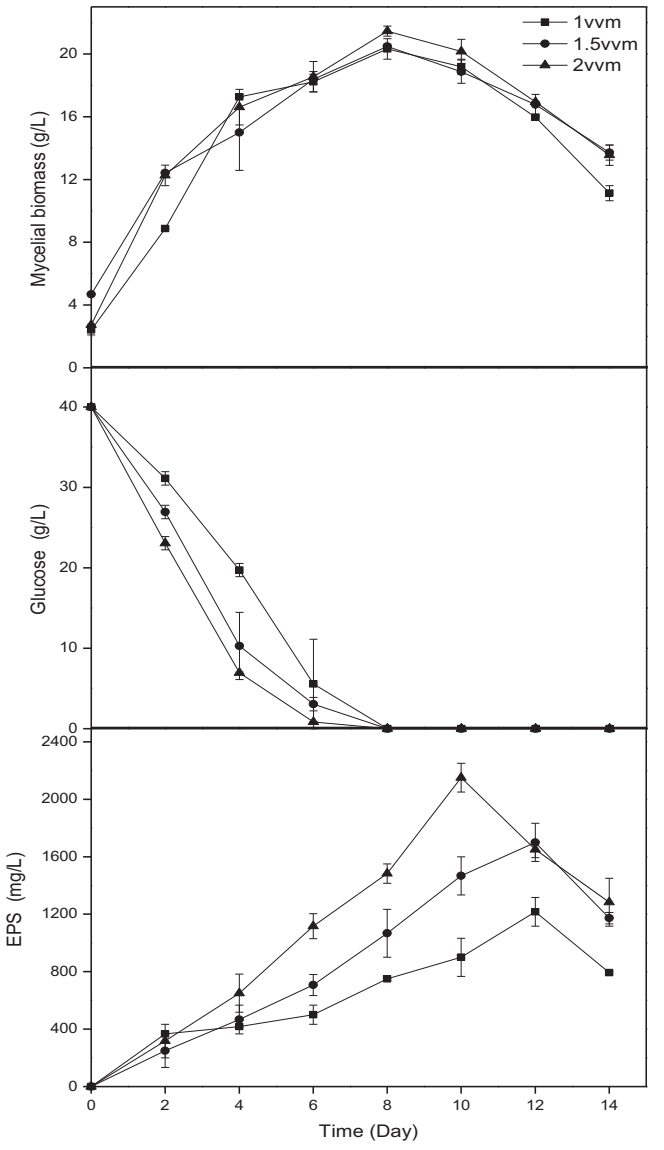

FIG. 3. Time courses of mycelial biomass, glucose consumption and EPS production of *C. militaris* with various aeration rates and an agitation rate of 150 rpm.

consume glucose more rapidly. Maximum EPS levels with aeration rates of 1 and 1.5 vvm were 1.217 g/L and 1.623 g/L, respectively, on day 12, while the highest EPS level of 2.15 g/L was observed after 10 days with an aeration rate of 2 vvm. The results also showed that the duration of EPS production was shorter under higher aeration rates.

Kim et al. (29) demonstrated that a greater amount of oxygen dissolved in the medium would increase EPS production by *Paecilomyces sinclairii*. Xu and Yun (44) also reported the EPS production from *Paecilomyces tenuipes* C240 was increased under higher aeration rates. From the studies of agitation and aeration, it was concluded that a higher agitation rate would cause higher shear stress, leading to a lower biomass and EPS production. On the other hand, a higher aeration rate would enhance the oxygen transfer rate, leading to a higher glucose consumption and EPS formation rates. In this study, the aeration at 2 vvm would cause more foaming and increase the difficulty of the operation. As such, an aeration of 1.5 vvm was used in subsequent experiments.

Repeated batch operations RB cultivation, an alternative to batch production, offers several advantages, including the reuse of microbial cells for subsequent fermentation runs with higher initial cell concentrations and less time required for operation processes (45). In addition, a higher productivity of microbial processes could be achieved. Several studies reported on the benefits of the RB process on metabolite production from microbes. Masuda et al. (46) demonstrated that RB operations effectively increased the biomass and cordycepin yield from *C. militaris*, however, they didn't mention about the EPS production via this RB cultivation. Rymowicz et al. (47) enhanced the productivity of citric acid from *Yarrowia lipolytica* and prolonged the length of the process with RB cultivation. Huang et al. (48) reported a 2.5-fold enhancement of hyaluronic acid from *Streptococcus zooepidemicus* via RB cultivation.

To test the effects of RB cultivation on EPS production from *C. militaris*, a 5 L stirred-tank bioreactor was used. As seen in Fig. 4, maximum EPS production was obtained on day 12 in the first batch. The culture broth was then harvested and new medium added. A maximum mycelial biomass of 21.3 g/L was harvested from the first batch. Due to no inoculum to the batches after the first run, the

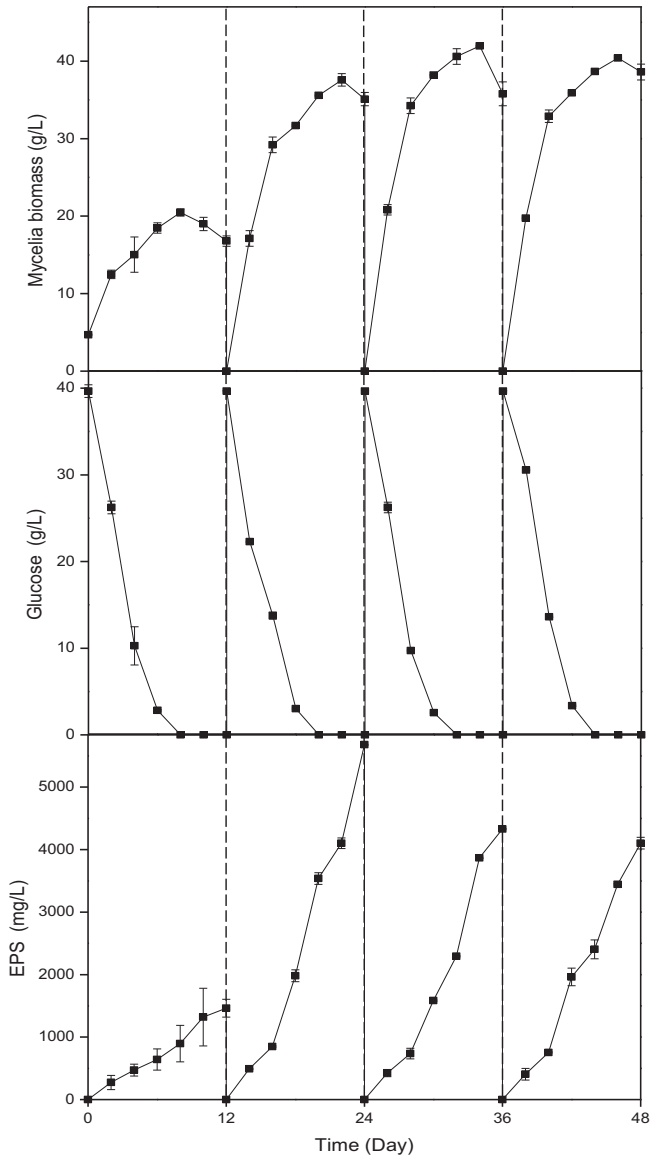

FIG. 4. Time courses of mycelial biomass, glucose consumption and EPS production of *C. militaris* by repeated batch cultivation under an agitation rate of 150 rpm and aeration rate of 1.5 vvm. The dotted lines signify the replacement of culture medium. The initial biomass and EPS for the batch after the first run were set to zero to indicate the supplemented fresh medium.

initial biomass levels were set to zero in Fig. 4. This meant the anchored cells would automatically release when they contacted with the added medium. It was found that the biomass increased to 42.2 g/L in the second batch, a remarkable two-fold increase. While the mycelial biomass reached its maximum on day 8 in the first batch, in subsequent batches the maximum biomass was obtained on day 10 after replacing the culture medium. It should be noted that whenever the medium was replaced, many mycelial cells remained attached to the inner walls of the bioreactor. As the attached mycelia could not be harvested, they served as inoculum for the next batch. As a result, due to the high levels of inoculum, the mycelial biomass grew quickly in the medium. This may explain why the biomass increased almost 2-fold compared to the first batch.

EPS level was about 1.448 g/L in the first batch, while in the second batch, an EPS level of 5.713 g/L was obtained with a productivity of 476 mg/L/day, as shown in Table 2. EPS yields were then maintained at 4.1–4.3 g/L for the third and fourth batches. Interestingly, the increase in EPS production was much higher than the level of biomass enhancement.

Mycelial cells attached on the inner surface might play a key role in the higher EPS production. When replacing medium for RB operations, the residual mycelia could act as seeding for the reactor, suggesting that abundant nutrition could be used for greater biomass and EPS production. The mycelia grew more efficiently thanks to sufficient glucose supplementation. As such, some of the glucose originally intended for cell growth was instead put towards EPS production, resulting in an increase in EPS. This may explain the significant increases in biomass and EPS production in the second batch. In the third and fourth batches, EPS production decreased a bit, however a more stable production was observed. The aging of the attached mycelial cells might slightly lower EPS production over long term production.

As compared to the productivity data of EPS in flask (94–162 mg/L/day in Table 1), the EPS productivity in bioreactor was calculated individually and the data are in range of 83–210 mg/L/day. The medium composition and the operation conditions would make the differences. On the other hand, the productivity data of repeated batch operation were evaluated and listed in Table 2. Although the amount of EPS was the highest in the second batch, the average EPS productivity for the first two batches was 298 mg/L/day. Even with the aging of the attached mycelial cells, the average EPS productivities for the first three and four repeated operations were 320 mg/L/day and 326 mg/L/day, respectively. It seems that with more repeated batches, the average EPS productivity reaches a constant, which is better than that for running the first two batches. Furthermore, repeated batches could simplify cultivation procedures, resulting in less work and cost savings.

TABLE 2. Maximum EPS production and EPS productivity of repeated batch operations.

Items	Repeated batch operation cycle			
	1	2	3	4
EPS production (g/L)	1.448	5.713	4.338	4.132
EPS productivity (mg/L/day)	121	476	362	344
Average EPS productivity[a] (mg/L/day)	121	298	320	326

[a] The average productivity is calculated as (total EPS concentration/the whole cultivation time).

TABLE 3. Comparison of maximum EPS production from *C. militaris* using different strategies.

Cultivation strategy	Reactor	EPS		Ref.
		Maximum production (g/L)	Productivity (mg/L/day)	
Dark conditions	Flask	1.4	140	30
Culture medium and pH optimization	Flask	1.5	92	18
Ultraviolet mutagenesis	Flask	1.9	-[a]	23
Culture medium optimization	Flask	2.0	280	33
Blue light illumination	Flask	2.4	240	30
Two-stage culture	Flask	3.2	267	26
Metal ions and surfactant additions with two-stage culture	Flask	3.3	273	28
Culture medium, pH and temperature optimization	Fermentor	3.4	243	25
Optimized batch culture	Flask	2.3	220	This study
Repeated batch	Fermentor	5.7	476	This study

[a] The data were not enough for estimation.

Comparison of EPS production from *C. militaris* A comparison of EPS production from *C. militaris* in the literature is shown in Table 3. Shih et al. (18) harvested 1.5 g/L of EPS from *C. militaris* by optimizing cultivation conditions in a flask. Lin et al. (23) employed ultraviolet light to irradiate *C. militaris* and obtained an EPS-overproducing mutant; the EPS yield of this mutant could reach 1.9 g/L in flask cultivation. Kho et al. (30) described how the use of blue light during *C. militaris* cultivation could result in an EPS production of 2.4 g/L. Cui and Zhang (28) reported that 3.3 g/L of EPS could be obtained by adding metal ions and surfactant during a two-stage cultivation. Kim et al. (25) proposed an optimization on medium and cultivation conditions in a 5-L fermentor and obtained an EPS yield of 3.4 g/L. In addition, as observed from the productivity data in Table 3, the higher ones were 273 mg/L/day in flask by Cui and Zhang (28) and 247 mg/L/day in fermentor by Kim et al. (25). The EPS production and productivity are two critical factors affecting the production cost from the engineering point of view. In this study, RB cultivation was applied to promote EPS production from *C. militaris*. A maximum EPS yield of 5.7 g/L was obtained with a productivity of 476 mg/L/day in the second operation batch. For a long-term cultivation operation, the average EPS productivity could reach a value around 320 mg/L/day, which was significantly higher than that of previous studies.

The results in this study demonstrated that RB operations are efficient for obtaining high EPS production and productivity in a submerged culture of *C. militaris*, and may be used on a large-scale fermentor in bio-industrial applications.

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