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Ganodiesel: A New Biodiesel Feedstock from Biomass of the Mushroom *Ganoderma lucidum*

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Abstract: There is a pressing demand for new sustainable eco-friendly approaches to producing green energy worldwide. This study represents the novel production of biodiesel feedstock from the medicinal mushroom *Ganoderma lucidum* QRS 5120 using state-of-the-art biotechnology tools. Response surface methodology (RSM) was used to enhance *G. lucidum* production in a repeated-batch fermentation strategy. By referring to the broth replacement ratio (BRR) and broth replacement time point (BRTP), RSM that was formulated using a central composite design (CCD) resulted in a significant model for all tested variables, which are exopolysaccharide (EPS), endopolysaccharide (ENS) and biomass, with BRR (%) of 60, 75 and 90, and BRTP (days) of 11, 13 and 15. The model was validated using the optimised conditions, and the results showed 4.21 g/L of EPS (BRR 77.46% and BRTP 12 days), 2.44 g/L of ENS (BRR 60% and BRTP 12.85 days), and 34.32 g/L of biomass (BRR 89.52% and BRTP 10.96 days) were produced. Biomass produced from the *G. lucidum* was subsequently used as feedstock for biodiesel production. Approximately 20.36% of lipid was successfully extracted from the dried *G. lucidum* biomass via a solvent extraction and subsequently converted to Ganodiesel through a transesterification process. The Ganodiesel produced fulfilled most of the international standards, i.e., US (ASTM D6751-08) and EU (EN 14214). Overall, this study demonstrates the optimised *G. lucidum* production and its lipid production as a new biodiesel feedstock.

Keywords: biodiesel; bioreactor biomass; *Ganoderma lucidum*; mushroom cultivation; repeated-batch fermentation

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1. Introduction

The consumption of food, water, energy and electricity has risen dramatically due to the rapid population growth linked with the commensurate need for improvements in the lifestyle around the world [1,2]. The global population is estimated to reach 10 billion people by 2050, which will place increasing pressure on energy and food production [3,4]. Currently, fossil fuels dominate the world's demands for energy and the economy, especially in the transportation sector. A high consumption of fuel is greatly affecting the environment [2,5]. Moreover, fossil fuels are non-renewable, with day-on-day depletion. Therefore, bio-based fuels such as biodiesel have been proposed as an alternative to fossil

fuels as they are sustainable, cost-effective, free of toxic chemicals (such as sulphur) and have greater lubricity (for automobiles) [6].

Although it is greener to utilise biodiesel than fossil fuel, the production of biodiesel also faces technical challenges [7]. For instance, it relies upon high yields of materials that may affect market demand, i.e., oil from crops [6,8]. Consequently, it has been estimated that using crops such as sunflower seed or rapeseed requires large-scale plantations and long durations to achieve existing biodiesel goals. For these reasons, it is important to find new sources of raw materials (the feedstocks) that can reduce the production price of biodiesel without competing with food security [9]. Recently, researchers tend to focus on biodiesel production from various feedstocks including plant-based oils, animal fats, waste oils, algal oils or even biomass [8]. However, there is an apparent gap in evidence-based research on the smart use of fungi-based feedstock to meet this pressing opportunity. Fungi are fast-growing and have been used for decades for biotech applications, thus, the production of biodiesel from fungal species could serve as a potential resource for new energy [10,11].

Ganoderma lucidum has been extensively used in traditional Asian medicine for more than 2000 years [12,13]. This mushroom is edible and has many health benefits such as for the treatment of various diseases, most commonly cancer [12–16]. *G. lucidum* undergoes four stages of its life cycle; (1) spores, (2) spore germination, (3) mycelium and (4) fruiting body. *G. lucidum* can easily be cultivated from its mycelium in a short period with the help of biotechnological practices and a suitable set of nutrients. A longer cultivation duration is required if growing *G. lucidum* using its fruiting body or spores [14,17]. Based on a recent study, *G. lucidum* mycelium can be cultivated using submerged fermentation (SmF), which takes 3 to 6 months to be fully completed [12,18,19]. A high biomass yield could be obtained after 10 days of the fermentation process, which can reduce the time needed to produce by-products and prevent the risk of contamination. Moreover, the active ingredients of *G. lucidum* are usually extracted for medicinal purposes. Apart from medicinal benefits, *Ganoderma lucidum* is also used as a reliable source of biomass and polysaccharides [11,20]. There is a growing awareness of the transformative potential of medicinal mushrooms, including the use of *G. lucidum*, in supporting and enabling green enterprises where its disruptive innovation potential will be actualised and accelerated through the forging of collaborative partnerships internationally between subject-matter experts in academia and industries, using connected innovation hubs [3,4].

In order to evaluate biomass production, a response surface methodology (RSM) can be applied using the broth replacement ratio (BRR) and broth replacement time point (BRTP) as the key parameters. By looking at the interaction and correlation between the main parameters, RSM is used to find the optimum conditions for the cultivation of polysaccharides and biomass production [21]. On the other hand, repeated-batch fermentation (RBF) is conducted, and it is a modification of the fermentation technique. This technique involves extracting a portion of the medium and, without changing the existing culture, thus replacing a fresh medium for continuous growth [22,23]. RBF offers many benefits, such as the formation of a long-term supply and a reduced workload, resulting in great savings of time and manpower [24–26].

Therefore, the extraction of lipids from *G. lucidum* for biodiesel production requires knowledge of the appropriate extraction techniques and factors that can affect the lipid yield. There are three extraction methods, namely physical, mechanical and chemical. These include Soxhlet extraction (SXE), solvent extraction (SVE), supercritical fluid extraction, aqueous enzyme extraction and pressurised liquid extraction [27]. Lipid extraction consumes around 90% of the energy used in biodiesel production and is costly, making the process difficult [28]. Thus, the implementation of green extraction techniques that can extract lipids with less duration, energy and solvent consumption without losing lipid quality is the key factor for cost-effective and environmentally sustainable processes. Earlier studies have shown that the amount of oil yielded from extraction methods is influenced by the sample size, temperature, solvent volume, solvent types and time [29,30].

The identification of the BRR and BRTP in producing the highest polysaccharide and biomass concentration from the mycelium of *Ganoderma lucidum* QRS 5120 will be the primary aim of this study. Therefore, it focuses on different extraction strategies; SXE, SVE and ultrasonic-assisted extraction (UAE), for the extraction of *G. lucidum* mycelia biomass lipids (GMBLs) cultured through SmF. The yield of the GMBL will be identified and compared to determine the most effective extraction technique for GMBLs. Thus, maximum yield with the shortest fermentation cycle can be achieved to reach an industrial level and meet the global market demand to produce biomass lipids.

2. Materials and Methods

2.1. Microorganism and Medium

G. lucidum strain QRS 5120 was obtained from Functional Omics and Bioprocess Development Laboratory, University Malaya. Medium compositions for both seed culture and repeated-batch fermentation were (in g/L): yeast extract (1), glucose (30), NH_4Cl (4), $MgSO_4$ (0.5), K_2HPO_4 (0.5) and KH_2PO_4 (0.5) [21,23].

2.2. Stock Culture and Inoculum Preparation

Stock culture of *G. lucidum* (Figure 1a) was prepared by cutting its mycelium from the mother plate into a size of 1 × 1 cm and sub-culturing it onto the potato dextrose agar (PDA). The inoculated culture was incubated at 30 °C for 7 days and stored in a 4 °C chiller until further use. The inoculum was prepared by cultivating the first seed for 10 days and then the second seed for 7–15 days with BRTP set at 30 °C and 100 rpm. From a seven-day plate (Figure 1b), three mycelial agar squares were cut into a size of 1 × 1 cm and inoculated in 100 mL of prepared medium. After 10 days, formed mycelium in the first seed culture was collected and homogenised using a sterile Waring blender. Then, 20 mL (20% v/v) of the first seed culture was transferred to another flask containing 80 mL and incubation was continued for another 7–15 days. The second seed culture was collected and homogenised for the fermentation process.

2.3. Batch Fermentation

Batch fermentation was conducted using an incubator shaker with the conditions set at an initial pH of 4.0, a temperature of 30 °C, an agitation speed of 100 rpm and an aeration rate of 2.0 vvm for regulated dissolved oxygen. To optimise the BRR, three different predetermined BRRs [60%, 75% and 90%] were studied. In addition, three batch fermentation growth curves obtained from previous research [11] were chosen to identify the appropriate BRTP as shown in Figure 1c. There are three main phases in each curve: the logarithmic, transition and stationery, which represent an increasing, highest and stabilising concentration, respectively.

2.4. Optimisation of Medium Compositions Using RSM

The optimisation of medium compositions to produce *G. lucidum* was conducted using RSM. To perform the RSM, a CCD was used to construct the experimental set using Design Expert version 12.0 software by StatEase®, Minneapolis, MN, USA. A BRR of 60% and BRTP of 7 days were set as the lowest variable, while a BRR of 90% and BRTP of 15 days were set as the highest variable. Meanwhile, the α-value was set to 1.0. EPS yield (g/L), ENS yield (g/L) and biomass concentration (g/L) were set as the response parameters of this study. At the end of the process, an analysis of variance (ANOVA) was generated to evaluate the optimisation model and validated using the optimised experimental parameters.

2.5. Repeated-Batch Fermentation Using Optimised Condition

Repeated-batch fermentation (RBF) was carried out until the maximum possible number of cycles to achieve the maximum biomass concentration until the fermentation broth reached the highest viscosity. The process was conducted by refilling the current mixture of medium fermentation with 80 mL of fresh medium for every cycle. As the main

purpose of RBF is to eliminate the lag phase, five days of the feeding interval were chosen for the full fermentation cycle.

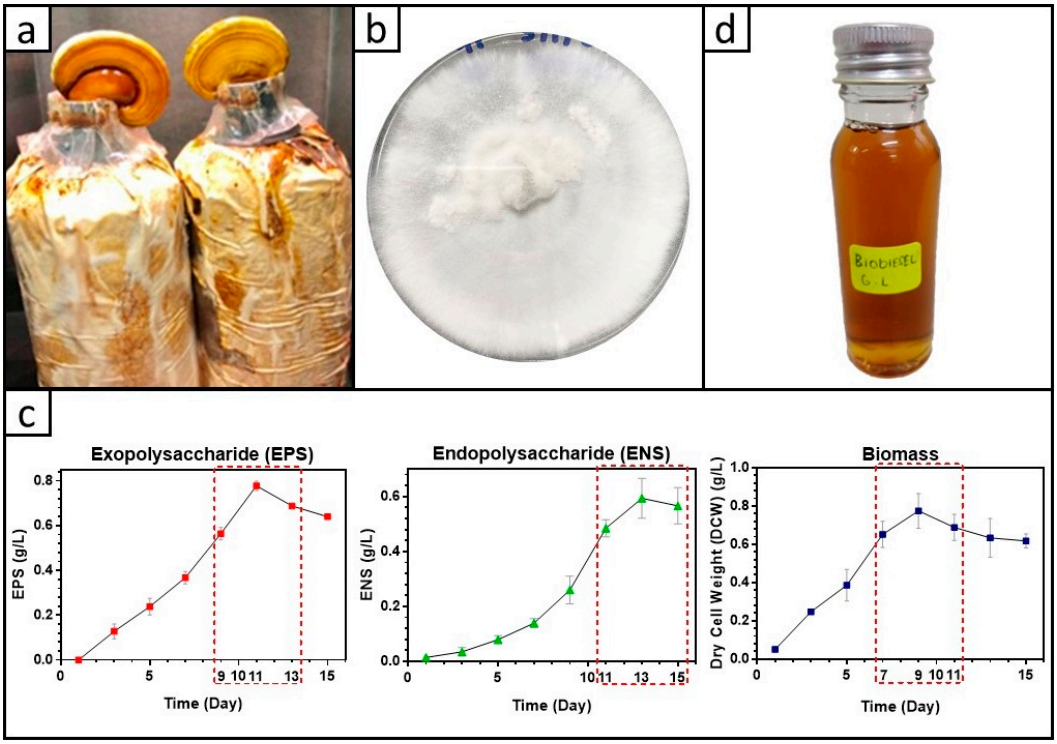

Figure 1. (**a**) Fruiting body of *Ganoderma lucidum* strain QRS 5120 located at the Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Universiti Malaya; (**b**) Mycelia state of the *G. lucidum* on a PDA plate after 7 days; (**c**) Growth curves discovered from previous studies were chosen as broth replacement time points (BRTPs) (red dashed box) for exopolysaccharide (EPS) 9–11–13, endopolysaccharide (ENS) 11–13–15 and biomass 7–9–11. Red circles = EPS production, Green triangles = ENS production, Purple squares = Biomass production; (**d**) Biodiesel extracted from *G. lucidum* QRS 5120.

2.6. Extraction of Lipid

The extraction of lipids from *G. lucidum* mycelial biomass was conducted using three different extraction techniques, which are Soxhlet extraction (SXE), solvent extraction (SVE) and ultrasonic-assisted extraction (UAE). Prior to the extraction process, mycelium was dried at 60 °C for 3 days in a drying oven. Then, the dried biomass obtained was ground into a fine powder. All three extraction techniques used hexane as a solvent and 5 g of *G. lucidum* mycelia biomass with the heating temperature set at 60 °C. After extraction, the lipids of *G. lucidum* (Figure 1d) were collected by filtering, followed by the evaporation of the lipid mixture using a rotary evaporator to remove the solvent [27].

2.6.1. Soxhlet Extraction

SXE was carried out using Soxhlet equipment with 5 g of Ganoderma biomass powder added in 50, 100 and 150 mL of hexane heated at 60 °C for 3, 6 and 9 h.

2.6.2. Solvent Extraction

SVE was conducted using 500 mL of a sample bottle containing 100, 150 and 200 mL of hexane mixed with 5 g of biomass powder and put on a hot plate at 60 °C with magnetic stirring of 200 rpm for 1, 2 and 3 h.

2.6.3. Ultrasonic-Assisted Extraction

UAE was performed using an ultrasonic water bath equipped with a 500 mL sample bottle containing 5 g of biomass powder mixed with 100, 200 and 300 mL of hexane and put in an ultrasonic water bath at 60 °C for 1, 3 and 5 h.

2.7. Ganodiesel Production through Transesterification

Using modified transesterification techniques [28], extracted lipids were converted to biodiesel. Acid-catalysed transesterification was conducted in accordance with the European Standards (EN 14103) in a 1000 mL round-bottomed glass flask. The reaction product was evaporated in a rotary evaporator at 70 °C for 20 min followed by gravity separation that separated Ganodiesel in the form of fatty acid methyl ester (FAME) from glycerol as a by-product. FAME was later washed with distilled water to neutralise the catalyst, then purified using silica gel and dehydrated with anhydrous sodium sulphate before gas chromatography (GC) analysis. FAME was filtered using a disposable polytetrafluoroethylene (PTFE) filter fitted to a glass syringe. The mixture of 1.5 mL of oil extract and 1 mL of 15% H_2SO_4 in methanol was then poured into a boiling tube. The tube was placed in a heating block at 70 °C for two hours. After heating, 1 mL of distilled water was added to the tube before aggressively shaking the mixture. The solution was left to allow the oil layer to separate. The FAME layer was further diluted with n-hexane before being injected for Gas Chromatography (GC) analysis. The FAME was examined with a GC–MS (Shimadzu, Nakagyo-ku, Kyoto, Japan) equipped with an Agilent Zorbax C18 column (80 Å, 3.5 µm, 1 × 150 mm, Agilent, Santa Clara, CA, USA).

2.8. Analytical Methods

2.8.1. Determination of Extracellular Polysaccharide Yield

EPS precipitation was collected by adding a 4:1 ratio of 95% ethanol (v/v) to the supernatant and kept at 4 °C overnight. EPS precipitate was filtered through filter paper that had been pre-dried and weighted and rinsed with 5 mL of 95% ethanol twice. The weight of EPS was recorded after drying in a food dehydrator to a constant weight (Figure 2a).

2.8.2. Determination of Endopolysaccharide Yield

ENS was isolated from the pellet mycelium after being drained from the extracted fermentation broth. Filtered mycelium was dried using a food dehydrator overnight and weighed to measure the amount of distilled water needed. The extraction of ENS was performed by rinsing dried mycelium with the ratio of 1:20 (w/v) of distilled water and then heating at 121 °C for 30 min. Then, the ENS was further treated by mixing the supernatant with ethanol at the ratio of 4:1 and kept overnight at 4 °C. The formed precipitate was filtered using pre-dried and weighed filter paper, followed by drying to a constant weight in the food dehydrator (Figure 2a) (Supplementary Materials, Table S1).

2.8.3. Determination of Biomass Concentration

The filtration of harvested fermentation broth through a Büchner funnel filter set attached to a water pump produced biomass. The mycelial biomass was washed repeatedly (three times) with distilled water, followed by a drying process in a food dehydrator to a constant weight. The weight of the dried mycelial biomass (Figure 2a) was recorded.

2.8.4. Determination of Lipid Yield

The lipid remained dissolved in the hexane solvent after the extraction process. By using a rotary evaporator, the excess solvent was eliminated by applying the concept of heat and pressure [27,30]. The mass of the recovered lipid after the removal of solvent was weighed. The extracted lipid yield (%) was estimated by using the equation below [30]:

$$Lipid\ yield\ (\%) = mi/ms \times 100 \quad (1)$$

where the coefficient mi (g/L) is the mass of recovered lipid while ms (g/L) is the mass of dried material (5 g of *G. lucidum* mycelial biomass) used for the extraction of SXE, SVE and UAE.

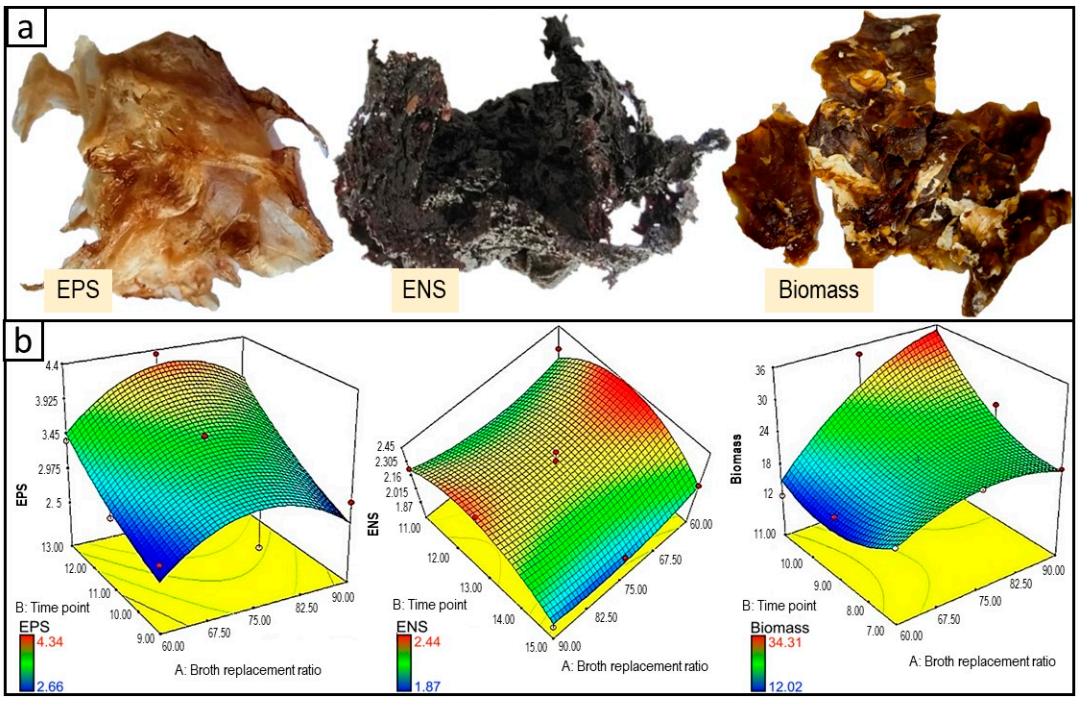

Figure 2. Dried EPS, ENS and biomass extracted from *Ganoderma lucidum* QRS 5120 (**a**), and response surface curve (3D plot) showing the EPS, ENS, biomass production and interaction between broth replacement ratio (BRR) and broth replacement time point (BRTP) from *Ganoderma lucidum* (**b**).

2.9. Kinetic Calculations

By following [31], the kinetic parameters were calculated.

$$EPS/ENS/Biomass\ productivity, P_{EPS}/P_{ENS}/P_X\left(\frac{g}{L}day^{-1}\right) = \frac{X_{max}-X_0}{the\ time\ for\ product\ recovery\ at\ certain\ cycle\ in\ repeated-batch\ culture} \quad (2)$$

where the X_{max} is the maximum cell concentration achieved at the stationary phase and X_0 is the initial cell concentration at inoculation.

2.10. Statistical Analysis

Triplicates for each sample were analysed and GraphPad Prism Version 9 by Dotmatics (GraphPad Software, San Diego, CA, USA) was used to calculate the mean and standard deviation with displayed error bars. Optimum BRR and BRTP for EPS, ENS and biomass were then assessed through response surface methodology (RSM).

Hence, the equation below demonstrates the influential factors and correlation, which are based on a quadratic second-order model for the responses:

$$Y = b_0' + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j>1}^{n} b_{ij} X_i X_j \quad (3)$$

where

Y: predicted response
b_0': constant coefficient
b_i: linear coefficient
b_{ij}: interaction coefficient
b_{ii}: quadratic coefficient
$X_i X_j$: coded values

Meanwhile, statistical analysis for lipid extraction from *Ganoderma lucidum* mycelial biomass was carried out by Minitab version 18 software. Extraction techniques (SXE, SVE, UAE) were conducted in triplicates. Analysis of variance (ANOVA) was used to calculate the mean ± S.D.

2.11. Determination of Biodiesel Properties

The biodiesel characteristics of *Ganoderma lucidum* fatty acid methyl esters (GLFAMEs) were examined by conducting a variety of tests following international guidelines [32,33]. Mini Pour/Cloud Point Tester MPC-102 was used to determine the cloud and pour point at −60 °C to 51 °C, which was subsequently evaluated according to ASTM D6749 and ASTM D2500, respectively. The automatic Kinematic Viscosity Measuring System AKV-201 was used to measure the kinematic viscosity at 40 °C, as mentioned in ASTM D445. Following ASTM D637, the measurement for the cold filter plugging point was conducted using Automated Cold Filter Plugging Point Tester AFP-102. By referring to the ASTM D4530, a test for carbon residue was carried out using Micro Carbon Residue Tester ACR-M3. The ignition point test was performed using Pensky-Martens Closed Cup Automated Flash Point Tester APM-7 by referring to the ASTM D93. Certified instrumentations provided by TANAKA Scientific Ltd., Tokyo, Japan, were used to carry out all the tests. In addition, the stability of the oxygen has also been tested using Rancimat 743 (Metrohm, Herisau, Switzerland) following ASTM 14,112. Additionally, tests for ester content, monoglyceride, diglyceride, triglyceride, total glycerol content, acid number, iodine value and water content were measured in accordance with European Standard Methods [32].

3. Results

3.1. Optimization

The influences of BRR and BRTP on the production of EPS–ENS–biomass from *Ganoderma lucidum* strain QRS 5120 mycelium were analysed using RSM. Tables 1 and 2 show the experimental designs and responses. The tables summarise that thirteen sets of conditions for culture were applied for RSM optimisation. This was defined by CCD and will be used to assess the coefficients using non-linear regression analysis. The determination of the model coefficient was analysed using analysis of variance, and the study reported the significance was $p < 0.05$. Based on Table 1, EPS and ENS concentrations were found to be the highest during day 13 of the BRTP with 4.34 g/L and 2.44 g/L, respectively. A total of 75% BRR at day 11 BRTP was the optimised variable for biomass production with the actual response of 34.31 g/L, as mentioned in Table 2.

Table 1. Experimental design and responses for production of EPS and ENS from *G. lucidum* mycelia. The experiments for the actual responses were conducted under controlled conditions.

Run No.	EPS (g/L)				ENS (g/L)			
	Variables		Responses		Variables		Responses	
	BRR (%)	BRTP (Day)	Actual	Predicted	BRR (%)	BRTP (Day)	Actual	Predicted
1	75	11	3.63	3.56	75	11	2.02	2.10
2	75	11	3.63	3.56	60	13	2.36	2.44
3	75	11	3.63	3.56	75	13	2.29	2.30
4	90	11	2.91	3.15	75	13	2.25	2.30
5	75	13	4.34	4.19	60	11	2.26	2.18
6	60	11	2.80	2.9	75	15	1.98	1.92
7	75	9	2.66	3.15	75	13	2.35	2.30
8	90	9	2.96	2.68	90	15	1.87	1.94
9	60	9	2.75	2.54	75	13	2.43	2.30
10	75	11	3.63	3.56	90	11	2.24	2.23
11	90	13	3.79	3.83	90	13	2.44	2.38
12	60	13	3.37	3.48	60	15	2.11	2.11
13	75	11	3.63	3.56	75	13	2.21	2.30

Table 2. Experimental design and responses for production of biomass from *G. lucidum* mycelia. The experiments for the actual responses were conducted under controlled conditions.

Run No.	Biomass (g/L)			
	Variables		Responses	
	BRR (%)	BRTP (Day)	Actual	Predicted
1	60	11	12.02	14.70
2	75	9	20.39	21.82
3	60	7	18.05	17.71
4	75	9	20.39	18.91
5	75	7	22.44	21.82
6	75	9	20.39	21.82
7	90	9	26.26	27.45
8	60	9	15.57	16.20
9	75	9	20.39	21.82
10	90	11	32.38	34.78
11	90	7	20.73	20.11
12	75	11	34.31	24.74
13	75	9	20.39	21.82

3.1.1. Optimization of EPS Production

Table 3 shows the ANOVA for EPS production and the 3D plot is expressed in Figure 2b. According to the table, the expected determination of the coefficient (R^2 = 0.8404) suggests that this model can express 84.04% of the response variability and as $p < 0.05$, thus it proved the significance of the model. The validity of the model is indicated by the value of the modified coefficient determination (Adj. R^2 = 0.727), which was adequate in comparison to the expected R^2 value. In the equation below, the model was regressed in terms of actual EPS variables and expressed as a final equation in terms of absolute factors.

$$EPS = (-8.65135) + 0.34713 \times BRR - 0.46309 \times BRTP + 1.75000e^{-3} \times BRR \times BRTP - 2.38774e^{-3} \times BRR^2 + 0.026940 \times BRTP^2 \quad (4)$$

Table 3. Experimental results of the CCD quadratic model using ANOVA for EPS production from *Ganoderma lucidum*.

Source	Sum of Squares	DF	Mean Square	*F* Value	Prob > *F*	
Model	2.560	5	0.510	7.39	0.0103 *	significant
A: BRR	0.091	1	0.091	1.32	0.2889	
B: BRTP	1.630	1	1.630	23.55	0.0018 *	significant
AB	0.011	1	0.011	0.16	0.7019	
A^2	0.800	1	0.800	11.50	0.0116 *	significant
B^2	0.032	1	0.032	0.46	0.5183	
Residual	0.490	7	0.069			
Lack of Fit	0.490	3	0.160			not significant
Pure Error	0	4	0			
Cor Total	3.05	12				
Std. Dev. = 0.26		R^2 = 0.8408		Adeq Precision = 9.232		
Mean = 3.36		Adjusted R^2 = 0.727				

* Significant value.

3.1.2. Optimization of ENS Production

Table 4 shows the ANOVA for the production of ENS, and Figure 2b displays the 3D plot. The model's R-squared was 0.8341, which means it could represent 83% of the response. The *p*-value was 0.0117, showing the model was significant as $p < 0.05$. It clearly shows that more variances can be explained if the number of R^2 is high so that the optimum model can be generated. The adjusted R^2 was 0.7155, indicating that the model was significant and showed consent to the expected R^2 value. If more non-significant

variables are added to the model, the difference between the R^2 and the adjusted R^2 will thus be greater. An equation as shown below has been formulated and regressed in terms of the actual factor of ENS, by considering the significant terms from the model:

$$ENS = (-8.46282) - 0.049201 \times BRR + 1.99825 \times BRTP - 1.83333e^{-3} \times BRR \times BRTP + 4.73563e^{-4} \times BRR^2 - 0.073362 \times BRTP^2 \quad (5)$$

Table 4. Experimental results of the CCD quadratic model using ANOVA for ENS production from *Ganoderma lucidum*.

Source	Sum of Squares	DF	Mean Square	*F* Value	Prob > *F*	
Model	0.310	5	0.062	7.04	0.0117 *	significant
A: BRR	5.40×10^{-3}	1	5.40×10^{-3}	0.62	0.4578	
B: BRTP	0.052	1	0.052	5.98	0.0445 *	significant
AB	0.012	1	0.012	1.38	0.2779	
A^2	0.031	1	0.031	3.59	0.1002	
B^2	0.240	1	0.240	27.19	0.0012 *	significant
Residual	0.061	7	8.75×10^{-3}			
Lack of Fit	0.031	3	0.010	1.39	0.3664	not significant
Pure Error	0.03	4	7.48×10^{-3}			
Cor Total	0.37	12				
Std. Dev. = 0.094		R^2 = 0.8341		Adeq Precision = 8.237		
Mean = 2.22		Adjusted R^2 = 0.7155				

* Significant value.

3.1.3. Optimization of Biomass Production

Table 5 shows the ANOVA for mycelium biomass production, with a 3D graph in Figure 2b. The predicted coefficient determination demonstrated that this model can explain 83.74% (R^2 = 0.8374) of the response variability. The model ($p < 0.05$) is applicable. The model's validity was suggested by the R^2 value of 0.7213, which was in reasonable agreement with the predicted R^2 value. The model was regressed and expressed as an equation in terms of the actual biomass variables by considering the relevant terms.

$$Biomass = +77.50102 + 1.30728 \times BRR - 27.91759 \times BRTP + 0.14733 \times BRR \times BRTP - 0.015057 \times BRR^2 + 1.01806 \times BRTP^2 \quad (6)$$

Table 5. Experimental results of the CCD quadratic model using ANOVA for biomass production from *Ganoderma lucidum*.

Source	Sum of Squares	DF	Mean Square	*F* Value	Prob > *F*	
Model	375.44	5	75.09	7.21	0.0110 *	significant
A: BRR	189.62	1	189.62	18.21	0.0037 *	significant
B: BRTP	50.98	1	50.98	4.90	0.0626	
AB	78.15	1	78.15	7.50	0.0289 *	significant
A^2	31.70	1	31.70	3.04	0.1246	
B^2	45.80	1	45.80	4.40	0.0742	
Residual	72.89	7	10.41			
Lack of Fit	72.89	3	24.30			not significant
Pure Error	0	4	0			
Cor Total	448.34	12				
Std. Dev. = 3.23		R^2 = 0.8374		Adeq Precision = 10.332		
Mean = 21.82		Adjusted R^2 = 0.7213				

* Significant value.

3.1.4. Verification of Optimised Conditions

Verification for a statistical model of the highest EPS–ENS–biomass is shown in Table 6 by applying the optimised variables. Various experiments were performed to validate the

strength and precision of the model according to the Equation EPS/ENS/biomass. Under optimised conditions, EPS production gained was 4.21 g/L, ENS production was 2.44 g/L and 34.32 g/L of mycelium biomass was obtained. These correspond to the prediction values of the EPS, ENS and biomass (g/L); 4.19, 2.44 and 34.78, respectively. Therefore, the validity of the model (Equation EPS/ENS/biomass) is verified for EPS, ENS and biomass production. However, due to different broth replacement time points for EPS, ENS and biomass (BRTP: refer to Section 2.3), the maximum production for the combination run was inapplicable and only an individual response for each run was achievable in this study.

Table 6. Verification of model with the optimised variables.

Run	Variables		Responses		
	BRR	BRTP	EPS (g/L)	ENS (g/L)	Biomass (DCW g/L)
EPS	77.46	13.00	4.21	–	–
ENS	60.00	12.85	–	2.44	–
Biomass	89.52	10.96	–	–	34.32

– Not available.

3.2. Repeated-Batch Fermentation Using Validated EPS–ENS–biomass Condition

3.2.1. Effect of BRR

Figure 3 displays the growth curves of EPS–ENS–biomass production in a repeated-batch fermentation. The growth curves included the extracted EPS–ENS–biomass for all six cycles (R1–R6). R0 was derived from the previously defined growth profile (Figure 1d). Using the pre-optimised conditions, 75%, 60%, and 90% were set as BRR for EPS, ENS, and biomass, respectively. The BRR procedure involved the removal and addition of fresh media to the flasks. Samples withdrawn in 120 h (5 days) intervals were analysed for their polysaccharide and biomass content.

3.2.2. Effect of BRTP

A study on the effect of time points was conducted based on the pre-optimised conditions. The broth replacement was carried out on day 13 for EPS and ENS, while biomass broth replacement occurred on day 11. According to Table 7, the production of EPS–ENS–biomass was observed and a total of six batches in a row could be conducted using the RBF process. It demonstrated the capability of *G. lucidum* cells toward this technique.

Table 7. EPS–ENS–biomass productivity from pre-optimised conditions in a repeated-batch fermentation strategy at day 13 (75%), day 13 (60%) and day 11 (90%).

BRTP (Day)	BRR (%)	Kinetics [a]	RBF Cycles [b]						Sum (R1–R6)
			R1	R2	R3	R4	R5	R6	
13	75	P_{EPS} (g/L day^{-1})	0.3467	0.0720	0.0813	0.1413	0.1080	0.0413	0.7906
13	60	P_{ENS} (g/L day^{-1})	0.0087	0.1160	0.0813	0.0927	0.0613	0.0833	0.4433
11	90	P_X (g/L day^{-1})	0.6880	0.7480	0.6600	0.5860	1.0160	1.1200	4.8180

[a] P_{EPS} (g/L day^{-1}) = EPS productivity, P_{ENS} (g/L day^{-1}) = ENS productivity, P_X (g/L day^{-1}) = biomass productivity. [b] Cycles repetition (R1–R6)

3.3. Evaluation of Lipid Yield

The extraction of GMBLs using SXE, SVE and UAE were evaluated. It was observed that all three techniques were able to extract GMBLs with different lipid yields obtained. The amount of lipid yield was not as high as expected. The highest GMBL yield, of 20.36%, was achieved using SVE (3 h/200 mL), followed by 18.8% through SXE (6 h/150 mL) and 7.5% through UAE (5 h/200 mL). Based on research, 1 g of mycelium of *G. lucidum* has a lipid content of approximately 1.67% (Hou et al., 2017). Therefore, Table 8 shows statistical analysis using analysis of variance for all techniques. Two factors have been tested upon the

yield of GMBL which were the solvent volume and extraction time as well as the interaction between both independent variables.

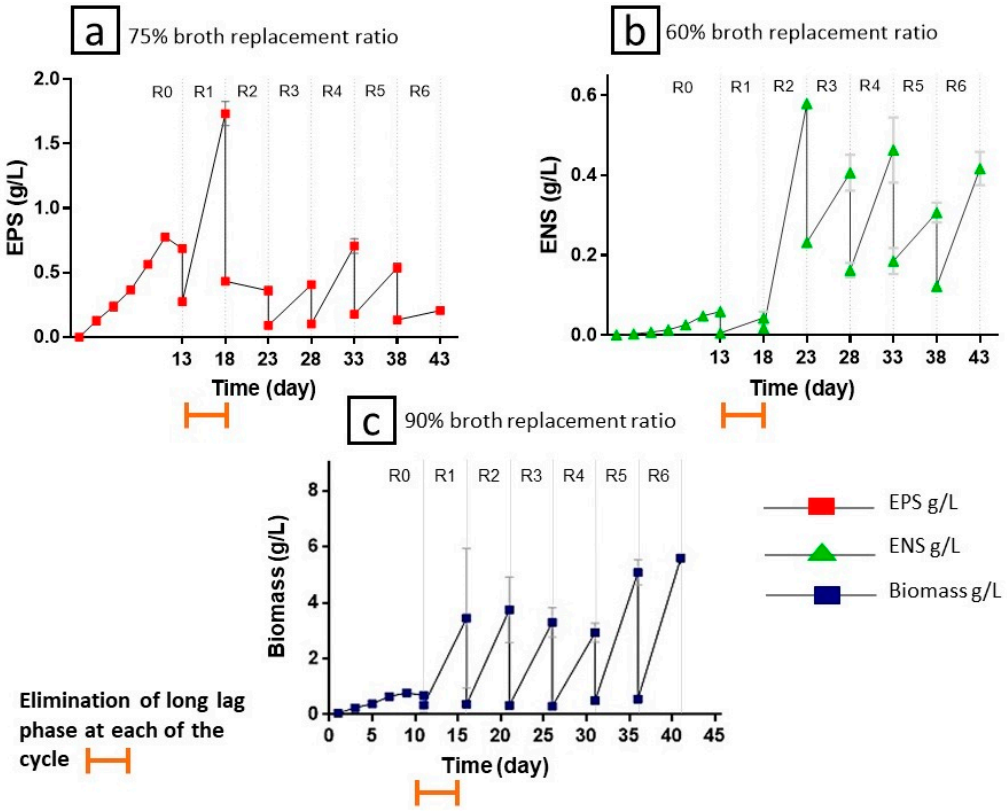

Figure 3. Production of EPS (**a**), ENS (**b**) and biomass (**c**) from the mycelium of *Ganoderma lucidum* QRS 5120 in a repeated-batch fermentation strategy from the pre-optimised conditions (EPS, 75% broth replacement ratio (BRR); ENS, 60% BRR; and biomass, 90% BRR).

Table 8. Experimental results of SXE, SVE and UAE using ANOVA.

Extraction Techniques [a]	Source	DF	Sum of sq	Mean sq	*F*-Value	*p*-Value
(a) SXE	Solvent volume	2	14.560	7.2800	67.55	0.000
	Extraction time	2	139.253	69.6267	646.02	0.000
	Solvent volume Extraction time	4	22.897	5.7242	53.11	0.000
	Error	9	0.970	0.1078		
	Mean = 12.12		R^2 = 0.9945			
	Variance = 10.45		Adjusted R^2 = 0.987			
	Std. Dev. = 3.03		Predicted R^2 = 0.9782			
(b) SVE	Solvent volume	2	9.906	4.9532	31.60	0.000
	Extraction time	2	137.224	68.6118	437.73	0.000
	Solvent volume Extraction time	4	43.766	10.9415	69.80	0.000
	Error	9	1.411	0.1567		
	Mean = 13.44		R^2 = 0.9927			
	Variance = 11.31		Adjusted R^2 = 0.9261			
	Std. Dev. = 3.36		Predicted R^2 = 0.9707			

Table 8. *Cont.*

Extraction Techniques [a]	Source	DF	Sum of sq	Mean sq	*F*-Value	*p*-Value
(c) UAE	Solvent volume	2	1.0844	0.54222	9.76	0.006
	Extraction time	2	19.1878	9.59389	172.69	0.000
	Solvent volume Extraction time	4	1.4056	0.35139	6.33	0.010
	Error	9	0.5000	0.05556		
	Mean = 9.24		R^2 = 0.9775			
	Variance = 5.34		Adjusted R^2 = 0.9574			
	Std. Dev. = 2.31		Predicted R^2 = 0.9098			

[a] SXE, Soxhlet Extraction; SVE, Solvent Extraction; UAE, Ultrasonic-assisted Extraction.

3.4. Effect of Solvent Volume and Extraction Time

The volume of hexane and time of extraction affects the yield of the GMBLs. Lipid extraction using SXE recorded a highest yield of 150 mL (6 h) and a lowest yield of 50 mL (3 h), SVE demonstrated a high of 200 mL (3 h) and a low of 100 mL (1 h), and UAE a high of 200 mL (5 h) and a low of 100 mL (1 h). The results obtained clearly show that lipid yield increased with the increase of the solvent volume and extraction time.

4. Discussion

4.1. Optimization of EPS–ENS–biomass Production

BRTP (B) and BRR^2 (A^2) of EPS, as mentioned in Table 3, show that both factors influence their production at $p < 0.05$ compared to others (A, B^2 and AB). Figure 2b illustrates the correlation effect between the BRR and BRTP for EPS production. From the figure, an increase in the BRTP leads to an increase in the production of EPS, and BRR at all ranges records the lowest EPS production. According to this, it was shown that the BRTP influenced the production of EPS and there was no correlation between the BRR and BRTP. Maximum production of EPS (red area) was 75% on day 13 with 4.34 g/L of EPS achieved.

According to the ENS model (Table 4), both the BRTP (B) and quadratic terms of BRTP (B^2) show the effect on the production of ENS as $p < 0.05$. However, BRR (A) and other quadratic terms (A^2 and AB) present a negative result. Figure 2b demonstrated the combined effect of BRR and BRTP on the production of ENS. From the figure, the BRTP on day 13 shows the most production of ENS and is least affected by the BRR. By this, it was concluded that the production of ENS was strongly affected by the broth replacement time point (BRTP) and there was no interaction between the BRR and BRTP. In contrast with EPS and biomass, the ENS model showed two red areas which indicate the optimised production of ENS. The highest distribution of red area fell on day 13 (60%) compared with the least red area, also on day 13 (90%). This also might be due to the insignificance of BRR which does not influence ENS production. Therefore, the highest production of ENS (2.44 g/L) obtained was on day 13, with 60% BRR.

Therefore, BRR (A) and a quadratic term (AB) of the biomass model (Table 5) present significant values as both terms record *p*-values of less than 0.05. However, BRTP (B) and other quadratic terms (A^2 and B^2) show a negative effect on the production of biomass. Figure 2b explained the relationship between BRR and BRTP. According to the figure, all ranges of BRTP record the lowest production of biomass. Nevertheless, BRR and BRTP show a linear correlation as the production of biomass increased when their value increased. From this, it can be summarised that there was a relationship between BRR and BRTP. The maximum production of biomass (34.31 DCW g/L) achieved was at 90% on day 11.

However, the total error Sum of Square (SS) and Mean of Square (MS) has indeed been zero for EPS and biomass as shown in Tables 3 and 5, respectively. All these values were very small and insignificant. The explanation for these findings may have been because the model's SS value of regression was much higher than the residual SS value. The null value of pure error, both SS and MS, showed that the lack of fit (LOF) test had no *p*-value and F value. Adequate Precision calculated the ratio of signal to noise and it is preferable

if this value is greater than 4. As the model for EPS and biomass had a 9.232 and 10.332 ratio, respectively, the signal was adequate, and this shows that the model is applicable for navigating the design area.

4.2. Repeated-Batch Fermentation Using Validated EPS–ENS–biomass Condition

Effect of Broth Replacement Ratio and Broth Replacement Time Point

As mentioned in Figure 3, fermentation for EPS–ENS–biomass production can be tolerated for up to six cycles (R6) when they show sustained continuous growth of the fungus during the RBF. The repeated-batch cell cycle has demonstrated its ability with repeated use. In this repeated-batch fermentation study, the lag phase has been reduced by sampling at five day intervals. Therefore, it resulted in the continuous production of EPS, ENS and biomass. The amount of EPS–ENS–biomass produced could be similar to or higher than that of batch fermentation (R0). As mentioned by Naritomi et al., productivity from repeated batch culture is higher rather than productivity from the standard batch culture [26]. Pre-optimised BRRs and BRTPs were used during the RBF process, with EPS (75%, day 13), ENS (60%, day 13) and biomass (90%, day 11).

According to Figure 3, the lowest development of EPS, ENS and biomass were observed on the sixth (R6), first (R1) and fourth (R4) days, respectively. The lowest production was due to some fungi attempting to cope with the new environment and conditions as new media were introduced, making some of the fungi enter the phase of death caused by stress. Meanwhile, in the first (R1), second (R2) and sixth (R6) cycles, the highest performance of EPS, ENS and biomass was recorded. This is due to the capability of the fungus to stabilise and preserve its production until the environmental viscosity was high for further growth. As similar cells of fungus (*G. lucidum*) have been used throughout the RBF process, which was in between the first cycle (R1) and the sixth cycle (R6), it was crucial to replace the volume at an optimised time point by changing the media to maintain the level toxicity of the EPS–ENS–biomass concentration. However, towards the end of the cycle for the present study, the EPS–ENS–biomass was still being produced. Therefore, future work can be conducted to identify the highest cycle for their production.

In general, the consistency of EPS and ENS productivity was recorded after the second cycle (R2) with (g/L day^{-1}) 0.0413–0.1413 and 0.0813–0.1160, while biomass was observed along with the six batches (R1-R6) with 0.5860–1.1200 g/L day^{-1}. Overall, total P_{EPS}, P_{ENS} and P_X (g/L day^{-1}) recorded were 0.7906, 0.4433 and 4.8180 with average readings of 0.1318, 0.0739 and 0.8030, respectively, from the six cycles. Furthermore, the highest productivity was reported for EPS (0.3467 g/L day^{-1}) at the first cycle (R1), ENS (0.1160 g/L day^{-1}) at the second cycle (R2) and biomass (1.1200 g/L day^{-1}) at the sixth cycle (R6).

4.3. Evaluation of Lipid Yield

Table 8 shows the fitness models analysed which were 0.9945 for SXE, 0.9927 for SVE and 0.9775 for UAE. These indicate that the lipid yield was strongly affected by the volume of solvent and time for extraction. The table displays that there were significant effects and interactions between the factors for all three extraction techniques (p-value < 0.05). A few factors have been suggested by previous findings as affecting the yield of lipids, including type and volume of solvent, extraction time and temperature [29,30]. According to Table 9, a comparison between the three different techniques used in the extraction of GMBL can be summarised. SXE and SVE have been recorded to be effective in extracting lipids and are widely used in many studies [34,35]. In extracting GMBLs, SVE (14.57–20.36%) and SXE (9.44–18.80%) were observed to have a higher yield compared with the use of UAE (4.30–7.50%).

In SXE and SVE, the transfer of heat between solid and liquid occurs from the outside to the inside of the sample membrane; meanwhile, the mass transfer happens and vice versa (from the inside to the outside) [27,36]. On the contrary, UAE utilises the frequency of electrostatic interactions arising from the development of high-intensity wave propagation. The UAE process involves a physical mechanism whereby the penetration of the cell walls

and washing out of the cell material takes place before it becomes disrupted [36,37]. Low lipid yield in UAE may be caused by the lack of transmission consistency and being exposed to the sonic power which affects the biomass cell wall.

Table 9. Comparison between three lipid extraction techniques on GMBL yield [27,34,36–38].

Aspects	Extraction Techniques		
	SXE	**SVE**	**UAE**
Procedure	An appropriate size of cellulose thimble was chosen for the sample before being placed in the Soxhlet extractor. The solvent in a round bottom flask heated with a mantle	Sample soaked in the solvent with a magnetic stirrer in a flat bottom flask and then heated by a hot plate	Sample soaked in solvent, and placed in an ultrasonic water bath equipment
Solvent	hexane	hexane	hexane
Sample size	1–5 g	1–5 g	1–5 g
Temperature	60 °C	60 °C	60 °C
Solvent volume	50–150 mL	100–200 mL	100–300 mL
Extraction time	3–9 h	1–3 h	1–5 h
Advantages	No filtration is needed, easy to use	Short extraction duration	Moderate extraction duration, easy to use
Disadvantages	Long extraction duration	Filtration needed	High solvent amount, filtration needed
Lipid yield	9.44–18.80%	14.57–20.36%	4.30–7.50%
Quantity	Moderate	High	Low

4.4. Effect of Solvent Volume and Extraction Time

Based on the results obtained, a high solvent volume will help to accelerate the chemical reaction resulting in enhanced lipid production. Indeed, one study found a correlation between solvent volume and extraction time, in which a high solvent quantity was caused by the higher extraction time required [39]. Additionally, the author reported that a sufficient volume of the solvent is needed to guarantee the complete immersion of sample particles during the extraction process. Hexane has been selected for this analysis because it can be quickly recovered, is non-polar, has low latent vaporisation heat and has high solvent selectivity [29,40,41]. Alternatively, an optimal solvent volume and extraction time could be determined to enhance the yield of GMBLs.

In SXE, the lipid yield decreased when the time was set to the highest at 9 h (50 mL to 150 mL), while when the time was set up from 3 h to 6 h the lipid yields increased. This is because SXE reached an optimal time (3 h–6 h) and therefore after 6 h there was not much lipid to be obtained. Therefore, the lower duration is more efficient compared to the higher duration. In comparison to SXE and UAE, SVE had the shortest time duration (between 1 h to 3 h) and the smallest volume of solvents (100 mL to 200 mL) for the highest lipid production. The higher yield of GMBLs in SVE could be due to the use of a magnetic stirrer that continuously mixed the biomass sample with hexane solvent throughout the extraction process, while in SXE and UAE the sample was static [36,42].

4.5. Comparison of the Current Study with the Literature

4.5.1. Comparison of *G. lucidum* Optimization Using SmF

Table 10 presents the latest statistical optimisation in determining the best technique for achieving the efficient production of EPS, ENS and biomass from *Ganoderma lucidum* through shake-flask fermentation. As mentioned, it indicates that only three studies using *Ganoderma lucidum* in EPS, ENS and biomass production have previously been published using SmF.

As stated, the method of cultivation using RBF has been shown to doubly increase the productivity of EPS compared to the normal batch fermentation technique performed by Supramani, Ahmad [21]. They used the same ideal glucose concentration of 26.5 g/L and pH of 4, and were designed using RSM. Meanwhile, a low concentration of EPS was achieved when using an orthogonal matrix (1.723 g/L) and Taguchi orthogonal matrix (0.42 g/L), as studied by Chang, Tsai [43] and Yuan, Chi [44], and concentrations of just 0.42 g/L and 1.723 g/L EPS were reported, respectively.

Therefore, the study of Supramani, Ahmad [21] was the only one to produce ENS, but different cultivation methods and batch fermentation were applied in their research. Meanwhile, in the current study, RBF has been used and optimised by RSM for ENS production, and it showed the efficiency of this technique compared to their result. This can be seen in Table 10, where the present study generated the highest ENS with 2.44 g/L; meanwhile, 1.52 g/L was the result from Supramani. As a result, the current study suggests that the best productivity for ENS production could be achieved with numerous advantages.

In addition, referring to Table 10, Chang, Tsai [43] mentioned that under several optimum conditions, biomass production was significantly enhanced. Yuan, Chi [44] in their findings reported that the production of biomass was significantly increased by the optimal media. RSM was used in both the study of Supramani, Ahmad [21] and the present study. However, high biomass production was achieved in this study, demonstrating that the method proposed was effective compared to other works in the literature.

Table 10. Comparison of *Ganoderma lucidum* optimization using SmF with the previous studies.

Ganoderma lucidum Strains	Optimization Method	Cultivation Mode	Cultivation Method	Total Prep. Time (Days)	Initial pH	Glucose Concentration (g/L)	Agitation (rpm)	EPS (g/L)	ENS (g/L)	Biomass (g/L)	References
Ganoderma lucidum QRS 5120	Response surface methodology	Shake Flask	Repeated-batch fermentation	5 (up to 6 cycles)	4	26.5	100	4.34	2.44	34.31	Current study
Ganoderma lucidum QRS 5120	Response surface methodology	Shake Flask	Batch fermentation	10	4	26.5	100	2.64	1.52	5.19	[21]
Ganoderma lucidum CCRC 36124	Taguchi's orthogonal array	Shake Flask	Batch fermentation	7	6.5	12.1	160	0.420	NA [a]	18.70	[43]
Ganoderma lucidum CAU 5501	Orthogonal matrix	Shake Flask	Batch fermentation	4	-	50	150	1.723	NA [a]	7.235	[44]

[a] NA, Not Available.

4.5.2. Comparison of Ganoderma Lipid Profile

Lipids from *Ganoderma lucidum* were extracted with hexane as the extraction solvent using a Soxhlet apparatus based on a modified method [45]. Supernatants produced from the extract centrifugation for 20 min at 1500 rpm were then filtered and dried (sodium sulphate anhydrous). Therefore, using a rotary evaporator, supernatants were concentrated at 30 °C under a vacuum and the weight was recorded. Lipid profiling was analysed in accordance with ASTM E2881 [46].

The fatty acids in GMBLs primarily comprised 66.1 wt% oleic acids (C18:1), 16.2 wt% palmitic acid (C16:0) and 13.2 wt% linoleic acids (C18:2), as shown in Table 11. Therefore, a total of 95.5 wt% fatty acids contents were accounted for from all three fatty acids. In comparison, *Ganoderma austral* and *Ganoderma applanatum* mainly consisted of 49.5 wt% and 58.2 wt% linoleic acids (C18;2), respectively [47,48]. In this study, lipids produced from *G. lucidum* contained the highest saturated fatty acid, including palmitic acid (16:0) compared with lipids from *G. austral* and *G. applanatum*. According to Lin and Chiu [49], high saturated fatty acids cause a slow deterioration rate of lipids due to their high oxidative and thermal stability. Therefore, they are beneficial for long-term storage or surrounding high temperatures as they can delay the fuel degradation process.

Table 11. Comparison of Ganoderma lipid profile with previous studies.

Fatty Acid	Structure	*Ganoderma lucidum* (wt%)	*Ganoderma austral* (wt%)	*Ganoderma applanatum* (wt%)
Palmitic	16:0	16.2	10.7	10.2
Palmitoleic	16:1	2.2	1.9	2.0
Stearic	18:0	2.3	2.7	2.5
Oleic	18:1	66.1	30	15.5
Linoleic	18:2	13.2	49.5	58.2
References		This study	[47]	[48]

4.6. Biodiesel Properties of Ganoderma lucidum FAME

A comparison of biodiesel properties between GLFAMEs and international standards is shown in Table 12 [46]. Generally, GLFAMEs fulfilled the most necessary biodiesel properties according to the standard. By referring to the standards of the US (ASTM D6751-08) and the EU (EN 14214), our study found that GLFAMEs fell within the range including the ignition point (153.5 °C), kinematic viscosity (3.8 mm^2/s), water content (345 mg/kg), total glycerol content (0.15%), ester content (97.5%), iodine value (112 $g(I_2)/100$ g) and acid number (0.38 mg(KOH)/g). However, the oxidation stability was low (6 h) and this may be due to the 81.5% unsaturated fatty acids in *G. lucidum* oil which is considered to be high. This property does not satisfy the EU standard that requires more than 8 h of oxygen stability. However, it still passes the EU standard compliance countries, which is the US standard (>3 h), therefore the addition of antioxidants to GLFAMEs for longer storage capacity is recommended. On the other hand, the positive outcome of highly unsaturated fatty acids content in GLFAMEs could be seen from its expected excellent performance at low temperatures, represented by the good values of pour point (−3.0 °C), cold filter plugging points (−2.0 °C) and cloud point (−1.0 °C).

Table 12. FAME Properties of *Ganoderma lucidum* methyl esters in comparison with international guidelines [a].

Properties	Method	Unit	GLME (Biodiesel)	US (ASTM D6751-08)	EU (EN 14214)
Kinematic viscosity (40 °C)	ASTM D445	mm^2/s	3.8	1.9–6.0	3.5–5.0
Carbon residue	ASTM D4530	wt%	0.10	≤0.05	<0.30
Pour point	ASTM D2500	°C	−3.0	-	-
Cold filter plugging point	ASTM D6371	°C	−2.0	-	-
Ignition point	ASTM D93	°C	153.5	≥130	>101
Cloud point	ASTM D6749	°C	−1.0	-	-
Oxidation stability	EN 14112	h	6	-	8
Ester content	EN 14103	wt%	97.5	-	>96.5
Monoglyceride	EN 14105	wt%	0.05	-	<0.80
Diglyceride	EN 14105	wt%	0.03	-	<0.20
Triglyceride	EN 14105	wt%	n.d. [b]	-	<0.20
Total glycerol content	EN 14105	wt%	0.15	<0.24	<0.25
Water content	EN ISO12937	mg/kg	345	<500	<500
Acid number	EN 14104	mg(KOH)/g	0.38	<0.50	<0.50
Iodine value	EN 14111	$g(I_2)/100$ g	112	-	<120

[a] CEN (2019); ASTM (2008). [b] n.d., not detectable.

5. Conclusions

The implementation of CCD RSM to optimise EPS–ENS–biomass production from *Ganoderma lucidum* strain QRS 5120 was verified using SmF. The maximum production of EPS (4.34 g/L^{-1}), ENS (2.44 g/L^{-1}) and mycelial biomass (34.31 g/L^{-1}) was achieved

under the optimised conditions of a 75% BRR, with day 13 and day 11 at 60% and 90%, respectively. All EPS, ENS and mycelial biomasses were accomplished within up to six cycles in a RBF strategy, as shown by continuous growth in the sixth cycle. Nevertheless, the RBF strategy offered high productivity of EPS, ENS and biomass, and reduced the fermentation period. Meanwhile, the total amount of GMBLs obtained from all three extraction methods was low. The SVE (20.36%) and SXE (18.8%) techniques extracted a higher amount of GMBL compared with the UAE method (7.5%). In SVE, the GMBL yield was enhanced as the amount of hexane (100–200 mL) and extraction time (1–3 h) increased. To maximise the lipid yield, the shortest time (3 h) and lowest volume of hexane solvent (200 mL) were needed in SVE. Among the extraction techniques used, SVE could be suggested as an effective extraction strategy to acquire lipids, as it requires a short extraction period with a moderately low solvent volume. The findings of this study will enhance the production of *Ganoderma lucidum* strain QRS 5120 and the capability to produce lipids as a new biodiesel feedstock. Therefore, the present study may be extended to different kinds of fungal fermentation for the effective production of EPS, ENS and biomass, and is ideal for the process of upscaling, allowing it to be introduced on an industrial scale to meet market demands. However, future research work should focus on other novel extraction techniques the optimisation of the extraction parameters in improving the lipid yield from *G. lucidum* mycelial biomass.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su141710764/s1, Table S1: Raw data from *G. lucidum* QRS 5120.

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