



Efficient biomass-exopolysaccharide production from an identified wild-Serbian *Ganoderma lucidum* strain BGF4A1 mycelium in a controlled submerged fermentation



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ARTICLE INFO

Keywords:

Wild-Serbian *Ganoderma lucidum*
Fungal identification
Exopolysaccharide
Biomass
RSM
Submerged fermentation

ABSTRACT

A wild-Serbian medicinal mushroom *Ganoderma lucidum* strain BGF4A1 (GLSB) was isolated from Mount Avala, Serbia and morphologically identified based on its brown-liquorish cap and woody stipe. Molecularly, GLSB (642.8 bp) was sequenced and found to be 99% similar to the Serbian-originated *G. lucidum* strain BEOFB 434 and *G. lucidum* strain BEOFB 431. The isolate belongs to the *G. lucidum* species as the sequence dissimilarities (K_{mic}) value between both sequences of the same fungal species was 0.001. In submerged-liquid fermentation, biomass and exopolysaccharide (EPS) production of GLSB was optimised using response surface methodology. The interactions between three variables: initial pH (4–6), temperature (20°C–30°C), and glucose concentration (10 g/L–50 g/L) were analysed using a central composite design. An analysis of variance revealed that the model was significant for all parameters investigated ($p < 0.05$). Temperature and glucose concentration were found to significantly influence mycelial biomass production, whereas for EPS production only glucose concentration had a significant effect. The model for biomass and EPS was validated by implementing the optimised conditions (pH 5.26, 50 g/L glucose, and 30°C) and was found to generate the highest biomass (3.12 g/L) and EPS (1.96 g/L). An efficient EPS-biomass production blueprint was thus established using optimised parameters for large-scale cultivation of Serbian *G. lucidum* strains.

1. Introduction

The well-known medicinal mushroom *Ganoderma lucidum* is considered staple food among Japanese (Rei-shi) and Chinese (Lingzhi) populations, and its use is widespread throughout Asia where it has been used as a traditional medicine over two millennia (Shah and Modi, 2018), primarily to treat and prevent various diseases (Hsu and Modi, 2018; Kashimoto et al., 2006; Wan-Mohtar et al., 2017).

Ganoderma species have beneficial health effects attributable to

various bioactive properties (Hsu and Cheng, 2018; Lai et al., 2019; Wan-Mohtar et al., 2016; Wan Mohtar et al., 2016a). Nowadays, *Ganoderma* is widely cultivated and commercialised using submerged liquid fermentation (SLF) over solid-state fermentation (SSF) due to high demand in the global market (Liu et al., 2010). SSF can take several months to cultivate the fruiting body of *G. lucidum* and may be associated with complications arising from factors such as culture environment and quality (Supramani et al., 2019b; Tang et al., 2011). However, SLF appears to be more suitable for producing the exopolysaccharide

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<https://doi.org/10.1016/j.bcab.2019.101305>

Received 18 May 2019; Received in revised form 6 August 2019; Accepted 23 August 2019

Available online 27 August 2019

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(EPS) and requires a shorter fermentation time (Wan-Mohtar et al., 2016; Wan Mohtar et al., 2016b).

Some studies have reported the optimisation of culture conditions in SLF of higher fungi to improve biomass and EPS yields (Ahmad et al., 2013; Hsu et al., 2016; Supramani et al., 2019a). Utilisation of the conventional “one-factor-at-a-time” (OFAAT) method appears to be ineffective in the long term because of a shortage of specific information on interactions and correlations between independent variables (e.g., initial pH, agitation or glucose concentration), given that only one variable is changed at a time while the others remain constant. Further, this method is time consuming and complicated as it requires multiple experimental trials. Response surface methodology (RSM) thus represents an alternative solution to OFAAT as it can examine several factors simultaneously, reducing cost, time, and labour requirements. According to Shah and Modi (2018), RSM can eliminate insignificant parameters and focus specifically on the critical factors. In the current study, the optimal growth of mycelia was determined by evaluating several parameters such as temperature, initial pH, and glucose concentration.

Previous studies of *G. lucidum* from the Serbian region focused only on the fruiting bodies and bioactive composition following EPS production (Rašeta et al., 2017; Stojković et al., 2014). Thus, the present study aimed to optimise the culture conditions of identified Serbian *G. lucidum* in shake flasks to obtain a high yield of mycelial biomass and EPS using RSM. The optimised parameters may be used for future studies of large-scale submerged cultivation of Serbian *G. lucidum*.

2. Materials and methods

2.1. Fungal source

Wild-Serbian *Ganoderma lucidum* strain BGF4A1 (GLSB) was found at the base of a wild oak (*Quercus robur* L.) in the mountainous region of Avala (511 m above sea level), as shown in Fig. 1(a). The specimens and mycelial culture are maintained in the herbarium of the Department of Industrial Microbiology, University of Belgrade Faculty of Agriculture for culture collections and future stock requirements.

2.2. Fungal molecular identification

Cultures were subcultured onto potato dextrose agar (PDA, Oxoid Limited, Hampshire, UK), incubated at 26 °C for 7 days, and stored at 4 °C to maintain viability and prevent contamination. Cultured mycelium was finely ground in liquid nitrogen and stored at μ -20 °C. The gDNA extraction was performed by following method of Zhou et al. (2007). The PCR procedure was as described by Liu et al. (2010) and Tamura et al. (2004) with slight modifications. Purification and sequencing were performed according to the method of Supramani et al. (2019). Targeted PCR yields were subjected to 1% agarose gel PCR Purification Tool (Tiangen Biotech Co., China) analysis for 1 h at 80 V, and sequenced using a BigDye® Terminator v3.1 sequencer (Applied Biosystems Co., USA). Sequences were aligned using Clustal Omega and were compared with related sequences of fungal species using BLAST software (NCBI) (adapted from <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The evolutionary distance (K_{nuc}) from the neighbour-joining analysis was computed using the Maximum Composite Likelihood method (Tamura et al., 2004) in Molecular Evolutionary Genetics Analysis (MEGA-X) (Kumar et al., 2018). Next, a phylogenetic tree was generated and the closest K_{nuc} of the isolated commercial fungus was classified as the same species. The GLSB strain was verified using A plasmid Editor (ApE) software (v2.0.55, May 2018; adapted from <http://jorgensen.biology.utah.edu/wayned/ape/>) in supplementary data 1.

2.3. Batch fermentation

The inoculum was prepared in a 500-mL Erlenmeyer flask containing 200 mL medium. The composition of the medium used in all stages of fermentation was as follows (g/L): [yeast extract (YE) 1, KH_2PO_4 (monopotassium phosphate) 0.5, K_2HPO_4 (dipotassium phosphate) 0.5, MgSO_4 0.5, NH_4Cl (ammonium chloride) 4], unless specified.

2.4. Optimisation of media using response surface methodology

In the present study, a central composite design (CCD) was selected for the optimisation of three independent variables as shown in Table 1. The selected outcomes were mycelial biomass (g/L) and EPS (g/L). A complete factorial CCD design for the factors and levels of each variable,

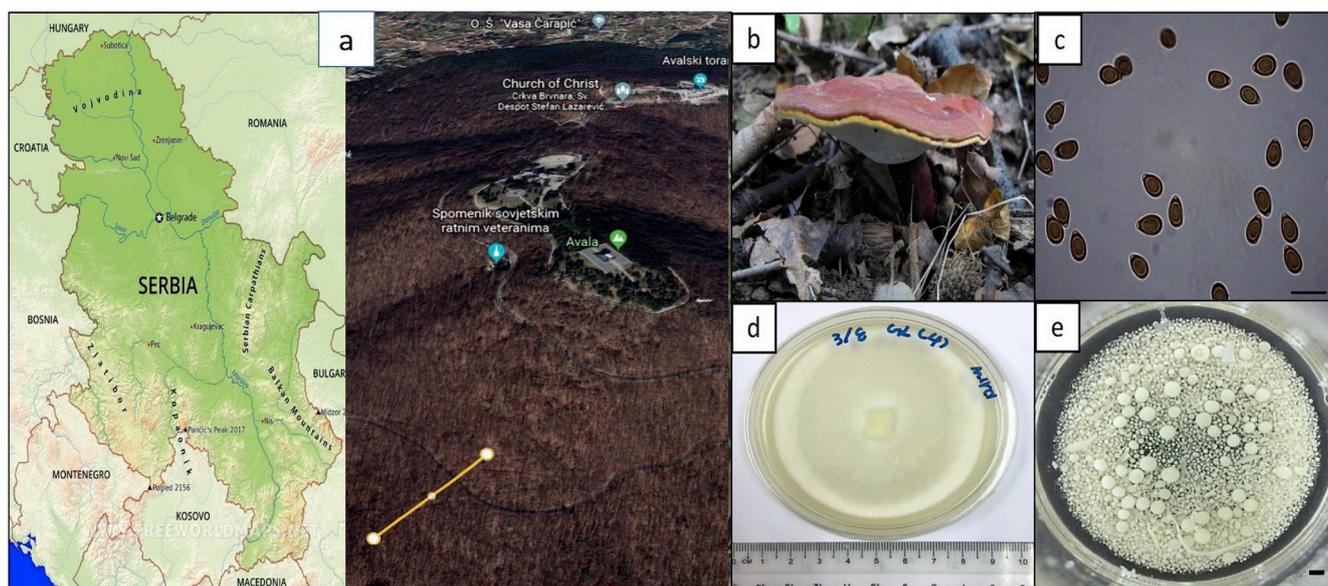


Fig. 1. (a): The locality of wild-Serbian GLSB found at the mountain of Avala (yellow bar indicates 165-m distance at the coordinates 44°41'25"N 20°30'51"E). Source: Google ; (b): fruiting body of wild-Serbian *G. lucidum* BGF4A1 (GLSB); (c): double-walled basidiospores of wild-Serbian GLSB under a microscope (100 × under oil immersion) (bar = 10 μm); (d): wild-Serbian GLSB grown on potato dextrose agar; (e): wild-Serbian GLSB grown in a shake flask at day 10 of second seed culture (bar = 1 cm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Experimental range and levels of independent variables.

Independent variables	Range and levels		
	-1	0	1
Initial pH	4	5	6
Glucose (g/L)	10	30	50
Temperature (°C)	20	25	30

and both outcomes, is presented in Table 2. All analytical tests were conducted in triplicate to ensure reproducibility. The optimal values of the parameters were analysed by analysis of variance (ANOVA) and plotted as three-dimensional (3D) response surface analysis using Design-Expert Software (version 7.0; © Stat-Ease, Inc. 2019. Design-Expert® Software is a registered trademark of Stat-Ease, Inc.). The interactions obtained were analysed by the following second-order polynomial equation, Eq. 1 (supplementary data 2).

2.5. Mycelial biomass and EPS measurement

Mycelial biomass (Fig. 1(e)) of *G. lucidum* (second seed culture) was measured according to Supramani et al. (2019a) while the harvested fermentation broth was mixed with cold ethanol (95%) at a ratio of 1:4 and incubated overnight at 4 °C. The precipitate was then centrifuged (8000 rpm, 10 min), filtered through GF/C filter paper (pre-dried and weighed) and washed twice with ethanol (95%). Crude EPS was then estimated (Wan Mohtar et al., 2018).

3. Results and discussion

3.1. Phylogenetic tree

The studied sequence was analysed and inputted with other top-10 related reference taxa retrieved from the NCBI GeneBank to generate a phylogenetic tree, which was constructed based on the Maximum Composite Likelihood method as shown in Fig. 2. The analysis (Fig. 2) showed that the studied strain was placed in the same clade (Clade A) with two other *G. lucidum* strains (BEOFB 434 and BEOFB 431), which

Table 2
Experimental design using RSM with CCD with responses for *G. lucidum* mycelium biomass and EPS production.

Run Order	Variables			Responses	
	Initial pH	Temperature	Glucose	Mycelium biomass (g/L)	EPS (g/L)
		°C	(g/L)	Actual value	Actual value
1	5	25	30	1.38	1.03
2	5	30	30	2.92	1.50
3	4	30	10	1.90	0.73
4	5	25	30	1.81	1.61
5	5	25	30	1.40	1.41
6	4	20	10	1.14	0.63
7	6	30	10	2.48	0.69
8	6	20	10	1.81	0.48
9	5	25	30	2.29	1.62
10	5	25	10	1.91	0.50
11	6	20	50	1.83	0.93
12	5	25	30	2.46	1.38
13	5	20	30	1.96	1.55
14	4	30	50	2.97	0.88
15	6	25	30	1.42	0.91
16	6	30	50	2.45	1.84
17	5	25	50	3.41	2.25
18	5	25	30	2.19	1.76
19	4	20	50	1.95	1.51
20	4	25	30	0.92	0.58

originated from Serbia. The sequence was found to be closely related to *G. lucidum* BEOFB 434 and BEOFB 431 strains with 99% similarity on the BLAST database. Meanwhile, in ApE software, GLSB showed 620 matches and 22 mismatches when aligned with *G. lucidum* (accession number: MG91100.1), which confirmed the GLSB strain as a *G. lucidum* species.

3.2. Optimization using RSM

The effects of temperature, initial pH, and glucose concentration on biomass and EPS production from *G. lucidum* mycelium were evaluated. Twenty distinct sets of culture conditions were used for optimisation in RSM (Table 2).

3.3. Mycelium biomass production optimization

ANOVA for mycelium biomass production is shown in Table 3. The model was significant, as the value of “Prob > F” was 0.0196 (<0.05). This showed that the response variable of the quadratic model was significant at a 95% confidence level. The coefficient determination ($R^2 = 0.7856$) stipulated that 78.56% of the variability in the response can be explained by the model, while the remaining variability was not. The model for biomass yield was regressed by considering the actual variables and is expressed in Eq. 2 (supplementary data 2).

From the model, both temperature (B) and glucose concentration (C) showed a significant effect at $p < 0.05$. Among the three variables, temperature exhibited the highest significant ($p = 0.0104$), followed by glucose concentration ($p = 0.0252$) and pH ($p = 0.4067$). Both quadratic terms of initial pH (AA) and glucose concentration (CC) also showed a significant effect ($p < 0.05$) on mycelium biomass yield. However, negative effects were shown by initial pH (A) and quadratic terms (AB, AC, BC, and B²).

The quadratic models were represented as response surface 3D graphs (Fig. 3), with the combination effect of initial pH, temperature, and glucose concentration. One factor was maintained at a constant value corresponding to the other two factors that were varied within the experimental range. Fig. 3(a) shows the effect of temperature (B) and glucose concentration (C), Fig. 3(b) shows the effect of initial pH (A) and glucose concentration (C), and Fig. 3(c) shows the effect of initial pH (A) and temperature (B) on mycelial biomass production. Fig. 3(a) shows that the effects of glucose and temperature on production of mycelial biomass were more important than that of initial pH. Meanwhile, Fig. 3 (b)–(c) shows that the initial pH was not significant for mycelial biomass production. However, both figures show that the yield of biomass production was reduced at lower or higher initial pH, and that the suitable initial pH appeared to be a pH of 5.00. Therefore, the temperature and glucose concentration are the significant factor for biomass production. The maximum yield of biomass exhibited by the sample was observed at initial pH of 5.28, 49.77 g/L glucose concentration and temperature at 29.92 °C.

3.4. EPS production optimization

ANOVA for EPS production is shown in Table 4. The predicted coefficient determination indicates that 77.30% ($R^2 = 0.7730$) of the variability in the response can be explained using this model. The model was significant for further analysis ($p < 0.05$). The model for EPS production was regressed by considering the actual variables and is expressed in Eq. 3 (supplementary data 2).

From the model, EPS production was dependent only on glucose concentration (C). The other variables, temperature and initial pH, were found to be insignificant for EPS production with $p > 0.05$. Among the studied variables, glucose concentration demonstrated the highest significant ($p = 0.0022$), followed by temperature ($p = 0.6251$) and pH ($p = 0.6340$). The quadratic terms of initial pH (AA) also showed a significant effect at $p < 0.05$ on the yield of EPS production. However,

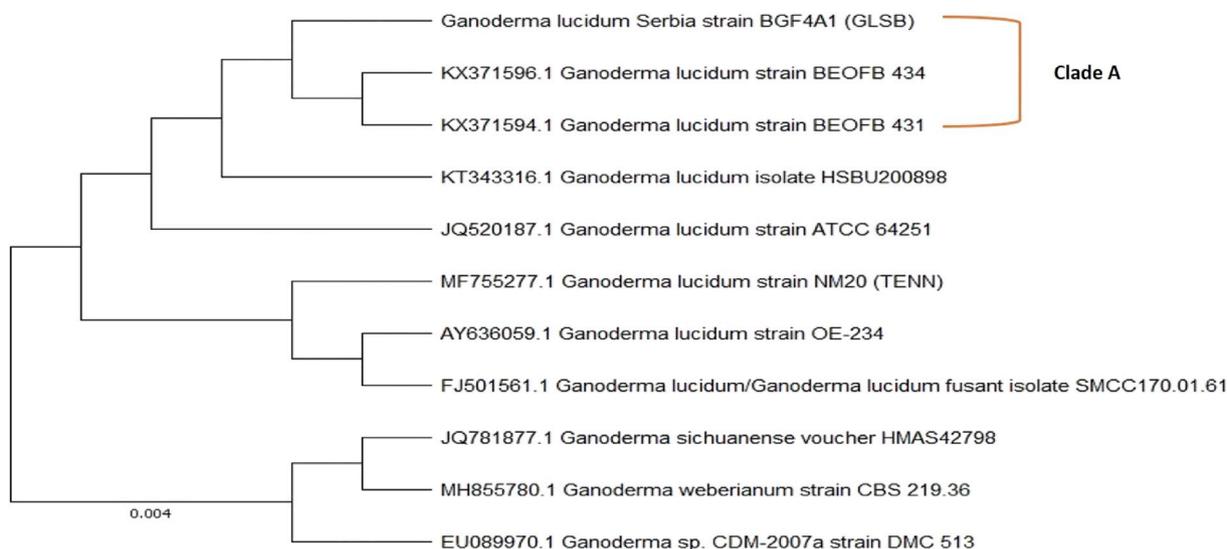


Fig. 2. Phylogenetic tree of *Ganoderma lucidum* strain BGF4A1 (GLSB) generated by neighbour-joining with evolutionary distance (K_{nuc}).

Table 3

Analysis of variance for the experimental results of the CCD quadratic model for mycelium biomass.

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Model	5.985715066	9	0.665079452	4.071172726	0.0196	Significant
A: pH	0.1225449	1	0.1225449	0.750138128	0.4067	
B: Temperature	1.6192576	1	1.6192576	9.912014813	0.0104	Significant
C: Glucose	1.1296321	1	1.1296321	6.914854133	0.0252	Significant
AB	0.029646125	1	0.029646125	0.1814738	0.6791	
AC	0.450775125	1	0.450775125	2.759344601	0.1277	
BC	0.005356125	1	0.005356125	0.032786624	0.8599	
A ²	2.140834778	1	2.140834778	13.10476235	0.0047	Significant
B ²	0.416521841	1	0.416521841	2.549668846	0.1414	
C ²	1.005510278	1	1.005510278	6.155063144	0.0325	Significant
Residual	1.633631134	10	0.163363113			
Lack of fit	0.560091801	5	0.11201836	0.521724527	0.7538	Not significant
Pure error	1.073539333	5	0.214707867			
Cor total	7.6193462	19				

Std. dev. = 0.40418203.

R² = 0.785594316.

Adequate precision = 7.255077177.

Mean = 2.0297.

Adj R² = 0.5926292.

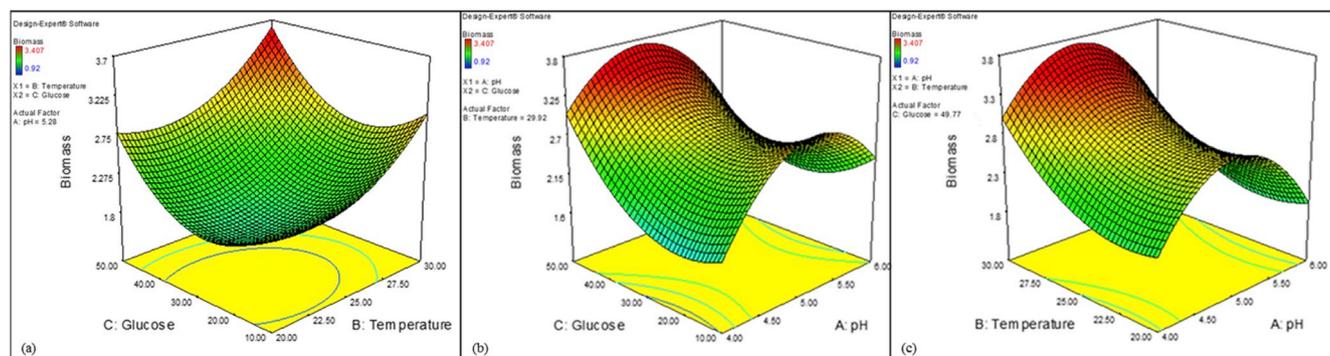


Fig. 3. Response surface (3D graph plot) showing the combined effects of (a): temperature and glucose concentration (b): initial pH and glucose and (c): initial pH and temperature of wild-Serbian *G. lucidum* (BGF4A1) on mycelial biomass production.

negative effects were shown by initial pH (A), temperature (B), and quadratic terms (AB, AC, BC, B² and C²).

Fig. 4(a) shows a positive coefficient of glucose concentration (C), indicating a linear effect with increased glucose concentration (C), and

resulting in increased EPS production while temperature (B) had no significant effect on EPS production. Meanwhile, Fig. 4(b) also shows that EPS production was affected by glucose concentration (C), as the maximum value of EPS (2.253 g/L) was at high glucose concentration

Table 4
Analysis of variance for the experimental results of the CCD quadratic model for EPS production.

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Model	3.907001848	9	0.434111316	3.782953663	0.0249	Significant
A: pH	0.0276676	1	0.0276676	0.241102327	0.6340	
B: temperature	0.02916	1	0.02916	0.254107471	0.6251	
C: glucose	1.9236996	1	1.9236996	16.76359536	0.0022	Significant
AB	0.334153125	1	0.334153125	2.911893196	0.1187	
AC	0.041905125	1	0.041905125	0.365171651	0.5591	
BC	0.000136125	1	0.000136125	0.001186227	0.9732	
A ²	1.098100023	1	1.098100023	9.569115908	0.0114	Significant
B ²	0.060310023	1	0.060310023	0.525556494	0.4851	
C ²	1.00227E-05	1	1.00227E-05	8.73405E-05	0.9927	
Residual	1.147545952	10	0.114754595			
Lack of fit	0.811992619	5	0.162398524	2.419861579	0.1772	Not significant
Pure error	0.335553333	5	0.067110667			
Cor total	5.0545478	19				

Std. dev. = 0.338754476.
 $R^2 = 0.772967633$.
 Adequate precision = 6.860330024.
 Mean = 1.1901.
 Adj $R^2 = 0.568638502$.

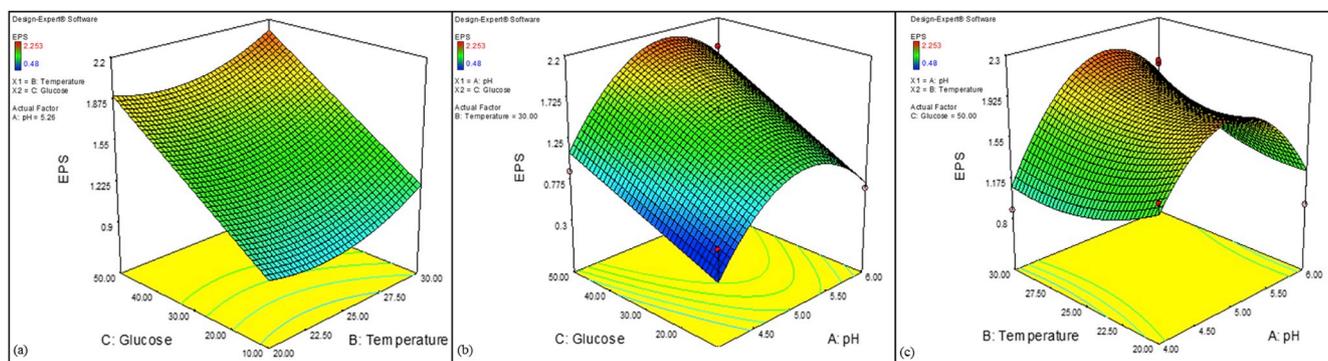


Fig. 4. Response surface (3D graph plot) showing the combined effect of (a): temperature and glucose concentration (b): initial pH and glucose concentration and (c): initial pH and temperature of wild-Serbian *G. lucidum* (BGF4A1) on EPS production.

(50 g/L), whereas, pH was not significantly effecting EPS production. Fig. 4(c) shows the 3D plot of initial pH (A) and temperature (B), which both had no significant effect on EPS production at the quadratic level. The results of this experiment supported those of showing that glucose had a positive effect on EPS production (Supramani et al., 2019b). The optimum conditions for maximum yield of EPS production were therefore determined to be initial pH of 5.26, concentration, temperature of 30 °C and 50 g/L of glucose.

3.5. Optimized conditions verification

To verify the effectiveness of the model, the biomass and EPS yields were measured and compared with the predicted values of responses under the statistically optimal conditions (Table 5). The validation experiment was performed in triplicate. The predicted values for mycelial biomass and EPS production were 3.45 g/L and 2.11 g/L, respectively, which were in line with the experimental values of 3.09 g/L and 1.98 g/L (10.43% and 6.16% difference, respectively, between the

Table 5
Validation of the model using optimized conditions.

Run	Variables			Response	
	pH	Temperature	Glucose	Biomass (g/L)	EPS (g/L)
Biomass	5.28	29.92	47.99	3.09 ± 0.1	–
EPS	5.26	30.00	50.00	–	1.98 ± 0.3
Biomass + EPS	5.26	30.00	50.00	3.12 ± 0.3	1.96 ± 0.4

values). Thus, the validity of the model under Eq 2 and Eq 3 was justified for biomass and EPS production as the average error of deviation was <15% (Milkey et al., 2014).

3.6. Comparison of current study with the literature

Recent statistical optimisation approaches to determine suitable parameters for efficient mycelial biomass and EPS production using *Ganoderma lucidum* in controlled shake-flask fermentation are shown in Table 6. As reported, only two previous studies applied distinct statistical approaches other than RSM by presenting the mycelial biomass and EPS production as responses. According to studies Chang et al. (2006) and Baojing et al. (2012) under optimal conditions of prepared medium, the biomass and EPS yields were significantly increased. Meanwhile, both the current study and study Supramani et al. (2019) demonstrated a high EPS yield but low mycelial biomass using the same optimisation technique (Table 6). However, the production of EPS relative to its biomass by Serbian *Ganoderma* is 12% higher than that of Malaysian *Ganoderma*. The present study therefore demonstrated higher efficiency in producing EPS compared with previous studies. Hence, RSM represents an effective statistical optimisation approach for improving the biomass and EPS yields from *G. lucidum*.

4. Conclusion

In this present study, RSM with CCD was applied for statistical optimisation of mycelial biomass and EPS production of *G. lucidum* by

Table 6

Comparison with published optimization processes using *Ganoderma lucidum* in submerged-liquid fermentation.

Origin	Optimization approach	Cultivation mode	Initial pH	Glucose concentration (g/L)	Temperature °C	Biomass (g/L)	EPS (g/L)	References
Serbia	Response surface methodology	Shake flask	5.26	50	30	3.12	1.96	<i>Current study</i>
Malaysia	Response surface methodology	Shake flask	4	26.5	–	5.19	2.64	Supramani et al. (2019a)
Taiwan	Taguchi's orthogonal array	Shake flask	6.5	12.1	34	18.70	0.420	Chang et al. (2006)
China	Orthogonal matrix	Shake flask	–	50	30	7.235	1.723	Baojing et al. (2012)

SLF. Both temperature and glucose concentration ($p < 0.05$) were found to be significant variables for higher yield of mycelial biomass production by 3.12 g/L, while only glucose concentration showed a significant effect by producing 1.98 g/L of EPS. From the quadratic model, optimal growth conditions generated for maximum mycelial biomass and EPS production were identified at a temperature of 30 °C, initial pH of 5.26, and 50 g/L of glucose concentration.

Conflicts of interest

All authors declare no conflicts of interest in this paper.

Acknowledgements

We are grateful to the University of Malaya for funding under Ministry of Education Malaysia (Department of Higher Education) RU003I-2017 and Fundamental Research Grant Scheme (FRGS: FP066-2018A).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2019.101305>.

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