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Efficient biomass-endopolysaccharide production from an identified wild-Serbian *Ganoderma applanatum* strain BGS6Ap mycelium in a controlled submerged fermentation

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ABSTRACT

A rare wild-Serbian *Ganoderma applanatum* strain BGS6Ap (GASB) was morphologically identified based on its basidiocarp with brownish-red cap and woody stipe. Molecularly, GASB DNA was extracted, sequenced (651 bp) and subjected to phylogenetic analysis with evolutionary distance (K_{nuc}). A plasmid Editor (ApE) software was used to verify the sequence and GASB was found to be 99% similarity to the commercial *G. applanatum* (GA) ATCC44053. The production of GASB's biomass and endopolysaccharide (ENS) in different temperature was screened at 20 °C, 25 °C and 30 °C via one-factor-at-a-time (OFAAT) scheme in a submerged liquid fermentation (SLF). Lower temperature was chosen due to GASB's natural habitat, which belongs to temperate continental climates with the influence of air currents from the Carpathians and the Mediterranean. Using the OFAAT data, the SLF was further optimised using response surface methodology (RSM) to generate biomass-ENS based on temperature (20°C–30 °C), agitation rate (100–200 rpm), glucose concentration (10 g/L–50 g/L) and initial pH (4–6). The highest production of biomass (17.51 g/L) and ENS (3.47 g/L) was generated using optimal conditions of 25 °C, 150 rpm, 10 g/L of glucose and pH 6. To study the structural composition, ENS confirmed spectral linkages in FTIR (2925 cm^{-1}, 1623 cm^{-1}, 1073 cm^{-1}, 890 cm^{-1}) and NMR (δ 4.30, 4.80, 5.20 ppm) as endo-β-D-glucan. This approach could serve as a blueprint for novel mycelial biomass and endo-β-D-glucan production of GASB which can be further studied on different temperate basidiomycetes.

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1. Introduction

Ganoderma applanatum (GA) is a medicinal macrofungi, belonging to the phylum Basidiomycota, found throughout Asia and Eastern Europe (Elsayed et al., 2014). The *G. applanatum* (Pers.) Pat., is usually found growing on barks of rotting trees (Lee et al., 2007). *G. applanatum* (Pers.) Pat has been used as medicinal compound to treat several diseases due to its triterpenoids (Nishitoba et al., 1989), α-D-glucan and β-D-glucan (Mizuno et al., 1981), ganoderic acid (Chouiter et al., 2008), applanoxidic acid (Nishitoba et al., 1989; Tokuyama et al., 1991), β-amyrin acetate (Tokuyama et al., 1991), β-amyrenone (Nishitoba et al., 1989), aldehyde (Chen et al., 1990), friedelin (Protiva et al., 1980) and other bioactive compounds (Jo et al., 2009).

Previous studies on similar mushroom species focused on the bioactive composition of fruiting bodies with no recent studies on optimisation using submerged-liquid fermentation (SLF) (Hassan et al., 2019; Rašeta et al., 2016; Stojković et al., 2014; Zwietering et al., 1990). Peintner et al. (2019) reported GA from the temperate continental climate, while Mediterranean, temperate and tropical climates influence the growth of basidiomycetes and polypore's (Ghobad-Nejhad et al., 2019). Variations of biochemical parameters of *Ganoderma* spp. are associated with different locations such as the temperate regions in Serbia (Karthikeyan et al., 2009; Stojković et al., 2014). The chemical composition and bioactivity of mushrooms from temperate regions showed better antibacterial, antifungal, antioxidant, and antitumor activity that proved to be highly reliant on its location of isolation (Peintner et al., 2019; Stojković et al., 2014; Zwietering et al., 1990).

Therapeutic properties of GA are mainly found from the extracted mycelial (biomass) polysaccharides called exopolysaccharides (EPS) and endopolysaccharides (ENS) (Lee et al., 2007). Production of biomass and ENS has proven to be more efficient and economical using SLF compared to production of fruiting bodies via solid state fermentation (SSF) (Lee et al., 2007; Wan-Mohtar et al., 2016). SSF utilises nutrient-rich and easily recycled substrates such as bran or paper pulp. However, it requires long fermentation period and is prone to contamination due to high water activity that cannot be easily controlled (Subramaniyam et al., 2012). Recent studies proved that SLF decreases fermentation time by rapidly producing biomass and ENS in the culture after utilizing nutrients provided in the free-flowing liquid media, thus easing purification steps (Subramaniyam et al., 2012).

The current cultivation techniques used are all grown using solid state fermentation that lasts several months (Balakrishnan, 1996; Grajek, 1987; Yang et al., 2003). It has been reported that liquid fermentation in general, produces large amounts of uniform mycelial biomass as a source of bioactive compounds (Omar et al., 2011). Besides, it is an effective and efficient method for fast growth of mycelial biomass and efficient production of ENS within a confine space with minimal chance of contamination (Ahmad et al., 2013). In addition, SLF may need frequent or constant substrate supplementation as that nutrients would be rapidly depleted (Subramaniyam et al., 2012; Supramani et al., 2019a,b).

Recent studies demonstrated that optimisation of culture conditions in SLF using response RSM yields higher mycelial biomass and ENS in a fast cycle compared to SSF (Ahmad et al., 2013; Wan-Mohtar et al., 2016c). One-factor-at-a-time (OFAAT) is a conventional strategy used to optimise culture conditions, but has several drawbacks such as multiple launches over a longer period of time, unable to assess more than one interaction between variable and response, possibly miss optimal setting of factors and bias to time trends (Czitrom, 1999; Frey and Jugulum, 2006; Frey and Wang, 2006). RSM is comprised of fundamental experimental design for linear response models, including the depiction of techniques for determining optimal operating conditions that includes a cluster of mathematical and statistical methods applied during the development of an adequate functional relationship between interest

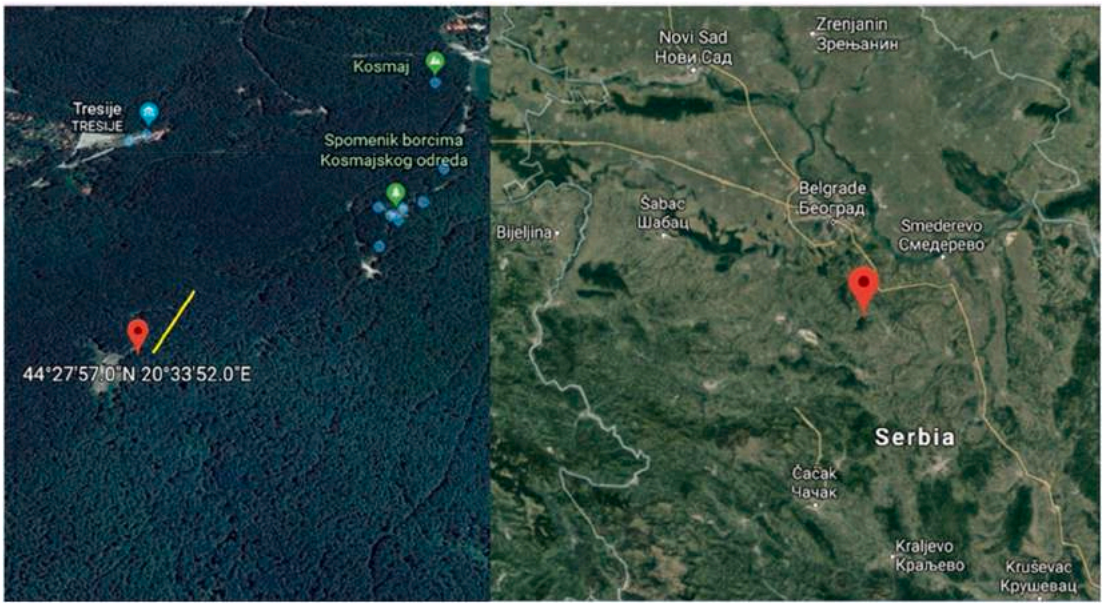


Fig. 1. Location of wild-Serbian *G. applanatum* found on Mountain Kosmaj (yellow bar indicates the altitude at 147-m distance at the coordinates 44°27′57′′N 20°33′52′′E) Source: Google Earth, 2020. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

responses, x, and several related control variables designated y1, y2, …, yk (Bezerra et al., 2008; Khuri et al., 2010).

The aim of the current study is to develop optimised media conditions using RSM to obtain optimum biomass and ENS production from a rare GASB found in the temperate continental Belgrade mountain reserve, Serbia. Prior to SLF, the mushroom species was subjected to morphological identification of fruiting body, molecular and phylogenetic analysis using Molecular Evolutionary Genetic Analysis (MEGA-X) software and A plasmid matching (ApE) software. The isolated strain was cultured at a controlled cold-temperature incubator to imitate natural habitat in the temperate continental regions. The optimised parameters were initial pH, glucose concentration, agitation rate and temperature.

2. Materials and method

2.1. Fungal source

The GASB was harvested from a fallen oak tree, on the temperate continental Belgrade climate of Mountain Kosmaj (44º27′57′′N 20º33′52′′E) in Serbia (Fig. 1) with an altitude of 147 m. The fruiting body and mycelial culture were maintained in the herbarium of the Department of Industrial Microbiology, University of Belgrade (Faculty of Agriculture) for future culture and stock requirements. The fungus was sub-cultured onto potato dextrose agar (PDA) upon receiving and incubated at standard 30 °C laboratory temperature until the mycelium covered the entire PDA plate (ThermoScientificTM OxoidTM CM0139B). Plates were stored at 4 °C and pure culture was preserved on PDA slants (ThermoScientificTM OxoidTM CM0139B) (Wan-Mohtar et al., 2017).

2.2. Fungal isolation and phylogenetic tree analysis

2.2.1. Preparation of mycelium for DNA extraction

GASB mycelium was grown on a PDA plate (ThermoScientificTMOxoidTM CM0139B) and selected parts of the mycelium were removed from the agar with a sterile scalpel blade knife (1 cm H x 1 cm W x 1 cm L). The mycelium was ensured to be liberated from traces of agar and placed in a pre-cooled pestle and mortar. It was grounded to a fine powder using liquid nitrogen, freeze-dried and was stored in an Eppendorf tube (Eppendorf no. 0030125185, Hamburg, Germany) at −20 °C (Usuldin et al., 2020; Wan-Mohtar et al., 2019).

2.2.2. DNA extraction of mycelium

Thirty miligrams of finely powdered mycelium was transferred aseptically into lysis buffer (500 μL) in a sterile 1.5 mL Eppendorf tube. It was resuspended several times until the suspension turned foamy. One μL of 10 mg/mL RNase A (R1253, Thermo Scientific, Waltham, MA, USA) was added into the tube and incubated (37 °C, 5 min) for purification of DNA. Next, NaCl (165 μL, 5 mol/L) solution was added, and the tube was inverted multiple times. Then, chloroform and phenol (400 μL each) were added, and the tube was gently inverted multiple times until the solution became cloudy. The solution was subjected to centrifugation (14000×*g*, 20 min, 4 °C) to remove protein, excess cell debris, and polysaccharides. The supernatant was transferred into a new 1.5 mL Eppendorf tube, followed by adding 95% ethanol (1:2 ratio) to precipitate the DNA. The mixture was subjected to centrifugation (13,000 rpm, 10 min, 4 °C) and the supernatant was removed. Lastly, the DNA pellet was washed thrice with 70% (300 μL) ice-cold ethanol and centrifuged again at 13,000 rpm for 1 min. The supernatant was removed, and the resulting DNA pellet was air-dried. The DNA pellet was then dissolved using 1x Tris-EDTA buffer (50 μL) (Thermo Fisher no. 17890, Invitrogen, Waltham, MA, USA), and stored at −20 °C (Usuldin et al., 2020; Wan-Mohtar et al., 2019).

2.2.3. PCR amplification

For fungal identification, two internal transcribed spacer universal primers (ITS1 and ITS4) were used for the polymerase chain reaction (PCR) (Ogbe et al., 2019). Twenty-five μL of the reaction mixture was added into PCR tubes (Eppendorf no. 0030124537, Hamburg, Germany), containing 1 μL of isolated DNA, 0.5 pmoL of both primers, deoxynucleotides triphosphates (dNTPs, 200 μM), 0.5 U DNA polymerase (Promega no. M3005, Madison, OH, USA), PCR buffer (ThermoFisher no. 14966123, Platinum II Green PCR Buffer) and water. The PCR was performed as follows: 25 cycles (98 °C for 2 min) for initial denaturation; (98 °C for 15 s; 60 °C for 30 s; 72 °C for 30 s) for annealing and extension, lastly followed by one cycle (72 °C for 10 min) for final extension of amplified DNA. A 16-capillary 3100 Genetic analysers (Applied Biosystems) were used to purify and precisely sequence the PCR products. BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used following the protocols supplied by the manufacturer (Usuldin et al., 2020; Wan-Mohtar et al., 2019).

2.2.4. Phylogenetic analysis and verification of species

The amplified PCR product acquired was submitted to NCBI BLAST analysis. The sequences with significant alignment were listed and top-10 hits from BLAST were chosen for Multiple Sequencing Alignment (MSA) obtained using Clustal Omega. Neighbouring-joining (NJ) used in Molecular Evolutionary Genetics Analysis (MEGA-X) were the exact fungal species computed using evolutionary distance (*Knuc*). Then the phylogenetic tree was generated, and any known species closest to the *Knuc* were considered the same species. Verification of species was done by comparing the mismatches using A plasmid Editor (ApE) software between the sequence of the GASB (genomic DNA) gDNA and sequence closest to the *Knuc* species (Supramani et al., 2019a,b; Usuldin et al., 2020).

Table 1
Experimental range and levels of independent variables.

Independent variables	Range and levels		
	−1	0	1
Glucose (g L^{-1}	10.0	30.0	50.0
pH	4.0	5.0	6.0
Temperature (°C)	20	25	30
Agitation (rpm)	100	150	200

Table 2
Illustrates the levels and range of variables used for this study.

	Variables				Responses	
Run No.	Agitation (rpm)	Temperature (°C)	Initial pH	Glucose (g/L)	Biomass (g/L)	ENS (g/L)
1	200	25	5	30	6.7697	0.3140
2	100	30	4	10	5.7753	1.2300
3	150	25	5	30	14.1513	1.0827
4	100	20	6	50	5.0350	0.6550
5	100	20	4	50	8.0267	0.9700
6	100	30	4	50	5.2870	1.2253
7	200	30	6	50	5.1107	0.2400
8	150	25	5	50	8.6817	3.8200
9	100	30	6	10	5.0820	1.6593
10	100	20	4	10	3.4433	0.7400
11	200	20	6	10	14.5190	1.6383
12	200	20	4	10	3.5450	0.9373
13	150	25	5	30	19.6290	1.2300
14	150	30	5	30	3.4153	0.3440
15	200	30	6	10	8.8507	1.4700
16	150	25	6	30	11.5500	0.3600
17	200	20	4	50	5.3700	0.5077
18	150	25	5	30	18.1510	1.1063
19	150	20	5	30	8.8507	0.1900
20	150	25	5	10	11.5300	4.4100
21	200	20	6	50	4.3733	0.4423
22	150	25	5	30	15.6953	1.0377
23	100	25	5	30	7.4003	0.4900
24	150	25	5	30	15.6847	1.1513
25	100	30	6	50	3.9647	0.6800
26	150	25	4	30	8.0897	0.1600
27	200	30	4	50	4.5073	0.3800
28	150	25	5	30	15.5867	1.1407
29	200	30	4	10	4.5860	0.9890
30	100	20	6	10	13.4610	1.1543

2.3. Submerged-liquid fermentation

A preliminary study was conducted. GASB cultivation was done and consists of two culture seeds that were cultivated for 20 days at 20 °C–30 °C, 100 rpm, 30 g/L glucose concentration at pH 4. The media was fixed at the following conditions: 20°C–30 °C, 100–200 rpm agitation, 10 g/L–50 g/L glucose concentration, and initial pH 4–6. First seed culture: Two square-plugs made from 14-day old growth (5 mm × 5 mm x 5 mm) were inoculated into an Erlenmeyer flask containing 100 mL of fermentation medium. First seed culture was homogenised using a sterile hand blender for 20 s on day 20. Second seed culture: Inoculum from first seed (20%) used with fresh fermentation media, and the mixture was allowed to ferment for a total of 20 days before analysis. The fermentation medium was maintained by using the same exact concentrations (g/L) of Yeast Extract 1, K_2HPO_4 0.5, KH_2PO_4 0.5, $MgSO_4$ 0.5, and NH_4Cl 8. Each experiment was conducted in three different conditions (Wan-Mohtar et al., 2016, Wan-Mohtar et al., 2017).

2.4. Optimisation of media using response surface methodology (RSM)

A complete factorial central composite design (CCD) was used to optimise the factors (initial pH, agitation rate, glucose concentration, temperature) and levels of production of each variable (biomass and ENS) (Table 1). Thirty experiments were generated using CCD in Design Expert 7.0 software as listed in Table 2. All experiments were conducted in triplicate for 20 days to ensure maximum yield of responses whereby it was analysed using ANOVA (analysis of variance) and plotted as (3D) three-dimensional models.

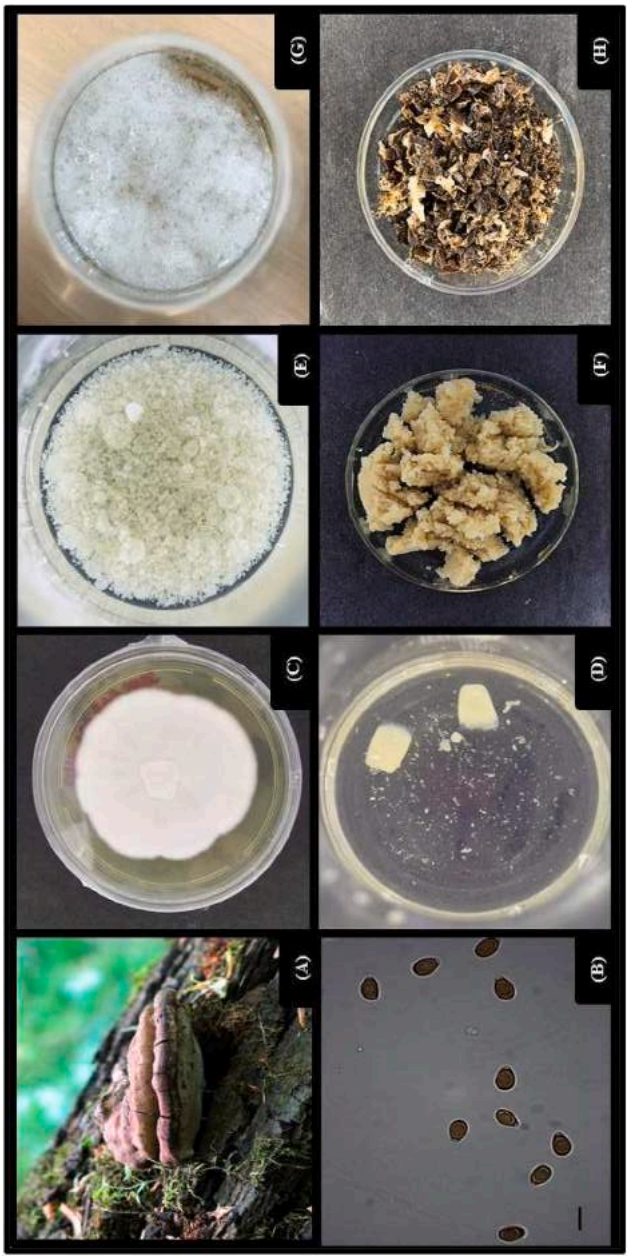


Fig. 2. (A) wild-Serbian *G. applanatum* fruiting body in Mountain Kosmaj, Serbia. (B) Basidiospores of wild-Serbian *G. applanatum* under a microscope (100 × using immersion oil) (Bar = 10 μm). (C) Mycelium of wild-Serbian *G. applanatum* on PDA plate at day 7. (D) First seed culture of wild-Serbian *G. applanatum* in SLF (Day 10) and (E) wild-Serbian *G. applanatum* in SLF at day 20. (F) shows the mycelium after filtration from (E). (G) shows the extracted ENS and (H) macroscopic morphology of dried biomass.

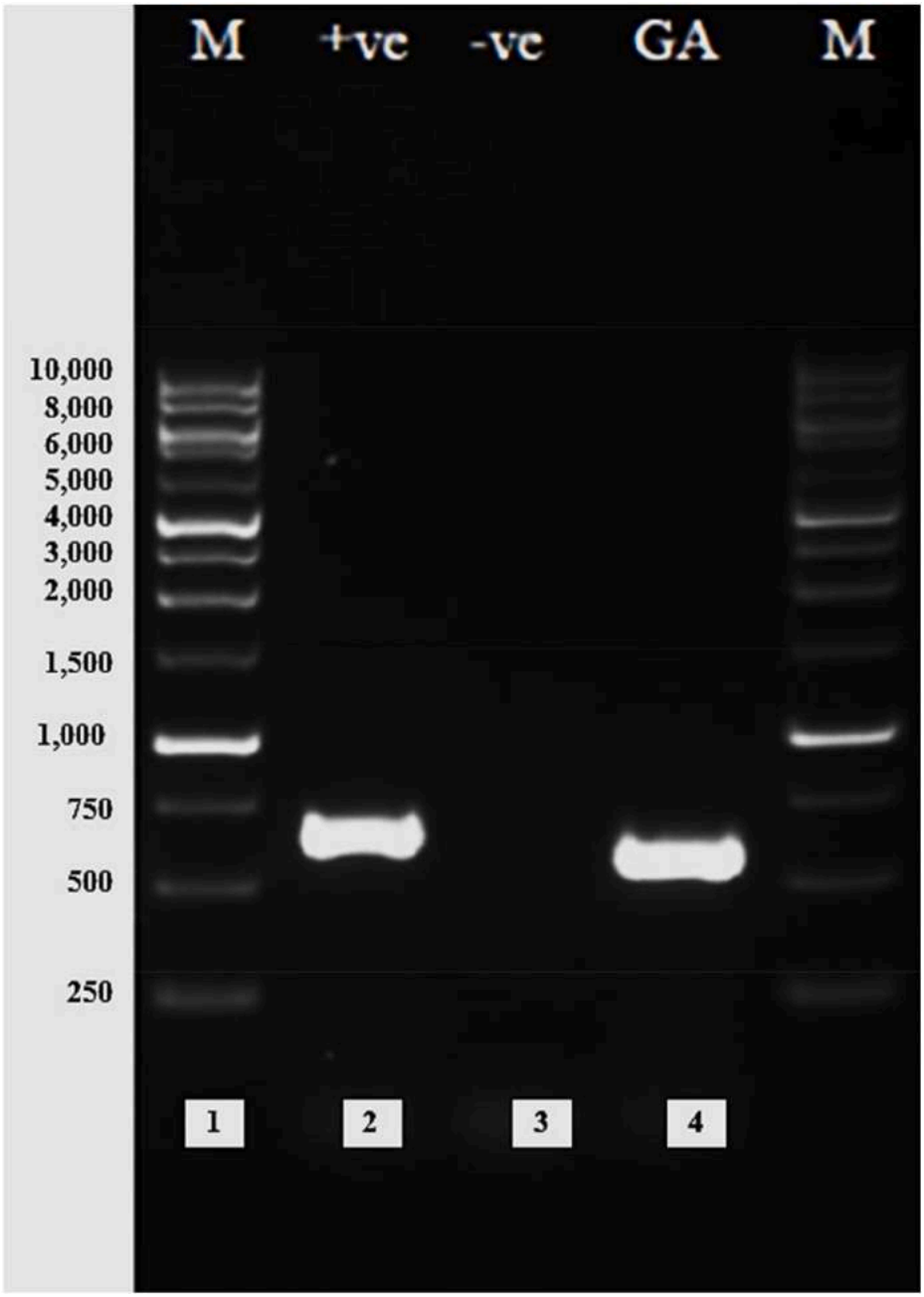


Fig. 3. Agarose gel electrophoresis showing the wild-Serbian *G. applanatum* strain BGS6Ap mycelium (GASB) PCR product. Lane 1 represent the 10 kb DNA ladder; lane 2 represent positive control (+ve) fungal gDNA; lane 3 represents the negative control (-ve) of no template PCR control and lane 4 corresponds to GASB PCR product.

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

The interactions between variable and responses were studied using the empirical model that is followed by second-order quadratic model. Based on Eq (1), Y is the forecast response, $b_0^{'}$ is the constant coefficient, b_i is the linear coefficient, b_{ii} is the quadratic coefficient, b_{ij} is the interaction coefficient, and X_iX_j are the coded values as shown in Eq (1) (Sohedein et al., 2020; Wan-Mohtar et al., 2017).

2.5. Mycelial biomass and endopolysaccharide (ENS) measurement

Mycelial biomass from Fig. 2E was processed and weighed from the second seed culture of GASB as shown in Fig. 2F & H. Next, 1 g

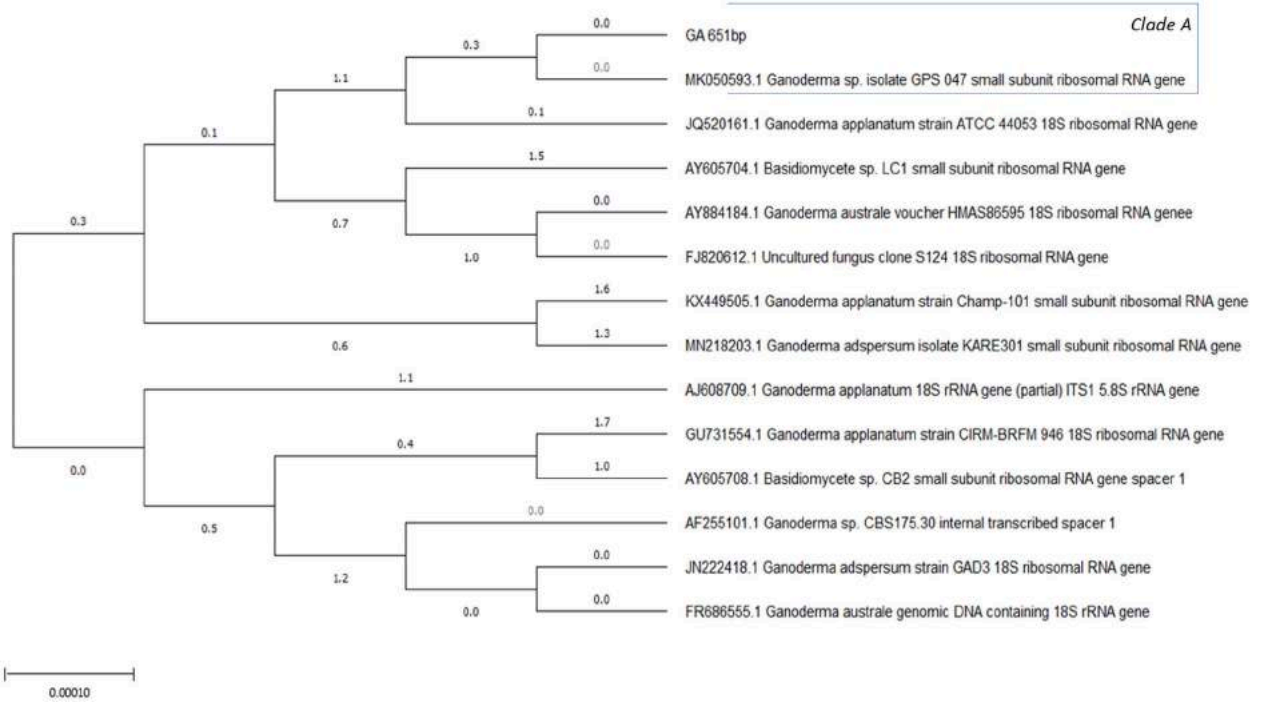


Fig. 4. Phylogenetic tree of wild-Serbian *G. applanatum* strain BGS6Ap represented as GA651bp generated by neighbour-joining with evolutionary distance (*Knuc*). GASB (Serbia) was placed in Clade A with two other *Ganoderma* sp. strains (GPS 047: Iran and ATCC 33053: Japan) with similar temperate continental climate.

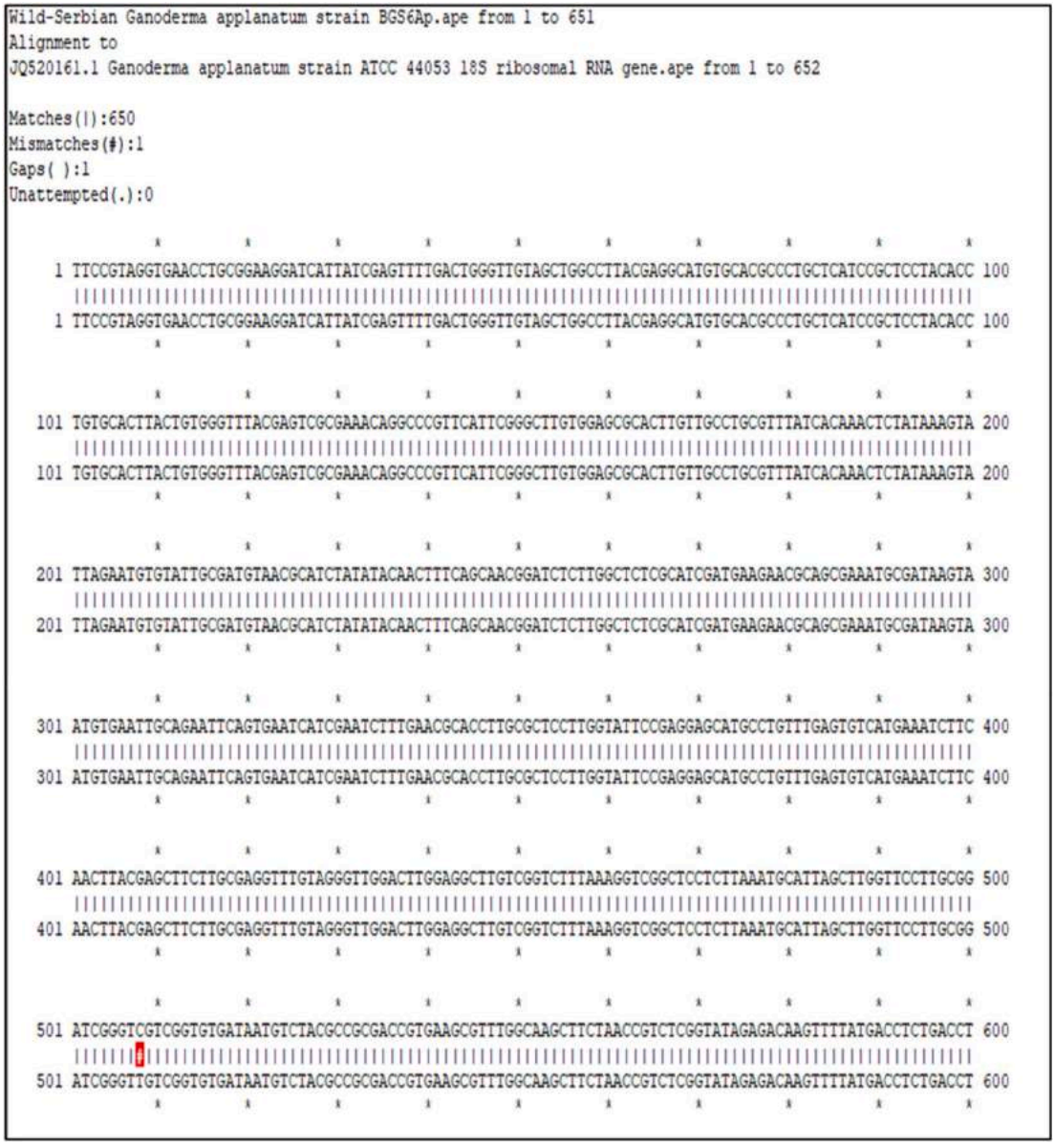
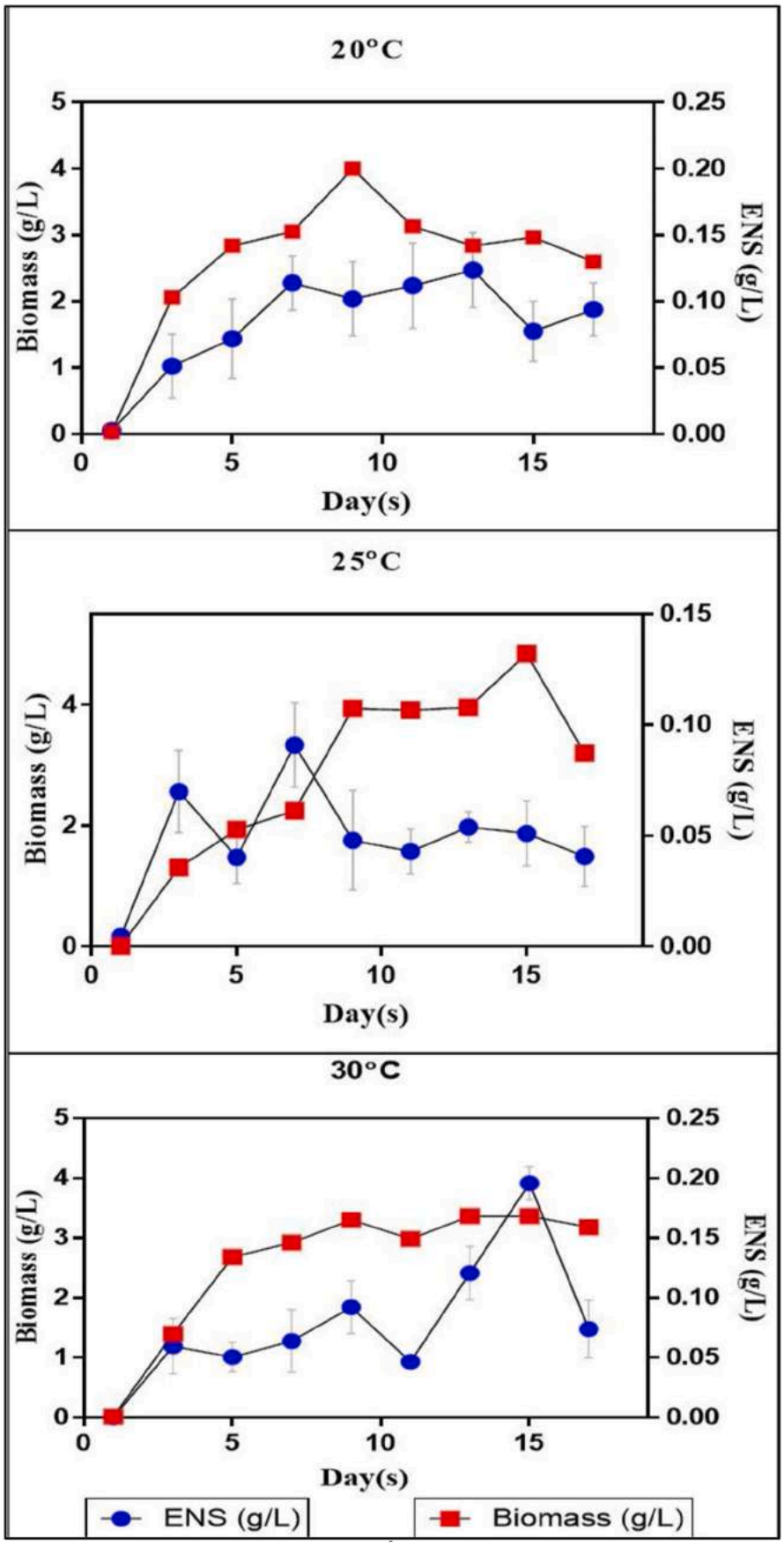


Fig. 5. Species verification using A plasmid Editor (ApE) software via sequence alignment of wild-Serbian *G. applanatum* strain BGS6Ap (GASB) and *Ganoderma* sp. ATCC 44053 strain.

of mycelium was suspended in 20 mL of distilled water and was heated at 121 °C for 30 min. The mixture was filtered out via filtration using a filter paper that was placed in a filter funnel, to obtain the supernatant. ENS was precipitated by adding 1:4 of 99.9% ethanol mixed with the supernatant that had been produced overnight. A precipitate was separated by centrifugation at 12,000×*g* for 10 min, followed by centrifugation at 12,000×*g* for another 10 min. The precipitate was washed twice with 5 mL of 95% ethanol, dried, and then eluted with 95% ethanol. It was sequestered in desiccators and weighed to determine the constant weight of ENS (Ubaidillah et al., 2015).

2.6. Fourier-transform infrared spectroscopy (FTIR)

FTIR of crude ENS (0.5 g) was characterized using the laboratory equipment Agilent Cary 630 equipped with diamond ATR (Attenuated Total Reflectance). The crude exopolysaccharides were characterized using FTIR analysis, and the frequency range are measured as wave numbers in the range of 4000–650 cm^{-1}. The crude ENS were subjected to analysis using a real-time Micro-Lab software (Usuldin et al., 2020). The glucan laminarin (*Laminaria digitata*, Sigma-Aldrich, Dorset, UK) was used as a standard for β-1, 3-D-glucan.



(caption on next page)

Fig. 6. Growth curve analysis of wild-Serbian *G. applanatum* strain BGS6Ap in shake flask fermentation and effect of temperature 20 °C, 25 °C, 30 °C on the production of biomass-ENS using one-factor-at-a-time (OFAAT) technique.

2.7. Characterization of endopolysaccharide using ^{1}H NMR spectroscopy

Using 700 MHz NMR spectrometer (Ascend™ 700, Bruker, Germany) the analysis was performed. Crude ENS (10 mg) was mixed with 500 μL of deuterium oxide (D_2O) at room temperature. The mixture was vortexed and sonicated for 15 min until crude polysaccharide completely dissolved. Next, the mixture was subjected to centrifugation at 10000 rpm for 10 min. The supernatant was transferred to a 5 mm NMR tube (Norell, 211 Sigma Aldrich, Canada) for analysis. The glucan laminarin was used as a comparison standard for β-1,3-D-glucan. All experiments were conducted at 25 °C. A pre-saturation pulse sequence (PRESAT) was performed to remove the large signal of HOD. HOD is a form of double water peaks observed in deuterated HNMR solvents. (Supramani et al., 2019a, b; Usuldin et al., 2020).

3. Result and discussion

3.1. Wild-Serbian Ganoderma sp. molecular identification

Molecular identification was performed on GASB by DNA isolation and PCR amplification using ITS1 and ITS4 primers. Fig. 3 showed the amplified GASB PCR product (lane 4) with a size between 500 and 750 bp in similar range to the positive control (lane 2). Sequence from GASB PCR product was subjected to NCBI BLAST analysis to align and compared the product with the top 10 related species in the GenBank, and a phylogenetic tree was constructed by composite likelihood method. Fig. 4 showed a comprehensive phylogenetic analysis showing the closest evolutionary distance (K_{nuc}) values. Based on the reference database, GASB was placed in the same clade (Clade A) with two other *Ganoderma* sp. strains (GPS047 and GA ATCC 44053) from Iran and Japan, both grown in temperate continental climates. Species verification between GASB (651 bp) and GA strain ATCC 44053 18s ribosomal RNA gene (632 bp) was performed using a plasmid matching software (ApE) as shown in Fig. 5. Only one base pair was mismatched at base pair 508 (GASB) and base pair 509 (GA ATCC44053). Moreover, only one unknown base pair was found between the two species, hence it was concluded that GASB has 99% similarities with GA strain ATCC 44053.

3.2. Analysis of wild-Serbian G. applanatum strain BGS6Ap growth curve

SLF was conducted using OFAAT technique to demonstrate the initial effect of temperature on biomass and ENS production prior to implementation of RSM. The variable of temperature was studied as a preliminary study because GASB is found at Mountain Kosmaj; the temperate continental climate is characterized by slightly warm summers and more humid while winters are very cold and windy (Obratov-Petković et al., 2006). The temperature on the mountain is usually below 20 °C, only occasionally reaching 27 °C (Obratov-Petković et al., 2006).

Fig. 6 illustrates, low temperatures between 20 °C successfully follows the traditional fungal growth pattern. Lag phase is observed from day 0 to day 2 indicating that the macrofungi is adapting to the surrounding environment. Day 3 to day 9 (3.9963 g/L ± 0.03) demonstrated exponential phase followed by stationary and death phase observed until day 17 (2.06037 g/L ± 0.07). Meanwhile, ENS production was slow for the first ten days because GASB mycelium was still adapting to the surrounding environment and is in the exponential phase (Wagner et al., 2003; Wan-Mohtar et al., 2016). Highest ENS (0.1237 g/L ± 0.03) was observed on day 13. After that, a decrease in ENS was observed until day 17.

Temperatures between 25 °C and 30 °C generate irregular growth patterns and has proven the least suitable for the growth of this temperate continental fungi GASB. At 25 °C, the fungi go through exponential phase from day 3 to day 9 and then through stationary phase from day 9 to day 13 before reaching its highest cell growth by day 15 (4.8437 g/L ± 0.07) moving into second-generation pellet growth. The biomass was slow growing until day 13 with comparatively lesser first-generation time interval compared to temperature 20 °C (day 17). ENS was observed highest on day 7 (0.0910 g/L ± 0.02). Fermentation process at 30 °C had irregular fungal growth curve pattern until day 17, the slowest to adapt to the environment because GASB was subjected to an environment outside of its range considering that the highest temperature from its locality is 27 °C. The highest cell growth was observed at day 15 (3.37013 g/L ± 0.06) before initial death phase on day 17. ENS (0.1960 g/L ± 0.02) were found to be highest on day 13 before declining.

In Fig. 6, there are two significant types of pellet growth known as 1st-generation and 2nd-generation during the exponential phase. The detachment of pellets occurs as described by Wan-Mohtar et al. (2016) and Wagner et al. (2004); ENS production is usually low when 1st-generation pellets formed but ENS production in 2nd-generation pellets is higher because they are formed from the 1st-generation pellets which are actively growing compared to newly inoculated mycelium that requires time to adapt and thrive in freshly cultured media. The growth curve analysis took far longer than others for this species and there were no nutrient replacements added to restore the depleted nutrient level. The unmodified slow-growing stage contributes to the growth of the 2nd-generation pellets by adhering to the pellet surface (Wan-Mohtar et al., 2016).

3.3. Optimisation of mycelial biomass production using RSM

Thirty distinct sets of culture conditions were used for optimisation in RSM (Table 2). The effects of temperature, agitation rate,

Table 3
Analysis of variance (ANOVA) experimental results for the actual responses using CCD quadratic model for mycelial biomass production of wild-Serbian *G. applanatum* strain BGS6Ap.

Source	Sum of squares	DF	Mean Square	F value	Prob > F	
Model	490.49	14	35.03	2.6317	0.0365	significant
A-Agitation	0.00	1	0.00	0.0001	0.9921	
B-Temperature	22.32	1	22.32	1.6768	0.2149	
C-pH	30.20	1	30.20	2.2687	0.1528	
D-Glucose	23.20	1	23.20	1.7428	0.2066	
AB	1.63	1	1.63	0.1223	0.7314	
AC	6.05	1	6.05	0.4541	0.5106	
AD	2.80	1	2.80	0.2102	0.6532	
BC	12.52	1	12.52	0.9401	0.3476	
BD	2.84	1	2.84	0.2132	0.6509	
CD	53.55	1	53.55	4.0223	0.0633	
A^2	24.11	1	24.11	1.8108	0.1984	
B^2	41.50	1	41.50	3.1174	0.0978	
C^2	0.26	1	0.26	0.0194	0.8912	
D^2	0.00	1	0.00	0.0002	0.9898	
Residual	199.69	15	13.31			
Lack of Fit	179.51	10	17.95	4.4483	0.0566	not significant
Pure Error	20.18	5	4.04			
Cor Total	690.18	29				
Std. Dev. =	**3.6486**	**R^2 =**		**0.7107**		
Mean =	**8.8707**	**Adjusted R^2 =**		**0.4406**		

(* Significant value).

initial pH, and glucose concentration on yield of biomass- ENS from GASB were evaluated for 20 days for each experimental design in triplicates.

The ANOVA for GASB mycelial biomass production is shown in Table 3. The model was significant as the value of "Prob > F″ was 0.0365 ($p < 0.05$). The predicted coefficient determination demonstrates that 71.07% ($R^2 = 0.7107$) of the variability in the response can be described using this model while the remaining variability cannot be explained. The adjusted coefficient determination value (Adj. $R^2 = 0.4406$) implies the significance of the model within the reasonable agreement with predicted R^2 value. Agitation (A), temperature (B), initial pH (C) and glucose (D) shows negative effect on the production of mycelia biomass followed by the quadratic terms (AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2) However, the three-dimensional plots (3D) (Fig. 7) showed the combined effect of quadratic terms (CD, B^2) had some positive effect towards the biomass yield. The model shows significant impact of biomass growth as temperature decrease below 25 °C. At this stage of mycelia growth, higher rpm was required to yield better biomass output. The maximum mycelial biomass was reached at glucose concentration (10 g/L), temperature 23.52 °C, 157.64 rpm, and pH 6. The regression model based on the actual factor of biomass can be expressed using Eq. (2).

$$\text{BIOMASS} = -141.93060 + 0.28538 \times \text{Agitation} + 8.34843 \times \text{Temperature} + 9.77150 \times \text{pH} + 0.36243 \times \text{Glucose} + 1.27609\text{E}-003 \times \text{Agitation} \times \text{Temperature} + 0.012294 \times \text{Agitation} \times \text{pH} - 4.18190\text{E}-004 \times \text{Agitation} \times \text{Glucose} - 0.17689 \times \text{Temperature} \times \text{pH} + 4.21190\text{E}-003 \times \text{Temperature} \times \text{Glucose} - 0.091470 \times \text{pH} \times \text{Glucose} - 1.22010\text{E}-003 \times \text{Agitation}^2 - 0.16009 \times \text{Temperature}^2 - 0.31540 \times \text{pH} - 7.35292\text{E}-005 \times \text{Glucose}^2 \quad (2)$$

3.4. Optimisation of endopolysaccharide production using RSM

The ANOVA for GASB-ENS production is shown in Table 4. The predicted coefficient determination demonstrates that 99.17% ($R^2 = 0.9917$) of the variability in the response can be described using this model. The model was significant ($p < 0.05$). The adjusted coefficient determination value (Adj. $R^2 = 0.9840$) implies the significance of model within the reasonable agreement with predicted R^2 value. Glucose (D) followed by the quadratic terms (AD, CD, A^2, B^2, C^2, D^2) shows the significantly strong effect ($p < 0.0001$) on the production of ENS (Table 4). A (agitation), C (pH) and quadratic terms (AB, AC) show a significant effect ($p < 0.05$) on ENS yield. However, the negative impacts were shown by temperature (B) and glucose (D) in addition to quadratic terms (BC, BD). The three-dimensional plots (3D) (Fig. 8) show that more combined quadratic terms have some positive effect on ENS production. The maximum mycelial biomass was reached at glucose concentration (10 g/L), temperature (25.25 °C), 150.99 rpm, pH 5.12. The model shows significant impact of biomass growth as the temperature increases approximately 25 °C. At this stage of mycelial growth, higher

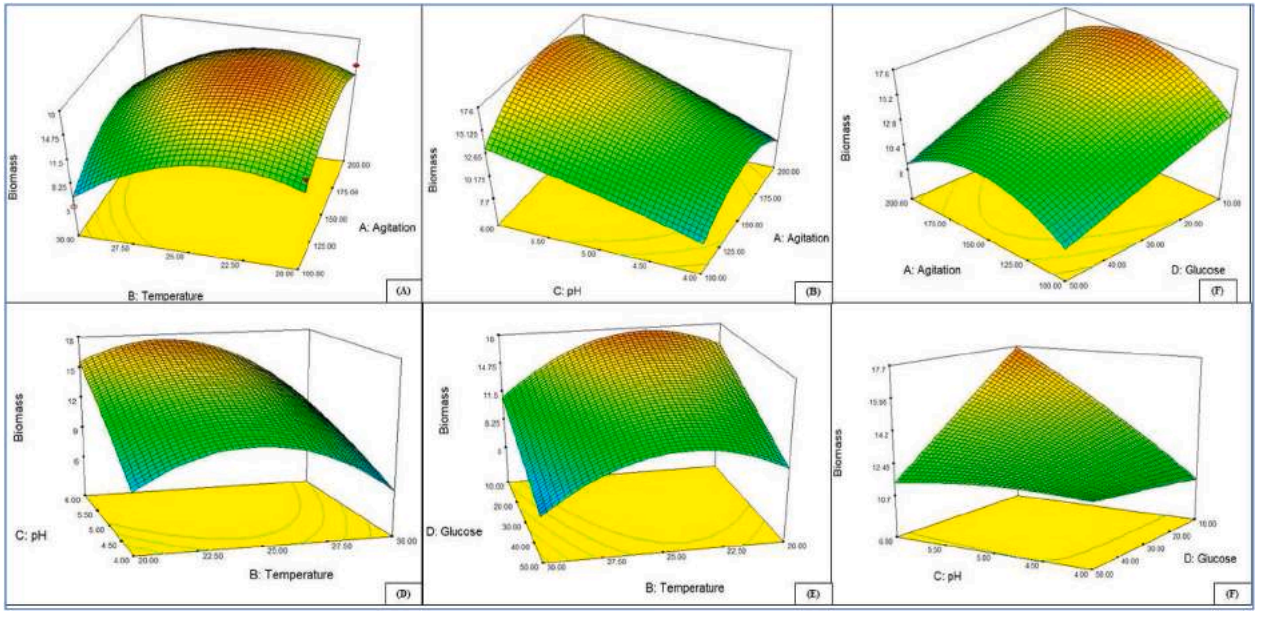


Fig. 7. Response surface profile (3D plot) of mycelial biomass production from wild-Serbian *G. applanatum* strain BGS6Ap displaying the interaction between (A) temperature and agitation, (B) agitation and pH, (C) glucose and agitation, (D) temperature and pH, (E) temperature and glucose and (F) glucose and pH.

Table 4
Analysis of variance (ANOVA) experimental results for the actual responses using CCD quadratic model for ENS production of wild-Serbian *G. applanatum* strain BGS6Ap.

Source	Sum of squares	DF	Mean Square	F value	Prob > F	
Model	25.31	14	1.81	128.279	< 0.0001	significant
A-Agitation	0.20	1	0.20	14.009	0.0020*	
B-Temperature	0.05	1	0.05	3.806	0.0700	
C-pH	0.07	1	0.07	5.303	0.0360*	
D-Glucose	1.57	1	1.57	111.047	< 0.0001*	
AB	0.19	1	0.19	13.149	0.0025*	
AC	0.06	1	0.06	4.375	0.0539*	
AD	0.31	1	0.31	21.683	0.0003*	
BC	0.02	1	0.02	1.153	0.2999	
BD	0.05	1	0.05	3.819	0.0696	
CD	0.60	1	0.60	42.374	< 0.0001*	
A^2	2.24	1	2.24	158.888	< 0.0001*	
B^2	2.94	1	2.94	208.380	< 0.0001*	
C^2	2.98	1	2.98	211.129	< 0.0001*	
D^2	20.07	1	20.07	1423.941	< 0.0001*	
Residual	0.21	15	0.01			
Lack of Fit	0.19	10	0.02	4.366	0.0587	not significant
Pure Error	0.02	5	0.00			
Cor Total	25.53	29				
Std. Dev. =	**0.1187**	**R^2 =**		**0.9917**		
Mean =	**1.0585**	**Adjusted R^2 =**		**0.9840**		

(* Significant value).

rpm is required to yield better ENS output. The regression model based on the actual factor of ENS can be expressed using Eq. (3).

$$\text{ENS} = -58.11283 + 0.11817 \times \text{Agitation} + 2.25422 \times \text{Temperature} + 11.04462 \times \text{pH} - 0.34870 \times \text{Glucose} - 4.30517\text{E}-004 \times \text{Agitation} \times \text{Temperature} + 1.24167\text{E}-003 \times \text{Agitation} \times \text{pH} - 1.38208\text{E}-004 \times \text{Agitation} \times \text{Glucose} - 6.37417\text{E}-003 \times \text{Temperature} \times \text{pH} - 5.80042\text{E}-004 \times \text{Temperature} \times \text{Glucose} - 9.66042\text{E}-003 \times \text{pH} \times \text{Glucose} - 3.71890\text{E}-004 \times \text{Agitation}^2 - 0.042589 \times \text{Temperature}^2 - 1.07173 \times \text{pH}^2 + 6.95818\text{E}-003 \times \text{Glucose}^2 \quad (3)$$

3.5. Verification of optimised conditions

The effectiveness of the model was verified by comparing and measuring the predicted values of biomass and ENS under statistically optimal conditions (Table 5). Validation experiments were conducted using 250 mL shake flasks and in triplicate under controlled conditions. The predictive ability of the model was verified using Eq. (2) and Eq. (3). The predicted values for mycelial biomass and ENS production were 17.78 g/L and 4.41 g/L, respectively, which were in-line with the experimental values of 17.66 g/L and 4.33 g/L. The difference between the respective values were 12% and 8%; therefore, the validity of the model can be justified as the average deviation of less than 15% (Milkey et al., 2014).

3.6. Characteristic of endo-β-glucan from the mycelium of wild-Serbian G. applanatum strain BGS6Ap using fourier-transform infrared spectroscopy (FTIR) and ^1H Nuclear magnetic resonance (NMR) spectroscopy

FTIR spectroscopy in Fig. 9B depicted spectral characteristics classifying the organic group of endo-β-D-glucan from GASB in comparison to the standard glucan laminarin (Fig. 9A). The endo-β-D-glucan displays absorptions of polysaccharides from 3000 cm^{-1} to 4500 cm^{-1} at a broad-stretched peak suggesting –OH group in the vibration-induced sugar residue. The low peak was observed at 2924 cm^{-1} and 2925 cm^{-1} which was related to the stretching C–H vibrations in the sugar ring. Meanwhile, C = O vibration between 1625 cm^{-1}–1640 cm^{-1} was demonstrated. The absorbance at 1073 cm^{-1}–1077 cm^{-1} indicates the presence of C–O–C and –OH suggesting the presence of β-glucan in the formation of a pyranose structure with the presence of 5 carbon atoms and two sets of double bonding with one oxygen molecule (Liu et al., 2014; Osinska-Jaroszuk et al., 2014). Absorbance at 892 cm^{-1} and 890 cm^{-1} verified the β-glucan linkages at 'anomeric region' (950 –700 cm^{-1}) (Osinska-Jaroszuk et al., 2014; Wan-Mohtar et al., 2016). Thus, these structural confirmations mimic the stretching vibration of bands that ensures the effectively synthesized presence of β-glucan from GASB.

The existence of β-glucans in glucose monomers is further confirmed by ^1H NMR spectroscopy (Lundquist et al., 1992). Proton ^1H NMR spectra represents broad fingerprint profiling of biomolecules (Pomin, 2012). GASB-ENS ^1H NMR spectroscopic analysis was performed at 25 °C in d_2O solvent as shown in Fig. 10 verifying the existence and structure of endo-β-D-glucan against the laminarin

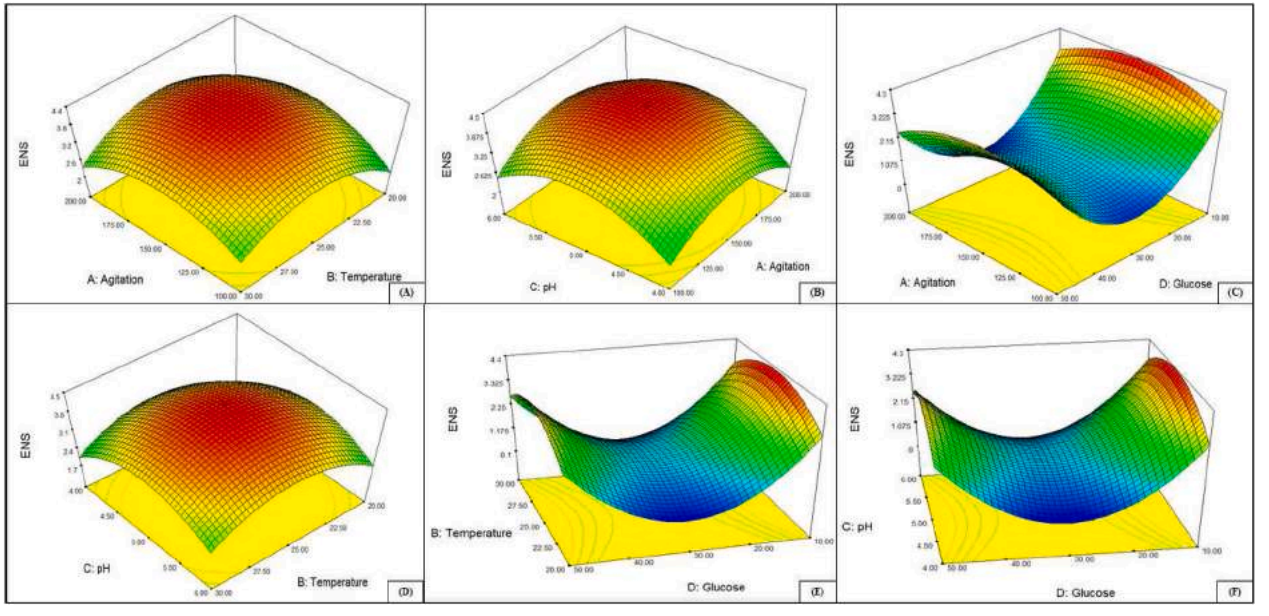


Fig. 8. Response surface profile (3D plot) of ENS production from wild-Serbian *G. applanatum* strain BGS6Ap displaying the interaction between (A) temperature and agitation, (B) agitation and pH, (C) glucose and agitation, (D) temperature and pH, (E) temperature and glucose and (F) glucose and pH.

Table 5
Validation of model using verified conditions.

Run	Variables				Responses	
	Temperature (°C)	pH	Glucose (g/L)	Agitation (rpm)	Biomass (g/L)	ENS (g/L)
Biomass	24	6	10	158	17.66	–
ENS	25	5.12	10	151	–	4.33
Biomass + ENS	24	5.55	10	154	16.24	4.10

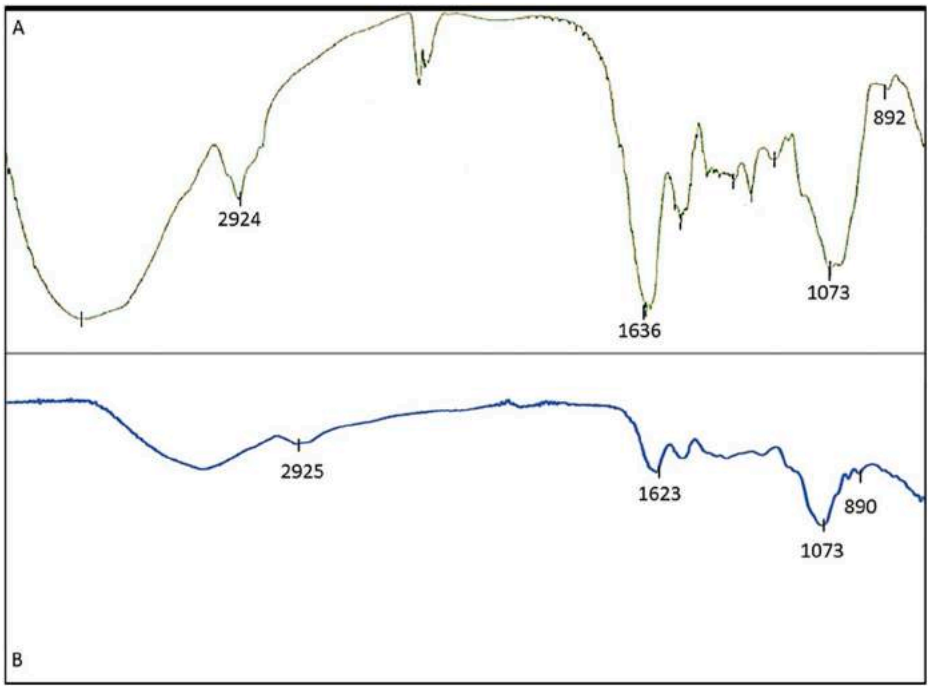


Fig. 9. Comparison of β-glucan FTIR spectra. A: glucan standard from laminarin and B: endo-β-D-glucan derived from batch fermentation of wild-Serbian *G. applanatum* strain BGS6Ap.

standard (β-1,3-D-glucan). Former findings have reported the chemical shift of β-D-glucan in ^{1}H NMR spectra is in the 'anomeric region' between 4.0 and 6.0 ppm with β-anomeric protons appear in the range of 4.0–5.0 ppm (Hu et al., 2016). In the ^{1}H NMR spectra of GABS-ENS (Fig. 10), as compared to the similar pattern of ^{1}H NMR standard laminarin, the most down-field signal at 5.20 ppm indicated as OH-2. Meanwhile, the peak appeared up-field at 4.60 ppm and 4.10 ppm has been assigned as OH-4 and OH-6, respectively. The current work is comparable to previous work by Wan-Mohtar et al. (2016b), which analysed laminarin and sulfated glucan of *Ganoderma lucidum* at signal 5.08 ppm (OH-2), 4.50 ppm (OH-4) and 4.40 ppm (OH-6). Hence, the β-type spectrum in Fig. 10(1) and (2) indicates the presence of glycosidic bonds in ENS as verified by Liu et al. (2014) and Supramani et al. (2019).

3.7. Comparison of current study with literature

A comparison for biomass and ENS production of *Ganoderma* sp. in literatures is shown in Table 6 as well as three studies including the current study that utilised RSM as an optimisation tool. Based on Table 6, lower glucose concentration and temperature provide better mycelial biomass and ENS production compared to its counterpart wild-Serbian *G. lucidum* (GLSB) found on Avala Mountain in Belgrade (Hassan et al., 2019). The first conducted studies were the Taguchi orthogonal array (TOA) and the orthogonal matrix (OM), however, all previous research showed only the optimisation of *G. lucidum* (GL) with different strains and no mycelium optimisation study for GA (Chang et al., 2006; Yuan et al., 2012). The maximum biomass obtained by GASB was relatively high in comparison to other *Ganoderma* spp. Serbia and China are both countries that belong to the temperate continental climate zone, so a similar amount of

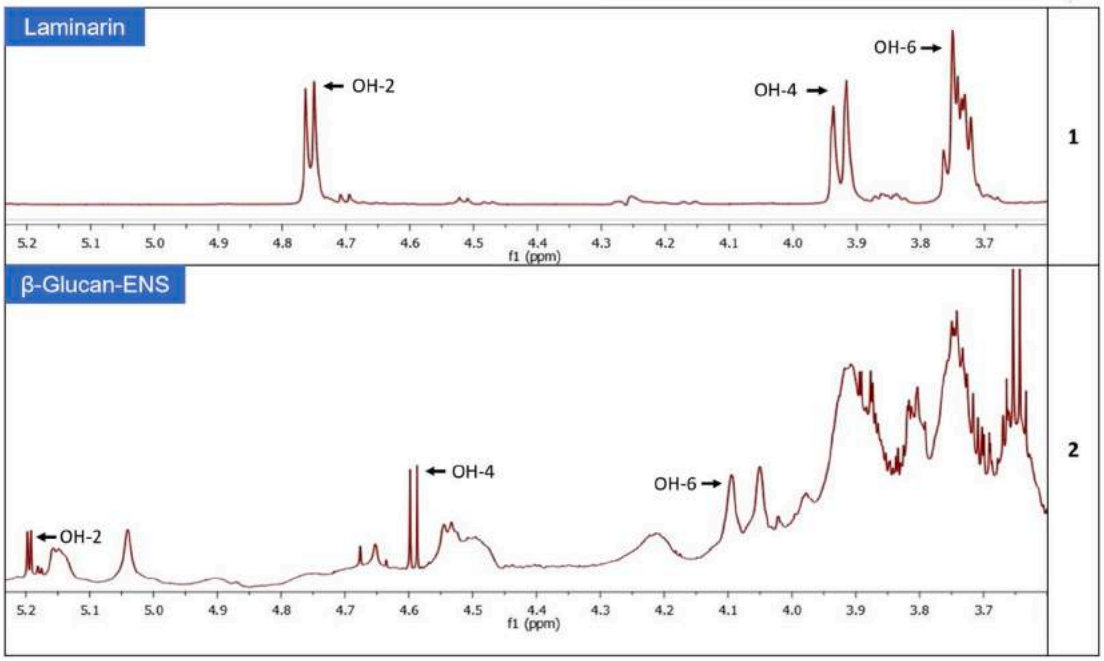


Fig. 10. Comparison of β-glucan ^{1}H NMR spectra of endo-β-D-glucan derived from batch cultures of wild-Serbian *G. applanatum* strain BGS6Ap (GASB) and *Laminaria digitata* standard in D_20-d_6.

biomass is emerging (Chang et al., 2006; Rašeta et al., 2016; Stojković et al., 2014). It has been proven that temperature plays a significant role in the production of biomass, but the technique used was Taguchi's orthogonal array which requires many experiments and takes a long time compared to RSM. Chang et al. (2006) verified that Taguchi's is less favourable as it requires prior knowledge before experimenting as a two-factor interaction system (Stone et al., 1994).

Orthogonal matrix is the method used to optimise media conditions using a set of experimental values and responses obtained from multiple OFAAT studies to find suitable range (Yuan et al., 2012). Yuan et al. (2012) and Chang et al. (2006) stated that biomass and polysaccharide production were remarkably improved by cultivation of mycelium in optimal media with subsequent optimal operating conditions. In Table 6, the GLSB followed the optimal temperature condition using 20 °C, 25 °C and 30 °C variables despite being the same locality with the current GASB probably due to the inability of *G. applanatum* to acclimatise in warmer conditions (>25 °C) compared to more resistant *G. lucidum* (>30 °C, 50 g/L glucose) (Hassan et al., 2019). Studies showed that the annual percentage of moisture content in Malaysia is 80% and in Serbia moisture content is 70% but the major difference is that water activity in the surrounding is higher in Malaysia and relatively low in Serbia (Peng et al., 2006; Pukhkal et al., 2015). The humidity and precipitation in the surrounding environment could have caused major increase in the production of biomass-ENS. The optimised key factors stated in this research, are the most recent using RSM on glucose concentration, agitation rate, pH, and temperature in improvement of biomass and ENS production, using mycelium in SLF. These optimised conditions could be employed to achieve high biomass and ENS when upscaled and served as a blueprint for fungal bioreactor fermentation for temperate continental climate mushrooms.

4. Conclusion

In conclusion, this research focused on the molecular and morphological identification of the wild-Serbian *G. applanatum* strain BGS6Ap, in addition to optimizing the cultivation condition for elevated biomass and ENS production in SLF. RSM was used to optimise media conditions to produce biomass-ENS based on the growth curve study, whereby the optimum growth conditions of the quadratic model provided maximum output of biomass-ENS at initial pH 6, 10 g/L of glucose concentration, agitation rate 153 rpm and temperature 24 °C. Utilizing RSM to optimise GASB's media composition resulted in overall biomass 19.93 g/L and ENS 4.33 g/L under optimised conditions. Clearly, temperature was the key factor affecting temperate GASB production of biomass and ENS relative to initial pH, agitation levels and glucose concentration. Lastly, the extracted ENS through batch fermentation of GASB mycelium was verified as endo-β-D-glucan via FTIR and ^{1}H NMR spectroscopy. Throughout this study, GASB showed adaptability and resilience to the RSM approach for a sustainable biomass and ENS production, hence RSM approach could be used in extended fermentation studies.

Table 6
Comparison of *Ganoderma* sp. statistical optimisation using shake flask fermentation with the literature.

Display	Species	Origin	SO	Initial pH	Glucose concentration (g/L)	Temperature (°C)	Agitation (rpm)	Biomass (g/L)	ENS (g/L)	Reference
	*G. applanatum* strain BGS6Ap	Serbia	RSM	6	10	24	153	17.51	3.47	*This study*
	*G. lucidum* strain BGF4A1	Serbia	RSM	5.26	50	30	–	3.12	–	Hassan et al. (2019)
	*G. lucidum* strain QRS 5120	Malaysia	RSM	4	26.5	–	100	5.19	1.52	Supramani et al. (2019)
NA	*G. lucidum*	Taiwan	TOA	6.5	12.1	34	160	18.70	–	Chang et al. (2006)
NA	*G. lucidum* strain CAU 5501	China	OM	–	50	30	150	7.24	–	Yuan et al. (2012)

*NA- Not Available *SO-Statistical Optimisation *TOA- Taguchi Orthogonal Array *OM- Orthogonal Matrix.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2021.102166.

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