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MYCELIAL GROWTH KINETICS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF A SUBMERGED CULTURE OF THE MEDICINAL FUNGI GANODERMA RESINACEUM GA1M ISOLATED FROM BULGARIA

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ABSTRACT

The aim of this study was the evaluation of the basic bioprocess parameters by applying mathematical modelling of the growth kinetics of *Ganoderma resinaceum* GA1M isolated from Bulgaria as well as assessment of the antioxidant and antimicrobial activity of the obtained biomass. The models of Monod, Moser, Andrews and Verhulst in combination with the Luedeking–Piret were applied for kinetics modelling of the cultivation process. The *in vitro* antioxidant activity was evaluated by applying four different methods. The disk diffusion method and broth microdilution methods were employed to determine the antimicrobial activity of the mycelium biomass extracts. Mathematical modeling of the kinetics of the cultivation process was performed and basic bioprocess parameters were determined. The obtained experimental data were in agreement with the applied mathematical models. The water extract of the mycelium biomass showed the highest antioxidant potential and the highest total phenolic content. The methylene chloride, methanol and ethanol extracts of the biomass demonstrated antimicrobial activity.

Keywords: Growth kinetics, *Ganoderma resinaceum*, medicinal fungi, antioxidant activity, antimicrobial activity

INTRODUCTION

Mushrooms of the genus *Ganoderma* Karst. (*Ganodermataceae, Basidiomycota*) are well-known ecologically important and pathogenic white-rot wood decay bracket macrofungi (**Baby *et al.*, 2015; Jargalmaa *et al.*, 2017**). Their natural habitat is the tropical and warm temperate regions where they grow on dead or living trees at injury sites causing white rot and forming hard fruiting bodies (**Ritcher *et al.*, 2015; Seo *et al.*, 2000**). About 400 species from the genus *Ganoderma* have been described worldwide and most of them were located in the tropics (**Jargalmaa *et al.*, 2017; Ritcher *et al.*, 2015**). Only seven *Ganoderma* species have been reported in Central Europe - *Ganoderma applanatum, G. lipsience, G. adspersum, G. resinaceum, G. pfeifferi, G. lucidum, G. carnosum* (**Kotlaba *et al.*, 1997; Sokol, 2000; Bernicchia, 2005; Ryvarden and Melo, 2014; Beck *et al.*, 2020**). The described *Ganoderma* species in Bulgaria are *G. adspersum, G. applanatum, G. lucidum, G. pfeifferi* and *G. resinaceum* and are usually found on deciduous trees in lowlands, most often near rivers (**Stoychev and Naydenov, 1984**).

Due to their thick, corky and tough fruiting bodies lacking fleshy texture characteristics the representatives of *Ganoderma* are generally not referred to as edible mushrooms (**Jonathan *et al.*, 2008; Jong and Birmingham, 1992**). Over the past two millennia, they have been recognized as medicinal mushrooms and used mostly in the formulation of nutraceuticals and functional foods to promote longevity and treat a variety of human illnesses (**Saltarelli *et al.*, 2009; Liang *et al.*, 2019; Peterson, 2006; Chen *et al.*, 2012; Postemsky *et al.*, 2014; 2016**). The phytochemical characterizations of different *Ganoderma* species reveal their potential to produce more than 400 bioactive metabolites including polysaccharides (**Nie *et al.*, 2013; Sudheer *et al.*, 2019**), triterpenoids, sterols, ergosterol (**Baby *et al.*, 2015**), enzymes (**Kumakura *et al.*, 2019; Yang *et al.*, 2019**), proteins (**Xu *et al.*, 2011; Lin *et al.*, 2011**), phenolic compounds (**Sudheer *et al.*, 2019; Kumari *et al.*, 2016**), amino acids (**Zhang *et al.*, 2018**), lipids and fatty acids (**Hsu and Cheng, 2018**), vitamins and minerals (**Yang et al., 2019; Ahmad, 2018**). They have beneficial properties for the prevention and treatment of a variety of socially important diseases such as hypertension, diabetes, hepatitis, cancers, and AIDS (**Baby *et al.*, 2015; Paterson, 2006; Shi *et al.*, 2020; Mothana *et al.*, 2000**).

Ganoderma resinaceum Boud. belongs to the white-rot fungi (*Basidiomycota, Polyporales*) and is most commonly found as a saprophyte on wood trunks, but also as a parasite on dying trees, mainly in forests in the parts of Europe with warmer climate (**Náplavová *et al.*, 2020**). The species has more or less global distribution from the tropics to the southern part of the temperate zone. In Bulgaria, this fungus grows most often on deciduous trees and in rare cases on conifers. This species from the genus *Ganoderma* has been used in traditional medicine in Asia and some parts of West Africa due to its numerous therapeutic effects, such as improving lung, kidney, spleen, and stomach function, and it is also used as a sedative for nerves (**Oke *et al.*, 2022; Náplavová *et al.*, 2020; Chen *et al.*, 2017; Oyeteyo, 2011**). Different mushroom species within a genus usually produce different chemical compounds (**Loyd *et al.*, 2018**). For example, the results of **Chen *et al.* (2017)** showed that *G. lucidum* and *G. resinaceum* may appear similar in respect of the fruiting body and spore morphology but there were differences in the polysaccharide and triterpenoid content. The concentration of polysaccharides in *G. resinaceum* was lower in comparison with *G. lucidum*, while its triterpenoid content was higher. Since the research interest has been focused mainly on the *G. lucidum* complex (**Papp *et al.*, 2017**), there is still scarce scientifical information about the cultivation technics of *G. resinaceum* and its bioactivity potential. Currently, *G. resinaceum* is considered promising for experimental cultivation and bioactive metabolite profiling because this species is quite widespread in the warm parts of Central Europe (**Bleha *et al.*, 2022**). Despite the efforts for developing a continuous cultivation process, currently, the industry relies mainly on batch and fed-batch cultivation techniques. The mathematical modelling of such processes provides scientists with adequate and useful information regarding the optimal process parameters. In the papers discussing the mathematical modelling of the mycelium growth kinetics, various models are applied, for example, Monod, Couteis, Moser, Hands-Woolf etc. (**Benkortbi *et al.*, 2007, Olorunnisola *et al.*, 2008**).

In the current study, *Ganoderma resinaceum* GA1M isolated from Bulgaria was grown by submerged cultivation for mycelium biomass obtaining. The models of Monod, Moser, Andrews and Verhulst (model of a logistic curve) in combination with the Luedeking–Piret model were applied for kinetics modelling of the cultivation process. The antioxidant and antimicrobial activity of different biomass extracts were investigated and the potential of this locally isolated medicinal mushroom to be used in the formulation of nutraceuticals was revealed.

MATERIAL AND METHODS

Mushroom

The macrofungal strain *Ganoderma resinaceum* GA1M is a part of the fungal collection of the Department of Biotechnology, University of Food Technology,

Plovdiv, Bulgaria. The strain was maintained at 4 °C on Mushroom Complete medium (MCM), containing g/L: glucose 20.0, KH_2PO_4 0.5, K_2HPO_4 1.0, $MgSO_4$ 0.5, peptone 2.0, yeast extract 2.0, Agar 2.0, pH 4.8-5.2 and subcultured every 30 days.

Submerged cultivation of *Ganoderma resinaceum* GA1M

For biomass obtaining *G. resinaceum* was cultivated under submerged conditions in MCM. Each 500 mL Erlenmeyer flask contained 100 mL of the medium and was inoculated with a suspension of vegetative cells from 7-day-old culture. The cultivation took place on a rotary shaker at 220 rpm, 28 °C and continued for 7 days. Filtration was used for the separation of the biomass from the broth. The mycelium biomass was washed with distilled water, lyophilized and ground. The cultural broth was subject to EPS isolation.

Isolation of exopolysaccharides

The isolation of the exopolysaccharides was performed by mixing the filtrate with ethanol in a 1:4 ratio followed by cooling at 4°C overnight (**Long *et al.*, 2021**). The recovery of the precipitated EPS was made via centrifugation at 6000 rpm for 20 min at 4°C. The obtained crude EPS was dried in a laboratory dryer at 30°C for 12 h and was expressed as grams per litre.

Determination of glucose concertation

The determination of the glucose concentration was conducted according to the PAHBAH method, described by **Lever (1993)**, which is based on the reaction of the glucose with the p-hydroxybenzhidrazine in alkaline conditions where ozazones are formed with an absorption maximum at 410 nm. The reaction mixture contained 250 µL cultural broth and 750 µL PAHBAH reagent. The reagent should be freshly prepared by mixing 9 parts 0.5M NaOH and 1 part 5% p-hydroxybenzhidrazine in 0.5M HCl. The analysis was performed in 1.5 mL tubes which were incubated at 100°C for 5 min. After the reaction time was over the tubes were cooled to room temperature and the absorption at 410 nm was measured. The glucose concertation was determined with a standard curve.

Modelling of the process kinetics

For the kinetics modelling of the cultivation process the models of Monod, Moser, Andrews and Verhulst (model of a logistic curve) in combination with the Luedeking–Piret model, and the following systems of differential equations were used (**Xu *et al.*, 2011; Zhong and Tang, 2004; Carmen *et al.*, 2022; Olorunnisola *et al.*, 2008**):

Monod model

$$\begin{cases} \frac{dX}{d\tau} = \mu_{max}\frac{S}{K_{sx}+S}\cdot X \\ \frac{dP}{d\tau} = q_{pmax}\frac{S}{K_{sp}+S}\cdot X \\ \frac{dS}{d\tau} = -\frac{1}{Y_{x/s}}\mu_{max}\frac{S}{K_{sx}+S}\cdot X - \frac{1}{Y_{p/s}}q_{pmax}\frac{S}{K_{sp}+S}\cdot X \end{cases}$$

Moser model

$$\begin{cases} \frac{dX}{d\tau} = \mu_{max}\frac{S^n}{K_{sx}+S^n}\cdot X \\ \frac{dP}{d\tau} = q_{pmax}\frac{S^n}{K_{sp}+S^n}\cdot X \\ \frac{dS}{d\tau} = -\frac{1}{Y_{x/s}}\mu_{max}\frac{S^n}{K_{sx}+S^n}\cdot X - \frac{1}{Y_{p/s}}q_{pmax}\frac{S^n}{K_{sp}+S^n}\cdot X \end{cases}$$

Andrews model

$$\begin{cases} \frac{dX}{d\tau} = \mu_{max}\frac{S}{K_{sx}+S+\frac{S^2}{K_{sxi}}}\cdot X \\ \frac{dP}{d\tau} = q_{max}\frac{S}{K_{sp}+S+\frac{S^2}{K_{spi}}}\cdot X \\ \frac{dS}{d\tau} = -\frac{1}{Y_{x/s}}\mu_{max}\frac{S}{K_{sx}+S+\frac{S^2}{K_{sxi}}}\cdot X - \frac{1}{Y_{p/s}}q_{max}\frac{S}{K_{sp}+S+\frac{S^2}{K_{spi}}}\cdot X \end{cases}$$

Verhulst (model of a logistic curve) in combination with the Luedeking –Piret model

$$\begin{cases} \frac{dX}{d\tau} = [\mu_m - \beta X]X \\ \frac{dP}{d\tau} = KX + q_{p0}\frac{dX}{d\tau} \\ -\frac{dS}{d\tau} = \gamma\frac{dX}{d\tau} + \delta X \end{cases} \quad \begin{matrix} X = \frac{X_H e^{\mu_{max}(t-t_{lag})}}{1-\frac{X_H}{X_{KP}}(1-e^{\mu_{max}(t-t_{lag})})} \\ P = P_0 + K(X-X_H) + q_{p0}\left(\frac{X_K}{\mu_m}\right)\ln\left(1-\frac{X_H}{X_K}(1-e^{\mu_m\tau})\right) \\ S = S_0 - \gamma X_0\left[\frac{e^{\mu_m\tau}}{1-\frac{X_0}{X_m}(1-e^{\mu_m\tau})}-1\right] - \delta\frac{X_m}{\mu_m}\ln\left[1-\frac{X_0}{X_m}(1-e^{\mu_m\tau})\right] \end{matrix}$$

where: μ_{max} – maximum specific growth rate, h^{-1}; q_{pmax} – the maximum specific rate of product biosynthesis, h^{-1}; K_{sx} – Monod saturation constant for the biomass equal to that substrate concentration where $\mu=0{,}5.\mu_{max}$, g/dm^3; K_{sxi} – substrate inhibition constant for the biomass, g/dm^3; K_{sp} – Monod saturation constant for the product, equal to that substrate concentration where $q_p=0{,}5.q_{pmax}$, g/dm^3; K_{spi} – substrate inhibition constant for the product, g/dm^3; n – parameter, demonstrating the change in the curve`s form through time; $1/Y_{x/s}$ и $1/Y_{p/s}$ – metabolite (trophic) coefficients; X,P,S – current concentration of biomass, product, and substrate, g/dm^3; $X_H(X_0)$ and $X_{кр}(X_к,X_m)$ – initial and final concertation of biomass, g/dm^3; β – intrapopulation competition coefficient, $dm^3/(g.h)$; K – constant, describing the product yield from the biomass in the stationary phase, g/(g.h); q_{p0} – constant, describing the product yield from the biomass in the exponential phase, g/(g.h); δ– substrate uptake constant per unit biomass in the stationary phase, g/(g.h); γ – substrate uptake constant by growing biomass and substrate required for maintaining the vitality of the biomass, g/(g.h), τ_{lag} – duration of the lag phase, h; τ – cultivation duration, h.

The differential equation systems of the Monod, Moser and Andrews models were solved through the Runge-Kutta of 4th order method and the identification of the parameters was done by minimizing the squared differences between the experimental and model data using Microsoft Excel 2019 (**Choi *et al.*, 2014; Kemmer and Keller, 2010**). The parameters in the model of Verhulst (model of a logistic curve) in combination with the Luedeking–Piret model were identified using nonlinear regression in CurveExpert Professional.

Preparation of mycelium biomass extracts

For the determination of total phenolic content (TPC) and antioxidant activity water, ethanol and methanol extracts of the biomass were used. The extracts were prepared as follows: 30 mL of each solvent (distilled water, 80% ethanol, or methanol) were added to previously weighed (1.5 ± 0.05 g) biomass. The samples were then placed in a laboratory shaker at 150 rpm for 24 h at 25 °C followed by centrifugation at 6000 rpm and 4 °C for 15 min. The supernatants were collected and stored at -18 °C and another portion of solvent was added to the residue for second extraction at the same conditions. The extraction procedure was performed in triplicate and the obtained extracts of each solvent were joined together and stored at -18 °C.

For antimicrobial activity determination, the biomass extracts were prepared as follows: The biomass (0.5 ± 0.05 g) was mixed with 20 mL of each solvent used (methylene chloride, methanol, ethyl acetate, ethanol, butanol, and water). The samples were then placed in a laboratory shaker at 150 rpm for 24 h at 25 °C followed by centrifugation at 6000 rpm and 4 °C for 15 min. The supernatants were collected and stored at -18 °C and another portion of each solvent was added to the biomass for second extraction at the same conditions, followed by centrifugation and collection of the extracts. This extraction procedure was performed in triplicate. The extracts were combined and evaporated under vacuum at 40 °C until they were completely dry. Dimethyl sulfoxide (DMSO) was used as a solvent for the dissolution of the dry extracts to a final concentration of 10 mg DW/mL.

Determination of TPC

The analysis of TPC was performed according to the method, described by **Kujala *et al.* (2000)** with minor modifications. Each reaction mixture contained Folin-Ciocalteu reagent (0.5 mL), 7.5% Na_2CO_3 (0.4 mL), and 0.1 mL of the obtained extract. After vortexing, the mixture was incubated at 50 °C for 5 min followed by measurement of the absorbance at 765 nm. The TPC was expressed as mg gallic acid equivalents (GAEs) per gram dry weight (g DW).

Determination of *in vitro* antioxidant activity

DPPH˙ Radical Scavenging Assay

The method of **Brand-Williams *et al.* (1995)** with slight modifications described by **Mihaylova *et al.* (2014)** was applied for the determination of the hydrogen donation ability and scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals by the extracts. The DPPH solution with concentration $4x10^{-4}$ M was freshly prepared in methanol, mixed with each extract separately in a 2:0.5 (v/v) ratio and after 30 min incubation, the absorbance was monitored at 517 nm. The DPPH radical scavenging activity was presented as a function of the Trolox concentration with equivalent antioxidant activity – Trolox equivalent antioxidant capacity (TEAC) and expressed as µM TE/g DW.

ABTS$^{\bullet+}$ Radical Scavenging Assay

The method of **Re *et al.* (1999)** was used for the determination of the radical scavenging activity of the extracts against 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$). The mixture of 7 mM stock ABTS solution with 2.45 mM $K_2S_2O_8$ (final concentration) was incubated in darkness and room temperature for 12-16 h in order for the ABTS radical cation (ABTS$^{\bullet+}$) to be formed. The final absorbance of 0.7 ± 0.02 at 734 nm was reached after diluting the ABTS$^{\bullet+}$ solution with ethanol. This diluted solution (1 mL) was added to the samples (0.01 mL) and the absorbance at 734 nm was recorded after 6 min incubation at 30°C. The results are expressed as the TEAC value (μM TE/g DW).

Ferric-Reducing Antioxidant Power (FRAP) Assay

The slightly modified procedure of **Benzie and Strain (1999)** was performed for establishing the ferric-reducing antioxidant power of the obtained extracts. The FRAP reagent was daily prepared and warmed up to 37 °C. The reaction mixture contained 2.85 mL of the reagent with 0.15 mL of each sample and was incubated for 4 min at 37°C. The absorbance was measured at 593 nm and the results were expressed as μM TE/g DW.

Cupric Ion-Reducing Antioxidant Capacity (CUPRAC) Assay

For the CUPRAC assay, 1 mL of 10 mM $CuCl_2$ solution was mixed with 1 mL of 7.5 mM methanolic $C_{14}H_{12}N_2$ solution, 1 mL ammonium acetate buffer (1 M) with pH 7.0, and 0.1 mL of the samples. The mixture was taken to a final volume of 4.1 mL with distilled water and mixed well. After 30 min incubation, the absorbance was measured at 450 nm against a reagent blank (**Apak *et al.*, 2004**). Trolox was used as standard and the results were expressed as μM TE/g DW.

Determination of antimicrobial activity

The following microbial strains were used for the determination of the antimicrobial activity of the obtained mycelium extracts: *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 19433, *Salmonella enterica* ssp. *enterica* ser. *enetritidis* ATCC 13076, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 8787, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 13883, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633. The strain *Proteus vulgaris* G was provided by the microbial collection of the Department of Microbiology at the University of Food Technologies, Plovdiv. The strains were grown on LBG agar with the following composition (g/L): peptone from casein – 10.0; yeast extract – 5.0; glucose – 10.0; NaCl – 10.0; agar – 15.0, and pH prior sterilization 7.0.

Disk diffusion method

The extracts were screened for antimicrobial activity according to the **CLSI method (2012)**. Microbial suspensions with concentration of 1.10^8 CFU/mL were prepared for each test-strain using saline solution. Sterile paper disks (d=6mm), soaked with tested extract were placed on the surface of Mueller Hinton agar (Merck), and inoculated with the test microorganisms. The Petri dishes were incubated at 30°C for 24 h for the bacilli and at 37°C for 18 h for the rest of the test-microorganisms. Then the diameter of the inhibition zones was measured.

Broth microdilution method

The minimum inhibitory concentration for each extract was evaluated according to the **CLSI method (2012)**. The extract was subjected to serial two-fold dilutions in Mueller-Hinton broth (Merck) using a 96-well microtitration plate. Then, each well was inoculated with a microbial suspension with concentration of 5.10^5 CFU/mL. After mixing, the plates were incubated at 30°C for 24 h for the bacilli and at 37°C for 18 h for the rest of the test microorganisms. The MIC is the lowest concentration of extract which completely inhibited the growth of the test-microorganism**.**

Statistical analysis

All the experiments were conducted in triplicate and the values were expressed as mean ± SD. Statistical significance was detected by analysis of variance (ANOVA, Tukey's test; the value of $p < 0.05$ indicated a statistical difference

RESULTS AND DISCUSSION

Submerged cultivation

The fungus *Ganoderma resinaceum* GA1M used in this research was collected from a forest near Maritza River, Bulgaria in May 2019, molecularly identified, and deposited in the GenBank under accession number MW996753 (**Angelova *et al.*, 2022**). The strain was subject to submerged cultivation where the dynamics of the changes in the biomass, glucose, and exopolysaccharide (EPS) concentrations during the cultivation process were monitored, showing the effect of the cultivation time on the strain growth. The results are presented in table 1. The biomass concentration increased constantly during the process reaching a final value of 9.43 g/L at 312 h. The same increase was also registered regarding the exopolysaccharide concentration reaching 0.91 g/L and remaining constant until the end of the cultivation.

Table 1 Changes in the concentrations of biomass, glucose, and exopolysaccharide during the cultivation

Time, h	Glucose, g/L	Biomass, g/L	EPS, g/L
72	16.30 ± 0.75	2.55 ± 0.11	0
120	14.10 ± 0.49	2.91 ± 0.19	0.17 ± 0.03
168	10.81 ± 0.56	3.29 ± 0.19	0.45 ± 0.09
216	8.40 ± 0.42	6.58 ± 0.59	0.52 ± 0.11
264	6.39 ± 0.29	8.43 ± 0.73	0.71 ± 0.13
312	3.81 ± 0.13	9.43 ± 0.83	0.91 ± 0.21
360	1.20 ± 0.09	9.42 ± 0.82	0.91 ± 0.19

The substrate concentration decreased constantly during the cultivation and at the end of the process, the glucose was almost completely utilized by the strain proven by the low residual concentration in combination with the limited pH variation during the cultivation. The results obtained during the cultivation process were used for modelling the kinetics of the cultivation.

Modelling of the kinetics of the cultivation process

The up-scaling of a biotechnological process from laboratory to industrial scale as well as its management could not be accomplished without an understanding of the process kinetics. For this reason, mathematical modelling of the kinetics of the researched cultivation process was performed and the basic bioprocess parameters were determined. The obtained experimental data were compared to the model-generated data and the results are given in fig.1 to fig 4. The kinetic parameters are given in table 2.

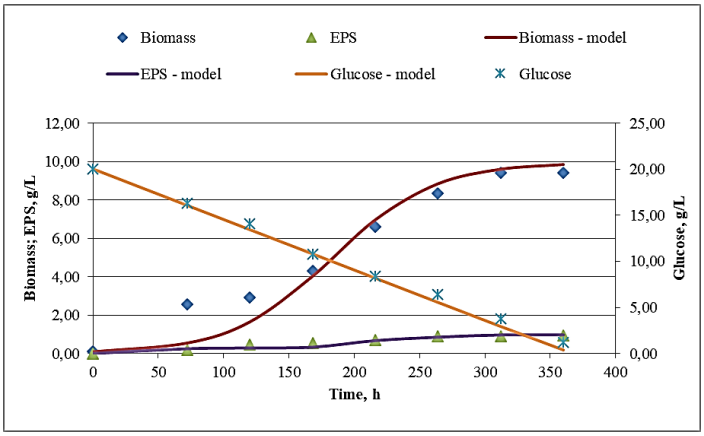

Figure 1 Comparison of Monod model predictions to the experimental data in the cultivation of *Ganoderma resinaceum*

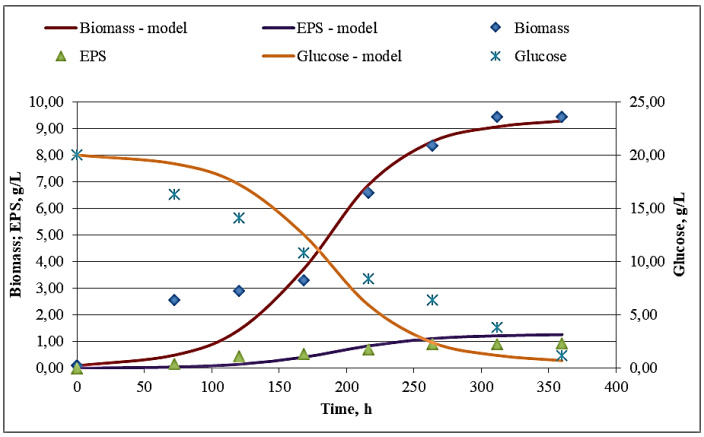

Figure 2 Comparison of Moser model predictions to the experimental data in the cultivation of *Ganoderma resinaceum*

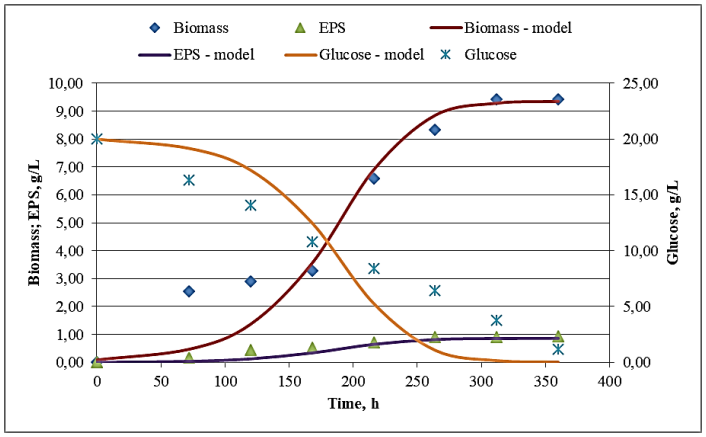

Figure 3 Comparison of Andrews model predictions to the experimental data in the cultivation of *Ganoderma resinaceum*

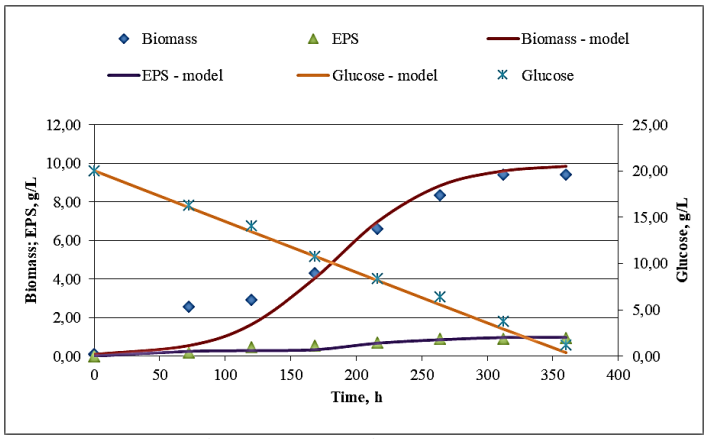

Figure 4 Comparison of Verhulst (model of a logistic curve) model in combination with the Luedeking–Piret model predictions to the experimental data in the cultivation of *Ganoderma resinaceum*

Table 2 Kinetic constants in the applied models used to describe the growth kinetics of *G. resinaceum* GA1M

	Monod	Moser	Andrews	Logarithmic equation and Ludeking-Piret model
μmax, h^{-1}	0.153	0.035	0.036	0.015
KSX, g/dm^3	103.85	61.51	29.19	-
KsXi, g/dm^3	-	-	126.19	-
qpmax, h^{-1}	0.010	0.003	0.005	-
KSP, g/dm^3	68.81	26.87	53.49	-
KSPi, g/dm^3	-	-	60.96	-
n	-	1.62	-	-
1/Yx/s	1.8921	1.8412	1.9565	-
1/Yp/s	0.6237	0.7526	2.1320	-
Yx/s	0.5285	0.5431	0.5111	-
Yp/s	1.6033	1.3287	0.4690	-
Xk, g/dm^3	-	-	-	9.95
β, $dm^3/(g.h)$	-	-	-	0.0015
K, g/(g.h)	-	-	-	$1.0.10^{-7}$
q_{p0}, g/(g.h)	-	-	-	$3.2.10^{-4}$
γ, g/(g.h)	-	-	-	11.9
δ, g/(g.h).	-	-	-	0.0051
R^2 (biomass)	0.9945	0.9769	0.9768	0.9809
Error (biomass)	1.44	1.40	1.44	1.45
R^2 (product)	0.9561	0.9598	0.9408	0.9641
Error (product)	2.98	2.94	2.98	2.37
R^2 (substrate)	0.8508	0.9168	0.9237	0.9995
Error (substrate)	0.14	0.19	0.13	0.13
Sopt (biomass), g/dm^3	-	-	60.69	-
Sopt (product), g/dm^3	-	-	57.10	-

The obtained experimental data were in agreement with the applied mathematical models. The correlation coefficients in all cases were in the range of 0.8505 to 0.9992 combined with low values for the calculated errors in the range of 0.13 to 2.98 meaning that the used models were adequate and could be applied for analysis of the cultivation process as well as for its management and forecasting.

With the Monod model, the values for the maximum specific growth rate (0.136 h^{-1}) and maximum specific rate of product synthesis (0.010 h^{-1}) were significantly higher compared with the values from the other applied models. With the Moser and Andrews models, the values for μ_{max} are 0.035 and 0.036 h^{-1}, respectively, and the q_{pmax} values were 0.003 and 0.005 h^{-1}, respectively. On the other hand, the calculated value for the substrate saturation constant for the biomass was abnormally high (103.85 g/L), and illogical from a biological point of view because such high substrate concentration would inevitably lead to substrate inhibition. For other strains used for the synthesis of ganoderic acid the value for the saturation constant, calculated by the Monod equation equaled 17.52 g/L (**Zhong and Tang, 2004**). A similar trend was observed for the saturation constant in the process of pleuromutilin synthesis by *Pleurotus mutilis*, where K_{SX} = 1.89 g/L (**Benkortbi *et al.*, 2007**). In comparison with the Andrews model, the calculated value for K_{SX} was 29.19 g/L and this low value was due to the fact that the Andrews model considers the substrate inhibition during the cultivation process.

The determination of the theoretical substrate concentration of the initiation of substrate inhibition of the culture growth was performed with the Andrews model (**Olorunnisola *et al.*, 2008**). The data from table 2 shows that K_{SXi}=126.19 g/L and this value is higher than the substrate inhibition constant for the product (60.96 g/L), thus the product synthesis could be inhibited with a higher rate with the increase of the substrate concentration in the medium regardless of the fact that EPS are built of glucose monomers. The advantage of the Andrews model is the possibility for calculation of the theoretical optimal substrate concentration which does not cause substrate inhibition of the culture growth and EPS synthesis:

$$\mu = \mu_{max}^{opt} \Rightarrow S_{opt} = \sqrt{K_{sx}K_{sxi}}$$
$$q = q_{max}^{opt} \Rightarrow S_{opt} = \sqrt{K_{sp}K_{spi}}$$

This is of great importance when the composition of the medium is being optimized. The optimal substrate concentration for biomass production was higher (60.69 g/L) compared to the optimal concentration needed for EPS production (57.10 g/L). The similar values for K_{SPi} and S_{opt} for the product synthesis prove that the process of EPS production by *G. resinaceum* is highly dependent on substrate inhibition. The high theoretical values for the optimal substrate concentration for the growth of the fungus are an indication of the need for further research in order to optimize the composition of the used medium.

The metabolic (trophic) coefficients - $1/Y_{x/s}$ and $1/Y_{p/s}$, describing the consumption of the substrate in the formation of biomass and product are also interesting and could easily be calculated since they represent the reciprocal values of the economic coefficients - $Y_{x/s}$ and $Y_{p/s}$. When the models of Monod and Moser were used most of the substrate was utilized for biomass formation (table 2), but when the Andrews model was applied the values of these two coefficients were almost even, proving that the substrate consumption for biomass production and EPS synthesis was comparable. The most appropriate models describing the dependence of the growth and the biosynthesis of metabolic products by substrate concentration are those of Moser and Andrews. The major disadvantage of the mentioned above models is the absence of a clear biological sense and the impossibility of describing the process kinetics caused by the constantly increasing biomass concentration and the effect of the limited volume of the medium on the growth and metabolite synthesis (**Bouguettoucha *et al.*, 2011; Feng *et al.*, 2016**). Also, they don't provide information about at what development stage the metabolites are produced, which is important when the process will be up-scaled. Thus, kinetics modelling according to the Verhulst model in combination with the Luedeking–Piret model was applied. In comparison with the other used models, this one calculated a lower value for the maximum specific growth rate – 0.015 h^{-1} (table 2). The lower value for the intrapopulation competition coefficient indicated that the culture was growing well in limited space and the competition for substrate did not lead to cell death. According to the model the EPS was mostly produced by cells in the exponential growth stage, where the substrate was utilized more rapidly. This was confirmed by the high value of the q_{p0} parameter–$3.2.10^{-4}$ g/(g.h), in comparison with the K parameter – $1.0.10^{-7}$ g/(g.h). A similar trend was observed with the synthesis of triterpenes and polysaccharides by other *Ganoderma* species (**Zang *et al.*, 2010; Feng *et al.*, 2013; Balamurugan *et al.*, 2021**), and also with the biosynthesis of EPS by the basidiomycetes *Armillaria luteovirens* Sacc QH and *Lentinus edodes* (**Xu *et al.*, 2011 Hatamain *et al.*, 2022**). The future experiment should be focused on exploring a fed-batch cultivation process where the culture is maintained in exponential stage leading to higher product yields. In conclusion, the dependence of the growth rate and the biomass and EPS production on the substrate concentration was better described by the Moser and Andrews models while the Verhulst model in combination with the Luedeking–Piret model fully described the phases of fungal growth and EPS synthesis.

Determination of TPC and antioxidant activity

Mycelium biomass extracts were evaluated for their total phenolic content and antioxidant potential (table 3). The TPC was in the range of 1.11 ± 0.01 and 9.95 ± 0.06 mg GAE/g DW, indicating the highest potential of the water extract. Water

as a solvent is preferable from the point of view of non-toxicity, low cost, environmental friendliness, and high efficiency in respect of *G. resinaceum* mycelium biomass **(Castro-Puyana *et al.*, 2017)**. Similar results have been reported by **Angelova *et al.* (2022)** for aqueous extracts of other edible mushrooms.

Table 3 Total phenolic content (TPC, mgGAE/g DW) and *in vitro* antioxidant potential (CUPRAC, FRAP, ABTS, and DPPH assays, μMTE/g DW) of *G. resinaceum* GA1M mycelium biomass extracts

Sample	TPC	ABTS	DPPH	FRAP	CUPRAC
Water extract	9.95 ± 0.06	49.10 ± 0.35	3.60 ± 0.08	29.76 ± 0.80	43.25 ± 1.48
Ethanol extract	1.37 ± 0.03	16.79 ± 0.65	under LOQ	1.93 ± 0.09	14.08 ± 0.22
Methanol extract	1.11 ± 0.01	27.46 ± 0.19	under LOQ	2.93 ± 0.13	19.22 ± 0.77

under LOQ - under the limit of quantification

Regarding the antioxidant potential, the four *in vitro* methods showed the same trend. The highest potential was found unequivocally in the aqueous extract and the reported values ranged from 16.79 ± 0.65 to 49.10 ± 0.35 μMTE/g DW in the ABTS assay, from 1.93 ± 0.09 to 29.76 ± 0.80 μMTE/g DW according to FRAP analysis and from 14.08 ± 0.22 to 43.25 ± 1.48 μMTE/g DW according to CUPRAC analysis, resp. According to the DPPH assay, the results were significant only for the water extract. The variation between the values confirmed the need to run several *in vitro* antioxidant assays **(Shahinuzzaman *et al.*, 2020).** The evaluated potential indicates the ability of *G. resinaceum* extracts to counteract oxidation in various aspects.

Determination of the antimicrobial activity

The disk diffusion method produces fast and reliable results regarding the presence or absence of antimicrobial activity, which makes it suitable for screening. Ten test-microorganisms were used and 9 of them were pathogens or conditional pathogens associated with food and cosmetic products. The results from the test are presented in table 4.

Table 4 Antibacterial activity of mycelial biomass extracts of *G. resinaceum* GA1M

Test-microorganism	Extracts*					
	MCE	ME	EAE	EE	BE	WE
E. coli ATCC 8739	9/20**	18**	19**	-	-	-
E. faecalis ATCC 19433	18**	16**	18**	18**	15**	-
S. enterica spp. *enterica* ser. *enteridis* ATCC 13076	-	-	14**	-	-	-
L. monocytogenes ATCC 8787	-	-	-	-	-	-
P. aeruginosa ATCC 9027	15**	19**	16**	18**	17**	-
P. vulgaris G	-	9	-	-	-	-
K. pneumoniae ATCC 13883	-	-	-	-	-	-
S. aureus ATCC 25923	-	-	-	-	-	-
B. subtilis ATCC 6633	-	-	-	-	-	-
B. cereus ATCC 11778	-	-	-	10	-	-

* MCE – methylene chloride extract; ME – methanol extract; EAE – ethyl acetate extract; EE – ethanol extract; BE – butanol extract; WE – water extract

** single colonies in the inhibition zone

- no inhibition zones

The mycelium biomass extracts of *G. resinaceum* obtained with MCE demonstrated antibacterial activity towards *E. coli* ATCC 8739 with a clear inhibition zone of 9 mm. The ME inhibited completely the growth of *P. vulgaris* and the EE – that of *B. cereus* (9mm and 10 mm inhibition zones, respectively). Some of the cells of *Escherichia coli*, *Salmonella*, and *Pseudomonas aeruginosa* were susceptible to the activity of most extracts, which resulted in the presence of single-cell colonies in inhibition zones with a large diameter (reaching 20 mm in the test of MCE against *E. coli* ATCC 8739).

The growth of *L. monocytogenes*, *S. aureus*, and *B. subtilis* was not affected by the obtained extracts. The water extract showed no antimicrobial activity against the tested microorganisms. According to **Gao *et al.* (2003),** *Ganoderma lucidum* as well as other *Ganoderma* sp. are often used in combination with chemotherapeutics for the treatment of diseases with bacterial origin due to the fact that the polysaccharides in the fungal biomass are the key metabolite in the antibacterial activity of the fungus.

For the determination of the minimal inhibition concentration (MIC), only the extracts which demonstrated significant antimicrobial activity were selected. The obtained results are summarized in table 5.

Table 5 MIC of *G. resinaceum* mycelial biomass extracts towards test-microorganisms

Test-microorganism	MCE	ME	EE
E. coli ATCC 8739	1.17	-	-
E. faecalis ATCC 19433	-	-	-
S. enterica spp. *enterica* ser. *enteridis* ATCC 13076	-	-	-
P. aeruginosa ATCC 9027	-	-	-
P. vulgaris G	-	5.00	-
B. subtilis ATCC 6633	-	-	-
B. cereus ATCC 11778	-	-	6.25

The obtained data clearly shows a wide range of minimal inhibition concentrations with the used extracts. The MCE demonstrated the highest antimicrobial activity towards *E. coli* and the MIC was respectively 1.17 mg/mL. The MIC of the ME towards *P. vulgaris* was 5.00 mg/mL, and a concentration of 6.25 mg/mL of the EA was sufficient to suppress the growth of *B. cereus*.

In the available literature, information on the antimicrobial activity *of Ganoderma resinaceum* is scarce. There is data for other species from the same genus, which confirms, that the antimicrobial activity of various extracts is moderate to weak. **Chan and Chong (2020)** reported MICs ranging from 0.625 to 5.00 mg/mL for *G. boninense*. **Quereshi *et al.* (2010)** determined even higher values of MIC (4.33 21.30 mg/mL) for *G. lucidum*. However, component derivatives from *Ganoderma* species showed much higher activity in the investigation of **Haleno *et al.* (2013)**, and the combination of extracts with antibiotics had additive effects in the work of **Yoon *et al.* (1994).**

CONCLUSION

In this study, *Ganoderma resinaceum* GA1M was submerged cultivated and the models of Monod, Moser, Andrews and Verhulst in combination with the Luedeking–Piret model were applied for the kinetics modelling of the cultivation process. The obtained data demonstrated that the used models were adequate and basic bioprocess parameters were determined. These parameters would allow for upscaling of the process of biomass accumulation and EPS synthesis. Further research in this area could be focused on a fed-batch cultivation process. Water, methanol and ethanol extracts of the biomass were used for the determination of the total phenolic content and the antioxidant potential of the mushrooms' mycelium. The water extract demonstrated the highest antioxidant potential and phenolic content. The antimicrobial activity was evaluated towards ten test microorganisms, where the MCE demonstrated the highest antimicrobial activity towards *E. coli*, ME towards *P. vulgaris* and the EA suppress the growth of *B. cereus*, and the water extracts exhibited no antimicrobial activity in the tested concentrations. The findings of this study give us reasons to conclude that *Ganoderma resinaceum* has potential and after additional surveys it could be used for the development of dietary supplements with antioxidant and antimicrobial properties.

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