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Research Article

Modeling *Grifola frondosa* fungal growth during solid-state fermentation

Grifola frondosa (maitake) is an edible and medicinal mushroom. Considering its increasing popularity, there are limited references for its cultivation. Previous studies demonstrated that carpophore formation is correlated directly with mycelial biomass. The development of a mathematical model for its growth under solid-state fermentation (SSF) may help to predict the potential of different substrates for maitake production. *G. frondosa* growth and basidiome development was studied, using oak sawdust and corn bran as substrates. The fungal biomass content was determined by measuring *N*-acetyl-D-glucosamine (NAGA). It increased steadily for the first 80 days, to a maximum in coincidence with the first fruiting (60.5 μg NAGA/mg dry sample). Two mathematical models were selected to evaluate *G. frondosa* development, measuring reducing sugars consumption and NAGA synthesis, as an indirect assessment of fungal growth. Both models showed a good fit between predicted and experimental data: logistic model ($R^2 = 0.8896$), two-stage model ($R^2 = 0.8878$), but the logistic model required a minor number of adjustment parameters.

Keywords: *Grifola frondosa* / Mathematical modeling / NAGA / SSF

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1 Introduction

Grifola frondosa (Dicks.: Fr.) SF Gray, also known as maitake, is a white rot basidiomycete (belonging to the family Polyporaceae, order Aphyllophorales), found in the temperate forests of Asia, Europe and eastern North America. *G. frondosa* has gained in popularity among consumers, not only because of its taste and flavor, but also because of its reported medicinal value [1]. Its active compounds primarily belong to the group of polysaccharides (especially 1,6-β-D-glucans and 1,3-β-D-glucans), glycoproteins and proteins. Medicinal effects of *G. frondosa* are numerous, including anticancer activity, immune system stimulation, effects on angiogenesis, reduction of benign prostatic hyperplasia, antibacterial and antiviral effects, effects on lipid metabolism and hypertension, antidiabetic activity, vitality and performance enhancement, antioxidant effects and beneficial cosmetic effects on skin [2]. As the demand for *G. frondosa* fruit bodies and/or mycelium biomass is constantly increasing, artificial cultivation has become essential. Solid-state fermentation (SSF) is one way of meeting the rising demand for fungal mycelium and its bioactive metabolites [2, 3]. Since the fungus grows on wood in nature, mixtures of lignocellulosic materials have been utilized as substrates in the commercial production of the mushroom. A common substrate used for its production is sawdust supplemented with rice bran or wheat bran in a 5:1 ratio, respectively [4]. *G. frondosa* is also capable of utilizing a variety of hardwood species on different substrate forms, e.g. stumps, logs and wood chips. Outdoor cultivation of *G. frondosa* in Japan commonly employs sterilized blocks of sawdust amended with corn and wheat bran [5]. Nevertheless, considering the increasing popularity of this mushroom, there are limited reference texts available for cultivating maitake [6] and knowledge of the bioconversion of lignocellulosics by *G. frondosa* during cultivation is still very limited. More detailed information would be helpful to improve the cultivation conditions for efficient production of the mushroom [2]. Recently, *G. frondosa*, was cultivated on substrates composed of olive oil press cakes [7], coffee spent ground [8] and spent brewery grains [9], with different supplements. The experiments demonstrated that of *G. frondosa* mycelium could become an efficient biotechnological process for the production and isolation of fungal β-polysaccharides [2].

SSF exhibits some advantages for fungal cultivation in comparison with submerged fermentation: lower production costs and higher yields. Filamentous fungi form a mycelium. i.e. a dense interconnected network of tubes called hyphae.

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Abbreviations: NAGA, *N*-acetyl-D-glucosamine; **PDA**, potato dextrose agar; **RH**, relative humidity; **SSF**, solid-state fermentation

Due to the complexity of its growth habit, the role of fungi in an environment exhibiting spatial and temporal heterogeneity is very difficult to investigate by experimental methods alone. Mathematical modeling is now proving to be a very powerful and successful complementary tool [10].

Assessing biomass content is very complicated, because it is almost impossible to separate the fungus from its solid substrate. Due to the structure and porosity of the solid substrate the mycelium penetrates and binds to it. Therefore, the fungal growth cannot be directly estimated as dry mass. Instead, the rate of substrate colonization can be determined by measuring ergosterol content [11], levels of enzyme production [12], or quantifying the production of *N*-acetyl-D-glucosamine (NAGA), a component of chitin, which constitutes the fungal cellular wall structure [13–15].

Commonly, SSF involves cultivation of filamentous fungi on natural solid substrates in which the carbon source constitutes part of their structure. In most cases, to track what happens in the processes of SSF, empirical sub-models should be used; however, resulting in complex sub-models due to the heterogeneity of the processes [16]. Several kinetic profiles have been reported to describe the process of SSF, including linear, logistics and two-phase models [17]. Some of the empirical equations associated with these models are shown in Table 1 (Eqs. 1–8).

The two-stage model describes an exponential (acceleration) phase followed by a deceleration stage [18]. The deceleration phase expression is represented by the Eq. (4b), where t_a is the time when this stage begins, and L is the relationship between the specific velocities of development of both phases. This deceleration is described by an exponential decay reflected in the specific velocity of development, with a constant velocity of the first order (k). One constraint when applying this model is that the exponential period is typically short. Nevertheless, this is the indicated model when adjustments of processes are required with few experimental data [19, 20].

The present paper focuses on *G. frondosa* mycelium growth kinetics during SSF using low-cost agro industrial by-products as substrates: oak sawdust and corn bran. The major substrate ingredient oak sawdust is a residue from the industry devoted to the production of oak barrels for the liquor manufacturing. The aim of this work was the evaluation of different mathematical models designed to describe *G. frondosa* development under SSF, measuring its substrate consumption and NAGA synthesis, as an indirect assessment of fungal growth, so as to provide practical guidelines leading to diminish uncertainty while selecting substrate formulations for this fungus commercial cultivation.

2 Materials and methods

2.1 Microorganism and culture conditions

G. frondosa (PSUMCC 922) was obtained from the Pennsylvania State University Mushroom Culture Collection, USA, and maintained on potato dextrose agar (PDA) at 4°C with periodic transfer. The mycelium was transferred from the stock culture to the center of a Petri dish containing PDA and incubated at 25°C for 21 days. The experiment was conducted under greenhouse conditions, in Manizales, Colombia, located at 2250 m above sea level, where the average annual temperature is 17°C and the relative humidity (RH) 70%.

2.2 Spawn preparation

Five pieces of 1.0 cm^2 of PDA medium colonized by the mycelium were inoculated in a 500-mL glass jar, in which half of the volume contained corn grains. Moisture content of the grains was adjusted to 33–40% of the fresh weight. The jars were incubated at 25°C for 25 days, until total substrate colonization.

2.3 Substrates and fruiting conditions

The substrate consisted of (dry weight basis): 75% of oak sawdust (25% moisture content), 23% of corn bran (15% moisture content), 1% sucrose (2% moisture content) and 1% of calcium carbonate. The substrates were packed in polypropylene bags and autoclaved at 121°C for 1 h. The moisture content was calculated in relation to the dry weight of the components. Each bag of 32 cm height and 12 cm diameter contained 1 kg of substrate (58% moisture content). One square hole of 2.54 cm^2 was made at the top of each bag and covered with a micro porous breather strip to facilitate gas exchange. The bags containing the substrate were aseptically inoculated with 3% (wet substrate basis) of spawn. The experiment was replicated ten times.

Table 1. Differential and integrated forms of the various empirical growth equations that have been applied to SSF systems[a)]

	Differential form		Integrated form	
Linear	$\frac{dx}{dt} = k$	(1)	$x = kt + x_0$	(5)
Exponential	$\frac{dx}{dt} = \mu x$	(2)	$x = x_0 e^{\mu t}$	(6)
Logistic	$\frac{dx}{dt} = \mu x\left(1 - \frac{x}{x_m}\right)$	(3)	$x = \frac{x_m}{1+((x_m/x_0)-1)e^{-\mu t}}$	(7)
Two-phase	$\frac{dx}{dt} = \mu x, \quad t < t_a$	(4a)	$x = x_0 e^{\mu t}, \quad t < t_a$	(8a)
	$\frac{dx}{dt} = [\mu L e^{-k(t-ta}]x, \quad t \geq t_a$	(4b)	$x = x_A \exp\left[\frac{\mu L}{k}(1 - e^{-k(t-t_a)})\right], \quad t \geq t_a$	(8b)

[a)] x, microbial biomass; t, time; k, velocity constant of linear development; μ, specific velocity of constant development; x_0, initial biomass; x_m, maximum amount of possible biomass. t_a, L and k are parameters for the two phase's expression which are explained in the text.

During the first week (adaptation phase to the new substrate), the cultures were maintained at 25°C and 60% RH, to facilitate a faster growth. Then, they were incubated at 20 ± 1°C for approximately 23 days more, until the complete colonization of the substrate. The third step before primordial formation, which includes the development of a dense white mycelial mat (mycelial coat), that progressively acquires an orange brown color and ends with the production of an orange exudate, required 40–45 days at 18–20°C and 60–65% RH. A cold shock to 10°C for 24 h (induction) was essential to facilitate primordial initiation. While the mycelium could grow in the dark, a low level of light (50–100 lx) was necessary afterward for basidiomata development. The period of basidiomata development, which was initiated when the primordia begins to grow and differentiate to form small pilei and stipes (7–10 days) required one air exchange (around 10 min) every 12 h, 16–18°C and 70–80% RH. At this stage, holes were cut in the bags exposing the developing primordia. The next phase, until harvesting, lasted for 3–5 days and required an air renewal of 10 min every 2 h (to hold CO_2 concentrations below 700 ppm) and 70–80% RH. The crop cycle (total time required for spawn run, primordial formation and mature basidiomata harvest) was of 81–96 days (until first crop). About 15–20 additional days were required for a second crop [8]. Biological efficiency (BE) was determined as the ratio of kilogram of fresh mushrooms harvested per kilogram of dry substrate and expressed as a percentage.

2.4 Quantification of the NAGA content and reducing sugars

Substrate samples were collected at different incubation periods (20, 30, 45, 60, 75 (vegetative growth), 87 and 107 days (both cycles if fruiting)). Entire solid cultures were dried at 90°C, until constant weight, ground in a mortar, and stored until they were used for chitin determination. The fungal biomass content of dried solid cultures was estimated by NAGA released from chitin after hydrolysis with 6 N HCl. Analytical grade NAGA served as reference [13]. Biomass content was indirectly determined by measuring glucosamine in the solid substrate and recalculated to dry biomass concentration (mg fungal biomass per g of dry substrate), taking into account glucosamine content of *G. frondosa* mycelium grown in 100-mL Erlenmeyer flasks with 25 mL of medium, containing glucose (30 g/L), yeast extract (6 g/L), $SO_4Mg.5H_2O$ (0.5 g/L), K_2HPO_4 (0.5 g/L). The water-soluble compounds were extracted by hydrolysis with hot water for 3 h [21], the reducing sugars were determined in the filtrate by the Somogyi–Nelson method [22].

3 Results and discussion

3.1 Parameters estimation and kinetic models

G. frondosa growth and basidiomata development along 107 days during SSF on a substrate based on oak sawdust plus corn bran was investigated. The biological efficiency obtained was 35.3% with a crop cycle of 12–14 wk [8]. Growth was characterized by measuring NAGA (released from chitin) and reducing sugars produced by enzymatic substrate hydrolysis. The substrate was fully colonized after 25–30 days. Figure 1 shows that reducing sugars produced by enzymatic substrate hydrolysis reached their highest value after the same period, and then declined sharply, probably consumed during *G. frondosa* growth. Lignocellulosic materials possess lignin as barrier to microorganisms; this complex molecule is unable to serve them as the sole carbon and energy source. As a lignin degrading fungus [23] *G. frondosa* is able to reach other carbon sources present in wood (cellulose and hemicellulose), which are more readily utilizable sources of carbon. During primary metabolism, the fungus produces the extracellular enzymes necessary to degrade these polysaccharides to soluble molecules that are transported to the intracellular space and stored as glycogen; later, NAGA content increases to the final of the incubation period, reflecting probably the associate preparation for fruiting. The onset of fruiting is observable after a stimulus such as physiological stress [24]. As shown in Fig. 1, NAGA content in *G. frondosa* increased steadily for the first 87 days, to a maximum in coincidence with the first fruiting (60.5 μg NAGA/mg dry solid). NAGA content in the carpophore was 77.5 μg NAGA/mg dry solid. As mycelium of *G. frondosa* contains 104 μg NAGA/mg, actual biomass could be estimated, attaining a maximum of 0.58 mg/g dry solid. In a previous work, when growing *G. frondosa* strain GF3 in an horizontal bioreactor on corn and olive press cake supplemented with olive oil and mineral additives, biomass increased to a maximum of 0.20 μg glucosamine/mg solid substrate after 10 days of cultivation, but remained nearly constant the subsequent 27 days [2]. The differences attained in our study may be attributed to the strain as well as the nature of the substrate used, considering that both of them have a pronounced effect on NAGA and therefore on biomass quantity in solid culture conditions [14].

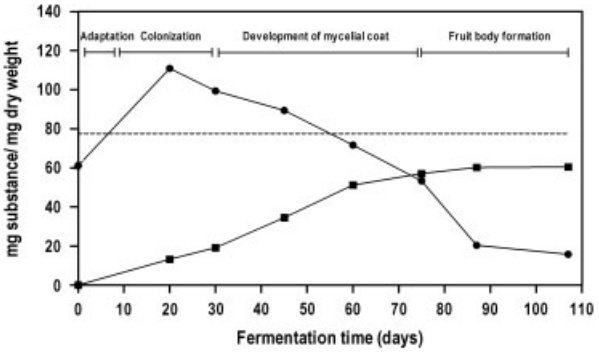

Figure 1. Time course of reducing sugars consumption and NAGA production during *G. frondosa* fermentation on oak sawdust. Biomass (μg NAGA/mg dry substrate) (■); reducing sugars (μg/mg) (●). Growth phases of *G. frondosa* during SSF: adaptation phase (days 1–7), complete colonization of the substrate (days 7–30), development of the mycelial coat (days 30–75) and fruit body formation (days 76–107: day 87 first crop, day 107 second crop).

SSF cultivations are usually discontinuous processes where substrate shortage may limit biomass growth. Experimental results showed that undersupply of substrate in the medium (evidenced by the lack of reducing sugars) resulted in decreasing rates of fungal growth until cessation. This was a conclusive factor when we tried to depict the experimental results applying mathematical models. Among four empirical kinetic profiles for *G. frondosa* growth in SSF, developed and tested experimentally (linear, exponential, logistic and two phases), a good fit between the logistic (Model I) and two-phase models (Model II) and the experimental data were found. They describe a tendency to a finite growth and the additional expression that equilibrates these models is the variation of substrate concentration (reducing sugars) represented in time by Eq. (9) [25]. Given that the selected models include only the decline in the amount of substrate, time zero was taken for this correlation, the experimental values obtained on 20th fermentation day ($G_0 = 13.33$ μg NAGA/mg dry sample and $S_0 = 110.85$ μg reducing sugars/mg dry sample). Eqs. (9)–(13) represent the adjustment for the SSF of *G. frondosa* on oak sawdust substrate.

$$\frac{dS}{dt} = -\alpha\frac{dX}{dt} \tag{9}$$

Logistic Model (Model I)

$$\frac{dG}{dt} = \vartheta G\left(1 - \frac{G}{G_m}\right) \tag{10}$$

$$\frac{dS}{dt} = -\alpha\frac{dG}{dt} \tag{11}$$

Where G represents NAGA concentration (μg NAGA/mg dry sample), S: substrate concentration (μg reducing sugars/mg dry sample), t: fermentation time (days), α represents the correlation between the amounts of substrate and NAGA (is a yield coefficient, its units are g/g), ϑ is the specific rate of NAGA production (per day). For this model, constants α and ϑ are used as setting parameters, and the value for NAGA maximum concentration, G_m, was taken from that obtained experimentally from the carpophores ($G_m = 77.5$ μg NAGA/mg dry sample).

Two-stage Model (Model II)

$$\frac{dG}{dt} = \begin{cases} \vartheta_1 G & t < t_a \\ \vartheta_1 e^{-k(t-t_a)} G & t \geq t_a \end{cases} \tag{12}$$

$$\frac{dS}{dt} = \begin{cases} -\alpha\vartheta_1 G & t < t_a \\ -\alpha\vartheta_2 e^{-k(t-t_a)} G & t \geq t_a \end{cases} \tag{13}$$

For Model II, α, ϑ_1, ϑ_2 *y* k, are used as adjustment parameters, where α represents the correlation between the amounts of substrate and NAGA (is a yield coefficient, its units are g/g), ϑ_1 and ϑ_2 are specific rates of NAGA production in the acceleration and deceleration stages respectively, and k is the constant rate of the first order reaction. The time change for the slope was set at 45th day of fermentation for this work, indicating the start of the deceleration phase ($t_a = 25$ days).

Table 2. Adjusting parameters of the models I (logistic) and II (two-phase) with the experimental data

Model I (logistic)	Model II (two-phase)
α = 1.6715 μg reducing sugar/μg NAGA data of production	α = 1.5596 μg reducing sugar/μg NAGA data of production
ϑ = 0.0426/day	$\vartheta_1 = 0.0264$/day $\vartheta_2 = 1.6921$/day $k = 0.0327$

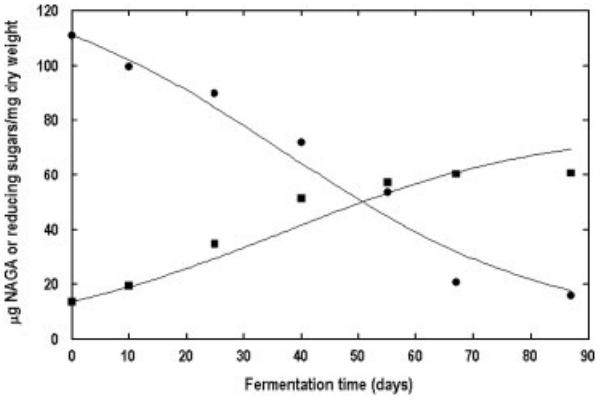

Figure 2. Experimental data adjustment to Model I (logistic model), where (●) represents the kinetics of reducing sugar consumption, and (■) the kinetics of NAGA synthesis. Solid lines represent the model data, the descending line corresponds to substrate disappearance data (reducing sugars) and the ascending line to the data of NAGA. Time zero: the experimental values obtained on the 20th fermentation day ($G_0 = 13.33$ μg NAGA/mg dry sample and $S_0 = 110.85$ μg reducing sugars/mg dry sample)

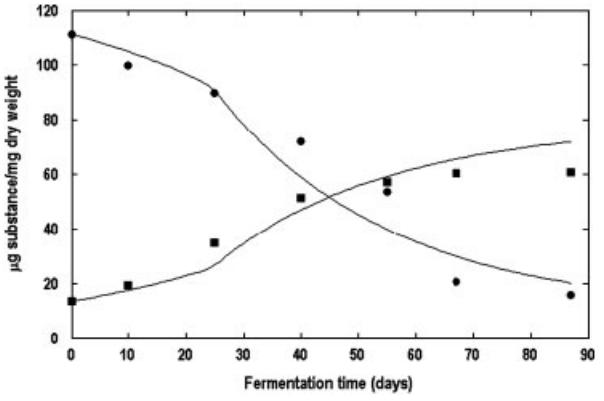

Figure 3. Experimental data adjustment to Model II (two-stage model), where (●) represents the kinetics of reducing sugar consumption in experimental data, and (■) the kinetics of NAGA synthesis. The solid lines represent the data model, the descending line corresponds to substrate disappearance data (reducing sugars) and the ascending line corresponds to NAGA synthesis, also reflects the slope change at the 25th fermentation day, as the start of the deceleration phase of the process. Time zero: the experimental values obtained on the 20th fermentation day ($G_0 = 13.33$ μg NAGA/mg dry sample and $S_0 = 110.85$ μg reducing sugars/mg dry sample)

Table 3. Statistical inference on the parameters of the models to choose the one that best fits the experimental data from SSF

Model	Parameter	Value	Reliable intervals	Interpretation
Model I (logistic)	α	1.6715 μg/μg	$1.3951 < \alpha < 1.9479$	The reliable interval is significant (interval does not include zero)
	ϑ	0.0426 dia^{-1}	$0.0351 < \vartheta < 0.0501$	The reliable interval is significant (interval does not include zero)
Model II (two- phase)	α	1.5596 μg/μg	$1.3084 < \alpha < 2.0298$	The reliable interval is significant (interval does not include zero)
	ϑ_1	0.0264/day	$0.0178 < \vartheta_1 < 0.0414$	The reliable interval is significant (interval does not include zero)
	ϑ_2	1.6921/day	$-1.6564 < \vartheta_2 < 1.7186$	The reliable interval is not significant (interval includes zero)
	k	0.0327	$-0.0131 < k < 0.0448$	The reliable interval is not significant (interval includes zero)

3.2 Adjustment of the experimental data to the selected kinetic models

The Software Matlab® was applied to experimental data adjustment to the selected kinetic models. Table 2 displays the adjusting parameters for the two models, α is similar for both models and the specific rate for Model I is similar to the ϑ_1 adjusted for the Model II. Figures 2 and 3 compare experimental and predicted data of the two models. Both models showed a good fit between predicted and experimental data: logistic model ($R^2 = 0.8896$), two-stage model ($R^2 = 0.8878$). However, the logistic model is more advantageous because it requires only two parameters for the adjustment, while the two-phase model requires four parameters. Considering that there was not a noteworthy variation in the adjustment of the two selected models; a statistical inference was conducted to determine which of the two models best approaches to the experimental data of the work. Table 3 shows the data for statistical parameters inference for both models, represented as the reliable intervals of each parameter. In the logistic model, the reliable intervals are significant for each of the parameters, since they do not pass through zero. While parameters ϑ_2 and k for the two-phase model, are not significant. Statistically a model without relevant parameters presents drawbacks in the description of the experimental data and therefore it is advisable to remove it. Hence, for monitoring the growth *G. frondosa* in SSF, the two-phase model is reduced to a logistic model. The logistic equation is an unstructured model that is largely descriptive, empirical and based on experimental observations. It includes a limitation on growth. It is shown that *G. frondosa* ran out of reducing sugars at approximately 85 days of fermentation. Since fungal growth also ceased at this time point, it is suggested that the absence of carbon substrate limited further growth.

4 Concluding remarks

Two mathematical models were selected to describe *G. frondosa* growth in SSF, and tested experimentally: logistic and two-phase. The reducing sugars present in the environment were used as the substrate, and the NAGA production, as an indirect assessment of the fungal growth. Both models were compared, given that they have been used by other authors [17, 18]. These two models were chosen because the experimental data obtained describe a trend with finite growth, limiting maximum NAGA production to that contained in the carpophore. A good fit between both models and the experimental data was found, but the logistic model required a minor number of adjustment parameters. Considering that previous cultivation studies on *Pleurotus* revealed that carpophores' formation was directly related to the spread of the mycelium into its solid substrate [26, 27], adjusting a mathematical model for *G. frondosa* growth under SSF may help to predict the potential of different substrates not only for mycelium growth, but also for maitake production.

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