Submerged Fermentation of *Ganoderma tsugae* for the Optimized Production of Exopolysaccharides

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Abstract: *Ganoderma tsugae*, commonly known as the hemlock reishi, has historically played a significant role in Eastern traditional medicine. Notably, *Ganoderma* species are known to have medicinal properties for potential commercial use, including cholesterol reduction, lowering blood pressure, antivirals, and antitumor therapies. To capitalize fully on these medicinal benefits for commercial use, the *Ganoderma* mycelium and its potential therapeutic metabolites, including biologically active polysaccharides, must be produced at scale. One promising approach is to employ stirred-tank bioreactors for the submerged fermentation of mycelium. The goal of this project is to maximize exopolysaccharide (EPS) production from the submerged fermentation of *G. tsugae* mycelium by locating which experimental variables are optimal. Experimental variables included media formulations and enrichments, temperature, pH, and agitation speed. Mycelium cultivation involved growing *G. tsugae* on Potato Dextrose Agar (PDA) plates, which were then used to inoculate liquid cultures in baffled shake flasks. Samples were taken every two to four days and assayed for biomass, reducing sugars, and exopolysaccharides (EPSs). The highest biomass and exopolysaccharide production was observed in a lactose-based media with a constant temperature of 28°C, pH of 5.5, and an agitation of 120 rpm. These optimized parameters resulted in a peak biomass yield of 11.4g/L and a peak exopolysaccharide yield of 1.68g/L.

Keywords: polysaccharides, *Ganoderma tsugae*, *Ganoderma lucidum*, submerged fermentation, mycelium, bioprocessing, biomanufacturing

Introduction

Over the last three decades, fungi and their chemical compounds have gained recognition from pharmaceutical industries for their effects in medicinal and dietary nutritional settings. One such fungus, *Ganoderma tsugae*, has been studied along with its close relative, *Ganoderma lucidum*, for its exopolysaccharide production. Both *G. tsugae* and *G. lucidum* excrete large amounts of these sugars as the most abundant of the bioactive compounds within them [1]. Polysaccharides from these species are known to possess anti-tumor effects but may depend on specific aspects such as molecular weight, galactose, and bound protein [2], [3]. Other *in vitro* effects of *G. tsugae* include antioxidant, anti-inflammatory, and cytotoxic properties against cancer cell lines [4-7].

Submerged fermentation of mycelium is an effective way to produce polysaccharides in large quantities compared to harvesting fruiting bodies due to the ease of transforming mycelium as a powder, as well as other factors such as maintaining a uniform distribution of the substrate, reducing contamination, and diminishing the time of growth [8]. Previous research included meta-analyses on submerged mycelium production, media formulation, and *G. lucidum* optimization in the year 2022. While other studies have identified a range of culture conditions for mycelium fermentation, additional studies are required in order to identify conditions for optimizing the production of specific bioactive compounds. The goal of the project was to optimize conditions for exopolysaccharide production. Media formulations were maximized, and media variations were tested in different batches through a sterile step-by-step plug-plate-flask-bioreactor procedure. *G. tsugae* was eventually considered over *G. lucidum* as *G. tsugae* was found to produce larger quantities of polysaccharides in lactose. This research into optimizing exopolysaccharide production of *G. tsugae* has potential commercial applications such as a nutritional supplement or food filler and an economical solution for downstream waste products.

Methods

Cultures of *G. tsugae* were obtained from Empire Medicinals, 125 Tech Park Drive, Rochester, New York, 14623, USA, and grown on Potato Dextrose Agar (PDA plates) assembled in the laboratory from PDA powder bought from VWR. To maintain cultures for use, plate-to-plate transfer of *G. tsugae* was performed by sterilely transferring a plug from a confluent culture to the center of a new PDA plate. New PDA plates were incubated at room temperature until confluency and then stored in a refrigerator.

Plugs from PDA plates were taken from the edge of the growth zone and were used to inoculate baffled flasks of liquid media. Five plugs were used to inoculate 250mL baffled shake flasks containing 100mL of media. Ten plugs were used to inoculate 500mL baffled shake flasks containing 200mL of media. Plugs were created using sterilized inoculation straws for size consistency. The shake flask media contained 20 grams per liter of lactose or glucose, 2.5 grams per liter of malt extract, 2.5 grams per liter of yeast extract, 0.5 grams per liter of KH_2PO_4, 0.5 grams per liter of Na_2HPO_4, 0.5 grams per liter of $MgSO_4$, 0.5 grams per liter of $(NH_4)_2SO_4$, and 250 micrograms per liter of ampicillin (bought from VWR). Seed cultures were incubated at 28°C shaking at 180 rpm to keep mycelium from anchoring to flask surfaces. Seed culture purity was monitored periodically with Phenol Red Broth (PR tubes), Trypticase Soy Agar (TSA plates), and microscopic examination of wet mount slides stained with Lactophenol Cotton Blue (LPCB).

After seven days of growth, flask cultures were used to inoculate either a New Brunswick BioFlo/CelliGen 115 stirred-tank bioreactor, model number BF-115 3-liter (2-liter working volume) or a Sartorius, model number 88438125-liter (4-liter working volume) stirred-tank bioreactor containing liquid media that is of the same composition as of the shake flask media and two drops per liter of Pluronic 60 to limit wall growth and foam. Samples were taken every 2-4 days and assayed for biomass (dry weight), reducing sugars (DNS assay), and exopolysaccharides (EPSs) using a phenol sulfuric acid method [9]. Batch runs testing different temperatures (25, 28, and 32°C), pH (4.5, 5.5, and 6.5), and agitation speeds (80, 100, 120, and 150 rpm) were performed. A secondary formulation of media was also tested using the conditions 28°C, 120 rpm, and 5.5pH.

The standard liquid media for the 3-liter and 5-liter stirred-tank bioreactors was formulated with the following ingredients: 2.5g/L yeast extract, 0.5g/L potassium phosphate monobasic (KH_2PO_4), 0.5g/L sodium phosphate dibasic (Na_2HPO_4), 0.5g/L magnesium sulfate ($MgSO_4$), and 2.5g/L malt extract. Each ingredient was fully dissolved using dH_2O and adjusted to 5.5pH via 1N NaOH and 1N H_2SO_4. Media to be used in a bioreactor was also given pluronic 60 to reduce foaming (2 drops per liter). The secondary media used only for the 2x experiment contained 2 times the amount of yeast and malt (5.0g/L). All media was then sterilized prior to scaling up to stirred-tank bioreactors.

To assess biomass, 10mL of homogenous samples were taken and centrifuged at 3,500 rpm for 15 minutes and the supernatant was retained for DNS and EPS assays. The remaining biomass was placed in a pre-weighed aluminum boat and then heated in an oven at 72ºC to evaporate all the liquid before being weighed.

To assess the amount of reducing sugars, lactose, or glucose as a food source, the DNS assay was utilized. From the supernatant retained in the biomass assay, a homogenized sample was diluted 1:40 with dH_2O to ensure the sample concentration was in range of the glucose/lactose standards of known concentration. The diluted samples along with the standards were then measured for reducing sugar concentration using the DNS method [9].

To assess EPS production, 2mL of supernatant retained in the biomass assay was drawn out and placed in a falcon tube. 8mL of chilled pure ethanol was added for a 1:4 dilution and sequestered in a freezer for at least 24 hours to precipitate out the exopolysaccharides. Once precipitated, the samples were centrifuged at 3,500 rpm for 15 minutes. The ethanol supernatant was decanted out, and the remaining pellet was resubmerged in 2mL of hot dH_2O and vortexed to a homogenized state. In order to ensure the EPS concentration of the sample was within the range of the **β**-glucan standards of known concentration, the samples were diluted 1:10 with dH_2O. The diluted samples, along with the standards, were then measured for EPS concentration using the phenol-sulfuric acid method [9].

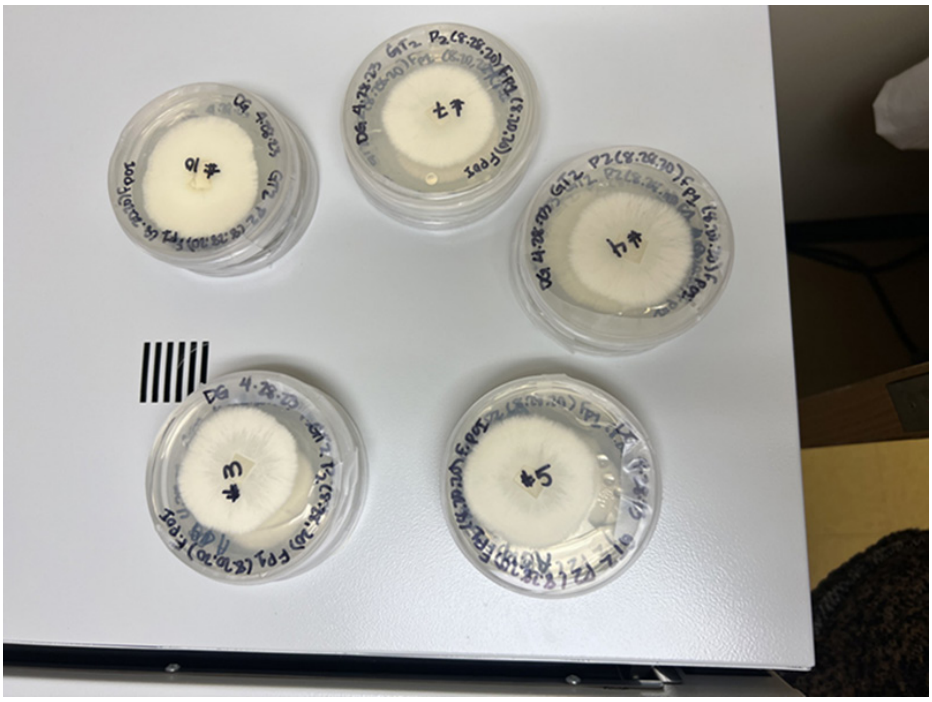

Fig. 1. 5 PDA stock plates of G. tsugae after 7 days of growth.

Fig. 2. 7-day seed flasks of G. lucidum (left) and G. tsugae (right). The elongated pellet morphology of G. lucidum is distinctly different from the circular, spider-like morphology of G. tsugae.

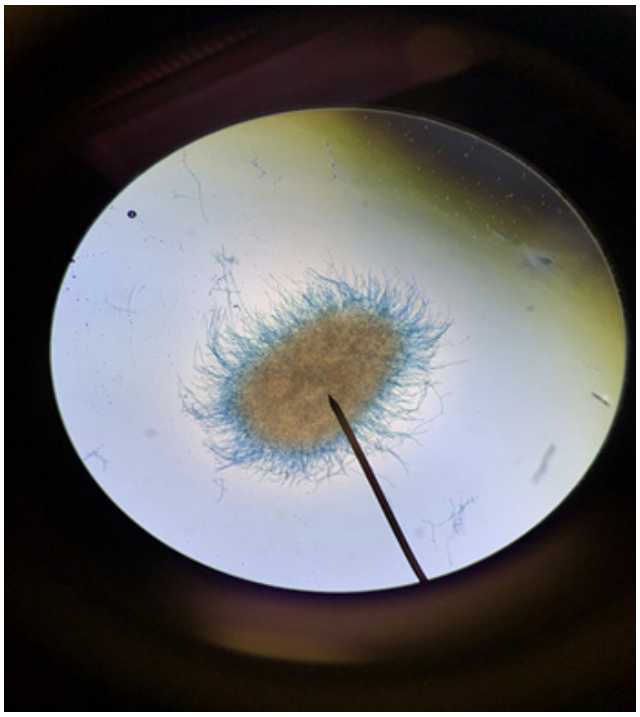

Fig. 3. G. tsugae stained with lactophenol cotton blue viewed under a microscope at 400x magnification. Lactophenol cotton blue stains chitin present in fungal cell walls.

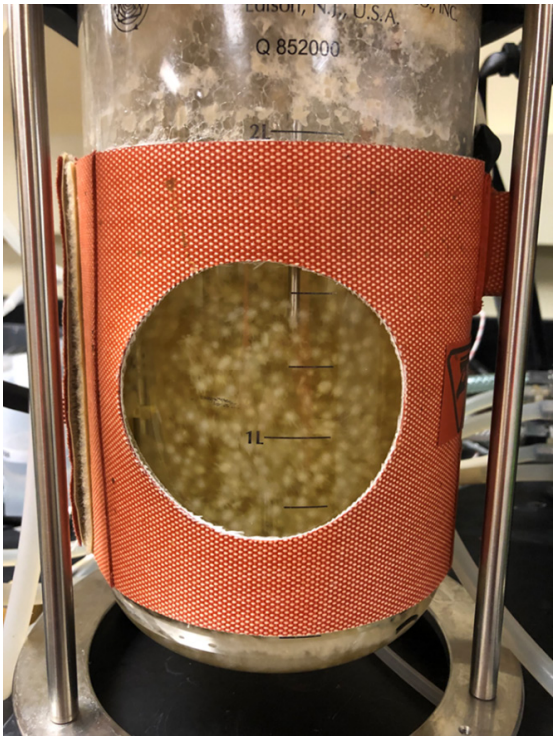

Fig. 4. G. tsugae fermented in a bioreactor after 7 days of fermentation.

Results and Discussion

The utilization of different reducing sugars as a food source, glucose, and lactose, were tested for *G. tsugae* in batches 2 and 14, respectively. Conditions included a constant temperature of 28°C, pH of 5.5, and agitation of 100 rpm over a 14-day bioreactor run. *G. tsugae* in glucose was found to have a peak EPS yield of 0.79 g/L, while *G. tsugae* in lactose was found to have a peak EPS yield of 1.21 g/L. Furthermore, both EPS and biomass production were consistently higher throughout the test run utilizing lactose rather than glucose.

The optimized food source was determined for G. *tsugae*, and other parameters were then fine-tuned for an optimized EPS yield. Optimized parameters included a 7-day shake flask incubation of *G. tsugae* in a lactose-based media at 180 rpm in a shaker, then inoculated and controlled at a constant temperature of 28°C, pH of 5.5, and agitation of 120 rpm over a 7-day run in a bioreactor (Fig. 4). These parameters resulted in a peak EPS yield of 1.68g/L and peak biomass of 11.4g/L on day 3 as seen in batch 35.

Table 1. Comparison of EPS Production (g/L) by Batch over Time (Days where samples weren't taken are denoted by "-")

Batch Number	Day 0	Day 3	Day 4	Day 6	Day 7
Batch 35 (28°C, 120 rpm, pH 5.5	0	1.68	-	1.02	0.92
Batch 34 (32°C, 120 rpm, pH 5.5)	0	-	0.45	0.39	0.34
Batch 36 (25°C, 120 rpm, pH 5.5)	0	-	0.51	0.08	0.1
Batch 37 (28°C, 80 rpm, pH 5.5)	0	0.36	-	0.72	0.44
Batch 40 (28°C, 150 rpm, pH 5.5)	0	0.81	-	0.49	0.24
Batch 44 (28°C, 120 rpm, pH 4.5)	0	0.12	-	0.14	0.15
Batch 45 (28°C, 120 rpm, pH 6.5)	0	0.38	0.34	0.30	0.32
Batch 58 (28°C, 120 rpm, pH 5.5, 2X media)	0	-	0.84	0.45	0.97

Table 2. Comparison of Biomass Production (g/L) by Batch over Time (Days where samples weren't taken are denoted by "-")

Batch number	Day 0	Day 3	Day 4	Day 6	Day 7
Batch 35 (28°C, 120 rpm, pH 5.5	0	11.4	-	10.85	8.55
Batch 34 (32°C, 120 rpm, pH 5.5)	0	-	5.85	6.30	3.90
Batch 36 (25°C, 120 rpm, pH 5.5)	0	-	0.44	0.57	0.52
Batch 37 (28°C, 80 rpm, pH 5.5)	0	2.20	-	3.10	3.20
Batch 40 (28°C, 150 rpm, pH 5.5)	0	4.70	-	4.85	3.95
Batch 44 (28°C, 120 rpm, pH 4.5)	0	1.45	-	0.90	1.10
Batch 45 (28°C, 120 rpm, pH 6.5)	0	6.30	5.75	1.35	3.00
Batch 58 (28°C, 120 rpm, pH 5.5, 2X media)	0	-	6.85	5.10	6.50

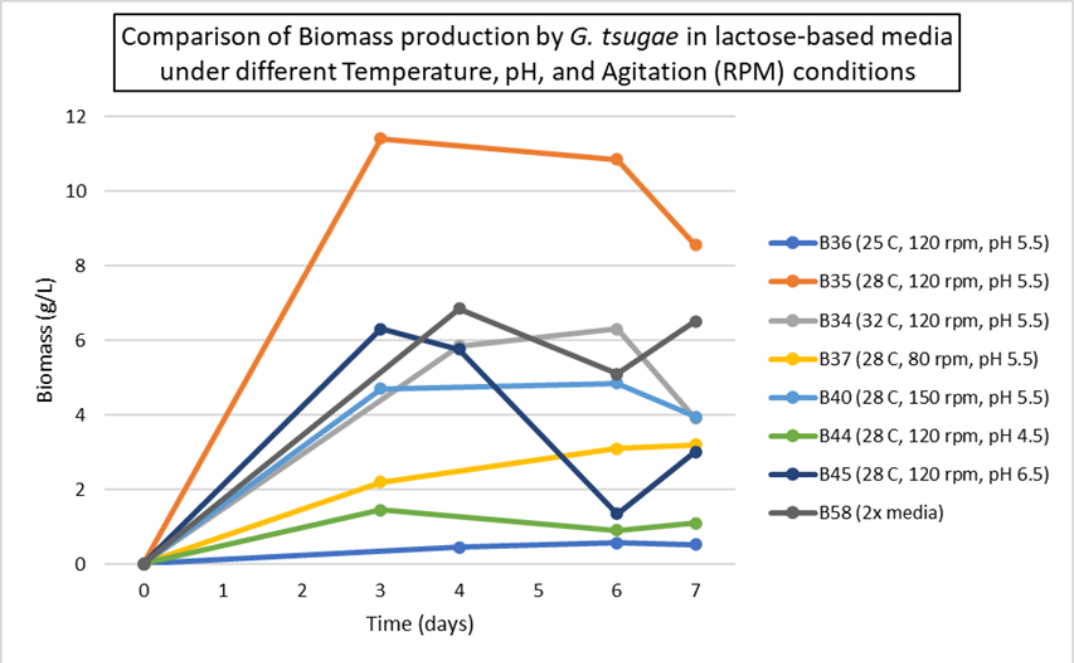

Fig. 5. The measured dried biomass for each batch over a 7-day run is plotted. Changes to the amount of dried biomass over time indicates growth or death depending on the direction of the change. Most batches peak at day 3 or 4, then decrease. This indicates that the fungi are in a growth or exponential phase until day 3 or 4, then enters a death phase. Two batches, B45 and B58, show a secondary exponential phase starting on day 6. B45 was a high pH test and B58 had twice the amount of nitrogen sources in the media. Further research is required to confirm those variables are the cause, but inducing a secondary exponential phase could have industrial applications.

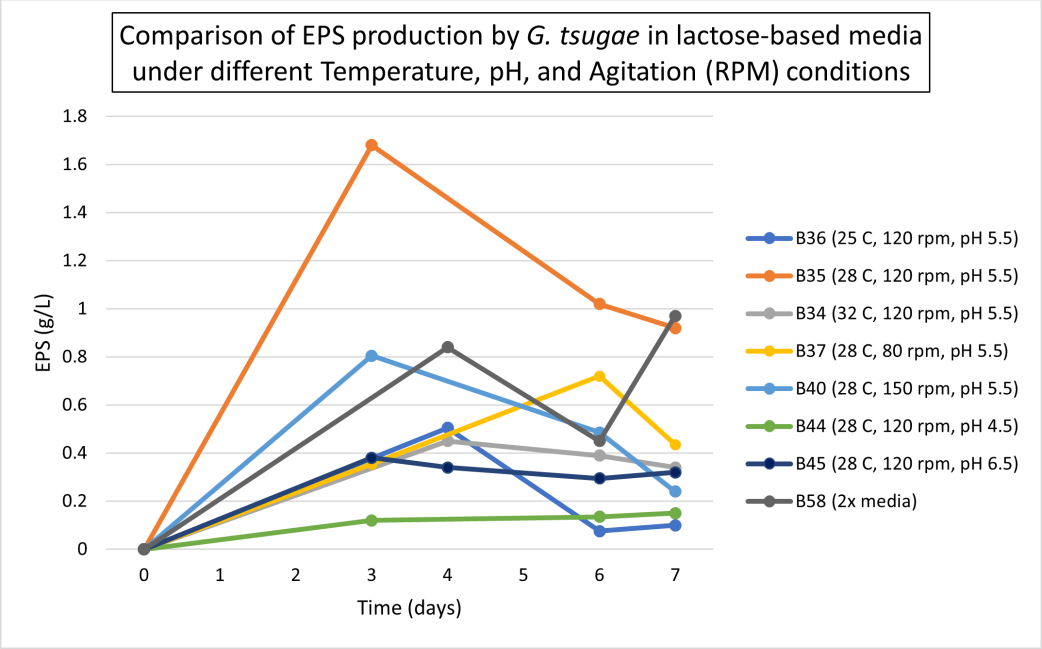

Fig. 6. The measured EPSs for each batch over a 7-day run is plotted. Like biomass, most batches have an EPS peak at day 3 or 4, then a decrease. This correlation between biomass and EPS production indicates that EPSs are created during the growth phase of the fungus life cycle. One possible explanation for the decrease is that when the fungi are in the death phase the fungi consume the EPSs as a food source. Like the biomass results (Fig. 5), B58 with double the nitrogen sources show a secondary phase of growth for EPSs. Further research is required to confirm and explain this result.

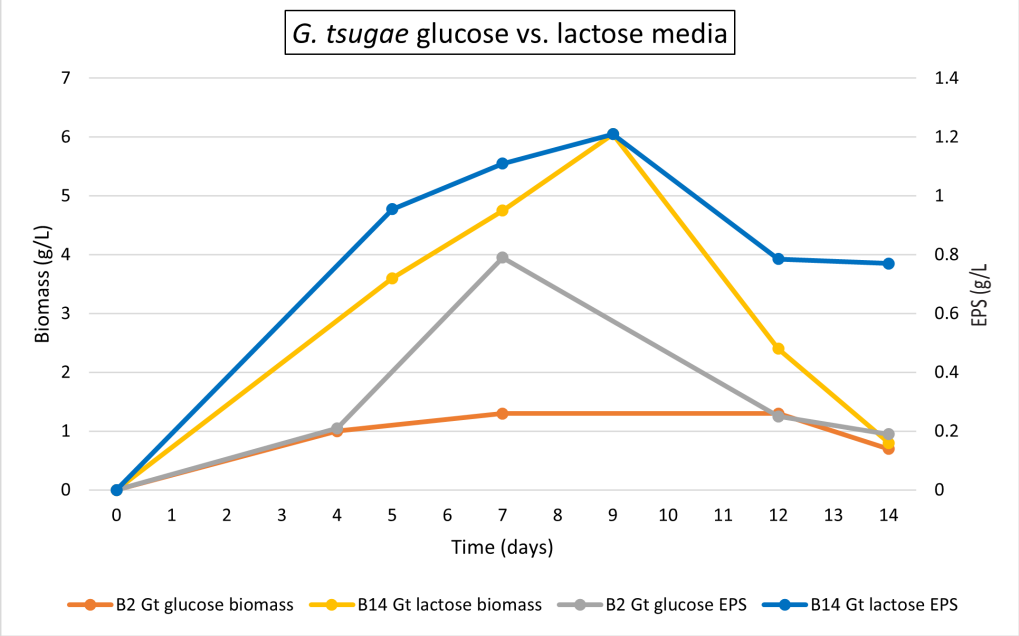

Fig. 7. G. tsugae was grown in two different media formulations containing different sugar sources, glucose and lactose. When grown in lactose, G. tsugae produces more biomass and EPS than when grown in glucose. These batches utilized standard media formulation at 28°C, 100 rpm and 5.5 pH.

It was found that when yeast and malt were doubled (2x) in media formulation (Fig. 9), although EPS production was ultimately sub-optimal, EPS production underwent a secondary exponential phase on days 6-7 with an increase of 0.52 g/L while utilizing the following conditions: 28°C, 120 rpm, 5.5 pH. This is thought to have occurred due to a death phase on days 4-6. Biomass was analogous on those days with an increase of 1.4 g/L. This second exponential phase was also observed in days 6-7 on batches that contained a constant pH different from 5.5 (6.5 and 4.5).

A 7-day two-phase pH strategy was deployed in a 5L bioreactor. The pH started at 5.5 and was increased to 6.5 on day 3 by adjusting the pH set value on the computer connected to the corresponding control tower containing acid and caustic for pH control. Optimal conditions of 28°C and an agitation of 120 rpm remained constant otherwise. While total EPS results were lower than the optimal parameters, EPS data continuing to rise is noteworthy.

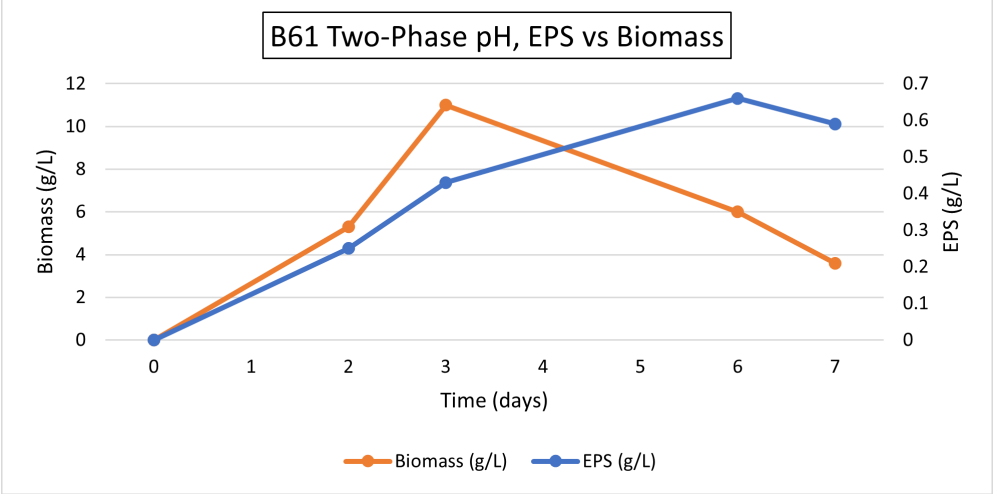

Fig. 8. G. tsugae was grown in a lactose-based media with a temperature of 28°C and 120 rpm. The pH control was done in two phases: starting at 5.5 pH, and then on day 3 the pH was changed to 6.5. This was done because batch 45, using a pH of 6.5, had a secondary exponential growth phase on day 6 (see Fig. 5). By starting with the current optimized pH of 5.5, and then switching over to 6.5 it was hypothesized that a secondary growth phase could be induced to further maximize EPS production. While biomass did not see a secondary growth phase, EPS production continued to rise.

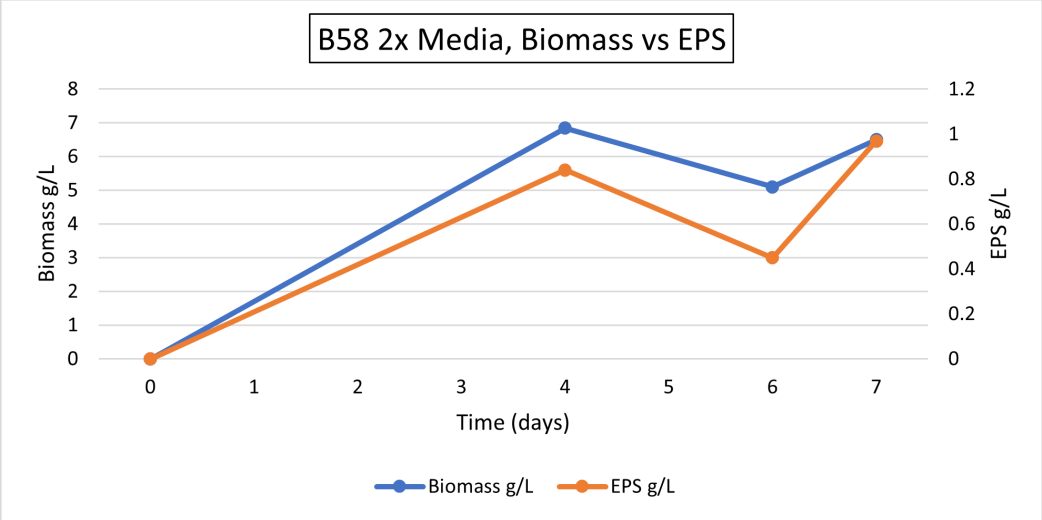

Fig. 9. G. tsugae was grown in a lactose-based media containing twice the amount of nitrogen sources with a temperature of 28°C, 5.5 pH, and 120rpm. Of note, on day 6 the batch underwent a second exponential growth phase producing more EPS.

The optimal conditions for *Ganoderma tsugae* biomass were found to be sensitive to rpm. This is theorized to be because the pellet shearing within the reactor influences the surface area to volume ratio within the submerged mycelium cells and affects the intake of nutrition. In lower rpm, *G. tsugae* was found to have a longer exponential phase, but media consumption was likely hampered due to large pellets lowering the surface area to volume ratio. As rpm increased, resulting in more breakage of the pellets, the surface area to volume ratio for the pellet cells increased, likely allowing for a better media consumption rate. Any rpm past 120 resulted in shearing the pellets, slowing growth and yields.

The optimal pH was shown to be 5.5, though days 6-7 did present signs of a potential second exponential phase. Also, low temperature and low rpm were considered lag phases, where the optimal peak was never reached until around day 6. A change in target pH rather than constant was considered as it was found to be beneficial with other submerged fungi [10] and led to time-critical experimentation in a two-pH strategy. The shake flask used for batch inoculation only progressed to day four and was believed to have affected the EPS growth rate. Contrarily to the EPSs, the Biomass was found to be comparable to optimal conditions from day 1-3 as expected. Beyond day 3, the EPSs were found to have risen, leading to the hypothesis that the age of the inoculated shake flask must be considered when describing optimal conditions for EPS. Additionally, a two-phase, 2x media strategy could also be employed because the 2x media experimentation resulted in a boost in both biomass and EPS production for days 6-7.

In the initial stages of experimentation, a media formulation was designed based on a comprehensive meta-analysis of relevant literature. To enhance the growth conditions and optimize nutrient availability, iterative adjustments by doubling (2x) yeast and malt from the original formulation were performed. This refined composition aimed to augment fungal growth and overall total polysaccharide production by increasing nitrogen sources.

While biomass is a necessary foundation for producing polysaccharides, the kinetics showed that EPSs and biomass can vary in their connectivity between strains. Other studies have focused on *G. lucidum* optimization; however, this study found that polysaccharide production was greater in G. *tsugae*. Batch 35 conditions were found to be potentially favorable in the large-scale commercialization of *G. tsugae* due to significantly higher biomass and EPS production than other batches.

Optimal EPS production by *G. tsugae* was measured in a lactose-based media with a peak concentration of 1.2g/L under initial conditions and a peak concentration of 1.68g/L under optimized parameters (Table 1). Previous research has shown that biomass production between the two species in focus was not proportional to EPS production. Peak biomass production of *G. lucidum* (28°C, 100 rpm, 5.5pH) was measured at 7.6g/L in a glucose-based media, while *G. tsugae* (28°C, 100 rpm, 5.5pH) biomass production was optimized in a lactose-based media with a peak biomass of 6.4g/L. However, EPS production was higher in said batches for *G. tsugae* (1.2g/L) compared to *G. lucidum* (0.8g/L) despite the lower biomass. Because of this data, *G. tsugae* was chosen as the successor to the project due to its ability to generate a larger quantity of polysaccharides. Batches were tested in which one variable was changed at a time from temperature, rpm, and pH. After this, it was found that the optimal conditions resulted in an even higher polysaccharide production at 1.68g/L, a 112.5% increase from the *G. lucidum* initial conditions (Fig. 10). *G. tsugae* has been found to succeed in lactose, a common waste product in food production and manufacturing. This was not the case with *G. lucidum*, as previous batches were found to grow less in lactose compared to glucose.

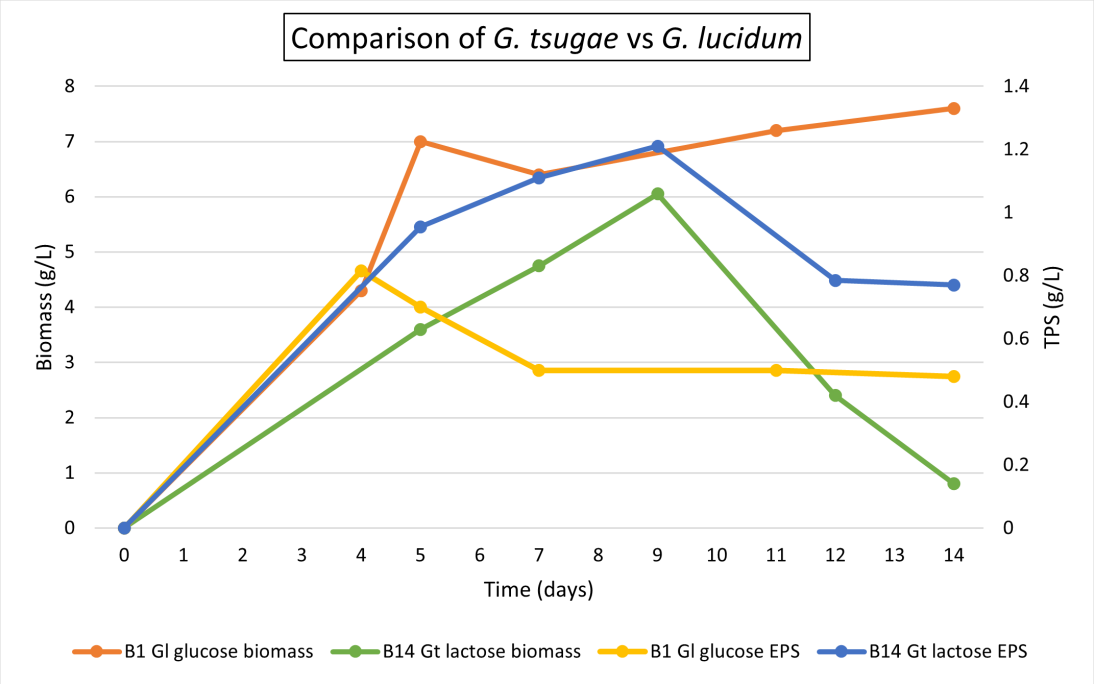

Fig 10. G. tsugae trends in its optimal media and G. lucidum trends in its optimal media against time. Though biomass was sampled higher for G. lucidum on day 5, EPS was not proportional. EPS was found to be highest in G. tsugae when using a lactose-based media.

Pluronic 60 was used as an anti-foaming agent to prevent masses of wall growth in the bioreactor. This was also believed to have lent itself to additional receivable exopolysaccharides in sample taking. This is because the mycelium excretes an extracellular matrix composed, in part, of polysaccharides for pellet cohesion. In the presence of Pluronic 60, it is hypothesized that the mycelium produces more of the extracellular matrix, and thus polysaccharides, to achieve the same level of pellet cohesion. It is important to note, however, that ultrafiltration does not work with Pluronic 60 due to its high molecular weight along with exo-polysaccharides. Therefore, crystallization may have to be used instead of ultrafiltration for downstream processing of target polysaccharides.

Conclusion

The highest biomass and polysaccharide production was observed in a lactose-based media with a constant temperature of 28°C, pH of 5.5, and an agitation of 120 rpm. These optimized parameters resulted in a peak biomass yield of 11.4g/L and a peak polysaccharide yield of 1.68g/L. Experimentation in this research concluded that lactose was a usable medium for *G. tsugae*. The implications of this research have presented a worthy competitor to *G. Lucidum* and will allow for optimum upscale processing of submerged *G. tsugae* fermentation. Lactose, a common waste product, can be utilized by *G. tsugae* to achieve EPSs and biomass, which can be leveraged in a variety of ways, such as in culinary and medicinal industries. Further research will require fine-tuning the optimal pH for each biomass and EPS production, respectively.

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Disclosures. The authors declare no conflicts of interest.

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