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THE CONTENTS OF GANODERIC ACIDS IN MYCELIUM OF DIFFERENT *GANODERMA* SPECIES (GANODERMATACEAE) OBTAINED BY DIFFERENT METHODS OF CULTIVATION

The effect of different cultivation methods on the content of ganoderic acids of 7 species, 10 strains of the Ganoderma genus (Ganoderma applanatum 1899; Ganoderma carnosum 2502; Ganoderma lucidum 1904; Ganoderma resinaceum 2477, 2503; Ganoderma sinense 2516; Ganoderma tsugae 1848, 2024, 2566, Ganoderma oregonense 2560) genus fungi from the IBK Mushroom Culture Collection M.G. Kholodny Institute of Botany of National Academy of Sciences of Ukraine was investigated. It has been shown that the submerged cultivation method is more efficient for the accumulation of ganoderic acids for five strains. In the mycelium of the strain G. sinense 2516 was the highest content of ganoderic acids – 25.2 ± 1.5 mg / g. The productivity (yeld) of ganoderic acids synthesis is much higher with using the submerged culture cultivation method for mycelium of all used species and strains since the use of this method provides the accumulation of much more biomass in comparison with the static liquid cultivation method. The highest yield amount of ganoderic acids was in the mycelium of the G. tsugae 2024 and G. tsugae 2566 species, namely: 0.35 ± 0.019 and 0.36 ± 0.028 g / l. It was proved that the modified extraction method significantly reduces the extraction time of ganoderic acids. Extraction time is reduced from 14 to 2 days. For the G. sinense 2516 and G. tsugae 2024 strains was determined content of the ganoderic acids and their yield in dynamics of grows in the submerged culture on 6, 8, 10, 12, 14, 16, 18 and 20 day of cultivation. The highest amount of the ganoderic acids content was accumulated by the mycelium of the strain G. sinense 2516 – it was 26.4 ± 1.5 mg / g on the 14th day of cultivation. The highest yield of the ganoderic acids was in G. sinense 2516 on 14th day, and G. tsugae 2024 mycelium on the 16th day of cultivation with the next numbers 0.6 ± 0.031, 0.62 ± 0.033 and 0.62 ± 0.027 g/l.

Keywords: Ganoderma, ganoderic acids, submerged cultivation, Ganoderma tsugae, Ganoderma sinense.

Introduction. *Ganoderma* P. Karst. is a genus of polypore fungi, growing on different types of both conifers and deciduous trees. Species of *Ganoderma* are well-known for their medicinal effects, and in Asian countries they have been used in traditional medicine for over 2000 years [1]. It was discovered that not only fruit bodies, but also mycelium of fungi of this genus contain biologically active compounds [2]. Among them are polysaccharides, proteins, amino acids, cytokines and more than 150 different triterpenoids, including ganoderic acids [3, 4, 5]. According to the PubChem database, there are currently over 60 types of ganoderic acids [https://pubchem.ncbi.nlm.nih.gov]. Ganoderic acids show antitumor, antiviral (including HIV), anti-inflammatory, antihistamine activity [6, 7]. Such properties make these substances promising for medical and pharmaceutical applications. However, most of the global studies were focused on *Ganoderma lucidum* (Curtis) P. Karst. as a source of ganoderic acids, while other species have not been sufficiently studied [8]. Therefore, the study of ganoderic acids from different species and strains of *Ganoderma* and the effect of cultivation conditions on their amount in the fungal biomass is promising and necessary.

Material and methods. Strains from the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany NASU [9] cultivated by static and submerged cultivation methods were selected for the study: *Ganoderma applanatum* 1899 (Pers.) Pat., *G. carnosum* 2502 Pat., *G. lucidum* 1904 P. Karst., *G. resinaceum* 2477, 2503 Boud., *G. sinense* 2516 J.D. Zhao, L.W. Hsu & X.Q. Zhang, *G. tsugae* 1848, 2024, 2566 Murrill, *G. oregonense* 2560 Murrill.

The inoculum was initially prepared by cultivating mycelium on a glucose-yeast-peptone agar medium at 26 ± 0.1 °C containing (g/l): glucose – 25, peptone – 3, yeast extract – 3, $MgSO_4$ – 0,25; KH_2PO_4 – 1; K_2HPO_4 – 1; agar-agar – 22; pH – 6,0. Glucose Peptone Yeast (GYP) was used for static and submerged cultivation (g/l): glucose – 25, peptone – 3, yeast extract – 3, MgSO4 – 0,25; KH2PO4 – 1; K2HPO4 – 1; pH – 6,0.

Liquid static cultivation was conducted under the following conditions: temperature 26 ± 0.1 °C, 50 ml of GYP in 250 ml Erlenmeyer flasks for 14 days. Inoculation was performed with 5 discs of mycelium grown on a glucose-yeast-peptone medium. Mycelium discs 5 mm in diameter were cut out with a sterile steel tube at a distance of 8-10 mm from the edge of active colony growth.

Submerged cultivation was conducted on a laboratory shaker under the following conditions: temperature 26 ± 0.1 °C, agitation speed 120 rpm, 100 ml of GYP in 500 ml Erlenmeyer flasks for 14 days. Inoculation was conducted with homogenized inoculum, which was grown in Petri dishes on a glucose-yeast-peptone agar medium. The inoculum was put into flasks in the amount of 10% of the nutrient medium.

To study the dynamics of biomass and productivity of synthesis of ganoderic acids, mycelium was grown by submerged cultivation method. Biomass was taken on 6, 8, 10, 12, 14, 16, 18, and 20 days of cultivation. After cultivation, the mycelium was separated from the nutrient medium by filtration through a nylon filter and washed with a potassium-phosphate buffer. The mycelium was dried to a constant weight at 60 ± 0.1 °C. The amount of dry biomass was calculated in g/l considering the mass of inoculum.

Ganoderic acids (GAs) were extracted from biomass by two methods – according to the classical method [10]: in the initial stage, the mycelium biomass (0.1 g) was extracted in 3 ml 70% methanol by temperature 4 ± 0.1 °C for 7 days; the procedure was repeated twice; after mycelium removal by centrifugation, the supernatant was evaporated at 50 °C under vacuum. The residue was dissolved in distilled water, after which chloroform extraction was performed. GAs from chloroform extract were further extracted with 5% $NaHCO_3$. At the next stage, the pH of the $NaHCO_3$-phase was brought to 2.5 with 2N HCl, after which GAs were extracted from NaHCO3-phase with chloroform. After chloroform removal on vacuum evaporator, the residue containing GAs was dissolved in absolute methanol. Concentration of GAs was measured at 245 nm on spectrophotometer SF 46 LOMO (USSR). A modified version of the classic extraction method was also used. At the initial stage, the mycelium biomass (0.1 g) was extracted in 3 ml 70% methanol at 26 ± 0.1 °C and 120 rpm, for 24 hours. The procedure was repeated twice. Further stages of extraction were carried out according to the classical method. The productivity of GAs synthesis was determined as the amount of ganoderic acids in biomass (g) per unit of nutrient medium (l) during the time of cultivation [11].

The number of repeats was 4. Statistical analysis was performed using Microsoft Excel software.

Results and discussion. When comparing the amount of GAs in mycelium of different species and strains of *Ganoderma* fungi in different cultivation conditions, the higher concentrations were generally observed while using submerged cultivation methods (Fig. 1).

The strains of *G. tsugae* 1848 and *G. sinense* 2516 contained 58% and 42.9% more GAs, respectively, when cultivated by submerged method, as compared to static liquid. Strains of *G. resinaceum* 2477, 2503, *G. oregonense* 2502 and *G. carnosum* 2502 produced approximately the same amount of GAs under static liquid and submerged cultivation conditions (the values are within statistical error limits). Mycelia of *G. tsugae* 2024, 2566, *G. applanatum* 1899, and *G. lucidum* 1904 grown by submerged cultivation had significantly higher amounts of GAs then those grown under static liquid conditions (Fig. 1). The highest level of GAs, 25.2 ± 1.5 mg/g, was found in the mycelium of *G. sinense* 2516, grown by submerged cultivation. As for the strain diversity of species, the GAs content of both strains of *G. resinaceum* was practically the same under both cultivation conditions. At the same time, *G. tsugae* strains had differences in the GAs content in the mycelium grown under static liquid conditions. Namely, the mycelium of *G. tsugae* strain 2566 accumulated 23.3% more GAs than *G. tsugae* 2024 and 56.7% more than *G. tsugae* 1848 (Fig. 1).

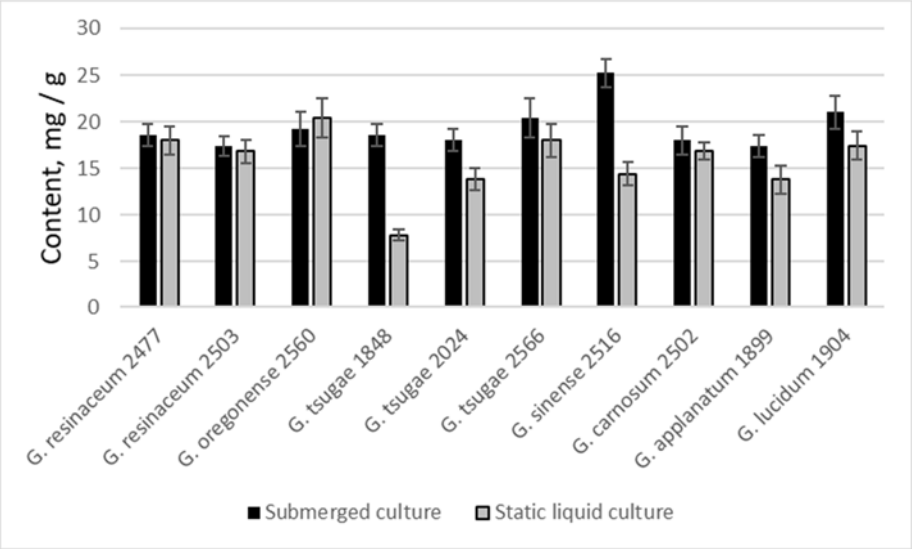

Fig. 1. The content of ganoderic acids in the mycelium of various species and strains of *Ganoderma* fungi under static and submerged cultivation conditions

All strains, except *G. oregonense* 2560, had a significant advantage in the productivity of GAs synthesis by mycelium when grown under submerged cultivation conditions (Fig. 2). This is due to the fact that most species and strains accumulate significantly higher amounts of biomass in submerged culture, which was described in our previous study [11]. The highest productivity of GAs synthesis ≈ 0.35 g / l, was in *G. tsugae* 2024 and 2566 strains grown in submerged culture, with the difference between them being in the range of statistical error (Fig. 2). Also the highest yield of endopolysaccharides synthesys was in mycelium of *G. tsugae* 2024, that was demonstrated in our previous experiment [11]. These results are due to the fact that strain *G. tsugae* 2024 acummulated the highest level of biomass, than other strains.Based on the results of this part of the study, strains of *G. sinense* 2516 (with the highest GAs content) and *G. tsugae* 2024 (with the highest biomass accumulation and productivity of GAs synthesis) in submerged culture were selected for further research. The application of the modified method of GAs extraction allowed to significantly reduce the total time of GAs extraction without loss of substance in comparison with the classical method. Therefore, in our further research a modified method was used.

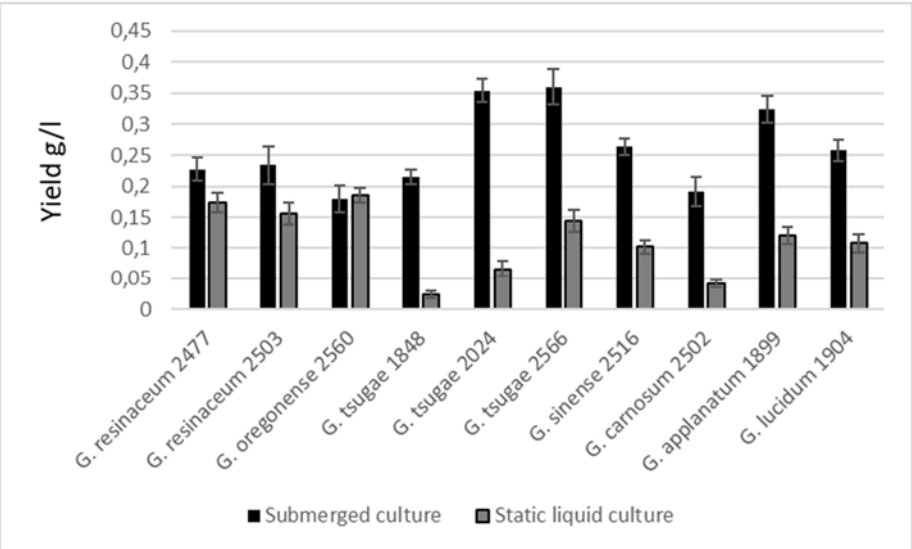

Fig. 2. Productivity of synthesis of ganoderic acids by the mycelium of various species and strains of *Ganoderma* fungi under static and submerged cultivation

As the diagram in Fig. 3 shows, the mycelium GAs content of *G. tsugae* 2024 and *G. sinense* 2516 strains increased gradually from the 6th day to a peak on the 14th day of cultivation, and after that, it began to decline. On the 6th day of cultivation, the amount of GAs in mycelium of both species was almost equal, and increased significantly already on the 8th day of cultivation, by 56.5% and 63.3% in *G. tsugae* 2024 and *G. sinense* 2516, respectively. In all other days there was a gradual increase in the content of GAs, with a dominant strain of *G. sinense* 2516. On the 14th day, the GAs content in the mycelium of both strains reached its maximum, while in *G. tsugae* 2024 this parameter was 9% lower than for *G. sinense* 2516. After that the trend of higher content of GAs was maintained in *G. sinense* 2516 (Fig. 3) In addition, on the 16th, 18th, and 20th day of cultivation, the content of GAs in *G. sinense* 2516 mycelium varied within the statistical error and was lower than that on the 14th day of cultivation. On the 16th day of cultivation, the GAs content in mycelium of *G. tsugae* 2024 was as high as on the 14th day of cultivation, but decreased and remained almost unchanged on the 18th and 20th days of cultivation.

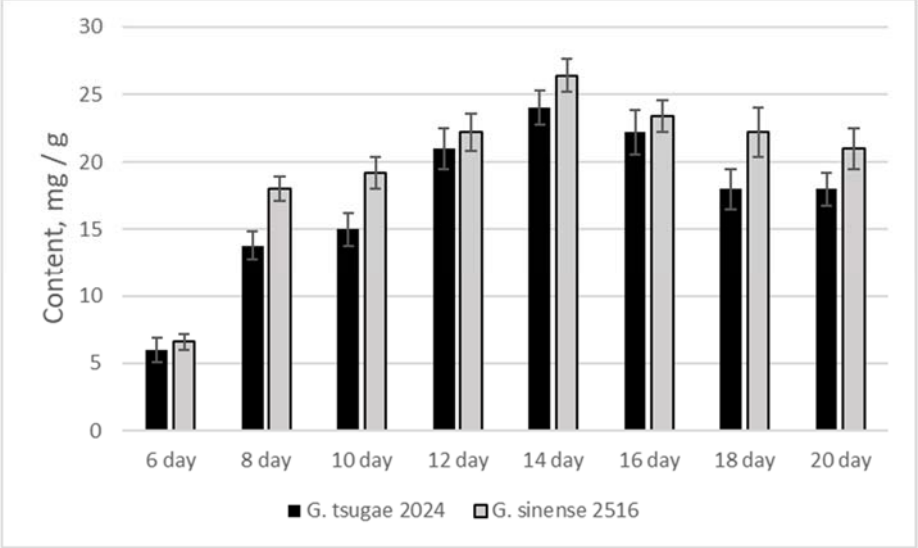

Fig. 3. The dynamics of ganoderic acids content in the mycelium of *G. tsugae* 2024 and *G. sinense* 2516

Data analysis in Fig. 4 indicates that both studied strains, *G. tsugae* 2024 and *G. sinense* 2516 showed extremely low yield of GAs synthesis on the 6th day of cultivation. This is related both to the small amount of biomass and the low level of GAs. On the 8th day of cultivation, the yield of GAs synthesis by both strains increased remarkably, although in *G. sinense* 2516 this value was almost twice as high as in *G. tsugae* 2024. However, on the 10th, 12th, and 14th days this parameter was almost the same for each of the strains (the values lied within the statistical error limits). The peak of productivity in *G. sinense* 2516 occurred on the 14th day and gradually decreased up to the 20th day of cultivation, which was related to the reduction of biomass and GAs content. The difference between the yield of *G. sinense* 2516 on the 14th and 6th days of cultivation (the highest and lowest value) was 98% (Fig. 4). For the mycelium of *G. tsugae* 2024, the peak of productivity occurred on the 16th day of cultivation, but on the 14th, 18th, and 20 days of cultivation the value varied within the statistical error, which was associated with an increase in the biomass accumulation of the mycelium of this strain. The difference between the synthesis productivity of *G. tsugae* 2024 on the 14th and 6th days of cultivation (the highest and lowest value) was also 98% (Fig. 4).

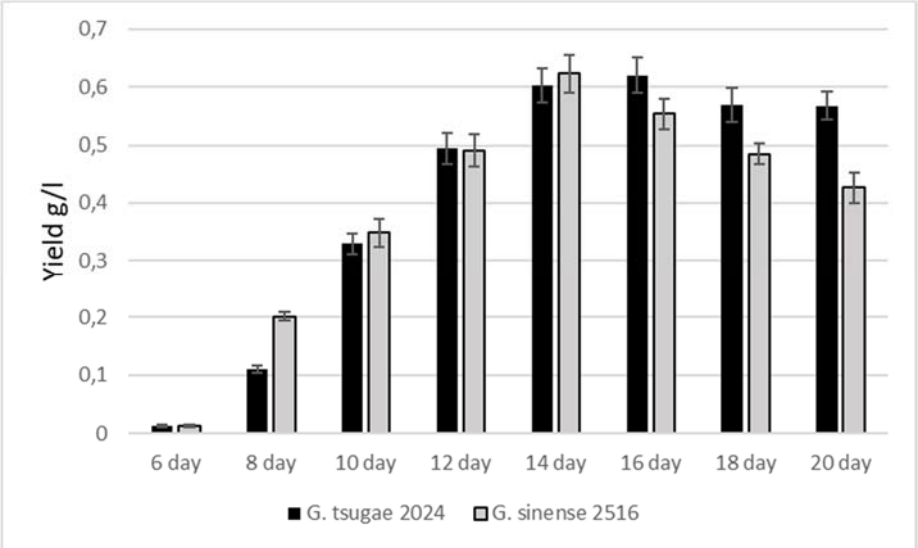

Fig. 4. Dynamics of synthesis productivity of ganoderic acids by *G. tsugae* 2024 and *G. sinense* 2516

Analysis of literature data on the content and productivity of GAs in the mycelium of different species and strains of the genus *Ganoderma*, including the impact of different cultivation conditions shows that the majority of researchers used strains of *G. lucidum* in their studies [14].

Fang and Zhong report GAs content of 18.6 mg/g and GAs synthesis yield of 0.267 g/l under submerged cultivation conditions for *G. lucidum* [12], which is 29.5% and 57.1% lower, respectively than similar parameters in the highest results, what we got in our study: GAs content of *G. sinense* 2516 is 26,4 mg/g, and productivity of *G. tsugae* 2024 is 0.623 g/l. In another study, Zhang and Tang [13] published the results on the positive effect of light exposure on *G. lucidum* mycelium in submerged culture and GAs content was 31 mg/g, which is 14.8% more than the same parameter in our experiment, but the productivity was 0.466 g/l [13], which is 25% lower than the maximum value in our study. According to Tang et al. [15], the GAs content and productivity were higher, but they were using a bioreactor for *G. lucidum* cultivation. The two-stage cultivation method in flasks allowed to obtain very high values for the specified parameters, GAs content was 44.7 mg / g, and the productivity of GAs synthesis was 1.427 g/l in mycelium of *G. lucidum* [16], which was 50% and 56.3% higher than the maximum values obtained in our study.

Wei et al. [8] in their study screened different species for GAs content and optimized cultivation conditions for the selected *G. lucidum* strain. As a result, after selecting the optimal nutrient medium and using 300 l bioreactor, the values of 20 mg/g on the GAs content and productivity of 0.677 g/l were obtained. Therefore, the highest GAs content of *G. sinense* 2516 strain we used in the study was 24.2% higher than that of the above researchers, but the maximum yield what we got was 7% lower. It should be noted that the cultivation time in our study was 5 days longer. It was, because using a bioreactor is better way to cultivation mycelium biomass then the Erlenmeyesr`s flasks with laboratory shaker.

Based on the results of the experiment, it is advisable to consider strains *G. tsugae* 2024 and *G. sinense* 2516 from the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany NASU as promising producers of valuable biologically active substances – ganoderic acids.

Conclusion. It was found that the submerged cultivation method has an advantage over the static cultivation method in terms of GAs accumulation parameter for 5 out of 10 studied strains and species (for 2 strains of *G. tsugae*, *G. sinense* 2516 as well as *G. lucidum* 1904 and *G. applanatum* 1899).

It was proved that the submerged cultivation method has a significant advantage over the static culture in terms of GAs synthesis productivity for all strains and species used in the study, except *G. oregonense* 2560.

It was established that our modified method of GAs extraction allows to significantly reduce the total time of their extraction from mycelium.

It was proved that the mycelium of the different strains of one specie (*G. tsugae*) could accumulate different number of GAs in the same conditions of cultivation.

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ВМІСТ ГАНОДЕРОВИХ КИСЛОТ У МІЦЕЛІЇ РІЗНИХ ВИДІВ РОДУ *GANODERMA* (GANODERMATACEAE), ОТРИМАНОГО РІЗНИМИ СПОСОБАМИ КУЛЬТИВУВАННЯ

У ході дослідження перевірено вплив різних способів культивування на вміст ганодерових кислот 7 видів, 10 штамів грибів роду Ganoderma з Колекції культур шапинкових грибів (IBK) Інституту ботаніки імені М. Г. Холодного НАН України: Ganoderma applanatum 1899; Ganoderma carnosum 2502; Ganoderma lucidum 1904; Ganoderma resinaceum 2477, 2503; Ganoderma sinense 2516; Ganoderma tsugae 1848, 2024, 2566, Ganoderma oregonense 2560. Доведено, що для 5 видів глибинний спосіб культивування є ефективнішим для накопичення ганодерових кислот. Визначено, що найбільший вміст ганодерових кислот був у міцелії штаму G. sinense 2516 – 25,2±1,5 мг/г. Продуктивність синтезу ганодерових кислот набагато вища за використання глибинного способу культивування для міцелію всіх видів і штамів, завдяки тому, що застосування вказаного способу забезпечує накопичення значно більшої кількості біомаси порівняно з методом поверхневого культивування. Найбільша продуктивність синтезу ганодерових кислот була отримана для міцелію видів G. tsugae 2024 та G. tsugae 2566 зі значеннями 0,35±0,019 та 0,36±0,028 г/л. Доведено, що модифікований спосіб екстракції дозволяє значно скоротити час екстракції ганодерових кислот. Порівняно з класичним методом час екстракції зменшується із 14 до 2 діб. Перевірено вміст ганодерових кислот і продуктивність їхнього синтезу для штамів G. sinense 2516 та G. tsugae 2024 в динаміці, вирощених у глибинній культурі, на 6, 8, 10, 12, 14, 16, 18 та 20 добу культивування. Найвищу кількість ганодерових кислот накопичував міцелій штаму G. sinense 2516 – 26,4±1,5 мг/г на 14 добу культивування. Найбільша продуктивність синтезу гародерових кислот була у G. sinense 2516 та G. tsugae 2024 на 14 добу культивування та G. sinense 2516 на 16 добу, і складала 0,6±0,031, 0,62±0,033 та 0,62±0,027 г/л відповідно.

Ключові слова: Ganoderma, ганодерові кислоти, глибинне культивування, Ganoderma tsugae, Ganoderma sinense.

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СОДЕРЖАНИЕ ГАНОДЕРОВЫХ КИСЛОТ В МИЦЕЛИИ РАЗЛИЧНЫХ ВИДОВ РОДА *GANODERMA* (GANODERMATACEAE), ПОЛУЧЕННОГО РАЗЛИЧНЫМИ СПОСОБАМИ КУЛЬТИВИРОВАНИЯ

В ходе проведенного исследования было проверено влияние различных способов культивирования на содержание ганодеровых кислот 7 видов, 10 штаммов грибов рода Ganoderma из коллекции культур шляпочных грибов (IBK) Института ботаники имени М. Г. Холодного НАН Украины: Ganoderma applanatum 1899; Ganoderma carnosum 2502; Ganoderma lucidum 1904; Ganoderma resinaceum 2477, 2503; Ganoderma sinense 2516; Ganoderma tsugae 1848, 2024, 2566, Ganoderma oregonense 2560. Доказано, что для 5 видов глубинный способ культивирования является эффективным для накопления ганодеровых кислот. Определено, что наибольшее содержание ганодеровых кислот было в мицелии штамма G. sinense 2516 – 25,2±1,5 мг/ч. Производительность синтеза ганодеровых кислот гораздо выше при использовании глубинного способа культивирования для мицелия всех видов и штаммов, благодаря тому, что использование указанного способа обеспечивает накопление значительно большего количества биомассы по сравнению с методом поверхностного культивирования. Наибольшая производительность синтеза ганодеровых кислот была получена для мицелия видов G. tsugae 2024 и G. tsugae 2566 со значениями 0,35±0,019 и 0,36±0,028 г/л. Доказано, что модифицированный способ экстракции позволяет значительно сократить время экстракции ганодеровых кислот. По сравнению с классическим методом экстракции время уменьшается с 14 до 2 суток. Было проверено содержание ганодеровых кислот и производительность их синтеза для штаммов G. sinense 2516 и G. tsugae 2024 в динамике, выращенных в глубинной культуре, на 6, 8, 10, 12, 14, 16, 18 и 20 сутки культивирования. Наивысшее количество ганодеровых кислот накапливал мицелий штамма G. sinense 2516 – 26,4±1,5 мг/г на 14 сутки культивирования. Наибольшая производительность синтеза гародеровых кислот была в G. sinense 2516 и G. tsugae 2024 на 14 сутки культивирования и G. sinense 2516 на 16 сутки, и составляла 0,6±0,031, 0,62±0,033 и 0,62±0,027 г/л соответственно.

Ключевые слова: Ganoderma, ганодеровые кислоты, глубинное культивирование, Ganoderma tsugae, Ganoderma sinense.