Том 50 2016 Вып. 5

ФИЗИОЛОГИЯ, БИОХИМИЯ, БИОТЕХНОЛОГИЯ

УДК 582.284.5 : 579.222

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THE EFFECT OF ZINC CITRATE AND ZINC SULFATE ON THE GROWTH AND BIOMASS COMPOSITION OF MEDICINAL MUSHROOM *GANODERMA LUCIDUM*

АЛЬ-МААЛИ Г. А., БИСЬКО Н. А., ОСТАПЧУК А. Н. ВЛИЯНИЕ ЦИТРАТА ЦИНКА И СУЛЬФАТА ЦИНКА НА РОСТ И СОСТАВ БИОМАССЫ ЛЕКАРСТВЕННОГО ГРИБА *GANODERMA LUCIDUM*

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A comparative study has been carried out on the impact of zinc citrate and zinc sulfate on the growth and biomass composition of mycelium of medicinal fungus *Ganoderma lucidum* cultivated in liquid media. It was demonstrated that sulfates and citrates of zinc have different effects on the mycelial growth, content of lipids, ash, polysaccharides and influence the amino acids and fatty acids composition. Zinc citrate significantly increases yield of biomass and crude protein, changes the amino acids compositions and enhance the content of essential amino acids in the mycelium of *G. lucidum* strain 1900. Zinc citrate and zinc sulfate affect the content of lipids and ash differently: zinc citrate enlarges the percentage of ash and doesn't affect the percentage of lipids, vice versa zinc sulfate reduces the content of ash and increases the percentage of lipids. At the same time, zinc citrate significantly increases the amount of stearic acid in the mycelium, relative to the trial with zinc sulfate and control trial without zinc.

Key words: *Ganoderma lucidum*, amino acids, ash, fatty acids, lipids, polysaccharides.

В статье приведены результаты исследования влияния цитрата и сульфата цинка на рост и биохимический состав биомассы мицелия ценного лекарственного гриба *Ganoderma lucidum*, выращенного на жидкой среде в условиях глубинной культуры. В ходе эксперимента было показано, что сульфат и цитрат цинка имеют различное влияние на накопление биомассы, содержание липидов, золы и полисахаридов. Установлено, что цитрат и сульфат цинка влияют на аминокислотный и жирнокислотный состав биомассы *G. lucidum* (штамм 1900). Наличие цитрата цинка в питательной среде способствовало продуктивности синтеза биомассы, сырого протеина, увеличивало содержание незаменимых аминокислот и зольность мицелия. Под действием сульфата цинка уменьшалась зольность биомассы и увеличивалось содержание липидов в мицелии *G. lucidum.* Присутствие цитрата цинка в питательной среде вызывало увеличение содержания стеариновой кислоты в составе липидов биомассы в отличие от сульфата цинка и контроля без цинка.

Ключевые слова: *Ganoderma lucidum,* аминокислоты, жирные кислоты, зола, липиды, полисахариды.

Ganoderma lucidum, or Reishi is a basidiomycete white rot fungus which is traditionally used in China, Japan and other countries in the Asian Region. It is used to treat numerous diseases, such as cancer, immunological disorders, inflammation etc. (Chen et al., 2006; Paterson, 2009). The effectiveness of Reishi has been attributed to polysaccharide fractions and triterpenes, which have various positive effects on disease control with little side effects (Sandiya et al., 2009; Suarez-Arroyo et al., 2013; Wasser, 2014). Moreover, *G. lucidum* has been used as an excellent source for lignocellulose degrading enzymes, such as laccase and Mn peroxidase (Wang, Ng, 2006).

Macro- and micronutrients, especially essential biometals, are often used to increase the yield of medicinal mushroom biomass and their biological activity in the modern industrial cultivation (Cui et al., 2013). This is due to the fact that trace elements are involved in numerous intracellular biochemical processes. A number of authors noted that some trace elements, including zinc, have a positive effect on biosynthesis of intracellular and extracellular polysaccharides (Zou, 2005; Xiao, 2006; Zhi-ling, 2009), ga-

noderic acid (Xu et al., 2013, 2014) and amino acids composition (Zou, 2005) in some medicinal mushrooms. It should be noted the special role of zinc in many key metabolic pathways, including synthesis of amino acids, metabolism of RNA and DNA, signal transduction, and gene expression. Zinc is the only metal which appears in all enzyme classes.

The aim of our research was to study the influence of zinc citrate and zinc sulfate on the growth and biomass composition of medicinal mushroom *G. lucidum*.

Materials and Methods

Strain and cultural conditions. The studied strain of *G. lucidum* 1900 was obtained from Culture Collection of Mushrooms from M. G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Kyiv (Buchalo et al., 2011). Zinc citrate was obtained from Institute nanobiotechnologies and resource conservation of Ukraine, Kyiv.

In this study we used glucose-peptone-yeast extract medium (GPY) with a following composition of (g/L): glucose — 25, peptone — 3, yeast extract — 3, K_2HPO_4 — 1, KH_2PO_4 — 1, $MgSO_4 \times 7H_2O$ — 0.25, distilled water — 1000 ml, pH 6.5. Various concentrations of zinc citrate or zinc sulfate, containing equivalent compositions of metal, were added to media. Control medium did not contain zinc. Mycelium of this strain was grown in a submerged culture on a rotary shaker (120 rpm) at 26 °C and in 250 ml Erlenmeyer flasks, containing 50 ml of liquid media. The biomass was harvested after 9 days of cultivation in the liquid medium. Inoculum obtained under similar conditions in 5 days. We used 5 ml of inoculum for inoculation flasks with liquid medium.

Determination of dry weight and content of total nitrogen, crude protein, lipids, total carbohydrates, endopolysaccharides and ash. The biomass was harvested after 9 days of cultivation in the liquid medium, filtered, washed, dried to a constant weight at 105 °C and weighted. Total nitrogen content (N_{total}) in the mycelium determined by Kjeldahl method, crude protein content was determined as $N_{total} \times 6.25$ (AOAC, 1995). The ash was obtained by the standard method (AOAC, 1995). Lipids were extracted from undried mycelium by a modified method of Bligh and Dyer (Manirakiza et al., 2001). Amount of total carbohydrates was calculated, using the following formula: $M_c = M_b - (M_{cp} + M_l + M_a)$, where M_c — weight of total carbohydrates, M_b — weight of biomass, M_{cp} — weight of crude protein, M_l — weight of lipids, M_a— weight of ash, g/L.

Endopolysaccharides were extracted by the standard method (Mizuno et al., 1999). Number of endopolysaccharides was determined by gravimetric method after drying them at 105 °C.

Amino acid detection method. Amino acid composition was analysed by high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride and o-phthalic anhydride.

Sample preparation: 0.1 g of the mycelium was placed in vial and 2 mL of 6N HCl were added. Hydrolysis was carried out for 24 hours at 110 °C. 0.5 mL of hydrolyzate obtained from centrifugation was evaporated and washed by distilled water 3 times. After evaporation, the extract was dissolved with 0.5 mL of distilled water and filtrated with 0.2 μm regenerated cellulose filter membrane. Obtainment of fluorescent derivative was carried out by an automatic programmed procedure before the samples were inserted in chromatography column.

The conditions for detection of amino acids were as follows: high-performance liquid chromatograph Agilent 1200 (Agilent technologies, USA); chromatography column: Zorbax AAA, 150 mm × 4.6 mm × 3 μm; Mobile phase A: 40 mM Na_2HPO_4, pH 7.8; B — acetonitrile : methanol : water (45 : 45 : 10, v/v/v); temperature of column thermostat is 40 °C. Detection of derivatized amino acids was implemented, using fluorescence detector. Identification of amino acids was performed by comparing the retention times with a mix of standard amino acids (Agilent 5061-3334) (Henderson et al., 2000; Jámbor, Molnár-Perl, 2009a, 2009b).

Fatty acids detection method. The methyl ethers of fatty acids were obtained by a standard method (Christie, 1989). The methyl ethers of fatty acids were determined by gas chromatography-mass spectrometry (GC/MS) Agilent 6890N/5973 inert. chromatography column: HP-5MS, 30m × 0.25 mm × 0.25 μm.

Chromatographic conditions: the carrier gas was helium at a flow rate of 1 mL/min. The injector was kept at 250 °C. The temperature gradient was 150—250 °C, at the rate of 40 C/min.

Mass spectrum conditions: ion source: electron ionization (EI); electric energy: 70 eV, chromatogram was obtained by SCAN mode in the range of 40—700 *m*/*z*. The identification of the components of the studied samples was performed, using the library of mass spectra NIST 02 and standard mix of methyl esters of fatty acids (Supelco, USA). Amount of each fatty acid was calculated as a percentage of total fatty acids.

Statistical analysis. Values are mean of three independent experiments done in triplicate and are expressed as mean ± errors. Data were statistically analyzed by t test using OriginPro 8.5.1, Origin-Lab Corporation, USA. Differences between means at 5 % ($p < 0.05$) level were considered to be significant.

Results and Discussion

Effect of different concentration of zinc citrate and zinc sulfate. Previously, we studied the effect of citrate and sulfate of different metals (iron, copper, manganese and zinc) on mycelial growth of *G. lucidum* 1900. Analysis of the results of this study showed that only zinc citrate significantly affected on increment of biomass of studied strain. Also, in this phase of our research we studied the effect of different concentrations of zinc (citrate and sulfate form) on the growth of mycelium. The results obtained indicate the concentration of 1 mg/L of Zn^{2+} (citrate form) was optimal for mycelial biomass synthesis (Fig. 1). Thus, mycelium of *G. lucidum* 1900 on GPY-zinc citrate medium with concentration of 1 mg/L of Zn^{2+} increased the biomass by 28.3 % relative to the control medium. Whereas

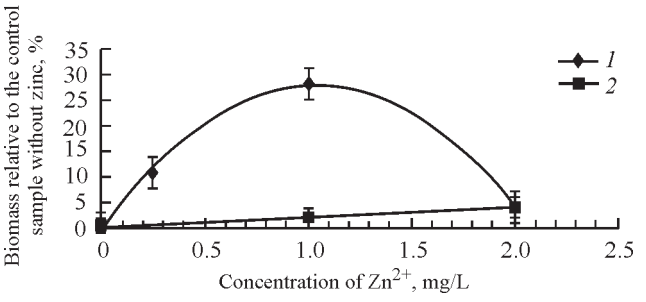

Fig. 1. The influence of zinc citrate (*1*) and zinc sulfate (*2*) on the synthesis of biomass of *Ganoderma lucidum* on GPY medium.

the amount of biomass harvested from the GPY-zinc sulfate medium (with concentration of 1 mg/L of Zn^{2+}) was the same as in the control medium without zinc. So, this concentration of zinc (1 mg/L) was chosen for further study, which compared the effects of zinc sulfate and zinc citrate on the growth of mycelium *G. lucidum* 1900.

Biochemical composition of mycelium. Analysis of the main components of the biomass showed the following changes. Zinc citrate and zinc sulfate equally affected the content of crude protein in the mycelium. In both cases, crude protein content in mycelium was growing by approximately 5 % relative to the control medium without zinc (Table 1). It is likely, this effect is due to the action of zinc ions.

Zinc citrate and zinc sulfate equally reduced the total amount of carbohydrate approximately by 4.5 %, relative to the control medium (Table 1). And in both case, the fraction of endopolysaccharides was reduced in relationship to the control trial. So, their content in mycelium decreased 1.5 times on GPY-zinc citrate medium and 1.2 times on GPY-zinc sulfate medium relative to the control medium. Noted, that Zhi-ling reported that zinc sulfate (0.1—0.2 ‰) significantly increase the contents of extracellular polysaccharide of *G. lucidum* (Zhi-ling, 2009).

Instead, adding zinc citrate to the culture medium had no affect on the total lipid content in mycelium relative to the control medium without zinc. At the same time, the presence of zinc sulfate (Zn^{2+} 1 mg/L) on the GPY medium increased the total content of lipids in the mycelium (approximately twice relative to control medium without zinc and relative to medium with zinc citrate).

Most dramatic is the effect of sulfate and zinc citrate on the amount of ash in the mycelium. So, the percentage of ash in *G. lucidum* mycelium on GPY-zinc sulfate medium decreased relative to the control medium by nearly 1.55. In contrast, addition of zinc citrate to the GPY medium increased percentage of ash in the mycelium *G. lucidum*.

So, zinc citrate and zinc sulfate have the same positive effect on crude protein and carbohydrate content of *G. lucidum* 1900 biomass. But they affect the content of lipids and ash differently: zinc citrate enlarges the percentage of ash and doesn't affect the percentage of lipids, vice versa zinc sulfate reduces the content of ash and increases the percentage of lipids.

Productivity. It should be noted, that given the significant growth of biomass in the case of zinc citrate, productivity of crude protein (gram per liter of used substrates) is raised by 49.7 % in relationship to the control medium (Fig. 2), as opposed to productivity of crude protein on the GPY-zinc sulfate medium, which is increased by 20 % (Fig. 3). Productivity of carbohydrates (gram per liter of used substrates) on GPY-zinc citrate medium is increased by 19 % relative to the control medium (Fig. 2), whereas on GPY-zinc sulfate medium we do not observe statistically significant changes in this productivity (Fig. 3). But in both cases, as with zinc citrate and zinc sulfate, we observed significant reduction of the productivity of endopolysaccharides synthesis.

Although, zinc citrate has a positive effect on the yield of lipids (productivity is increased by approximately 31 %), zinc sulfate behaved more effectively. Thus, the productivity of lipids on the GPY-zinc sulfate medium is increased by 94.5 %.

Amino acids content. Amino acids composition of the mycelium depends on the occurrence of zinc citrate or zinc

Table 1

The influence of zinc citrate and zinc sulfate on the biomass production and biochemical parameters of *Ganoderma lucidum* on GPY medium

Parameters	Control medium	GPY-zinc citrate medium	GPY-zinc sulfate medium
Biomass, g/L	7.79 ± 0.23	9.99 ± 0.23	8.14 ± 0.64
Increase of mycelial biomass relatively to control medium, %	0	28.34	4.53
Crude protein, g/L	2.00 ± 0.01	2.96 ± 0.07	2.38 ± 0.03
Crude protein, % biomass	25.67	29.6	30.06
Total carbohydrates, g/L	5.17	6.17	4.93
Total carbohydrates, % biomass	66.35	61.81	62.12
Endopolysaccharides, g/L	0.63 ± 0.04	0.53 ± 0.02	0.54 ± 0.02
Endopolysaccharides, % biomass	8.04	5.28	6.78
Lipids, g/L	0.13 ± 0.02	0.21 ± 0.01	0.32 ± 0.04
Lipids, % biomass	2.1	2.1	4.03
Ash, g/L	0.46 ± 0.02	0.65 ± 0.05	0.30 ± 0.02
Ash, % biomass	5.88	6.49	3.79

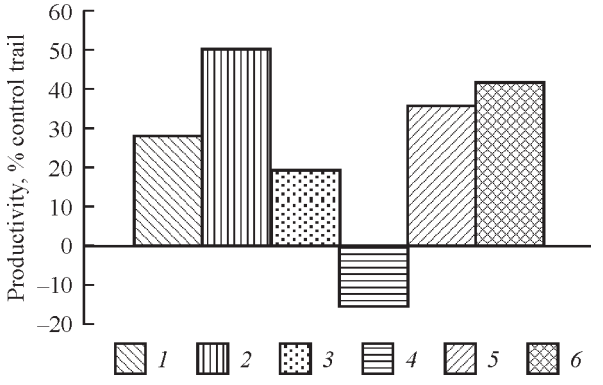

Fig. 2. Productivity of biomass and some biochemical compounds of *Ganoderma lucidum* 1900 during cultivation on GPY-zinc citrate medium relative to the control trial.

1 — biomass, *2* — crude protein, *3* — total carbohydrates, *4* — endopolysaccharides, *5* — lipids, *6* — ash.

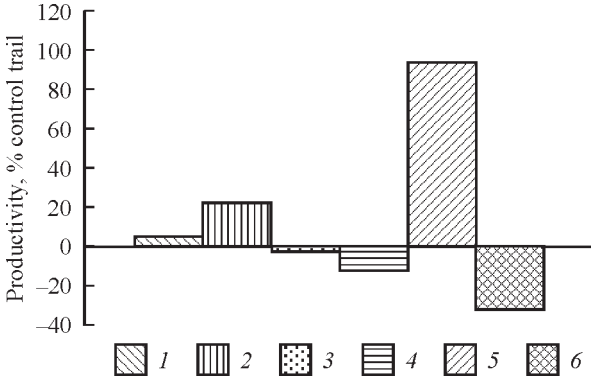

Fig. 3. Productivity of biomass and some biochemical compounds of *Ganoderma lucidum* 1900 during cultivation on GPY-zinc sulfate medium relative to the control trial.

1 — biomass, *2* — crude protein, *3* — total carbohydrates, *4* — endopolysaccharides, *5* — lipids, *6* — ash.

sulfate in the medium. It is necessary to note, that zinc citrate and zinc sulfate modified amino acid composition of the mycelium differently. Both compounds have the same effect on the amount of L-serine. Thus the amount of L-serine in mycelium on GPY-zinc sulfate medium is decreased 1.2 times and on GPY-zinc citrate medium 1.4 times relative to the control medium (Table 2). Moreover, zinc sulfate increased the content of L-proline in the mycelium, as opposed to zinc citrate which did not affect the amount of L-proline. At the same time, the amount of L-histidin, L-phenylalanine and L-lysine is increased in the mycelium on GPY-zinc citrate medium, but the amount of L-arginine is reduced in relationship to the control medium. Thus the amount of total essential amino acids in the mycelium is increased on GPY-zinc citrate medium respective to the control medium. Conversely, the addition of zinc sulfate to the GPY medium didn't affect this score (Table 2). But, Zou reported that amount of total essential amino acids is markedly increased in mycelium of *Agaricus brasiliensis* on the medium with zinc sulfate (Zou, 2005).

So, zinc citrate significantly increases yield of crude protein, changes the amino acids compositions and enhance the content of essential amino acids in the mycelium of *G. lucidum* 1900.

Table 2

The influence of zinc citrate and zinc sulfate on the amino acids content in mycelium of *Ganoderma lucidum* on GPY medium

Amino acid	Control medium	GPY-zinc sulfate medium	GPY-zinc citrate medium
L-Aspartic acid	9.28 ± 0.21	9.64 ± 0.18	9.57 ± 0.23
L-Serine	13.83 ± 0.36	11.27 ± 0.21	9.86 ± 0.25
L-Glutamic acid	7.69 ± 0.35	8.22 ± 0.24	8.32 ± 0.31
L-Histidin	1.8 ± 0.09	1.99 ± 0.11	2.85 ± 0.17
L-Glicine	7.14 ± 0.31	7.72 ± 0.28	6.57 ± 0.28
L-Threonine	6.69 ± 0.27	7.24 ± 0.21	7.49 ± 0.15
L-Arginine	5.99 ± 0.30	5.58 ± 0.24	4.88 ± 0.17
L-Alanine	8.6 ± 0.21	8.98 ± 0.27	8.4 ± 0.27
L-Tyrosine	2.35 ± 0.17	1.74 ± 0.11	2.34 ± 0.13
L-Valine	4.59 ± 0.22	4.62 ± 0.22	4.99 ± 0.19
L-Methionine	0	0	0
L-Phenilalanine	4.70 ± 0.24	4.46 ± 0.20	6.03 ± 0.14
L-Isoleucine	4.88 ± 0.23	4.83 ± 0.14	5.38 ± 0.23
L-Lysine	10.52 ± 0.32	10.77 ± 0.26	12.06 ± 0.25
L-Leucine	4.15 ± 0.15	3.59 ± 0.24	3.48 ± 0.22
L-Proline	7.79 ± 0.21	9.34 ± 0.22	7.78 ± 0.11
L-Cystein	0	0	0
Sum of essential amino acids	35.53	35.51	39.43

Fatty acids content. We detected 10 fatty acids on the *G. lucidum* 1900 biomass: myristic acid (C14 : 0), pentadecanoic acid (15 : 0), palmitic acid (C16 : 0), cis- and trans-form of palmitoleic acid (C16 : 1), margaric acid (C17 : 0), stearic acid (C18 : 0), oleic acid (C18 : 1 cis-9), linoleic acid (C18 : 2 cis, cis-9, 12), lignoceric acid (C24 : 0). Quantity of predominant fatty acids was unchanged during all trials (GPY without zinc, GPY-zinc citrate, GPY-zinc sulfate). Thus, the amount of palmitic acid is approximately 22 %, oleic acid — 38 % and linoleic acid — 26—27 %. Amount of stearic acid (6.17 ± 0.15 %) is increased 3.7 times relative to the control medium and 5.2 times relative to the GPY-zinc citrate medium. Amount of the rest of identified fatty acids was insignificant and did not exceed 1 % of each.

So, zinc citrate increases the yield of lipids by 31 % and insignificant modifies the fatty acids composition. In that time, zinc sulfate significantly rises productivity of lipids and has no effect on fatty acids compositions.

Conclusion

For the first time, it was demonstrated that sulfates and citrates of zinc have different effects on the mycelial growth, content of lipids, ash, polysaccharides and influence on amino acids and fatty acids composition. So, zinc citrate and zinc sulfate affect the content of lipids and ash differently: zinc citrate enlarges the percentage of ash and doesn't affect the percentage of lipids, vice versa zinc sulfate

reduces the content of ash and increases the percentage of lipids. Also zinc citrate significantly increase the amount of stearic acid the mycelium of *G. lucidum* 1900. In that time, zinc citrate modifies the fatty acids composition, relative to the trial with zinc sulfate. Zinc citrate significant increases yield of crude protein, changes the amino acids compositions and enhance the content of essential amino acids in the mycelium of *G. lucidum* 1900. Also the amount of total essential amino acids in the mycelium is increased on GPY-zinc citrate medium respective to the control medium and medium with zinc sulfate.

We are grateful to V. G. Kaplunenko (Institute of nanobiotechnologies and resource conservation of Ukraine, Kyiv) for synthesizing and providing zinc citrate.

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Received 10 12 2015