UDC 579.017.8

S.L. Golembiovska, T.V. Dvornyk, L.M. Yanchenko, B.P. Matseliukh

Institute of Microbiology and Virology, NASU, 154, Zabolotny str.,
Kyiv, Ukraine, e-mail: golembiovska@ukr.net

THE PRODUCTIVITY OF MUTANT STRAIN *STREPTOMYCES GLOBISPORUS* 1912-4LCPHP7 IN THE DIFFERENT CONDITIONS

Aim. *To determine the cause of decrease of carotenoid synthesis of lycopene in liquid medium from 4.2 to 2.8 mg/g of dry biomass in strains of Streptomyces globisporus 1912-4Lcp Hp7 and to select medium components and optimal conditions that contribute to the stabilization of the process.* ***Methods.*** *The selection of the producer was carried out to the feature of early synthesis of lycopene. Selected culture was cultivated in different liquid conditions. The biomass was determined gravimetrically, the amount of lycopene – with spectrophotometric method.* ***Results.*** *The reason for the decreased activity of the producer of lycopene S. globisporus 1912-4Lcp Hp7 of liquid cultivation was culture dissociation with formation of uncolor variants, frequency of 1 x 10^{-3} that observed after sieving suspension mycelium on agar medium. As it turned out, their accumulation was caused by the adding of corn flour into the cultivation medium, which, combined with oatmeal is used to nowadays, because it was this source of carbon and energy that contributed to the accumulation of biomass producer. The conditions for the cultivation of producer were defined: pH 7,0, 28°C, V_{inc}10 ml, V_{med}100 ml and concentration of salts: $MgSO_4$ x $7H_2O$ – 0,1%, $(NH_4)_2MoO_4$ – 0,05%, which stabilized the process of biosynthesis of lycopene in the liquid conditions. It is shown that lycopene biosynthesis also contributes to using sodium casein and temperature of 37 °C and $KMnO_4$, which is used for selection of the culture.* ***Conclusions.*** *It was found the reason for the decrease of lycopene biosynthesis in strains S. globisporus 1912-4 Lcp Hp7 and defined medium, optimal conditions and salt, which stabilized the process of biosynthesis of lycopene in liquid cultivation conditions.*

Key words: streptomyces, strain producer, biosynthesis of lycopene.

Lycopene (ψ -carotene, $C_{40}H_{56}$) is a the precursor of all color C_{40}-carotenoids, including β-carotene, has purple-pink color. Its molecule has 11 conjugated double bonds, which connect and neutralize free electrons and prevent damage of the cells. According to clinical studies lycopene reduces growth of cancer cells, especially reproductive cells and lycopene therapy improves reproductive function [10].

The high content of lycopene is in tomatoes (0.4–0.9 mg/g dry weight, depending on the sort), as well as in grapefruit, persimmon and watermelon. Except the plants, lycopene is formed fungi and microorganisms. Its industrial producers is *Blakeslea trispora* (0.8–1.0 g/l), in which there have been blocked enzymes that convert lycopene to beta-carotene by diphenylamine [8].

Mutants with constitutively form carotenoids lycopene and beta-carotene were obtained in colorless strain *S. globisporus* 1912 in the Department of Genetics of Microorganisms in 1998, among which the attention was attracted to the variant *S. globisporus* 1912-4 Lcp [5]. It accumulates only one carotenoid lycopene in an amount of 1.5–2.2 mg/g dry biomass (DBM). In 2010, in order to obtain highly productive and stable mutants – lycopene producers for this strain used a series of mutagenesis. As a result, there were selected a mutant strain of *S. globisporus* 1912-4 Lcp Hp7, which obtained the ability to accumulate 4.2 mg/g DBM after of treatment inoculum by hydrogen peroxide in amount of 0.5% after 24 h of liquid cultivation [1]. Such accumulation of lycopene was the highest among described in the literature carotenoid biosynthesis streptomycetes, but during three years synthesis of lycopene has decreased to 2.8 mg/g DBM. It can be concluded that in spite of the constitutive character the synthesis of lycopene and presence of stress-factor (H_2O_2), a producer needs regular amplification of measures of gene expression of carotenogenesis.

Therefore, our aim was to determine the cause of decreased synthesis of lycopene in strain *S. globisporus* 1912-4Lcp Hp7 in liquid medium and to choose the best component structure of medium and cultivation conditions to stabilize the carotenogenesis process.

Materials and methods

For work there were used lycopene producer *S. globisporus* 1912-4Lcp Hp7. Selection of the culture medium was carried out to using standard culturing for streptomycetes natural flour: corn, oats, soybean meal, they were compatible combinations and products of grain processing: wheat and rye bran. Their main composition is given in Table 1 [4].

Table 1

The composition of the main components of natural substrates (%) [4]

Raw material	Protein	Lipid	Mono-saccharide	Carbohy-drates	Na^+	K^+	Ca^{2+}	Mg^{2+}	P^{5+}	Fe^{2+}
Corn flour	7.2	1.5	1.3	68.9	0.04	1.47	0.20	0.36	1.09	0.03
Soy flour	34.9	17.3	5.7	3.5	0.06	16.07	3.48	2.26	6.03	0.15
Oat flour	10.0	6.2	1.1	36.5	0.37	4.21	1.17	1.35	3.61	0.05
Wheat bran	12.5	1.9	3.4	61.3	Traces	Traces	0. 39	0.94	3.36	0.04
Rye bran	10.7	1.6	5.6	63.4	0.19	Traces	0.43	0.75	2.56	0.04

For monomediums we brought respectively flour 40 g/L. In medium from soybean we added 10 g/l of starch to balance the carbohydrates with present in it high rate protein. Combined medium consisted of 20 g/l of the substrates. Salts NaCl (5.0 g/l) and $CaCO_3$ (3.0 g/l) were brought in all medium.

Feature selection of the culture of early and intensive lycopene synthesis was carried out on agar medium, followed by siftings individual colonies on agar surface plates. The most productive superficial mycelium (7–10 day) have been put in the conical Erlenmeyer flask (750 ml) and have been grown for 40–48 h in liquid continuous. Substrate served with corn-soybean liquid culture medium. The inoculum in the amount of 10% has been contributed to the appropriate fermentation medium and cultured on a shaker at 260 rev/min for 72 h 28 °C. The initial value pH was regulated by HCl or NaOH. The studied temperatures were 21, 28 and 37 °C.

The influence of organic salts in concentration of 0,5 % were determined on liquid corn-oatmeal medium. The effect of metals on the biosynthesis of lycopene was observed on agar medium after making in holes 0,1 ml 1% solution of metal salt: $Ca(NO_3)_2$, $CaCl_2$, $MgCl_2$, $FeCl_2$, $ZnSO_4$, $MgSO_4$, $CuSO_4$, $FeSO_4$, $(NH_4)_2MoO$ and except $KMnO_4$ – 0,05% after 48 hours of incubation at 28 °C. Then optimal concentrations of suitable salts were determinated.

The biomass accumulation was determined after washed by distilled water of medium, centrifugated at 5000 rev/min for 10 minutes, dried at 60 °C to constant weight and was weighed. Then biomass (10 mg) was ground with quartz sand in a porcelain pounder, lycopene was extracted by acetone and selected solution was centrifuged at 12000 rev/min for 3–5 min. The quantitative content of lycopene (X, g) was defined by the formula [7]:

$$X = A \times y/100E,$$

A – absorption of the sample at 472 nm on a spectrophotometer Beckman DU-8B*;* *n* – the amount of pigment solution, ml*; E* – extinction coefficient of lycopene 3450.

Results and discussion

To begin, it should be noted that the method of obtaining in 2010 the mutant strain S. *globisporus* 1912-4 Lcp Hp7 was specific. Nearly lethal dose of hydrogen peroxide (2%) was put in liquid corn-soy media for 24 h culturing strain *S. globisporus* 1912-4 Lcp, was grown one day and was seeded on similar agar medium. The 28 variants with able to early synthesis of lycopene (48 h) were selected from the surface of agar, but only a mutant strain of *S. globisporus* 1912-4 Lcp Hp7 was stable to this sign. It was observed that after cultivation with the introduction of hydrogen peroxide mycelium had visually intense color than colonies on agar. It was established that accumulation of lycopene was 4.2+0.05 mg/g DBM in making hydrogen peroxide (2%) for 24 h, without making peroxide – 2.8+0.05 mg/g DBM. Contributed to the intensification of the lycopene synthesis and unlimited biomass concentration of hydrogen peroxide (0.5%) was picked up. It was thought that peroxide increases of synthesis of the lycopene in producer cells and limits the growth colorless variants.

Through this practice, while lycopene yield was consistently high and did not need to change methods of cultivation. But later we began to observe a gradual decrease in carotenoid synthesis to 2.8 mg/g DBM. It has been suggested that besides intracellular antioxidant components (lycopene) the producer increased the synthesis of extracellular enzymes protection. Its ability to neutralize oxygen free radicals in the medium, reduce their impact on the cells that promoted the normal growth of colorless variants. This hypothesis was suggested but not investigated.

Therefore, based on the property of the strain *S. globisporus* 1912-4 Lcp Hp7 to accumulate lycopene in an amount of 4.2 mg/g DBM it was decided to begin to define the optimal culture media for this. As a result of cultivation it was determined that the accumulation of biomass has contributed the corn meal, due to its high content of carbohydrate composition (Table 2). The synthesis of lycopene was the highest on media with soy and especially oat. This parameter may indicate the content material stress or carotenoids predecessors in the seeds of these plants. Last comes to mind because of the high lipid content in these natural substrates they are used to enhance carotenogenesis for cultivation of yeast [6]. Unfortunately, the cultivation of producer on cheap substrates – wheat and rye bran were justified hope. Probably, it did not satisfy microorganisms in the required amount of nutrients (Table 1).

Table 2

Accumulation of biomass and synthesis of lycopene by strain *S. globisporus* 1912-4 Lcp Hp7

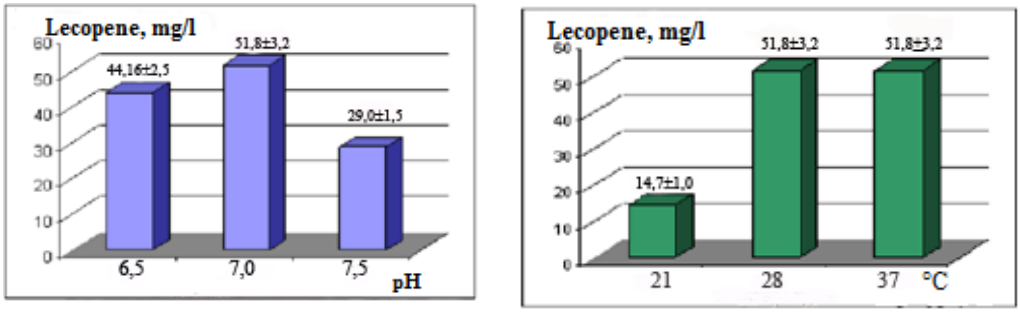
Medium	Biomass, g/l	Lycopene, mg/g dry biomass	Lycopene, mg/l medium
Corn	14.8±1.0	2.6±0.05	38.48±1.0
Soy	10.0±1.0	3.5±0.05	35.00±1.0
Oat	14.3±1.0	3.5±0.05	50.05+1.0
Corn-soy	14.8±1.0	2.8±0.05	41.44±1.0
Corn-oat	14.8±1.0	3.5±0.05	51.80+1.0
Soy-oat	13.0±1.0	3.5±0.05	45.50±1.0
Wheat bran	8.0 ± 1.0	1.5±0.05	12.00±1.0
Rye bran	6.5 ± 1.0	1.0±0.05	6.5±1.0

After cultivation of producer into liquid medium the suspension of mycelia was selected and put on similar agar medium. So visually the cause of the difference activities in media with corn flour and bran were identified. In the first case, the reason was the formation of uncolor colonies, and in the second – a decrease of the synthesis of the metabolite. The frequency of accumulation of uncolor colonies with using cornmeal was averaged $1x10^{-3}$, which had a negative effect on the result. On the other hand, it is impossible to refuse from making corn flour, as it contributes to

the development of surface mycelium and the accumulation of biomass for inoculum. In addition, surface mycelium on corn media has grown the best and was facilitated for getting the accumulation of cultivated material. Surface mycelium on soy and oat medium developed hardly. So corn-oat medium was used for further studies.

After several cultivation in liquid corn-oat medium it was observed that it eventually lost its effectiveness. It was therefore decided, without changing the media, to explore necessary conditions substances that would help to stabilize carotenoid synthesis in submerged conditions.

The analysis of the growth conditions and lycopene synthesis by *S. globisporus* 1912-4 Lcp Hp7 were started from determination of the initial pH and temperature of cultivation (Fig. 1a). It is known that rate of pH 7.0–8.0 contributes to lycopene accumulation, whereas the products of metabolism of streptomycetes are lower pH [6].



а б

Fig.1. Yield of lycopene from *S. globisporus* 1912-4 Lcp Hp7 with different pH (a) and temperature of cultivation (b)

It is necessary to note that the medium contained a sufficient amount of the calcium carbonate ($CaCO_3$), which limited the formation of H^+ ions and prevented shift pH in the acid area. Synthesis of lycopene appeared to be very sensitive to small variations pH. Favorable for the accumulation of carotenoid pigment was neutral initial pH. A slight shift of the neutral reduced productivity of the culture. The fact of low yield of lycopene after culturing mutant at 21 °C is very interesting. It is only 7 °C lower than the standard temperature of streptomycetes cultivation (28 °C) [4]. The maximal synthesis of lycopene has been expected at 37 °C due to the stress effects described in the literature [8]. This fact can be used to restore the activity of the producer in the event of another decrease if it is economically feasible.

It is known that organic acids have a significant impact on carotenoid biosynthesis [3]. Organic acids were used as sodium salts (Table 3). Ammonium salts in some cases have reduced the synthesis of lycopene in the producer Hp7. It has previously been shown by us [2]. At first we were analyzing the pH after cultivation. Potassium-sodium tartrate 4-aqueous did not contribute to the synthesis of lycopene. Adding of other sodiums have led to mean pH 8.0–9.0 that has been productively.

Table 3

The effect of organic compounds on the performance of *S. globisporus* 1912-4 Lcp Hp7

Salts of organic acids	pH	Biomass. g/l	Lycopene. mg/g dry biomass	Lycopene. mg/l medium
Control	8.0	14.8±1.0	3.5±0.05	51.80±1.0
Sodium acetate	9.0	15.5±1.0	3.2±0.05	49.60±1.0
Sodium citrate	8.5	16.5±1.0	3.2±0.05	52.80±1.0
Sodium tartrate 4-aqueous	5.5	Traces	Traces	Traces
Sodium casein	9.0	15.0±1.0	4.2±0.05	63.00+1.0
Sodium succinate	8.5	16.8±1.0	3.2±0.05	53.76±1.0

It was detected that salt can be used to enhance the synthesis of biomass. Citrate and succinate sodiums have increased biomass by at least 10%, acetate salt by 5%, but have decreased the synthesis of lycopene. The productive effect on the synthesis of lycopene has got sodium casein, it may indicate the contents of stress substances or precursors of carotenoids. On the other hand, the salt is an expensive substrate, and it can be kept in mind when growing biotech demand producer.

Table 4

The effect of nonorganic salts on synthesis of lycopene and growth of *S. globisporus* 1912-4 Lcp Hp7

Salts	Growth strain	Lycopene biosynthesis
$Ca(NO_3)_2$	Not affected	Not affected
$CaCl_2$	Not affected	Not affected
$MnCl_2$	Not affected	Not affected
$FeCl_3$	Delays in 7 mm	Absent
$ZnSO_4$	Suppresses	Absent
$MgSO_4$	Enhances	Enhances
$CuSO_4$	Delays in 12 mm	Absent
$FeSO_4$	Delays in 5 mm	Absent
$KMnO_4$	Enhances	Enhances
$(NH_4)_2MoO_4$	Enhances	Enhances

Cultivation is economically viable with the introduction of nonorganic salts. In order to determine the effect of metal ions on the biosynthesis of carotenoids were tested salt: $Ca(NO_3)_2$, $ZnSO_4$, $MgSO_4$, $CaCl_2$, $MnCl_2$, $FeCl_3$, $FeSO_4$, $KMnO_4$, $(NH_4)2MoO_4$, $CuSO_4$ [3, 6, 8]. Salts of $Ca(NO_3)_2$, $CaCl_2$, $MnCl_2$ did not affect the strain performance. $FeCl_2$, $ZnSO_4$, $CuSO_4$, $FeSO_4$ inhibited the growth of the strain. $MgSO_4$, $KMnO_4$, $(NH_4)_2MoO_4$ have been suitable. The last two are known as oxidants, and they may contribute to the synthesis of lycopene as stress factors.

$(NH_4)_2MoO_4$ is used as fertilizer for plants [9]. In further studies, it was found necessary concentration of metal salts for the cultivation Hp7: $MgSO_4 \times 7H_2O$ – 0.1%; $(NH_4)_2MoO_4$ – 0.05%; $KMnO_4$ – 0.001%. Their combined using has activated synthesis of lycopene and 4.2 mg/g DBM, which was characteristic for obtained producer in 2010. $(NH_4)_2MoO_4$, $KMnO_4$ improve redox potential, that has affect for aeration and the ability to obtain good results at medium volume 100–150 ml, while still working volume did not exceed 60 ml.

Permanganate as a toxic substance was decided to use only for the selection of the producer, and in the case of performance degradation it could be used for stimulation of the synthesis of lycopene.

Thus, available components for the performance of the strain *S. globisporus* 1912-4 Lcp Hp7 have been analyzed, it should continue to be useful in the case of reducing its activity. At present, selection and obtaining of inoculum producer and cultivation is carried out by using the medium of the following composition (g/l): corn flour – 20.0; oat flour – 20.0; NaCl – 5.0; $CaCO_3$ – 3.0; $MgSO_4$ – 0.1%; $(NH_4)_2MoO_4$ – 0.05% and conditions: initial pH 7, 28 °C, V_{in}10 ml, V_{med}100 ml.

С.Л. Голембіовська, Т.В. Дворник, Л.М. Янченко, Б.П. Мацелюх

Інститут мікробіології і вірусології НАНУ, вул. Заболотного 154,
Київ, МСП, ДО3680, Україна, e-mail: golembiovska@ukr.net

ПРОДУКТИВНІСТЬ МУТАНТНОГО ШТАМУ *STREPTOMYCES GLOBISPORUS* 1912-4 LCP HP7 ЗА РІЗНИХ УМОВ КУЛЬТИВУВАННЯ

Реферат

Мета. *Визначення причини зниження синтезу каротиноїда лікопіну в умовах глибинного вирощування з 4,2 до 2,8 мг/г сухої біомаси у штама Streptomyces globisporus 1912-4Lcp Hp7 та підбір компонентного складу середовища і оптимальних умов, які б сприяли стабілізації цього процесу.* **Методи**. *Селекцію продуцента здійснювали за ознакою раннього синтезу лікопіну. Селекціоновану культуру глибинно вирощували за різних умов. Біомасу визначали ваговим методом, кількість лікопіну – спектрофотометрично.* **Результати**. *Причиною зниження активності продуцента лікопіну S. globisporus 1912-4Lcp Hp7 в умовах глибинного вирощування виявилася дисоціація культури з утворенням безпігментних варіантів з частотою 1 х 10^{-3}, що спостерігали після розсіву суспензії міцелію на агаризованому середовищі. Як з'ясувалося, їх накопичення викликане внесенням*

в середовище культивування кукурудзяного борошна, яке в поєднанні з вівсяним застосовують і по сьогоднішній день, тому що саме це джерело вуглецю та енергії сприяло накопиченню біомаси продуцента. Визначено умови культивування продуцента: pH 7,0, 28°C, $V_{інок}$10 мл, $V_{серед}$100 мл та концентрації солей: $MgSO_4$ x $7H_2O$ – 0,1%, $(NH_4)_2MoO_4$ – 0,05%, які стабілізують процес біосинтезу лікопіну в умовах глибинного вирощування. Показано, що лікопіногенезу сприяє також внесення казеїновокислого натрію і температура 37 °C та $KMnO_4$, який застосовують для селекції культури. **Висновки**. *Встановлено причину зниження біосинтезу лікопіну у штаму S. globisporus 1912-4 Lcp Hp7 та визначено середовище, оптимальні умови і солі, які стабілізують процес біосинтезу лікопіну у продуцента в умовах глибинного вирощування.*

Ключові слова: стрептоміцети, штам продуцент, синтез лікопіну.

С.Л. Голембиовская, Т.В. Дворник, Л.М. Янченко, Б.П. Мацелюх

Институт микробиологии и вирусологии НАН Украины, ул. Заболотного 154, Киев, МСП, ДО3680, Украина, E-mail: Golembiovska@ ukr.net

ПРОДУКТИВНОСТЬ МУТАНТНОГО ШТАММА *STREPTOMYCES GLOBISPORUS* 1912-4 LCP HP7 В РАЗЛИЧНЫХ УСЛОВИЯХ КУЛЬТИВИРОВАНИЯ

Реферат

Цель. *Определение причины снижения синтеза каротиноида ликопина в условиях глубинного выращивания с 4,2 до 2,8 мг/г сухой биомассы штамма Streptomyces globisporus 1912 4Lcp Hp7 и подбор компонентного состава среды и оптимальных условий, которые бы способствовали стабилизации этого процесса. Методы. Селекцию продуцента осуществляли по признаку раннего синтеза ликопина. Селекционированую культуру глубинно выращивали при разных условиях. Биомассу определяли весовым методом, количество ликопина – спектрофотометрически.* **Результаты.** *Причиной снижения продуктивности штамма S. globisporus 1912 4Lcp Hp7 в условиях глубинного выращивания оказалась диссоциация культуры с образованием беспигментные вариантов с частотой 1 x 10^{-3}, которую наблюдали после рассева суспензии мицелия на среде с агаром. Как выяснилось, их накопление вызвано внесением в среду культивирования кукурузной муки, которую в сочетании с овсяной применяют и по сегодняшний день, потому что именно этот источник углерода и энергии способствовал накоплению биомассы продуцента. Определены условия культивирования продуцента: pH 7,0, 28 °C, $V_{инок}$10 мл, $V_{серед}$100 мл и концентрации солей: $MgSO_4$ x $7H_2O$ – 0,1%, $(NH_4)_2MoO_4$ – 0,05%, которые стабилизируют процесс биосинтеза ликопина в условиях глубинного выращивания. Показано, что биосинтезу ликопина способствует также внесение казеиновокислого натрия и температура 37 °C, а также $KMnO_4$, который применяют для селекции культуры.* **Выводы.** *Установлена причина снижения биосинтеза ликопина у штамма S. globisporus 1912-4 Lcp Hp7 и определены среда, оптимальные условия и соли, которые стабилизируют этот процесс у продуцента в условиях глубинного выращивания.*

Ключевые слова: стрептомицеты, штамм продуцент, синтез ликопина.

REFERENCES

1. *Golembiovska S.L., Lavrenchuk V.J., Matselyukh B.P.* Selection of high-performance mutants in lycopene // Achievements and problems of genetics, breeding and biotechnology. – 2012. V. 4 – P. 334–338.

2. *Golembiovska S.L., Tymoshenko S.G., Matselyukh B.P*. Influence of carbon and nitrogen sources on biosynthesis of lycopene in *Streptomyces globisporus* 4LCP // Microbiol. Journal – 2010. – V. 72, № 6. – P. 46–51.

3. *Kiritsa Elena.* The directed synthesis of carotinoids by yeasts and the perspectives of its use: Dissertation of Dr. in biology. Chisinau – 2005, p.129.

4. *Practical* medium for aktynomyces and fungi. Directory. / ed. By S.M.Semenova. – M: Мир, 1978. – 140 p.

5. *Matselyukh B.P., Lutchenko V.A., Polishchuk L.V* Synthesis of carotenoids by mutant strains of *Streptomyces globisporus* 1912 // Microbiol. Journal – 2003. – V65, N 6. – P. 24–30.

6. *Feofilova E.P.* Fungal carotenoids: biological functions and practical use // Prikl Biokhim. Mikrobiol. – 1994 – V 30(2) – P. 181–196.

7. *Britton G.* General carotenoid methods // Meth. Enzymol. – V. 111. Steroids and Isoprenoids. Part B. / Ed. J.H. Law, H.C. Rilling. – Orlando, San Diego etc.: Academ. Press. – 1985. – P. 113–149.

8. *Choudhari S.M., Ananthanarayan L., Singhal R.S.* Use of metabolic stimulators and inhibitors for enhanced production of beta-carotene and lycopene by Blakeslea trispora // Bioresour. Technol. – 2008. – 99, № 8. – P. 3166–3173.

9. http://selo-delo.ru/8-zemelnie-resursi?start=47

10. *Stahl W.N.* Lycopene: a biological important carotenoid for human. // Arch. Biochem. Biophys. – 1996. – V. 336. – P. 1–9.

Стаття надійшла до редакції 22.04.2014 р.