K.S. Ogirchuk, N.K. Kovalenko, O.A. Poltavska

Institute of Microbiology and Virology after D.K. Zabolotny NASU,
154, Zabolotny str., Kyiv, SMEs, D03680, Ukraine, tel.: +38(066)056 21 39
e-mail: ekaterina.kiev@gmail.com.

OPTIMIZATION OF LACTOBACILLUS ACIDOPHILUS 55 CULTIVATION IN A MEDIUM WITH SODIUM SELENITE

Aim. *Optimization of culture conditions of the probiotic strain Lactobacillus acidophilus 55 in the medium with sodium selenite.* ***Methods****. Studies of the influence of sodium selenite, inoculum size and cultivation time on the concentration of viable cells of L. acidophilus 55 were performed using a three-level factorial experiment on Box-Behnken (BBD).* ***Results.*** *It has been found that the inoculum size is the factor that has the greatest effect on the concentration of viable cells. The optimum conditions to maximize the concentration of viable cells in the presence of selenium ions were: the content of sodium selenite in the medium of 8.3%, the amount of inoculum 5.3% and 18 hours of cultivation time.* ***Conclusions****. When L. acidophilus 55 was cultured at optimized conditions, the highest concentration of viable cells was* 2.5×10^8 *CFU/ml.*

Key words: Lactobacillus acidophilus, sodium selenite, optimization.

Selenium (Se) has received considerable attention as an essential micronutrient. Biological functions of selenium in the organism are mediated by a variety of seleno-proteins (iodotrin 5'-deiodinase, glutathione peroxidase, etc.) as the enzymes involved in key biological processes, such as the AOD, thyroid function, bone formation, immunity, maintaining of reproductive health and others [3, 7, 11]. In this regard, currently, the addition of selenium in the diet becomes very popular. Nutritional supplements based on organic selenium compounds that are safer and more bioavailable than its inorganic forms have received the highest recognition [3]. Various selenium-enriched biological products, including yeast, eggs, meat, wheat, fruits and vegetables have been developed [6, 8, 9, 12, 13].

At the same time, the effect of selenium on the representatives of the normal intestinal microbiota has hardly been studied. So, *Calomme M.R. et al* [4] first showed that different types of lactobacilli can accumulate Se in biomass comprising intracellular selenocysteine and are a potential source of organic selenium. There are few informations about selenium biotransformation ability of such species of lactic acid bacteria (LAB) as: *Lactobacillus delbrueckii subsp. bulgaricus, L. casei subsp. casei, L. plantarum*, *Bifidobacterium sp., Streptococcus thermophilus, Enterococcus faecium* [1, 2, 4, 14]. Similar data on Lactobacillus acidophilus, which is one of the most common types of lactobacilli – representatives of normobiota of gastrointestinal tract of humans, are absent. Such studies are actual and have the prospects of use in medicine and food industry.

The purpose of this study was to determine the optimal conditions of cultivation of *L. acidophilus* strain 55 in the medium with sodium selenite, using statistical methods of multifactorial experiment planning (Box-Behnken).

Materials and Methods

The object of the study was active strain *Lactobacillus acidophilus 55*, isolated from human intestine. Cultivation of the strain was carried out on MRS medium at 37 °C.

To determine the tolerance to lactic acid bacteria to Se^{4+}, overnight culture of the test strain has been cultured in 50 ml vials, on MRS medium supplemented with sodium selenite (Na_2SeO_3). Na_2SeO_3 solution in sterile distilled water was added to the sterile culture medium to the final concentration of sodium selenite 1; 2; 3; 4; 6; 8; 20; 26 mg/l [4, 5].

Input data to determine the optimal parameters of growth was determined by cultivating the lactobacilli in 200 ml flasks containing 50 ml of MRS, supplemented with sodium selenite at final concentration 1; 8 or 16 mg/l in the medium. The medium was inoculated with 18-hour culture of lactobacilli grown in MRS and standardized to 1.5×10^8 CFU/ml. The inoculum was added into the flasks with the medium in the amount of 3, 5 or 8%. The results have been processed by counting the colony forming units (CFU) of *Lactobacillus* grown on solid MRS medium after 48 hours of growth.

Dependence of the concentration of viable cells *L. acidophilus 55* (Y) from the concentration of sodium selenite in the culture medium (X_1), inoculum size (X_2) and fermentation time (X_3) was assessed using regression analysis methods in the theory of experiment planning on Box-Behnken [10]. This approach allows in the frame of the same model to estimate the linear and quadratic effects of influence of factors (X_1, X_2 and X_3) on optimization index (Y) and express it as a regression equation:

$$y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j<i} a_{ij} x_i x_j \ .$$

were a_0 – constant, a_i – linear coefficient, a_{ii} – quadratic coefficient, a_{ij} – interactive coefficient.

The statistical data processing (calculation of the regression coefficients, regression analysis of variance ANOVA and the construction of response surfaces) was conducted by using the program Statistica 6.0 (StatSoft Ink., 2002). The obtained coefficients were considered as statistically significant at $p\leq0.05$.

Results and Discussions

At the beginning of the study the ability of the strain *Lactobacillus acidophilus* 55 to grow on the medium containing various concentrations of selenite ions has been determined. The ability to accumulate biomass and active acidity of the medium at the end of cultivation were the main parameters characterizing the tolerance of lactobacilli to Se^{4+}. The evaluation of the influence of sodium selenite in a wide concentration range (1–26 mg/l) on the gowth of the culture showed that the presence of sodium selenite in the medium at concentrations of 1, 4, 6 and 8 mg/l does not significantly affect the growth of the culture. At the concentrations of 2 and 3 mg/l weaker stimulating effect was observed (Fig. 1), while higher concentrations (20 and 26 mg/l) inhibited the accumulation of lactic acid bacteria biomass.

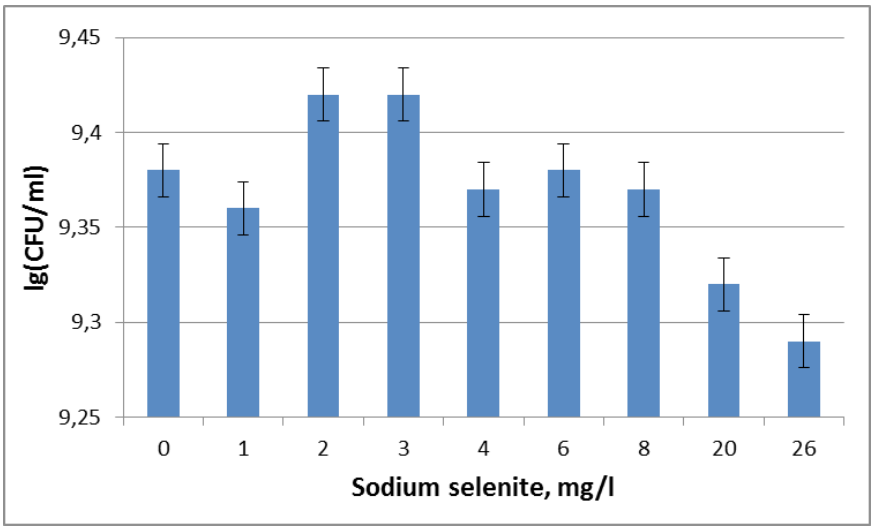

Fig. 1. Biomass accumulation by *L. acidophilus 55* strain in the medium with varying concentrations of sodium selenite

The intensity of acid formation by culture of *L. acidophilus* 55 decreased in the presence of sodium selenite at the concentrations of 20 and 26 mg/l and after 24 hours of growth the pH of the culture medium was detected within 4.0–4.5 (Fig. 2). For other concentrations of sodium selenite studied pH of the culture medium was reduced to 3.5.

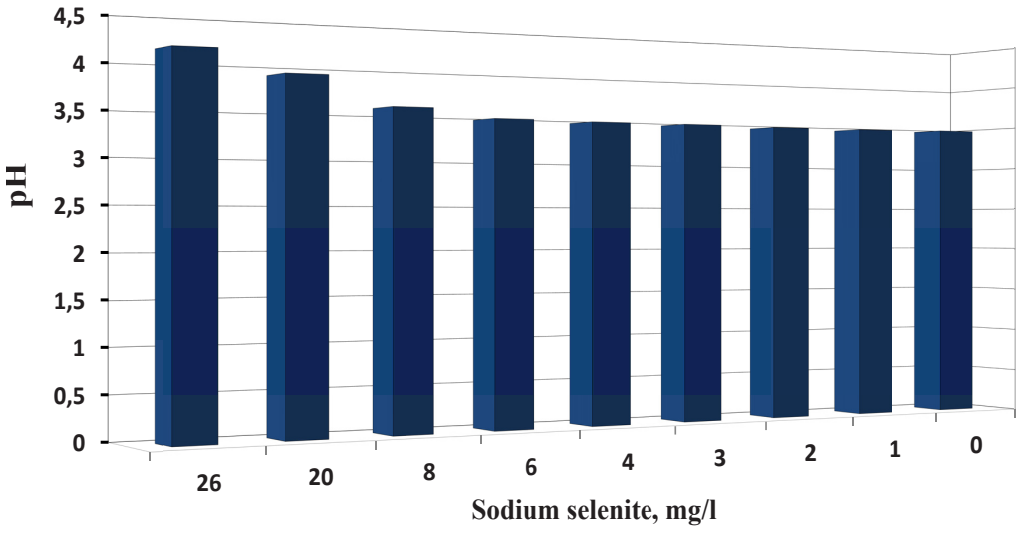

Fig. 2. The pH of the culture medium *L. acidophilus* 55 in the medium with varying concentrations of sodium selenite

Thus, the concentrations of selenium 20 and 26 mg/l were excluded from further analysis because they suppressed the growth of lactic acid bacteria and acid production.

In the further investigation *in silico* optimization of the parameters of cultivation of *L. acidophilus* 55 in the presence of various concentrations of sodium selenite (1–16 mg/l) was carried out. The investigations were carried out on Box-Behnken (Table 1). The design of experiment, as well as the variants of factor combinations and the results obtained are shown in Table 2.

Table 1

Optimization factors and their values used in Box-Behnken design

Factors	Factor levels, coded values		
	-1	**0**	**+1**
Sodium selenite concentration (X_1), mg/l	1	8	16
Inoculum size (X_2), %	3	5	8
Fermentation time (X_3), hour	6	18	30

Table 2

Planning matrix of the experiment of Box-Behnken design

№	Factors			Parameter optimization
	X_1	X_2	X_3	*lgCFU/ml*
1	1	3	18	9.10
2	16	3	18	7.84
3	1	8	18	7.48
4	16	8	18	7.57
5	1	5	6	7.74
6	16	5	6	7.72
7	1	5	30	7.60
8	16	5	30	7.60
9	8	3	6	7.74
10	8	8	6	7.84
11	8	3	30	7.48
12	8	8	30	7.00
13-15	8	5	18	7.68 ± 0.075

Note: X_1 – Sodium selenite concentration, mg/l,
X_2 – inoculum size, %,
X_3 – fermentation time, hour

The level of significance of the effects (linear, quadratic and interaction effects) was determined by the analysis of variance (ANOVA), which showed that each of the optimization factors studied (concentration of sodium selenite (X_1), inoculum size (X_2) and the fermentation time (X_3)) had significant ($p \leq 0,05$) influence on the concentration of viable cells of lactic acid bacteria (Table 3).

Table 3

Results of ANOVA of depending of CFU of *Lactobacillus acidophilus* 55 from the factors and their interaction ***

Factors	Sum of squares	Degree of freedom	Average amount	F	p
(1) Sodium selenite, mg/l (L)*	0.22	1	0.22	38.5	0.03
Sodium selenite, mg/l (Q)**	0.15	1	0.15	26.2	0.04
(2) inoculum size, % (L)	0.83	1	0.83	146.9	0.01
(3) Time, hour (L)	0.17	1	0.17	29.4	0.03
Time, hour (Q)	0.33	1	0.33	57.8	0.02
1L by 2L	0.46	1	0.46	80.9	0.01
1L by 2Q	0.25	1	0.25	43.9	0.02
1Q by 2L	0.32	1	0.32	56.7	0.02
Error	0.01	2	0.01		
Total sum of squares	2.63	14			

*L – linear effects,
**Q – quadratic effects,
«by» – designation of the combined effect of several factors.
*** Factors and options combined action of factors that do not have significant influence on the estimated figure excluded from the analysis and are not presented in the table.

For visual assessment of the effects of ANOVA the Pareto chart, on which the effects are located according to absolute value descending has been shown in Fig. 3. This diagram shows that the linear effect of the amount of inoculum has the highest reliable influence, and the quadratic effect of concentration of sodium selenite has the lowest reliable influence.

During the analysis of raw data the regression equation has ben obtained, which has the form of a quadratic polynomial of the second order, taking into account only statistically significant effects:

$$Y = 11,56 - 0,57\,X_1 - 1,27\,X_2 + 0,10\,X_3 + 0,01\,X_1^2 - 0,002\,X_3^2 + \\ + 0,15\,X_1X_2 - 0,01\,X_1X_2^2 - 0,003\,X_1^2X_2$$

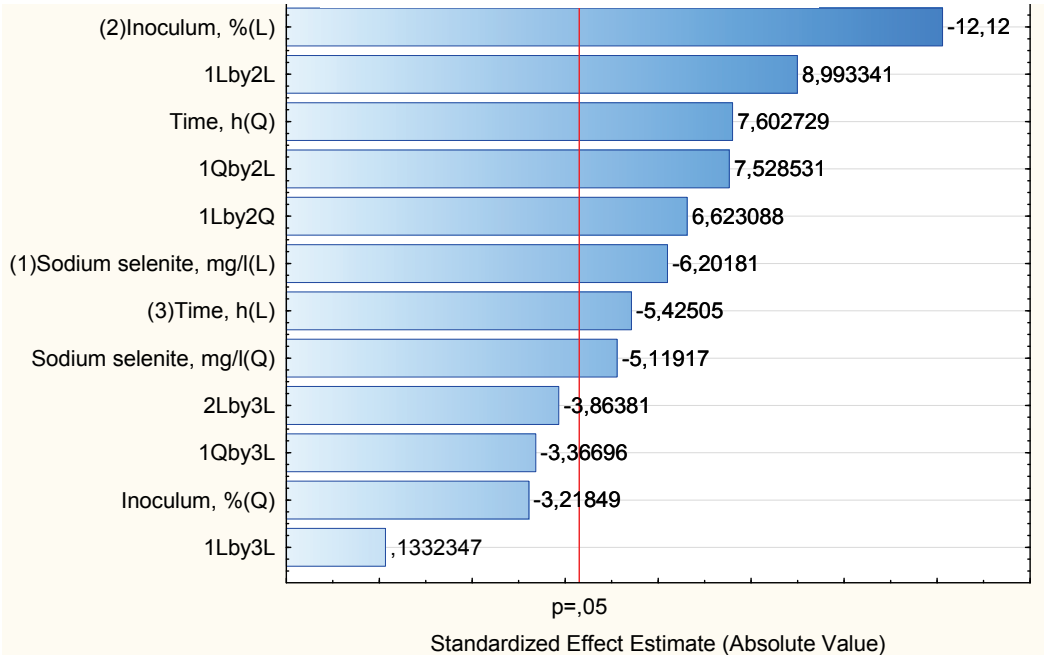

Fig. 3. Optimization factors influence on the concentration of viable cell *Lactobacillus acidophilus* 55

According to the resulting model contributed amount of inoculum was the most important factor ($F = 146.9$, $p \leq 0.01$), with the increase of which the concentration of viable cells of lactobacilli should decrease linearly. Likewise, increase of the concentration of sodium selenite, with constant other factors, should reduce the concentration of viable cells. At the same time, the coordinated increase in both factors will lead to the increase in the concentration of viable cells, which clearly indicate the need to maintain the balance between the concentration of inoculum and sodium selenite in the culture medium. Probably, such need arises from the fact that a large number of inoculum contributes to the rapid acidification of the culture medium, which leads to the destruction of bacteria. At the same time the positive effect of the interaction between these two factors can be explained by the decrease of toxic effects of inorganic selenium by lowering its concentration in the medium in terms of one CFU of lactobacilli at inoculation of large amounts of inoculum.

According to the response surface of concentration of viable cells of culture *L. acidophilus* 55 (Fig. 4), sodium selenite itself has a certain effect, inhibiting the growth of lactobacilli and the maximum concentration of viable cells will be achieved in the absence of sodium selenite in the culture medium. However, if the culture medium still contains sodium selenite, to obtain the maximum concentration of viable cells strict concentration ratio “sodium selenite concentration: inoculum: cultivation time” is needed.

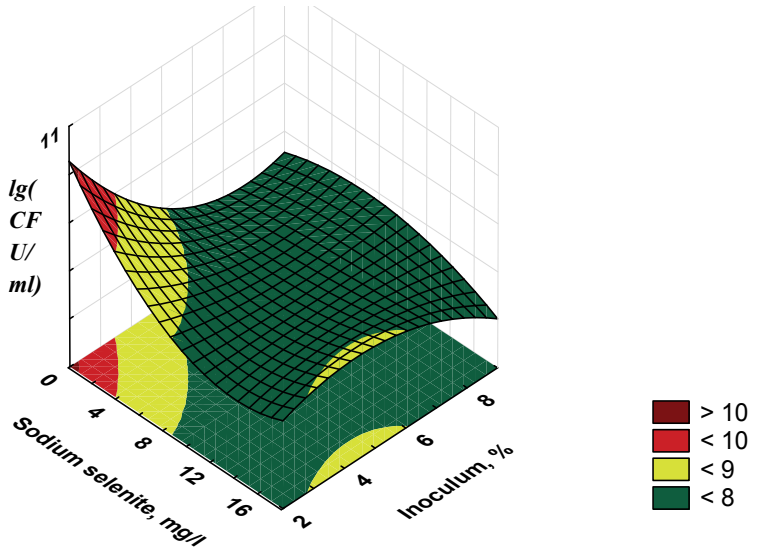

Fig. 4. Response surface concentration of viable cells *Lactobacillus acidophilus 55* as a function of the inoculum size and the concentration of sodium selenite for fixed time of 18 h of fermentation

Assessing the effect of the concentration of the inoculum and sodium selenite on the concentration of viable cells, the cultivation time was an important factor ($F = 57.8$, $p \leq 0.02$) as well, indicating that the greatest effect on the interaction of factors studied is achieved after brief cultivation (6–18 h), and after definite time period (after 18 h), the concentration of viable cells will decline sharply (Fig. 5).

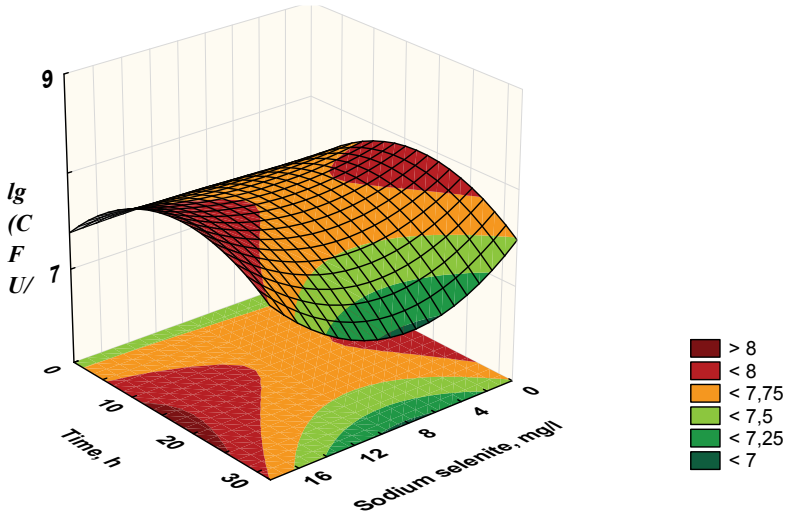

Fig. 5. Response surface concentration of viable cells as a function of culture time and the concentration of sodium selenite at fixed value by the average inoculum size level

The optimal design values for the studied factors were: sodium selenite 8.3 mg/l at 5.3% inoculum at 18 h of cultivation (Fig. 6). Deviations from the calculated data invariably leads to reduction in the titer of viable cells.

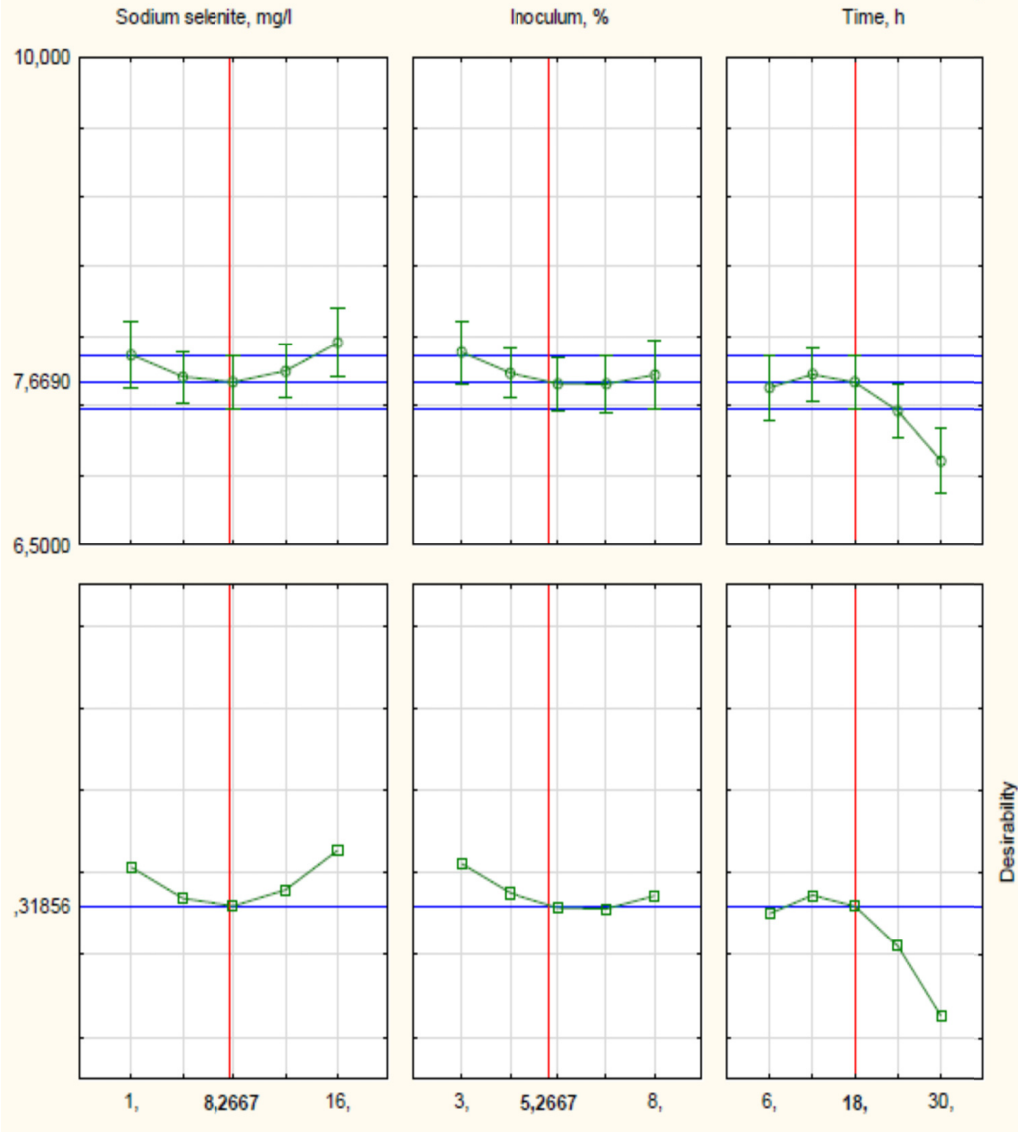

Fig.6. Profiles of the predicted values for the optimal concentration of viable cells of *Lactobacillus acidophilus* 55

To confirm the calculated data, concentrations of viable cell of *L. acidophilus* 55 at cultivation in the medium with 4, 8 and 64 mg/l of sodium selenite at 5% inoculum within 18 hours (Fig. 7) has been determined. It is shown that in the presence of 8 mg/l sodium selenite concentration of viable cells of *L. acidophilus* 55 is 17%

higher than when cultivated with 64 mg/l but 4% lower than in the medium without selenite. These results confirm the results of a visual analysis of the response surface of concentration of viable cells of *Lactobacillus acidophilus* 55 (Fig. 4)

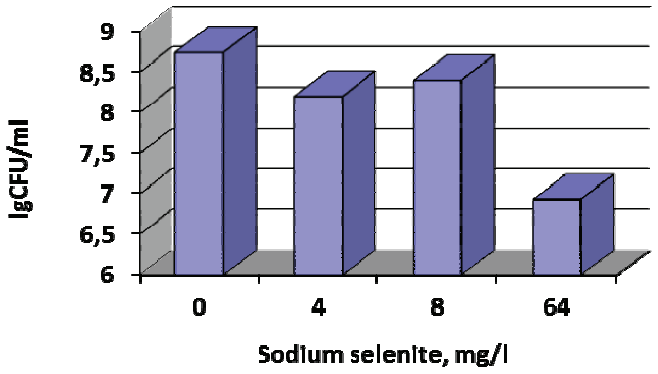

Fig.7. Concentration of viable cells *L. acidophilus* 55 under optimized conditions of culturing in the medium with varying concentrations of sodium selenite

The given result of the work showed that the optimal concentration of viable cells (2.5×10^8 CFU/ml) can be prepared by culturing *L. acidophilus* 55 for 18 h in the presence of 8 mg/l sodium selenite, when making a 5% inoculum.

К.С. Огирчук, Н.К. Коваленко, О.А. Полтавская

Институт микробиологии и вирусологии им. Д.К. Заболотного НАН Украины,
ул. Академика Заболотного, 154, Киев, МСП, Д03680, Украина, тел.: +38(066)056 21 39,
e-mail: ekaterina.kiev@gmail.com

ОПТИМИЗАЦИЯ УСЛОВИЙ КУЛЬТИВИРОВАНИЯ *LACTOBACILLUS ACIDOPHILUS* 55 В СРЕДЕ С СЕЛЕНИТОМ НАТРИЯ

Реферат

Цель. *Оптимизация условий культивирования пробиотического штамма Lactobacillus acidophilus 55 в среде с селенитом натрия.* **Методы.** *Изучение влияния селенита натрия, количества вносимого инокулюма и времени культивирования на концентрацию жизнеспособных клеток L. acidophilus 55 проводили с применением трехуровневого факторного эксперимента по плану Бокса-Бенкена (BBD).* **Результаты.** *Установлено, что количество инокулята является фактором, который оказывал наибольшее влияние на концентрацию жизнеспособных клеток. Оптимальными условиями для получения максимальной концентрации жизнеспособных клеток в присутствие ионов селена были: содержание селенита натрия в среде 8,3%, количество вносимого инокулюма 5,3% и время культивирования 18 ч.* **Выводы.** *При культивировании L. acidophilus 55 в оптимизированных условиях, максимальная концентрация жизнеспособных клеток составила $2,5\times10^8$ КОЕ/мл.*

Ключевые слова: Lactobacillus acidophilus, селенит натрия, оптимизация.

К.С. Огірчук, Н.К. Коваленко, О.А. Полтавська

Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, МСП, Д03680, Україна, тел.: +38(066)056 21 39,
e-mail:ekaterina.kiev@gmail.com

ОПТИМІЗАЦІЯ УМОВ КУЛЬТИВУВАННЯ *LACTOBACILLUS ACIDOPHILUS* 55 В СЕРЕДОВИЩІ З СЕЛЕНІТОМ НАТРІЮ

Реферат

Мета. *Оптимізація умов культивування пробіотичного штаму Lactobacillus acidophilus 55 в середовищі з селенітом натрію.* **Методи.** *Вивчення впливу селеніту натрію, кількості інокулюму, що вноситься і часу культивування на концентрацію життєздатних клітин L. acidophilus 55 здійснювали з застосуванням трьохрівневого факторного експерименту за планом Бокса-Бенкена (BBD).* **Результати.** *Встановлено, що кількість інокуляту є фактором, який виявляв найбільший вплив на концентрацію життєздатних клітин. Оптимальними умовами для отримання максимальної концентрації життєздатних клітин за присутності іонів селену були: вміст селеніту натрію в середовищі 8,3%, кількість інокулюму, що вноситься 5,3% та час культивування 18 годин.* **Висновки.** *При культивуванні L. acidophilus 55 в оптимізованих умовах, максимальна концентрація життєздатних клітин становила 2,5×10^8 КОЕ/мл.*

Ключові слова: Lactobacillus acidophilus, селеніт натрію, оптимізація.

REFERENCES

1. *Alzate A., Fernandez-Fernandez A., Perez-Conde M. C., Gutierrez A. M. et al.* Comparison of biotransformation of inorganic selenium by *Lactobacillus* and *Saccharomyces* in lactic fermentation process of yogurt and kefir // J. Agric. Food Chem. – 2008. – 56. – P. 8728–8736.

2. *Andreoni V., Moro Luischi M., Cavalca L., Erba D., Iappellano S.* Selenite tolerance and accumulation in the *Lactobacillus* species // Ann. Microbiol. – 2000. – 50. – P. 77–88.

3. *Biminger M., Pilawa S., Flohe L.* Trends in selenium biochemistry // Nat. Prod. Rep. – 2002. – 19. – P. 693–718.

4. *Calomme M.R., Van den Branden K., Vanden Berghe D.A.* Selenium and *Lactobacillus* species // J. Appl. Bacteriol. – 1995. – 79. – P. 331–340.

5. *Calomme M.R., Van den Branden K., Vanden Berghe D.A.* Seleno-lactobacillus: An organic selenium source // Biol. Trace Element Res. – 1995. – 47. – P. 379–384.

6. *Carvalho K.M., Gallardo-Williams M.T., Benson R.F., Martin D.F.* Effects of selenium supplementation on four agricultural crops // J. Agric. Food Chem. – 2003. – 51. – P. 704–709.

7. *Ebert R., Jakob F*. Selenium deficiency as a putative risk factor for osteoporosis // International Congress Series 1297. – 2007. – P. 158–164.

8. *Fisinin V.I., Papazyan T.T., Surai P.E.* Producing selenium-enriched eggs and meat to improve the selenium status of the general population // Crit. Rev. Biotechnol. – 2009. – 29. – P. 18–28.

9. *Lintschinger J., Fuchs N., Moser J., Kuehnelt D., Goessler W*. Selenium-enriched sprouts. A raw material for fortified cereal-based diets // J. Agric. Food Chem. – 2000. – 48. – P. 5362–5368.

10. *Palaniraj R., Nagarajan P*. Statistical Analysis of Experimental Variables for the Production of Lactic acid using *Lactobacillus casei* from Waste Potato Starch by Box-Behnken Design // Int. J. Chem. Tech. Res. – 2012. – 4, № 3. – P. 1049–1064.

11. *Schrauzer G.N.* Anticarcinogenic effects of selenium // Cellular and Molecular Life Sciences. – 2000. – 57. – P. 1864–1873.

12. *Suhajda A., Hegoczki J., Janzso B., Pais I., Vereczkey G.* Preparation of selenium yeasts I. Preparation of seleniumenriched *Saccharomyces cerevisiae* // J. Trace Elem. Med. Biol. – 2000. – 14. – P. 43-47.

13. *Turakainen M., Hartikainen H., Seppanen M.M*. Effects of selenium treatments on potato (*Solanum tuberosum L.*) growth and concentrations of soluble sugars and starch // J. Agric. Food Chem. – 2004. – 52. – P. 5378–5382.

14. *Xia S.K., Chen L., Liang J.Q.* Enriched Selenium and Its Effects on Growth and Biochemical Composition in *Lactobacillus bulgaricus* // J. Agric. Food Chem. – 2007. – 55. – P. 2413–2417.

Стаття надійшла до редакції 25.12.2013 р.