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THE COMPARISON OF SIDEROPHORES CONTENT IN BACTERIA ISOLATED FROM BLACK SEA MUSSELS

Aim. *This research was carried out for determination of ability to produce siderophores by strains of Pseudomonas aeruginosa and Bacillus sp. isolated from Black Sea mussels and for studying characteristics of their synthesis at mono- and co-cultivation of researched strains.* ***Materials and methods.*** *In the study we used four strains of Pseudomonas aeruginosa and two strains of Bacillus sp. Monocultivation and co-cultivation were carried out with these strains on a LB medium. CAS (chrome azurol S) analysis was used to determine the content of synthesized siderophores, the measurement was carried out in spectrophotometer SmartSpec Plus at 630 nm.* ***Conclusions.*** *This study showed that marine strains of P. aeruginosa can produce more siderophores than marine strains of Bacillus sp. At monocultivation, strain P. aeruginosa M1 was able to produce the largest amount of siderophores with value of SU (siderophores units) 65 ± 4% and the smallest one strain B. atrophaeus MH4 with value of SU 21 ± 1%. Co-cultivation provides an increase in production of siderophores in each strain, that is the result of special interactions between different microorganisms. And through it, the combination B. subtilis MC3+P. aeruginosa M1 demonstrated the highest content of siderophores with value of SU 81 ± 6%, the lowest content was shown by combination B. atrophaeus MH4+P. aeruginosa M3 with value of SU 41 ± 4%. And such results showed that co-cultivation is the useful method for obtaining more content of siderophores from already famous strains.*

Key words: siderophores, marine bacteria, Pseudomonas, synthesis, co-cultivation.

Siderophores are low-molecular compounds that have high ability to chelate metal ions, especially ions of iron. Many kinds of organisms can produce siderophores, among them are plants, fungi, bacteria and even certain animals. By these organisms siderophores play many roles necessary for normal living [10]. These substances supply metals to the cell, that is the most important function of them, but also many siderophores have antibiotic activity, can regulate expression of genes, provide pathogenicity and correct forming of biofilms [7, 8].

There are a few types of siderophores in general that contain in a structure one type of special chemical groups necessary for metal binding: catecholates, hy-

droxamates, hydroxycarboxylates. Also are three types of mixed siderophores with different chemical groups and one with untypical structures. Each type of siderophores has their own force of chelation and special traits, for example amphiphilicity, photoreactivation and others [3, 5].

Special fermentative complexes are responsible for biosynthesis of siderophores. They are called NRPS (nonribosomal peptide synthetases) and NIS (NRPS independent synthetases) that are coded by BGC's (biosynthetic gene clusters). And the main way of increasing the synthesis of siderophores is activation of BGC's expression [2; 4].

Considering all this, the aim of this study was a determination of ability to produce siderophores by strains of *Pseudomonas aeruginosa* and *Bacillus* sp. isolated from Black Sea mussels and a study of characteristics of their synthesis at mono- and co-cultivation of researched strains.

Materials and methods

Six strains of bacteria isolated from Black Sea mussels were used in this study: *Pseudomonas aeruginosa* M1, *P. aeruginosa* M3, *P. aeruginosa* M4, *P. aeruginosa* PA01, *Bacillus subtills* MC3 and *Bacillus atrophaeus* MH4. Monocultivation and co-cultivation (Table 1) were carried out with these strains for obtaining a lot of bacteria`s biomass.

Table 1

Scheme of co-cultivation of marine bacteria

Variant of co-culture	Abbreviated marking	Variant of co-culture	Abbreviated marking
B. subtilis MC3 + *P. aeruginosa* M1	MC3+M1	*B. atrophaeus* MH4 + *P. aeruginosa* M1	MH4+M1
B. subtilis MC3 + *P. aeruginosa* M3	MC3+M3	*B. atrophaeus* MH4 + *P. aeruginosa* M3	MH4+M3
B. subtilis MC3 + *P. aeruginosa* M4	MC3+M4	*B. atrophaeus* MH4 + *P. aeruginosa* M4	MH4+M4
B. subtilis MC3 + *P. aeruginosa* PA01	MC3+M4	*B. atrophaeus* MH4 + *P. aeruginosa* PA01	MH4+M4

The nutrient medium for cultivation of bacteria was LB that consisted of 15 g peptone, 10 g yeast extract, 5 g NaCl and 1 L marine water. Prepared medium was sterilized at 121 °C for 15 minutes.

Both types of cultivation were carried out in conical flasks. One flask contained 95 ml LB medium and 5 ml suspension of daily bacterial culture (10^6 cells/ml) at monocultivation. At co-cultivation, one flask contained 95 ml LB medium, 2.5 ml suspension of daily culture of first strain of bacteria (10^6 cells/ml) and 2.5 ml suspension of daily culture of second strain of bacteria (10^6 cells/ml). Cultivation was carried out at 30 °C for 72 h with mixing at 150 rpm.

After cultivation, the process of centrifugation was performed in order to obtain the supernatant. Cultural fluid was centrifuged at 3000 rpm for 20 minutes.

For qualitative and quantitative analysis CAS (chrome azurol S) method was used. Special reagent was prepared for this method that consisted of 7.5 ml 2 mM chrome azurol S, 6 ml 10 mM HDTMA (hexadecyltrimethylammonium bromide), 1.5 ml $FeCl_3$ solution (1 mM $FeCl_3 \times 6H_2O$ in 10 mM HCl), 50 ml dH_2O and buffer (4 g piperazine, 6.5 ml 10 M HCl, 10 ml dH_2O) [6].

CAS analysis was carried out in a 96-well tablet, where 100 µl supernatant and 100 µl CAS reagent were placed into one hole. For the control sample 100 µl pure LB medium was placed into one hole, instead of supernatant [1]. The sense of this method consists in selective binding of iron ions with siderophores. First, all iron ions are bound with chrome azurole S and the color of complex is blue, but when siderophores chelate iron, chrome azurole S changes its color and becomes orange. Formula of the process:

FeCAS + L > FeL + CAS, where L is a ligand (siderophore), CAS is chrome azurol S.

Hence the content of siderophores is determined through an intensity of the change in colors. Measurement of OD (optical density) in samples was performed in the spectrophotometer SmartSpec Plus (Bio-Rad, Hungary) at 630 nm. The content of siderophores was determined by the value of the measured optical density and was expressed in SU (siderophores units). The formula was used to find value of SU:

SU (%) = [(Ar-As)/Ar] × 100, where Ar is absorption of control sample, As is absorption of experimental sample.

Statistical analysis of data was carried out in RStudio. Arithmetic mean of values ($\bar{X}$), standard error ($S\bar{X}$) and Student`s test was calculated.

Results

The results of determining the production of siderophores by monocultures of marine bacteria are shown in Table 2.

Table 2

The comparative content of siderophores in monocultures of researched bacteria

Microorganism	Content of siderophores, SU, %	Microorganism	Content of siderophores, SU, %
P. aeruginosa M1	65 ± 4	*B. subtilis* MC3	31 ± 2
P. aeruginosa M3	36 ± 3	*B. atrophaeus* MH4	21 ± 1
P. aeruginosa M4	57 ± 5		
P. aeruginosa PA01	41 ± 3		

The obtained data showed that strain *P. aeruginosa* M1 is capable to the largest production of siderophores among researched organisms with a SU value of 65 ± 4%. The lowest result was shown by the strain *B. atrophaeus* MH4 with a SU

value of 21 ± 1%. In general, all *Pseudomonas* strains showed higher results in the synthesis of siderophores than strains *Bacillus*, the difference between the average SU values of strains of each genus being 20 ± 3% ($p < 0.051$) what is a significant value. However, *P. aeruginosa* M3 showed the lowest result among *Pseudomonas*, which may be similar to the result of *B. subtilis* MC3.

The results of determining the content of siderophores in co-cultures of marine bacteria are presented in Table 3.

Table 3

The comparative content of siderophores in co-cultures of researched bacteria

Variant of co-culture	Content of siderophores, SU, %	Variant of co-culture	Content of siderophores, SU, %
B. subtilis MC3 + *P. aeruginosa* M1	81 ± 6	*B. atrophaeus* MH4 + *P. aeruginosa* M1	70 ± 5
B. subtilis MC3 + *P. aeruginosa* M3	54 ± 4	*B. atrophaeus* MH4 + *P. aeruginosa* M3	41 ± 4
B. subtilis MC3 + *P. aeruginosa* M4	76 ± 5	*B. atrophaeus* MH4 + *P. aeruginosa* M4	63 ± 6
B. subtilis MC3 + *P. aeruginosa* PA01	52 ± 4	*B. atrophaeus* MH4 + *P. aeruginosa* PA01	49 ± 5

Co-cultivation showed significant increase in production of siderophores as compared with monocultivation. The lowest result obtained from *B. atrophaeus* MH4+*P. aeruginosa* M3 was 20% higher than the lowest result among monocultures – *B. atrophaeus* MH4. That is why, in general, it can be seen that co-cultivation is more effective. But despite the mixing of organisms, an unidirectionality with monocultures remains almost everywhere. The combination *B. subtilis* MC3 + *P. aeruginosa* M1 with a SU value of 81 ± 6% showed the highest ability to siderophore`s production among co-cultures, when monocultures of these organisms also showed the highest results of producing among their genera.

At the next stage of the work, a comparative analysis of the expected and real content of siderophores in co-cultures was carried out. The results are shown in Fig. 1 and Fig. 2.

Expected results of co-cultivation were calculated as half of the sum of the siderophores` content from two respective monocultures. Real results in all cases of co-cultivation of *B. subtilis* MC3 with *P. aeruginosa* exceeded expected results by an average of 28% ($p < 0{,}025$). The largest difference was shown by the combination of *B. subtilis* MC3 + *P. aeruginosa* M4, where the difference between expected result and real one was 37%. On the contrary, the smallest difference was shown by the combination of *B. subtilis* MC3 + *P. aeruginosa* M3 with SU value of 20%.

In the case of co-cultivation of *B. atrophaeus* MH4 with different strains of *P. aeruginosa*, a similar situation develops. The expected results are smaller than the real ones, but the difference between them is smaller than in co-cultures with

B. subtilis MC3 and it is on average 20% ($p < 0,045$). The largest difference was shown by the combination of *B. atrophaeus* MH4 + *P. aeruginosa* M1 with SU value of 27%, and the smallest by the combination of *B. atrophaeus* MH4 + *P. aeruginosa* M3 with SU value of 13%.

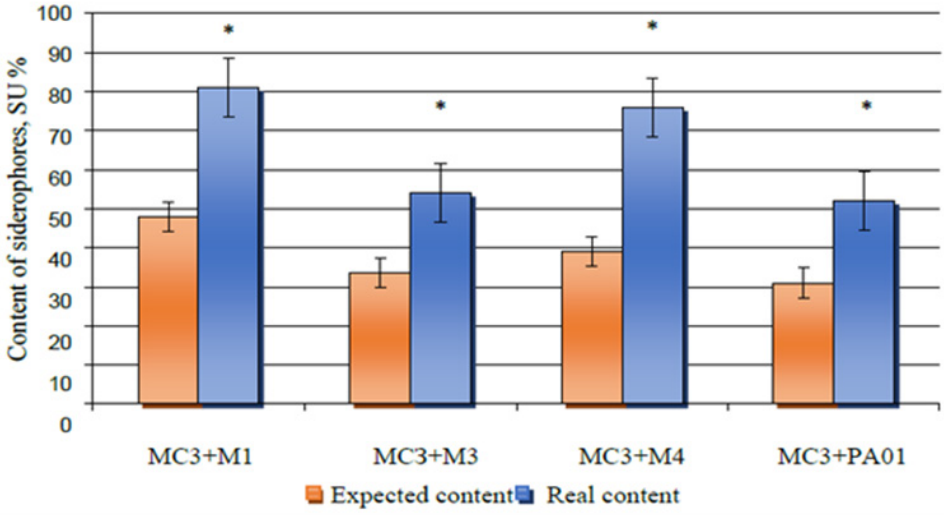

Fig. 1. Comparison of real content of siderophores in co-cultures B. subtilis MC3 with the following strains of P. aeruginosa with the expected

Note: * – the difference is reliable in comparison with the expected data

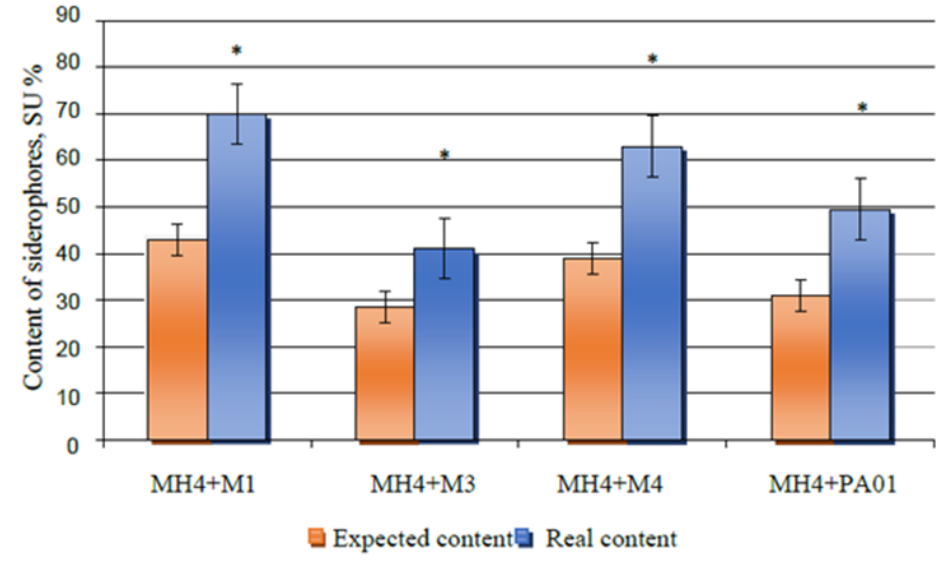

Fig. 2. Comparison of real content of siderophores in co-cultures B. atrophaeus MH4 with the following strains of P. aeruginosa with the expected

Note: * – the difference is reliable in comparison with the expected data

Conclusions

Siderophores are very important molecules that provide normal life of organisms in conditions of low availability of iron and other metals in the environment. This study demonstrated the aptitude of marine bacteria isolated from Black Sea mussels to synthesize siderophores. In general, all strains of *P. aeruginosa* synthesize more siderophores than strains of *Bacillus* sp. At monocultivation, strain *P. aeruginosa* M1 was able to produce the largest content of siderophores with value of SU 65 ± 4 % and the smallest content strain *B. atrophaeus* MH4 with value of SU 21 ± 1%. At co-cultivation, all results increased significantly in each used strain. The combination *B. subtilis* MC3 + *P. aeruginosa* M1 shown the highest content of siderophores with value of SU 81 ± 6%, the lowest content was shown by combination *B. atrophaeus* MH4 + *P. aeruginosa* M3 with value of SU 41 ± 4%. Average difference between mono- and co-cultivation is 20% compared to the results. Also, the co-cultivation demonstrated higher results than it expected. The real content of siderophores was higher by an average of 30% than expected content in the co-cultures with *B. subtilis* MC3 and *P. aeruginosa*, in co-cultures with *B. atrophaeus* MH4 and *P. aeruginosa* by an average 20%.

Such results are caused by the special interaction between two organisms happening during co-cultivation. This interaction is allelopathy, mutual inhibition of growth that promotes big production of secondary metabolites and siderophores also [9]. And by creating such conditions, we received valuable results, which are useful for work in the field of co-cultivations of *Bacillus* with *Pseudomonas* strains.

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ПОРІВНЯННЯ ВМІСТУ СИДЕРОФОРІВ У БАКТЕРІЙ, ВИДІЛЕНИХ З ЧОРНОМОРСЬКИХ МІДІЙ

Реферат

Мета. *Дослідження було проведене для визначення здатності продукувати сидерофори штамами Pseudomonas aeruginosa та Bacillus sp. виділеними із чорноморських мідій та для вивчення особливостей їх синтезу при моно- та ко-культивуванні у досліджуваних штамів.* ***Матеріали та методи.*** *В дослідженні було використано 4 штами Pseudomonas aeruginosa та два штами Bacillus sp. Проводилось моно- та ко-культивування цих штамів на середовищі LB. Для визначення кількості синтезованих сидерофорів використали CAS (chrome azurol S) метод, вимірювання проводилося в спектрофотометрі SmartSpec Plus при 630 нм.* ***Висновки.*** *Дослідження показало, що морські штами P. aeruginosa продукують більше сидерофорів, ніж морські штами Bacillus sp. При монокультивуванні найбільшу кількість сидерофорів був здатний продукувати штам P. aeruginosa M1 із значенням SU (siderophores units) 65 ± 4 %, а найменшу – штам B. atrophaeus MH4 із значенням SU*

21 ± 1%. Ко-культивування забезпечує збільшення продукції сидерофорів у кожного штаму, що є результатом особливої взаємодії між різними мікроорганізмами. Й через це комбінація B. subtilis MC3 + P. aeruginosa M1 продемонструвала найвищий вміст сидерофорів із значенням SU 81 ± 6%, найменший – комбінація B. atrophaeus MH4 + P. aeruginosa M3 із значенням SU 41 ± 4%. Результати показали, що ко-культивування є корисним методом для отримання більшого вмісту сидерофорів у вже відомих штамів.

Ключові слова: сидерофори, морські бактерії, Pseudomonas, синтез, ко-культивування.

СПИСОК ВИКОРИСТАНОЇ ЛІТЕРАТУРИ

1. *Arora N. K., Verma M.* Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria // 3 Biotech. – 2017. – V. 7, № 6. – A. 381.
2. *Bailey D., Alexander E., Rice M. R.* Structural and functional delineation of aerobactin biosynthesis in hypervirulent *Klebsiella pneumoniae* // Journal of Biological Chemistry. – 2018. – V. 293, № 20. – P. 7841–7852.
3. *Chen J., Guo Y., Lu Y.* Chemistry and biology of siderophores from marine microbes // Marine Drugs. – 2019. – V. 17, № 10. – P. 562–590.
4. *Chen R., Wong H. L., Kindler G. S., MacLeod F. I., Ferrari B. C.* Discovery of an abundance of biosynthetic gene clusters in shark bay microbial mats // Frontiers in Microbiology. – 2020. – V. 11. – A. 1950.
5. *Hider R. C., Kong X.* Chemistry and biology of siderophores // The Royal Society of Chemistry. – 2010. – V. 27, № 5. – P. 637–657.
6. *Himpsl S., Mobley H*. Siderophore detection using chrome azurol S and cross-feeding assays // Methods in Molecular Biology. – 2019. – V. 2021. – P. 97–108.
7. *Johnstone T. C., Nolan E. M.* Beyond iron: non-classical biological functions of bacterial siderophores // Dalton Transactions. – 2015. – V. 44, № 14. – P. 6320–6339.
8. *Krewulak K. D., Vogel H. J.* Structural biology of bacterial iron uptake // Biochem. Biophys. Acta – Biomembranes. – 2008. – V. 1778, № 9. – P. 1781–1804.
9. *Marmann A., Aly A., Lin W.* Co-cultivation – a powerful emerging tool for enhancing the chemical diversity of microorganisms // Marine Drugs. – 2014. – V. 12, № 2. – P. 1043–1065.
10. *Sah S., Singh R.* Siderophores: structural and functional characterization – a comprehensive review // Agriculture (Poľnohospodárstvo). – 2015. – V. 61, № 3. – P. 97–114.

REFERENCES

1. Arora NK, Verma M. Modified microplate method for rapid and efficient estimation of siderophores produced by bacteria. 3 Biotech. 2017;7(6):381.
2. Bailey D, Alexander E, Rice MR. Structural and functional delineation of aerobactin biosynthesis in hypervirulent *Klebsiella pneumoniae*. Journal of Biological Chemistry. 2018;293(20):7841–7852.

3. Chen J, Guo Y, Lu Y. Chemistry and biology of siderophores from marine microbes. Marine Drugs. 2019;17(10):562–590.
4. Chen R, Wong HL, Kindler GS, MacLeod FI, Ferrari BC. Discovery of an abundance of biosynthetic gene clusters in shark bay microbial mats. Frontiers in Microbiology. 2020;11:1950.
5. Hider RC, Kong X. Chemistry and biology of siderophores. The Royal Society of Chemistry. 2010;27(5):637–657.
6. Himpsl S, Mobley H. Siderophore detection using chrome azurol S and cross-feeding assays. Methods in Molecular Biology. 2019;2021:97–108.
7. Johnstone TC, Nolan EM. Beyond iron: non-classical biological functions of bacterial siderophores. Dalton Transactions. 2015;44(14):6320–6339.
8. Krewulak KD, Vogel HJ. Structural biology of bacterial iron uptake. Biochem. Biophys. Acta – Biomembranes. 2008;1778(9):1781–1804.
9. Marmann A, Aly A, Lin W. Co-cultivation – a powerful emerging tool for enhancing the chemical diversity of microorganisms. Marine Drugs. 2014;12(2):1043–1065.
10. Sah S, Singh R. Siderophores: structural and functional characterization – a comprehensive review. Agriculture (Poľnohospodárstvo). 2015;61(3):97–114.

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