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SYNTHESIS CHARACTERISTICS OF SIDEROPHORES OF DIFFERENT TYPES FORMED BY PSEUDOMONADS UNDER DIFFERENT CULTIVATION CONDITIONS

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Abstract. *The aim of this work was to develop a method of obtaining siderophores and to study the influence of different cultivation conditions, in particular the composition of nutrient media, on the spectrum and level of their synthesis by some members of the Pseudomonas genus. Strains of Pseudomonas chlororaphis ONU 306, Pseudomonas fluorescens ATCC 13325 and Pseudomonas aeruginosa ATCC 10145 produced more intensely chelating compounds during cultivation in King B liquid medium. The corresponding values were higher than 9% for P. aeruginosa ATCC 10145) to 47% (for P. chlororaphis ONU 306). The spectrum of compounds of the siderophore series formed by the studied strains of pseudomonads mainly consisted of substances of hydroxamate and catechol type.*

Key words: *siderophores, pseudomonads, CAS-reagent, nutrient medium*

Introduction

Most aerobic and facultative anaerobic microorganisms synthesize at least one type of siderophores [1]. Today, according to the peculiarities of the structure, there are 3 main groups of siderophores: one of them contains phenolates and catecholates (enterobactin), the other is hydroxamate ligands (ferrichrome) [2]. There is also a third group - with mixed ligands (pyoverdine). In the molecules of catechol siderophores short peptides are acylated with 2,3-dihydroxybenzoic acid, in carboxylate along with amino acids there are also residues of hydroxycarboxylic acids [11].

In terms of chemical structure, many siderophores are modified peptides in which certain groups participate in the formation of the iron-binding center. Hydroxamate siderophores contain residues of ornithine or lysine, to the terminal amino groups of which are attached various substituents [10]. Based on the side chain of the functional group, hydroxamate siderophores are divided into three categories: ferrioxamines, ferricchrome and aerobactin. They are found in *Escherichia coli*, *Pseudomonas* spp., *Klebsiella pneumoniae* [1].

Due to the fact that all siderophores differ significantly in their structure, there is no single system for their isolation [2].

Of the bacterial iron chelators, the most studied today are siderophores of gram-negative bacteria, especially members of the genus *Pseudomonas* [3].

The aim of this work was to determine the influence of different cultivation

conditions, in particular the composition of nutrient media, on the spectrum and level of synthesis of siderophores by some species of pseudomonads.

Materials and methods

The study was done on the basis of the Biotechnological Research and Training Center of ONU named after I.I. Mechnikov. *Pseudomonas* strains were used in the work: *Pseudomonas chlororaphis* ONU 306, *Pseudomonas fluorescens* ATCC 13325 and *Pseudomonas aeruginosa* ATCC 10145, which were obtained from the collection of cultures of microorganisms of the Department of Microbiology, Virology and Biotechnology.

Maintenance of microorganisms was carried out on a nutrient medium King B, which contained (g/l): peptone - 20; $MgCl_2$ - 1.5; K_2SO_4 - 1.8; yeast extract - 2; glycerol - 10. The pH of the medium was 7.4 ± 0.2. The minimum medium MM9 of the following composition (g/l) was also used in the work: K_2HPO_4 - 0.5; NH_4Cl - 1; $MgSO_4 \times H_2O$ - 0.2; NaCl - 0.5; glucose - 10.0; gluconic acid - 2.5; malic acid - 2.5; casamic acids - 0.5. The pH of the medium was 7.0 ± 0.1. When using a dense variant of nutrient media, 1.5% agar-agar was added to them, after which it was autoclaved at 1.0 atm (King B medium) or 0.5 atm (MM9 medium) [7].

To prevent iron contamination, all glassware was soaked in 10% nitric acid solution and then washed with distilled water [9].

Previously studied strains of microorganisms were cultured on minimal medium at 22° C for 24 hours. After that, using a sterile saline solution prepared cell suspensions, the optical density of which at 600 nm was equal to 2.0. 2.0 ml were taken from the appropriate samples and added to 100 ml of the liquid version of the minimum medium. Cultivation was carried out under similar conditions, with constant shaking 150 rpm. Cells in the exponential growth phase were collected by centrifugation (11,000 g, 10 min), washed with sterile saline and re-introduced into fresh minimal medium containing 29 mg/l of ferric chloride ($FeCl_3$). The final culture step was performed for three days, during which 5 ml of cell suspension was taken every 24 hours and siderophores were determined. In this case, the growth of crops occurred under the above conditions.

The CAS method with chrome azurol S (CAS) in the modification of Alexander and Zuberer was used to determine the ability of the studied strains of bacilli to produce siderophores [9]. The basis of the CAS-method is the interaction of the formed bacterial siderophores with the CAS-reagent. A mixture prepared with this reagent and distilled water was also used for comparison. Samples of culture fluid containing siderophores were able to change the color of CAS - reagent from the original (dark blue) to orange [4].

Two methods were used to study the type of microbial siderophores: in the case of siderophores of the catechol type, the Arnow method [5], and the hydroxamate type method - the Atkin method [3].

To detect siderophores by the first method, 1.0 ml of the test sample was sequentially mixed with 1.0 ml of 0.5M HCl, 1.0 ml of nitrite-molybdenum reagent and 1.0 ml of 1M NaOH. The sample was incubated at room temperature for 5 min to develop color. For blank, 1.0 ml of distilled water was used instead of the test sample of culture fluid. The nitrite-molybdenum reagent was prepared by dissolv-

ing 10 g of $NaNO_2$ and 10 g of Na_2MoO_4 in 100 ml of distilled water. If the microorganisms produced catechole-type siderophores, the resulting mixture developed an orange-pink color that was stable for 1 h [4].

Determination of hydroxamate-type siderophores by the Atkin method to 0.05 ml of the test sample was added a 5 mm solution of $Fe(ClO_4)_3 \times nH_2O$ in 0.1 M $HClO_4$. In the presence of siderophores of hydroxamate type, a yellow color was formed.

Statistical processing of research results. All experiments were performed twice, the number of repetitions in each was 5. The data presented are given as the arithmetic mean ± standard deviation.

Results and discussion

The first stage of the study was to determine the growth dynamics of strains of *P. chlororaphis* ONU 306, *P. fluorescens* ATCC 13325 and *P. aeruginosa* ATCC 10145 in liquid media, MM9 and King B. The obtained data show that microorganisms quickly adapted to the composition of nutrient media. In both cases, 24 hours after the start of cultivation, the development of the stationary phase of development of the studied pseudomonads was observed.

The largest increase in biomass was recorded for *P. aeruginosa* ATCC 10145: the optical density of the respective suspensions was almost 4 times higher than the corresponding non-aeriginosa strains. It was also determined that when culturing microorganisms in King B medium, the duration of the stationary phase of development was only, on average, 12 hours.

Thus, starting from 36 hours in the suspension was not only the cessation of bacterial growth, but, conversely, there was cell death. This is evidenced by the obtained decrease in the optical density of the suspension of pseudomonad cells.

With regard to the nutrient medium MM9, the term of the stationary phase of growth of the studied strains was longer. Thus, starting from 24 hours of cultivation and during the next two days, the number of cells in the suspensions of *Pseudomonas* sp. did not change.

It is known from the literature that microorganisms most actively begin to produce secondary metabolites, in particular siderophores, during the stationary phase of development [6, 8].

Thus, at the next stage of research, the type of siderophores and the level of their production were determined on the first day of cultivation of pseudomonads, which corresponded to the beginning of the stationary phase of growth of these microorganisms. Based on the obtained data, we can assume that the most intensive production of siderophores occurred during the cultivation of the studied microorganisms in King B. The concentration of iron chelators in this case exceeded the corresponding values for the medium MM9 from 23% (in *P. chlororaphis* ONU 306) to 66% (in *P. aeruginosa* ATCC 10145).

According to the intensity of the synthesis of siderophores at the beginning of the stationary phase, the studied producer strains can be arranged as follows:

P. fluorescens ATCC 13325> *P. chlororaphis* ONU 306> *P. aeruginosa* ATCC 10145.

However, when King B medium was used for cultivation of pseudomonads,

instead of MM9, the last two strains changed places, *P. aeruginosa* ATCC 10145 became a more efficient producer of siderophores.

Determining the type of siderophores synthesized by pseudomonads revealed the presence of representatives of two classes of biological chelators - siderophores of hydroxamate and catechol type (Table 1).

Table 1

Type of siderophores produced by the studied strains of pseudomonads

A variant of the nutrient medium	Microorganism-producer	Type of siderophores	
		hydroxamate	catechol
MM9	*P. chlororaphis* ONU 306	+	-
	P. fluorescens ATCC 13325	+	-
	P. aeroginosa ATCC 10145	+	+
Кінг В	*P. chlororaphis* ONU 306	++	++
	P. fluorescens ATCC 13325	++	++
	P. aeroginosa ATCC 10145	++	++

Note: ++ - qualitative reaction when determining the type of siderophores was manifested immediately after adding the appropriate components to the nutrient medium; + - signs of the reaction developed at the end of the incubation period; - - the reaction did not take place, ie the production of the corresponding group of siderophores was not carried out.

However, if the cultivation of microorganisms in MM9 medium caused mainly the appearance of siderophores of the hydroxamate type, then when using King B, two types of chelators were observed. In the latter case, there was a more intensive synthesis of compounds.

Conclusions

Thus, using different types of nutrient media, in particular MM9 or King B, in the cultivation of pseudomonads, you can significantly affect the production of siderophores. In this case, there are not only changes in the intensity of education, but also their spectrum.

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