

ACCUMULATION BY *Streptomyces massasporeus* CNMN-Ac-06 STRAIN OF BIOMASS AND LIPIDS DURING CULTIVATION ON COMPLEX MEDIUM WITH 4-AMINO BENZOIC ACID

Anastasia GARBUZNEAC*, Svetlana BOORTSEVA**, Tamara SIRBU**, Maxim BYRSA**, Natalia CHISELITA**, Oleg CHISELITA**

* State University of Moldova, Chisinau, Republic of Moldova

** Institute of Microbiology and Biotechnology of Technical University of Moldova, Chisinau, Republic of Moldova

Correspondence author: Maxim Byrsa, Institute of Microbiology and Biotechnology of Technical University of Moldova, 1 Academiei Str., MD-2028, Chișinău, Republica Moldova, phone: 0037322739609, e-mail: maxim.birsa@imb.utm.md

Abstract. The researches have been carried out to study the effect of 4-aminobenzoic acid on the growth, lipid synthesis, and fractional composition of lipids in the biomass of *Streptomyces massasporeus* CNMN-Ac-06 strain. Under laboratory conditions, during cultivation of *S. massasporeus* CNMN-Ac-06 strain, various concentrations of 4-aminobenzoic acid were added to the SP-I complex medium. The maximum amount of biomass in comparison with the control sample was obtained by cultivation of the strain on SP-I complex medium supplemented with 4-aminobenzoic acid in concentration of 1.37 g/L – 24.99±1.1 g/L, and the amount of total lipids in it was 1.96±0.08 g/L, by 32.28% more in comparison with the control sample. An increase in the phospholipid fraction was obtained on the SP-I complex medium supplemented with 4-aminobenzoic acid in a concentration of 2.74 g/L – 24.39% (when in control sample – 20.70%). Triglyceride and sterol fractions increased on the SP-I complex medium supplemented with 4-aminobenzoic acid in concentration of 1.37 g/L – 18.46% and 20.54%, respectively (when in control sample – 10.55% and 6.80%, respectively).

Key words: *Streptomyces*; complex medium; 4-aminobenzoic acid; biomass; lipids; lipid fractions.

INTRODUCTION

Determining the effect of food components, including those based on metabolites of microbial origin, on body functions, establishing the mechanism of these effects is one of the urgent tasks of human and animal physiology, medicine, microbiology, as well as practical zootechnics, and veterinary medicine [14, 16, 18, 19]. Among microorganisms, one of the most productive and promising groups in terms of obtaining biologically active substances of various chemical nature and scope are actinobacteria of the genus *Streptomyces* [28, 33, 34, 45, 48].

Streptomycetes are actinobacteria, widespread in nature and integral participants in the life of the biosphere. Acting in a variety of geochemical processes, they are able to modify many compounds – both natural and xenobiotic, having a significant impact on the environment. The unique ability of streptomycetes to synthesize secondary metabolites, such as antibiotics, enzymes, herbicides, anticancer agents, vitamins, immunomodulators, lipids, and plant growth factors, has been used for a long time and with great success in various branches of human practical activity [6, 7, 24].

Strains of various species of actinobacteria of the genus *Streptomyces* are well established as producers of compounds with pharmaceutically relevant properties, such as anti-inflammatory, antiviral, antimicrobial, anticancer activity. Therefore, studies in this regard are undoubtedly relevant and are of both theoretical and practical interest [4, 29, 31, 32, 43, 51, 53-55].

Microorganisms have a high cell growth rate with simple cultivation methods. Actinobacteria of the genus *Streptomyces* are capable of accumulating surprisingly large amounts of intracellular fatty acids

from simple carbon sources such as glucose under conditions of limited growth. Accumulation occurs predominantly during the stationary phase. This way, different actinobacteria have the ability to accumulate lipids under certain cultivation conditions. Microbial lipids contain a large amount of polyunsaturated fatty acids and can potentially serve as a source of it [52].

In a number of studies on model animals, the neuroprotective properties of metabolites of such strains of streptomycetes as *Streptomyces purpeofuscus*, *Streptomyces nitrosporeus*, *Streptomyces griseoflavus*, *Streptomyces exfoliates*, and their ability to prevent neurodegeneration provoked by oxidative stress, have been revealed. Inhibitors of lipid peroxidation of cell membranes were isolated from the biomass of streptomycetes: aestivophoenins A and B, benzastatins H and I, mescengricin, carquinostatins B, and their significance as powerful neuroprotective substances under conditions of lipid peroxidation induction was shown [16, 25, 27-29, 49, 50]. Moreover, some of the metabolites of streptomycetes (lactacystin, anhydroexfoliamycin, inubosins A, B and C etc.), which have a neuroprotective effect by using various models of neurodegeneration, have the ability to stimulate neuritogenesis, affecting the ultrastructural organization of various neuronal formations of the brain and the differentiation of neural structures of stem cells [2, 16, 21, 34, 35, 41, 48, 56].

Recently, special attention has been paid to the study of the influence of the components of the nutrient medium, temperature regimes, aeration, mixing, on the growth and development of strains of biologically active substances producers. In the literature, there are works that consider the ability of streptomycete strains to activate the production of biomass, and especially compounds such as lipids, phospholipids, vitamins,

when the composition of the most important components of the nutrient medium changes: sources of carbon, nitrogen, phosphorus etc. [3, 26].

The decisive criterion for the effectiveness of the cultivation medium is the content of substances in it that can be used for the synthesis of biologically active substances by a microorganism [13, 17]. Increasing attention of microbiologists and biotechnologists is attracted by the use of plant materials as the basis of nutrient media. For example, complex or organic media, in which the main source of carbon is corn, wheat, pea, soy flour, etc., are widely used [7, 8, 10].

In modern science, there is a constant search for new microorganisms synthesizing biologically active substances with a different spectrum of action, including the creation of effective combined preparations for their use in a number of areas. To do this, it is necessary to have data on their composition, method of preparation, intermediate products resulting from the metabolism of drugs, the mechanism of action on the physiological functions of organisms, and the effect obtained during their use [1, 47].

According to the literature, the addition of 4-aminobenzoic acid (PABA, vitamin B₁₀) to the nutrient medium contributed to an increase in the production of various benzastatins by streptomycetes, which are powerful neuroprotective substances [28, 29]. PABA, as well as its derivatives, have a wide range of biological effects, are involved in metabolic processes, and are an important growth factor for many microorganisms capable to synthesize vitamins, that inhabit the intestines of animals and take part in maintaining the balance of gut microbiota. The stimulating effect of 4-aminobenzoic acid on the growth and development of young animals, its positive effects on the stability and physiological state of the animal organism in adverse environmental conditions, found in some studies, is associated with such an indirect effect [1, 30, 47].

To argue necessity of research in this way, an investigation on evaluation for effect of PABA on lipid accumulation in *Schizochytrium limacinum* SR21 was analyzed. According to results, the lipid yield was increased by 56.84% with PABA at a concentration of 200 mg/L. The analysis showed that PABA in this concentration was optimal, redirecting the metabolic flux to lipid synthesis. After fed-batch fermentation, PABA increased lipid content by 35.03%. The yields of docosahexaenoic acid and eicosapentaenoic acid were increased by 33.28% and 42.0%, respectively [36]. At the moment, the mechanism of the stress response of microorganisms due to the addition of benzoic acid derivatives to the cultivation media remained unclear [38].

The aim of the research was to determine the action of 4-aminobenzoic acid on accumulation of biomass and lipid synthesis of the strain *Streptomyces massasporeus* CNMN-Ac-06 during growth on a complex medium.

MATERIALS AND METHODS

As object of the study served *Streptomyces massasporeus* CNMN-Ac-06 strain from the National Collection of Non-pathogenic Microorganisms of the Institute of Microbiology and Biotechnology of Technical University of Moldova, isolated from the soil of the central part of the Republic of Moldova.

Inoculum was cultivated for 3 days on platform shaker (180-200 rpm), at 27°C in liquid mineral medium Dulaney (g/L): glucose – 20.0, (NH₄)₂HPO₄ – 7.5, NaCl – 5.0, K₂HPO₄ – 2.0, MgSO₄*7H₂O – 1.0, CaCl₂ – 0.4, ZnSO₄*7H₂O – 0.01, FeSO₄*7H₂O – 0.01, pH=7.0.

In laboratory conditions, to obtain biomass, the *inoculum* in an amount of 8% was cultivated in the following nutrient media, where soybean and corn flour were the main carbon source (g/L): 1) SP-I complex medium (control): corn flour – 20.0, soy flour – 10.0, glucose – 10.0, NaCl – 5.0, CaCO₃ – 1.0, pH=7.0-7.2; 2) SP-I + 0.685 PABA: corn flour – 20.0, soy flour – 10.0, glucose – 10.0, NaCl – 5.0, CaCO₃ – 1.0, 4-aminobenzoic acid – 0.685, pH=7.0-7.2; 3) SP-I + 1.37 PABA: corn flour – 20.0, soy flour – 10.0, glucose – 10.0, NaCl – 5.0, CaCO₃ – 1.0, 4-aminobenzoic acid – 1.37, pH=7.0-7.2; 4) SP-I + 2.74 PABA: corn flour – 20.0, soy flour – 10.0, glucose – 10.0, NaCl – 5.0, CaCO₃ – 1.0, 4-aminobenzoic acid – 2.74, pH=7.0-7.2.

Cultivation was carried out for 5 days on platform shaker (180-200 rpm), at 27°C. The biomass was separated from the supernatant liquid by centrifugation. The amount of total biomass was determined after dehydration, by obtaining of absolute dry biomass (ADB).

The extraction of intracellular lipids from the biomass of streptomycetes was carried out by the Folch method, in the modification described earlier [9].

The quantitative composition of lipids was determined by thin-layer chromatography on Sorbfil plates (100x150 mm) in the solvent system: hexane : diethyl ether : glacial acetic acid (73:25:5). A 10% solution of phosphomolybdic acid in ethanol was used as a developer. The number of individual lipid fractions was determined densitometrically [7].

Statistical data processing was performed using Microsoft Office Excel 2010. Data are displayed as mean ±SEM. The statistical significance was evaluated using a one-way ANOVA with Tukey post hoc test. A p value <0.05 was considered statistically significant. Data were analyzed using PAST 3.26 statistical software [22].

RESULTS

Studies were carried out to determine the amount of biomass and the content of lipids in *S. massasporeus* CNMN-Ac-06 during growth on the SP-I complex medium with the addition of 4-aminobenzoic acid in three concentrations.

As a result of the studies, it was found that during cultivation of the *S. massaporeus* CNMN-Ac-06 strain on the SP-I complex medium, the biomass yield was 7.99 ± 1.12 g/L, and the content of total lipids in it was 1.48 ± 0.19 g/L. During cultivation on the SP-I + 0.685 PABA medium, the biomass yield showed a better result than on the control medium – 10.91 ± 1.07 g/L, and the amount of total lipids – 1.49 ± 0.14 g/L. The highest biomass yield was obtained by cultivation the strain on SP-I + 1.37 PABA medium – 24.99 ± 1.1 g/L, and the amount of total lipids in it was 1.96 ± 0.08 g/L, which was by 32.28% more in comparison with the control (Table 1).

Comparing the ability to produce the main and secondary lipid fractions by the studied strain during cultivation on the SP-I complex medium supplemented with 4-aminobenzoic acid in various concentrations, it

can be noted that the amount of physiologically important lipid fractions (phospholipids, sterols, and triglycerides) has changed significantly.

As could be seen in fig. 1, the addition of PABA to the SP-I complex medium in concentrations of 0.685 – 2.74 g/L caused changes in the amount of the phospholipid fraction. So, for example, when PABA was added to the medium in an amount of 0.685 g/L, the quantity of phospholipids was 21.48%, and when PABA was added to the medium in an amount of 2.74 g/L – 24.39%, while 20.70% of phospholipids in control.

Cultivation of the studied strain of streptomycetes on a complex medium with the addition of PABA contributed to a noticeable increase of sterols in lipids, which was 10.67%; 13.38%, and 20.54%, while 6.80% in the control, respectively.

Table 1. Accumulation of biomass and lipids by the *Streptomyces massaporeus* CNMN-Ac-06 strain during cultivation on the SP-I complex medium supplemented with 4-aminobenzoic acid

Culture medium	Biomass		Total lipids	
	ADB, g/L	% / control	Lipids, g/L	% / control
SP-I (Control)	7.99 ± 1.12	100	1.48 ± 0.19	100
SP-I + 0.685 PABA	10.91 ± 1.07	136.54	1.49 ± 0.14	100.84
SP-I + 1.37 PABA	$24.99 \pm 1.1^{**}$	312.76	$1.96 \pm 0.08^{**}$	132.28
SP-I + 2.74 PABA	$15.41 \pm 0.78^*$	192.86	$1.85 \pm 0.12^{**}$	124.94

Note: *p<0.05; **p<0.01

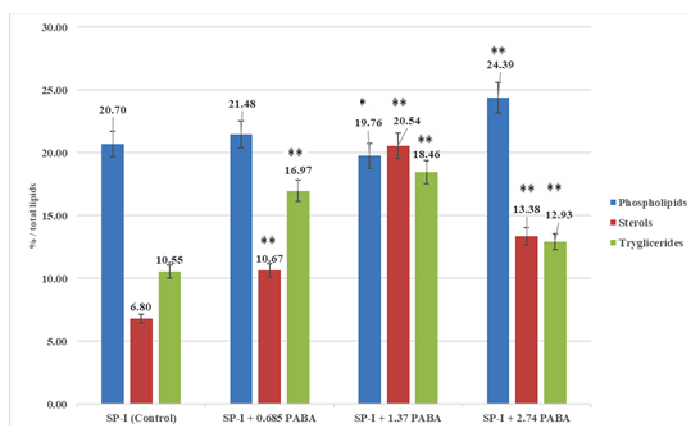


Figure 1. The quantity of main lipid fractions of the *Streptomyces massaporeus* CNMN-Ac-06 strain after cultivation on the SP-I complex medium supplemented with 4-aminobenzoic acid (Note: *p<0.05; **p<0.01)

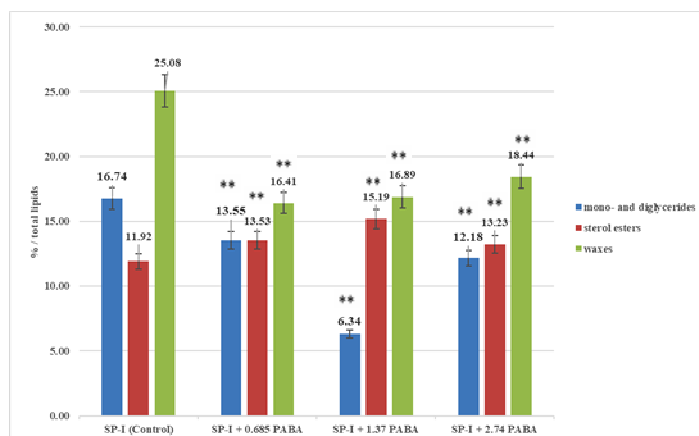


Figure 2. The quantity of secondary lipid fractions in the *S. massaporeus* CNMN-Ac-06 strain after cultivation on the SP-I complex medium supplemented with 4-aminobenzoic acid (Note: **p<0.01)

The lipid fraction, triglycerides, also showed changes in quantity, in comparison with control. So, for example, the growth of the strain on SP-I complex medium with the addition of 0.685 g/L PABA increased the quantity of triglycerides to 16.97%, while 10.55% in the control.

The presence of PABA in the complex medium in the amount of 2.74 g/L changed the quantity of triglyceride fraction in the lipids of the studied strain to 12.93%. The best results were noted during cultivation of the strain on a complex medium with the addition of 1.37 g/L PABA: the amount of triglyceride fraction in the total lipids of the strain was 18.46%, while 10.55% in the control. Analysis of the content of the main lipid fractions showed that during cultivation of *S. massasporeus* CNMN-Ac-06, the maximum quantity of phospholipids (24.39%) was registered on SP-I + 2.74 PABA medium; triglycerides (18.46%), and sterol fraction (20.54%) – on SP-I + 1.37 PABA medium (Fig. 1).

The change in the content of secondary lipid fractions in the biomass of the studied strain of streptomycetes after cultivation on SP-I complex medium supplemented with different concentrations of 4-aminobenzoic acid is shown in fig. 2. Analyzing the nature of the separation of lipids into fractions on a thin layer chromatogram, it should be noted the presence of clear spots of each fraction. However, it was noted that the fractions of mono- and diglycerides were one spot, which was smaller in size on the plates than in the control (after recalculation in experimental lipid samples – 13.55%, 6.34%, 12.18%; while 16.74% in control). As could be seen in fig. 2, the presence of 4-aminobenzoic acid in the complex medium in 3 different concentrations, the proportion of the sterol ester fraction increased slightly in comparison with the control, while the wax fraction decreased significantly – 16.41-18.44%, while 25.08% in control.

DISCUSSION

It is known that for active growth and development, any representative of the microbial world needs certain conditions, and most importantly, a nutrient medium. Since the middle of the 20th century, a scientific approach to the preparation of balanced nutrient media has been developed, taking into account the needs of individual strains of microorganisms in individual components. Numerous data obtained by us earlier revealed a significant dependence of the ability to accumulate biomass and synthesize lipids by streptomycetes during cultivation (temperature, pH of the medium, time), and especially on the composition of the medium [7].

In a number of research papers of the last two decades of the 20th century and the beginning of the 21st century, the results of the study of lipids and their components, including phospholipids, sterols, triglycerides, and fatty acids, especially unsaturated fatty acids, are presented. Issues related to the

metabolism and biological role of lipids are also considered; fatty acid metabolism; synthesis of phospholipids and their topological features; generalizes data on physiological functions and properties, prospects for their use in biology, and medicine [5, 15, 23, 37, 39, 40]. Among the nutrient media proposed for the cultivation of actinobacteria, complex or organic media are widely used, in which the main source of carbon is flour (soybean, corn etc.), as well as various additives (corn extract, baker's yeast, yeast hydrolysate etc.), and mineral salts. Despite the existence of a large number of compositions for the preparation of nutrient media for the cultivation of actinobacteria, issues related to increasing the amount of biomass obtained and reducing its cost, and increasing the yield of certain metabolites of interest in each individual case, are still relevant [7, 46, 56].

According to literary sources, it is known that among the various substances that make up the cells of streptomycetes, a special role belongs to lipids. These compounds have different biological activity: antibacterial (in relation to a number of gram-positive and gram-negative bacteria, yeasts), immunological, growth-stimulating, antitumor etc. [11, 23, 37]. Lipids are considered not only reserve substances, but also the most important biological component of the cell. Particular attention of researchers is drawn to phospholipids and sterols, lipid fractions with high biological activity.

At present, it is known that one of the conditions for the successful cultivation of microorganisms, which ensure the maximum accumulation of biomass and the synthesis of physiologically active substances, is the selection of nutrient media. According to the literature, the media for the cultivation of microorganisms should contain all necessary nutrients in a fairly easily digestible form. The optimal composition of the medium should correspond to the balance of nutrients and nutritional needs of the microorganism cell.

For the cultivation of microorganisms, the nutrient medium must include a certain qualitative and quantitative composition of the components or individual elements necessary for the constructive and energy metabolism of the body: sources of nitrogen, carbon, phosphorus, a number of microelements, vitamins, growth substances etc. [44].

Among the nutrient media proposed for the cultivation of actinobacteria, complex media remain relevant, in which the main carbon source is soybean and corn flour, as well as mineral salts and various additives (corn extract, baker's yeast etc.) [37, 50, 56]. Analyzing the ability of streptomycetes to synthesize physiologically active lipid fractions during their cultivation on complex media of different composition, it should be noted that the composition of nutrient media plays an important role [9, 15, 20, 23, 37]. So, for example, earlier studies were carried out on the formation of biomass of the *S. canosus* CNMN-Ac-02 strain during cultivation on various complex nutrient media, where the largest amount of it was obtained

during cultivation on P medium, which includes corn flour, soluble starch, and mineral salts (12.76 g/L) [42]. During cultivation of this strain on M-I medium containing only corn flour and mineral salts, the amount of biomass obtained was only 4.37 g/L. The results obtained during cultivation on the PM medium containing corn flour, phosphate salts, and other components showed that the amount of total lipids increased by 17.6% in comparison with their amount in the biomass during growth on the M-I medium – 6.22%. According to the literature data, scientists used cyanobacterial biological products in their studies as possible stimulators of growth and lipogenesis of streptomycetes. The addition of some of these biological preparations to the nutrient medium has a different effect on the growth of biomass and lipid synthesis of *Streptomyces canosus* CNMN-Ac-02. The stimulating effect on the increase in the amount of biomass under the action of a biological product obtained from *Porphyridium cruentum* was also evaluated, which increased the amount of biomass in the *Streptomyces canosus* CNMN-Ac-02 strain by 4.78-17.5%, and when using a biological product from *Spirulina platensis*, the biomass of this strain increased by 8.06% [12].

Our previous studies have shown that the largest amount of biomass of the strain *Streptomyces massasporeus* CNMN-Ac-06 was obtained after cultivation on SP-I complex medium containing soy and corn flour (10.56±1.29 g/L), and the percentage of total lipids was 15.85%. Experiments have shown that the growth and lipogenesis of the studied strain change and depend on the composition of nutrient media.

The conducted studies showed that the maximum amount of biomass in comparison to the control was obtained during cultivation on SP-I complex medium with the addition of 4-aminobenzoic acid at concentration of 1.37 g/L – 24.99±1.1 g/L, and the amount of total lipids in it – 1.96±0.08 g/L, which amounted by 32.28% more in comparison to the control.

Analyzing the data on the ability of streptomycetes to synthesize physiologically active lipid fractions after cultivation on complex nutrient media, it should be noted that when 4-aminobenzoic acid was added in different concentrations to the SP-I complex medium, the maximum quantity of phospholipids was 24.39% on SP-I + 2.74 PABA medium; triglycerides – 18.46% and sterol fraction – 20.54% on SP-I + 1.37 PABA medium. In the study of secondary lipid fractions of the biomass of the *S. massasporeus* CNMN-Ac-06 strain, monoglycerides showed a high percentage during cultivation on SP-I + 0.685 PABA medium – 13.55%; sterol esters on SP-I + 1.37 PABA medium – 15.19%; and waxes on SP-I + 2.74 PABA medium – 18.44%.

The results obtained show the prospects for using 4-aminobenzoic acid to stimulate the accumulation of biomass and increase the amount of physiologically important lipid fractions during the cultivation of

streptomycetes on various complex nutrient media [28, 29].

Thus, the conducted studies showed that the composition of the medium plays a significant role in the productivity of biomass and lipids in the studied strain, as well as in stimulating the synthesis of metabolites with neuroprotective and neurotropic effects. The amount of physiologically important lipid fractions can be increased by cultivation of *S. massasporeus* CNMN-Ac-06 strain, isolated from the central part of the Republic of Moldova, on the SP-I complex medium with the addition of 4-aminobenzoic acid at various concentrations.

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