

CONTENT of ORGANIC ACIDS in the CULTURAL MEDIUM of *Bacillus subtilis* IMV B-7023 at CULTIVATION WITH DIFFERENT SOURCES of the PHOSPHORUS NUTRIENT

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ABSTRACT

The strain *Bacillus subtilis* IMV B-7023 is part of the complex bacterial preparation Azogran for crop production. This bacterium has the ability to increase the availability of phosphorus for plants through the synthesis of different organic acids. Using GS-MS, the qualitative and quantitative composition of organic acids in the culture medium of bacilli was investigated depending on the source of phosphorus nutrition in the nutrient medium. It was established that *B. subtilis* IMV B-7023 synthesizes the greatest amount of organic acids at cultivation in a glucose-mineral medium with calcium glycerophosphate after 24 hours. Among them: acetic, isobutyric, butyric, isovaleric, valeric, caproic, enanthic, caprylic, pelargonic. The content of the acetic acid was 32.5 µg/mL and prevailed over other acids. In the presence of calcium glycerophosphate as the sole source of carbon and phosphate nutrition, the concentration of butyric acid increased by 14.5 µg/mL, valeric – by 17.0 µg/mL, caproic – by 36.6 µg/mL, caprylic – by 9.3 µg/mL, and pelargonic – by 13.1 µg/mL, compared with the previous variant. In the presence of Ca₃(PO₄)₂, *B. subtilis* IMV B-7023 produced the acetic and isobutyric acids. The concentrations of these compounds reached a maximum (300.0 and 90.0 µg/mL) after 48 hours of cultivation of bacteria, and then decreased.

Keywords: *Bacillus subtilis*; organic acids; calcium glycerophosphate; calcium orthophosphate

INTRODUCTION

The world scientific community pays considerable attention to the development of new technologies of biopreparations, that based on use highly effective strains of bacteria that stimulate plant growth and development (plant growth promoting rhizobacteria, PGPR) (Van der Ent *et al.*, 2009). Ecological compatibility of biologics contributes to their active introduction into agricultural practice.

One of the most promising objects in the industrial production of preparation for various fields of human activity, including plant growing, are bacteria of the genus *Bacillus* (Siddiqui, 2006). Compared to other artificially introduced into agrobiocenosis free-living microorganisms, their advantage lies in the ability to avoid competitive pressure on the part of aboriginal microflora (Maksimov *et al.*, 2011).

Strain *B. subtilis* IMV B-7023 is a component of a highly effective complex bacterial preparation Azogran (Patent № 54923A). Bacterium synthesizes a complex of biologically active compounds (Skorochod *et al.*, 2013; Tserkovniak *et al.*, 2009a,b), whose components may affect plant growth and development (Roy *et al.*, 2003), and provide protection against abiotic and biotic stress factors (Roy *et al.*, 2005; Roy *et al.*, 2012; Skorochod *et al.*, 2011). Investigated bacilli are characterized by high activity in mobilization and mineralization of poorly soluble phosphorus (P) compounds, which promotes the subsequent availability of this important macroelement to plants (Roy *et al.*, 2004).

One of the dominant mechanisms responsible for the formation of available forms of P in soil are mobilized by organic acids (OA) synthesized by phosphate mobilizing microorganisms (Cheng *et al.*, 2017; Rodriguez *et al.*, 1999). These metabolites through their hydroxyl and carboxylic groups chelate the cations bound to phosphate and then make it available for the plant use (Sagoe *et al.*, 1998).

However, today there is insufficient information on the dependence of the synthesis of OA by bacilli on the source of their phosphate nutrition. Since identification of OA can be important in elucidating the formation of strategies by highly effective strains of bacteria in relation to their positive effects on plants. Accordingly, the purpose of the work was to investigate the synthesis of

organic acids by the strain *B. subtilis* IMV B-7023 for its cultivation in nutrient media with organic and inorganic compounds phosphorus

MATERIAL AND METHODS

Bacterial strains and nutrient media

The object of the study was strain *B. subtilis* IMV B-7023, isolated from black soil (Cherkassy region, Ukraine). The strain is supported at the Depository of the Zabolotny Institute of Microbiology and Virology, NAS of Ukraine (Patent № 54923A). The strain was cultivated in liquid nutrient media of the following composition:

I. glucose-mineral medium with organic source of phosphorus nutrition (Menkina, 1950), (g/L): (NH₄)₂SO₄ – 0.5, MgSO₄·7H₂O – 0.3, KCl – 0.3, CaCO₃ – 5.0, MnSO₄·7H₂O – 0.001, FeSO₄ – 0.001, glucose – 10.0, calcium glycerophosphate – 2.0 (pH 7.0-7.4);

II. modified mineral medium (Menkina, 1950), in which no glucose was added, and calcium glycerophosphate was the only source of carbon and phosphorus nutrition;

III. glucose-mineral medium with inorganic source of phosphorus nutrition (Muromtsev, 1957), (g/L): MgSO₄·7H₂O – 0.2; K₂SO₄ – 0.2; (NH₄)₂SO₄ – 0.5; glucose – 10.0; Ca₃(PO₄)₂ – 2.0; yeast autolysate – 2.0; pH 6.5 – 7.0.

All the reagents used were analytical grade and obtained from CHEMLABORREACTIV, Ltd. (Kyiv region, Ukraine). Chemicals were used as received without further treatment.

The strain *B. subtilis* IMV B-7023 was cultured under batch conditions with shaking at 240 rpm in Erlenmeyer's flasks of 750 mL volume containing 100 mL of the corresponding nutrient medium. The growing time was 24 – 72 hours at a temperature of 28 ± 1°C. The number of viable cells (colony-forming units (CFU)) was determined by the method of seeding a suspension of bacteria on an agar potato medium from serial ten-fold dilutions.

Obtaining of the culture medium of bacteria *B. subtilis* IMV B-7023

The culture liquid of *B. subtilis* IMV B-7023 after completion of this strain growth was freed from the cells of bacterium by centrifugation on the centrifuge OPn-8 (joint stock company “TNK DASTAN,” Kirgizstan) during 15 min at 5000g. In the obtained culture medium (CM) of *B. subtilis* IMV B-7023, the content of organic acids and concentration of PO₄³⁻ were determined (Lurie, 1974).

Chromatographic Analysis

Qualitative and quantitative detection of organic acids was performed using a gas chromatograph with a mass spectrometric detector (GC-MS) manufactured by Agilent, model 6890N / 5973inert equipped with the NIST02 mass-spectrums library used to identify the compounds (Center for Collective Use of Scientific Instruments of the NAS of Ukraine). The system is meant to be used in the modes of electron ionization and chemical ionization, thereby enabling to run high-quality qualitative and quantitative analyses, determine the molecular weight of the reviewed objects and identification of unknown compounds. The concentration of organic acids was determined by the peak areas that were obtained for authentic standards.

Statistical Analysis

Microsoft Excel (Microsoft Corporation, USA) was used to analyze the data on the average of the three replicates (±SE) obtained from the three independent experiments. Differences were compared with the statistical significance at a P level less than 0.05 (P < 0.05). The Kolmogorov-Smirnov test was used to assess the normality of the distribution of each treatment (Lakin, 1990; Zar, 1984).

RESULTS AND DISCUSSION

Synthesis of organic acids by bacterium *B. subtilis* IMV B-7023 during its cultivation in a glucose-mineral medium with calcium glycerophosphate

Soil is a peculiar dynamic system that is an ecological niche of the biological activity of various organisms (Bagyaraj et al., 2000) the functioning of which is determined by the free flow of various micro and macro elements. Among them, an important place is given to phosphorus (P). It is an integral component in

ensuring the processes of growth and development of living organisms. However, due to the low availability of soluble minerals (apatite, hydroxyapatite) and organic (inositol phosphate, phosphoesters) forms of phosphorus in the soil, plants are limited in its assimilation (Gamalero et al., 2011; Vessey, 2003).

The solubilization of insoluble forms of P is an extremely important problem for increasing the consumption of this macroelement by crops (Alam et al., 2002; Nautiyal, 1999). Microorganisms are responsible for this process in soil, in particular bacteria, which make up about 50% of microbiota, capable of dissolving various forms of phosphorus (Khan et al., 2009). They form a peculiar group under the general name of phosphate-solubilizing bacteria (FSB), which include representatives of the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Micrococcus*, *Flavobacterium*, *Burkholderia*, *Achromobacter*, *Erwinia* and *Agrobacterium* (Rodríguez et al., 1999). A significant part of them function in the rhizosphere and is able to colonize the roots of plants, thus ensuring the growth and development of the latter. Therefore, these FSB are also known as plant growth promoting rhizobacteria (PGPR) (Hayat et al., 2010).

Initiators of transformation process of P_{insoluble form} to P_{soluble form} are low molecular organic acids, which are produced by phosphate-solubilizing bacteria (Chen et al., 2006). According to the literature (Hinsinger, 2001; Plassard et al., 2010), OA are can promote mobilizing both mineral and organic phosphates, which release phosphate groups. However, there is little information about factors influencing the synthesis and the composition of OA which are produced by bacteria depending on the initial source of phosphorus. Such studies should be conducted with microorganisms, which are actively involved in mobilization, and mineralization of phosphorus containing compounds. Therefore, as the main object of experimental work, the strain *B. subtilis* IMV B-7023 was chosen (Roy et al., 2004). We found that at presence of organic compound of P – calcium glycerophosphate and glucose in the nutrient medium, the studied bacterium synthesized 9 organic acids after 24 hours of cultivation (Figure 1). Among them, in a quantitative index, acetic acid prevailed – 32.5 µg/mL, which, along with isobutyric acid, was determined throughout the period of bacillus cultivation (Table 1). It was established that at 48 h of cultivation of the strain *B. subtilis* IMV B-7023 were produced, in addition to the two above-mentioned OA, valeric, enanthic, caprylic, pellargonic acids. At the same time, at 72 hours in the CM of investigated strain, most of the OA were not determined, except for acetic and isobutyric acids, which concentration dropped to 19.0 and 1.0 µg/mL, respectively (Table 1).

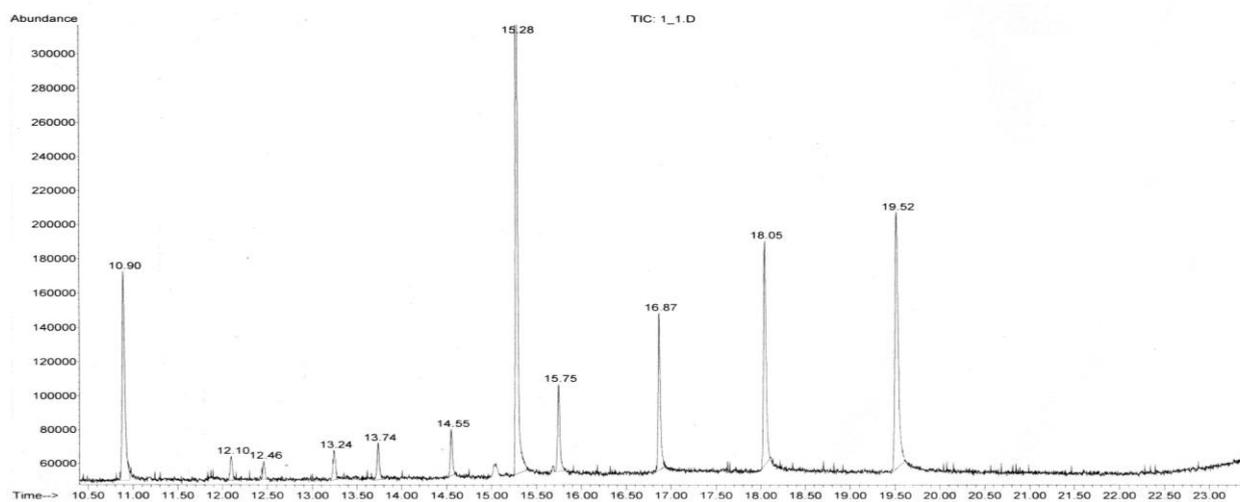


Figure 1 GC-MS spectra of organic acids CM of the bacterium *Bacillus subtilis* IMV B-7023 at its cultivation in a nutrient medium with glucose and calcium glycerophosphate

Table 1 The content of organic acids in the culture medium of the bacterium *Bacillus subtilis* IMV B-7023 at its cultivation in a glucose-mineral medium with calcium glycerophosphate

RT, min	Organic acid	Content OA (µg/mL), at cultivation of bacterium for an, hours		
		24	48	72
10.90	Acetic	32.5 ± 3.2	15.0 ± 1.1	19.0 ± 1.3
12.46	Isobutyric	5.5 ± 0.4	2.0 ± 0.2	1.0 ± 0.1
13.24	Butyric	4.5 ± 0.3	-	-
13.74	Isovaleric	2.0 ± 0.2	-	-
14.55	Valeric	6.0 ± 0.4	2.0 ± 0.2	-
15.75	Caproic	1.4 ± 0.1	-	-
16.87	Enanthic	2.4 ± 0.2	2.0 ± 0.2	-
18.04	Caprylic	3.7 ± 0.3	4.0 ± 0.3	-
19.52	Pelargonic	5.9 ± 0.4	6.0 ± 0.4	-

*: «-» – not identified.

Synthesis of organic acids by bacterium *B. subtilis* IMV B-7023 during its cultivation in a modified mineral medium with calcium glycerophosphate

It was shown that at cultivation of bacillus in a nutrient medium that did not contain glucose, and calcium glycerophosphate was the only source of carbon and phosphorus nutrition, the composition of carboxylic acids in CM of bacterium was almost identical to the previous version (Figure 2). However, the concentrations of most of them significantly differed and were much higher than when the investigated strain *B. subtilis* IMV B-7023 was cultured in a glucose-mineral medium (Table 1 and 2). In particular, butyric acid – by 14.5 µg/mL, valeric – by 17.0 µg/mL, caproic – by 36.9 µg/mL, caprylic – by 9.3 µg/mL and pelargonic – by 13.1 µg/mL (Table 1 and 2). An exception was that the content of acetic acid dropped 1.7 times (Table 1 and 2) and not was identified of isobutyric and isovaleric acids. At the same time at the cultivation of *B. subtilis* IMV B-7023 in a medium where calcium glycerophosphate was the only source of phosphoric and carbon nutrition – the bacterium does not synthesized isomers of butyric and valeric acids. Carbohydrates can be substrate for the formation of some organic acids (acetic, propionic, butyric, valeric) (Fleming et al., 1986), at

the same time isobutyric acid is derivate of valine and isovaleric acid – of leucine (Nakae et al., 1965). In the synthesis of both amino acids, glucose is involved (Teresava et al., 1990; Tsuchida et al., 1986). Since modified medium does not contain this carbohydrate, valine and leucine may not be produced in sufficient a

quantity that makes it impossible the synthesis of isoforms valeric and butyric acids.

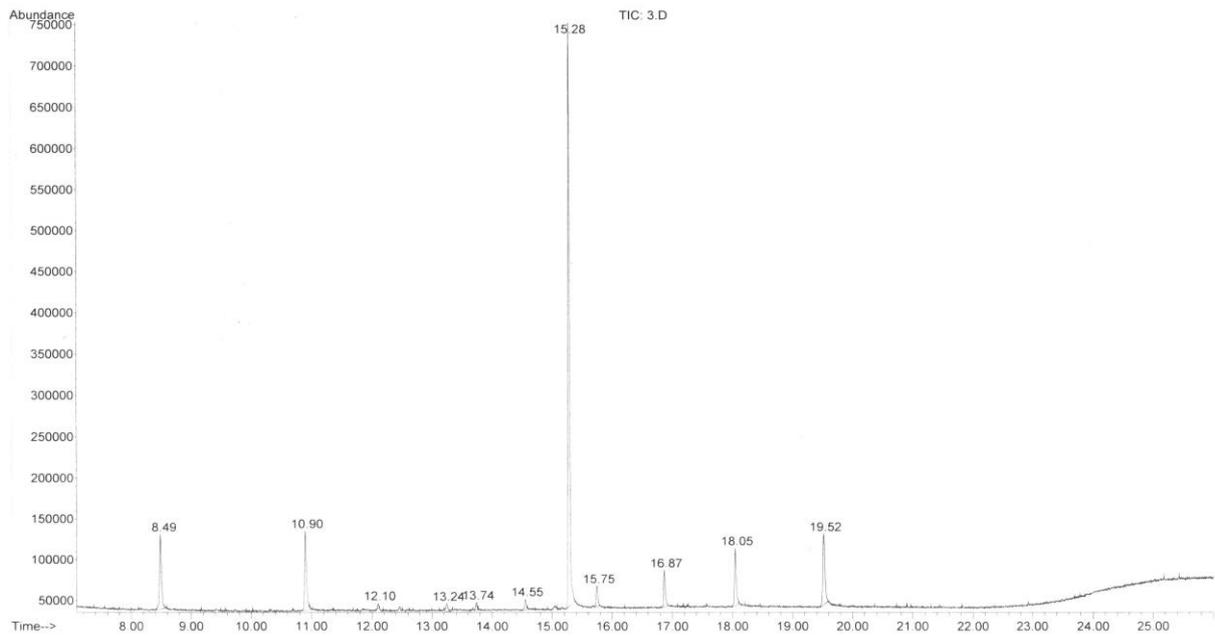


Figure 2 GC-MS spectra of organic acids CM of the bacterium *Bacillus subtilis* IMV B-7023 at its cultivation in a nutrient medium with calcium glycerophosphate

Table 2 The content of organic acids in the culture medium of the bacterium *Bacillus subtilis* IMV B-7023 during its cultivation in a mineral medium with calcium glycerophosphate and without glucose

RT, min	Organic acid	Content OA (µg/mL), at cultivation of bacteria for an, hours		
		24	48	72
10.90	Acetic	19.0 ± 1.2	15.0 ± 1.1	19.0 ± 1.3
13.24	Butyric	19.0 ± 1.3	-	-
14.55	Valeric	23.0 ± 1.4	-	0.7 ± 0.05
15.75	Caproic	38.0 ± 3.1	-	-
16.87	Enanthic	7.0 ± 0.6	-	-
18.04	Caprylic	13.0 ± 1.1	1.0 ± 0.1	-
19.52	Pelargonic	19.0 ± 1.4	2.0 ± 0.2	-

*: «->» – not identified.

Synthesis of organic acids by bacterium *B. subtilis* IMV B-7023 at its cultivation in glucose-mineral medium with Ca₃(PO₄)₂

The qualitative and quantitative composition of OA in the culture medium of bacterium *B. subtilis* IMV B-7023 significantly differed at its cultivation in a nutrient medium with an inorganic phosphorus compound – Ca₃(PO₄)₂. Only acetic and isobutyric acids have been identified (Figure 3, Table 3). These compounds have been identified throughout the entire period of cultivation. The concentration of acetic acid was increased to 300 µg/mL after 48 hours of cultivation of *B. subtilis* IMV B-7023, while in the case of isobutyric – practically not changed (90.0 µg/mL). However, after 72 hours, the content of both OA decreased slightly (Table 3). It should be noted that representatives of the genus *Bacillus* at their cultivation with calcium orthophosphate synthesize from 2 to 6 of OA (Chen et al., 2006; Zaidi et al., 2016).

In our studies with inorganic compound of P, a decrease in the pH of the nutrient medium was observed, consistent with Sharma et al. (2012) and Mehta et al. (2015), which indicated that Ca-phosphate solubility increased with decreasing pH. The decline of the latter indicates the production by rhizobacteria of organic acids that is obviously the main mechanism in the solubilization of mineral phosphates (Mehta et al., 2001).

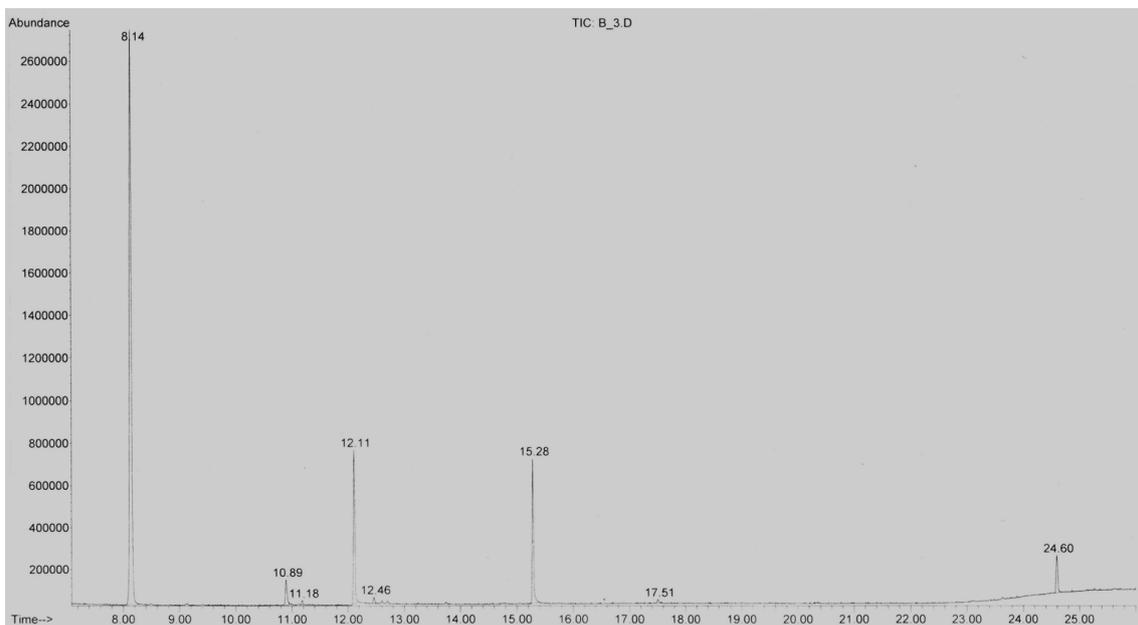


Figure 3 GC-MS spectra of organic acids in CM of the bacterium *Bacillus subtilis* IMV B-7023 during its cultivation in a nutrient medium with calcium orthophosphate

Table 3 The content of organic acids in the culture medium of the bacterium *Bacillus subtilis* IMV B-7023 at its cultivation in a glucose-mineral medium with calcium orthophosphate (Ca₃(PO₄)₂)

RT, min	Organic acid	Content OA (µg/mL), at cultivation of bacterium for an, hours		
		24	48	72
10.89	Acetic	19.0 ± 2.0	300.0 ± 40.0	200.0 ± 12.0
12.46	Isobutyric	92.0 ± 8.0	90.0 ± 8.7	80.0 ± 7.6

Synthesis by bacillus of acetic and isobutyric acids at high concentrations was accompanied by a decrease in the pH of the nutrient medium from 6.7 to 4.3, which caused a negative effect on growth activity. The amount of phosphate ions in the culture medium *B. subtilis* IMV B-7023 increased by almost 8 times after 48 h cultivation (Table 4).

Table 4 Physiological activity of *Bacillus subtilis* IMB B-7023 at cultivation in a nutrient medium with Ca₃(PO₄)₂

Term of cultivation, hours	pH	Number of viable cells (CFU/mL)	PO ₄ ³⁻ , mg/L
0	6.7	(6,5 ± 0,1) · 10 ⁷	66,0 ± 5,7
24	4.7	(1,3 ± 0,1) · 10 ⁹	376,7 ± 23,6
48	4.5	(1,4 ± 0,1) · 10 ⁷	446,7 ± 9,8
72	4.3	(2,1 ± 0,2) · 10 ⁶	433,3 ± 16,8

CONCLUSION

Thus, the quantitative and qualitative composition of organic acids synthesized by strain *B. subtilis* IMV B-7023 depended on the composition of the nutrient medium. The largest number of OA differing in structure of the C-C chain were identified in the culture medium of bacillus cultured in the classical glucose-mineral medium (with glucose and an organic compound of phosphorus – calcium glycerophosphate) or in its modified form (with calcium glycerophosphate but without glucose). The *B. subtilis* IMV B-7023 at cultivation with inorganic compound of P – Ca₃(PO₄)₂ synthesized only acetic and isobutyric acids, whose concentrations were at times higher than in medium with calcium glycerophosphate.

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