Full Length Research Paper

Accumulation of Phenolic Compounds in the Cultural Media of Phosphate-Mobilizing Bacteria of Genus Bacillus Cohn

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Abstract. Bacteria belonging to the genus Bacillus are capable to produce of phenolic compounds. The most noticeable concentrations of phenolic compounds in the cultural media these bacteria were determined at cultivation of bacteria in nutrient medium with gelatin (Bacillus subtilis IMV V-7023 – 94.1 μg/mL, B. subtilis 11 – 86.6 μg/mL and B. cereus v. mycoides 16 – 86.1 μg/mL). At cultivation of B. subtilis IMV V-7023 in the liquid mineral medium with calcium orthophosphate and glucose during three–twenty four hours the content of phenolic compounds reached 58.3 μg/mL, in the medium with calcium glycerophosphate and glucose – 27.0 μg/mL and in the same medium without glucose – 10.2 μg/mL. Accumulation in the cultural media of the studied strains of bacilli of compounds of the phenolic nature depended from the composition of nutrient media both and from the strains characteristic of bacteria.

Keywords: bacilli; culture conditions; glucose; nutrient mediums; phenolic compounds

1. INTRODUCTION

The Bacillus genus is a heterogeneous group of Gram-positive, facultative anaerobic, endospore-forming bacteria spread into the environment, even though soil is generally accepted as its natural reservoir. These bacteria produce biologically active extracellular agents: antibiotics, enzymes, amino acids, plant growth regulators, toxins and other substances (Priest et al., 1993). Many of these connections are characterized by phenol nature. The compounds of phenolic nature are the extensive group of the organic substances, very heterogeneous in the chemical structure and which contain in their composition aromatic ring (Ar) with one or several hydroxyl groups (Ar(OH)n). In basis to classification of phenols it is fixed:

(*) nature of Ar – simple phenols, naphtols and oxy derivatives of other aromatic connections;

(*) numbers of OH-group that is related to the aromatic ring – mono- and polyphenols;

(*) amounts of fragments of Ar(OH)n – mono- and polynuclear phenols. Separately distinguish stericly hindered or the hindered phenols in that the nearest to the hydroxyl group deputies are tert-alkylen (Roginsky, 1988). These substances are active metabolites of cellular exchange and play an important role various physiological processes. Synthesis of these metabolites is characteristic of practically all plant cells (Fedotova et al., 2012; Fedotova et al., 2013; Zaprometov, 1996). In literature there aren’t enough data on synthesis of phenolic substances by microorganisms. It is known that these substances in the cells of bacteria perform the role of antioxidants (Skorochod et al., 2012) and they also participate in the protection of plants from phytopathogenes (Kiprushkina et al., 2014; Manzhelesova et al., 2012). Some phenolic compounds, synthesized by microbial cell, possess antagonistic activity (Manzhelesova et al., 2012; Tserkovniak et al., 2009).

By us it was shown that phosphate-mobilizing bacteria of Bacillus genus are capable synthesize substances of phenolic nature (Skorochod et al., 2013; Tserkovniak et al., 2009). Thus, in the cultural media of B. subtilis IMV V-7023 established existence of 3-hydroxy-4,4-dimethyltetrahydro-2-furanone, 5-hydroxymethyl-2-furancarboxaldehyde, 1-(4-acetylphenyl)-1-ethanone, n-nonadecane, phenylactic and 4-hydroxyphenylacetic acids. In the cultural media of B. pumilus 3 these substances aren’t found out that testifies about the strains distinctions of a range of synthesizable phenolic compounds by bacteria of Bacillus genus (Tserkovniak et al., 2009).
Considering that phosphate-mobilizing bacilli are perspective for creation of bacterial preparations for plant growing, by the purpose of the work was a study of the accumulation of phenolic connections by these bacteria in the dependence on conditions of their cultivation.

2. MATERIAL AND METHODS

2.1. Chemicals

Chemicals were from Sigma unless otherwise stated. All chemicals and solvents used were of analytical grade.

2.2. Bacterial strains

Bacterial strains of Bacillus genus used in this study are listed in Table 1 are capable of mineralizing the phosphorus-containing organic and solubilizing the insoluble inorganic substances of phosphorus. These microorganisms are isolated from the chernozem soil of the Ukraine (Roy et al., 2001) also were presented by the species following: B. megaterium, B. subtilis, B. cereus v. mycoides and B. pumilus. For comparison used a B. subtilis IB-22 (Patent RU 2178970, 2002), isolated from the model of the typical chernozem, selected in the territory of the Republic of Bashkortostan, kindly provided by Dr. A. I. Melentiev (laboratory of applied microbiology of the Institute of Biology, Ufa Sci. Centre of RAS [Russian Academy of Science].

<table>
<thead>
<tr>
<th>Table 1: Bacillus species and strains used in the present work</th>
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<tr>
<td><strong>Bacillus species</strong></td>
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<td>B. megaterium</td>
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<td>B. subtilis</td>
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<td>B. cereus v. mycoides</td>
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<td>B. pumilis</td>
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2.3. Bacterial culture conditions

Bacterial strains used in this study were grown in the nutrient mediums of next composition (g/L):

I (with calcium glycerophosphate) = (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 6.8 – 7.2;

II (with gelatin) = maltose – 1.0; gelatin – 10.0; KH₂PO₄ – 1.60; MgSO₄·7H₂O – 0.75; ZnSO₄·7H₂O – 0.25; (NH₄)₂SO₄ – 0.50; yeast autolysate – 0.15. The cultivation of bacilli was conducted in the microbiological test tubes, which contain on 20 ml of nutrient medium. The microbiological test tubes were incubated at 28 °C and 240 rpm for 96 h (Varbanets et al., 2008).

In a number of experiments the bacteria grew up in the nutrient medium of the third type, containing Ca₃(PO₄)₂ (Mehta et al., 2001). Strains were grown in 750 mL Erlenmeyer flasks with 100 mL of liquid nutrient medium. Flasks were incubated at 28 °C and 240 rpm for 96 h.

Bacterial growth in the cultural liquid was determined by method of serial tenfold cultivations with the subsequent seeding on a potato agar. pH-value the cultural media defined by potentiometry.

Cultural liquids are 16 strains the bacteria of Bacillus genus freed from the bacteria by centrifugation on the centrifuge OPn-8 (joint stock company “TNK DASTAN”, Kirgizstan) during 15 min at 8000 rpm. In the obtained cultural media the total phenolic compounds were determined (Skorochod et al., 2014).

2.4. Protein quantification

Protein in the cultural media of bacteria was quantified using the Bradford method (Bradford, 1976) with bovine serum albumin (Sigma) as the standard.
2.5. The influence of the concentration of the source of carbonic power and energy in the nutrient medium on synthesis of B. subtilis strain IMV V-7023 of phenolic compounds

*B. subtilis* IMV V-7023 grew up in the nutrient medium with calcium glycerophosphate in which concentration of glucose was set at the level from 1.0 to 20.0 g/L, and also in the same nutrient medium without glucose, where calcium glycerophosphate it was the only source of carbon and phosphorus.

2.6. Determination of total phenolic compounds

The content of the soluble compounds of phenol nature in the cultural medium of bacilli (Tserkovniak et al., 2009; Skorochod et al., 2013; Skorochod et al., 2014) was evaluated according to the Folin-Ciocalteu method (Folin et al., 1927; Wrolstad et al, 1999) in the modification of Singleton and Rossi (Singleton et al., 1965). A calibration curve was calculated using pure gallic acid. The total phenol content was expressed as gallic acid equivalents (GAE) in μg of gallic acid per ml cultural media are 16 strains the bacteria of *Bacillus* genus.

Data presented are average of three measurements.

Table 2: The dependence of accumulation a phenolic compounds by bacteria *B. subtilis* IMV V-7023 from concentration of glucose in a nutrient medium

<table>
<thead>
<tr>
<th>The concentration glucose in a nutrient medium, g/L</th>
<th>pH</th>
<th>The content of protein, μg/mL</th>
<th>Total phenolics contents, μg/mL</th>
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<tr>
<td>1.0</td>
<td>7.7</td>
<td>0.4±0.01</td>
<td>10.3±0.11</td>
</tr>
<tr>
<td>5.0</td>
<td>7.7</td>
<td>2.2±0.10</td>
<td>14.8±0.50</td>
</tr>
<tr>
<td>10.0</td>
<td>6.6</td>
<td>14.0±0.10</td>
<td>19.4±1.20</td>
</tr>
<tr>
<td>15.0</td>
<td>6.8</td>
<td>8.6±0.07</td>
<td>15.4±0.40</td>
</tr>
<tr>
<td>20.0</td>
<td>7.7</td>
<td>2.7±0.04</td>
<td>14.6±0.70</td>
</tr>
</tbody>
</table>

2.7. Statistical analysis of the data

Each result is indicated as the mean value of at least three independent experiments ± the standard error of the mean (SEM). Results were analyzed by means of program packet Microsoft Excel (Microsoft Office 2003, Microsoft Co.) and Statistics 6 (Stat Soft Inc.2001).

3. RESULTS AND DISCUSSIONS

3.1. Influence of various compositions of nutrient mediums on accumulation of phenolic compounds by phosphate-mobilizing strains of bacilli

It is established that accumulation of phenolic compounds by bacteria of *Bacillus* genus is defined by their strains special characteristics. At cultivation of bacteria in the nutrient medium with calcium glycerophosphate and glucose the most active producers of phenolic connections were *B. megaterium* 1 and *B. megaterium* 2. The content of these substances in their cultural mediums made 67.3 and 67.7 μg/mL, respectively. In these conditions of *B. pumilus* 7 accumulated of phenolic compounds – 55.2 μg/mL. Their smallest contents was observed in the cultural mediums are *B. megaterium* 9 (trace quantities), *B. pumilus* 4 – 15.3 μg/mL and *B. cereus* v. *mycoides* 14 – 13.6 μg/mL (Figure 1).

The calcium glycerophosphate is a source of calcium and phosphate ions. Previously we found that when cultivation *B. megaterium* 1 and *B. megaterium* 2 in nutrient medium with glucose and calcium glycerophosphate for 3 days, in cultural liquids this bacteria the amount of phosphate ions was 96.0 and 40.0 mg/L. The largest accumulation of phosphate ions observed in cultural liquid of *B. megaterium* 9 – 204 mg/L (Roy et al, 2001). According to literature data (Knobloch et al., 1983), low concentrations of ions phosphate accompanies an increase in content of phenolic compounds, and high – lower.

In a nutrient medium with gelatin and maltose the studied strains of bacteria accumulated significant amounts of compounds of the phenolic nature. The high concentrations of phenolic compounds was noted at *B. subtilis* IMV V-7023 and *B. subtilis* 11 (94.1 and 86.6 μg/mL), and also at *B. cereus* v. *mycoides* 16 (86.1 μg/mL). According to our previous researches at cultivation of *B. subtilis* IMV V-7023 in a nutrient medium with gelatin, proteolytic activity of bacteria increased in 28 times in comparison with the proteolytic activity of this strain when cultured in a media with calcium glycerophosphate (Roy et al, 2014). It is possible to assume, that the possible cleavage of proteins, produced significant amounts of
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Amino acids, which are taking part in the synthesis of phenolic substances. These studies are at an early stage and should be continued in the future. The low concentrations of these substances observed at B. megaterium 9 (64.1 µg/mL), B. pumilus 4 (65.0 µg/mL) and B. cereus v. mycoides 10 (47.1 µg/mL). Thus, accumulation of phenolic compounds by the studied phosphate-mobilizing bacteria is defined not only their strains features, but also cultivation conditions (Figure 1).

![The medium with calcium glycerophosphate and glucose](image1)

**Fig. 1:** The total phenolic contents in the cultural mediums of phosphate-mobilizing strains of bacilli at their cultivation in nutrient mediums of various compositions

### 3.2. Influence of type the source of carbon and phosphorous nutrition on the accumulation of the compounds of phenolic nature by the B. subtilis IMV V-7023

B. subtilis IMV V-7023 (Ukr. Patent No. 54923 A, 2003) is included in composition of complex bacterial preparation for the plant growing “Azogran”. It has a stable composition during prolonged storage, improves nitrogenous and phosphorous nutrition of plants, stimulates their growth and development by the biologically active substances of bacterial origin, protects plants against pathogens and increases their yield crop on 18-37% (Kurdish, 2010). It was of interest to investigate the dependence of the accumulation of phenol compounds by these bacteria in the dependence on the type of the source of carbon and phosphorous nutrition. It is established that at cultivation of bacteria in a nutrient medium with calcium glycerophosphate without glucose the content of compounds of the phenolic nature in cultural media didn’t exceed 10.2 µg/mL (Figure 2). At cultivation in the analogous in composition nutrient medium, but with the addition 10.0 g/L of glucose bacteria synthesized the greater amount of phenolic compounds – 27.8 µg/mL after 48 h and 27.0 µg/mL after 72 h. Feasibility study of the cultural medium B. subtilis IMV V-7023, obtained in the stationary phase of growth after cultivation in the nutrient medium with calcium glycerophosphate and glucose showed the presence in it of L-phenylalanine and L-tyrosine. The amount of these compounds when grown bacteria in these conditions have been increasing after 24 and 48 h, cultivation. After 72 h. their content was reduced further again increased by 96 h. Synthesis of cells L-phenylalanine and L-tyrosine in a nutrient medium where calcium glycerophosphate was the sole source of carbon and phosphorus supply increased gradually (Tserkovniak et al., 2009). The L-phenylalanine and L-tyrosine are predecessors of synthesis of substances of the phenolic nature (Harborne, 1967). The greatest concentrations of phenolic substances (58.3 µg/mL) observed at cultivation of B. subtilis IMV V-7023 within 72 h in the nutrient medium with glucose and almost insoluble inorganic phosphate (Ca₃(PO₄)₂) which was for cells a source of phosphorous nutrition (Figure 2). According to research by Ruiz et al (2003), calcium clearly exerted a positive influence on the activities of enzymes (phenylalanine ammonia-lyase, polyphenol oxidase and peroxidase) involved in the
metabolism of the phenolics. Thus, a favorable factor for synthesis of phenolic compounds by this strain is availability of glucose in a nutrient medium.

![Graph](image)

**Fig. 2:** Accumulation of compounds of the phenolic nature by bacteria *B. subtilis* IMV V-7023 at cultivation with a different source of carbon and phosphorous nutrition

Note:
I – a nutrient medium with inorganic almost insoluble phosphate (Ca₃(PO₄)₂) and glucose;
II – a nutrient medium with calcium glycerophosphate and glucose;
III – a nutrient medium with calcium glycerophosphate and without glucose

3.3. The dependence of synthesis of compounds of the phenolic nature by the *B. subtilis* IMV V-7023 from concentration of glucose in a nutrient medium

According to literary data, biosynthesis of compounds of the phenolic nature happens in two basic ways – shikimate and acetate-malonate biosynthetic pathways (Harborne, 1967). By what pathway synthesis of these substances by bacteria of *Bacillus* genus is carried out, it is authentically not known. However, the initial products of the biosynthesis of the phenolic compounds – phosphoenolpyruvate, erythrose-4-phosphate (shikimate biosynthetic pathway), acetyl-CoA (acetate-malonate biosynthetic pathway) are formed during glycolysis of sugars (Harborne, 1967).

It was of interest to study the dependence of synthesis of compounds of the phenolic nature by the *B. subtilis* IMV V-7023 from concentration of glucose in a nutrient medium. The results of researches showed that at increase in concentration of glucose from 1.0 g/L to 10.0 g/L the content of phenolic compounds in the cultural media increased from 10.3 to 19.4 μg/mL, and protein increased from 0.4 to 14.0 μg/mL (Table 2). Further increase the concentration of glucose in a nutrient medium to 15 and 20 g/L was followed by decrease in the content of these substances (15.4 – 14.6 μg/mL, and 8.6 – 2.7 μg/mL, respectively). The pH-value of cultural media on 48 h the cultivation of *B. subtilis* IMV V-7023 decreased, reaching the minimum values at concentration of glucose in a nutrient medium of 10 g/L, and at its bigger contents again increased (Table 2).

4. CONCLUSION

Thus, most noticeable concentration of phenolic substances, the bacteria accumulated at their cultivation in the nutrient medium with gelatin (*B. subtilis* IMV B-7023 – 94.1 μg/mL, *B. subtilis* 11 – 86.6 μg/mL and *B. cereus* v. *mycoides* 16 – 86.1 μg/mL). It was shown that accumulation of phenolic compounds by bacteria *B. subtilis* IMV B-7023 at cultivation within three days in the mineral medium with calcium orthophosphate and glucose reached 58.3 μg/mL, in the nutrient medium with calcium glycerophosphate and glucose – 27.0 μg/mL and in the same nutrient medium without glucose – 10.2 μg/mL. The received results testify that accumulation in a nutrient medium of compounds of the phenolic nature the studied bacilli is defined not only their strains features, but also cultivation conditions.

4.1. Recommendations

The prospect for the obtained results consists in the selection the compounds of phenol nature of the
strains bacteria of Bacillus genus highly productive on the synthesis and the creation of effective biological preparations for the plant growing, and it is also possibly and other spheres of human activity.

4.2. Conflicts of Interest

All the authors have contributed equally in completing the research work. Further, there are no conflicts of interests amongst the authors.

Acknowledgments

The authors are grateful to Dr. A. I. Melentiev (laboratory of applied microbiology of the Institute of Biology, Ufa Sci. Centre of RAS [Russian Academy of Science]) for kindly provided strain of B. subtilis IB-22.

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Anton Ocheretyanko, engineer of the Department of Microbiological processes on solid surfaces in the Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. After completion of University (speciality is Human and Animal Physiology Human and Animal Physiology) got a specialist. The university investigated the influence of synthetic artificial sweeteners on cholepoietic liver function. Currently doing research quantitative and qualitative composition of phenolic compounds that are synthesized by soil bacteria of the genus Bacillus and Azotobacter.

Iryna Skorochod, junior scientist of the Department of Microbiological processes on solid surfaces in the Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. After completion of University (speciality is biology-chemistry) got the Master's of biology degree. Her scientific interests include antioxidant systems of different microorganisms, biological redox, low molecular weight antioxidants of bacteria, the influence of nanoparticles on protector complex of bacteria. Iryna Skorochod is a member of Ukrainian biochemical society. She is an author of scientific works on studying of antioxidant systems of bacteria, to influence of nanoparticles on a protector complex of microorganisms. Presently works on dissertation.

Alla Roy (was born on October 10, 1946. In. Kyiv), senior staff scientist of the Department of Microbiological processes on solid surfaces in the Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. In 1976 protected candidate's dissertation. Alla Roy is the expert in the field of ecology, physiology and morphology of microorganisms, soil microbiology, and biotechnology. She is a member of Vynograds'kyj Society of Microbiologists of Ukraine, forming part FEMS. Alla Roy is the author of 147 scientific publications, including 2 reviews, 9 patents, 2 publications in foreign editions.

Dr. Ivan Kurdish is a doctor of biological sciences, professor. He is a leader of Department of Microbiological processes on solid surfaces in the Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. It was born in 1942. After studies in the Taras Shevchenko National University of Kyiv from 1970 works in the Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. In 1978 protected candidate's dissertation, in 1993 – doctoral dissertation. His research interests related to research of ecology, physiological and biochemical properties of methanotrophic, nitrogen-fixing and phosphate-mobilizing bacteria for their co-operating with the different factors of environment, including with solid materials and nanomaterials. It is shown them, that heterogeneous cultivation of representatives the different physiology groups of microorganisms is at a presence nanomaterials accompanied them by pin cooperation and, as a rule, considerably induce physiological and biochemical activity of bacterial populations, protects cells from negative influence of extreme factors of environment. Under his guidance the worked out biotechnology of decline of methane release is in coal mines, high-efficiency complex bacterial preparation for a plant-grower that is applied in industry, is created. Dr. Ivan Kurdish is author of 280 scientific works, including four monographs and row of inventions. He is the member of editorial boards of three scientific journals. He is a vice-president of Vynograds'kyj Society of Microbiologists of Ukraine.