Estimation of the Ability to Decompose Sodium Benzoate by a Bacterium Isolated from Biohumus *Eicenia Fetida*

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Abstract. The study presents growth kinetics of a BG28 bacterial strain isolated from vermicompost (generated with the use of *Eisenia fetida* worms). The strain was given a codename BG28. It was revealed that the isolate is capable of using high concentrations of sodium benzoate (up to 15 g/l) as a substrate. At the same time, the rate and division constants of a culture that grows at 5 g/l, 10 g/l, and 15 g/l do not differ. However, the mean and specific increment of BG28 at the end of the logarithmic growth phase is significantly higher in the variant with 5 g/l. It was testified, that with the shortest duration of the logarithmic growth phase on a medium with 5 g/l, the substrate loss constant is higher than in other variants. The difference in the kinetic parameters of the culture at 5 g/l and 10 g/l sodium benzoate is insignificant. With an increase of the substrate concentration to 15 g/l, the duration of the logarithmic growth phase increased significantly, but the sodium benzoate decrease constant was found to be the lowest. During the study of the individual properties of BG28, it was revealed that the strain is capable of growing on benzoic acid. On this basis, it can be assumed that the culture contributes to the degradation of plant residues during vermicomposting. Besides, the isolate grows on a mineral nutrient medium with polyethylene glycol 6000 and liquid paraffin. In the process of the individual properties estimation, it was revealed that the strain is capable of local suppression of the *Alternaria brassicicola* VKM F-1864 mold growth when co-cultivated on agar nutrient medium.

1 Introduction

Eisenia fetida worms are capable of rapid decomposition of organic matter (plant and animal residues) in the native soil. This property allows them to be used in vermicomposting processes meaning biodegradation of organic waste (by-products of the production of vegetables, fruits, various drinks, wood, sewage sludge, cattle breeding and the sugar industry) [1, 2, 3, 4, 5, 6, 7].

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The biochemical and physiological activity of earthworms is one of the main factors of their utilization efficiency, since they are capable of synthesizing cellulase, chitinase, protease, amylase, glucoseoxidases and phosphatases [8].

Vermicomposting is a complex process involving the microbial community. According to some data, *Entomoplasma somnilux* and *Bacillus lichenoformis* turned out to be dominant among the bacteria in the intestines of earthworms. Other microorganisms included the genera *Aeromonas*, *Clostridium*, *Ferrimonas*, *Photobacterium* and *Paenibacillus* [9]. As follows, earthworms exist in an abundance of microorganisms, many of which form symbiotic relationships with *E. fetida* (for example, the stimulating effect of *Paenibacillus motobuensis* culture on the growth of worms and the formation of vermicompost conglomerates.) [10]. Though, there are also disease-causing species among the microbiota.

Having adapted to these conditions, the worms have developed an effective immune system. The first line of defense is the so-called antibiotic barrier, which consists of mucous secretions secreted by special glands in the structure of the worm's skin [11]. *E. fetida* is capable of the antibacterial substances formation which is another mechanism of resistance to pathogenic mechanisms [12]. Among these compounds are vermipeptides, which have antibacterial activity against both gram-positive and gram-negative bacteria, as well as fungi [13]. The ability of earthworms to interact with bacteria which have a positive effect on the vital activity of *E. fetida* and the ability to protect themselves from the negative effects of pathogens. This is an important component of achieving conjugation of species in a biotic community, the entire metabolic potential of which is aimed at obtaining energy during the conversion of complex plant material into readily available connections. As it follows, earthworms are an important component of soil biota, causing serious biological effects on the soil ecosystem and coexist with microorganisms in either antagonistic or symbiotic forms of relationship.

Sodium benzoate is the sodium salt of benzoic acid, which is an aroma intermediate in several plant metabolic pathways. Its physiological role may lie in adaptive mechanisms to reduce oxidative stress [14, 15]. Benzoate is not only a component of plant residues, but an intermediate in the transformation and decomposition of substances such as phenylalanine, mandelic acid and alkylbanzole by microorganisms [16].

Sodium benzoate is one of the synthetic additives widely used in the food industry as a preserving agent. Though, the phytotoxic properties of sodium benzoate are also known. This salt reduced the germination ability, growth and accumulation of rice biomass. At the same time, an increase in the concentration of benzoate led to the destruction of cell membranes, the water status of plants and the degradation of photosynthetic pigments [17].

Therefore, the sodium salt of benzoic acid is both a common compound in plant residues and a convenient model compound in the researches of the destruction of aromatic hydrocarbons by microorganisms.

The aim of this experiment was to determine the kinetic growth parameters (growth rate constant, division rate constant, doubling time, mean and specific increment, duration of the logarithmic growth phase, substrate loss constant) of the isolated bacteria from vermicompost, designated BG28, depending on the concentration of sodium benzoate and to evaluate the ability of the culture to suppress the phytopathogenic *Alternaria brassicicola* VKM F-1864 mold.

2 Materials and methods

A vermicompost sample with masses of 1g was added to 100 ml of sterile water to isolate bacteria capable of using sodium benzoate as a growth substrate. 1 ml of the suspension was diluted to 108 and passage with 100 μl on a mineral nutrient medium with sodium

benzoate as a carbon source (composition: sodium benzoate - 0.1%; KNO_3 - 4 g; KH_2PO_4 - 0.6; Na_2HPO_4 - 1.4; $MgSO_4$ - 0.8; agar - 2%; distilled water - 1000 ml). The colony staining medium green was isolated and further investigated.

Gram staining, mobility and capsule detection were performed using standard testing methods. [18].

To determine the formation of catalase in an isolated culture, a 3% solution of hydrogen peroxide was applied to its colony. Catalase test is considered positive if gas bubbles are released [18].

The proteolytic activity of bacteria was determined by its ability to hydrolyze casein (for its detection, milk agar was used, consisting of sterile defatted by centrifugation at 8000 rpm, milk and 2% agar) and gelatin. Casein hydrolysis is detected by the clarification zone of the medium by the stroke of the culture, while for gelatin - liquefaction [18]. The presence of lipase in bacteria was studied by inoculating a 3% peptone nutrient medium, into which 10 ml (g / l) Tween-80 was added; peptone - 10 g; NaCl - 5 g; $CaCl_2 \cdot H_2O$ - 0.1 g. [18]. The hydrolysis products of Tween-80 react with calcium chloride, forming turbidity. The presence of urease was determined on a nutrient medium, in which carbamide was used as a carbon source (composition (g/l): $(NH_2)_2CO$ - 5.0, K_2HPO_4 - 0.5, Na citrate - 5.0). Urea was added after autoclaving. A litmus paper soaked in sterile water was placed under the plug. The presence of urease was judged by the release of ammonia, which changes the color of the litmus test to blue. The reaction of ammonium sulfate with sodium hydroxide can act as a control, as a result of which ammonia is released (detected using a commercial Nessler reagent (Ural Chemical Plant LLC, Russia), when added, the medium turns yellow in the presence of ammonia) [18].

The ability of the culture to form H_2S was determined with a moistened filter paper in a 5% solution of lead acetate in a strong solution of sodium hydroxide, fixed under a stopper during cultivation in a liquid 3% peptone medium. Blackening of a piece of paper indicated the formation of hydrogen sulfide [18]. The presence of indole was determined by the Salkovsky reaction. For this, a few drops of strong sulfuric acid were added to 1 ml of 0.2% KNO_3. Indole is making it cherry red [18]. Starch hydrolysis was assessed by treating an agar plate with Lugol's solution. For this, 3-5 ml of Lugol's solution was poured onto the surface of the medium. The medium containing starch turns blue, and the hydrolysis zone remains colorless [18].

The ratio of the isolate to oxygen was determined by inoculation with an injection into an agar column. For this, 3% peptone agar was poured into 8 ml tubes. Autoclaved and inoculated into the cooled molten medium. the growth of a culture in the thickness of the medium characterizes its relation to O_2 [18].

The ability of microorganisms to use organic compounds as the only carbon sources was studied by inoculation on a medium containing sodium benzoate, citrate, succinate, glucose, fructose, lactose, sucrose, mannitol, phenol glycine, L-cysteine, β-alanine, L - glutamic acid, DL-phenylalanine, ornithine hydrochloric acid, L-arginine hydrochloric acid, leucine, norleucine and methionine as a carbon source at a concentration of 1 g/l. The inoculations were incubated at 25℃ for 3 days. By the nature of the growth, the ability of the strain to grow on the substrate was judged.

The kinetic parameters of culture growth in a liquid mineral nutrient medium (without agar) were carried out at 5 g/l, 10 g/l, and 15 g/l sodium benzoate. For this, 100 µl of the initial suspension of BG28 cells was introduced into the medium (100 ml) in duplicate. After seeding, the initial optical density was measured and repeated every day at a wavelength of $\lambda = 600$ nm.

The growth rate constant was calculated by the formula [19]:

$$\mu = \frac{(lg\, x_1 - lg\, x_0)}{lg\, e(t_1 - t_0)}, \quad (1)$$

where, x_0 and x_1 are the OD values of the cell suspension corresponding to the growth times t_0 and t_1. The fission rate constant was calculated by the formula [19]:

$$v = \frac{(lg\, x_1 - lg\, x_0)}{lg\, 2(t_1 - t_0)}, \quad (2)$$

where x_0 and x_1 are the OD values of the cell suspension corresponding to the growth times t_0 and t_1.

The biomass doubling time, or the generation time, was calculated by the formula [18]:

$$g = \frac{1}{v}, \quad (3)$$

where, g is the generation time, v is the specific growth rate.

Average culture growth was calculated using the formula [20]:

$$X = (10^{lgG_{(x+1)}} - 1) \cdot 100\%, \quad (4)$$

where

$$lgG_{(x+1)} = \frac{\sum lg(x+1)}{n}, \quad (5)$$

where n is the number of repetitions of measurements.

The lag phase duration was calculated by the formula [21]:

$$L = T * v, \quad (6)$$

where

$$T = t_1 - \frac{(lnx_1 - lnx_0)}{\mu}, \quad (7)$$

Specific increment was calculated using the formula [22]:

$$R = \frac{x_1 - x_0}{x_0}, \quad (8)$$

The rate constant for the of the substrate decrease was calculated by the formula [21]:

$$K = \frac{lnC_0 - lnC_1}{t_1 - t_0}, \quad (9)$$

where C_0 and C_1 are the initial and the final optical density of sodium benzoate at a wavelength of λ - 226 nm, respectively, t_0 and t_1 are the cultivation time during which the first measurement of optical density and the final one falls.

The difference method of statistical analysis was used in order to reliability calculation [23].

Evaluation of the antagonistic properties of the bacteria BG28 against *Alternaria brassicicola* VKM F-1864 was carried out by the method of agar discs, based on the ability of antibiotic substances to diffuse into the nutrient medium with the test culture. BG28 bacteria was sown on a nutrient medium (3% agar peptone) with a lawn and incubated for a day at a temperature of 25°C.

Then, with a forstner bit (d = 11), agar disks were cut out from the BG28 dish and transferred to a Petri dish with a frozen Sabouraud nutrient medium, which was previously inoculated with the *A. brassicicola* VKM F-1864 fungus using the lawn method. The crops were incubated for several days at a temperature of 25°C.

3 Results and Discussion

A bacterial strain was isolated from vermicompost and designated as BG28.

Colonies of the isolate, on 3% peptone nutrient medium at 25°C, convex, round, smooth with a smooth edge. The isolated bacterium is represented by gram-negative, non-spore-forming, motile rods.

The strain does not hydrolyze casein and does not exhibit lipolytic activity. Hydrolyzes gelatin and starch. Tests for catalase, phosphatase and oxidase activity are positive. Does not form H_2S and indole when cultivated on liquid 3% peptone. Forms ammonia. Urease test is positive. With respect to oxygen, the strain is aerobic.

Culture BG28 grows on a mineral nutrient medium containing D-glucose, raffinose, arabinose, fructose, L-cysteine (weak growth), β-alanine, L-glutamic acid, ornithine hydrochloric acid, L-arginine, leucine, methionine as a carbon source (weak growth), and does not grow on medium with sucrose, galactose, lactose, mannitol, maltose, glycine, DL-phenylalanine and norleucine. The strain is able to grow on a nutrient medium with sodium benzoate and benzoic acid, but is not able to grow on a medium with 3-chlorobenzoic acid and 4-chlorobenzoic acid.

In the course of studying the growth kinetics of the isolate on a mineral nutrient medium with sodium benzoate as a substrate, it was found that the growth rate constants (μ) at a substance concentration of 5 g/l, 10 g/l and 15 g/l do not differ. The fission rate constant (v) of the same variants turned out to be similar. Accordingly, the differences in the generation time (g) of the variants also do not differ.

It was found that the specific increase in BG28 when cultivated in a mineral nutrient medium with 5 g/l is higher than with 10 g/l and 15 g/l by 73% and 75%, respectively. The average growth of cultures per day, at which the culture enters the phase of slow growth, corresponds to the specific one: the value of the variant grown at 5 g/l is higher than that of the others by 66% (for 10 g/l) and 71% (for 15 g/l).

In the course of calculating the duration of the logarithmic growth phase of strain BG28, it was shown that its values increase with an increase in the concentration of sodium benzoate in the medium. At the same time, the loss constant (K) of the substrate decreases as the concentration increases from 5 g/l to 15 g/l.

Strain BG28 uses sodium benzoate to form a colored substance. On this basis, it can be assumed that the biodegradation of the benzoic acid derivative proceeds along the meta-pathway of cleavage. In this case, the breaking of the aromatic ring between the hydroxylated and non-hydroxylated carbon atoms is catalyzed by dioxygenases (pyrocatechin-2,3-dioxygenase). The cleavage product in this case is 2-hydroxymuconic acid semialdehyde, which is then converted into pyruvate, acetaldehyde, or other metabolic intermediates [23].

There is evidence that the vast majority of microorganisms are capable of decomposing sodium benzoate at concentrations up to 5 g/l [24]. However, the *Rhodococcus opacus* 1CP strain was able to grow on a nutrient medium with a benzoic acid salt at concentrations up to 10 g/L [25]. Based on this, it can be argued that the BG28 bacterium grows at high concentrations of sodium benzoate - 10-15 g/l. However, 5 g/l is a more optimal concentration than 10 g/l and 15 g/l. So, despite the fact that the growth and division rate constants are the same for all variants, the average and specific growth was higher in the variant with 5 g/l of sodium benzoate in the medium. At the same time, the substrate disappearance constant at a concentration of 5 g/l is higher than at 10 g/l and 15 g/l. At the same time, the shortest duration of the logarithmic phase of the culture on a nutrient medium is observed at 5 g/l of sodium benzoate in the medium.

According to literature data, the group of Gram-negative aerobic, non-spore-forming, motile rod-shaped bacteria that are capable of growing on 3% peptone agar includes

representatives of such genera as *Acetobacter*, *Acidiphilium*, *Acidovorax*, *Acinetobacter*, *Afipia*, *Agrobacterium*, *Agromonas*, *Alcaligenes*, *Alteromonas*, *Aminobacter*, *Aquaspirillum*, *Azomonas*, *Azorhizobium*, *Azotobacter*, *Beijerinckia*, *Bradyrhizobium*, etc. Among the genera under consideration, there are data on the ability to decompose sodium benozoate in representatives of *Acinetobacter* sp. [24], *Zoogloea* sp. [25], *Halomonas* sp., but the bacteria of the genus *Pseudomonas* are best known as degraders of sodium benzoate [26].

The ability of the strain to use high concentrations of sodium benzoate attracts attention, since this benzoic acid derivative is used as a preservative and has been reported to have antagonistic efficacy against yeast [27] and bacteria (for example, representatives of the genus *Salmonella*, *Campylobacter*, *Listeria*, *Escherichia* and *Staphylococcus* [28].

It is known that phenolic compounds are widely distributed in plants. Thus, benzoic acid and its derivatives are involved in plant signaling mechanisms, which are activated upon damage by pathogenic organisms [29]. Soil microorganisms utilize plant residues, and the presence of special systems in microbes that destroy aromatic hydrocarbons is an integral part of the biodegradation of such compounds [30]. The BG28 culture can participate in the processes of vermicomposting of plant residues, together with *Eisenia fetida*, for example, in the processes of biodegradation of phenylalanine and some of its derivatives.

In the course of studying the individual properties of strain BG28, it was found that the isolate is capable of growing on a nutrient medium containing glycerol, liquid paraffin, and polyethylene glycol 6000. The data obtained can be used in a further assessment of the prospects for using the strain for the biodegradation of simple hydrocarbons.

It was also found that the BG28 bacterium is characterized by antagonistic activity against the phytopathogenic fungus *A. brassicicola* VKM F-1864, as evidenced by the absence of fungal growth around agar disks with this bacterium.

4 Conclusions

The conversion of matter and energy in nature is carried out by many macro- and micro-organisms. When organisms live together in the same space, they exist within certain forms of relationships (for example, antagonism, metabiosis, symbiosis, etc.), which are the main parameters for the formation of complex communities, forming a trophic system. The totality of organismal elements of the biota should be characterized by conjugation. It is easier to achieve by focusing the entire system on the destruction and transformation of one complex substrate, where both micro- and macroorganisms complement the trophic specificity of each individually. Based on this, the isolated BG28 strain can act as a decomposer of phenylalanine and sodium benzoate in plant residues that are macerated and primarily decomposed during vermicomposting in *Eisenia fetida* worms.

In the course of studying the individual properties of the BG28 culture, it was revealed that the strain presumably belongs to the genus *Pseudomonas*. The isolate was able to grow on liquid paraffin, glycerol and polyethylene glycol 6000, as well as in the presence of high concentrations of sodium benzoate. So, at 5 g/l, 10 g/l and 15 g/l, the values of the growth rate constant and the division rate constant do not differ, while the average and specific growth showed that the highest values are in the variants growing at 5 g/l of sodium benzoate. However, at the shortest duration of the logarithmic phase at 5 g/l, the loss constant of the substrate in terms of optical density is slightly lower than that of the variant that was incubated at 10 g/l of sodium benzoate (about 16%). Based on this, it can be assumed that the BG28 bacterium is able to efficiently utilize high concentrations of benzoic acid salt. In turn, staining indicates that the cleavage occurs along the meta-pathway.

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