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Study of Pesticide-Degrading Properties of Bacteria Using 2,3,5-Triphenyltetrazolium Chloride as an Indicator of Their Dehydrogenase Activity

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Abstract—The paper presents a method of introducing water-insoluble active ingredients of commercial pesticides into selective media as the only source of organic food for *Bacillus mycoides K*1 and *Pseudomonas putida Pn6* bacterial strains, with the aim to assess the degrading activity of the bacteria.

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The first stage of bacterial degradation of xenobiotics involves oxidative metabolism reactions catalyzed by dehydrogenases [1, 2]. Therefore, activity of such enzymes in bacteria is evidence of degrading properties of the latter.

Tetrazolium salts are widely used for activity assessment of dehydrogenases (enzymes catalyzing redox reactions). Dehydrogenases reduce these salts into formazanes which are colored compounds. It was found that such usual reducers as cellular and tissue glutathione, ascorbic acid, and cysteine do not convert tetrazolium salts into formazanes at pH lower than 9.0. Tetrazolium salts are also not reduces by sugars, because the latter are only active at pH higher than 11. In cellular redox reactions, tetrazolium salts function as acceptors of electrons from the substrate being oxidized. This principle forms the basis of a method for the detection of oxidative enzymes by means of tetrazolium salts [2].

The test for the dehydrogenase activity of bacteria toward xenobiotics is recommended to perform with a 1–5% aqueous solution of triphenyltetrazolium chloride [1–3]. The method is based on the reduction of a colorless salt 2,3,5-triphenyltetrazolium chloride to a red-colored triphenylformazane under the action of dehydrogenases involved in the degradation of xenobiotics [1]. Qualitative assessment of the ability of bacteria to degrade xenobiotics is performed by visual observation of color changes of the reaction system, which is considered as evidence of the dehydrogenase activity of bacteria. This procedure allows one to simultaneously test a great number of samples and find out fairly rapidly whether the tested bacteria exhibit degrading activity or not.

To find out whether bacteria are capable of degrading pesticides, one should know whether the specific substrate of bacterial dehydrogenases is directly the active ingredient of commercial pesticides as the specific substrate. The case in point is that most commercial pesticide formulations have a fairly complex chemical composition and contain, along with the active ingredient, organic additives as emulsifiers, stabilizers, polymers, and preservatives. Therefore, the probability that bacteria use these components instead of the active ingredient casts some doubt in the positive results of the test for commercial pesticide formulations. For unambiguous results of this test, a pure active ingredient that contains no admixtures should be tested.

In the present work we set ourselves the task to find out whether the positive 2,3,5-triphenyltetrazolium chloride test is a response for the bacterial degradation

of the active ingredient of a pesticide rather than of organic components of its commercial formulation.

As model pollutants we chose the herbicide Gesagard (prometryn active ingredient) and the insecticide Molniya (λ-cyhalothrin active ingredient). In the experiments we used the State Standard Samples (SSSs) of prometryn (State Standard 7667-99) and λ-cyhalothrin (State Standard 7732-99), produced at the BLOK-1 Research and Production Cooperative (Moscow). Thus, in our experiments bacteria could not use any other substrates but the active ingredients of pesticides.

In preparing samples for experiments we had to solve the problem how to introduce the pesticides to be studied into liquid or agar media as the only source of carbon in view of the fact that these pesticides are scarcely soluble in water [4]. To this end, we developed method for introducing pure active pesticide ingredients as the limiting component of selective bacterial culture media.

In view of the fact that the active pesticide ingredients are insoluble in water but are readily soluble in organic solvents like xylene, toluene, carbon tetrachloride, and chloroform, we prepared saturated solution of the prometryn and λ-cyhalothrin SSSs in chloroform.

Chloroform is heavier than water, hydrophobic, and harmful for bacteria, before the model systems were added to water or contacted with bacteria, the solvent was evaporated. In some experiments, after chloroform had been removed, a highly dispersed aqueous suspension of SSS crystals was prepared.

Preparation of Highly Dispersed SSS Suspensions

Taling into account that the 2,3,5-triphenyltetrazolium chloride test is a qualitative reaction, and the effect is assessed visually, all components of SSS suspensions were taken in arbitrary amounts. Suspensions were prepared individually for each SSS.

First concentrated chloroform solutions of prometryn and λ-cyhalothrin SSSs were prepared. A small portion of each solution was poured into a sterile beaker or flask and scattered uniformly over the bottom of the vessel. The vessel was left open in a hood for 45 min under UV light for chloroform to evaporate completely. As a result, the bottom of the vessel was coated with a thin layer of uniformly scattered extremely fine SSS crystals. A little sterile water was added, and the mixture was magnetically stirred to obtain a highly dispersed uniform aqueous suspension of SSS crystals in water.

As the model pesticide-degrading bacteria we chose *Pseudomonas putida* Pn6 bacterial strain with prometryn *Bacillus mycoides K*1 bacterial strain with λ-cyhalothrin. The main selective medium was a liquid and a solid M9 synthetic agar medium [5], to which 1 mL of 5% aqueous solution of triphenyltetrazolium chloride was added. As a source of carbon we used active pesticide ingredient SSSs introduced into the reaction system as an aqueous suspension or a chloroform solution.

For bacterial inoculation of degrading strains, a suspension of these strains with cell concentrations of about 10^{10} cells/mL was used.

Bacteria in Petri dishes were incubated in a thermostat at 28–30°C. Incubation in a liquid medium was performed in a temperature-controlled shaker at 28–31°C.

Assessment of the Dehydrogenase Activity of Strains on a Solid Minimal Selective Medium with a Water-Soluble Limiting Component

A ternary system containing an active pesticide ingredient SSS, triphenyltetrazolium chloride as a redox indicator, and a suspension of degrading strains was used. Experiments were performed by two procedures.

Procedure 1. M9 agar medium containing triphenyltetrazolium chloride was dispensed in Petri dishes. After the medium has solidified, 0.1 mL of a chloroform SSS solution was applied on its surface and scattered with a sterile spatula. Chloroform evaporated to leave a film of uniformly scattered fine SSS crystals on the surface of the medium. The Petri dishes were divided into two groups: experiment and control. On the agar surface in the Petri dishes of the experimental group we applied 3 separate drops (by 0.05 mL) of a pure bacterial suspension of the corresponding strain. In the Petri dishes of the control group, no bacteria were added, and these media were used to control the degree of bacterial contamination of the tetraphenyltetrazolium chloride in the medium.

Procedure 2. M9 agar medium containing triphenyltetrazolium chloride was dispensed in Petri dishes. After the medium has solidified, 0.1 mL of a chloroform SSS solution was applied on its surface and

scattered with a sterile spatula. The contents of the Petri dishes were incubated in a thermostat for 3 days at 28–30°C. After that the Petri dishes were divided into two groups: experiment and control. On the agar surface in the Petri dishes of the experimental group we applied 3 separate drops (by 0.05 mL) of a highly dispersed aqueous SSS suspension. In the Petri dishes of the control group, no carbon source was added. Incubation was then continued for an additional 7 days.

In the experiment by procedure 1, large bacterial colonies have grown on the spots where bacterial suspension was applied on the thin SSS film. In the control group no coloration was observed. Consequently, colored colonies grown in the experimental group represent the generation of introduced model strains.

In the experiment by procedure 2, too, red coloration was observed in the spots where a highly dispersed aqueous SSS suspension was applied on the bacterial lawn. In the control group, no coloration was observed.

Thus, both in the experiment by procedure 1 and in the experiment by procedure 2, coloration was observed only in sites, where all the three components of the tested system are contacted with each other. This result provides evidence showing that the strains exhibit the dehydrogenase activity toward the substrate.

Thus, using our developed procedure involving forming sites of contact of degrading agents with a pure, water-insoluble substance we obtained clear evidence showing that the model degrading strains are capable of direct oxidation of active pesticide ingredients.

Assessment of the Dehydrogenase Activity of Strains on a Liquid Minimal Selective Medium with a Water-Insoluble Limiting Component

Chloroform solutions of prometryn and λ-cyhalothrin SSSs (0.2 mL) were placed in sterile flasks (50 mL) and uniformly scattered over the bottom of the flasks. The flasks were left open in a hood under UV light for 45 min for chloroform to evaporate completely. As a result, the bottom of the vessel was coated with a thin layer of uniformly scattered extremely fine SSS crystals. A sterile M9 minimal medium (25 mL) and 0.25 mL of a 5% aqueous solution of tetraphenyltetrazolium chloride (1% of the volume of the medium) and then 0.25 mL of bacterial suspension of the corresponding degrading strain were added to one flask (experimental flask).

In the experiments we used two parallel controls: M9 medium containing tetraphenyltetrazolium chloride and SSS but no bacterial suspension (positive control C+) and M9 medium containing tetraphenyltetrazolium chloride and bacterial suspension but no SSS (negative control C–). After incubation, the presence or absence of bacterial growth was evaluated visually.

In the experimental flask with the medium containing tetraphenyltetrazolium chloride, an SSS, and bacterial suspension, a loose red colored bacterial layer grew on the crystal surface. In the control flask C+ containing pollutant but no degrading agent, no changes in the shape and color of the fine SSS crystals on the flask bottom were observed. In the control flask C–, where the medium contained degrading bacteria but no pollutant, too, no coloration or biomass growth was observed.

Thus, on adhesion of bacteria on the surface of SSS crystals, a redox process and biomass growth are initiated in the site of contact, implying that water-insoluble pesticide SSS crystals are used as the only source of food in aqueous medium.

Both experiments show that the proposed approaches to the assessment of the dehydrogenase activity of degrading agents by means of the tetraphenyltetrazolium chloride test can be used to detect sparingly water-soluble xenobiotics.

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