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Proceeding Paper

Indicators of Microbial Corrosion of Steel Induced by Sulfate-Reducing Bacteria Under the Influence of a Supernatant from Bacterial Cultures of Heterotrophic Bacteria with Biocontrol Properties †

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Abstract

Microorganisms take an active part in the processes of microbiologically influenced corrosion, which is protected against by using bactericides—often toxic compounds—with inhibitory properties. There are many studies of eco-friendly "green" biocides/inhibitors, in particular those based on microbial metabolites. Indicators for the processes of microbial corrosion of steel 3 induced by the sulfate-reducing bacteria *Desulfovibrio oryzae* NUChC SRB2 under the influence of the strains *Bacillus velezensis* NUChC C2b and *Streptomyces gardneri* ChNPU F3 have not been investigated, which was the aim of this study. The agar well diffusion method (to determine the antibacterial properties of the supernatants) was used, along with the crystal violet (to determine the biomass of the biofilm on the steel) and gravimetric methods (to determine the corrosion rate). A moderate adhesiveness to steel 3 was established for *D. oryzae* due to its biofilm-forming ability. The presence of a supernatant on cultures of *S. gardneri*, *B. velezensis* and their mixture (2:1) did not reduce the biofilm-forming properties of *D. oryzae*. Compared to the control, a decrease in the corrosion rate was recorded for the variant of the mixture of the studied bacterial culture supernatants. This indicates the potential of this mixture for use in corrosion protection in environments with sulfate-reducing bacteria, which requires further research.

Keywords: *Bacillus velezensis*; *Desulfovibrio oryzae*; "green" biocides/inhibitors; microbiologically influenced corrosion; *Streptomyces gardneri*

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1. Introduction

Microorganisms play an important role in the processes of microbiologically influenced corrosion [1–3]. Bactericides with inhibitory properties are used for protection against microbiologically influenced corrosion, and they are often toxic compounds [4–6]. There are many studies of eco-friendly "green" biocides/inhibitors—in particular, those based on microbial metabolites—for the prevention of microbial corrosion [7–13]. A number of scientists have considered the artificial addition of secondary metabolites of heterotrophic bacteria to eliminate corrosion in environments with corrosion hazards [7–9,14]. In particular, the following secondary bacterial metabolites have shown corrosion-inhibiting properties: exopolysaccharides [15–19], γ-polyglutamic acid [20], antibiotics indolicidin, bactenecin, probacterin [21] and peptide gramicidin S [22].

Biocontrol strains of *Bacillus velezensis* are known for their ability to form biologically active compounds—or secondary metabolites—that have both antibacterial properties (bacillizin, dificidin, fengycin, bacillaen, macrolactin, surfactin, plantazolicins, mersacidin, subtilin, locillomycins, bacillomycins) and plant growth-stimulating properties that control phytopathogens (bacillibactin), including nematodes (hexahydropyrrolo [1,2-a]pyrazine-1,4-dione) [23]. The effect of the *B. velezensis* strain NUChC C2b, *Streptomyces gardneri* strain ChNPU F3 and *Streptomyces canus* strain NUChC F2 on the ability of *Peribacillus* (*Bacillus*) *simplex* strain ChNPU F1, isolated from the soil ferrosphere, to form biofilms on a glass surface was investigated [23]. For strains of *Streptomyces gardneri*, the production of thiopeptide antibiotics and proactinomycin A, B and C is known [23]. However, the formation of biofilms by SRB on steel in the presence of these bacteria with biocontrol properties has not been studied but is of significant practical interest, as it could expand knowledge about "green" inhibitors/biocides of microbially induced corrosion, which could reduce the toxic load on natural ecosystems while simultaneously preserving metal structures. Currently, indicators of the microbial corrosion of steel induced by *D. oryzae* under the influence of *B. velezensis* NUChC C2b and *S. gardneri* ChNPU F3 have not been investigated, which was the purpose of this study.

2. Materials and Methods

2.1. Bacterial Strains

Pure cultures of the bacterial *D. oryzae* strain NUChC SRB2 (MT102714.1 in the GenBank) and *S. gardneri* strain ChNPU F3 (KX349221 in the GenBank), previously isolated from the soil ferrosphere and identified [24], and *B. velezensis* strain NUChC C2b (MN749356.1 and MN749357.1 in the GenBank) from the collection of the Department of Biology at the T.H. Shevchenko National University "Chernihiv Colehium" were used for this research.

A bacterial suspension with an optical density of 0.5 McFarland standard was prepared in a sterile isotonic sodium chloride solution from a 5-day culture of SRB in a liquid medium of Postgate's "C" (without $FeSO_4$), which was used for further studies.

From cultures of heterotrophic bacteria in the meat–peptone broth (MPB)—*S. gardneri* ChNPU 3 (age 10 days), *B. velezensis* NUChC C2b (age 5 days)—supernatants were prepared by centrifugation for 20 min at 10,000 rpm. The resulting supernatants were used in further studies.

2.2. Investigation of the Sensitivity of Sulfate-Reducing Bacteria to Supernatants from Cultures of Heterotrophic Bacteria with Biocontrol Properties

To study the sensitivity of the sulfate-reducing bacteria *D. oryzae* NUChC SRB2 to supernatants from cultures of heterotrophic bacteria with biocontrol properties, we used the diffusion method with wells in an agar medium. To do this, wells were cut to the thickness of the Postgate's "C" agar medium and inoculated with a test culture of bacteria using a sterile metal tube with a diameter of 7 mm, which reached the bottom of the Petri dish. 0.05 mL of the investigated supernatant from the appropriate culture of heterotrophic bacteria was added to the formed wells. A solution of sterile MPB was used as a control solution. To create anaerobic conditions, the inoculated medium in the Petri dish was covered with glass and the spaces between the glass and the dish were filled with sterile molten agar-agar (using the method of Shturm L.D. in Duda's V.I. modification) [25]. This was repeated 3 times. The Petri dishes were placed in a thermostat at a temperature of 29 ± 2 °C. Every day for 14 days, the dishes were examined for the appearance of a sterile zone around the wells (absence of a black coloration in the medium), which would indicate the inhibition of the development of SRB and the SRB's sensitivity to the investigated supernatant solutions. The experimental scheme is presented in Figure 1.

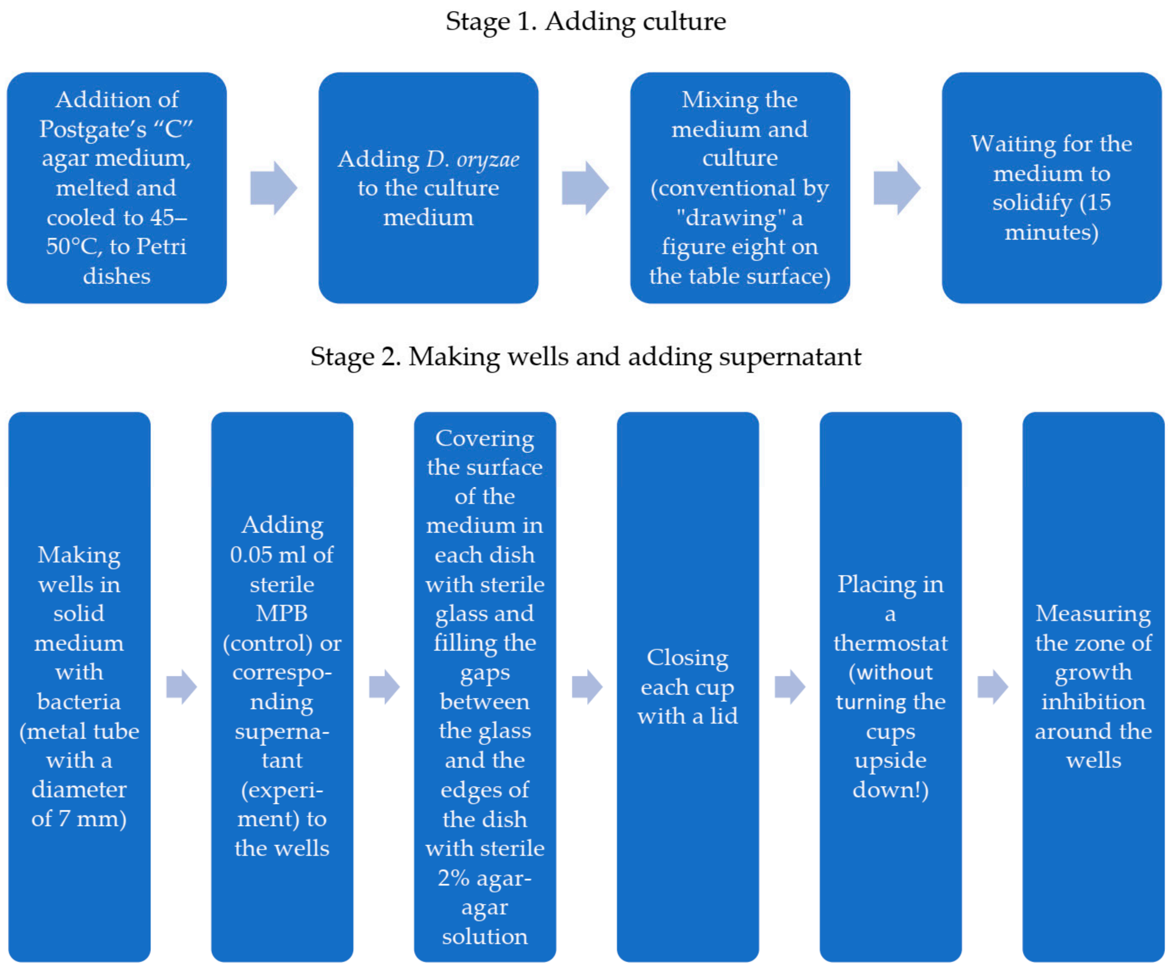


Figure 1. Scheme of the experiment to determine whether the supernatants of heterotrophic bacteria with biocontrol properties have antimicrobial properties against a culture of anaerobic sulfate-reducing bacteria.

2.3. Investigation of the Corrosion Activity of Sulfate-Reducing Bacteria

The experiment was carried out during the cultivation of the studied SRB (2% of the volume) under anaerobic conditions, at a temperature of 29 ± 2 °C, in the Postgate's "C" liquid medium (76% of the volume) with an addition of either 22% of the volume of MPB (variant SRB2) or a supernatant from the MPB culture of strains: *S. gardneri* ChNPU F3 (variant SRB2 + F3), *B. velezensis* NUChC C2b (variant SRB2 + C2b) or mixtures of the supernatants of the specified strains *S. gardneri*:*B. velezensis* (2:1) (variant SRB2 + F3 + C2b). Sterile Postgate's "C" medium with the addition of MPB was used as a control. Samples of steel 3 (20 × 20 × 2.5 mm) were added into the control and test tubes. Samples were pre-treated with sandpaper, weighed on analytical scales and sterilized with ethyl alcohol. The duration of exposure of the samples in the respective media was 120 days (2880 h). This was repeated 4 times. The specified experimental scheme was used previously in the study of microbial corrosion indicators in some medical preparations [26]. The scheme of the experiment is presented in Figure 2.

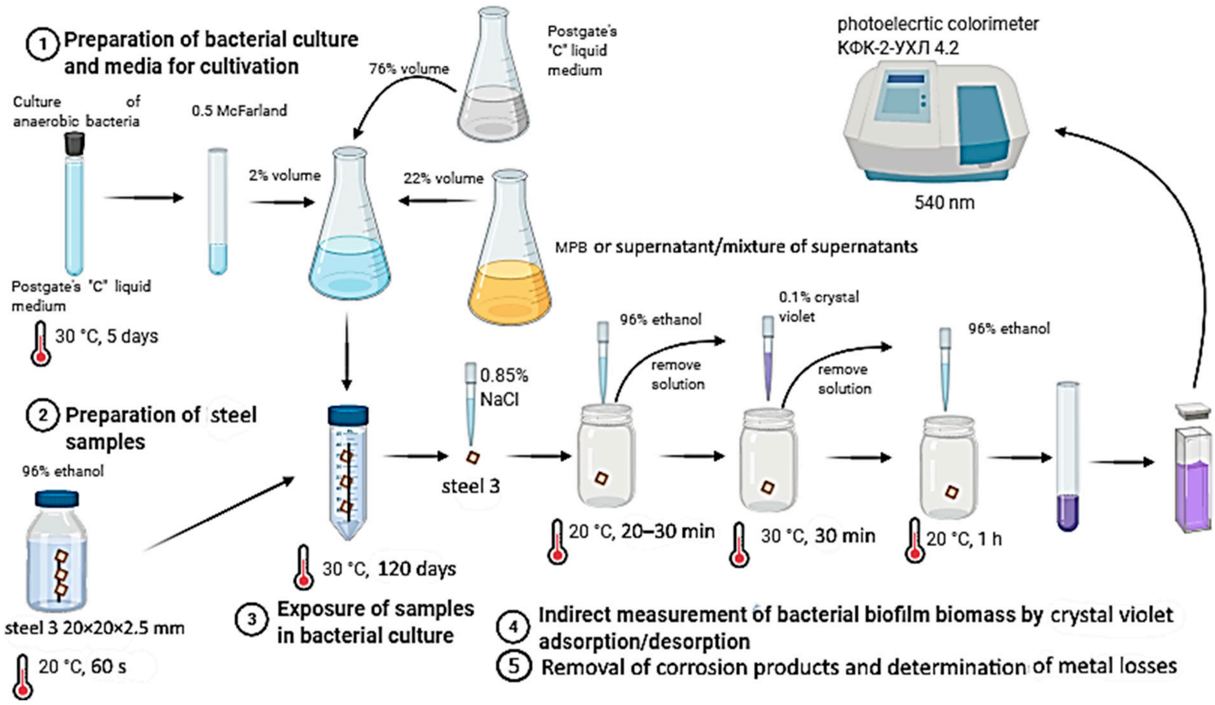


Figure 2. Scheme of the experiment [26], adapted by us to study the indicators of microbial corrosion of steel under the influence of supernatants of heterotrophic bacteria with biocontrol properties.

Corrosion activity of *D. oryzae* strain NUChC SRB2 against steel 3 was investigated based on its ability to form biofilms, the biomass of those biofilms on the surface of steel samples (method with crystal violet) [27] and its effect on corrosion rate (gravimetric method) [11].

2.3.1. Method with Crystal Violet

To determine the biomass of the biofilm, we used the crystal violet dye adsorption/desorption method with the methodology given in the work [28]. To do this, after exposure, the samples were washed with a sterile NaCl solution (0.9%), the biofilm was fixed on them with a 96% solution of ethyl alcohol (for 20–30 min), then the alcohol was drained and the samples were dried at room temperature. Next, the samples were dyed with a 0.1% aqueous solution of crystal violet at a temperature of 37 °C for 30 min, the dye was then drained and the samples were washed under running water until there was no color in the washing water, at which point they were dried at room temperature. Next, 96% ethyl alcohol was added to the dried stained samples for 60 min at room temperature to desorb the dye adsorbed by the biofilm. The optical densities of the obtained alcohol solutions of crystal violet were determined on a photoelectrocolorimeter at a wavelength of 540 nm [28].

2.3.2. Gravimetric Method

The metal corrosion rate (W, mg/m^2 $\times$ h) was determined by the weight loss of the samples [11]:

$$W = \frac{m_1 - m_2}{s \times t}, \quad (1)$$

where s is the surface area (m^2) of the sample, m_1 and m_2 are the sample weights before (m_1) and after (m_2) corrosion (mg) and t is the immersion time (h).

To determine the change in the mass of the metal sample before and after exposure, the samples were weighed on analytical balances. After exposure, the corrosion products were removed from the samples with an eraser, wiped with 96% degreasing alcohol and immersed in the rust converter for 1 h (during the treatment time, the solution was rubbed with a brush, as specified in the manufacturer's instructions). Then the samples were washed, dried and weighed on analytical balances.

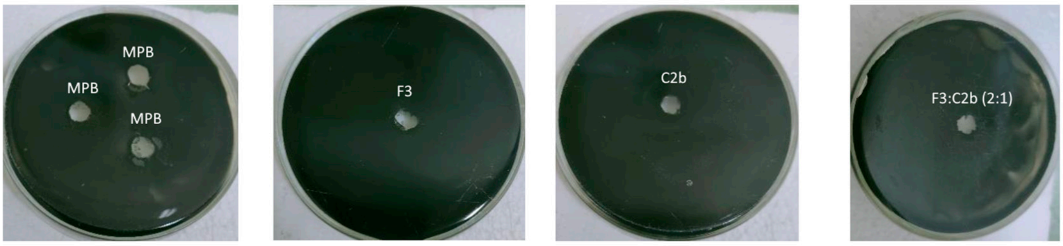
2.4. Statistical Data Processing

When processing the obtained data, methods of mathematical statistics were used, including calculating the arithmetic mean (M) and the standard error of the arithmetic mean (m). The reliability of the arithmetic mean differences between the control and the experiment was assessed by Student's *t*-test for significance. Statistical processing of the research results was carried out for a significance level of 0.05.

3. Results and Discussion

The search for effective "green" antimicrobial compounds has explored microbial metabolites [7–10,13], plant extracts [29] and animal metabolites [30,31]. An important direction is the search for such compounds to prevent microbial deterioration of materials [6,11–13,32].

The sensitivity of the SRB *D. oryzae* strain NUChC SRB2 to supernatants from the cultures of the heterotrophic bacteria *S. gardneri* ChNPU F3 and *B. velezensis* NUChC C2b—which previously showed biocontrol properties [23]—and their mixtures was investigated. The results are presented in Figure 3.



a b c d

Figure 3. The results of the study on the sensitivity of *D. oryzae* NUChC SRB2 to the supernatant of the studied cultures (the experiment with the wells): (**a**) with MPB in the wells; (**b**) with the supernatant from the MPB cultures of *S. gardneri* ChNPU F3 in the well; (**c**) with the supernatant from the MPB cultures of *B. velezensis* NUChC C2b in the well; (**d**) with the mixture of supernatants from the specified strains of *S. gardneri*:*B. velezensis* (2:1) in the well. It can be seen that the SRB are developing, and there are no zones of growth inhibition.

It was established that the strain *D. oryzae* NUChC SRB2 did not show sensitivity to the studied supernatants of bacterial cultures. The obtained data coincide with the results of a study on the sensitivity of SRB to the metabolites of heterotrophs that used a method that flooded the colonies of the antagonist (*B. velezensis* strains NUChC C1 or NUChC C2b) with an agar medium containing the test culture (*D. oryzae* strains NUChC SRB1 or NUChC SRB2) [25].

The results of the study on the influence of supernatants from cultures of bacteria that are known for their biocontrol properties on the corrosion activity of the *D. oryzae* strain NUChC SRB2 are presented in Figures 4–7.

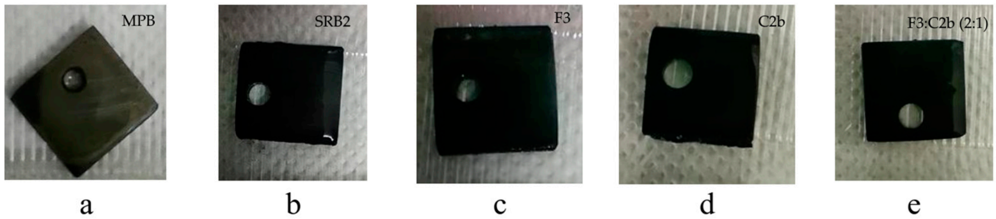


Figure 4. The samples of steel 3 after the experiment (120 days): (**a**) control (sterile liquid medium of Postgate's "C" with the addition of MPB); (**b**) *D. oryzae* NUChC SRB2; (**c**) *D. oryzae* NUChC SRB2 under the influence of the supernatant from the MPB culture of *S. gardneri* ChNPU F3; (**d**) *D. oryzae* NUChC SRB2 under the influence of the supernatant from MPB culture of *B. velezensis* NUChC C2b; (**e**) *D. oryzae* NUChC SRB2 under the influence of the mixture of supernatants of *S. gardneri*:*B. velezensis* (2:1).

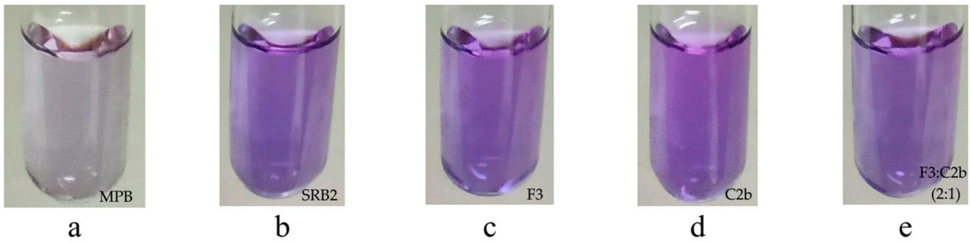


Figure 5. Alcohol solutions of crystal violet desorbed from the biofilm that formed on the surface of the steel samples: (**a**) control (sterile liquid medium of Postgate's "C" with the addition of MPB); (**b**) *D. oryzae* NUChC SRB2; (**c**) *D. oryzae* NUChC SRB2 under the influence of the supernatant from the MPB culture of *S. gardneri* ChNPU F3; (**d**) *D. oryzae* NUChC SRB2 under the influence of the supernatant from the MPB culture of *B. velezensis* NUChC C2b; (**e**) *D. oryzae* NUChC SRB2 under the influence of the mixture of supernatants of *S. gardneri*:*B. velezensis* (2:1).

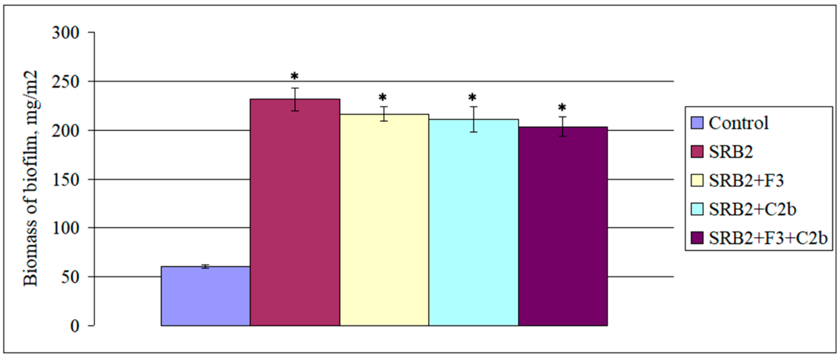


Figure 6. Biomass of the biofilms on the steel samples. Note: the differences are significant * compared to the control at $p \leq 0.05$.

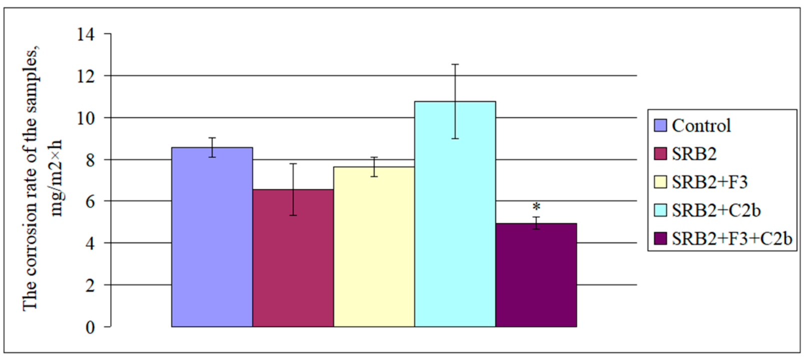


Figure 7. The corrosion rate of the steel 3 samples. Note: the differences are significant * compared to the control at $p \leq 0.05$.

It was established that, in all variants of the experiment, the SRB of the studied strain developed intensively, regardless of the absence or the addition of supernatants, and formed a clearly visible biofilm on the surface of the steel 3 samples (Figure 4). The biomass of the biofilm in the presence of SRB and in all variants of the experiment was the same, and the difference was statistically insignificant (Figures 5 and 6).

The difference in the corrosion rate of the steel samples in the presence of SRB *D. oryzae* NUChC SRB2 both without the supernatants (variant SRB2) and with their addition (variants SRB2 + F3 and SRB2 + C2b) was statistically insignificant. However, a significant reduction in the corrosion rate was noted for the SRB2 + F3 + C2b variant, in which a mixture of supernatants (2:1) of *S. gardneri* and *B. velezensis* cultures was added to the SRB culture. The studied species *S. gardneri* and *B. velezensis* are known as producers of a number of secondary metabolites, information about the biological properties of which is summarized in the work [23]. In particular, *S. gardneri* and *B. velezensis* metabolites with antimicrobial properties can be bacterial surfactants. The presence and high transcriptional activity of the genes of the siderophore bacillibactin synthesis operon were established for the strain *B. velezensis* NUChC C2b [25]. However, in this study, the antibacterial properties of the supernatants from the cultures of *S. gardneri* and *B. velezensis*, as well as their mixture, were not noted.

It is known that bacterial surfactants exhibit anticorrosive properties. Potential anticorrosion properties for amino acid-based surfactants (also known as biosurfactants), including sodium N-dodecyl asparagine, sodium N-dodecyl tryptophan and sodium N-dodecyl histidine, were noted by Fawzy et al. [14]. Siderophores are low-molecular-weight compounds that chelate Fe (III) ions, convert insoluble Fe (III) into the bioavailable form of Fe (II) and are synthesized by some bacteria, fungi and plants when iron ions are deficient in the environment [33]. Siderophores have the high anti-corrosion properties of steel [34–37], contributing to the passivation of metals [38–40]. Siderophores are eco-friendly compounds that are classified as "green" inhibitors [34]. Effective inhibition of corrosion caused by SRB was established using marine *Streptomyces* sp. [41]. It is known that chelating agents are able to inhibit stages 2 (the formation of microcolonies) and 3 (the maturation of a biofilm) of the biofilm formation process [42]. However, in our study, in the presence of supernatant from a culture of *B. velezensis* NUChC C2b with high transcriptional activity in siderophore bacillibactin synthesis operon genes, no antibiofilm properties were observed. In the case of using a mixture of supernatants, a slight anti-corrosion effect was recorded, which was possibly associated with the formation of a complex of secondary metabolites from the bacteria *S. gardneri* and *B. velezensis*. However, this assumption requires further research.

The limitations of the obtained results should be recognized as the use of laboratory research conditions and a pure culture of sulfate-reducing bacteria. These limitations are based on the fact that microbial corrosion processes in natural conditions occur under variable environmental factors and with the involvement of different ecological and physiological groups of microorganisms [43]. Sulfate-reducing bacteria are important participants in creating microbial damage to steel structures [2,44,45]. The importance of Fe (III) uptake by SRB is considered an important step in their transition from anaerobic to microaerophilic conditions of existence [46]. Analysis of 26 genomes from eight families of SRB revealed that all the SRB examined carried genes (feoA and feoB) for an iron uptake system to transport Fe (II) across the plasma membrane and into the cytoplasm. Genes for a canonical ABC transporter, which can transport iron siderophore or chelated iron species from the environment, were also found in the SRB genomes examined. Gram-negative SRBs have an additional mechanism for importing iron siderophore and chelated iron species, as they possess the TonB system, which can work in conjunction with any of the outer membrane porins annotated in the genome. It has been discussed that SRB may use the putative siderophore uptake system to import metals other than iron [46]. Data on secondary metabolites in microbially induced corrosion zones are limited [47]; therefore, the role of these compounds in regulating microbial communities remains uncertain. Understanding the role of microbial siderophores in the course and prevention of microbial corrosion processes requires further research.

4. Conclusions

In terms of its biofilm-forming ability, the studied strain of SRB was moderately adherent to steel 3. A decrease in the biofilm-forming properties of *D. oryzae* under the influence of the supernatants from the MPB cultures of *S. gardneri*, *B. velezensis* and their mixture was not detected. Similarly, the protective properties of the studied supernatants in relation to the microbial corrosion of steel influenced by *D. oryzae* was also not detected. Further research should focus on studying the rate of microbial corrosion under conditions involving natural corrosion-active communities with SRB and a supernatant mixture, given the established anti-corrosion potential of the latter.

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