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Properties of anaerobic bacteria from ferrosphere crucial for biofilm development

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Abstract. The purpose of this study was the investigation of biofilm-forming, hydrophobic, sulfidogenic and aggregative properties of sulfate-reducing bacteria *Desulfovibrio oryzae* strains in monocultures and associations with organic acid-producing bacteria *Anaerotignum propionicum*. Studies of biofilm formation on polypropylene surface by strains were carried out by a biofilm assay (indirect measurement of bacterial biofilm biomass by crystal violet adsorption/desorption), hydrogen sulfide production was determined by iodometric titration, aggregation - by aggregation test, hydrophobicity - by salt aggregation test. It was found that the studied strains of *D. oryzae* NUChC SRB1 and NUChC SRB2 are highly adhesive, have high sulfidogenic, aggregation and hydrophobic properties in the complete Postgate's medium (with Fe^{2+}). During the cultivation of the studied strains of *D. oryzae* with *A. propionicum* NUChC Sat1, a significant increase in aggregation (both in complete medium and without Fe^{2+}) and hydrogen sulfide production by sulfate-reducing bacteria were observed. These properties indicate the potentially high biodegradable activity of *D. oryzae* monocultures NUChC SRB1 and NUChC SRB2 and their associations with *A. propionicum* NUChC Sat1. The observed increase in the sulfidogenic activity of *D. oryzae* in association with *A. propionicum* promotes the corrosion hazard of the studied bacterial association.

Keywords: aggregation, *Anaerotignum propionicum*, biofilm, *Desulfovibrio oryzae*, hydrogen sulfide production, hydrophobicity.

Abbreviations:
SRB – sulfate-reducing bacteria.

1. Introduction

Biofilms are structured microbial communities surrounded by a biopolymer matrix (Nikolaev & Plakunov, 2007; Vorobey et al., 2012). The formation of biofilms on the surfaces of building materials, pipelines and other structures contributes to their damage (Andreyuk et al., 2005). Considerable attention of researchers of microbially induced corrosion is focused on the processes of formation of biofilms by sulfate-reducing bacteria (Andreyuk et al., 2005; Azeredo et al., 2017).

It is now known that hydrophobic, aggregative and adhesive properties of bacteria are crucial for biofilm development (Denkhaus et al., 2007; Azeredo et al., 2017; Zabielska et al., 2017). In particular, it was found that the lower layer of the biofilm, which is in contact with the metal,

contains exolipopolysaccharides and exopolysaccharides, which play an important role in adhesion. Under the exopolysaccharide matrix, the anode zone is formed, electron flow and ionization of iron, with the further accumulation of sulfides begin. The latter cooperate with exopolysaccharides, forming stable structures, which due to close contact with the metal form microgalvanic cells (FeS-Fe^0), where a new center of microbial corrosion appears (Andreyuk et al., 2005).

Therefore, the process of microbially induced corrosion is defined as a consequence of the activity of microorganisms in the form of a biofilm on a metal or other corroding surface. The ferrosphere (Andreyuk et al., 2005) is a zone of active development of corrosive microorganisms of the soil that is in direct contact with the metal surface of the underground structure. Previously, from the sulfidogenic community isolated from the soil ferrosphere, we isolated strains of sulfate-reducing bacteria *Desulfovibrio oryzae* NUChC SRB1 and NUChC SRB2 (Tkachuk et al., 2020) and a strain of organic acid-producing bacteria *Anaerotignum propionicum* NUChC Sat1 (Tkachuk et al., 2018), hydrophobic, aggregative and adhesive properties of which have not been studied.

Therefore, the aim of this study was the investigation of the biofilm-forming, hydrophobic, sulfidogenic and aggregative properties of *D. oryzae* strains in monocultures and associations with *A. propionicum*.

2. Material and Methods

2.1. Organisms and growing conditions

Five-day pure cultures of *A. propionicum* NUChC Sat1, *D. oryzae* NUChC SRB1 and *D. oryzae* NUChC SRB2 isolated from the sulfidogenic microbial community of soil ferrosphere were used for the study (Tkachuk et al., 2018, 2020). The nucleotide sequences were deposited in the GenBank with accession numbers MG924854.1, MT102713.1 and MT102714.1 respectively. Both monocultures of bacteria and their associations were used: SRB1 + Sat1, SRB2 + Sat1.
Bacteria were cultured for 6 and 14 days in Postgate's "C" liquid medium (with or without $FeSO_4$) in Eppendorf-type tubes (anaerobic conditions, 29 ± 2ºC).

2.2. Determination of the amount of hydrogen sulfide formed by sulfate-reducing bacteria

The total content of hydrogen sulfide, sulfide and hydrosulfide ions based on H_2S was studied during SRB (mono- and associative cultures) growing in Postgate's "C" liquid medium (14 days, temperature 29ºC) by iodometric titration (Lur'e, 1984).

2.3. Investigation of the intensity of the formation of biofilms by bacteria

A biofilm assay (indirect measurement of bacterial biofilm biomass by crystal violet adsorption/desorption) was used (Lagun et al., 2012). Suspensions with an optical density of 0.5 McFarland were prepared from cultures of the studied strains in sterile isotonic sodium chloride solution. In the study of biofilm formation by bacterial monocultures, the calculated initial concentration of bacterial cells in Postgate's "C" medium was 1×10^7 cells/ml (SRB1 and SRB2) and 1×10^3 cells/ml (Sat1). In the study of the formation of biofilms by bacterial associations (SRB1 + Sat1, SRB2 + Sat1), the calculated total initial concentration of bacteria in the medium was 2×10^7 cells/ml. Bacterial cultures in various combinations were grown in Eppendorf-type tubes (5 ml) in a thermostat (static conditions) at 29°C for 6 and 14 days. Staining of the formed biofilms was performed with a 0.1% aqueous solution of crystal violet at 30°C for 60 minutes.

Sterile Postgate's "C" medium was used as a negative control. The experiment was performed in triplicate. Measurements of the concentration of crystal violet in the control and experimental samples were performed at a wavelength of 540 nm on a photoelectrocolorimeter in a cuvette with an optical path length of 1.0 mm. To quantify the thickness of the formed biofilms, the concentrations of crystal violet in the washing alcohol solutions and the mass of the dye, which was sorbed by the biofilm, were used.

It was believed that the biomass of the formed biofilms is directly proportional to the concentration of crystal violet in the washing solutions, the mass of the biofilm was represented as the mass of the dye, which was absorbed by the biofilm during staining (Lagun et al., 2012). The strains were classified according to the scale proposed by Stepanović et al. (2000).

2.4. Investigation of the strains aggregation

Investigation of the strains aggregation was performed according to the aggregation test proposed by Del Re et al. (2000). Aggregation was calculated by the formula:

$$[1\text{-}(\frac{A0}{A})] * 100\%$$

,

where A0 is the optical density of the upper layer of the bacterial suspension after incubation for 2 hours at 30°C;

A - optical density before incubation.

2.5. Investigation of the hydrophobicity of the strains

The hydrophobicity of the strains was assessed by salt aggregation test, which is based on the formation of aggregates by bacteria in the presence of ammonium sulfate at concentrations from

0.2 M to 4.0 M (Nwanyanwu & Abu, 2013). The following scale for assessing the hydrophobicity of bacteria was used: high (<1.0 M), moderate (1.0-2.0 M), low (> 2.0 M).

2.6. Statistical analysis of experimental data

Statistical processing of the obtained results was performed using the statistical module of Microsoft Office Excel 2010. Methods of descriptive statistics were used - we calculated the arithmetic mean (M) and the standard error of the arithmetic mean (m) (Plohinskij, 1970). The Student's significance criterion (t) was calculated, and the 95% probability of differences ($p<0.05$) was considered statistically significant.

3. Results and Discussion

3.1. Biofilm formation and hydrogen sulfide production by strains

The results of study of biofilm formation (by absorption of crystal violet dye formed by biofilm) on polypropylene surface of Eppendorf-type tubes and hydrogen sulfide products are presented in Figures 1 and 2.

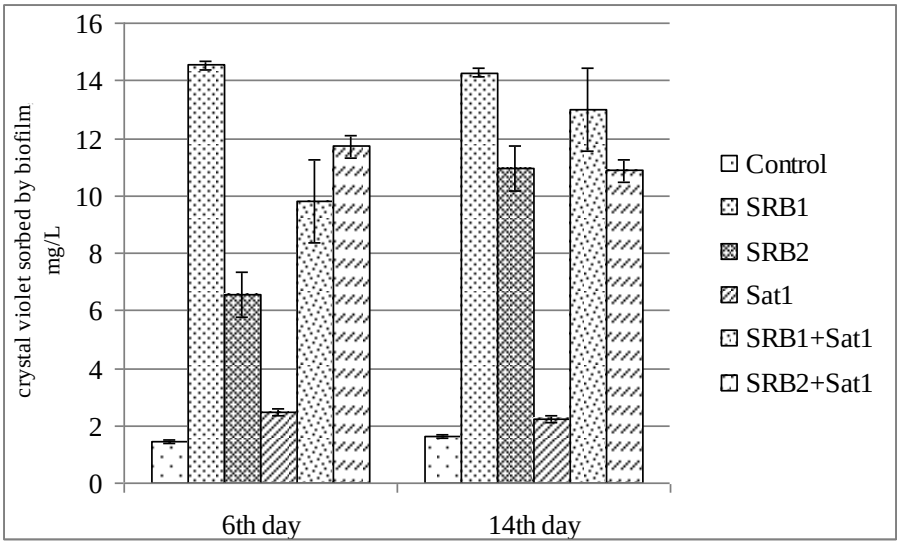

Figure 1. Comparative characteristics of biofilm-forming ability of the studied strains of *D. oryzae* and *A. propionicum* in mono- and associative cultures

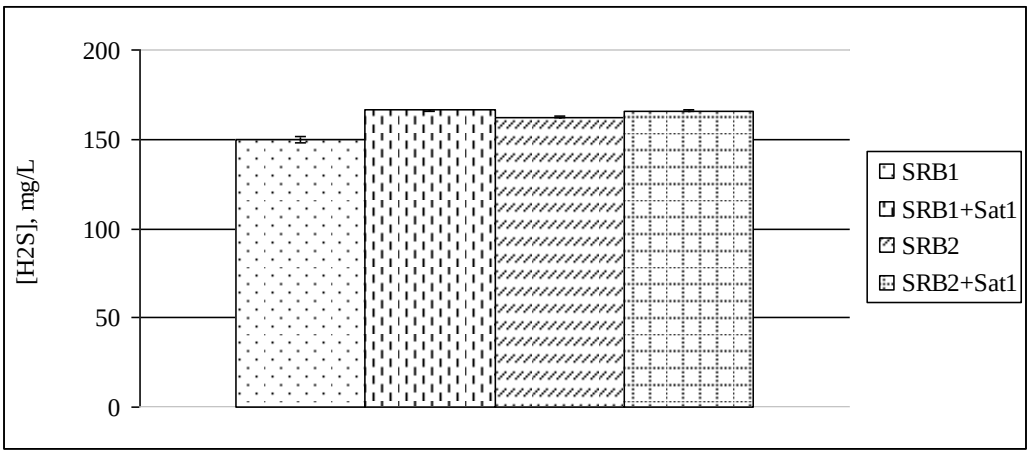

Figure 2. Production of hydrogen sulfide by the studied strains of *D. oryzae* (in monocultures and associations with *A. propionicum*) (iodometric titration, 14th day of cultivation)

The study of biofilm formation by strains confirmed the results of visual evaluation - strain SRB1 formed a stronger biofilm than strain SRB2, both on the 6th and 14th day of the experiment - 2.2 times and 1.3 times, respectively (Fig.1). Therefore, it was found that the studied strains of sulfate-reducing bacteria are capable of forming a biofilm and are highly adhesive on the scale of Stepanović et al. (2000).

In addition to differences in the intensity of biofilm formation, the studied strains of SRB also differed in H_2S production. Thus, it was found that strain SRB2 is more sulfidogenic than strain SRB1 (Fig. 2). It formed significantly more hydrogen sulfide (9.0%) than strain SRB1. According to the literature, the amount of hydrogen sulfide formed by sulfate-reducing bacteria is 170-300 mg/L (Abdulina et al., 2011; Purish et al., 2012, 2014; Hallbeck, 2014; Abdulina et al., 2015). In technogenic soils, the sulfidogenic activity of SRB increases to 450 mg/L (Abdulina et al., 2011). Thus, the production of hydrogen sulfide by the studied strains was not high and corresponded to the level for the natural zone of soils (not disturbed by technogenic intervention) (Abdulina et al., 2011).

The strain of organic acid-producing bacteria *A. propionicum* NUChC Sat 1 in monoculture was weakly adhesive and almost did not form a biofilm on both the 6th and 14th day of cultivation. This may be due to unfavorable cultivation conditions, namely Postgate's "C" medium, which is not elective for these bacteria. However, in the co–cultivation of sulfate-reducing bacteria *D. oryzae* with *A. propionicum*, significant changes in the intensity of biofilm formation on the 6th day of the experiment were observed. Thus, in the case of culturing the association of SRB1 with Sat1, the biomass of the formed biofilm was significantly lower (1.5 times) than in the cultivation of one strain of SRB1.

However, at this time, when co-culturing SRB2 with Sat1, the mass of the formed biofilm was significantly higher (1.8 times) than in SRB2 monoculture. On the 14th day of cultivation, the intensity of biofilm formation of the associations SRB1 and SRB2 with *A. propionicum* did not differ from that in SRB monocultures. However, the production of hydrogen sulfide by SRB strains in associations with *A. propionicum* on the 14th day of cultivation was significantly higher than in monocultures: by 11.4% (SRB1) and 2.2% (SRB2). However, there was no significant increase in the sulfidogenic activity of SRB, the production of hydrogen sulfide by the studied strains did not remain high - at the level typical of the natural soil zone (not disturbed by technogenic intervention) (Abdulina et al., 2011).

The possibility of a mutual growth of the association of bacteria *D. oryzae* and *A. propionicum*, which is caused by trophic interaction, was shown (Tkachuk et al., 2020). The participation of the studied associated bacteria in the corrosion process, along with the possible utilization of hydrogen (*D. oryzae*) and the formation of substrates-products of metabolism of SRB (hydrogen and organic acids), which are corrosive compounds (*A. propionicum*), may be supplemented by increased sulfidogenic activity of *D. oryzae* in the presence of *A. propionicum*. This increases the corrosion risk of this bacterial association. Increased physiological and/or genetic activity of SRB in the presence of associate bacteria has also been reported (Rozanova & Nazina 1989; AlAbbas et al., 2013; Baba et al., 2017).

3.2. Aggregation of the studied strains

Currently, there is an increase in the biofilm formation of bacteria in cases of their increased tendency to aggregation (Kragh et al., 2016). The researchers note that despite a large body of research about finding out the mechanisms of attachment of individual cells to surfaces in the early stages, the current view on the biofilm formation model bases on including the role of aggregates in biofilm initiation (Kragh et al., 2016). Therefore, we evaluated the ability of the studied strains of anaerobic bacteria to aggregation. The results of this study are shown in Table 1.

Under the condition of culturing bacteria in the Postgate's "C" medium without $FeSO_4$ aggregation properties in the studied strains of SRB were not observed - aggregation of 0% (Table 1). However, in the presence of $FeSO_4$ in the environment, high aggregation was observed: 71.0 ± 0.6% (SRB1) and 79.1 ± 0.1% (SRB2). Obviously, this fact is associated with the formation of iron sulfides, which precipitate and provide a high rate of aggregation. Due to the high adsorption properties of the formed sulfides to bacteria (Konishi et al., 1990; Vilinska, 2007), the latter can be adsorbed on their surface and deposited with them in the form of aggregates.

Table 1. Aggregation of the studied strains by the aggregation test on the 6th day of cultivation. (Note: the differences are significant * between 1 and 3, ** between 2 and 4 at $p \leq 0.05$ (t_{st} = 2.92-4.30-9.93)

№	A variant of the experiment	The aggregation, %	
		without $FeSO_4$	with $FeSO_4$
1.	SRB1	0	71.0±0.6
2.	SRB2	0	79.1±0.1
3.	SRB1+Sat1	18.7±0.3*	76.0±0.1*
4.	SRB2+Sat1	15.2±0.5**	76.9±0.1**

Picard et al. (2018) showed that more than half of the minerals of iron sulfide, which are formed in the presence of live SRB, are on the cell surface, and the rest are probably related to extracellular compounds. The presence of iron sulfide minerals, which are formed on the surface of microbial cells, raises the question of the role of extracellular and intracellular minerals of iron sulfide for microorganisms, for example, as protection against external stress. The mechanisms by which microbial cells are inlaid and the role of cell inlay have been little studied (Picard et al., 2018).

When co-culturing the studied sulfate-reducing bacteria *D. oryzae* with *A. propionicum* NUChC Sat1 aggregation of bacteria significantly increases and is 18.7% (SRB1 + Sat1) and 15.2% (SRB2 + Sat1) under the conditions of culturing bacteria in a medium without $FeSO_4$ (Table 1). The introduction of $FeSO_4$ provides slight changes in the aggregation index in the associations of *D. oryzae* NUChC SRB1 with *A. propionicum* NUChC Sat1 compared with monocultures of *D. oryzae* (Table 1).

3.3. Hydrophobicity of the studied strains

Bacterial cell surface hydrophobicity is one of the most important factors that influence bacterial adhesion (Dahlbäck et al., 1981; van Loosdrecht et al., 1987; Stenström, 1989; Zita & Hermansson, 1997).

The results of the study of the hydrophobicity of the studied strains are shown in Table 2. It was found that the hydrophobicity of the studied monocultures and associations belong to the category of bacteria with high hydrophobicity (Table 2). A number of experiments have

shown (Dahlbäck et al., 1981; van Loosdrecht et al., 1987; Stenström, 1989; Zita & Hermansson, 1997) that hydrophobic cells adhered to a greater extent than hydrophilic cells.

Our results of high hydrophobicity of SRB are consistent with their high adhesive properties. However, for the strain *A. propionicum* NUChC Sat1, despite its high hydrophobicity, adhesion properties were not observed (possibly due to unfavorable conditions of its cultivation), and for its cultivation it is necessary to choose the optimal nutrient medium.

Table 2. Hydrophobicity of the studied strains by salt aggregation test

A variant of the experiment	Hydrophobicity, M
SRB1	<1.0
SRB2	<1.0
Sat1	<1.0
SRB1+Sat1	<1.0
SRB2+Sat1	<1.0

The growth conditions may induce changes in the bacterial cell surface layers which will influence the overall degree of hydrophobicity and charge (Stenström, 1989; Rozgonyi et al., 1990). Stenström et al. (1989) showed that a large variability in cell surface characteristics such as cell surface hydrophobicity and charge may exist within the bacterial strains. This variability may express various amounts of lipopolysaccharides, different types and number of cell surface appendages, or capsular material. Since the expression of fimbriae and flagellae of bacteria may be under phase variation, this may add to the variability (Stenström, 1989; Rozgonyi et al., 1990).

4. Conclusions

Thus, the studied strains of *D. oryzae* NUChC SRB1 and NUChC SRB2 are highly adhesive on polypropylene surface, have high aggregation, hydrophobic and sulfidogenic properties in the complete Postgate's "C" medium (with Fe^{2+}). During the cultivation of the studied strains of *D. oryzae* with *A. propionicum* NUChC Sat1, a significant increase in aggregation (both in the complete Postgate's "C" medium and without Fe^{2+}) and production of hydrogen sulfide by reducing sulfate bacteria are remarkably significant. These properties indicate the potentially

high corrosion activity of *D. oryzae* monocultures NUChC SRB1 and NUChC SRB2 and their associations with *A. propionicum* NUChC Sat1.

The participation of the studied associated bacteria in the corrosion process, along with the possible utilization of hydrogen (*D. oryzae*) and the formation of substrates-products of SRB metabolism (hydrogen and organic acids), which are corrosive compounds (*A. propionicum*), may be supplemented by increased sulfidogenic activity of *D. oryzae* in the presence of *A. propionicum*. This determines the corrosion hazard of this bacterial association.

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