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Effects of phenolic compounds on the sulfidogenic activity of *Desulfotomaculum* sp. and *Desulfovibrio desulfuricans* bacteria

Natalia Verkholiak*, Taras Peretyatko, Andrii Halushka

Ivan Franko National University of Lviv, 4, Hrushevsky str., Lviv, 79005, Ukraine

*corresponding author e-mail: nataljaverkholjak@gmail.com

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Abstract. Purification of environment from the pollutants of organic and inorganic matter is one of the actual problems of nowadays. Compounds with benzene ring are among the most hazardous pollutants. The danger of getting the phenolic compounds into the environment is connected with their toxicity for biological objects and stability. One of the most effective methods of environment detoxification is the usage of microorganisms. The aim of this work was to determine the ability of *Desulfotomaculum* sp. AR1 and *Desulfovibrio desulfuricans* Ya-11 bacteria to utilize phenol derivatives as the carbon source and to study the effects of phenolic compounds on the sulfidogenic activity of these bacteria. Both bacterial strains grew in the media with pyrogallol and hydroquinone, but the biomass in the medium with hydroquinone was considerably lower. Inhibition of sulfate ion utilization by bacteria was found during the growth of *Desulfotomaculum* sp. AR1 and *D.* desulfuricans Ya-11 in the medium with phenolic compounds. Effectiveness of pyrogallol utilization by both bacterial strains was for 4 times higher, compared to the effectiveness of hydroquinone utilization. *Desulfotomaculum* sp. AR1 and *D.* desulfuricans Ya-11 bacteria are also capable for growth in the medium with sodium benzoate, utilizing this aromatic compound as carbon and energy source. Process of catechol degradation by *Desulfotomaculum* sp. AR1 bacteria under anaerobic conditions is ineffective.

Desulfotomaculum sp. AR1 and *Desulfovibrio desulfuricans* Ya-11 bacteria are capable for growth in the medium with phenolic compounds, utilizing them as the sole carbon source and removing them from the environment. ANOVA showed significant differences between the amount of utilized SO_4^{2-} in the control medium and medium with aromatic compounds. Presence of aromatic compounds in the medium inhibits the sulfate reduction in *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 bacteria, which results in the decrease of toxic hydrogen sulfide accumulation in the medium.

Usage of microorganisms with the aim of environment detoxification is a promising way of water biotopes remediation technologies.

Keywords: pyrogallol, hydroquinone, sulfate ion, bioremediation, hydrogen sulfide, catechol.

1. Introduction

Purification of environment from the pollutants with different chemical structure, particularly, phenolic compounds, is one of the actual problems of nowadays. Phenols get into the biosphere with the industrial and domestic waste, they are present in the wastewater of chemical-recovery, metallurgic, gas, wood, furniture, pulp and paper industry, in the products of oil refining, treatment of peat, etc. (Kopcha et al., 2010). High content of phenol, cresol, pyrocatechole, xylenole, derivatives of resorcin, hydroquinone and fluoroglucine is found in the wastewater of coxobenzol plants. Wastewater of gas generation stations and chemical-recovery plants contains up to 2.5 g/L of phenols. Volume of phenolic wastewater, which depends on the type and capacity of production, counts hudreds and thousands of cubic meters per day with phenolic compounds concentrations from 0.7 to 2 g/L (Dudnik & Yevtushenko, 2013). The highest permissible concentration (HPC) of phenols in the fishery water objects is 0.001 mg/L. Phenolics degrade with difficulty

in water and spread for hudreds of kilometers from the place of their disposal in rivers and for tens of kilometers in reservoirs (Dudnik & Yevtushenko, 2013).

Phenols have the anti-septic properties, owing to which they are widely used for the disinfection in medical and pharmaceutical industry, medical and sanatorium institutions, that is why they are also found in domestic wastes in small concentrations (Dudnik & Yevtushenko, 2013).

The danger of getting the phenolic compounds into the environment is connected with their toxicity for biological objects and stability (Kopcha et al., 2010). The main effect of phenolic compounds to algae is the intensification of oxidation and phosphorylation processes, which results in the significant consumption of energy, required for the assimilation processes. Inhibitory effect of phenols on the growth of green algae and cyanobacteria appears after different time. Hydroquinone, pyrocatechole (catechol) and caffeic acid have negative effects on the functional activity of algae during the first hours after their getting into the water. Phenolic pollution of water results in the loss of appearance of fish, mollusks, Crustacea and other water organisms. Presence of low concentrations of phenol in water gives muscle and fat tissues of hydrobionts the specific smell, which does not vanish after the temperature treatment. Bacteria, algae, protozoa, Crustacea and mollusks are more resistant to phenolic poisoning than fishes (Dudnik & Yevtushenko, 2013).

More than 2 billion m^3 of non-purified and insufficiently purified industrial wastewater is discharged into the water objects of Ukraine annually, which poses a threat to the ecological condition of environment and increases its stress (Demadis et al., 2007; Baltser & Pavlovich, 2012; Bargiel & Zabochnicka-Swiatek, 2018; Galkina & Dehtiar, 2019). Phenol-containing wastewater is traditionally purified from the mechanical admixtures in the settlers, than biochemically from phenols and other compounds in aerotanks with the next additional purification on the filters with the granular loading, and the stabilizatory treatment is performed with the inhibitors of corrosion and scaling. Dephenolization of wastewater at the chemical-recovery plants of other countries is performed by the extraction method, and their additional purification – by biological one (Demadis et al., 2007; Baltser & Pavlovich, 2012; Bargiel & Zabochnicka-Swiatek, 2018; Galkina & Dehtiar, 2019).

Microorganisms play a significant role in the process of phenols degradation in the environment. Biodegradation of phenolic compounds by microorganisms is one of the most promising, ecological and economically profitable methods of environment purification (Kopcha et al., 2010; Dalal et al., 2012).

The aim of our research was to determine the ability of *Desulfotomaculum* sp. AR1 and *Desulfovibrio desulfuricans* Ya-11 bacteria to utilize phenol derivatives as the carbon source and to study the effects of phenolic compounds on the sulfidogenic activity of these bacteria.

2. Study area

Sulfate-reducing bacteria *D. desulfuricans* Ya-11, isolated from Yavoriv Lake (49.94617, 23.47202) (Peretyatko et al., 2006), and *Desulfotomaculum* sp. AR1, isolated from Lviv city system of industrial and domestic wastewater purification (49.86279, 24.07431) (Verkholiak & Peretyatko, 2018), were used in this work (Fig. 1).

Lviv city wastewater is collected and transported through canalization collectors and pump stations system to the purification system, where their mechanical and biological purification is performed, after which the purified water is dumped to Poltva river, and the solid fraction is removed to the sludge ground (Oliferchuk et al., 2009). Large number of purification systems use biological method of purification in aerotanks with the usage of active sludge (Voloshyn et al., 2009). The predominant hydrobionts of active sludge are protozoa of *Ciliophora* phylum (*Chilodonella*, *Opercularia* and *Aspidisca* genera) (Shved et al., 2012). Nitrifying and nitrogen-fixing bacteria are the predominant groups among bacteria, but there are also other physiological groups, particularly, sulfate-reducing bacteria (Sholyak et al., 2013).

Discovery of Lviv region sulfur deposits (1956 – Rozdil open pit, 1969 – Yavoriv open pit, 1974 – Podorozhne open pit) provided the rapid development of mining and chemical industry of Ukraine and significantly changed the history and geography of region (Haidin & Zozulia, 2009; Taras, 2013). At the beginning of 90^{th}, native sulfur production became non-profitable and mining was almost stopped. Ukrainian government approved the project of Yavoriv sulfur open pit flooding and disturbed landscape remediation in 2003. Artificial lake with water area 10 km^2, shoreline length 12 km, depth over 100 m has been started to construct. Shklo river was directed into very large excavation of sulfur open pit for this purpose. In 2007, after the pouring of artificial well, it flew again by its own bed to Poland trough Yavoriv. As a consequence, deep and poor lake has formed. The project which expects the creation of recreation area, safe conditions for swimming, fishing, beach territories, medical sanatoria, hydropark and ornithological sanctuary is developed there. But a lot of problems which prevent this exist, particularly, the surrounding soil is infertile. SO_4^{2-} salt residue content vary from 330 mg/kg to 8000 mg/kg at HPC 160 mg/kg. Excelling of heavy metals content is also observed (Taras, 2013). Considering this, study of microbiota of environment with high sulfur and sulfur-containing compounds content, which can participate in their transformation, is important.

Figure 1. Points of water (Yavoriv Lake, Lviv region, Ukraine) and Lviv city (Lviv region, Ukraine) wastewater purification system active sludge samples collection for the isolation of sulfate-reducing bacteria

3. Materials and methods

Bacteria *D. desulfuricans* Ya-11 *Desulfotomaculum* sp. AR1 were grown in Postgate C medium with such content (g/L): potassium dihydrophosphate – 0.5; ammonium chloride – 1.0; sodium sulfate – 4.5; calcium chloride hexahydrate – 0.06; magnesium sulfate heptahydrate – 0.06; sodium lactate – 6; yeast extract – 1; ferrous sulfate heptahydrate – 0.004; sodium citrate dihydrate – 0.3; pH 7.6 (Postgate, 1984) and modified Postgate C medium without sodium lactate, but with pyrogallol (1.3 g/L) and hydroquinone (3.4 g/L).

Biomass was measured turbidimetrically using the photoelectrocolorymeter KFK-3 (λ=340 nm, 3 mm cuvette). SO_4^{2-} content was measured turbidimetrically (λ=520 nm, 10 mm cuvette) after the sedimentation by barium chloride according to HOST 26426-85. Glycerol was used as the stabilizer of suspension. Hydrogen sulfide content was measured in culture liquid colorymetrically using *p*-aminodimethyl amine dihydrochloride (λ=665 nm, 30 mm cuvette) (Sugiyama, 2002).

Hydroquinone and pyrogallol content was measured by high performance liquid chromatography (HPLC). Chromatographic system consisted of two Varian ProStar 210 pumps (Agilent Technologies, Singapore), Pursuits 5 C18 column (Agilent Technologies, Netherlands), 250×4.6 mm in Varian ProStar 500 column module (Agilent Technologies, Australia), Varian ProStar 335 UV-visible photodiode array detector (Agilent Technologies, Australia).

45:55 v/v mixture of 1st class water (obtained using water purification system Adrona Crystal E Bio with ultrafilter Milipore (Adrona, Latvia) and methanol (Sigma-Aldich, France) was used to measure hydroquinone content. Mobile phase flow was 1.5 ml/min, sample volume – 20 µl. Chromatograms were recorded at 295 nm. Column temperature was 35°C (Siddique et al., 2012).

Two eluents were used to measure pyrogallol content: 0.1% water solution of trifluoroacetic acid (Sigma-Aldich, eluent A) and methanol (Sigma-Aldich, France, eluent B). Chromatographic analysis was started from 80% A during 5 min. The analysis was continued in the linear gradient from 80% to 60% A during the next 5 min and to 40% A during the next 15 min. Mobile phase flow was 1 ml/min, sample volume was 20 µl. Chromatograms were recorded at 266 nm (Zhang et al., 2015). Equilibration time was 4 min, hold time – 10 min. Column temperature was 35°C.

Data analysis is based on the One-way ANOVA and Student's t-test. Difference between control and experimental values was considered significant at $P < 0.05$.

4. Results and Discussion

Mechanism of the anti-microbial action of phenols is the damage of cell wall integrity and bacterial proteins denaturation. Spectrum of anti-microbial action of phenols covers gram-positive and gram-negative bacteria, but bacteria of

Pseudomonas genus and spore-forming anaerobic microorganisms are less sensitive to them (Cueva et al., 2010).

The ability of spore-forming *Desulfotomaculum* sp. AR1 bacteria and non-spore-forming *D. desulfuricans* Ya-11 bacteria to grow in the medium with pyrogallol/hydroquinone as the carbon source was studied. Postgate C medium with sodium lactate was used as the control. According to the obtained results, both bacterial strains grew in the medium with pyrogallol, accumulating approximately 2 g/L of biomass, while in the medium with hydroquinone biomass was significantly lower – up to 0.5 g/L (Fig. 2).

Sulfate ion content after 10 days of *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 growth in media with pyrogallol and hydroquinone decreased for 2–3 mM, which is 5–7 times lower than in control (Table 1). Effectiveness of sulfate ion utilization by both bacterial strains in media with aromatic compounds was approximately 7–11%, and in medium with sodium lactate – 55–60%.

Effectiveness of pyrogallol and hydroquinone utilization was measured by HPLC. Obtained results concerning phenolic compounds utilization correlated with the results of bacterial growth study (see Fig. 2). Effectiveness of pyrogallol utilization by *Desulfotomaculum* sp. AR1 and *D.* desulfuricans bacteria was 40% after 7 days of growth (Fig. 3a), while of hydroquinone utilization – only 10% (Fig. 3b).

Choice of initial hydroquinone and pyrogallol concentrations in bacterial cultivation media was predetermined by different HPC of these compounds in water of domestic and drinking water supply objects and different toxicity of these compounds (hydroquinone belongs to the 4th class of hazard, pyrogallol – the 3rd). Initial pyrogallol and hydroquinone concentrations exceed HPC 10^4 times.

Table 1. Effectiveness of sulfate ion utilization by *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 bacteria in the media with pyrogallol and hydroquinone

Carbon source	Amount of utilized sulfate ion, mM		Effectiveness of sulfate ion utilization, %	
	Desulfotomaculum sp. AR1	*D. desulfuricans* Ya-11	*Desulfotomaculum* sp. AR1	*D. desulfuricans* Ya-11
pyrogallol	2.20±0.40***	2.00±0.13***	8.16	7.42
hydroquinone	3.06±0.15***	2.98±0.20***	11.35	11.05
sodium lactate (K)	16.08±0.14	14.72±0.27	59.62	54.58

Initial sulfate ion concentration = 26.97±0.43;
*** – P < 0.001 – probable changes compared to control

Results of the study of *Desulfotomaculum* sp. AR1 and *D. desulfuricans* growth in the media with different electron donors and acceptors are presented at Figure 4. According to the obtained data, both bacterial strains accumulated the same biomass in the medium with pyrogallol as electron donor and with/without sulfate ion as electron acceptor. Bacterial biomass in the control medium with sodium lactate without sulfate ion was inhibited for 10 times.

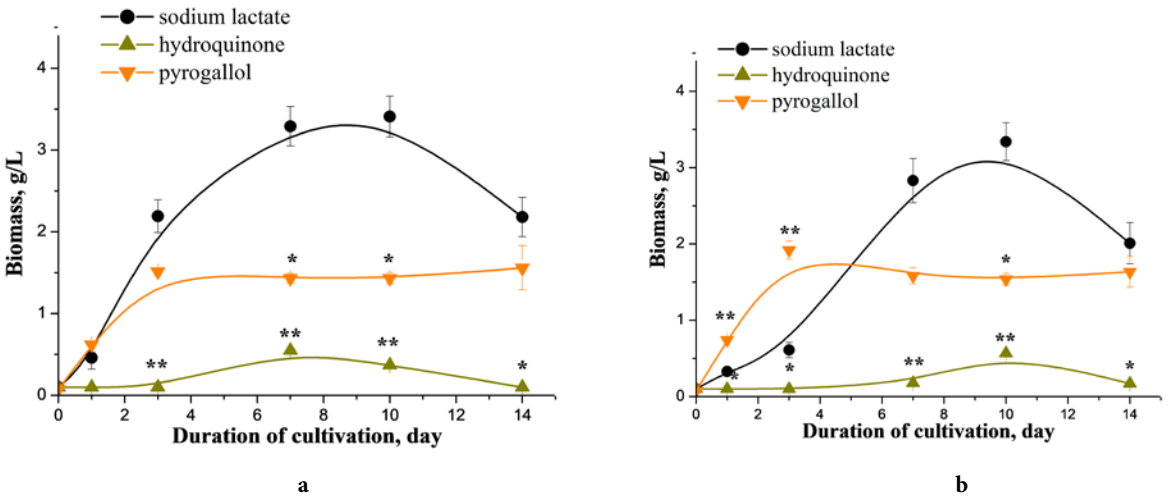

Figure 2. Accumulation of biomass by *Desulfotomaculum* sp. AR1 (a) and *D. desulfuricans* Ya-11 (b) bacteria in media with different carbon sources

* – P < 0.05 – reliable changes, compared to control;
** – P < 0.01 – reliable changes, compared to control

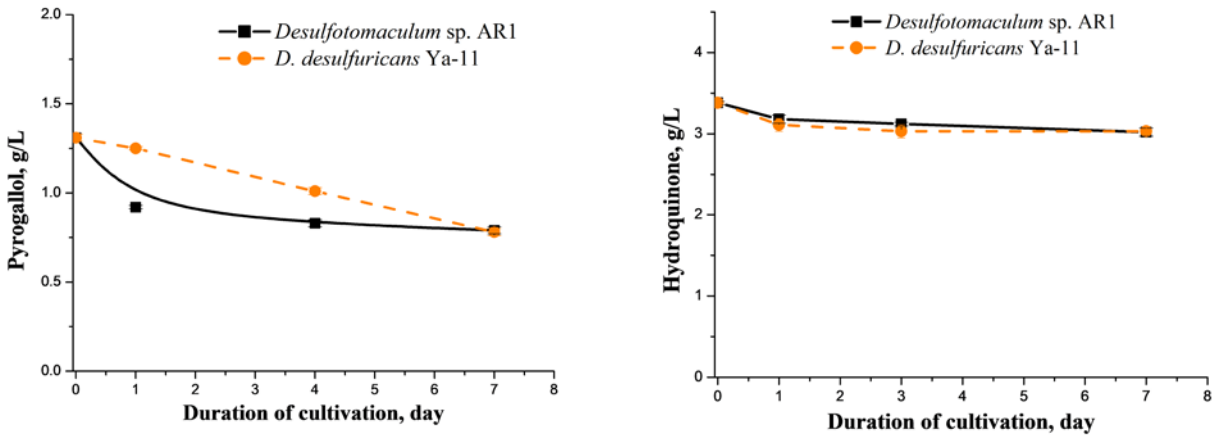

Figure 3. Changes in pyrogallol (a) and hydroquinone (b) concentrations during 7 days of *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 bacteria cultivation

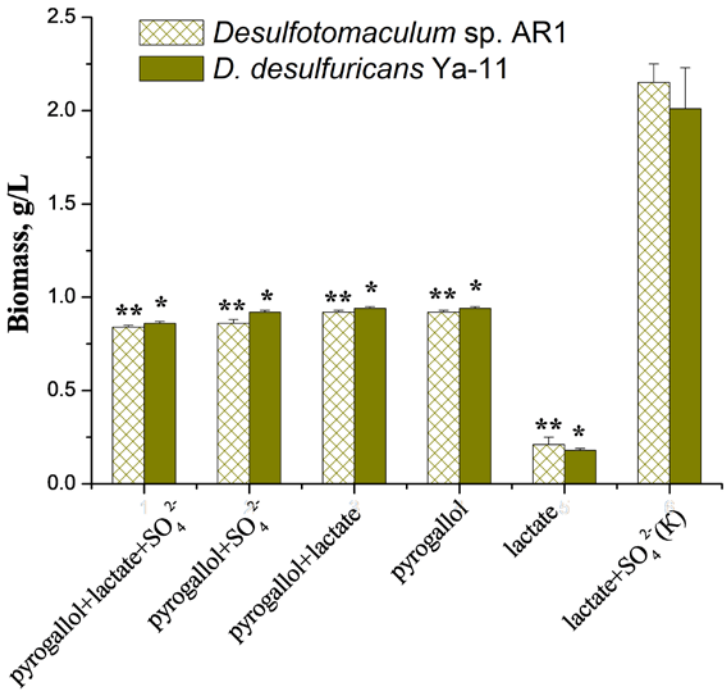

Figure. 4. Biomass of *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 bacteria in Postgate C medium with different electron donors and acceptors

* – $P < 0.05$ – reliable changes, compared to control;
** – $P < 0.01$ – reliable changes, compared to control

Perhaps, it is connected with the fact that some aromatic compounds can be electron acceptors in the biodegradation processes (Khomenkov et al., 2008). Keto-enolic tautomerization is also typical for phenols, in consequence, properties of electron acceptor are typical for quinones (Lastukhin & Voronov, 2006).

Benzoyl-CoA is formed as a result of anaerobic bio-oxidation of aromatic molecules which contain halogenated, metoxylated or carbonated side chains. Benzoyl-CoA is also the intermediate during the degradation of monohydroxylated aromatic substrates and some dihydroxylated compounds, particularly, catechol (Schink et al., 2000; Gibson & Harwood, 2002). Sodium benzoate, as the majority of aromatic acids, transforms to benzoyl-CoA by CoA-ligase (Auburger & Winter, 1992). Degradation of catechol – the main intermediate of aromatic compounds degradation at

the anaerobic conditions is the long process at anaerobic conditions, but it also results in benzoyl-CoA formation (Gorny & Schink, 1994; Schink et al., 2000).

Consequently, we have studied the ability of *Desulfotomaculum* sp. AR1 bacteria to grow in the medium with sodium benzoate or catechol as the unique carbon and energy source (Fig. 5). Bacterial biomass was almost twice higher in the medium with sodium benzoate, compared to the same during the growth of bacteria in the medium with catechol (Fig. 5a). Degradation of catechol – the main intermediate of aromatic compounds degradation at aerobic conditions – is the long process at anaerobic conditions (Gorny & Schink, 1994). Results also show that anaerobic bacteria also ineffectively degrade catechol, utilizing it as the sole carbon source. Concentration of sulfate ion in the medium with sodium lactate decreased for 23 mM during 14 days of bacterial growth, with benzoate – for 9 mM, with catechol – for 2 mM (Fig. 5b).

D. desulfuricans Ya-11 bacteria are also capable for growth in the medium with sodium benzoate, utilizing this aromatic compound as carbon and energy source. But biomass accumulation was insignificant at these conditions (for 6 times lower, compared to control) (Fig. 6a). *D. desulfuricans* Ya-11 bacteria reduced approximately 6 mM SO_4^{2-} at these conditions, which is 3 times lower, compared to control (Fig. 6b).

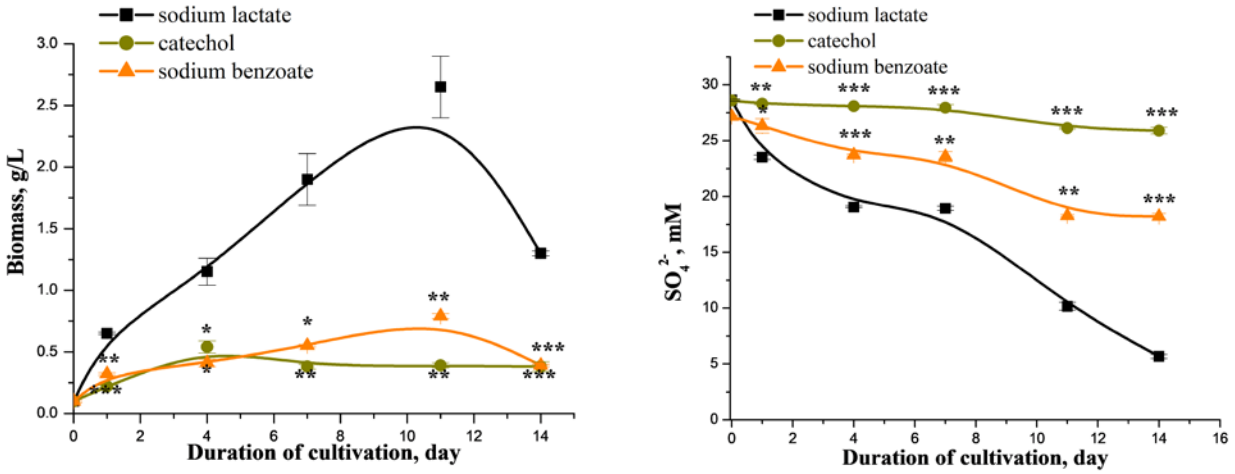

Figure 5. Accumulation of biomass (a) and utilization of sulfate ions (b) by *Desulfotomaculum* sp. AR1 bacteria in Postgate C medium with different electron donors

* – $P < 0.05$ – reliable changes, compared to control; ** – $P < 0.01$ – reliable changes, compared to control;
*** – $P < 0.001$ – reliable changes, compared to control

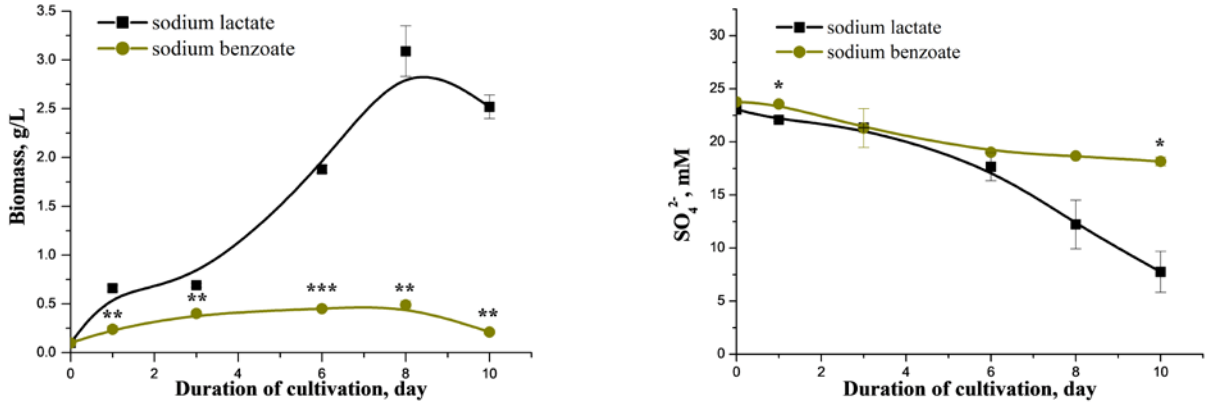

Figure. 6. Accumulation of biomass (a) and utilization of sulfate ions (b) by *D. desulfuricans* Ya-11 bacteria in Postgate C medium with different electron donors

* – $P < 0.05$ – reliable changes, compared to control; ** – $P < 0.01$ – reliable changes, compared to control;
*** – $P < 0.001$ – reliable changes, compared to control

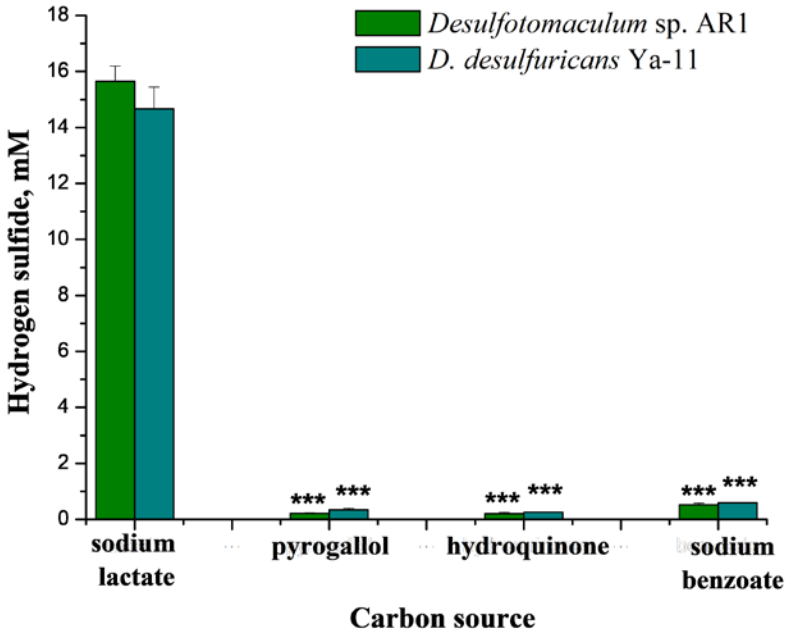

Figure 7. Concentration of hydrogen sulfide, produced by *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 bacteria, after 10 days of cultivation in media with different carbon sources
*** – $P < 0.001$ – reliable changes, compared to control

Hydrogen sulfide concentration during bacterial growth in the medium with aromatic compounds was not significant, it was 15 times lower than in control (Fig. 7).

ANOVA showed the considerable changes between the amount of utilized SO_4^{2-} in control medium and medium with aromatic compounds. Thus, the presence of aromatic compounds in the medium inhibits sulfate reduction in *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 bacteria, which results in the decrease of hydrogen sulfide accumulation.

Usage of microorganisms is a promising way of the purification of wastewater and remediation of water objects, because they rapidly react for the change of chemical composition of environment owing to the flexibility of metabolism.

5. Conclusion

Usage of microorganisms with the purpose of water purification from organic and inorganic compounds is the promising method of environment bioremediation. Sulfate-reducing bacteria *Desulfotomaculum* sp. AR1, isolated from Lviv city wastewater purification system, and *D. desulfuricans* Ya-11, isolated from Yavoriv Lake, are able to metabolize phenolic compounds, particularly, pyrogallol and hydroquinone, utilizing them as carbon and energy source. Studied bacteria removed 10% of hydroquinone and 40% of pyrogallol from the medium. *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 bacteria are able to metabolize sodium benzoate, decreasing its content in water.

Presence of phenolic compounds in the medium inhibits the sulfate reduction in *Desulfotomaculum* sp. AR1 and *D. desulfuricans* Ya-11 bacteria. As a result, they produce lower amount of toxic hydrogen sulfide.

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