Short communication

ISSN 2658-3518
LIMNOLOGY FRESHWATER BIOLOGY
www.limnolfwbiol.com

Bacterial biofilm formation in fuel

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ABSTRACT. Two strains of hydrocarbon-oxidizing bacteria isolated from jet fuel TS-1 were placed in fuel samples under different conditions (aerobic and anaerobic) and their ability to degrade hydrocarbons under these conditions was studied. In both conditions strains were able to develop and performed hydrocarbons degradation, but used different strategies. To adapt to the hydrocarbons of the oil cells used two strategies: planktonic form and biofilm. It has been shown that binary association begins to form biofilms in which it is possible to visualize both cell aggregates and microcolonies, as well as exometabolites excreted by strains during development.

Keywords: fuel biodamage; fuel biodegradation; microbial community; bacterial biofilm

1. Introduction

The development of oil industrial complex carries a great threat on the environment, which includes damages to the living organisms and soil poisoning, as well as chemical and biological pollution of the environment. Emergency oil spills are one of the priority problems in this area. Due to the difference in density, oil form a thin film floating on the surface of the water. In this case, it partially dissolves and form a stable emulsion, while heavier fractions settle to the bottom. More favorable condition of cells in such substances is biofilm, thus, cells tend to form biofilms, which helps them to withstand with fuel hydrocarbons. And cells excessive accumulation can lead to premature clogging of filters and equipment failure. In this work, we studied the biofilm formation in diesel fuel and TS-1 jet fuel with hydrocarbon-oxidizing bacteria (HOB) strain isolated from TS-1 previously.

2. Material and methods

Development of HOB and biofilm formation was observed under aerobic and anaerobic conditions in the Evans medium (EM) (Evans et al. 1970) in two types of fuel (diesel fuel and TS-1 jet fuel). Two strains were selected: *Sphingobacterium multivorum* Bi*2* (GenBank ID MG812313.1) *Sphingobacterium mizutaii* Bi*9* (GenBank ID MK968143) as active destructors according to the previous study (Shapiro et al., 2018).

To create aerobic conditions, 36 ml of fuel and 4 ml of EM with a suspension of bacteria (OD 0.2) were added to a sterile flask with a volume of 100 ml. To create anaerobic conditions, 36 ml of fuel and 4 ml of Evans medium with a suspension of bacteria (OD 0.2) were added to a sterile test tube. The samples were kept at room temperature for 14 days. Increasing the height of the column of liquid (fuel) and excluding the air space in the test tube prevents the access of oxygen from the air to the fuel-water interface.

Biodegradation was estimated visually, by biomass growth in mg/kg and according to Method for determining mechanical impurities in medium distillates of ST RK EN 12662-2011. This standard applies to liquid petroleum products with a kinematic viscosity of no more than 5 mm^2/s at 40°C. What is suitable for analyzing jet fuel, diesel in present study. According to this standard, "mechanical impurities" are insoluble substances, organic and inorganic, that have settled on the filter after filtration under test conditions. The biomass accumulated as a result of microbial activity is mechanical impurities.

For estimation the presence of exopolysaccharide in bacterial biofilm layer we stained the samples with 0.15% (w/v) ruthenium red (pH 7.0) and methylene blue (pH 7.0) during 10 min and studied cells under light microscope Leica DM 2500 (Leica, Germany).

3. Results

Bacterial growth in the form of biofilm in diesel fuel was observed on the 4th day under anaerobic conditions (Fig. 1B). In aerobic conditions, there was a turbidity of the medium and the formation of cell

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aggregates on the 5th and 6th days. In TS-1 jet fuel under anaerobic conditions, bacterial growth in the form of biofilms was observed on day 6. In the aerobic, there was a turbidity of the medium on the 7th day. The high growth level of HOB evaluated by OTU for both types of fuel indicated an increase in the HOB biomass due to bacterial cell division ($17.5x10^8$ for diesel fuel and $37.0x10^6$ for TS-1). Measuring of mechanical impurities (the dry weight of the water phase) in addition to the cell biomass, includes extracellular polymer compounds (polysaccharides, glycoproteins, lipids) in addition to the cells (340.5 for diesel fuel and 79.1 for TS-1).

Mechanical impurities in the form of a small sediment and microflocules were found in samples of contaminated TS-1 jet fuel. Microscopy of floccules and sediment revealed clusters of bacterial cells of different morphology (cocci and bacilli), forming microcolonies (Fig. 1A). Staining of samples of mechanical impurities of an infected fuel with ruthenium red, which specifically binds to acidic polysaccharides, showed that bacterial cells of floccules were immersed and united by polysaccharide matrices (Fig. 1C). Individual cells and microcolonies of bacterial cells are united into extracellular polymeric matrix (EPM).

4. Discussion

The genus *Sphingobacterium* belongs to the hydrocarbon-oxidizing group known as the hydrocarbon destructor (Das and Chandran, 2011; Bücker et al., 2014; Noparat et al., 2014; Satti et al., 2019). The development of HOB in fuels goes in different directions in aerobic and anaerobic conditions. The development of HOB in the form of biofilm indicates the presence of stressful conditions for the growth and development of microorganisms. Under these conditions, they combine and form a complex structure - biofilm in which their cells are integrated into the EPM synthesized by them, which protects them from the negative effects of the environment. The formation of the biofilm leads to the appearance in the fuel of clots of EPM, aggregates or floccules of cells, that is, mechanical impurities. In more favorable conditions, bacterial cells are characterized by planktonic growth, which is well visualized in the form of turbidity of the medium. In this case, microbial cells actively divided, they were physiologically active and used fuel hydrocarbons for growth during development, thus, changing its chemical composition.

The obtained data indicate that in anaerobic conditions, at the initial stages, the development of the HOB community occurs in the form of the biofilm, in order to experience stressful conditions and create more favorable conditions for development, and subsequently transition to the planktonic stage of growth. In aerobic conditions, HOB, being physiologically active, use fuel hydrocarbons for growth from the first hours, which could be visualized in the turbidity of the medium and chemical rearrangement of the fuel, causing a change in its composition. Features of microscopic organization of microflocules and TS-1 jet fuel sediment also allow us to speak about the formation of biofilm-type structures in the fuel by microorganisms.

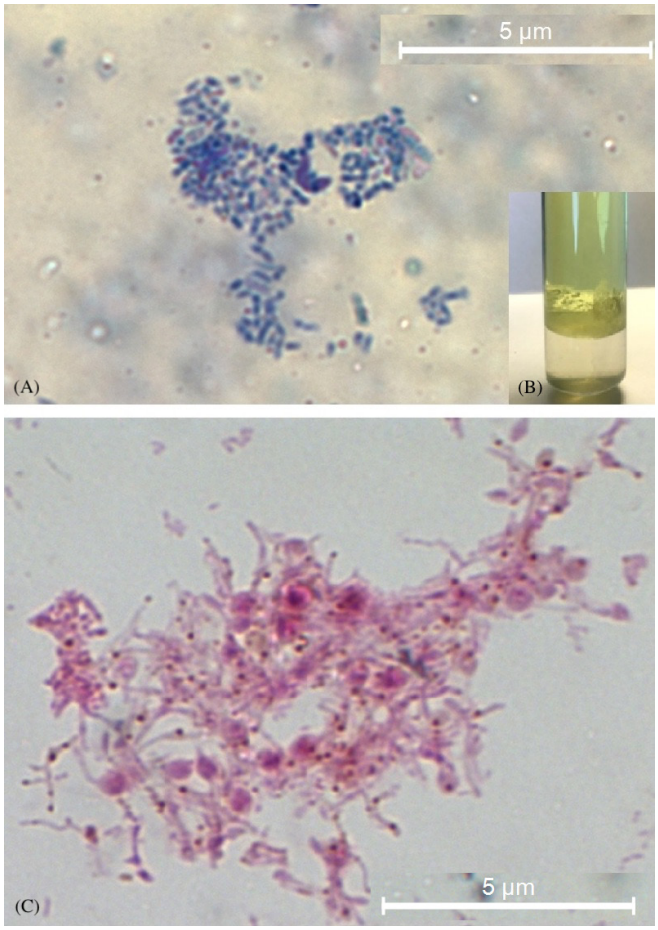

Fig.1. Cell aggregates stained with methylene blue (pH 7.0) (A); exopolysaccharide in bacterial biofilm layer stained with **0**.15% (w/v) ruthenium red (pH 7.0) forming strands (C); Bacterial biofilm layer on the surface boundary formed in diesel fuel under anaerobic conditions (B).

5. Conclusion

The association used in this work effectively grew in different oxygen concentration conditions. We have shown the effectiveness of isolated strains of the genus *Sphingobacterium* in the association as biodegrading agents in modeling conditions where HOB protect themselves from toxic fuel hydrocarbons forming biofilm.

Acknowledgements

This work was supported by Russian Foundation for Basic Research, project no. 18-29-05067. Authors have no conflict of interest.

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