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Oil Degradation by the Strain *Rhodococcus sp. HN4* with the Application of a Balanced Methanogenic Association of Microorganisms under Thermophilic Conditions

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Abstract This article presents the results of a study on the possibility of intensifying the process of destruction of oil and oil products by a balanced methanogenic association of microorganisms (obtained after fermentation under thermophilic conditions) (BTMAM) by introducing into them a strain of the bacterium Rhodococcus sp. HN4. The amount of inoculum of Rhodococcus sp. HN4 was 4% of the volume of the inoculated medium. The BTMAM used in the experiment was 5 and 10%. Oil with a density of 832 kg/m3 (20°C) at a concentration of 2% to 8% was used as a substrate. Cultivation was carried out in flat-bottom flasks with a capacity of 250 ml on a shaker (150 rpm) at a temperature of 28–30°C for 20 days. Thus, it has been established that the bacterial strain of the genus Rhodococcus, possessing a wide range of metabolic capabilities, contributes to other groups of microorganisms that are part of BTMAM to assimilate hard-to-reach substrates, in particular oil and its decay products. BTMAM in the community with the Rhodococcus sp. HN4 degrades oil with a high degree of degradation and in high concentrations of 6-8%. The spectrum of microorganisms that make up the association are not pathogens; they are producers of biologically active compounds, resistant to high oil concentrations, in the oil degradation process, and contribute to soil biologization.

Keywords Oil, a balanced association of microorganisms, Degradation, Microorganisms as oil degraders

1. Introduction

Advancements in the oil industry have caused environmental pollution resulting from the components of oil and oil products that are toxic to living organisms. Solutions are needed to mitigate pollution and make the oil industry more environmentally sustainable. One solution to this problem involves the development of effective, environmentally friendly, and safe methods of purification using biological products based on microorganisms. Compared with some physicochemical methods, bioproducts based on microorganisms are ecologically safe and effective (Silva-Castro G. A. et.al., 2014, Dados A. et.al., 2015). The effectiveness of the use of biological products in solving environmental problems also depends largely on environmental conditions (temperature, pH, humidity), and the degree of the pollutant. During degradation, it is necessary to maintain the appropriate concentration of nutrients, especially nitrogen, and phosphorus, in an optimal ratio; this is necessary to compensate for the imbalance caused by the high carbon content of crude oil during pollution, which can slow down the growth and activity of microorganisms (Bamforth et.al., 2005, Ayotamuno et.al., 2006).

Crop and livestock waste can be used to tackle the problem of environmental pollution from the oil industry. For example, several studies have shown the potential for using plant organic waste, such as sunflower and rice husks (Li P. 2002), banana peels (Agbor R. B. 2012), and husks from cocoa pods (Agbor R. B. 2012), as well as organic animal waste, including cow, goat, and poultry manure (Adesodun J.K et.al., 2008, Nwogu T. P. et.al., 2015, Agarry S.E. et.al., 2013). The microorganisms contained in organic waste possess broad enzymatic capabilities, and they have a bio-stimulating effect on the microflora of contaminated areas, thereby contributing to the solution of environmental problems (Delegan Y. A. et.al., 2016).

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The purpose of the work is to investigate the possibility of intensifying the process of destruction of oil and oil products by a balanced methanogenic association of microorganisms obtained after fermentation by introducing into them a strain of the bacterium Rhodococcus sp. HN4.

2. Materials and Methods

To degrade the oil, the *Rhodococcus sp. HN4* strain, which was isolated from soils contaminated with motor oil in a consortium with a balanced thermophilic methanogenic association of microorganisms (BTMAM), was obtained by successive fermentation of cattle manure and poultry manure (Tashbaev Sh.A. et.al., 2020). When studying the influence of the strain Rhodococcus sp. HN4 on the degree of oil degradation using a balanced methanogenic association of microorganisms obtained after fermentation, Rhodococ cultured on tryptone soy broth medium (HiMedia, India) was used for 48 hours. The amount of inoculation of Rhodococcus sp. HN4 introduced into the growing medium was 4% of the volume of the inoculated medium. BTMAM, which was used in the experiment, was previously diluted in sterile saline, and at a concentration of 5 and 10% was introduced into the medium. The moisture content of the applied BTMAM was 85–90% and pH = 7.6–7.9. Oil with a density of 832 kg/m3 (20°C) was used as a substrate. The oil was pre-sterilized in an autoclave for 30 min at 1 atm; after sterilization, at a concentration of 2% to 8%, it was added to Raymond's medium. BTMAM without the addition of Rhodococcus sp. HN4 was used as a control.

The cultivation was carried out in flat-bottomed flasks with a capacity of 250 ml on a shaker (150 rpm) at a temperature of 28–30°C for 20 days. After 20 days of cultivation, a dilution was made and the titer of microorganism cells was determined using the Koch method (Netrusov A.I. et.al., 2005). Nutrient agar (HiMedia, India) was used to calculate the total number of cells of the microbial association. Crops were incubated at 28–30°C for 5–6 days.

The degree of oil degradation was determined using the gravimetric method in relation to the total loss of oil in a liquid medium (Leonenko I.I. et.al., 2010). The analysis of the content of oil products in the medium was carried out as follows: After degradation, the samples were extracted with 50 ml of chloroform by shaking for 30 min. The chloroform extract was separated on a separating funnel and dried in a ventilated thermostat at a temperature of 70–75°C for 3–4 hours; it was then kept at room temperature for 18 hours, followed by weighing. The fractional composition of residual oil was assessed by chromatography using microcolumns filled with L40/100 silica gel (0.7 g each; Chemapol, Czech Republic), and the column was equilibrated with hexane. Samples of 0.005 g each, obtained by extraction during weight analysis, were dispersed in 1 ml of hexane, then applied to microcolumns and left for 20 min. Elution was carried out sequentially with hexane, benzene, and a benzene-ethanol mixture at a ratio of 1:1; 4 ml samples were then collected in tared test tubes. The resulting hexane, benzene, and benzene-alcohol fractions were evaporated in air and brought to constant weight in a ventilated drying cabinet at 75°C. The content of the components in the fractions was determined in reference to the weight of the initial sample. Finally, the genus and species affiliation of some strains belonging to the BTMAM association were determined from the spectrum of ribosomal peptides on matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF; Bruker Daltonics, USA).

3. Results and Discussion

The introduction of microbial associations to increase the efficiency of oil biodegradation is a promising and environmentally friendly method of remediating oil pollution in the environment. For active oil degradation, a combination of bacterial strains with wide enzymatic capabilities is required (Delegan Y. A 201653, Tashbaev Sh.A. et.al., 2020, Leonenko I.I. et.al., 2010, Ivanova A.A. et.al., 2015). It is known that bacteria of the genus Rhodococcus have a wide range of metabolic capabilities. They synthesize enzymes (dehydrogenases, peroxidases, oxygenases, alkyl sulfatase, nitrile hydratase, and phenol hydrolase) with broad substrate specificity and resistance to toxic compounds. Bacteria of the genus Rhodococcus are active biodegradants of hydrocarbons (aliphatic, aromatic, polycyclic, and heterocyclic) and their derivatives, which are difficult to access and toxic to other microorganisms. They are active producers of biosurfactants that emulsify oil and oil products and increase the bioavailability of pollutants. Currently, they form the basis of many biological products for cleaning oil pollution (Leonenko I.I. et.al., 2010, Netrusov A.I. et.al., 2005).

Given the characteristics of the *Rhodococcus* genus described above, the ability to degrade oil in high concentrations (2%–8%) of the *Rhodococcus sp. HN4* strain in conjunction with BTMAM was studied in this research. Previously, in the process of fermentation of chicken manure in thermophilic conditions, BTMAM was obtained, the microbial landscape was studied, and it was shown that the composition of BTMAM includes methane-forming, facultative, anaerobic, cellulose-decomposing, and nitrogen-fixing microorganisms. The number of which was: methane-forming $2.5*10^4$, cellulose-decomposing $2.5*10^4$, and denitrifying bacteria $5*10^3$, with the content of ammonifiers $4*10^3$. Since BTMAM was obtained in the process of thermophilic fermentation, it does not contain bacteria, pathogens belonging to the *Enterobacteriaceae* family, including the species *Escherichia coli*, the genera *Shigella* and *Salmonella*, as well as other pathogenic bacteria (Fig. 1).

It has been shown that BTMAM contains humic substances (humic acids, auxin, gibberellin, and fulvic acids). Several publications have reported that humic substances are necessary when eliminating pollution and restoring soils since their main functions are the restoration of many functions of the soil, the sorption of substances necessary for plants, and an increase in seed germination and yield (Tashbaev Sh.A. et.al., 2017, 2018). It is assumed that humic acids can also affect photosynthesis and respiration, exhibiting hormone-like activity (Golubkina N.A. et.al., 2010, Schnitzer M. et.al., 1978). Thus, the content of humic substances (humic acids and fulvic acids) in BTMAM was more than 20% (Fig. 2).

It is known that, in terms of chemical composition, chicken manure is one of the best types of organic fertilizers. It contains microelements, physiologically and growth-active substances, biologically active substances, and microelements that create favorable conditions for the development of microorganisms. Thus, in BTMAM, the content of phytohormones, in particular auxin and gibberellin, was 115 µg/g and 430 µg/g, respectively (Fig. 3. A). When studying the mineral composition of BTMAM, it was shown that BTMAM contains a large amount of both micro and macro elements Fig. 3(B). Thus, it has been shown that BTMAM is rich in organomineral composition.

In further research in a consortium with *the Rhodococcus sp. HN4*, 5, and 10% BTMAM were used for the degradation of oil in a concentration of 2 to 8%. As a control, BTMAM was used without the addition of *Rhodococcussp. HN4*. It should be noted that the oil degradation from the liquid culture medium was 83% in all variants of the experiment.

Studies have shown that *Rhodococcus sp. HN4* in monoculture is capable of degrading oil at a concentration of 2-4% with a degree of degradation of 60-64%, respectively. Whereas BTMAM is capable of degrading oil in concentrations of 2 and 4% with a degree of degradation of no more than 28% and 5%, respectively.

It was experimentally found that when the culture of *Rhodococcus sp. HN4* was added to 5 and 10% BTMAM, the degree of degradation increased significantly, and at an oil concentration of 2% was 77 and 81%, respectively, while at an oil concentration of 4% it was 75 and 78%, respectively. As a result of the studies carried out, it was found that BTMAM in consortium with the cell culture of *Rhodococcus sp. HN4* is capable of degrading oil in concentrations from 2 to 8%.

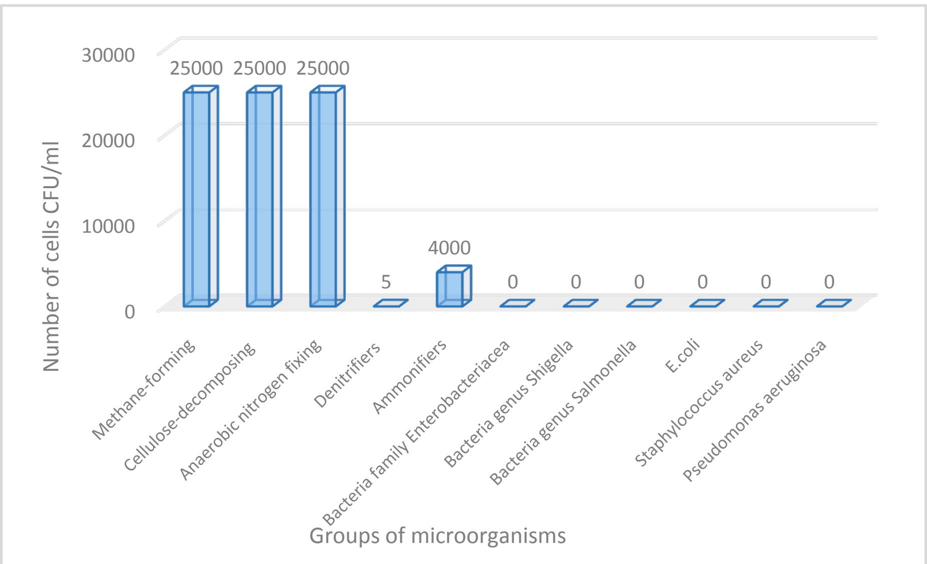

Figure 1. The main groups of microorganisms constitute the BTMAM

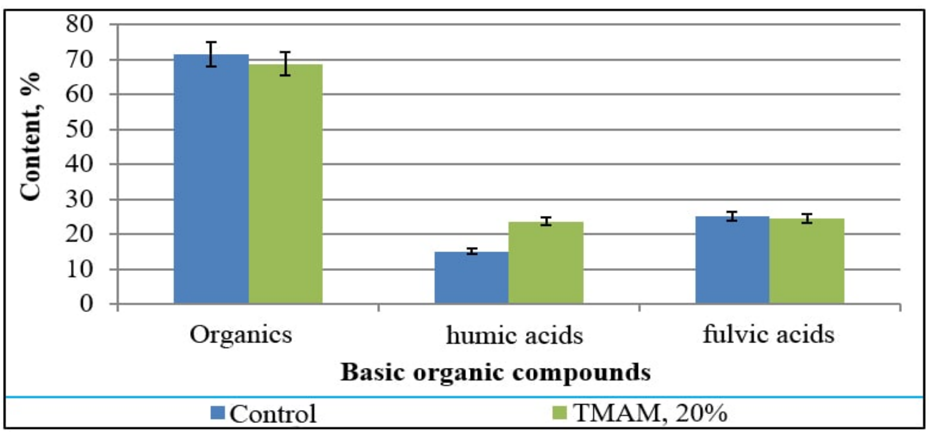

Figure 2. The organic composition of the obtained BTMAM

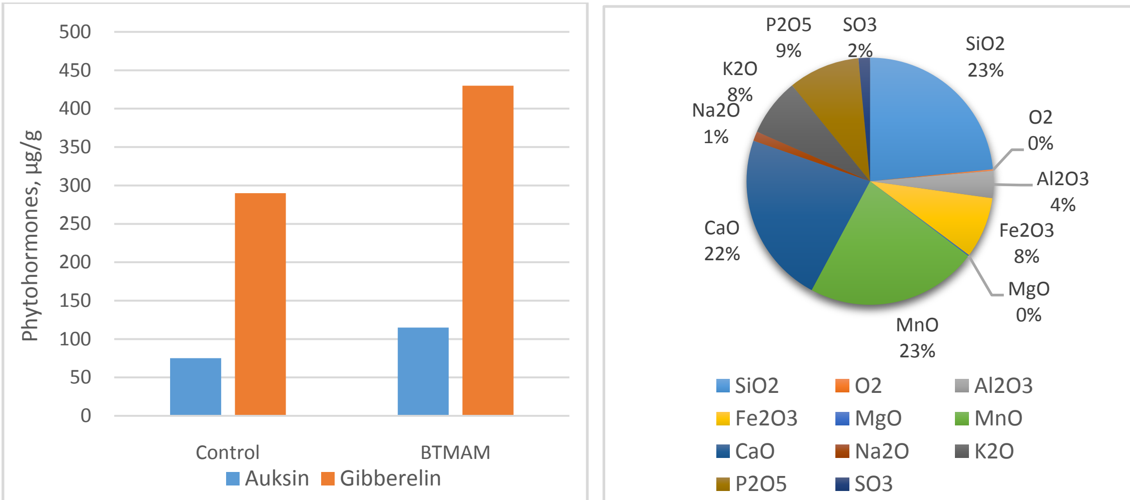

Figure 3. (A) Content of phytohormones in BTMAM, (B) Mineral composition of BTMAM

It was found that with an increase in oil concentration, the degree of oil degradation decreases, and in an oil concentration of 6%, the degree of degradation was no more than 51% and 42% with a residual oil concentration of 2.45 g/100 ml and 2.89 g/100 ml, respectively. Whereas for an oil concentration of 8%, the degree of degradation was significantly lower and amounted to 30-32% with a residual oil concentration of 4.65 g / 100 ml and 4.25 g/100 ml, respectively.

When determining the growth and development of strains after 20 days of cultivation, the cell titer was determined, which was for the bacterial strain *Rhodococcus sp. HN4* - $2.4*10^6$. For microorganisms that are part of the BTMAM association in the process of degradation 10^8-10^9 CFU/ml. In the process of further degradation of oil, after 30 days of cultivation, the degree of degradation increases, and for oil, in a concentration of 6-8% it was 62-48%, respectively, with a residual oil concentration of 1.8 g/100 ml and 3.45 g/100 ml.

Table 1. Oil degradation by the culture of *Rhodococcus sp. HN4* in consortium with BTMAM (within 20 days)

Consortium	Concentration of oil, %			
	2	4	6	8
	Degree of oil degradation, %			
Oil (control)	1.66	3.32	4.98	6.64
BTMAM	0.46 (28)	0.16 (5)	-	-
Rhodococcussp. HN4	0.99 (60)	2.12 (64)	1.49 (30)	1.06 (16)
5% BTMAM + *Rhodococcus sp. HN4*	1.27 (77)	2.49 (75)	2.53 (51)	1.99 (30)
10%BTMAM+*Rhodococcus sp. HN4*	1.34 (81)	2.58 (78)	2.09 (42)	2.39 (32)

To evaluate the intensity and direction of the biodegradation of oil pollution, the fractionation of residual hydrocarbons extracted from oil-polluted objects is often used. These data provide information on the ability of microorganisms included in the association to degrade any fractions of oil and oil products. Our research shows the fractional composition of 10% BTMAM + *Rhodococcus sp. HN4* (Table 2). The initial oil used as a substrate during cultivation was taken as a control.

It was shown that in the process of biodegradation with a content of 10% BTMAM + *Rhodococcus sp. HN4,* the degree of oil degradation at a concentration of 4% was 78%. The part not utilized by microorganisms was fractionated according to the described method. After partial biodegradation, the fractional composition of hydrocarbons between the fractions changed. Thus, the content of benzene and alcohol-benzene fractions were 8 and 6%, respectively.

Table 2. Fractional composition of residual hydrocarbons after biodegradation by a consortium of 10% BTMAM + *Rhodococcus sp. HN4*

Fractions of hydrocarbons	Content in the original oil (%)	Content after partial biodegradation (%)
Hexane	64	6
Benzene	19	8
Alcohol-benzene	15	6
Total (%)	98	20

Thus, based on the studies carried out, it was shown that *Rhodococcus sp. HN4* contributes to an increase in the degree of oil degradation. To understand the process of oil degradation by a bacterial consortium, it is necessary to study the interaction within mixed cultures. Apparently, bacteria of the genus *Rhodococcus* in the process of growth and development decompose compounds that are inaccessible to other members of the consortium and thereby facilitate the assimilation of metabolic products by bacteria that are part of the BTMAM. Perhaps one of the interaction mechanisms is associated with a synergistic interaction of microorganisms present in the association, that is, one of the types of microorganisms removes metabolic products that are toxic to other microorganisms, increasing the degree of oil degradation.

In the process of oil degradation, the microbial landscape of some genera of microorganisms included in BTMAM was studied. According to the results of MALDI-TOP, it was shown that the composition of BTMAM includes bacteria belonging to the species *Bacillus siralis, Bacillus megaterium, Bacillus amyloliquefaciens, Bacillus firmus, Bacillus flexus, Bacillus subtilis, as well as bacteria Lactobacillus sakei, Lactobacillus Plantarum, Solibacillus Silvestri, Hafnia alvei, Pseudomonas tolaasii*. The cell titer of these types of bacteria was 10^7-10^8 CFU/ml. According to the literature data, the spectrum of microorganisms included in the association are producers of biologically active compounds that are used to combat certain plant pathogens in agriculture, aquatic culture, and hydroponics. They are active against bacterial and fungal pathogens and have a prolonged effect on the degradation of xenobiotics (Netrusov A.I. 2015 et.al., Ivanova A.A. et.al., 2015).

Thus, it has been established that the bacterial strain of the genus *Rhodococcus*, possessing a wide range of metabolic capabilities, contributes to other groups of microorganisms that are part of BTMAM to assimilate hard-to-reach substrates, in particular oil and its decay products. BTMAM in the community with the *Rhodococcus sp. HN4* degrades oil with a high degree of degradation and in high concentrations of 6-8%. The spectrum of microorganisms that make up the association are not pathogens, they are producers of biologically active compounds, and they are resistant to high concentrations of oil, in the process of oil degradation they will contribute to soil biologization. Being a good organic substrate, it contains nitrogen, phosphorus, and potassium, which have great potential for improving bioremediation.

Author Contributions

All authors contributed to the article and approved the submitted version.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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