Selection of bacteria capable of biodegradation of organic nitrogen-containing compounds and metagenomic analysis of their living environment

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Abstract. Samples of karst lakes, rivers, industrial effluents, and natural soil were studied for selection of bacteria for biodegradation. A metagenomic analysis of the used natural and man-modified media was carried out. It has been established that the most unique are the compositions of the metagenomes of natural karst reservoirs, as well as microcenoses of industrial effluents. Most of the detected bacteria belong to Proteobacteria. As a result of selection, strains of microorganisms with high activity of nitrocellulose biodegradation, aromatic nitrates, quinoline derivatives and amides were obtained. In particular, Pseudomonas strains and R. erythropolis, effectively utilizing nitrobenzenes, nitrocellulose, quinolines, which were isolated from industrial effluents. At the same time, Pseudomonas strains were isolated from the natural karst lake, which quickly utilized the nitrocellulose. It was also found that natural soils contain a large number of prokaryotes that can utilize organic nitrates. The results confirm the view that natural environments, due to high metabolic diversity, are the richest source of producers of a wide variety of enzymes. An obvious reason for the proliferation of the metabolic systems of technogenic pollutants in natural environments is the presence in natural environments of some quantities of their structural analogues - nitrogen-containing plant metabolites and soil microflora, as well as lignin decomposition products.

1. Introduction

Synthetic nitrogen-containing compounds, such as organic nitrates, quinolines, amides, etc., are widespread environmental pollutants. The most common of them are organic nitrates, such as nitrocelluloses, nitrobenzenes, nitrotoluenes, nitrophenols, are widely used in industry for plastics, paints, varnishes, adhesives, pesticides, dyes, medicines, explosives, fungicides, analytical reagents, etc.

The danger of nitro compounds for human health is due to their high toxicity, as well as the possible formation of mutagenic metabolites. Various physical and chemical methods have been developed for the degradation of nitrogen-containing organic compounds. The most common are incineration and chemical processing. However, these methods are not environmentally friendly and are applicable only to dry concentrated waste, while much of the damage to the environment is caused by industrial runoff and soil pollution.

Many organic nitrates are resistant to biodegradation due to their high toxicity for most microorganisms (nitrophenols) or low availability (water-insoluble nitrocellulose, 2,4,6-trinitrotoluene, etc.). Because of this, they accumulate in the environment.
Due to the high relevance of research in the field of biodegradation of organic nitrates have developed over several decades [1]. The ability to biotransformation and biodegradation of nitro compounds is known in the strains of the species *Pseudomonas fluorescens*, *Mycobacterium, R. erythropolis* [2-6]. Aerobic and anaerobic processes of such decomposition are known. The main class of enzymes involved in the transformation of trinitrotoluene are nitroreductases; enzymes of the family of flavoproteins, oxidase, hydrogenase, and peroxidase can also participate in degradation [2].

In the last decade there has been a significant expansion of knowledge about the biotransformation of TNT at the genetic and biochemical level and the development of the application of these processes in the field of biotechnology. Research in the field of biotransformation of nitro compounds has received a new impetus through the use of modern molecular methods, including metagenomic studies [7-10].

Despite the long history of studying the processes of biotransformation of nitroorganic compounds, this trend remains relevant as a result of the practical relevance and sustainability of such substrates to biological and abiotic degradation.

Actual are the selection of new cultures of microorganisms - active biodestructors, the identification of new genes and enzymes of metabolism of organic nitrates, enzymological analysis [2,8,11].

2. Materials and research methods

2.1. Objects of study

Samples from karst lakes, bottom sediments of rivers, industrial effluent and natural soils of Perm region were used for isolate microorganisms-biodestructors of nitrogen-containing organic compounds.

With a low level of anthropogenic chemical load:

- Molebnoe Karst Lake and the failure of Kishertsy karst area);
- Bottom sediments of the river Suz'va and its tributaries, Nytvensky district of the Perm region;
- Sod-meadow soil, Nytvensky district of the Perm region.
- Bottom sediments of the Yazovaya River, Perm (industrial effluents of OAO Kamteks-Khimprom).

2.2. Substrates, media and culture conditions

The isolation of communities (consortia) and pure cultures transforming organic nitrogen-containing compounds was carried out on a mineral medium with the addition of a nitrogen-containing compound as the sole source of carbon and / or nitrogen.

As substrates nitrobenzen- and 1,3-dinitrobenzenes, 2,4–dinitrophenol, dinitrocellulose (colloxylin), quinoline, and 8-nitroquinoline were used.

Cultures were grown in liquid mineral medium N of the following composition, g / l: K₂HPO₄ - 1.0; KH₂PO₄ - 1.0; NaCl - 0.5 A trace element solution was added to the medium, g / l: MgSO₄ × 7H₂O - 0.5; CaCl₂ - 0.005; CoCl₂ × 6H₂O - 0.01; FeSO₄ × 7H₂O - 0.005 [12]. Ammonium chloride at a final concentration of 10 mM was used as a standard source of nitrogen, and glucose at a concentration of 0.1% was used as a source of carbon and energy. Pure cultures were isolated by plating on LB agar medium (Luria Bertani) containing (g / l): tryptone 10.0, yeast extract 5.0, NaCl 10, agar (Sigma) 15. Agar media were prepared by adding purified agar for microbiological media (Sigma) to the final concentration 1.5%.

Cultures were grown under conditions of periodic cultivation in Erlenmeyer flasks in 50 ml of nutrient medium on an orbital shaker with a rotation speed of 150 rpm at a temperature of 30 °C.

Isolates were isolated by the method of cumulative culture. After 30 days, they were seeded on Petri dishes with agar N medium containing selective substrates at a concentration of 10 mM. On days 7-14 of growth, the resulting colonies were selected, transferred to Petri dishes with fresh selective medium and with full LB medium.

The density of the culture was evaluated by the change in the optical density of the cell suspension at λ = 540 nm, considering the dilution.
Minimum inhibitory concentrations of substrates were determined by the growth intensity of the culture in the Luria – Bertani medium (LB) with different concentrations of chloroquine by serial dilution in a 96-well polystyrene plate (Eppendorf). The plates were incubated in a thermostat at a temperature of 30º C for three days and the optical density at 540 nm was measured on an Infinity M1000 plate spectrophotometer, Tecan, Switzerland. For the minimum inhibitory concentration took the highest concentration at which there was no significant increase in the density of the culture.

2.3. Identification of selected cultures

Taxonomic analysis of bacteria was carried out on the basis of standard cultural-morphological, biochemical, and chemotaxonomic characteristics according to the Berge bacterium determinant [13], and also using specialized guidelines. Also, for species identification, amplification of a fragment of the 16S rRNA gene with universal primers 27F (50-AGAGTTTGTCCTGGCTCAG-30) and 1492R (50-TACCTTGTTACGACTT-30), sequencing of the obtained PCR product and comparison of sequences deposited in the Ezio database of data has been used in the Ezio data database, has been used. www.ezbiocloud.net/). A metagenomic analysis of the samples studied was carried out on the 16S rRNA genes on the MiSeq platform (Illumina) [14].

2.4. Molecular genetics methods

DNA sequencing was performed using an Applied Biosystem 3500XL system using the Big Dye Terminator Cycle Sequencing Kit reagents according to the manufacturer's instructions. Comparison of gene sequences was carried out using the software packages VectorNTI11, ClustalW, as well as the original application YACWGUI 1.2.

DNA extraction was carried out by the alkaline method (heating): the material of the colony was transferred with a microbiological loop in 0.5 ml of 0.01 M NaOH, mixed on a vortex, kept in a solid-state thermostat at 97 ° C for 10 minutes, then in a freezer at -18ºC 10 minutes. Then the warm-up and cooling cycle was repeated twice more.

2.5. Biotransformation and analysis

Biotransformation products were determined by GC-MS methods on an 689 / 573T MSD apparatus (Agilent-Hewlett Packard), under the following conditions: extraction of samples with ethyl acetate; capillary column HP 5MS, column initial temperature - 60 ° C (3 min), heating to 315 ° at a rate of 7 ° / min; evaporator temperature 280 ° C, carrier gas helium, as well as using HPLC LC10, Shimadzu on a column Luna C18 (250 x 4.6 mm). The flow rate was 1 ml / min at 25 ° C. Detection was performed at 230 and 340 nm.

The amount of water-insoluble aromatic nitrates and nitrocellulose was determined gravimetrically on an analytical balance after drying to constant weight.

Mathematical processing of experimental data was performed using MS Office Excel and Statistica 5.0.

3. Results and discussion

3.1. Isolation of bacteria communities

It is known that among nitrogen-containing organic compounds of one series, amines, amides and nitriles are most easily subjected to biological utilization, while nitro compounds and nitrogen heterocycles are generally resistant to biodegradation due to lower bioavailability and, in some cases, high toxicity.

Among cultures of microorganisms capable of biodegradation of nitro compounds, anaerobic bacteria attract the particular attention of researchers. The reduction of nitro groups to nitroso derivatives, hydroxylamines or amines is catalyzed by nitroreductases through the sequential addition of pairs of electrons whose donors are co-substrates. It is believed that the degradation of most polynitroaromatic compounds is possible only under anaerobic conditions [10,11,15].
At the same time, the possibility of using aerobic processes attracts a higher rate of metabolism and growth of aerobic cultures, as well as the potentially greater ease of controlling aerobic processes. In this regard, in our work we used microorganisms capable of aerobic growth and transformation of substrates under aeration conditions.

The method of cumulative culture obtained on the basis of nitrogen-free salt medium N with the addition of nitrocellulose and nitroaromatic compounds (nitrobenzene, p-nitrophenol, quinoline, 8-nitroquinoline) as selective substrates, as a source of carbon and/or nitrogen, obtained communities of microorganisms capable of using nitro groups of organic compounds as a source of nitrogen. Investigated the activity of the obtained microbial communities in the process of biodegradation of a number of organic nitrates. The degree of biodegradation of the substrate, expressed as a percentage of the amount introduced into the medium and the growth of bacteria, given in units of optical density at a wavelength of 540 nm, was evaluated. The high activity of communities isolated from industrial effluents of Kamteks-Khimprom, in particular, during the decomposition of dinitrocellulose (Table 1) is shown.

From the literature, a similar result is known in the anaerobic process using sulfate-reducing bacteria of the genus Desulfovibrio [16,17], however, in the case of the consortium studied, utilization was observed in a wide range of physicochemical conditions.

The ability of selected microorganisms to utilize organic nitrates has been investigated. Cultures were grown on mineral medium containing up to 0.25% of aromatic nitrates as the sole source of nitrogen. It is established that the most optimal growth agent under these conditions is nitrobenzene, which provided the maximum biomass growth. At the same time, it was found that a consortium of bacteria isolated from industrial wastewater degrades mononitrophenol in a weakly alkaline medium (pH 7.8) at a concentration of more than 1 mmol / l in 5 hours, with the formation of aliphatic carboxylic acids, which significantly exceeds the rate of biodegradation, described in the literature for strains Arthrobacter, Burkholderia, and others [2,3,12,18]. For example, for the strain Burkholderia sp. FDS-1 - 9 hours at a concentration of 0.6 mmol / 1 MNF, pH 7 - transformation to hydroquinone [18].

| Substrate                          | Substrate biodegradation, % / Culture density, OD540 |
|-----------------------------------|------------------------------------------------------|
|                                   | Sample (community) number | 1 | 2 | 3 |
| 0.1% p-nitrophenol                | 22.12±3.01/ 22,12±3.01/ | 22.12±3.01/ 22,12±3.01/ |
| 0.1% p-nitrophenol + 0.1% glucose | 24.32±2.99/ 24,32±2.99/ | 24.32±2.99/ 24,32±2.99/ |
| 0.1% nitrobenzene                 | 37.79±3.15/ 37,79±3.15/ | 37.79±3.15/ 37,79±3.15/ |
| 0.1% dinitrocellulose             | 49.45±3.22/ 49,45±3.22/ | 49.45±3.22/ 49,45±3.22/ |
The community numbers correspond to the samples from which they were isolated:
1 - Sod-meadow soil, Nytvensky district of the Perm region;
2 - Bottom sediments of the Syuzyva River;
3 - Bottom sediments, industrial effluents of Kamteks-Khimprom JSC.

3.2. Metagenomic analysis
A metagenomic analysis of the media from which the active crops were selected: the technogenically altered environment (bottom sediments and industrial effluents of Kamteks-Khimprom JSC), as well as natural environments — karst lakes and rivers that do not have significant chemical pollutants (Suzyva, Nytvensky region), natural soil.

It has been established that the most unique is the composition of the metagenome of natural karst reservoirs, as well as microcenoses of industrial effluents containing “nitrotel”. As a result of metagenomic analysis, it was found that most of the detected bacteria in natural reservoirs belong to the Proteobacteria fillet - 61% of the total number of reads, mostly represented by the Betaproteobacteria class (37.5%), the Alphaproteobacteria class (10.3%), the Deltaproteobacteria class (5%), class Gammaproteobacteria (2%), class Epsilonproteobacteria (0.1%). The class Betaproteobacteria is represented by the orders of Burkholderiales (8%), the family Comamonadaceae (37.5%); order of Gallionellales (7.2%), the family Gallionellaceae (7.2%) and order Rhodocyclales (5%), the family Rhodocyclaceae (5%). A significant proportion of identified prokaryotes belonged to the phyla: Bacteroidetes (14.7%), Cyanobacteria (0.4%), Acidobacteria (1.1%), Chlorobi (0.6%). Sulfate-reducing bacteria belonged to the class Deltaproteobacteria (5%), represented by the orders Desulfuromonadales (0.6%), Desulfovibrionales (0.5%), Desulfobacterales (0.5%). Desulfuromonadales order is represented by the family of Geobacteraceae 0.3% (of the total number. The Desulfovibrionales family (0.5%) belongs to the Desulfivibrionales order (0.5%). The Desulfobacterales order is represented by the families: Desulfobulbaceae (0.2%), Desulfofoliaceae (2.2%).

3.3. Pure cultures
Active strains isolated using subculturing were identified by polyphasic taxonomy and analysis of 16S RNA genes as representatives of the proteobacteria Pseudomonas fluorescens (4 strains) and Pseudomonas denitrificans (4) and Pseudomonas sp. (one); actinobacteria of the genera Rhodococcus (15), in particular, species of Rhodococcus erythropolis (9 strains), Rhodococcus ruber (2), Rhodococcus rodii (4), genera Arthrobacter (7), Gordona (1). In particular, strains Pseudomonas fluorescens N-17, N19 and Pseudomonas putida N-26, as well as R. erythropolis N-4 and N-14, which efficiently utilize nitrobenzenes and nitrocellulose, were isolated from the effluent of nitrocellulose. At the same time, the strains Pseudomonas sp. N52, N53, quickly disposed of colloxylin. It has also been established that natural soil contains a large number of diverse cultures of prokaryotes capable of utilizing aromatic nitrates, albeit at a slower rate compared with isolates from industrial effluents. Despite the fact that the majority of organic nitro compounds — environmental pollutants — are of technogenic origin, a large number of bacteria and their communities capable of using these substances were sown from natural soils and waters that are not in contact with anthropogenic organic nitrates. This may be due in part to the fact that in open systems it is possible to transfer microorganisms from media that have industrial pollution, as well as the possibility of transferring trace amounts of nitroorganic compounds.

An obvious reason for the spread of such metabolic systems is the presence in natural environments of certain amounts of structural analogs of man-made pollutants, such as various nitrogen-containing plant metabolites and soil microflora, as well as lignin decomposition products, including its abiogenic
destruction. And, as is well known, natural environments, including rich soils, due to the highest metabolic diversity, are the richest source of producers of a wide variety of enzymes.

It has been shown that most of the studied actinobacteria are able to use the nitrogen of the nitro groups of organic nitroesters, as is known for other microorganisms and fungi [16-20]. It was established that the majority of bacterial cultures - active transformants had nitroreductase activity. Nitroesterase activity was detected only in two isolates, which did not show a high rate of biodegradation under the conditions of model experiments.

As a result of screening studies, genes characteristic of some representatives of the genus *Pseudomonas* were detected in 6 studied *Pseudomonas* cultures using the Ps_nfsA-F/R primers.

Analysis of the genes encoding the biodegradation enzymes of other nitrogen-containing compounds, in particular, amidases, was performed as previously described. Phylogenetic analysis of amidase genes was obtained, as well as the results of studies of the activity, thermal and pH stability of controlled enzymatic activities in the transformation of nitrocellulose and aromatic nitrates (nitrobenzenes, nitrotoluene).

It has been shown that most of the studied actinobacteria are able to use the nitrogen of the nitro groups of organic nitroesters, as is known for other microorganisms and fungi [18, 20-23].

**3.4. Minimal inhibitory concentrations and cultivation**

The complexity of the utilization of aromatic nitro compounds is caused by the influence of the following factors: the lower availability of nitro groups for enzymes caused by steric hindrances (nitro groups are associated with an aromatic ring); hydrophobicity, low solubility of aromatic nitriles in the aqueous phase; the difficulty of diffusion of biotransformation products and substrates into and out of the cell; toxicity of aromatic nitriles for cells.

To assess the effect of the latter factor on transforming cells, minimal inhibitory concentrations of a number of aromatic nitro compounds for the isolated bacteria were determined. It was found that, in general, strains of the genus *Rhodococcus* were the most resistant to the presented substrates. The most toxic for bacteria were nitrophenols and nitroquinoline (MIC for *Pseudomonas* strains - 31.3, for *Rhodococcus* - 62.5. Toluene nitrates were less toxic, apparently due to low solubility in aqueous medium. Colloxylin did not inhibit the growth of bacteria.

The ability of the isolated strains to grow and transform nitro compounds as the sole substrate (source of carbon and nitrogen) and with the addition of glucose (the aromatic substrate is the source of only nitrogen) was investigated.

It was established that the most active biotransformation of *Rhodococcus* strains was when using as a carbon source 0.1% acetate and 0.25% nitroaromatic compound or 1-5% colloxylin at an ambient temperature of 28-30 °C and slightly alkaline pH values of 7.2-8.

It was shown that highly active cultures of the strains *Pseudomonas* sp. N-52, *P. fluorescens* N-17, N19, *P. putida* N-26, *R. erythropolis* N-5 and N-14 in optimal conditions of 7.7 mg O_2 / l, pH 7.8–8.0, 25°C are able to transform colloxylin c from high speed - up to 0.5 g / 1 g dry weight of cells / day.

**4. Conclusions**

Thus, microbial communities and pure cultures capable of using the nitro groups of man-made organic compounds as a nitrogen source were selected. In particular, strains of *P. fluorescens*, *P. putida* and *R. erythropolis*, which effectively utilize nitrobenzenes, nitrocellulose, substituted quinolines and amides, were isolated.

It is also shown that microorganisms which possess metabolic systems for the disposal of nitroorganic compounds inhabit not only in the environment, polluting chemicals, but also in the environment of karst lakes, rivers, soils, remote from industrial centers and not tangible man-made stress. At the same time, the representatives of bacteria, active in the process of biodegradation of nitroorganic compounds, do not belong to the dominant genera identified as a result of metagenomic analysis. The results confirm the view that natural environments, as the best metabolic diversity, are the richest experience of producing a wide variety of enzymes. An obvious reason for the propagation,
the metabolic systems of technogenic pollutants in natural environments is the presence in natural environments of some quantities of their structural analogues - nitrogen-containing plant metabolites and soil microflora, as well as lignin decomposition products.

Highly active cultures of *Pseudomonas* sp. N-52, *P. fluorescens* N-17, N19, *P. putida* N-26, *R. erythropolis* N-5 and N-14, capable of utilizing the nitro-organic connections at high speed, are promising for use in local wastewater treatment of chemical plants.

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