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Diazotrophs Originated from Petrochemical Sludge as a Potential Resource for Waste Remediation

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Abstract: The presence of nitrogen-fixing bacteria in petrochemical sludges was established first by using of aseptically recovered cores drilled from about 0,3 m-deep layer of industrial sludge repositories, which are the result of permanent discharges of petrochemical solid wastes. Bacteria isolated on nitrogen-free medium were examined for the presence of nitrogenase gene (*nifH*) and the ability to biological fixation of atmospheric N_2, which corresponds to acetylene reduction at the level of 1-5 nmol C_2H_4 ml^{-1} h^{-1}. Five isolates were selected after testing their ability to degrade organic contaminants of wastes (polyethyleneglycol, naphthalene, hexadecane, sludge) and their potential as plant growth promoters. They produce indoleacetic acid at level 5-10 μg ml^{-1} and exhibit antagonistic activity against bacterial and fungal pathogens such as *Erwinia carotovora*, *Xantomonas campestris*, *Fuzarium solani, Alternaria alternata, Alternaria sambacinum.*

Key words: Diazotrophs · Petrochemical sludge · *nifH* · Acetylene reduction assay · PGPR

INTRODUCTION

The accumulation of industrial waste sludges is one of the most actual environmental problems of petrochemical and chemical industrial branches of Russia, as well as of many other countries. In comparison with combustion and other physicochemical methods of sludges treatment, the more economically and ecologically effective strategy is bioremediation allowing to detoxify the wastes and to involve them into industrial recycling. One of more perspective technologies in this field is phytoremediation based on plant-microbial interactions [1].

Some specific properties of sludges, first of all the high carbon to nitrogen ratio, make necessary of the supplementation of corresponding quantities of nitrogen fertilizers to improve metabolic processes [2]. At the same time, large amounts of mineral nitrogen are known to be toxic for plants and even for microorganisms [3].

As it was shown earlier [4], the aged (about 50 years-old) petrochemical sludge contains the metabolically diverse groups of surviving microorganisms which are able to perform the critical steps of specific sludge pollutants degradation. This phenomenon has been used as the background for creation of petrochemical sludge treatment biotechnology [5].

This work was undertaken with the purpose to gain knowledge about the principal possibility of diazotrophs existence in petrochemical sludges stored in industrial repositories.

MATERIALS AND METHODS

Petrochemical Sludges: Petrochemical sludges stored for about 50 years in special depositories of the factories "Nizhnekamskneftekhim" and "Kazanorgsynthes" (Russia) were used. For microbiological analyses the sludges were sampled aseptically.

Bacteria Isolation and Nitrogenase Activity Assay: Diazotrophic microorganisms were isolated using solid N-free selective medium (SM): (g l^{-1} distilled water): KH_2PO_4 – 0,2; K_2HPO_4 – 0,1; $MgSO_4*7H_2O$ – 0,2; NaCl – 0,2; $CaCO_3$ – 5; sucrose – 20; agar-agar – 20. Isolates were named as NK (Nizhnekamskneftekhim) and KOS (Kazanorgsynthes) according to their origin. The nitrogen fixing activity was measured by gas chromatography on the base of acetylene reduction assay [6]. Nitrogenase

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activity was expressed as nmol C_2H_4 ml^{-1} h^{-1} (1 ml incubation suspension contained 10^8 cells assessed by optical density (A_{600})).

***nifH* Gene Amplification:** Genomic DNA from bacterial isolates was purified using standard phenol/chloroform extraction and ethanol precipitation. Fragments of *nifH* genes were amplified using a nested PCR method as previously described by Widmer *et al.* [7]. Amplified fragments were visualized in 2% agarose gel. Gene Ruler 1000 bp was used as the DNA molecular mass marker.

Utilization of Sludges Organic Components: Isolates were examined for growth on SM supplemented with polyethyleneglycol (PEG) (0,3%), naphthalene (0,3%), hexadecane (1%) and petrochemical sludge (10%) as a sole source of carbon and energy. Growth of cultures on nitrogen (NH_4NO_3, 0,6 g l^{-1})- supplemented media was as control.

Indoleacetic Acid (IAA) Production: IAA produced by bacteria was assayed colorimetrically with ferric chloride-perchloric acid reagent ($FeCl_3$-$HClO_4$) as it was described previously [8]. This method estimated the quantities of indole compounds produced by bacteria during growth in medium containing precursor L-tryptophan. Concentrations values were calculated on a standard curve made with pure IAA (Sigma).

Antagonistic Activity: Antagonistic activity of the diazotrophs was tested against bacterial (*Erwinia carotovora*, *Xantomonas campestris*) and fungal (*Fuzarium solani, Alternaria alternata, Alternaria sambacinum*) phytopathogens. The development of pathogen inhibition zone on potato glucose agar medium (peeled potato 250 g, glucose 20 g, agar 20 g, distilled water 1 L) around the tested bacterial colony was observed.

RESULTS AND DISCUSSION

In search of bacteria able to fix atmospheric nitrogen, we were interested in diazotrophs originated from petrochemical sludges, their surviving in the sludge thickness, as well as the retaining of their nitrogen fixing ability. Investigation of two kinds of sludges allowed us to reveal the cultivable diazotrophs at the level up to some million cells per gram of dry sludge. Ten isolates representing different morphotypes were estimated on their response to the medium composition, namely, addition of NH_4NO_3 (0, 6 g l^{-1}), replacement of glucose by sludge or its constituents (Table 1). The obtained data allowed differentiating the isolates through the diazotrophic growth and diapason of utilizable xenobiotics.

The most active growth on N-free selective medium was observed in variant with the isolates NK1, NK9, NK15 and NK17, whereas the significantly lower growth was in case of NK5 and KOS11 (Table 1). The addition of mineral nitrogen to the SM exerted a stimulating effect. In some cases the growth on SM was comparable with growth on NBA (isolates NK1, NK17). The ability of most isolates to grow on medium supplemented with sludge or its components (instead glucose) is an important biotechnological property of isolated diazotrophs.

Table 1: Growth of isolates dependently on the medium composition

				SM (- glucose)				SM (- glucose) +N			
Isolates	SM	SM +N	NBA	Sludge	PEG	Naph	Hexadec	Sludge	PEG	Naph	Hexadec
NK1	+++	++++	+++++	++	+++	++	++	+++	+++	++	++++
NK5	+	+++	++++	-	++	+	+	+	+	+	++
NK9	+++	++++	+++++	+	+	+	+	+	+	+	+
KOS10	++	+++	+++++	+	+++	++	++	++	+	++	++++
KOS11	+	++	++++	+	++	+	+	+	+	+	+
NK15	+++	+++	++++	++	+++	++	++	+	+++	++	+++
NK17	+++	+++++	+++++	+	+++	++	+++	+++	++	++	++++
NK20	++	++++	+++++	+	++	++	++	+	+	+	+++
NK21	++	+++	++++	-	+	+	+	-	+	+	+
NK22	++	+++	++++	+	+	+	++	++	++	+	+++

SM – selective N-free media; NBA – nutrient broth agar; Naph – naphthalene; Hexadec - hexadecane

Table 2: The levels of nitrogenase activity and IAA production by the isolates

Isolates	NK1	NK9	KOS10	NK17	NK20
Nitrogen fixation activity, nmol C_2H_4 ml^{-1} h^{-1}	4,5	3,2	2,6	1,8	1,5
IAA production, μg ml^{-1}	10,3	9,8	7,6	3,8	2,1

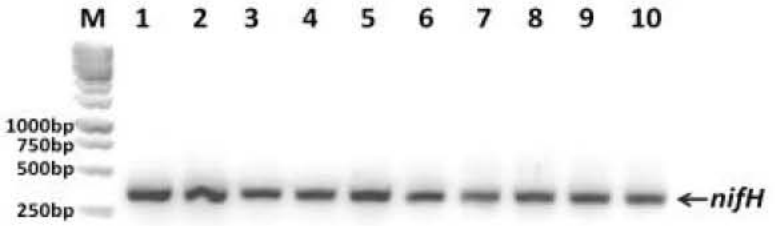

Fig. 1: PCR amplification of *nifH* gene fragment using genomic DNA from isolates. M – DNA ladder, 1-10 – individual isolates

Table 3: Antagonistic activity of isolates against pathogens (zone of growth inhibition, mm)

	Pathogen				
Isolate	*Erwinia carotovora*	*Xantomonas campestris*	*Fuzarium solanii*	*Alternaria alternata*	*Alternaria sambacinum*
NK1	13	20	20	29	12
NK9	5	7	0	2	3
KOS10	0	9	4	2	2
NK17	16	25	27	7	4
NK20	7	19	7	5	5

The functional gene *nifH* (nitrogenase reductase) is the prevailing marker gene for the detection of potential N_2 fixers. We established the presence of *nifH* gene in the genome of all investigated isolates (Fig. 1). For quantitative characteristic of nitrogenase activity widely spread acetylene reduction assay was used. Data of Table 2 demonstrate the scale of N_2 fixing activity of five more active isolates. The production of phytohormone IAA is considered to be one of key parameters which characterize the plant growth promoting rhizobacteria (PGPR) [9]. The isolates exhibiting a middle levels of nitrogenase activity (1,5-4,5 nmol C_2H_4 ml^{-1} h^{-1}) and IAA production (Table 2) are comparable, for example, with species of *Azotobacter* isolated from saline environments [10].

Along with the above features, the biotechnological potential of the investigated bacteria includes the antagonistic activity in respect to phytopatogenic bacteria and micromycetes from genera *Erwinia, Xanthomonas, Fusarium* and *Alternaria* (Table 3). It is known that PGPR antagonistic properties can be realized through antibiotics and siderophore production [11] and the chitinolytic mechanism (in case with micromycetes) [12].

CONCLUSIONS

Diazotrophic bacteria, which are first discovered in hazardous petrochemical wastes, share a complex of biotechnologically attractive features including N_2 fixation, indoleacetic acid production and suppressive activity against phytopathogens. It allows improvement of petrochemical sludge bioremediation technology by overcoming of N limitation and plant growth promotion (in relation to phytoremediation)

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