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ORIGINAL PAPER

Effect of plant extract on the degradation of nitroaromatic compounds by soil microorganisms

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Abstract Remediation of soils contaminated by nitroaromatic compounds and nitramines, i.e. explosives, is known as very important, complicated, and rapidly developing area of biotechnology. A search for optimal growth conditions for soil bacteria is of a great importance in order to isolate various xenobiotic degraders. Bacteria consortium A43 was isolated from soils contaminated with explosives. In the presence of carbohydrate and plant extract, an addition of TNT to the solidified minimal medium stimulated the growth of the tested bacteria, as compared to other bacteria consortium isolated from the same soils. Reducing sugars as carbohydrates, and cabbage leaf extract as a plant extract were used in these experiments. Cultivation of the A43 in liquid medium of the same content showed that addition of cabbage leaf extract alone to medium is much more efficient for TNT degradation by growing biomass as compared to addition of carbohydrate alone.

Keywords Cabbage leaf extract · Soil bacteria · TNT degradation

Abbreviations

CLE	Cabbage leaf extract
TNT	2,4,6-Trinitrotoluene
1,3-DNB	1,3-Dinitrobenzene
2-Am-4,6-DNT	2-Amino-4,6-dinitritoluene
2,3-DNT	2,3-Dinitrotoluene

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Introduction

Remediation of soils contaminated by nitroaromatic compounds and nitramines, i.e. explosives, is known as very important, complicated, and rapidly developing area of biotechnology. The use of naturally selected microbial populations to remove organic contaminants has been referred as intrinsic remediation or natural attenuation. The driving force behind natural attenuation lies in the benefit that microorganisms derive from the presence of the contaminant, which provides the energy and nutrients needed for microbial growth [14–16].

A search for optimal growth conditions for soil bacteria is of a great importance in order to isolate various xenobiotic degraders. For instance, *Pseudomonas* genus today constitutes one of the best-studied bacterial groups. Nevertheless, new *Pseudomonas* species are described after application of new media or isolation procedures [1, 2, 13]. It has been demonstrated that different media giving comparable plate counts can be used to select for different bacterial types, leading to different estimates of diversity for the same soil [1, 20]. In addition, bacteria's ability to degrade TNT and other explosives can be considerably influenced by various amendments added to the explosives-containing medium [4, 9].

Our preliminary results indicated to the positive role of a cabbage leaf extract as an amendment to the solidified growth medium upon isolation of bacteria with explosive-degrading ability. The experiments described below were performed with the aim to compare the TNT-degrading activity of bacterial consortium A43 in liquid medium amended with carbohydrates alone and cabbage leaf extract.

Materials and methods

The bacterial consortium A43 was originally isolated from explosive-contaminated soil based on its ability to grow with TNT and other nitroaromatic compounds as the sole nitrogen source. Microorganisms were identified as *Burkholderia cepacia* and *Pseudomonas* spp., using API® (BioMérieux). The M8* medium used for cultivation of bacteria isolated from contaminated soils was a modified M8 minimal medium [12, 17], i.e. medium contained, g/l: Na_2HPO_4, 60; KH_2PO_4, 30; NaCl, 5 (pH 6.9). Sucrose, glucose, lactose, cabbage leaf extract were added to the medium in concentrations indicated in the experiment scheme. The solidified M8* medium contained 1.5% agar. Cabbage leaf extract was prepared from white cabbage leaves. Leaves (500 g) were washed with tap water, boiled at 100 °C for 30 min, cooled, afterwards filtered (Millipore, 0.45 μm) and steamed for 15 min. The prepared extract was stored at +4 °C until used. The sterility of extract was verified by plating on solidified medium as a parallel sample in experiments. Trotyl (a source of TNT) was kindly provided by National Armed Forces of the Republic of Latvia. All other chemicals used were reagent or analytical grade.

The M8* medium, mentioned above, without agar, was used for experiments with the A43 in liquid medium. A total of 150 ml sterile polypropylene bottles contained 100 ml of M8* medium with various amendments, according to the experiment scheme. Medium was inoculated with 100 μl of an aerobic liquid culture (initial optical density at 560 nm was 0.01, which corresponded to the cell concentration $\approx 4 \times 10^5$ CFU/ml) of the A43 and incubated at +28 °C, for 7 days, periodically stirred (once in a day). The growth was monitored photometrically by measuring the turbidity at 560 nm (spectrophotometer model Jenway 6300, Barloworld Scientific Ltd, UK).

TNT and metabolites were detected and quantified by HPLC according to EPA method [21]. The standard mixtures of explosives MixA (EPA 8330, SUPELCO Bellefonte, PA), Nitroaromatics/ExplosiveMix1 and Nitroaromate-Nitroamine-Mix4 (Dr.Ehrenstorfer Reference Materials) were used for calibration. The concentration of carbohydrates was determined by HPLC (Agilent 1100 HPLC, Zorbax Carbohydrate column).

Three series of experiments were conducted in duplicate.

Results and discussion

Changes in concentration of 2,4,6-trinitrotoluene and its degradation products

Among the tested samples, the changes of medium content during a 7-day incubation of the A43 were noticeable. Addition of CLE to the medium resulted in a decrease of the TNT up to 90% (Fig. 1, sample No.3). The spectrum of TNT degradation products was relatively narrow, i.e. 1,3-DNT, 2-Am-4,6-DNT and 2,3-DNT. The mechanism of CLE effect on the TNT degradation by some soil bacteria is not clear. It can be presumed that some of CLE compounds are used by bacteria in TNT degradation process. CLE contains nitrogen compounds, in particular, quaternary ammonium (e.g. choline) [19]. In our study, the total nitrogen and carbon in cabbage leaf extracts prepared from the different cabbage cultivars, varied in the ranges of 0.22–1.00 vol% and 0.555–1.251 vol%, respectively [11]. It was reported that the soil bacterial consortium could not use TNT as a nitrogen source but required the addition of ammonium [5]. In turn, other authors consider that bacteria use TNT as a nitrogen source. Thus, an increased microbial growth was found in lake microcosms amended with TNT. However, negligible mineralization of TNT was detected, suggesting that TNT was not utilized as a carbon source, but as a nitrogen source [26]. Besides, enzymes reducing the nitro groups of the aromatic ring in TNT molecule were found for *E. coli*. Further formation of nitrites and hydroxylamino derivatives can result in the release of ammonium ions, which is used as a nitrogen source by *E. coli* for growth [10].

Addition of sucrose to the TNT-containing M8* medium resulted in conversion of a whole amount of TNT into 2-Am-4,6-DNT (Fig. 1, sample No.2). Such a conversion is well known as a typical TNT degradation product of

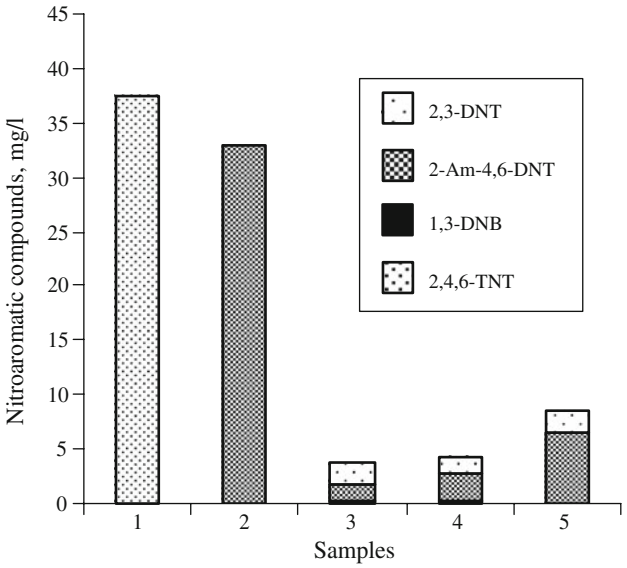

Fig. 1 Concentration of TNT degradation products in M8* liquid medium with different amendments after incubation of the A43 (+28 °C, 7 days). The samples *1–5* contained 40 mgTNT/l and an inoculum in M8* liquid medium; *2* amended with 2% sucrose; *3* amended with 2% CLE; *4* amended with 2% sucrose and 2% CLE; *5* amended with 1% sucrose and 1% CLE

microbial activity and is widely spread in environment. An inhibition of further TNT degradation in the sample with 2% sucrose might be explained by enhanced sucrose concentration. However, addition of both, 2% sucrose and 2% CLE (sample No.4), gave the results influenced the TNT degradation, similarly as in the sample containing the CLE only (sample No. 3). As it is shown on Fig. 1, there is a tendency for the TNT degradation to decrease with a decline of concentration of amendments added to the medium (samples 4 and 5). These results indicated a stimulated role of CLE in TNT degradation by bacterial consortium A43.

Effect of medium composition on the growth of bacteria

According to the "classification" of soil bacteria in terms of their response to the presence of toxic agent, there are few models of the relationship between bacteria and TNT. Thus, at least four types of bacterial behavior can be determined as a response to a toxicant. The first, bacteria are inhibited by toxicant; the second, bacteria resist the presence of toxicant; the third, bacteria resist and degrade toxic molecules without growth; the fourth, bacteria resist, degrade toxic molecules and use the products of degradation for the biomass growth. Obviously, there are many diverse specific nuances in the relationship within the four types of bacterial response to TNT, mentioned above. One of the most important factors influencing the bacterial behavior in the presence of TNT could be the environmental conditions, in particular, medium content.

During the experiments described in this paper, the TNT degradation and active growth of biomass were interrelated. It could indicate to the ability of the A43 to degrade TNT under certain conditions and to use the TNT degradation products for growth. Obviously, CLE plays a stimulating role for bacterial growth under the tested conditions, both, in the presence of TNT and without it (Fig. 2).

The growth-promoting effect of cabbage extract, in particular, *Streptococci*, was reported in 1921 [3]. The cabbage juice medium prepared using Chinese cabbage (*Brassica campestris*) was shown as a substrate for yeast biomass production. Relatively high concentration of reducing sugars is suitable for yeast culture. As for lactic acid bacteria, opinions on CLE effect on these bacteria growth are different. Thus, fresh juice of Cecile cultivar cabbage (*Brassica oleracea*) was inhibitory to the growth of lactic acid bacteria [8]. Most probably, the effect of CLE on the microbial growth is microorganism and cabbage cultivar-dependent. In addition, the medium composition and the protocol for CLE preparing can noticeably influence the above mentioned effect.

Regarding the effect of sucrose alone, as an amendment, it was not effective in terms of biomass growth (Fig. 2, sample No.2). The results of experiments with liquid M8*

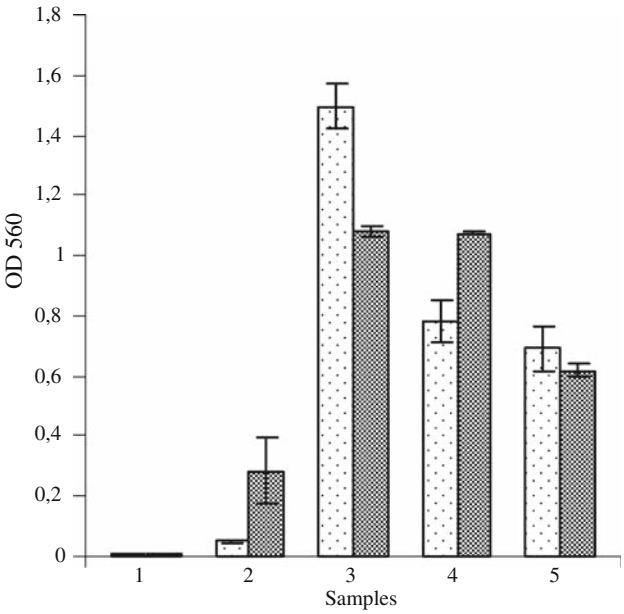

Fig. 2 Growth of A43 in the liquid M8* medium amended with different compounds (7 days, +28 °C). *Light bars* without TNT; *dark bars* with TNT. The samples *1–5* contained 40 mgTNT/l and an inoculum in M8* liquid medium; *2* amended with 2% sucrose; *3* amended with 2% CLE; *4* amended with 2% sucrose and 2% CLE; *5* amended with 1% sucrose and 1% CLE

medium were in a good agreement with those obtained on solidified M8* medium (results not shown). Regarding *B.cepacia*, it is known that some of strains are declared to use sucrose for their metabolism; other strains do not use it [22, 23]. Obviously, under certain conditions of the experiments described in this paper, sucrose was used by the growing culture of the consortium containing *B.cepacia*, only in the presence of other amendments added (Figs. 2, 3, samples No.4 and 5). The role of other bacteria in consortium, i.e. *Pseudomonas* spp. remained unclear.

Changes of the sugar concentration in medium during the bacterial growth

The measurement of concentration of sucrose, glucose and fructose in the samples before and after incubation provided additional information regarding the balance of sugars in tested samples.

As far as CLE contains both, polysaccharides, di- and monosaccharides, and bacteria can change the ratio of these compounds due to the enzymatic activity, the concentration of sugars available for bacterial consumption, can be permanently changed during incubation [18]. Some increase in the concentration of reducing sugars in medium can be caused by enzymatic activity mentioned above (Fig. 3, samples No.3 and 5). In the sample containing M8*, CLE and TNT, without inoculum, the ratio of sucrose, glucose and fructose was found to be 0.03:0.13:0.11, respectively.

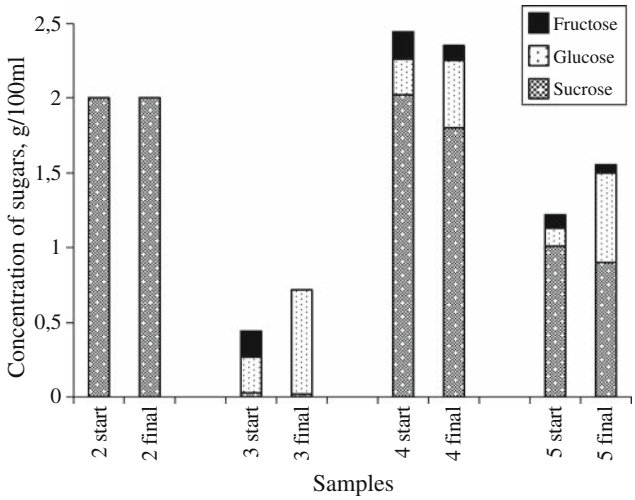

Fig. 3 Concentration of carbohydrates in the liquid M8* medium amended with different compounds after 7-day-incubation of A43 (+28 °C). The samples *1–5* contained 40 mgTNT/l and an inoculum in M8* liquid medium; *2* amended with 2% sucrose; *3* amended with 2% CLE; *4* amended with 2% sucrose and 2% CLE; *5* amended with 1% sucrose and 1% CLE

These data are in a good agreement with the data found in literature. According to Wenneberg et al. [25], the content of the soluble fraction of carbohydrates in blanched cabbages, and the concentration of sucrose, glucose and fructose was in the ratio 4:17:12 and 3:16:13 for different cultivars of white cabbage.

The microbial activity during incubation resulted in noticeable changes in the concentration of sugars originated from CLE. Thus, the sample No.3 after 7 days incubation contained sucrose, glucose and fructose in the ratio 0.02:0.7:0. Theoretically, glucose and fructose initially have to be in equimolar concentrations, taking into account (1) an initial sugar balance in the soluble fraction of CLE; (2) the result of sucrose hydrolysis. An enhanced concentration of glucose in the sample No.3 after incubation can indicate to the use of fructose by growing cells. An addition of sucrose to the samples No.4 and 5 also resulted in the enhanced amount of glucose after incubation (Fig. 3). The role of fructose as a carbon source for the use of TNT by *Pseudomonas* hybrid strain was reported by Duque and co-authors [6, 24]. This reference is in a good agreement with our hypothesis, which has to be verified in future experiments.

In most cases described to date, aerobic bacteria tend to transform the TNT molecule by reducing one or two nitro groups to hydroxylamino or amino groups and to produce different isomers of aminonitroaromatic compounds, which in turn usually accumulate in the culture medium without further metabolism. Only few aerobes able to use TNT as a nitrogen or carbon source have been reported, and mineralization of this compound has been described even less frequently. Elimination of the nitro group is required to decrease the electrophilic nature of the molecule and allow dioxygenases to use di- or mononitroaromatic compounds as suitable substrates [7]. This fact led researchers to isolate microorganisms able to use TNT as the sole nitrogen source in mineral medium supplemented with additional carbon sources [6]. The role of sucrose, glucose, starch, or molasses as an additional co-substrate for the metabolism of TNT is shown for anoxic processes and supposed to prove an oxygen removal by growing aerobes, and electron donation for nitro group reduction of TNT [7].

In our experiments, addition of cabbage leaf extract resulted in enhanced TNT degradation by bacteria consortium A43. Complex, partly not-reproducible (among different cultivars and harvests) composition of this amendment makes this study rather difficult.

Application of different organic amendments, e.g. compost, manure, pulp sludge, molasses, etc. for soil bioremediation has become a common practice worldwide. All of them are highly variable by bio-chemical composition. Moreover, development of microbial diversity in contaminated soil in the presence of organic amendment under real conditions can be unpredictable. Experiments on real scale should be supported by data obtained in model experiments under laboratory conditions. In future, it is supposed to investigate the promoting effect of cabbage leaf extract to the soil bacteria with explosives-degrading activity more detailed to use it in soil remediation technologies.

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