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ORIGINAL PAPER

Application of FT-IR spectroscopy for control of the medium composition during the biodegradation of nitro aromatic compounds

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Abstract Previous studies showed that cabbage leaf extract (CLE) added to the growth medium can noticeably promote the degradation of nitro aromatic compounds by specific consortium of bacteria upon their growth. For further development of the approach for contaminated soil remediation it was necessary to evaluate the qualitative and/or quantitative composition of different origin CLE and their relevance on the growth of explosives-degrading bacteria. Six CLE (different by species, cultivars and harvesting time) were tested and used as additives to the growth medium. It was shown that nitro aromatic compounds can be identified in the FT-IR absorption spectra by the characteristic band at 1,527 cm^{-1}, and in CLE by the characteristic band at 1,602 cm^{-1}. The intensity of the CLE band at 1,602 cm^{-1} correlated with the concentration of total nitrogen ($R^2 = 0.87$) and decreased upon the growth of bacteria. The content of nitrogen in CLE differed (0.22–1.00 vol.%) and significantly influenced the content of total carbohydrates (9.50–16.00% DW) and lipids [3.90–9.90% dry weight (DW)] accumulated in bacterial cells while the content of proteins was similar in all samples. Though this study showed quantitative differences in the composition of the studied CLE and the response of bacterial cells to the composition of the growth media, and proved the potential of this additive for remediation of contaminated soil. It was shown that analysis of CLE and monitoring of the conversion of nitro aromatic compounds can be investigated by FT-IR spectroscopy as well as by conventional chemical methods.

Keywords FT-IR spectroscopy · Cabbage · Soil bacteria · Nitro aromatics

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Introduction

Bioremediation is one of technologies used for the reduction of nitro aromatic explosive contamination [14]. Promoting the development/expansion of naturally occurring TNT degrading bacterial consortia is of essential importance and thus application of different organic amendments has become a common practice. Compost, manure, pulp sludge, etc., are used for this purpose [3]. Our previous study showed the stimulating effect of white cabbage leaf extract on the growth and degradation activity of soil bacteria in the presence of nitro aromatic compounds [10]. Considering the highly variable bio-chemical composition of organic amendments including plant extracts [9], it is necessary to evaluate their content and influence on the metabolism of bacteria as the macromolecular composition of microbial cells and fermentation broth is an integrated and quite changeable qualitative and quantitative indicator of the organism's physiological state, reflecting the influence of cultivation conditions on the cell regulatory mechanisms. FT-IR spectroscopy is an established time-saving method to characterize and analyze microorganisms and monitor biotechnological processes by changes in the content of biochemical components [6, 12, 13]. A considerable number of typical narrow absorption bands in the IR-spectra of organic substances permit not only to identify pure compounds, but also to perform a quantitative analysis of complex multi-component mixtures, such as microbial biomass

[2, 4, 5]. The analysis is carried out without separation into individual components and is frequently possible to determine not only the concentrations of the ''base'' substance, but also the quantities of admixtures.

The aim of present study was to evaluate the qualitative and/or quantitative composition of CLE extracts of different origin and their relevance on the growth and metabolism of explosives-degrading bacteria by means of FT-IR spectroscopy.

Materials and methods

Microorganisms and growth conditions

The consortium of Gram-negative bacteria further called A43 was originally isolated from the explosive-contaminated soil on the basis of its ability to grow in media with TNT and other nitro aromatic compounds as the sole nitrogen source. Microorganisms were identified as *Burkholderia cepacia* and *Pseudomonas* spp., using API® (BioMérieux). CLE were prepared from six different cabbage cultivars identified in Table 1.

CLE was prepared as follows: 500 g of leaves was washed with tap water, boiled at 100 °C for 30 min, cooled, liquid fraction filtered and steamed for 15 min. The extract was stored at 4 °C and the sterility was verified by plating on solidified medium as a parallel sample.

M8* liquid medium contained: Na_2HPO_4 60 g l^{-1}, KH_2PO_4 30 g l^{-1}, NaCl 5 g l^{-1}, and CLE 60 g l^{-1} (pH 6.9). TNT was added to the M8* medium before inoculation if required, according to the scheme of the experiment. Sterile liquid medium was inoculated with 100 µl of an aerobic liquid culture (initial optical density at 590 nm) of the A43 and incubated at +28 °C, for 6 days and stirred once a day. The culture growth was monitored photo metrically by measuring the turbidity at 590 nm (Jenway 6300, Barloworld Scientific Ltd., UK). Calibration of the optical density and cell concentration revealed linear according to an equation $y = 158.5x + 1.0305$. $OD_{590} = 0.233$ corresponded to the cell concentration $\approx 39 \times 10^7$ CFU ml^{-1}.

Three series of experiments were conducted in duplicate.

Table 1 Cabbage cultivars used for preparation of CLE

CLE sample number	Cabbage cultivars and harvest
1	White cabbage *Brassica oleracea* harvested in early 2007
2	White cabbage *Brassica oleracea* harvested in late 2006
3	White cabbage *Brassica oleracea* harvested in late 2006
4	Savoy cabbage *Brassica oleracea*
5	Chinese cabbage *Brassica rapa*
6	Red cabbage *Brassica oleracea*

Analytical methods

The concentration of reducing sugars was determined by HPLC (Agilent 1100) with Zorbax carbohydrate column (p.n.840300-908) at +30 °C using acetonitrile**:**water (70:30 with flow rate 1.2 ml min^{-1}) as the mobile phase, 10 µl sample volumes and using refractive index detector. Data were processed by Agilent Chemstation software. The concentration of carbon was measured by C analyzer ELTRA and total nitrogen was measured by Kjeldal method.

FT-IR absorption spectra were registered on a HTS-XT micro plate reader (BRUKER). Samples of CLE (5–30 µl) or bacterial biomass (10–20 µl) were dried on a 96 place silicon plate at 50 °C, spectra was collected over the wavelength range of 4000–600 cm^{-1} region (32 scans, resolution 6 cm^{-1}). Software OPUS 6.0 (BRUKER) was used for data processing, which was baseline corrected by rubber band method with CO_2 bands excluded. For semi-quantitative analysis integration mode "K", the vertical to the oblique line between the closest minimums, was used.

Quantitative analysis of bacterial biomass was performed using the determined molar extinction coefficients, and applying a system of Vierordt equations to evaluate the concentrations of the principal cell components [5]. The characteristic absorption bands used were as follows: 1,082 cm^{-1} for carbohydrates; 1,241 cm^{-1} for nucleic acids; 1,545 cm^{-1} for proteins; and 2,936 cm^{-1} for lipids.

Chemicals used were of reagent or analytical grade. Trotyl (a source of TNT) was provided by the National Armed Forces of the Republic of Latvia.

Results and discussion

FT-IR spectra of liquid medium with various amendments

Previously we showed that addition of CLE to the growth medium can noticeably increase the degradation of nitro aromatic compounds during the growth of microbial biomass [7]. In order to follow the possible conversion of TNT and/or CLE by bacteria, it is necessary to record the spectra of the initial growth media and select the specific/characteristic absorption bands indicating the exact components. The spectra of growth media M8* with various amendments are shown in Fig. 1, lines 4 and 5. In the spectra of medium M8*, the main absorption is in the region 1,000–1,200 cm^{-1}. In the spectra of CLE, a broad band with maximum at 1,602 cm^{-1} can be selected as a characteristic because it is not overlapped by the main bio-sample absorp-

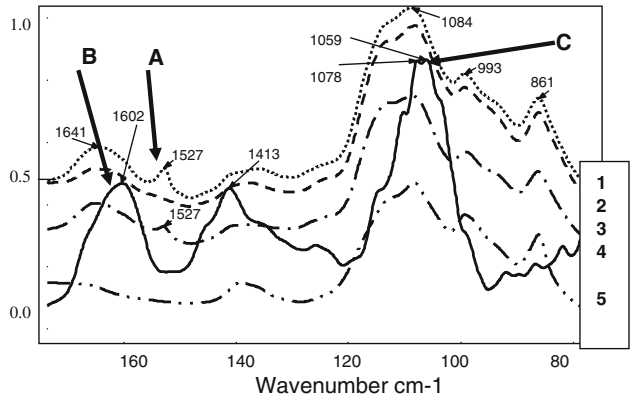

Fig. 1 The FT-IR absorption spectra of liquid M8* medium with amendments after cultivation of the consortium A43 (*lines 1–3*), CLE (*line 4*) and M8* medium without amendments before cultivation (*line 5*). At +28 °C for 6 days: *1* M8* amended with 6% CLE + 40 mgTNT (*arrow A* indicates to the specific band detected in samples containing nitroaromatic compounds); *2* M8* amended with 6% CLE; *3* M8* amended with 6% CLE + 15 mg l^{-1} TNT; *4* M8* amended with 6% CLE (*arrow B* indicates to the specific band for CLE, *arrow C* indicates the carbohydrates); *5* M8* medium

tion bands like Amid I and II (1,654 and 1,545 cm^{-1}, respectively.) The spectra of culture liquid after the growth of A43 did not show the characteristic cabbage band (Fig. 1, lines 1–3) and this can indicate the conversion of CLE by bacteria. This assumption is investigated in the following series of experiments described below.

Our previous results on FT-IR spectra of a nitro aromatic compound mixture water solution revealed two intensive bands with maximums at 1,535 and 1351 cm^{-1} (results not shown). In this study, the TNT additive in the liquid M8* medium showed separate, sharp absorption band at 1,527 cm^{-1}. Two different series of experiments revealed a band of nitro aromatic compounds in 1527–1535 cm^{-1} regions and thus can be used as a characteristic for further identification of these components in water (Fig. 1, lines 1, and 3).

Chemical composition of different CLE

As the composition of medium, mostly the content of carbon and nitrogen, influences the growth and metabolism of bacteria, it was necessary to evaluate the possible variations of the chemical composition of different CLE. The content of carbon, nitrogen and main carbohydrates was determined by conventional chemical methods and the results are shown in Table 2.

The data showed that the content of the main C and N sources can vary quite significantly not only among different cabbage species but also among cultivars and even harvests. Savoy origin CLE significantly differed by high content of nitrogen and N/C ratio, 0.843, among the studied samples. The lowest N concentration and N/C ratio, 0.18, was in CLE prepared from the red cabbage leaves. The content of carbon did not vary as much as the content of nitrogen and thus seems that further CLE discrimination could be based on the differences in the nitrogen concentration. Regarding the content of carbohydrates, the higher concentration of reducing sugars showed samples 2, 3, and 6, i.e., white and red cabbage harvested in 2006.

FT-IR spectroscopy was used as an alternative quick method for discrimination of CLE samples. The advantages of this method are easy sample preparation, just dried on a silicon plate (no pre-treatment, extractions or even grinding), and small sample amount (≤30 µl) and in few minutes the spectra gives information about the composition of the sample under study. FT-IR spectra of CLE are shown in Fig. 2. All spectra showed four main absorbance bands, 2,934 cm^{-1} (CH_2, CH stretching); 1,602 cm^{-1} (NH_2, NH_3, N–NO_2, C=C); 1,410 cm^{-1} (C–N amines); and 1,077 cm^{-1} (COC, C=O, P=O, PO_2) [1, 11], thus indicating the similarity of the chemical composition, i.e., qualitative identity. Evaluation of the spectra showed that the ratio among the bands at 1,604 cm^{-1}, 1,410 cm^{-1} and 1,077 cm^{-1} is different and, therefore, CLE can be discriminated by the quantity of the main biochemical components. As the absorbance of the spectra used for evaluation did not exceed 80%, the Lambert–Buger–Beer Law applies, the concentration of a substance and its absorbance of radiation at a certain frequency are directly proportional. Thus a semi-quantitative method based on integration of the characteristic cabbage band was used and the results are shown in Fig. 3. The linear regression analysis was used to verify the correlation between the integral of the CLE band at

Table 2 The content of carbon, nitrogen and reducing sugars in different CLE

CLE sample[a]	Carbon (vol.%)	Total nitrogen (vol.%)	N/C	Sucrose (g l00 ml^{-1})	Glucose (g l00 ml^{-1})	Fructose (g l00 ml^{-1})	Total reducing sugars (g l00 ml^{-1})
1	0.555	0.38	0.68	0.57	7.08	6.03	13.68
2	1.186	0.29	0.24	1.33	13.41	10.24	24.98
3	1.221	0.57	0.47	1.04	11.01	8.74	20.79
4	1.186	1.00	0.84	1.38	6.06	4.97	12.41
5	0.823	0.38	0.46	0.64	6.38	5.51	12.53
6	1.251	0.22	0.18	2.09	10.79	8.30	21.18

[a] Description of CLE samples (Table 1)

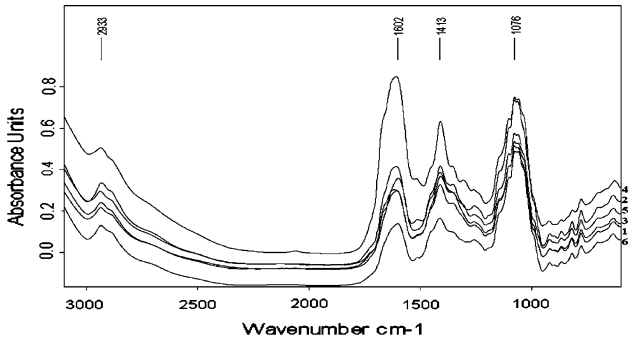

Fig. 2 FT-IR spectra of different CLE extracts (Table 1, description 1–6)

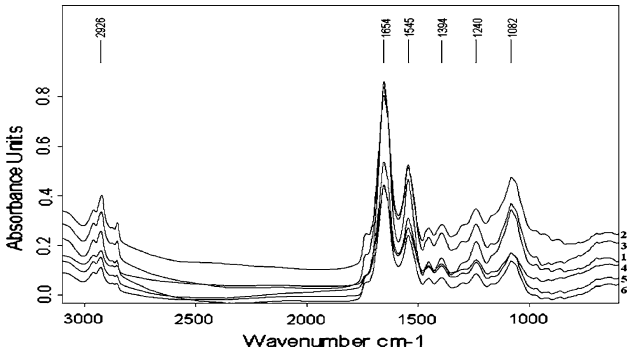

Fig. 4 FT-IR spectra of the A43 biomass grown in medium with different CLE additives (6 days, +28 °C), (Table 1, description 1–6)

1602 cm^{-1} and the content of total nitrogen for different CLE. The correlation was found to be $R^2 = 0.8682$ and is considered as significant. These results show that for discrimination of CLE on the basis of nitrogen content by both methods, conventional chemical and FT-IR, correlate and thereby proved that band at 1,602 cm^{-1} can be used as characteristic for CLE.

Growth of bacterial consortium and analysis of biomass

The bacterial consortium A43 was grown in liquid M8* medium amended with six different CLE to investigate the influence of variable qualitative and quantitative composition on the bacterial growth and the conversion of nutrients. The dynamics of bacterial growth (Fig. 3a) was strongly influenced by the CLE origin thus demonstrating the dependence on the CLE composition. In turn, the decrease of the integral of 1,602 cm^{-1} band during growth of the A43 was revealed for all tested CLE. Thus, as the intensity of the characteristic CLE peak at 1,602 cm^{-1} decreased during the growth of bacteria, it was shown that the bacterial consortium A43 converts CLE.

FT-IR spectra of biomass grown in M8* liquid medium with different CLE amendments were recorded to evaluate the influence of CLE composition on the metabolism of the A43. The spectra (Fig. 4) are typical for bio-sample spectra with the main absorption bands of carbohydrates at 900–1,200 cm^{-1} (C–O, C–O–C), proteins, Amid I and Amid II at 1,654 cm^{-1} (C=O) and 1,545 cm^{-1} (N–H), respectively, lipids at 2,850 and 2926 cm^{-1} (CH), and nucleic acids at 1,240 cm^{-1} (P=O, PO_2) [8, 11].

The content of the main biochemical components in microbial biomass reflects the influence of growth conditions on cell regulatory mechanisms, thus the concentration of carbohydrates, nucleic acids, proteins and lipids in microbial biomass after growth in different CLE amended medium was calculated and results are shown in Fig. 5. Quantitative FT-IR analysis of bacterial biomass showed less significant differences in the content of proteins (62.5–55.6% DW) while the content of lipids varied from 9.9 to 3.9% DW and carbohydrates from 16% DW (CLE 1) to 9.5% DW (CLE 4).

Conclusions

This study showed that nitro aromatic compounds in liquid M8* medium can be identified by the peak at 1,527 cm^{-1} in

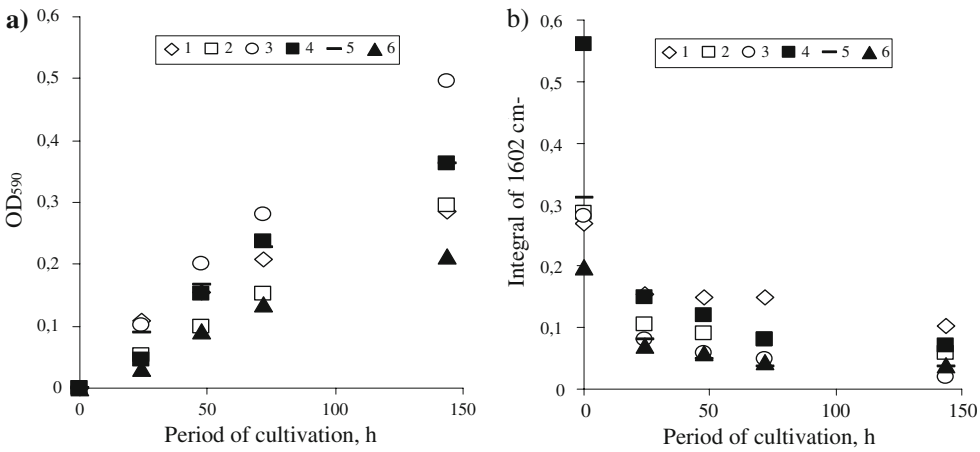

Fig. 3 Growth of the consortium A43 in liquid M8* medium amended with CLE (**a**), and the changes of the integral of 1,602 cm^{-1} band during the growth for 6 days, at +28 °C (**b**) (Table 1, 1–6)

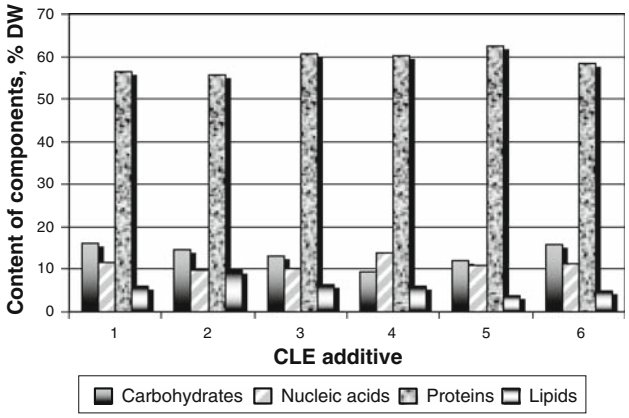

Fig. 5 Content of the main biochemical components of the A43 biomass grown in liquid M8* medium amended with different CLE (Table 1, 1–6)

the FT-IR absorption spectra and it can be used for monitoring of the degradation of nitro aromatic compounds while for characterization and discrimination of various CLEs can be used the band at 1,602 cm^{-1}. Evaluation of different origin CLEs showed variations in the content of nitrogen, carbon, glucose, fructose and sucrose and can be determined by conventional chemical methods as well as by FT-IR spectroscopy. The results of quantitative FT-IR analysis demonstrated the response of bacterial cells to the variations in the biochemical composition of growth medium.

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