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Capillary electrophoretic monitoring of microbial growth: determination of organic acids

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Abstract. Capillary electrophoresis (CE) with indirect UV detection was used for determination of organic acids produced by microorganisms in a liquid medium. The microbial culture broth has a complex composition including organic metabolites and a large amount of mineral salts. Analysis of such samples is troublesome. However, in the benzoate background electrolyte with addition of cetyltrimethylammonium bromide (CTAB), organic acids were successfully separated without a significant peak distortion due to the matrix effect. This electrolyte system was applied for a study of solubilization of mineral phosphates by rhizosphere fungi. It was demonstrated also that a background electrolyte containing pyridine-2,6-dicarboxylic acid as a complexing reagent and CTAB allows simultaneous determination of organic acids and metal cations in one electrophoretic run. This electrolyte composition provides a basis for more sophisticated application of CE: monitoring the consumption of heavy metals by bacterial culture.

Key words: metabolism, microorganisms, fungi, bacteria, heavy metals, organic acids, capillary electrophoresis, simultaneous separation.

INTRODUCTION

Organic acids are involved in many metabolic processes of cells. They are important metabolites of several biochemical pathways in microorganisms. As they are frequent main or by-products in different bioprocesses, a demand for monitoring organic acids often exists.

Microbiological systems where several compounds of different chemical nature appear or disappear in the medium during the bioprocess are of special interest. Solubilization of mineral phosphates by soil fungi and adsorption of heavy metals by bacterial culture represent typical examples of such processes.

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The ability of the rhizosphere microorganisms to dissolve calcium phosphate and apatites in a pure culture and in association with plant roots was attributed to organic acids and chelating metabolites accompanied by the reduction of media pH. However, the identification of the metabolites involved has been done in only a minority of the reported investigations [1–3]. In many works concerning investigations on phosphate solubilization in soil or liquid medium chemical methods in combination with colorimetry are generally used for the determination of phosphate [4] while thin-layer chromatography and ion chromatography are the main methods for analysis of organic acids. However, it is of high practical importance to follow and analyse all the changes in the concentrations of ions by one method and, desirably, by one run.

In recent years there has been an increasing interest in processes involving the interactions of heavy metals with microorganisms through the bacterial transformation and conversion of metallic ions by reduction in different polluted environments [5, 6]. As the bacteria-mediated consumption of heavy metals occurs most obviously through the cells' metabolic pathways, the need for monitoring not only cations of metals but also inorganic anions and organic acids is evident. By separating cations and anions simultaneously, it is possible to collect more information about the samples with less work and analysis time than by the traditional CE protocol.

Traditionally, in microbiology and biotechnology the determination of organic acids is performed by means of chromatographic methods including gas chromatography, ion chromatography, and high-performance liquid chromatography (HPLC). However, such methods often require special preparation of samples: derivatization, complexation, and other chemical transformation of analytes before the analysis. That is time- and labour-consuming and may cause significant losses of the analyte during these numerous operations. The methods of classical chemical and enzymatic analysis are in wide use and appropriate commercial kits for the determination of the exact compounds are available. However, usually these methods result in the determination of only one analyte or a whole unseparated group of similar chemical compounds that belong to one class.

In the last two decades capillary electrophoresis (CE) has proven to be a rapid, easy, and reproducible method for the determination of organic and inorganic anions with a minimum of tedious sample preparation. Much attention was paid to the proper choice of buffer system formulations meeting the requirements for the best separation of given analytes [7–12]. Saturated aliphatic organic acids have no chromophores in their structure and they absorb UV light insignificantly. That is why the indirect photometrical method became the most popular for their detection [13] as it determines the presence of an intensively absorbing probe in the composition of a background electrolyte (BGE). For decreasing the time of analysis, the electroosmotic flow (EOF) reversal is usually performed by adding an EOF cationic modifier to the CE buffer.

Numerous publications including detailed review articles on the application of CE to the analysis of organic acids in different real sample matrices can be found

(CE for the determination of short-chain organic acids in natural rubber latex [14], industrial samples [15], beverages [16–19], plants [20], etc.). To our surprise, there are not so many publications on the use of this method for the analysis and monitoring of bioprocesses although the excretion of organic acids is specific and characteristic of the great part of processes occurring in the presence of microorganisms. The following problems in this field have been studied employing CE facilities: the bacterial taxonometry [21] and identification [22], determination of inorganic anions in microbial fermentation processes [23], analysis of organic acids produced by fungi [24–26]. In fact, the CE monitoring of the processes that are caused by the activity of microorganisms, like brewing, fermentation, natural remediation, and waste management processes as well as microbially-mediated adulteration of food and beverages, could be definitely included into this list.

Simultaneous separation and detection of cations and anions in a single run is not easily achieved in CE, because particles with opposite charges tend to migrate in opposite directions under a given electric field. Several ways have been proposed for separating simultaneously cations and organic acids by using CE [27, 28]. One approach developed for this kind of separation was to use a single detector and a larger EOF to force the cations and anions to migrate in the same direction towards the detector. As it is not well understood how large the EOF should be to ensure that both the small cations and anions move towards the detector, this approach is difficult to handle. An original approach for simultaneous CE separation based on electromigration sample introduction from both ends of the capillary and indirect UV detection was developed by Padarauskas et al. [29]. The approach allowed fast simultaneous determination of cations and anions and was especially useful for fast routine analysis of samples with similar composition [30].

Another approach for simultaneous separation of cations and anions is the formation of the anionic complex between the auxiliary ligand and the analyte metal ions by precapillary or on-capillary complexation. When these methods are used, the primary consideration is the kinetics of the complexation equilibrium. Precapillary complexation of cations has been studied by using complexing agents such as ethylenediaminetetraacetic acid (EDTA) [31] and pyridine-2,6-dicarboxylic acid (PDC) [32]. On-capillary complexation requires fast kinetics for complex formation. This method is useful when sample treatment before measurement is not possible, like in on-line monitoring. Studies using PDC [33], 18-crown-6/1,2-dimethylimidazole [34], and EDTA [35] show that the on-capillary method allows the complexation or derivatization of analytes to happen in the capillary.

The aim of this study was to investigate the CE method performance for monitoring the products appearing in the liquid growth medium in the presence of microorganisms. The utility of the method has been evaluated in the following three cases. The first one is the on-line screening of organic acids production by *Lactobacillus plantarum*. The second example is investigation of the phosphate solubilizing (PS) activity of the soil fungi studied *in vitro* (simultaneous

determination of inorganic and organic anions). The third one is the study of the adsorption of heavy metals from the liquid medium by metal-consuming bacteria (simultaneous determination of the cations of divalent metals and the anions of organic acids).

EXPERIMENTAL

Instrumentation

All the measurements were done by using a self-built CE instrument. It consisted of a pneumatic autosampler, a high voltage supply, an UV-detector and a computer for recording the data. As a detector a CV^4 CE absorbance detector (ISCO, Lincoln, NE, USA) was used. Labview® version 4.1 software (National Instruments, Austin, TX, USA) was used to record the electropherograms. A fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with a total length of 80 cm and 120 cm and i.d. of 50 mm was used. A detection window was created at 40 cm for 80 cm capillary and at 90 cm for 120 cm capillary from the capillary inlet. Indirect UV detection was performed at a single wavelength of 235 or 254 nm for organic acid separation and at a single wavelength of 230 nm for simultaneous separation of organic acids and heavy metals. MATLAB® Version 6.0 Release 12 (The MathWorks, Inc., Natick, MA, USA) was used for data handling.

Chemicals

All chemicals were of analytical or reagent grade. Milli-Q water was used throughout the measurements. Inorganic and organic standards were prepared from their salts or free acids. Cetyltrimethylammonium bromide (CTAB), which was used as a modifier, was purchased from Sigma. Pyridine-2,6-dicarboxylic acid used as an electrolyte was purchased from Merck. Tryptose and the yeast extract for liquid media were purchased from International Diagnostics Group plc.

Electrophoretic procedures

Prior each run, a capillary was pre-conditioned with 0.1 M NaOH for 6 min and with an electrolyte for 6 min. For the separation of organic acids, 20 mM benzoic acid buffer with 4 mM CTAB was used as an electrolyte and the pH was adjusted to 5.4 with 0.1 M NaOH. For the analysis of organic acids originating from fungal culture broth, the BGE contained 10 mM benzoic acid and 1 mM CTAB. For simultaneous separation of heavy metals and organic acids 20 mM PDC with 4 mM CTAB was used as an electrolyte and the pH was adjusted to 5.7 with 1 M NaOH. All electrolytes were filtered through a Millipore® 0.45 μm membrane filter. Separation potential of −20 kV was used only for the separation

of organic acids and –24 kV for the simultaneous separation of heavy metals and organic acids. The capillary total length for the separation of organic acids was 80 cm and for the simultaneous separation of heavy metals and organic acids 120 cm.

Sample preparation

Organic acid producing bacteria

Gram-positive homofermentative bacteria *Lactobacillus plantarum*, which produce lactic acid at optimal conditions, were used as organic acid producing bacteria in this study. An inocolum of *L. plantarum* (3 mL) was added into the liquid medium (30 mL) in a small bioreactor. Samples of the solution containing organic acids produced due to bacterial metabolism were collected through a sample department, which was isolated from the reactor department with a membrane (Spectropor®), by a pneumatic autosampler. The liquid medium for bacteria contained (L^{-1}): glucose 1 g, tryptose 5 g, and yeast extract 2.5 g. The samples were analysed on-line with CE every 2 h.

Fungi cultures

Phosphate solubilizing fungi *Penicillium* sp. and *Aspergillus* sp., isolated from the soil of an oil shale ash mound in the Kohtla-Järve region in North-East Estonia, were cultivated in a mineral medium containing 200 mL of Kucey medium [36], 1 g of $Ca_3(PO_4)^2$ and 0.2 g of yeast extract. Kucey medium contained (L^{-1}) 0.1 g of NaCl, 0.4 g of NH_4Cl, 0.78 g of KNO_3, 0.12 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 10 g of glucose. The mineral medium was added to 1 L Erlenmeyer flask and an inoculum of fungi was added straight into the flask. The flasks were shaken at room temperature for 6 days on a rotary shaker at 150 pt min^{-1}. Every day 10 mL aliquots of samples were collected from each flask and centrifuged in polypropylene tubes at 7000 pt min^{-1} for 15 min. The supernatant was kept in sample tubes in a refrigerator before use. Prior the injection into the capillary the sample was filtrated through a 0.45 μm Millipore® filter. The samples were analysed off-line with CE.

Heavy metals consuming bacteria

The bacteria culture isolated from technogenic soil of ash mounds near Kohtla-Järve and Kiviõli and marked with a code A8 was cultivated on a petri dish and transferred to a 500 mL Erlenmeyer flask containing the liquid medium. The composition of the solution was the following (L^{-1}): cations of heavy metals such as copper, zinc, lead, iron, cadmium, and cobalt, 5 mM of each; glucose (1 g); tryptose (5 g); and yeast extract (2.5 g). Flasks were shaken at room temperature for 15 days on a rotary shaker at 100 pt min^{-1}. Samples of 5 mL were collected every day from each flask and kept in a refrigerator. The electropherograms of the samples were measured off-line.

RESULTS AND DISCUSSION

Lactobacillus plantarum

Two organic acids produced by *Lactobacillus plantarum* (Fig. 1) were obtained. Acids were identified as lactic and acetic acids by spiking with standards. The production of organic acids by the bacteria *L. plantarum* turned out to be fast in the beginning, but after the first day of the production of organic acids by bacteria the level of organic acids reached its maximum. This can be due to the limiting growth factors, such as the concentration of nutrients and changes in the pH.

Since most dissociation constants of common organic acids are less than 5, the pH of the benzoate buffer electrolyte was adjusted to 5.20. In this way it was confirmed that organic acids were mainly found in their anionic form and were capable of moving under the given electric field in the capillary. Benzoic acid

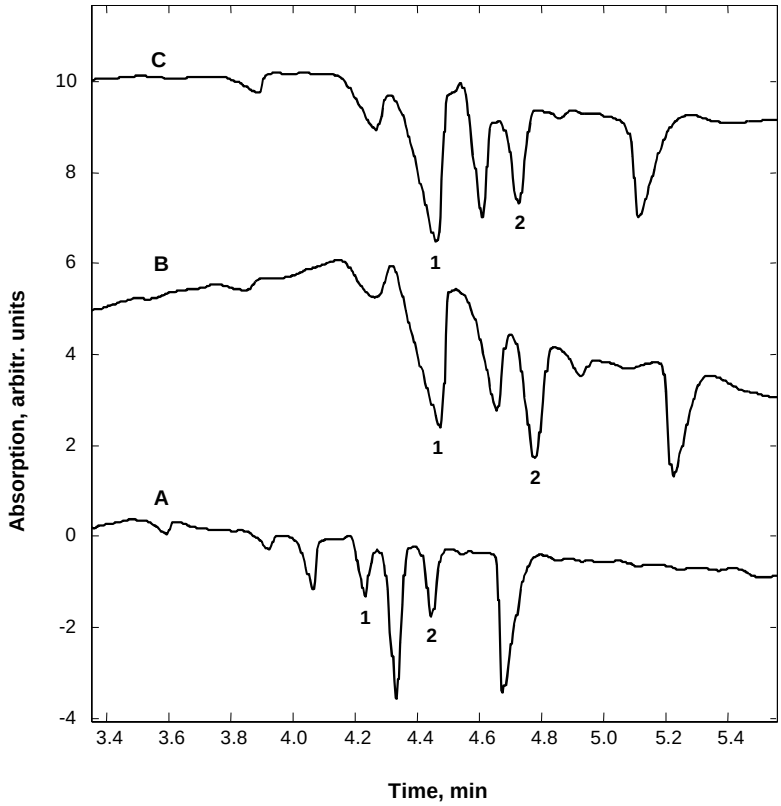

Fig. 1. Electropherogram of on-line monitoring of organic acids produced by bacteria *Lactobacillus plantarum*. Background electrolyte – 20 mM benzoic acid and 4 mM CTAB water solution, pH = 5.20; applied voltage – 17 kV; detection at 235 nm. A – after 2 h; B – after 26 h; C – after 96 h. Acids: 1 – acetic acid, 2 – lactic acid. Other peaks are unknown organic acids, which come from the liquid media.

concentration of 20 mM was used to ensure a sufficient peak separation and CTAB concentration of 4 mM was used to modify the EOF. Due to the nature of the sample, the reproducibility of migration times and peak areas was not as good as with standard samples. Nevertheless, as follows from Fig. 1, the pattern of the peaks of metabolites can be easily recognized even after 26 h and 96 h from the beginning of the process. The method for detecting organic acids was considered to be successful.

Solubilization of mineral phosphates by fungi

The benzoate electrolyte with addition of CTAB as an EOF modifier was chosen for the CE monitoring of phosphate solubilizing activity of some *Penicillium*- and *Aspergillus*-like fungi because of its simplicity and good performance for the separation of the low-mass organic acids.

Special care was taken to check whether the peaks of the anions of interest would overlap or be distorted by the peaks of the inorganic anions – components of the growing medium. Figure 2 shows a pherogram of the growing medium containing mineral substances, yeast extract, and glucose according to the formulation described in the Experimental section. The high salt content in the

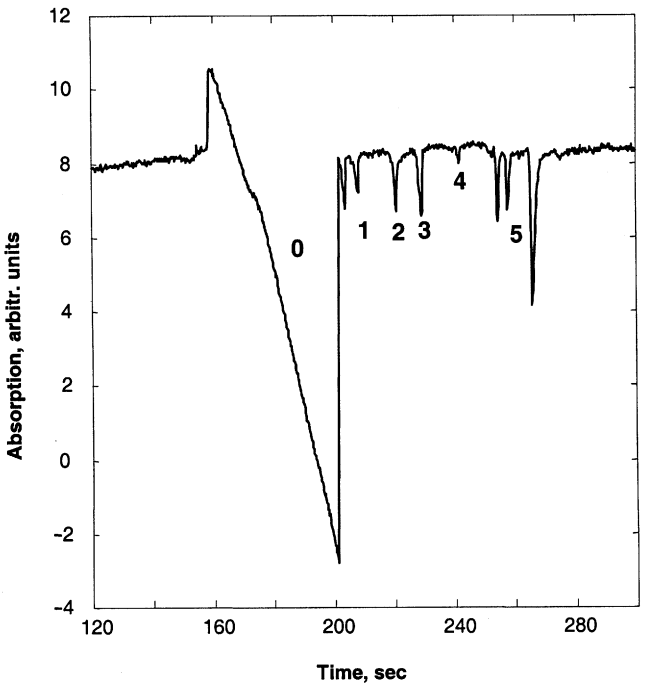

Fig. 2. Identification of organic acids from a growing medium containing yeast extract and mineral salts. Background electrolyte – 10 mM benzoic acid and 1 mM CTAB water solution, pH = 6.4; applied voltage – 20 kV; detection at 235 nm. Peaks: 0 – the bulk of inorganic anions, 1 – succinic, 2 – citric, 3 – acetic, 4 – lactic, 5 – 2-hydroxyisovaleric acid.

liquid medium caused by the initial conditions of the experiment (composition of the nutrients) does not seem to be a very disturbative factor for these measurements. Otherwise, successful separation and analysis of the inorganic anions present in the growing medium in high concentrations could be carried out after the appropriate dilution of the sample and possibly using another CE buffer (such as chromate) whose mobility matches better that of the fast anions. The small size, low molecular masses, and high mobilities of the inorganic anions cause their fast passage through the capillary and they are the first to elute in a large peak at 200 s. From the organic anions, only oxalate may partly overlap with this huge peak under the given experimental conditions. It is clear from Fig. 2 that organic anions of interest migrate according to their charge-to-mass ratio.

The benzoate buffer system allows not only detection of the products of the fungal metabolism such as organic acids formed from the carbohydrates but also checking simultaneously for the appearance of the peak of the dihydrophosphate anion released into the medium due to the PS activity of fungi. This makes the CE method of analysis especially attractive for the study of heterogeneous systems modelling the solubilization of minerals in the presence of acid-eluting microorganisms. Peaks of substances, namely lactic acid, gluconic acid, and dihydrophosphate, appearing in a culture broth after 5 days of incubation of *Penicillium*-like fungi are demonstrated in Fig. 3, pherogram B. Peak identification was done by comparison with the pherogram of the standard mixture of organic acids (line A in Fig. 3) and was confirmed by spiking with some standards. For a better matching with the matrix of the culture broth this standard mixture was prepared in the mineral salts solution – the basis of the growing medium. As the mobility of dihydrophosphate can be expected to be very sensitive to the pH of the BGE, the desirable resolution of the peaks of interest was attained by means of the proper changing of the acidity of the running buffer.

In Fig. 4 the results of the off-line monitoring of the phosphate solubilization by *Aspergillus* sp. fungi are presented. From this set of daily pherograms the appearance and growth of the dihydrophosphate peak as well as the appearance, fast increase, and the complete disappearance on the 6th day of incubation of the gluconate peak can be followed. The separation curves differ from day to day, reflecting changes in the culture broth and giving valuable information for better understanding of the PS process studied. When following the change of the content of organic acids and other substances during the growth of microorganisms it is of high value and importance to qualify the changes in the concentrations of different substances. As the peaks of the main metabolites and dihydrophosphate anion are well separated under the given conditions, the quantitative information on the concentrations of the analytes is available from the measurements of the peak areas and the appropriate calibration graphs.

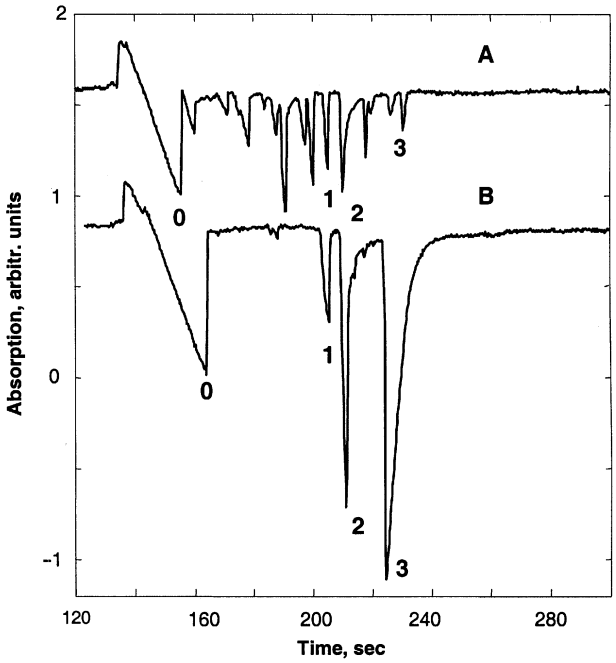

Fig. 3. Identification of organic acids from the *Penicillium*-type fungus F5 culture medium. Background electrolyte – 10 mM benzoic acid and 1 mM CTAB water solution, pH = 5.1; applied voltage – 20 kV; detection at 235 nm. A – standard mixture of organic acids in mineral salt solution, B – *Penicillium*-type fungus growing medium after 5 days of incubation. Peaks: 0 – the bulk of inorganic anions, 1 – lactate, 2 – dihydrophosphate, 3 – gluconate.

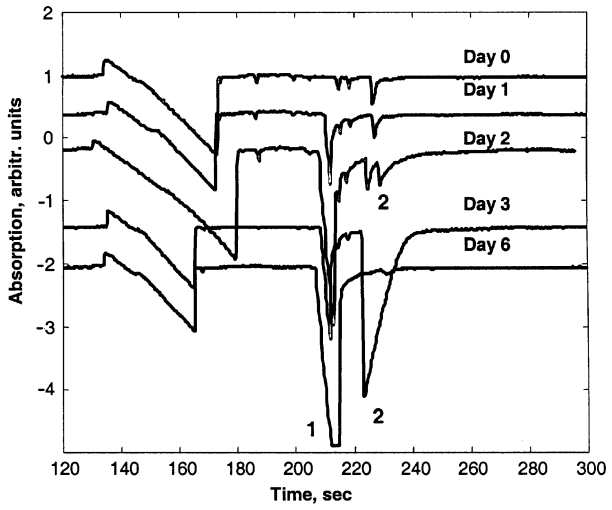

Fig. 4. Metabolic profiles obtained by CE during incubation of *Aspergillus* sp. fungi in the mineral medium containing calcium orthophosphate. Experimental conditions as in Fig. 3. Peaks: 1 – dihydrophosphate, 2 – gluconate.

Consumption of heavy metals by bacteria

Investigation of metal adsorption initiated by the activity of bacteria represents the most interesting case in a CE method development for rapid detection of changes occurring in the ionic composition of the liquid medium, as the decrease in the concentration of metal ions is usually accompanied by the excretion of bacterial metabolites. Hence, a reliable and simple analytical method for determination of these analytes is of high practical importance.

To achieve simultaneous CE separation of organic acids and heavy metals in a form of anionic complexes, a BGE on the basis of pyridine-2,6-dicarboxylic acid was chosen. PDC was found to absorb at the wavelength of 230 nm [37], where organic acids absorbance is low and hence indirect UV detection is possible. Complexes between PDC and heavy metals have a higher absorbance at this wavelength than those of the BGE and can be detected as positive peaks – characteristic of the direct UV detection mode. The pH value of the CE buffer was chosen to be 5.7, since studies by Soga & Ross [33] showed that this is an optimum pH for simultaneous separation of organic acids and heavy metals. The concentration of the electrolyte PDC was chosen to be 20 mM to obtain a better peak resolution for cations, also 4 mM CTAB was added to the separation buffer to modify the EOF. Typical electropherograms are shown in Figs. 5 and 6. In fact, they represent a set of negative as well as positive peaks.

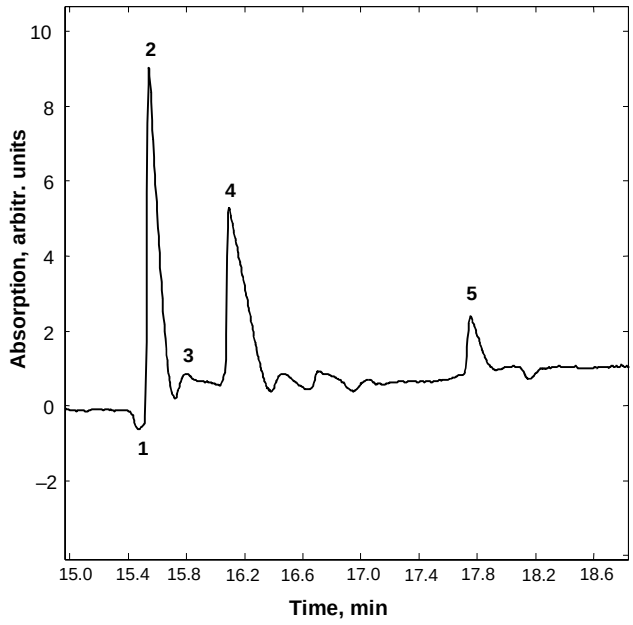

Fig. 5. Electropherogram of simultaneous separation of heavy metals and organic acids. Background electrolyte – 20 mM pyridine-2,6-dicarboxylic acid and 4 mM CTAB water solution, pH = 5.70; applied voltage – 24 kV; detection at 230 nm. Peaks: 1 – unidentified organic acid, 2 – Cu^{2+}, 3 – Pb^{2+}, 4 – Zn^{2+}, 5 – Fe^{3+}.

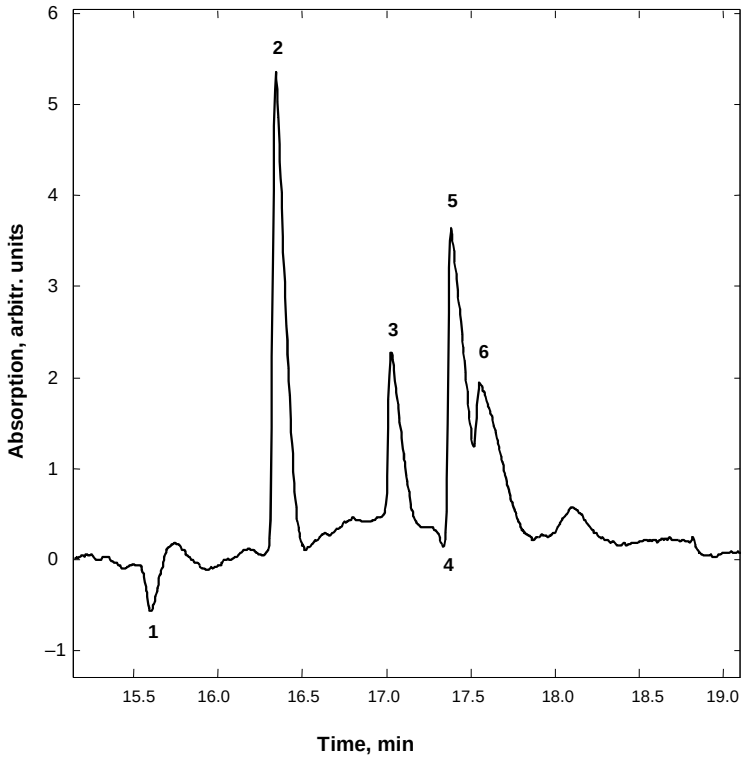

Fig. 6. Electropherogram of simultaneous separation of heavy metals and organic acids. Sample is from reaction with heavy metals consuming bacteria and liquid media. Experimental conditions as in Fig. 5. Peaks: 1 – unidentified organic acid, 2 – Cu^{2+}, 3 – Zn^{2+}, 4 – unidentified organic acid, 5 – Cd^{2+}, 6 – Co^{2+}.

For such a complex mixture as a growing medium with an addition of salts of heavy metals, the separation between all the cations and organic acids was not sufficient and the results of the detection of cations and organic acids were found to be dependent on the sample matrix. It was found that if organic acids were present, the cadmium and cobalt zones would migrate together due to their similar migration times. As the absorption of organic acids is negative in a given BGE, in the case of similar mobilities of analytes distortion of the peaks of metal complexes may occur due to the overlap with peaks of organic acids. This needs special care in tuning the separation conditions for quantitative analysis. The formation of metal complexes with other than the BGE probe organic ligands originating from the growing medium are not excluded either. Detection of lead was unsatisfactory due to the similar absorption of a lead complex with that of PDC. However, separation of cations such as copper, zinc, lead, and iron from a culture broth containing organic acids was found to be satisfactory (see Fig. 5) while the negative peaks of the main metabolites forwarded the set of metal species on a pherogram. By using on-column complexation and EOF reversal the total CE analysis of anions of organic acids and divalent metals was accomplished within 20 min.

CONCLUSIONS

A reliable and simple capillary electrophoresis method for the determination of organic acids from complex liquids of microbial processes was developed. In comparison with the conventional chromatographic techniques, CE has the following advantages: (1) very limited sample preparation, usually not more than filtration and dilution, (2) short analysis time, (3) good separation of organic acids and medium-sized inorganic anions without significant sample matrix interference, and (4) possibility for separation and detection of both metal cations and organic acids in one measurement. The CE method was applied to different case studies from microbiological practice. The protocol for the determination of dihydrophosphate and organic acids in 10 mM benzoate background electrolyte with 1 mM CTAB was readily used for the investigation of phosphate solubilizing actvity of soil fungi. Simultaneous determination of cations of metals and anions of organic acids using the BGE with a complexing agent, pyridine-2,6-dicarboxylic acid, provided new possibilities for monitoring heavy metal consumption from the environment by metal-consuming bacteria. From the results reported here it is apparent that CE is a good alternative to HPLC for the analysis of various ions from complex microbiological liquids. The CE method could be used as a convenient tool for the on-line monitoring of bioprocesses, since preconditioning of samples may be avoided and several analytes of interest could be qualified and quantified in one run.

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Mikroorganismide kasvu kapillaarelektroforeetiline seire: orgaaniliste hapete määramine

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Mikroorganismide elutegevuse tagajärjel vedelas keskkonnas tekkinud orgaaniliste hapete määramiseks kasutati kaudse UV detekteerimisega kapillaarelektroforeesi meetodit. Mikroorganismide kasvukeskkonda ei ole kerge analüüsida, sest see koosneb paljudest komponentidest, sisaldades sealhulgas orgaanilisi metaboliite ja suures koguses mineraalsooli. Analüüsiks kasutati tsetüültrimetüülammooniumbromiidi (CTAB) lisandiga bensoaatpuhvrit, mis võimaldas lahutada orgaanilisi happeid keerulistest segudest ilma piigi kuju muutumiseta keskkonnamaatriksi mõjul. Antud elektrolüütsüsteemi rakendati risosfäärist eraldatud seente toimel tekkiva mineraalse fosfaadi lahustumisprotsessi seireks. Kasutades taustelektrolüüdina kompleksimoodustajat püridiin-2,6-dikarboksüülhapet ja kapillaari pinna modifikaatorina CTAB-i, on võimalik samaaegselt ühe elektroforeetilise mõõtmise jooksul lahutada orgaanilisi happeid ja metallide katioone. Nimetatud lahutusprotokolli kasutati bakterite raskmetallide tarbimise uuringutes.