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Fermentation reactor coupled with capillary electrophoresis for on-line bioprocess monitoring

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Abstract

In this paper, a filter probe integrated into a computerized pneumatic sampler for capillary electroseparations was developed for an on-line monitoring of bioprocesses. The optimization of the performance of the coupled system was done by using a response surface modeling and three-level two-factor design. The resolution was found to be the most important parameter influencing the performance of an on-line analysis of the microbial metabolism. For the on-line analysis the migration time and detection limit were also found to be important parameters. Different parameters were combined by using an overall desirability function to find optimum conditions for all parameters. The equipment with an optimized separation protocol was used to monitor the bioaccumulation of Cu, Zn, Co and Cd (with detection limits 0.46, 0.37, 1.2, 0.84 mM correspondingly) by the *Rhodococcus* sp. bacteria isolated from the highly polluted technogenic soil of northeastern Estonia during a 2-week experiment.

Keywords: Optimization; On-line analysis; Experimental design; Capillary electrophoresis; Bioaccumulation; Heavy metals

1. Introduction

In the recent decade, bioprocess monitoring has been gaining in importance and interest. A detailed monitoring of bioprocesses is necessary in order to optimize the recovery process with regard to both the quantity and quality (e.g. biological activity) of products. In its ambitious form the bioprocess monitoring means effortless access to continuous, real time information about all variables relevant to a given process [1–3]. The on-line monitoring of bioprocesses affords real time information with less effort and with many possibilities of optimizing and controlling the processes. The on-line monitoring of microbial metabolism is necessary in order to obtain more information about factors that influence a microbial behavior.

The monitoring and structural elucidation of both known and unknown compounds in complex samples requires the combination of hyphenated analytical techniques which have not been yet commercially provided at the process analytical market by means of automated analytical systems. Until now the analytical tools used in monitoring bacterial processes have been either spectroscopy [4–6] or monitoring of single chemical compounds (by sensors, e.g. for oxygen or glucose). Spectroscopic methods may lack selectivity and monitoring only a couple of compounds during the process run might not be enough to understanding the process. The full power of contemporary separation methods has rarely been implemented. There are several reports on using high performance liquid chromatography (HPLC) to monitor bacterial processes [7–11], but HPLC is an inherently slow method, might lack efficiency and definitely requires sophisticated sample preparation procedures in the case of bacterial process monitoring. The on-line HPLC monitoring of bioprocesses has been critically discussed in the literature [2–13] as it appears not to be appropriate for process control. Yet it has been established in the biological and medical monitoring [14] and approaches have been made in the development of a selective HPLC based on-line monitoring in wastewaters [15,16] and bioreactors [17–21].

Capillary electrophoresis (CE) is a powerful separation method, due to its high resolution and short analysis time. Due to the low consumption of the running electrolyte and small sample need it has become one of the main separation methods together with HPLC and GC. However, CE offers some advantages as expensive chromatographic columns are unnecessary and method development can be easier and faster than HPLC

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as there is no need to switch and equilibrate new columns. The application of an on-line CE instrument in the determination of inorganic anions and organic acids in water samples taken from pulp and paper machines, as well as in the separation of diuretics and catecholamines were described by Siren et al. [22–24]. Possibilities of the on-line monitoring of bacterial metabolism by using capillary electrophoresis and micellar electrokinetic chromatography have been studied previously, for example, in the consumption of phosphates [25] and phenols [26]. Among many others, an important process from the environmental point of view is the consumption of heavy metals by microorganisms. Heavy metal tolerant microorganisms are used for treatment of polluted sites either by bioremediation or bioaugmentation. Heavy metals like copper, zinc and cobalt are part of the microbial metabolism and monitoring the concentration of these metals may afford an important insight into the mechanism of heavy metals consumption by them. Commonly, organic acids are part of the microbiological metabolism as well and are often produced by different microbes. Therefore, monitoring organic acids and heavy metals simultaneously in real time could give valuable information on the course of the process under study. The simultaneous separation of anions like organic acids and small cations like heavy metals by using capillary electrophoresis has been studied and many different separation methods have been suggested [27–29]. For on-line analysis, disadvantages of these methods are the need for complex formation procedures before analysis or the injection procedure which is performed from both ends of the capillary and therefore requires a complicated injection device.

In our work, a computerized process analytical system has developed based on capillary electrophoresis with UV detection. A microfiltration based sampling probe was combined with the pneumatic sampler developed in our laboratory [30] for a continuous on-line monitoring of a bioaccumulation process of heavy metals. The analytical system was optimised by the use of a test mixture containing several heavy metal ions as well as several organic acids as model metabolites of the process. It was an objective of this work to identify a suitable separation protocol that allows simultaneous separation of anions like organic acids and small cations like heavy metals. Pyridine-2,6-dicarboxylic acid (PDCA) was chosen as a background buffer component It has been successfully used for an on-capillary complexation of heavy metals. In this way it has been possible to simultaneously separate organic acids and heavy metals [31,32]. PDCA has also been used to separate two different oxidation states of chromium [33].

However, to apply this protocol to monitoring heavy metal consumption by bacteria further optimization was required because of the complexity of the sample consisting of many possible interferences. In this work, attempts were made to optimize the separation condition in a more systematic way using a three-level two-parameter experimental design procedure. The experimental design has been successfully applied to optimizing different separation protocols in capillary electrophoresis [34–39]. In this study, the experimental design procedure was improved further by using the desirability function which involves a simultaneous finding of the best resolution between peaks together with maximizing the signal-to-noise ratio of some target organic acid peaks during the shortest possible analysis time, i.e. several parameters simultaneously.

The functionality of the analytical system was demonstrated by monitoring the bioaccumulation of Cu, Zn, Co and Cd ions by bacteria isolated from the highly polluted technogenic soil of northeastern Estonia as well the appearance of organic acid as metabolites during this process.

2. Experimental

2.1. Chemicals

All the reagents were of analytical grade. The salts, $CuSO_4 \cdot 5H_2O$, $ZnSO_4$, $CdSO_4$, $Co(NO_3)_2 \cdot H_2O$ as well as the two organic acids, acetic and citric acid, were from MERCK. The pyridine-2,6-dicarboxylic acid (PDCA) used as a background buffer component was purchased from MERCK. The cetyltrimethylammonium bromide (CTAB), which was used as a modifier in the background buffer, was purchased from SIGMA. Milli-Q water was used to dissolve the salts and prepare the electrolyte.

2.2. The preparation of buffer, standard and culture media

A stock solution with a concentration of PDCA of 25 mM was prepared. For optimizing the separation protocol, nine buffer solutions were prepared with different concentrations of PDCA which ranged from 4 to 20 mM and the pH value ranging from 5.2 to 6.5 (Table 1). CTAB was used to reverse the direction of EOF. The concentration of CTAB was 4 mM and it was constant in each buffer system. The pH of the buffer was adjusted to the desired value by using 1 M NaOH. A 25 mM of PDCA and 20 mM of CTAB were used as stock solutions. The buffer solutions were prepared by diluting the stock solutions with Milli-Q water. The standard sample, containing 5 mM of each heavy metal and organic acid, was prepared by weighting a calculated amount of salts and by diluting them with Milli-Q water.

The gram positive bacterial culture was isolated from the highly polluted technogenic soil of northeastern Estonia and identified as *Rhodococcus* sp. The bacterial strain used, *Rhodococcus* A8 had a high range of substrate specificity degrading resorcinol, 5-methylresorcinol, *p*-butylphenol, *m*-cresol, phenol and sodium benzoate as a carbon and energy source while the highest concentration of phenol degraded was 32 mM [40]. It was cultivated on a Petri dish on Plate Count Agar (PCA) media consisting of tryptose (5 g/l), yeast extract (2.5 g/l), glucose (1 g/l), agar (15 g/l) and incubated for 6 days at 25 °C. For sample preparation the bacterial culture was additionally incubated for 4 days at 25 °C in a liquid PCA media consisting of tryptose (5 g/l), yeast extract (2.5 g/l), glucose (1 g/l). To avoid contamination and for sterilizing, the probe was washed with the ethanol and bioreactor and media were autoclaved at 120 °C 20 min before experiments.

Table 1
Response values measured for an individual experimental design value

pH	[PDCA] (mM)	Acetic acid peak height[a]	Migration time (minimum, Co peak)	Resolution (Cd/Co peaks)	Cu peak height[a]
5.20	4.00	5.6	19.25	0.00	17.3
5.20	12.0	9.1	16.42	0.80	25.3
5.20	20.0	10.8	16.59	1.97	15.8
5.85	4.00	6.6	20.50	0.68	15.3
5.85	12.0	8.5	17.49	0.60	15.3
5.85	20.0	10.2	17.47	0.86	16.8
6.50	4.00	13.5	18.97	0.57	20.9
6.50	12.0	4.3	15.06	0.96	8.2
6.50	20.0	9.1	15.76	1.07	16.4

[a] Arbitrary units.

2.3. Instrumentation

All the measurements were done by using a self-built capillary electrophoretic instrument. It consisted of a pneumatic autosampler, a high voltage supply, a detector and a computer for recording the data. For more details of the sampler operation and instrument see [30].

The reactor consisted of 1 l DURAN® ISO laboratory bottle (SCHOTT UK Ltd) with a bottle-neck attachment (Metrohm AB, Herisau, Switzerland part #6.1602.150 (bottle-neck attachment/GL 45 −3×10/32)). The bottle-neck attachment had three threaded connector openings. One connector was used to pressurize the bottle, the second to fix the support rod and the probe and the third opening was used to connect the probe volume with the pneumatic sampler. The probe itself was manufactured from the plastic cylinder (4.5 cm long, 0.8 cm i.d.) which was suspended into the biomass via the supporting rod so that the open end of it was just above the biomass level. The other end of the probe was closed by a 45 μm membrane filter and inserted into the biomass. The membrane protected from the penetration of the particle material into the probe volume. When the biomass sampling action was activated, the fermentation liquid in the probe flowed rapidly to the pneumatic sampler under the pressure. After the sampling action was completed the pressure inside the reactor forced fermentation liquid to filtrate through the membrane and fill the probe within couple of seconds.

The bioreactor and sampler schematics are presented in Fig. 1. Since the sampler has been described thoroughly in earlier publications [30], only a brief description is given here. The dimensions of the particular autosampler body used in this work are 4.7 cm × 1.6 cm × 1.6 cm and it was made of polyether ether ketone (PEEK). The bioreactor, washing liquid reservoir and the buffer vessel were connected to the sampler body with a PTFE tubing (100 mm long, 0.7 mm i.d.). The work of the sampler is based on the principle of a rapid exchange of the buffer to the sample (and vice versa) in a narrow input channel (25 mm long, 1.5 mm i.d., 44 μl volume) into which the capillary and high-voltage electrodes are inserted. The flow of the liquids is controlled by a personal computer that activates solenoid valves (not shown in Fig. 1) connected to the compressed air tank, thus providing pressure pulses necessary to activate the liquid flow. The channels are opened by PTFE films by liquid pressure and closed by air delivered through the solenoid valves. The pressure applied ranged from 30 to 80 kPa. If P3 is released to the atmosphere, the channel in front of the capillary is rinsed by the sample liquid and sampling occurs since certain amount of the sample penetrates to the capillary. Similarly, if P4 is released to the atmosphere, then the buffer fills the input channel. To analysis run both P3 and P4 are applied. The correct balancing of the liquid/air pressure is important and can be performed easily. It is evident from Fig. 1 that to execute the sampling process the pressures must satisfy the following relationship P1 = P2 < P3 = P4.

The measurement process was completely computerized taking advantage of the design of the sampler used in this work. So, besides the sample introduction rinsing of a capillary with the washing liquid (NaOH) and buffer after each electropherogram run was also performed automatically under computer control by applying pressures to the corresponding vessels. A Labview version 4.1 software (National Instruments, Austin, TX, USA) was used to control the sampling and record the data. A constant separation potential of −24 kV was used. The samples were injected under pressure for 0.1 s. The data were handled by MATLAB® Version 6.0, Release 12 (The MathWorks, Inc., Natick, MA USA).

The samples were separated in a capillary (Polymicro Technologies, Phoenix, AZ USA) with a total length of 125 cm (95 cm to the detector) and i.d. of 50 μm. Prior to each run, a capillary was pre-conditioned with 0.1 M NaOH for 4 min and with an electrolyte for 4 min. As a detector a CV^4 capillary electrophoresis UV detector (ISCO, Lincoln, NE USA) was used. The indirect UV detection was performed at a wavelength of 230 nm for the simultaneous separation of organic acids and heavy metals. At this wavelength organic acids are detected in an indirect mode against the strong absorption of PDCA and heavy metals, in a direct mode.

3. Results and discussion

3.1. Experimental design of the separation protocol

The separation conditions (pH and the PDCA concentration) proposed in Ref. [32] and obtained by an intuitive optimization of experimental parameters were not suitable for our purposes for the separation of an important pair of heavy metals—Co and

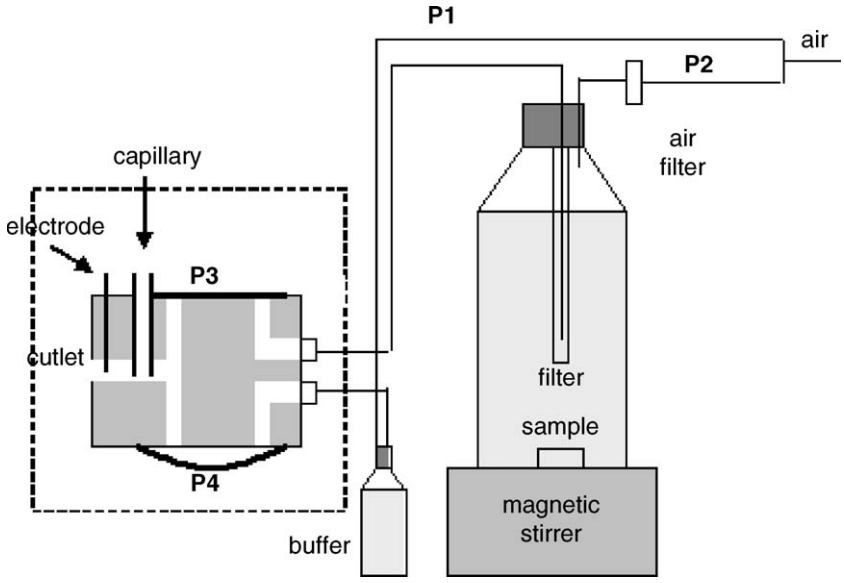

Fig. 1. A closed reactor system connected to capillary electrophoresis for monitoring bacterial processes. The sample is injected through the filter by a pneumatic injection system to the capillary. The pneumatic injection system is not in scale.

Cd. The concentrations of these metals are of great interest in the study of heavy metal consumption by bacteria. In this study, a standard procedure of the 3^2 factor design was used [41]. Of course the set of parameters to be optimized is not limited with pH and buffer composition. Other electrophoretic parameters such as capillary length or voltage could also be optimised but their effect can be easily predicted without using experimental design procedure and were considered to be optimal for the purposes of this work. Two experimental factors, pH and buffer concentration, were varied at three levels. The factors chosen were considered to have the most significant effect on the simultaneous separation of heavy metals and organic acids. The PDCA concentrations chosen were 4, 12 and 20 mM. A 20 mM was chosen as a maximum concentration of PDCA, because this value is near its solubility limit. The pH values chosen were 5.20, 5.85 and 6.50. The lowest value of pH was chosen as 5.20 because of the pK_{a3} value of 5.22 of PDCA [42]. The highest value of pH, 6.50, was chosen because at higher pH organic acids tend to form extra peaks when PDCA is used as a buffer component. The parameter settings ranges in the design are given in Table 1.

Four response factors were utilized to find optimum conditions. The first response factor was the resolution between the peaks of Cd and Co. These peaks were found to be the most difficult to separate. The second response factor was the peak height of acetic acid in order to obtain the best possible detection limits for organic acids. The third response factor selected was the migration time of the last peak to decrease the analysis time. The fourth response factor was the peak height of copper in order to obtain the best possible detection limits for heavy metals. The response values measured for individual experimental design experiments are given in Table 1.

When studying individual parameters and their optimum conditions it can be seen from Table 1, that an electropherogram with all original demands (satisfactory resolution, peak height and migration time) could not be obtained. To find an optimum point for all the responses simultaneously, a study of the desirability function was performed. The desirability approach is a popular method that assigns a given score to a set of responses and chooses the factor settings that maximize the overall score. The overall desirability is a geometric mean of all individual desirabilities' ranging from 0 to 1. The following formula [43] describes how the overall desirability is expressed by individual desirabilities by calculating a geometric mean from individual desirabilities'

$$D = (d_1, d_2 \ldots d_n)^{1/n}$$

where D is the overall desirability, d_i is the normalized desirability function of the ith response and n is the number of responses. To normalize a particular response within 0,. . .,1, the procedure proposed in Ref. [44] was used. By plotting the overall desirability values against two factors, pH and PDCA concentration, it was possible to find optimum conditions for all responses together. A plot of the overall desirability values against pH and PDCA concentration is shown in Fig. 2. From this Figure it was possible to find an optimum point for all four responses together. The highest point with a value of 0.7375 was found where the pH of the buffer is 6.50 and the PDCA concentration of the buffer, 17 mM.

3.2. Validation and transferability of the method

The validation of the separation method was done by using a PDCA buffer with a concentration of 17 mM and pH 6.50. Different persons ran the sample during several days in order to avoid environmental effects. The sample was run seven times and the reproducibility of the migration time and peak area for each peak was calculated. The external standard calibration was performed and the detection limits (LOD) were estimated by peak area standard deviations according to the recommendations of the US Code of Federal Regulations [45]. By this, the LOD is

Table 2
Performance data in optimum conditions

	Acetic acid	Citric acid	Cu	Zn	Cd	Co
Migration time (interday)[a]	1.67	1.63	1.09	1.39	1.01	0.99
Migration time (intraday)[a]	1.64	1.65	1.73	1.95	1.82	2.08
Peak area (interday)[a]	2.97	3.73	3.53	6.60	6.21	8.09
Peak area (intraday)[a]	2.99	3.75	3.69	7.29	7.19	11.11
b_1[b]	278063	298982	847378	198678	670252	180046
b_0[b]	443563	218162	853840	109611	−116498	−107690
CC[c]	0.985	0.872	0.987	0.956	0.933	0.900
Detection limit (mM)	0.21	0.19	0.46	0.37	1.2	0.84

[a] Percentage of relative standard deviation.
[b] Parameter of calibration line: peak area = $b_0 + b_1 \times$ concentration.
[c] CC, calibration correlation coefficient.

calculated as follows: LOD = $st_{n-1,0.99}$ where s is the standard deviation of the seven replicate measurements and $t_{n-1,0.99}$ is a t-distribution taken at a confidence level of 0.99 and degrees of freedom $n - 1 = 6$. The results are presented in Table 2.

To test the performance of the probe and sampler the method was applied to an on-line analysis of the real bioprocess. The process consisted in the consumption of heavy metals by the bacteria grown on Plate Count Agar liquid culture media, which consisted of tryptose, glucose, yeast extract and heavy metals (Cu, Zn, Cd and Co) at pH 5.0 and a temperature of 25 °C. For comparison, monitoring of the liquid culture without bacteria was also performed. The performance of the probe during the experiment was excellent. Neither clogging of the probe nor its malfunction in any other form was noticed during the monitoring run.

In Fig. 3 the electropherograms of the sample from the real process before and after optimization are presented together with an electropherogram of the standard sample in optimum conditions. Peaks were identified by spiking of standards. As one can see the resolution between Cd and Co peaks has definitely been improved after optimization. The electropherogram runtime appears to be relatively long and as such the speed of the CE over HPLC is not particularly pronounced in this case. On the other hand, in this work several parameters were optimized simultaneously with setting tolerable limits for the extreme values of the parameters. For the present process the electropherogram runtime was adequate. When a shorter analysis time is important, CE is more flexible than HPLC and the present protocol can be re-optimized. Shortening of the analysis time 2–3 times (compared to 17 min used in this work) can be easily achieved in common CE.

Fig. 4 shows how the concentration of different metals (copper, cadmium and cobalt) and organic acids change during the first 100 h compared to the background. Obviously, a certain amount of all the metals is irreversibly adsorbed by the media since the average amount of detected metals that was initially introduced into the reaction mixture is less than 5 mM. The reason for the rise of the graph for Cu and Co at the beginning of the experiment is not clear at the moment. Probably this is not related to the bacterial processes since it happens also in the

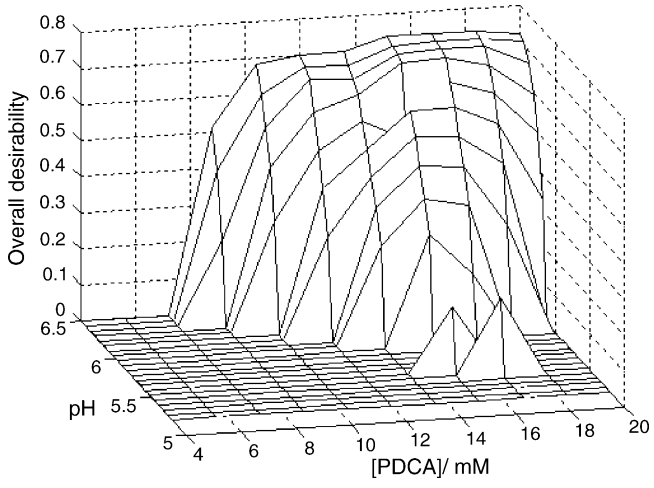

Fig. 2. Overall desirability values as a function of pH and the PDCA concentration.

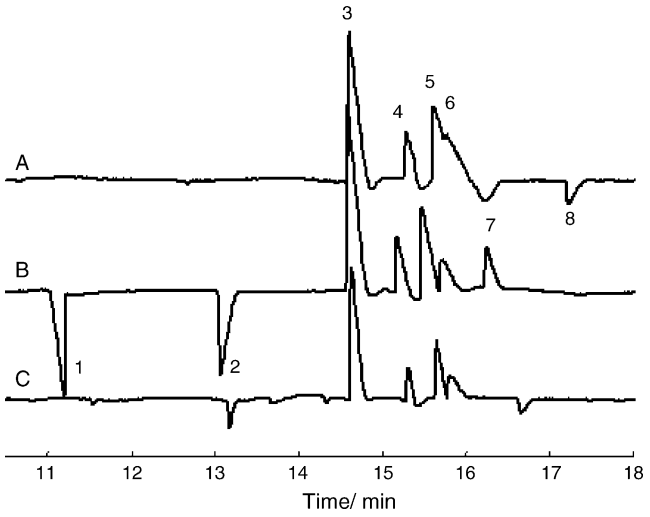

Fig. 3. (A) An electropherogram of on-line analysis using the real sample, separation not optimized; (B) an electropherogram of the standard sample using optimum conditions; (C) an electropherogram of on-line analysis using the real sample with optimum conditions. Peaks: 1, acetic acid; 2, citric acid; 3, copper; 4, zinc; 5, cadmium; 6, cobalt; 7, calcium (from organic acid salt); 8, unknown peak from culture media. Conditions for electropherogram A: buffer 20 mM PDCA and 4 mM CTAB at pH 5.70, −24 kV of applied voltage and UV detection at 230 nm. Conditions for electropherograms B and C: buffer 17 mM PDCA and 4 mM CTAB at pH 6.50, −24 kV of applied voltage and UV detection at 230 nm.

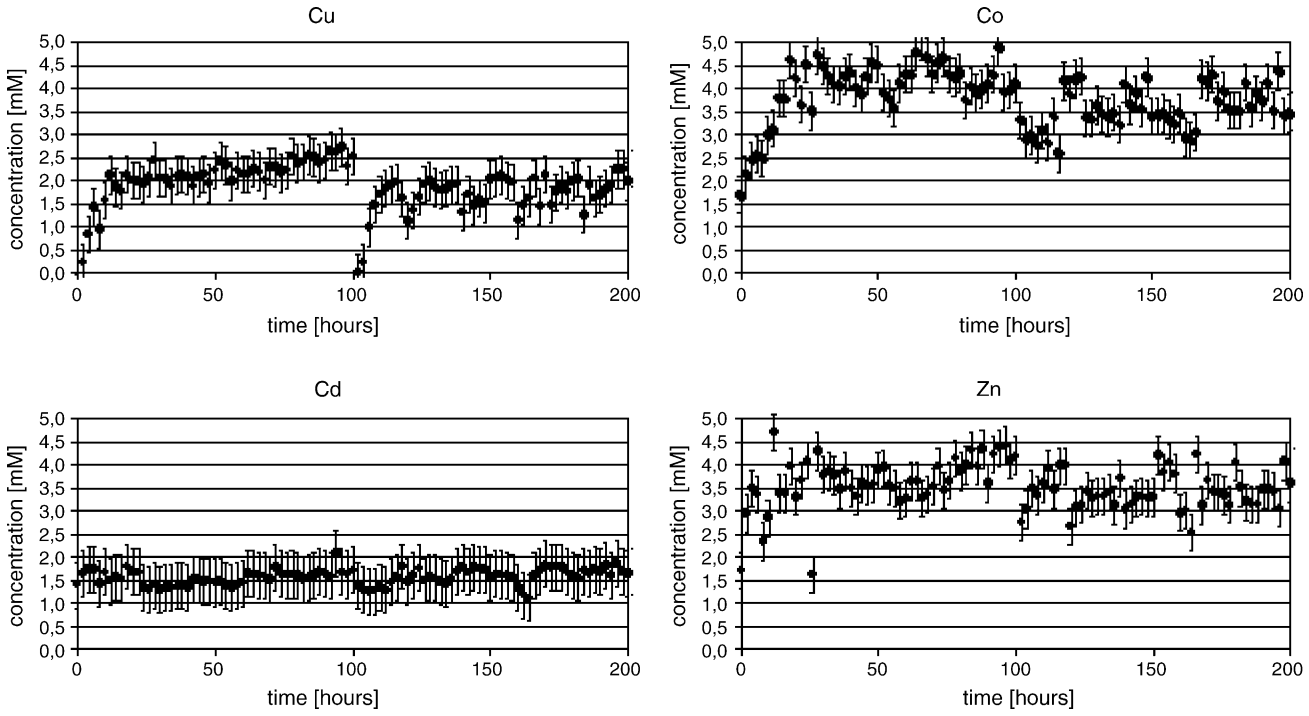

Fig. 4. The bioaccumulation of different metals by bacteria compared to the changes in the background culture media without bacteria. For better comparison *x*-axis for bacterial data have been shifted 100 h against the origin, i.e. 0–100 h: background and 100–200 h background with bacteria.

reactor without bacteria. There is a clear evidence of the bacterial effect of Cu consumption consistent with data reported earlier on 25% removal of Cu from culture media containing elevated concentrations of copper (2.3 mM), chromium, and arsenic by *Rhodococcus luteus* [46]. The bacterial effect can be recognized on the traces of Co and Zn as well but the error of determination (expressed by error bars on data points in Fig. 4) is too large to make any definite conclusions.

There seems to be no bacterial effect on Cd. The results might be due to the fact that Cu, Co and Zn are essential components of enzymes and vitamins in bacterial cells while Cd is a toxic heavy metal. Although the highest possible concentration of Cd that *Rhodococcus* bacteria generally tolerate is 100 μM [47] we observed bacterial activity even at the concentration of 1.5 mM. Bacterial cell viability was tested, taking samples from the reactor after 100 h run and cultivating them on Petri dishes with solid Plate Count Agar media (without heavy metals). The colonies grown were stained by Gram and the shape and colour checked by light microscope. No differences were observed, confirming that no secondary contamination occurred during the experiment. In addition to that the reactor was isolated from the surroundings by sterile air filter.

Fig. 4a–d indicate complex (stochastic) oscillations occurring in the bioreactor. This is probably due to two processes: the inorganic complexation of heavy metals with biomass components and bacterial metabolism. Periodic phenomena are widespread in biology, e.g. yeast glycolytic oscillations, the cell cycle, etc. [48]. The experimental conditions under which yeast cells exhibit sustained glycolytic oscillations are nonphysiological. NADH fluorescence oscillations emerged if starved cells in an anaerobic environment were suddenly fed glucose and subsequently inhibited with cyanide [49]. In continuous cultures metabolic overflow (combined effect of product inhibition and enhanced product formation) can also lead to unusual dynamic behavior, e.g. longer time period to reach a steady state and oscillatory transition from one steady state to another [50]. Thus organisms tend to react on stress conditions with appearance of metabolic products oscillations. Changes in the concentration of metabolites, acetic and citric acids were also observed during monitoring when the reaction mixture contained bacteria (especially increase in citric acid concentration was noticed at the beginning of the experiment which is also an evidence of bacterial metabolism). The average levels of these analytes were 1.1 ± 0.2 and 0.3 ± 0.2 mM, respectively, but the stochastic oscillations remained within the limits of uncertainties and monitoring traces are not presented here.

4. Conclusions

In this work, a method for studying heavy metals and organic acids in one run by using capillary electrophoresis was optimized by using the response surface modeling with a three-level factorial design, PDCA being used as a buffer. Separate optimum values were combined by using the desirability function. Difficulties in the separation of the peaks of cadmium and cobalt were eliminated, the migration time of the analytes was decreased and the best conditions for the peak height of organic acids and heavy metals were found. The response surface modeling was shown to be an effective tool for optimizing an analytical method for an on-line measurement of the metabolism of bacteria. As an example, the method was applied to monitoring the bioaccumulation of heavy metals (copper, zinc, cadmium and cobalt) by bacteria

isolated from the highly polluted technogenic soil of Estonia. By using capillary electrophoresis with a robust and simple sampling probe it was possible to monitor on-line the heavy metal accumulation by bacteria in a closed reactor system. Compared to HPLC the advantage of CE appeared to be a small sample size and opportunity to separate metals and organic acids in one run. We believe that the coupling a fermentation reactor with capillary electropherophoresis is suitable not only for studying of bioaccumulation of metals but also for on-line monitoring of wide range of bioprocesses.

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