Capillary electrophoresis for the monitoring of carboxylic acid production by *Gluconobacter oxydans*

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ABSTRACT

Determination of carboxylic acids in *Gluconobacter oxydans* fermentations of wheat straw hydrolyzate was carried out. This matrix is of complex composition containing carbohydrates, organic compounds (e.g., amino acids, toxins), and inorganic salts making the analysis challenging even with separation techniques. A method based on capillary electrophoresis with indirect UV detection was developed for the simultaneous quantification of 18 carboxylic acids. The background electrolyte solution of ammonia, 2,3-pyridinedicarboxylic acid, and Ca^{2+} and Mg^{2+} salts, containing myristyltrimethylammonium hydroxide as a dynamic capillary coating reagent, was validated for the robust and repeatable separation of the carboxylic acids. Intraday relative standard deviations in the optimized method were less than 1.6% for migration times and between 1.0% and 5.9% for peak area. Interday relative standard deviations were less than 5.0% for migration times and between 5.7% and 9.3% for peak area. With 11 nl injected, detection limits for the analytes were between 10 and 43 μmol/l. Detection limits ranged from 0.1 to 0.5 pmol at signal-to-noise ratio of 3. The results demonstrated that wheat straw hydrolyzate was a suitable substrate for *G. oxydans* with a product yield of 45% for the formation of xylonic acid from xylose and 96% for the formation of gluconic acid from glucose.

1. Introduction

Monitoring and control of processes in bioreactors is of critical importance in biotechnology [1]. The essential tasks in bioprocess monitoring are the determination of substrate uptake rate, specific growth rate of the organisms, and the product formation rate. In practice, the most widely measured and controlled parameters are pH, dissolved oxygen and carbon dioxide, temperature and pressure [2,3]. Carboxylic acids are involved in many metabolic processes of the cell and they are important metabolites of several biochemical pathways in microorganisms. Because they are frequently either the main products or significant by-products in bioprocesses, monitoring them is often essential [4].

There has been a considerable interest in the use of lignocellulosic materials as a renewable source of chemicals for over two decades now [5]. Lignocellulose is a highly promising raw material; it is a natural and cheap polymer that is abundantly present in agricultural waste (wheat straw, corn stalks, soybean residues, sugarcane bagasse), industrial waste (pulp and paper industry), forestry residues, and municipal solid waste. Lignocellulose is esti-

mated to account for about 50% of all biomass on Earth [6]. It consists of three main components: cellulose, hemicellulose, and lignin. Wheat straw is composed of 35–45% cellulose and 20–30% hemicelluloses with a relatively low lignin content (<20%) [7]. Cellulose and hemicellulose are composed of chains of carbohydrate molecules that can be hydrolyzed to monomeric sugars. Cellulose is composed of glucose and hemicellulose of galactose, mannose, glucose, xylose, and arabinose [8]. The pentose monosaccharide xylose together with the hexose monosaccharide glucose, are two of the most abundant sugars found in nature. Xylose is the predominant hemicellulosic sugar of hardwoods and agricultural residues, accounting for up to 25% of the dry biomass of some plant species. The abundance and ease of isolation of xylose makes it an important potential feedstock for the production of bulk chemicals such as carboxylic acids [9].

Most monomeric sugars can be metabolized by microorganisms, but an organism which is able to efficiently convert a variety of sugars (pentoses and hexoses) to useful products, and to tolerate toxins and stress conditions is required to exploit lignocellulosic material [6]. Bacterial metabolism of xylose characteristically generates multiple products, including alcohols (butanol, ethanol, isopropanol, 2,3-butanediol), carboxylic acids (acetic, butyric, formic, and lactic), polyols (arabitol, glycerol, xylitol), ketones (acetone), and gases (methane, carbon dioxide, hydrogen) [1]. *Gluconobacter oxydans* has an exceptional capacity for the oxidative

Table 1
Carboxylic acids and their structures.

Compound	Molecular weight (g/mol)	Structure	Compound	Molecular weight (g/mol)	Structure
Formic acid	46.026		Acetic acid	60.052	
Propionic acid	74.078		Glycolic acid	76.052	
Oxalic acid	90.036		Lactic acid	90.078	
Malonic acid	104.062		Maleic acid	116.072	
Succinic acid	118.088		Malic acid	134.088	
α-Ketoglutaric acid	146.098		Arabonic acid	166.13	
Xylonic acid	166.13		Citric acid	192.124	
Isocitrate	192.124		Galacturonic acid	194.14	
Gluconic acid	196.156		Galactaric acid	210.139	

transformation of carbohydrates to polyhydric alcohols, and carboxylic acids [5].

The most widely available and accessible sources of lignocellulosic biomass are agricultural residues [10]. Wheat straw is the major crop residue in Europe and the second largest agricultural residue in the world [11]. The wheat straw hydrolyzate used here was prepared as a xylose rich hydrolyzate, containing small amounts of glucose and arabinose. *G. oxydans* is able to convert glucose and xylose to gluconic acid and xylonic acid, respectively, and these two carboxylic acids can be further used as ligands, buffers, dispersants, etc.

Traditionally, carboxylic acids have been analyzed by gas chromatography (GC) [12,13] and either by liquid chromatography (LC) [14–17] or by its submethod ion chromatography (IC) [18,19]. However, these methods have some deficiencies. When using GC, traditionally the carboxylic acids have been analyzed after derivatization to make them volatile, and therefore direct quantification has not been used [20]. LC is time-consuming and limited by a narrow linear dynamic range, high limits of detection (g/l), and susceptibility to matrix interferences [14]. IC lacks selectivity for carboxylic acids, which are weakly retained onto ion exchange materials. Furthermore, it has fairly modest chromatographic efficiency and low duration of the stationary phases [21].

Capillary electrophoresis (CE) is a versatile technique with no special need for derivatization and no limitation on polar solvents, analytes or samples. Resolution and efficiency are high in optimized methods, creating great potential for rapid detection and quantification [22]. Usually, the separation of analytes by CE is based on their different electrophoretic mobilities, which are strongly influenced by the composition of the background electrolyte (BGE), pH, ionic strength, concentration of organic cosolvents, and electrolyte additives [23]. The technique offers great possibilities for the analysis of real life samples.

CE has been widely used in the analysis of carboxylic acids. Methods have been developed to determine them in a diversity of matrices, including juice [24–26], wine [20,27,28], beer [29,30], coffee [31] and dairy products [12,22,32]. Few studies have dealt with carboxylic acids in fermentation samples of lignocellulosic material [10,15,33,34], and only one study has been made by capillary electrophoresis [35]. Soga and Ross [36] established a CE method for the analysis of 23 different organic acids from soy sauce, nutrient tonic and pineapple. For our analysis purposes this method was not efficient enough. The high pH would have caused problems because of very high concentration of sugars in the fermentation samples. Sugars are ionized at pH above 12 and can be seen in the electropherogram. In lower pH values the separation was not sufficient enough. Moreno et al. [20] introduced a CE method for the analysis of 9 organic acids from must, wine, brandy and vinegar. They used sodium tetraborate buffer and Ca²⁺ and Mg²⁺ as the metals to complex the carboxylic acids. Combination of these two alkali earth metals enhanced the separation significantly. Even so, using tetraborate buffer was not applicable in this study because most of the organic acids presented in this article could not be detected using this electrolyte solution. Combining these two CE methods gave the right separation efficiency.

The aim of this work was to develop a CE method for the simultaneous separation, identification and quantification of 18
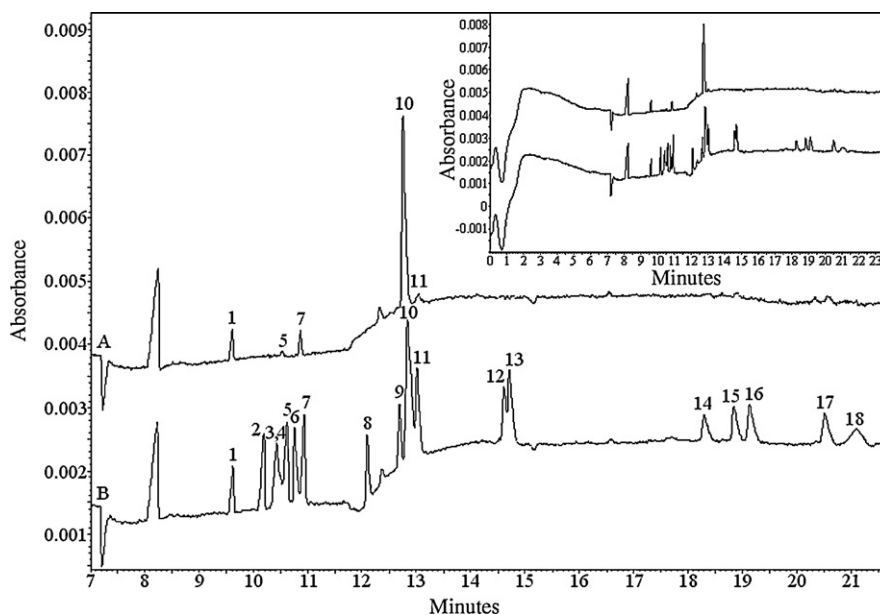
Fig. 1. (A) Blank cultivation medium; and (B) cultivation medium where the studied 18 organic acids have been added in concentration of 50 mg/l. Peak assignments: 1, formic acid; 2, malonic acid; 3, maleic acid; 4, α-ketoglutaric acid; 5, succinic acid; 6, oxalic acid; 7, malic acid; 8, isocitric acid; 9, galactaric acid; 10, acetic acid; 11, glycolic acid; 12, propionic acid; 13, lactic acid; 14, citric acid; 15, xylonic acid; 16, arabonic acid; 17, gluconic acid; and 18, galacturonic acid. Analysis conditions as described in the text.

carboxylic acids potentially present in microbial fermentation process samples. Identification and quantification of these acids is of great importance in controlling the bioprocess and in monitoring the behavior of microorganisms in order to estimate the reaction kinetics. The carboxylic acids of this study are presented in Table 1.

2. Experimental

2.1. Chemicals

All chemicals were of reagent grade. Chemicals for the BGE were 2,3-pyridinedicarboxylic acid (2,3-PDC) (Sigma–Aldrich, Steinheim, Germany), myristyltrimethylammonium hydroxide (MTAH) (Waters, Milford, USA), calcium chloride dihydrate and magnesium chloride hexahydrate (Riedel-de Haen, Seelze, Germany), and methanol (Rathburn, Walkerburn, Scotland). Gluconic acid (lactone), galacturonic acid, acetic acid, propionic acid, galactaric acid, D-lactic acid (lithium salt), and glycolic acid were purchased from Fluka (Buchs, Switzerland). Formic acid, malonic acid, and citric acid (citric acid monohydrate) were from Merck (Darmstadt, Germany). Isocitrate, maleic acid, malic acid, succinic acid, oxalic acid, and α-ketoglutaric acid were from Sigma–Aldrich. Xylonic acid (calcium salt) and arabonic acid were produced and purified at VTT (Espoo, Finland). 1000 mg/l stock solutions were prepared of the 18 carboxylic acids and calcium and magnesium salts. Sodium hydroxide (Akzo Nobel, Bohus, Sweden) was prepared as a 1 M stock solution.

2.2. Instrumentation

All the measurements were made with the P/ACE MDQ capillary electrophoresis system (Beckmann Coulter Inc., Fullerton, USA) equipped with a PDA detector. Untreated fused-silica capillaries (Teknolab, Norway) with inner diameter of 50 μm, outer diameter of 365 μm, and total length of 80 cm (70 cm to the detector) were used. Capillaries were conditioned before use with 0.1 M NaOH, Milli-Q purified water, and BGE for 20, 10, and 20 min, respectively. Voltage (20 kV) was then applied for 10 min. Between analyses, cap-

illaries were rinsed with 0.1 M NaOH and Milli-Q purified water for 1 min each and with BGE for 3 min. Data was collected and processed with 32Karat software (Beckmann Coulter Inc., Fullerton, USA). All of the solutions were prepared in Milli-Q purified water (Millipore, Bedford, USA).

2.3. Analysis conditions

BGE was prepared with 20 mM 2,3-PDC, 0.3 mM MTAH, 30 mg/l Ca^{2+} and 30 mg/l Mg^{2+} in methanol:water (10:90, v/v). The pH of the solution was adjusted to 9 with ammonia (25% (v/v), Merck). Before analysis, the BGE was degassed by ultrasound and filtered through a 0.45 μm Acrodisc GHP syringe filter (Pall Life Science, Ann Arbor, USA).

Standards and samples were injected by using 0.5 psi pressure for 15 s. Separation was carried out at −20 kV (reversed polarity) at constant capillary temperature 25 °C. Indirect UV detection was at 254 nm.

2.4. Microbial growth conditions

G. oxydans E97003 was maintained in 300–450 ml cultures in Infors 500 ml Multifors bioreactors at pH 5.6, 30 °C, agitation 500 rpm, and aeration of 1.3 ± 0.2 volume air [volume culture]$^{-1}$ min^{-1}. Cultures were inoculated with cells grown for 2 days in medium containing 5 g/l yeast extract, 5 g/l $(NH_4)_2SO_4$, 2 g/l $K_2HPO_4 \cdot 3H_2O$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 8 g/l glycerol, 1 g/l glucose and 1 g/l xylose to give an initial concentration of approximately 0.1 g/l biomass. Cultures were maintained in medium derived from wheat straw hydrolyzate (Abengoa Bioenergia Nuevas Tecnologias, Spain) supplemented with 5 g/l yeast extract. To avoid contamination and for sterilizing, the medium was autoclaved at 121 °C for 20 min.

2.5. Sample preparation

For standardization, five levels of calibration mixtures were prepared for the 18 carboxylic acids. The concentration range of the standard mixtures was 5–100 mg/l. Before analysis the fermenta-

Table 2
Performance of the method under optimized conditions. Analytes are prepared in yeast extract.

	Formic acid	Malonic acid	Maleic acid	α-Ketoglutaric acid	Succinic acid	Oxalic acid
Migration time (intraday)[a]	0.74	0.78	0.81	0.81	0.82	0.84
Migration time (interday)[b]	3.01	3.12	3.15	3.20	3.21	3.34
Peak area (intraday)[a]	5.34	5.91	4.79	5.11	3.02	4.01
Peak area (interday)[b]	9.34	6.93	7.70	9.00	5.71	6.64
Calibration equation	$y = 77.85x + 138.79$	$y = 76.522x + 97.892$	$y = 53.912x - 10.12$	$y = 53.702x - 155.72$	$y = 82.747x + 723.29$	$y = 73.784x - 77.377$
Calibration correlation coefficient	0.9996	0.9951	0.9984	0.9966	0.9987	0.9921
Detection limit (mg/l)	2	2	2	2	2	2
Quantification limit (mg/l)	5	5	5	5	5	5
	Malic acid	**Isocitric acid**	**Galactaric acid**	**Acetic acid**	**Glycolic acid**	**Propionic acid**
Migration time (intraday)[a]	0.85	0.94	0.99	1.01	1.01	1.13
Migration time (interday)[b]	3.37	3.41	3.49	3.55	3.62	3.78
Peak area (intraday)[a]	2.22	5.71	5.76	5.34	2.61	4.30
Peak area (interday)[b]	7.27	7.23	8.12	8.12	6.44	6.81
Calibration equation	$y = 63.709x + 71.634$	$y = 57.933x - 172.07$	$y = 48.6x - 82.184$	$y = 124.07x + 419.46$	$y = 92.3x - 60.602$	$y = 89.502x + 76.24$
Calibration correlation coefficient	0.9992	0.9992	0.9989	0.9968	0.9993	0.9987
Detection limit (mg/l)	2	2	5	2	2	2
Quantification limit (mg/l)	5	5	10	5	5	5
	Lactic acid	**Citric acid**	**Xylonic acid**	**Arabonic acid**	**Gluconic acid**	**Galacturonic acid**
Migration time (intraday)[a]	1.14	1.45	1.46	1.46	1.56	1.61
Migration time (interday)[b]	3.91	3.98	4.71	4.73	4.94	5.01
Peak area (intraday)[a]	4.28	4.79	5.89	3.76	5.18	0.99
Peak area (interday)[b]	7.26	7.31	6.43	7.95	7.04	6.30
Calibration equation	$y = 97.321x + 921.61$	$y = 93.506x - 278.23$	$y = 64.405x - 206.7$	$y = 70.445x - 90.896$	$y = 54.059x - 166.4$	$y = 62.454x - 313.05$
Calibration correlation coefficient	0.9988	0.9980	0.9937	0.9995	0.9988	0.9985
Detection limit (mg/l)	2	5	5	5	5	5
Quantification limit (mg/l)	5	10	10	10	10	10

[a] Percentage of relative standard deviation, $n = 8$.
[b] Percentage of relative standard deviation, $n = 4$.

tion samples were diluted to 1:5, 1:100, and 1:250 with Milli-Q purified water and centrifuged at 10 000 rpm for 10 min.

3. Results and discussion

3.1. Method validation

Determination of organic acids has often been achieved in CE with direct UV detection. However, all the analytes of interest do not exhibit sufficient UV absorption. Use of indirect UV detection by adding a UV absorbing reagent to the BGE is one way to overcome the problem [21]. From the wide variety of different UV absorbing chemicals, we chose 2,3-PDC due to research group's prior experiments [37].

MTAH, a surfactant that is commonly used in the analysis of carboxylic acids, was added as modifier to change the direction of the electroosmotic flow (EOF) [20,26,38,39]. Therefore, negative voltage could be applied to accelerate the separation of the carboxylic acids.

Cations of the alkali earth group have strong tendencies to form partially dissociated complexes in solution with the anions of carboxylic acids and other weak acids. The affinity of the association increases with the number of carboxylic acid groups of the acid anion [40]. Formation of these metal complexes is generally rapid, and the relatively weak complexes are in equilibrium with the noncomplexed components. This type of complex formation is widely exploited in chromatography to achieve selectivity. The most important parameters affecting complexation equilibrium are type and concentration of the metal and the pH of the electrolyte [41]. Ca^{2+} and Mg^{2+} ions were added to the BGE to enhance separation efficiency.

The addition of methanol, up to 20% to the BGE has been found to increase migration times and resolution. Methanol acts in the electrolyte solution used for the separation by reducing EOF. In addition its dielectric constant is lower than that of the water. Com-

plexation reactions and formation of high degree complexes can be promoted in a low dielectric constant medium thus providing additional possibilities for improving the separation [41].

Different compositions of the BGE were studied. Concentrations of 2,3-PDC, Ca^{2+}, and Mg^{2+} varied between 20 and 50 mM, 10 and 90 ppm and 10 and 60 ppm, respectively. pH of the BGE was studied between 8 and 10. Different analytical conditions were also examined. Voltage, analysis temperature, and detection wavelength were optimized in the ranges of 15–30 kV, 20–30 °C and 200–300 nm, respectively. In the optimized method, concentrations of 2,3-PDC, MTAH, Ca^{2+} and Mg^{2+} were 20 mM, 0.3 mM, 30 mg/l and 30 mg/l, respectively, in methanol–water mixture (10:90, v/v). The pH was 9 and it was adjusted after mixing all the components of BGE. Voltage was −20 kV, capillary temperature 25 °C and detection wavelength 254 nm.

In Fig. 1A is presented an electropherogram of cultivation medium that was used in this study. The performance of the developed method is presented in Fig. 1B where the 18 organic acids have been added to the cultivation medium. The exact composition of cultivation medium is presented in Section 2.4. Without addition of Ca^{2+} and Mg^{2+} as the metals to complex organic acids or using either of them, the separation would not be sufficient enough for identification and quantification of organic acids introduced in Table 1. Most of the organic acids could not even be separated from each other without using both metals for complexation.

3.2. Quantification

For the quantification of the carboxylic acids, calibration curves based on matrix-matched standards were prepared. Standards were added to yeast extract. Standards were calibrated for concentrations between 5 and 100 mg/l. Intraday relative standard deviations for migration times of the carboxylic acids were less than 1.3% and those for peak areas between 2.7% and 5.6%. Interday relative standard deviations for migration times of the carboxylic
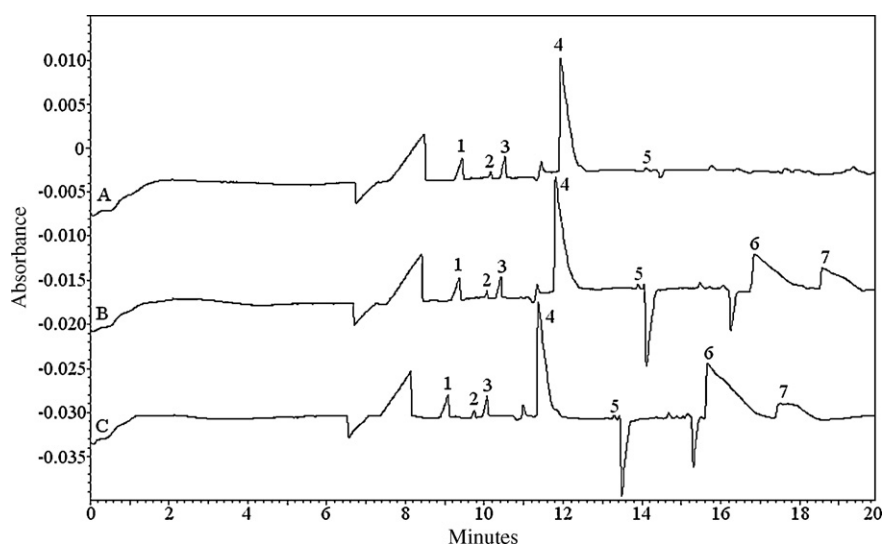
Fig. 2. Electrophoretic separation of carboxylic acids in wheat straw hydrolyzate. Fermentation time: (A) 0 h; (B) 19.8 h; and (C) 119.1 h. Peak assignments: 1, formic acid; 2, succinic acid; 3, malic acid; 4, acetic acid; 5, lactic acid; 6, xylonic acid; and 7, gluconic acid. Analysis conditions as in Fig. 1.

Table 3
Concentrations (mg/l) of carboxylic acids in wheat straw hydrolyzates.

	Sample id							
	52(1) 0[a]	52(2) 3.5[a]	52(3) 19.8[a]	52(4) 26.8[a]	52(5) 44.3[a]	52(8) 76.7[a]	BS52(6) 99.9[a]	BS52(7) 119.1[a]
Formic acid	960	960	980	1130	1050	1080	1200	1040
Succinic acid	130	130	140	150	160	190	200	180
Malic acid	770	590	610	1140	660	710	800	680
Acetic acid	6100	5600	6100	7000	6000	6100	6100	5400
Lactic acid	98	104	91	88	80	71	67	73
Xylonic acid	nd	2200	9700	10 800	15 800	16 900	17 200	17 400
Gluconic acid	nd	3000	5500	5300	5200	5100	5600	5100

nd, not detected.

[a] Fermentation time (h).

acids were less than 5.0% and those for peak areas between 5.7% and 9.3%. Detection limits for the standards were between 2 and 5 mg/l, i.e. 0.1–0.5 pmol. Correlation coefficients for the calibration curves were between 0.9921 and 0.9996.

The calibration curves, correlation coefficients, repeatabilities, and reproducibilities of the method are summarized in Table 2.

3.3. Analysis of fermentation samples

The optimized method was applied to the analysis of samples from a *G. oxydans* culture in wheat straw hydrolyzate, which was rich in xylose. Samples were diluted to three different dilutions before analysis: 1:5 (v/v) for the analysis of carboxylic acids in small concentrations (less than 100 mg/l), 1:100 (v/v) for high concentrations (100–10 000 mg/l) and 1:250 (v/v) for very high concentrations (>10 000 mg/l). Electropherograms for samples are presented in Fig. 2. The fermentation time in Fig. 2A is 0, in 2B it is 19.8 and in 2C it is 119.1 h. The negative peaks in Fig. 2B and C are most probably UV absorbing carboxylic acids, e.g., different phenolic acids or furan derivatives that are formed during fermentations. The formation of these compounds is highly avoidable in fermentations since they can act as inhibitors. The results are summarized in Table 3. As the results demonstrate, there was rapid increase in xylonic acid and gluconic acid concentration during fermentation (Fig. 3). Changes in the levels of other carboxylic acids were insignificant. Because every sample had different ion strength, migration times of organic acids varied between samples. However, the profile of the electropherograms and peak shapes remained the same

and the identification was easy. Just to make sure, the identification was assured by spiking the first sample. The metabolism of *G. oxydans* and its ability to convert certain sugars to certain organic acids is well known. Using this strain and these cultivation conditions, only 7 organic acids were formed.

It is worth noting that the hydrolyzate from the fermentation of wheat straw contained glucose (5.3 g/l) as well as xylose (39 g/l). Gluconic acid production was complete within less than 20 h, but xylonic acid production continued. The yield for the production of xylonic acid from xylose was 45%, and for the production of gluconic acid from glucose was 96%. Thus, *G. oxydans* was able to produce large amounts of the desired carboxylic acids from wheat straw hydrolyzate. The high concentrations of formic, malic and acetic acids were not metabolized by *G. oxydans*, but did not pre-
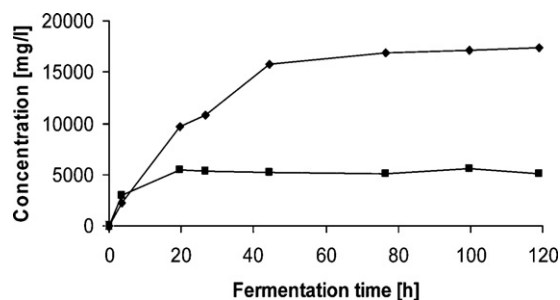
Fig. 3. Production of xylonic (♦) and gluconic (■) acids from wheat straw hydrolyzate by *Gluconobacter oxydans*.

vent conversion of glucose and xylose to gluconic and xylonic acids, respectively.

4. Conclusions

A capillary electrophoretic method for the simultaneous analysis of 18 carboxylic acids was developed for bioprocess monitoring. The method was successfully applied in the analysis of samples from a fermentation broth of complex composition. *G. oxydans* has an exceptional capacity for transforming carbohydrates to carboxylic acids, which could be used as ligands, buffer chemicals and dispersants.

Acknowledgements

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