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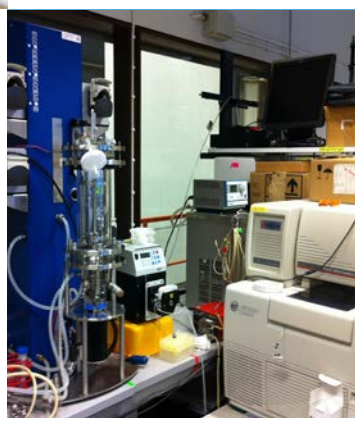
Dissertation
47

Capillary electrophoresis for monitoring carboxylic, phenolic and amino acids in bioprocesses

Heidi Turkia



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VTT SCIENCE 47

Capillary electrophoresis for monitoring carboxylic, phenolic and amino acids in bioprocesses

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Thesis for the degree of Doctor of Philosophy to be presented with due permission for public examination and criticism in Auditorium 1381, at the Lappeenranta University of Technology, on the 24.1.2014 at 10:00.

ISBN 978-951-38-8107-8 (Soft back ed.)
ISBN 978-951-38-8108-5 (URL: http://www.vtt.fi/publications/index.jsp)

VTT Science 47

ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)

JULKAISIJA – UTGIVARE – PUBLISHER

VTT
PL 1000 (Tekniikantie 4 A, Espoo)
02044 VTT
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Kopijyvä Oy, Kuopio 2013

Capillary electrophoresis for monitoring of carboxylic, phenolic and amino acids in bioprocesses

Kapillaarielektrofeesin käyttö karboksyyli-, fenoli- ja aminohappojen monitorointiin bioprosesseissa. **Heidi Turkia.** Espoo 2013. VTT Science 47. 103 p. + app. 36 p.

Abstract

Bioprocess technology is a multidisciplinary industry that combines knowledge of biology and chemistry with process engineering. It is a growing industry because its applications have an important role in the food, pharmaceutical, diagnostics and chemical industries. In addition, the current pressure to decrease our dependence on fossil fuels motivates new, innovative research in the replacement of petrochemical products. Bioprocesses are processes that utilize cells and/or their components in the production of desired products. Bioprocesses are already used to produce fuels and chemicals, especially ethanol and building-block chemicals such as carboxylic acids. In order to enable more efficient, sustainable and economically feasible bioprocesses, the raw materials must be cheap and the bioprocesses must be operated at optimal conditions. It is essential to measure different parameters that provide information about the process conditions and the main critical process parameters including cell density, substrate concentrations and products. In addition to offline analysis methods, online monitoring tools are becoming increasingly important in the optimization of bioprocesses.

Capillary electrophoresis (CE) is a versatile analysis technique with no limitations concerning polar solvents, analytes or samples. Its resolution and efficiency are high in optimized methods creating a great potential for rapid detection and quantification. This work demonstrates the potential and possibilities of CE as a versatile bioprocess monitoring tool. As a part of this study a commercial CE device was modified for use as an online analysis tool for automated monitoring. The work describes three offline CE analysis methods for the determination of carboxylic, phenolic and amino acids that are present in bioprocesses, and an online CE analysis method for the monitoring of carboxylic acid production during bioprocesses. The detection methods were indirect and direct UV, and laser-induced frescence. The results of this work can be used for the optimization of bioprocess conditions, for the development of more robust and tolerant microorganisms, and to study the dynamics of bioprocesses.

Keywords bioprocess monitoring, capillary electrophoresis, online capillary electrophoresis, carboxylic acids, phenolic acids, amino acids

Kapillaarielektroforeesin käyttö karboksyyli-, fenoli- ja bioprosesseissa

Capillary electrophoresis for monitoring of carboxylic, phenolic and amino acids in bioprocesses.
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Tiivistelmä

Bioprosessitekniikka on monitieteellinen teollisuudenala, joka yhdistää biologian ja kemian tietämyksen kemiantekniikkaan. Se on kasvava teollisuudenala, koska sen sovellukset ovat hyvin tärkeitä ruoka-, farmasia-, diagnostiikka- ja kemianteollisuudelle. Tämän lisäksi riippuvuutta öljystä tulee vähentää merkittävästi, mikä mahdollistaa uuden ja innovatiivisen tutkimuksen petrokemikaalituotteiden korvaamiseksi. Bioprosessit ovat prosesseja, jotka käyttävät soluja ja/tai niiden osia tuottamaan haluttuja tuotteita. Bioprosesseja on jo käytetty polttoaineiden ja kemikaalien tuottamiseen, erityisesti etanolin ja ns. building-block kemikaalien, kuten karboksyylihappojen, tuottamiseen. Jotta bioprosesseista saadaan tehokkaampia ja taloudellisesti kannattavampia, raaka-aineiden on oltava halpoja ja bioprosessien on toimittava optimaalisissa olosuhteissa. Erilaisten parametrien mittaaminen on välttämätöntä, sillä ne antavat tietoja prosessin tilasta ja kriittisistä prosessiparametreista, joita ovat biomassa, lähtöaineet ja tuotteet. Offline-mittausten lisäksi online-määritykset kasvattavat merkitystään bioprosessien optimoinnissa.

Kapillaarielektroforeesi (CE) on monipuolinen analyysitekniikka, jolla ei ole rajoituksia polaaristen liuottimien, analyyttien tai näytteiden kanssa. Optimoitujen menetelmien korkea resoluutio ja tehokkuus mahdollistavat nopean detektoinnin ja kvantifioinnin. Tämä työ osoittaa CE:n potentiaalin monipuolisena bioprosessimonitorointityökaluna. Osana tätä tutkimusta kaupallinen CE-laitteisto muutettiin toimimaan online-analysointilaitteistona prosessien automaattista monitorointia varten. Tämä työ kuvaa kolme offline-CE analyysimenetelmää karboksyyli-, fenoli- ja aminohappojen analysointiin sekä yhden online-CE analyysimenetelmän karboksyylihappojen monitorointiin bioprosessien aikana. Käytetyt detektointimenetelmät olivat epäsuora ja suora UV sekä laser-indusoitu fluoresenssi. Työn tuloksia voidaan käyttää bioprosessiolosuhteiden optimoinnissa, elinvoimaisempien ja kestävämpien mikro-organismien kehittämisessä sekä bioprosessien dynamiikan tutkimisessa.

Avainsanat bioprocess monitoring, capillary electrophoresis, online capillary electrophoresis, carboxylic acids, phenolic acids, amino acids

Preface

This work was carried out at VTT Technical Research Centre of Finland during the period 2008–2013. Financial support from the Finnish Centre of Excellence in White Biotechnology – Green Chemistry Research granted by the Academy of Finland (grant number 118573), the EU-project Nanobe which received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 227243, Laboratory of Chemistry at the Lappeenranta University of Technology and Graduate School for Chemical Sensors and Microanalytical Systems (CHEMSEM) is gratefully acknowledged.

I express my deepest gratitude to my supervisors Professor Heli Sirén and Dr. Sci. (tech.) Juha-Pekka Pitkänen. Your help, support and encouragement during the work was invaluable. The fruitful conversations and brainstorming were of great importance for the success of this study. In addition, your knowledge and enthusiasm has been inspirational for me. Vice President of Bio and Process Technology Dr. Anu Kaukovirta-Norja, Technology manager Dr. Kirsi-Marja Oksman-Caldentey, her predecessor Dr. Tiina Nakari-Setälä, and team leader Dr. Jaana Uusitalo are acknowledged for providing excellent working facilities and research equipment.

I thank Professor Mihkel Kaljurand and Professor Krist V. Gernaey for their excellent review of the thesis. Kathleen Ahonen and Michael Bailey are thanked for the language revision of the articles, and Michael Bailey for the language revision of the thesis. Your work upgraded the scientific value of my writings.

I thank my co-authors Sami Holmström, Toni Paasikallio, Merja Penttilä, Juha-Pekka Pitkänen, Heli Sirén and Marilyn Wiebe for your contribution. Working with you has been a privilege. All the supervisors and students at the CHEMSEM graduate school are thanked for the interesting and fruitful annual meetings and courses.

I would also like to thank all of my colleagues, especially Sami, Toni, Jouni, Susanne, Dorothee and everyone else in my team. Working with you has been a pleasure and I sure do hope to work with you in the future. I have had so many interesting conversations with the people in the morning coffee table of all imaginable subjects, thank you for making starts of mornings so much fun.

Finally I would like to thank my friends and family for their love and support. Especially my husband Ville is acknowledged for his never ending support and understanding. I love you so much.

Espoo, December 2013

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List of publications

This thesis is based on the following original publications which are referred to in the text as I–IV. The publications are reproduced with kind permission from the publishers.

I Turkia, H., Sirén, H., Pitkänen, J.-P., Wiebe, M., Penttilä, M., 2010. Capillary electrophoresis for the monitoring of carboxylic acid production by *Gluconobacter oxydans*. Journal of Chromatography A 1217, 1537–1542.

II Turkia, H., Sirén, H., Penttilä, M., Pitkänen, J.-P., 2013. Capillary electrophoresis for the monitoring of phenolic compounds in bioprocesses. Journal of Chromatography A 1278, 175–180.

III Turkia, H., Holmström, S., Paasikallio, T., Sirén, H., Penttilä, M., Pitkänen, J.-P., 2013. Online capillary electrophoresis for monitoring carboxylic acid production by yeast during bioreactor cultivations. Analytical Chemistry 85, 9705–9712.

IV Turkia H., Sirén, H., Penttilä, M., Pitkänen, J.-P., 2013. Capillary electrophoresis with laser-induced fluorescence detection for studying amino acid uptake of yeast during beer fermentation. Submitted to Journal of Chromatography B.

Author’s contributions

I The author carried out the method development and wrote the article together with the co-authors.

II The author invented the original idea, carried out the method development and wrote the article together with the co-authors.

III The author invented the original idea, assisted in the design and assembly of the online CE system, carried out the method development and wrote the article together with the co-authors.

IV The author invented the original idea, carried out the method development and wrote the article together with the co-authors.

Contents

Abbreviations

18C6	18-Crown-6-ether
2,3-PDC	2,3-Pyridinecarboxylic acid
AD	Amperometry detector
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BGE	Background electrolyte
CD	Conductivity detector
CE	Capillary electrophoresis
CHAPSO	3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate
CMC	Critical micelle concentration
CZE	Capillary zone electrophoresis
ED	Electrochemical detector
EOF	Electroosmotic flow
FDA	United States Food and Drug Administration
FID	Flame ionization detector
GABA	γ-Aminobutyric acid
GC	Gas chromatography
GLYR1	Glyoxylate reductase
GMO	Genetically modified organism
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
IC	Ion chromatography

ICL1	Isocitrate lyase
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LIF	Laser-induced fluorescence
LMW	Low-molecular-weight
M^3C	Measurement, monitoring, modeling and control
MEKC	Micellar electrokinetic chromatography
MLS2	Malate synthase
MS	Mass spectrometer/spectrometry
MTAH	Myristyltrimethylammonium hydroxide
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
OG-SE	Oregon green® 488 succinimidyl ester
PAT	Process Analytical Technology
PIPES	1,4-Piperazinediethane sulfonic acid
RI	Refractive index
RPLC	Reversed phase liquid chromatography
SDS	Sodium dodecyl sulfate
TCA	Tricarboxylic acid
UPLC	Ultra-performance liquid chromatography
UV/Vis	Ultraviolet/visible

List of symbols

A	Absorbance
α	Molar absorption coefficient
c	Concentration of an analyte
E	Electric field strength
ε	Dielectric constant
F_{in}	Flow rate into the bioreactor
F_{out}	Flow rate from the bioreactor
I.D.	Inner diameter of a capillary
l	Optical path length
η	Viscosity of the background electrolyte solution
O.D.	Outer diameter of a capillary
q	Charge of a molecule
r	Radius of a molecule
μ_{eo}	Electroosmotic mobility of a molecule
μ_{ep}	Electrophoretic mobility of a molecule
μ_{tot}	Total mobility of a molecule
v	Ion velocity
V	Volume
v_{eo}	Electroosmotic velocity of a molecule
ζ	Zeta-potential

1. Introduction

1.1 Overview of bioprocesses

A bioprocess can be defined as any process that uses cells or their components to produce desired products. For centuries bioprocesses have been utilized to produce bread, cheese, cultured milk products, soy sauce, vinegar, beer and wine even before the existence of microorganisms was known. In these traditional bioprocesses, natural microbial flora or a small amount of the previous fermented material were used as inoculum. Even today, good starter dough is treasured and passed from generation to generation. These processes are carried out with a mixed microbial culture, but in bioprocess industry, the use of mixed cultures is rare. Such applications include manufacturing of traditional foods, beverages and alcohols, waste water treatment and biogas production. [1]

The bio-based industry is a multidisciplinary industry that combines the knowledge of biology, chemistry and chemical engineering in order to develop and operate its processes. The applications of biotechnology have an important role in the food, pharmaceutical, diagnostics and chemical industries. [2] Global markets of some fine chemicals produced in bioprocesses are presented in Table 1.

Table 1. Global markets for fine chemicals produced by microorganisms. [3]

Chemical	2009 $ millions	2013 $ millions[a]
Amino acids	5 410	7 821
Enzymes	3 200	4 900
Organic acids (lactic acid 20%)	2 651	4 036
Vitamins and related compounds	2 397	2 286
Antibiotics	1 800	2 600
Xanthan	443	708
Total	15 901	22 351

[a] Estimate

The majority of chemicals are produced from petroleum. Petroleum is a non-renewable starting material, hence the petrochemical products are unsustainable,

and the production is polluting and has an effect on climate change. There is growing interest in the use of renewable feedstock, such as lignocellulose and algal biomass, for the production of chemicals. [4] Lignocellulose is biomass that is derived from plants, and it is estimated to account for about 50% of all biomass on Earth. It has a complex structure consisting of three main fractions: cellulose (~45% of dry weight), hemicellulose (~30%), and lignin (~25%). The proportions of the three fractions vary between different plant species. Cellulose and hemicellulose are polymers that are composed of glucose, and of xylose, glucose, galactose, mannose and arabinose, respectively. These polymers can be hydrolyzed to monomeric sugars that can be fermented by microorganisms. Lignin is an aromatic polymer that is a dehydration product of three monomeric alcohols: p-coumaryl alcohol, p-coniferyl alcohol and p-sinapyl alcohol. The structure of lignin varies considerably and hence it is not known exactly, and the lignin monomers cannot be utilized by current industrial microorganisms. [5]

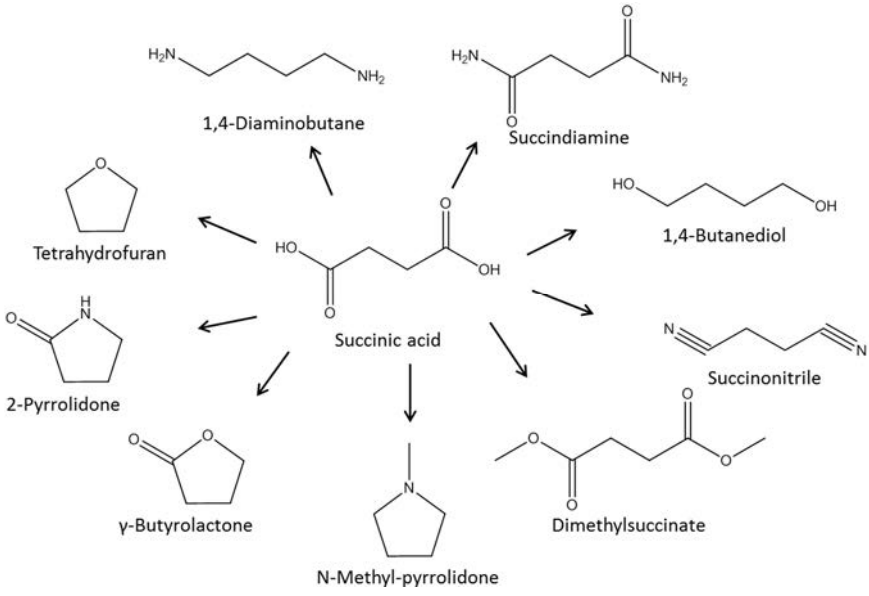

Figure 1. Compounds that can be derived from succinate by chemical conversion. [6]

Fuels and chemicals, especially building-block chemicals for the chemical industry, can be produced in bioprocesses. As an example, carboxylates are among the most important chemicals that are produced in microbial processes. Because of their functional groups, carboxylic acids are very important starting materials (e.g. building-block chemicals) for the chemical industry. For example succinic acid is used widely as a surfactant, detergent or antifoaming agent, as an ion chelator and in the food industry (as an acidulant, flavoring agent or anti-microbial agent) as well as in the pharmaceutical industry (antibiotics). Some of the compounds that can be derived from succinic acid by chemical conversion are presented in

Figure 1. Butanediol, tetrahydrofuran and γ-butyrolactone are common feedstocks for the chemical industry, both as solvents and for fiber and polymer production. Succinate can also be polymerized directly to form biodegradable polymers. Succinic acid can be produced from petroleum, but the process is too expensive to be used for the production of succinate for lower value applications. However, microbially produced succinate could be a solution to this problem. [6] Some companies that produce or plan to produce carboxylic and amino acids for commercial purposes in biotechnological processes are presented in Table 2.

Table 2. Companies producing bio-based building-block chemicals. [7]

Company	Country	Product	Capacity (t/a)	Start year
BioAmber	USA	Succinic acid	17 000	2013
	USA	Succinic acid	3 000	2009
Cargill/Novozymes	USA/Denmark	Acrylic acid	10	
CSM/BASF	the Netherlands /Germany	Succinic acid	15 000	2011
Evonik Industries	Germany	Methionine	580 000	2014
	Germany	Methylmethacrylic acid	10	
Galactic	Belgium	Lactic acid	1 650	2000
HiSun	China	Lactic acid	5 500	2008
Lanxess	Germany	Succinic acid	20 000	2012
Myriant Technologies/Davy Process Technology	USA/UK	Succinic acid	15 000	2013
NatureWorks	USA	Lactic acid	155 000	2015
	USA	Lactic acid	155 000	2005
OPX Biotechnologies/DOW	USA	Acrylic acid	20	2015
Perstorp	Sweden	Propionic acid and 3-hydroxypropionic acid	1 000	2012
PHB Industrial Brazil S.A.	Brazil	Polyhydroxybutyric acid	100	
Purac	the Netherlands	Lactic acid	100 000	2007
Roquette/DSM	France/the Netherlands	Succinic acid	10 000	2012
Segetis	USA	Levulinic acid	120	2009
Tianjin GreenBio Material Co.	China	Polyhydroxybutyric acid	10 000	2009
Tong-Jie-Lang	China	Lactic acid	100	2007
Verdezyne	USA	Adipic acid	40	2013
Wacker Chemie AG	Germany	Acetic acid	500	2010

t/a tons per year

There are indications that full transition from the petrochemical industry to bio-based chemical industry is possible but there are a variety of biological, technical, economic and ecological challenges to be met. These issues can be divided into five major engineering and decision-making challenges: (1) novel cell factories for

the production of building-block chemicals and a wide range of fine chemicals must be developed and optimized; (2) upstream and downstream processes must be improved, including conversion of biomass to fermentable carbon sources, and product separation and purification methods; (3) novel bioprocesses must be economically viable and competitive with the traditional approaches; (4) the ecological benefits of biochemical industry must be evaluated objectively; and (5) research efforts and funding should be focused in a more strategic way to enable the research on the most promising combinations of feedstock, technologies and potential products in both economic and ecological perspectives. [4]

In order to make the bioprocesses more feasible and profitable, methods in measurement, monitoring, modeling, and control (M^3C) are crucial. M^3C methodologies that are currently applied in industrial cell culture technologies include for example chromatographic techniques for culture media optimization, and online monitoring of bioreactor state variables (temperature, dissolved oxygen and carbon dioxide, pH, agitation, redox, conductivity and the intake of substrate and the formation of products and by-products (chromatography, *in situ* probes). [8] Methods for the measurement and monitoring of bioprocesses are discussed in Section 1.2 in more detail.

1.1.1 Cultivation modes

The International Union of Pure and Applied Chemistry (IUPAC) has defined that a bioreactor is “an apparatus used to carry out any kind of bioprocess; examples include fermenter and enzyme reaction”. [9] Bioreactors for growing microorganisms must be sterilizable and air tight in order to prevent contamination by external microbes. [10] In Figure 2 a general description of a stirred tank bioreactor is presented. It has a volume V and it is fed with a stream of fresh and sterile cultivation medium with a flow rate F_{in}. Simultaneously, liquid is removed from the bioreactor with a flow rate F_{out}. [11] These flow rates determine whether the cultivation is batch, fed-batch or continuous cultivation.

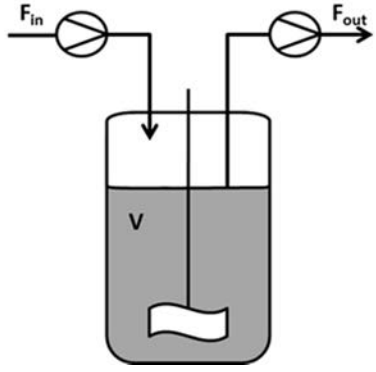

Figure 2. Schematic figure of a bioreactor. F_{in} presents flow going into the bioreactor and F_{out} flow from the bioreactor. Adapted from [11].

Batch cultivation. In batch cultivation, $F_{in}=F_{out}=0$ and volume is constant, which means that all the necessary ingredients (substrates, nutrients etc.) are added to the bioreactor in the beginning of cultivation. After this, the process is closed until the cultivation is ended after a given time whereupon the mixture of substrate together with the products is withdrawn. However, during the cultivation, gases (nitrogen, oxygen), anti-foaming agent and acid or base (pH adjustment) can be added to the media as in all cultivation modes, and samples are taken for bioprocess monitoring. Batch cultivation is the most simple experimental setup and is easy to perform. [9–11]

Fed-batch cultivation. In fed-batch cultivation, $F_{in}\neq 0$ and $F_{out}=0$ which means that the volume of the culture increases during cultivation when fresh medium is added to the bioreactor. This cultivation mode is probably the most common mode in industrial bioprocesses because it enables the control of substrate concentration at a certain level to maximize product formation and final concentration. In addition, fed-batch operation is often preferred to avoid issues with substrate inhibition. Especially in fed-batch cultivation, online monitoring of the process is essential for the control of liquid flow into the bioreactor (F_{in}). [10, 11]

Continuous cultivation. In continuous cultivation, $F_{in}=F_{out}\neq 0$ and volume is constant which means that fresh cultivation medium is added with same flow rate as cultivation medium is removed from the bioreactor. The most common operations of continuous bioreactor are chemostat and turbidostat. In a chemostat concentration of rate-limiting substrate defines the cell density and dilution rate determines the growth rate. In a turbidostat, the inlet flow rate is adjusted to keep the biomass concentration constant throughout the cultivation. Thus the cultivation is growth rate limited. In the chemical industry continuous processes are the most common processes and they are also becoming more important in bioprocess technology because they improve overall reactor productivity. [10, 11]

1.1.2 Microorganisms in bioprocesses

Yeast was one of the first microorganisms to be utilized by humans. It has been used for thousands of years to produce wine and beer through ethanol fermentation. In modern biotechnology, in addition to yeast, bacteria, molds, fungi and algae are also used as host organisms to produce desired products. Strain selection for production of desired products depends both on strain characteristics and on the product properties and application. [12]

In any industrial technology, raw material costs are in direct correlation with the final cost of products. Lignocellulosic material is much cheaper than starchy raw material (corn, wheat) but in addition to hexose sugars the microorganism must also be able to utilize pentoses, especially xylose. In order to be as cost efficient as possible, it is preferred that the microorganism is able to utilize pentoses and hexoses simultaneously. [13] Nowadays, genetically modified organisms (GMO) are widely used in biotechnological processes. For example, the production levels of enzymes can be increased or metabolic routes can be modified to enable

production of novel compounds or to eliminate side-products. Furthermore, genetic manipulation is essential when using raw materials that are not natural nutrients of the microorganism. Usually, a gene or genes are transferred to the host organism from other organisms. In this case, the functioning of the transferred gene(s) in an unfamiliar environment must be ensured. [14] The microorganisms used in this study are reviewed briefly in the following.

Yeast. Yeasts are unicellular eukaryotic microorganisms that reproduce vegetatively by budding or fission. Yeast identification and characterization is of great importance in biotechnology. For example it is essential to distinguish between *wild* yeast and *cultured* yeasts in industrial processes. In the brewing industry the presence of wild yeast may cause undesirable off-flavors in the final product, and during baker´s yeast propagation contaminating wild yeasts such as *Candida utilis* may easily outgrow strains of *Saccharomyces cerevisiae* because of their more efficient sugar utilization. [15]

Saccharomyces cerevisiae (baker´s yeast) is the most important commercially utilized microorganism, and it is regarded as a GRAS organism (*g*enerally *r*ecognized *a*s *s*afe). It has been used extensively because of its capacity for the ethanolic fermentation of carbohydrate feedstock. *S. cerevisiae* has two major pathways in its energy metabolism: glycolysis and aerobic respiration. Ethanol is an important compound in both pathways being an end product in glycolysis and a carbon source in aerobic respiration. [16] Ethanol red is a *S. cerevisiae* strain that has been developed for industrial ethanol production from glucose. Because of it has high tolerance for alcohol, the ethanol production yields can be up to 18% (v/v). The fermentation temperature range is relatively wide, 30–40 °C, and it utilizes less glucose for cell maintenance. It is also rather tolerant to high stress environments.

Kluyveromyces lactis was originally isolated from milk-derived products and because of its origin it has GRAS status for industrial use. *K. lactis* is primarily an aerobic organism but it can ferment glucose to ethanol, and is able to assimilate a wider variety of carbon sources than *S. cerevisiae*. These carbon sources include different sugars, alcohols, carboxylic acids and amino acids. *K. lactis* is able to use lactose as sole source of carbon and energy which makes it a suitable microorganism for a number of applications in the dairy industry, e.g. in yogurt production and in the production of chymosin that is used for cheese manufacturing. [17]

Gluconobacter oxydans. Aerobic microorganisms usually oxidize their carbon sources to carbon dioxide and water. During this process, energy and intermediate metabolites that are mandatory for biosynthesis are produced. *Gluconobacter oxydans* is an acetic acid bacterium. It is a Gram-negative, obligate aerobic and rod-shaped acidophilic organism that oxidizes its substrates incompletely even in normal growth conditions. High oxidation rates usually correlate with low biomass production which is favorable in the biotechnology industry. It is non-pathogenic and its natural habitats are sugary niches such as flowers and fruits. It can also be found from alcoholic beverages and soft drinks as an undesirable contaminant because it causes off-flavors and spoilage. [18]

G. oxydans can be utilized in biotechnology in various processes because of its incomplete oxidation of a wide range of sugars, alcohols and acids to corresponding aldehydes, ketones and organic acids. The products are almost always extracellular compounds and are produced in approximately equimolar yields which makes *G. oxydans* an important industrial microorganism. The most common applications are the production of L-sorbose from D-sorbitol (vitamin C synthesis); D-gluconic acid, 5-keto- and 2-ketogluconic acids from D-glucose; and dihydroxyacetone from glycerol. Strains of the *Gluconobacter* genus can also produce aliphatic, aromatic carbocyclic and thiocarboxylic acids that can be used as e.g. flavoring ingredients. *G. oxydans* is able to grow in solutions containing high sugar concentration and at low pH values. [18, 19] The *G. oxydans* strain used in this study was VTT E-97003, a *suboxydans* subspecies. It has been widely studied for its capabilities in xylonic acid production. [20–23]

1.1.3 Carboxylic acids in bioprocesses

Carboxylic acids are involved in many metabolic processes of the cell and they are important metabolites of several biochemical pathways in microorganisms. They are frequently either the main products or significant by-products in bioprocesses. [24] Probably the most well-known metabolic pathway is the tricarboxylic acid (TCA) cycle, in which the main metabolites are di- and tricarboxylic acids (Figure 3). The TCA cycle is also known as the citrate cycle or Krebs cycle and it is an important aerobic pathway for the oxidation of fuel molecules such as amino acids, fatty acids and carbohydrates. The cycle starts with acetyl-CoA, the activated form of acetate derived from glycolysis and pyruvate oxidation of carbohydrates and from β-oxidation of fatty acids. The two-carbon acetyl group in acetyl-CoA is transferred to the four-carbon compound oxaloacetate to form the six-carbon compound citrate. In a series of reactions two carbons from citrate are oxidized to carbon dioxide (CO_2) and the reaction pathway supplies NADPH or NADH for use in oxidative phosphorylation and other metabolic processes. The pathway also supplies important precursor metabolites including α-ketoglutarate. At the end of the cycle the remaining four-carbon component is transformed back to oxaloacetate. The enzymes that are used in the citric acid cycle are also presented in Figure 3. [25]

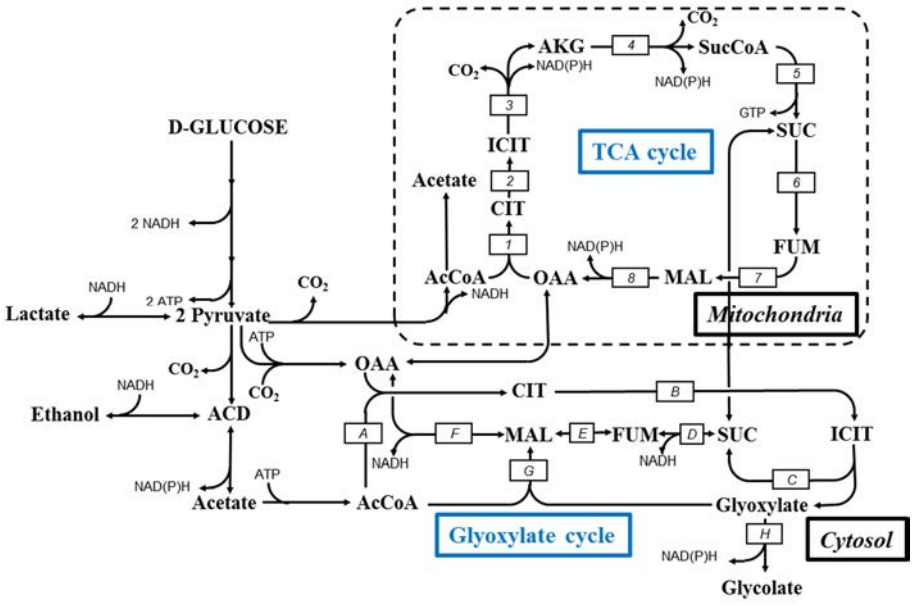

Figure 3. Main metabolic routes of carboxylic acids**.** AcCoA acetyl coenzyme A, ACD acetaldehyde, AKG α-ketoglutarate, ATP adenosine triphosphate, CIT citrate, CO_2 carbon dioxide, FUM fumarate, GTP guanosine triphosphate, ICIT isocitrate, MAL malate, NADH nicotinamide adenine dinucleotide, NADPH nicotinamide adenine dinucleotide phosphate, OAA oxaloacetate, SUC succinate, SucCoA succinyl coenzyme A. Enzymes of the TCA cycle: (1) citrate synthase, (2) aconitase, (3) isocitrate dehydrogenase, (4) α-ketoglutarate dehydrogenase, (5) succinyl CoA synthetase, (6) succinate dehydrogenase, (7) fumarase, (8) malate dehydrogenase. Enzymes of the glyoxylate cycle: (A) citrate synthase, (B) aconitase, (C) isocitrate lyase, (D) fumarate reductase/succinate dehydrogenase, (E) fumarase, (F) malate dehydrogenase, (G) malate synthase, (H) glyoxylate reductase.

The TCA cycle is the dominant metabolic route of yeast when using sugars as a carbon source in cultivations. When more simple compounds, such as acetate or ethanol, are used as substrate, the TCA cycle cannot produce enough biosynthetic precursors to maintain cell growth. Therefore, yeast employs a modified metabolic route of TCA called the glyoxylate cycle (Figure 3) which is able to convert two-carbon substrates into four-carbon dicarboxylic acids. As in the TCA cycle, acetyl-CoA reacts with oxaloacetate to produce citrate which in turn is converted to isocitrate. The glyoxylate cycle requires two additional enzymes. One is isocitrate lyase which converts isocitrate to succinate and glyoxylate. The other is malate synthase, that is used to produce malate from acetyl-CoA and glyoxylate. [26] In addition, glyoxylic acid can be converted to glycolic acid but the conversion is not efficient.

The difference between the TCA and glyoxylate cycles, in addition to the carbon source, is that the former occurs in the mitochondria and the latter in the cytosol and peroxisome of the cell. When glucose is utilized, it is converted to pyruvate that can enter the mitochondrial matrix. Pyruvate is oxidatively converted to acetyl-CoA, which enters the TCA cycle. When ethanol or acetate is used as carbon source, the conversion into acetyl-CoA occurs in cytosol, where it enters the glyoxylate cycle. In addition, lactic acid can be produced from pyruvate. [27] The interaction of these metabolic routes can also been seen in Figure 3.

The glyoxylate cycle can be further modified by metabolic engineering to convert glyoxylic acid to glycolic acid. Glycolic acid is one of the building-block chemicals that can be produced in bioprocesses. As depicted earlier, glycolic acid can be produced from glyoxylate but the conversion is not very efficient in yeast and the yield is low. In genetically modified *K. lactis* yeast the production of glycolic acid was enabled by deletion of the genes encoding malate synthase (MLS2) and by overexpressing the genes for isocitrate lyase (ICL1) and glyoxylate reductase (GLYR1) (Figure 4). [28]

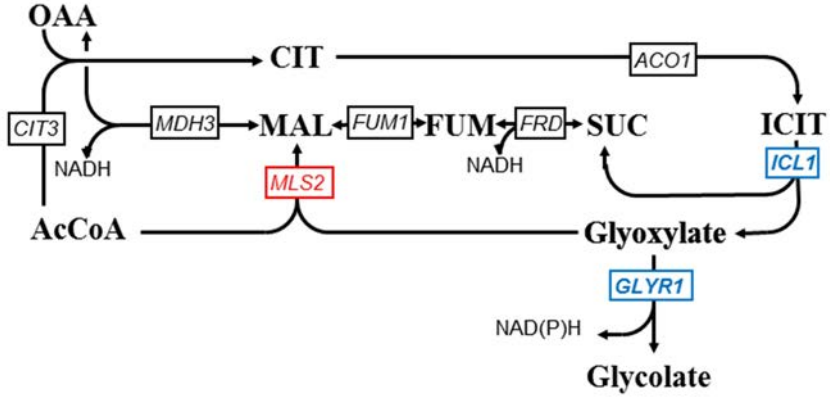

Figure 4. Engineered glyoxylate cycle. [28] Enzymes: CIT3 citrate synthase, ACO1 aconitase, ICL1 isocitrate lyase, FRD fumarate reductase/succinate dehydrogenase, FUM1 fumarase, MDH3 malate dehydrogenase, MLS2 malate synthase, GLYR1 glyoxylate reductase. The overexpressed enzymes are indicated in blue and the deleted enzyme in red.

Carboxylic acids are also formed during hydrolysis of lignocellulosic material. The most abundant carboxylates generated are acetic acid that is released from hemicellulose by de-acetylation and levulinic acid originating from cellulose and hemicellulose. Some formic acid is also produced from the same sources as levulinic acid. [29] These acids act as inhibitors in bioprocesses. Figure 5 illustrates the known inhibition mechanisms of weak acids in *S. cerevisiae*. In high concentrations they inhibit yeast fermentation by reducing biomass growth and ethanol yield. The main mechanisms of inhibition are presented in two theories: the uncoupling

theory and the intracellular anion accumulation theory. According to the uncoupling theory, the dissociated weak acid can diffuse from fermentation medium across the plasma membrane of the yeast, thus decreasing the cytosolic pH. Plasma membrane ATPase, which pumps protons out of the cell, is activated and it tries to increase intracellular pH. This causes ATP depletion in cytosol and leads to decreased biomass formation. However, in low acid concentrations, the ATP production is probably stimulated by the acids, leading to increased biomass formation and ethanol yield. The intracellular anion accumulation theory states that the anionic form of the acid is captured inside the cell and the undissociated acid will diffuse into the cell until equilibrium is reached. Formic acid is more inhibitory than levulinic acid, which in turn is more inhibitory than acetic acid. Weak acids have also been demonstrated to inhibit yeast growth by reducing the uptake of aromatic amino acids from the cultivation medium. [30]

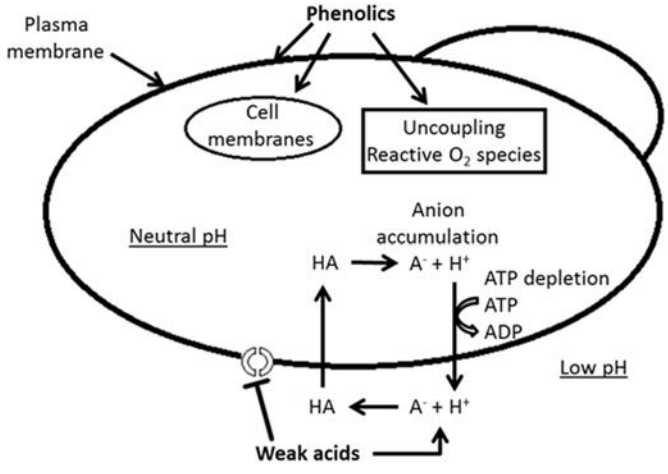

Figure 5. Known inhibition mechanisms of phenolic compounds and weak acids in *S. cerevisiae*. Adapted from [30]

1.1.4 Phenolic acids in bioprocesses

Aromatic compounds that are present in bioprocesses originate from the lignin breakdown and carbohydrate degradation during the hydrolysis of lignocellulosic material. They are present in very diverse forms and in low concentrations because of their low solubility in water. The concentrations are dependent on the harshness of the hydrolysis. Phenolic compounds have inhibitory effects on microorganisms even at low concentrations, and the compounds with the highest inhibitory effect are low molecular weight molecules. [29] Known inhibition mechanisms of phenolic compounds in *S. cerevisiae* are illustrated in Figure 5. The biomass yield, growth rate and ethanol productivity of the yeast are more

decreased than ultimate ethanol yields. It has been noted that low-molecular weight phenolic compounds are more inhibitory than high-molecular weight phenolic compounds and that the substituent position has an effect on compound toxicity. Furthermore, the higher hydrophobicity of the phenolic compounds correlates with reduced ethanol productivity in yeast. In general, aldehydes and ketones act as stronger inhibitors than acids, which are in turn more inhibitory than alcohols. The inhibition mechanisms of phenolic compounds have been studied, but the mechanisms are still not completely understood. Phenolic compounds may act on cell membranes, causing loss of integrity which leads to loss of membrane functionality as selective barrier and enzyme matrix. Weakly acidic phenolic compounds may destroy the electrochemical gradient by transporting the protons back across the mitochondrial membranes. They can also cause uncoupling and accumulation of reactive oxygen species in the cell. [30]

1.1.5 Amino acids in bioprocesses

In nature, nitrogen sources always occur in diverse and complex forms. Nitrogen is also one of the main elements in many macromolecules of living organisms, playing a central role in structure and function, and most organisms have detailed control mechanisms to maintain a constant supply of nitrogen. [31] Yeast is able to use a wide variety of compounds as nitrogen source, but it prefers ammonia or the amino acids asparagine, glutamine and glutamate. In the absence of these primary nitrogen sources or if they are present in concentrations low enough to limit growth, other nitrogen sources, such as nitrite, nitrate, amides, peptides and other amino acids can be utilized. [32]

In bioprocess technology, it has been noted that the amino acid composition of cultivation broth has an effect on biomass accumulation, productivity and viability of yeast during the cultivation. In addition, in beer fermentation amino acids affect the concentration of flavor-active higher alcohols, vicinal diketones, and esters. The total concentration and composition of amino acids is also important because the use of sugar supplements in cultivation medium reduces the nitrogen/carbon ratio, resulting in potential limited growth of yeast and the need for nitrogen supplement addition. Brewery (all-malt) wort is composed of a wide variety of natural nitrogen sources, of which amino acids are the most abundant. It contains all the physiologically active amino acids but there are differences in individual amino acid concentrations between different wort types. Amino acids are taken up sequentially, although the exact order of uptake is strain specific. [33]

1.2 Bioprocess monitoring

Monitoring and control of processes in bioreactors have gained well-earned attention in biotechnology. The most important measurements in bioprocess monitoring are the determination of substrate uptake rate, specific growth rate of the organism, and the product formation rate. In practice, the most widely

measured and controlled parameters are pH, dissolved oxygen and carbon dioxide, temperature and pressure. Cell density, substrates and products are the main critical process parameters in bioprocesses that also need to be monitored in order to enable more efficient, sustainable and controlled applications in biotechnology. Measurements of yield, final concentration of the product and side-products, and both volumetric (grams of product per volume per hour, g L^{-1} h^{-1}) and specific (grams of product per cell mass per hour, g g^{-1} L^{-1}) productivity are of great importance in the definition of successful process. [10]

1.2.1 Chromatography and electrodriven separations

1.2.1.1 Carboxylic acids

Carboxylic acids are intermediates or final metabolites of several biochemical pathways in living organisms, as well as products in industrial biotechnological processes. Therefore, the analysis of these compounds can serve as an indicator of a process and as a means of quality control. [34] Carboxylic acids are weak organic acids and they are partially dissociated in aqueous systems. According to their pK_a-value and pH of the solution, equilibrium is established between undissociated, uncharged molecules and their anionic form(s). [35]

Traditionally carboxylic acids have been analyzed by gas or liquid chromatography but an increasing number of articles describing capillary electrophoresis as an analysis technique have recently been published.

Gas chromatography (GC) is an analysis technique for the determination and separation of volatile and thermally stable compounds. In addition, with a derivatization procedure many compounds can be modified to enable their analysis with GC. In the gas phase analyte molecules move along with the carrier gas and they are affected by random diffusion and random collisions with the carrier gas molecules. The molecules can also move and diffuse in the stationary phase of the capillary. This procedure is influenced by the thickness of the stationary phase, and by the size and diffusion constant of the molecule. When a molecule diffuses to the surface of the stationary phase, it can detach from it and move back to the gas phase. The probability of a molecule being moved to the gas phase depends on its kinetic energy and molecular interactions with the stationary phase. The kinetic energy is dependent on the temperature. The separation of analyte molecules is defined by the number and frequency of the contacts with the stationary phase. On the other hand, the diffusion rate of analyte is dependent on the properties of the gas, analysis temperature and the molecular mass of the analyte. [36]

Detectors used in carboxylic acid analyses include flame-ionization detection (FID) and mass spectrometer (MS). In most cases, carboxylates are derivatized by silylation prior to the analysis. The derivatization procedures are laborious and time-consuming analysis steps. In bioprocess monitoring, GC has been used to analyse carboxylic acids in anaerobic cultivation of municipal solid waste [37, 38], microbial culture media [39], cultured maize embryos [42], foodstuffs such as beer,

wine and soy sauce [43], and in fermented soy bean paste [46]. In addition, it has been used to study carboxylic acids in tobacco [40], rye grass [41], and clinical samples [44, 45]. GC methods for the analysis of carboxylates in different matrices are presented in Table 3.

Table 3. GC methods for carboxylic acid analysis.

Detection	Derivati-zation	Application [a]	Analytes	Ref.
FID	-	**Aliphatic carboxylic acids in anaerobic cultivation of municipal solid waste**	Ace, But, Cap, Hep, Pro, Val	[37]
MS	-	**Aliphatic carboxylic acids in anaerobic cultivation of municipal solid waste**	Ace, But, For, Pro	[38]
	Silylation	**Organic acid profile of culture media from *Lactobacillus pentosus* and *Pediococcus lolli***	Cit, Gla, Lac, Pyr, Suc	[39]
	Silylation	Volatile organic acids in tobacco	Ace, Buta, Cap, Dec, Dod, For, Fur, Hep, Hex, Non, Oct, Pen, Pro, Tet	[40]
	Silylation	Organic acids in rye grass samples	Cit, Fum, Glu, Gly, Icit, Mal, Male, Oxa, Pyr, Suc, Tar	[41]
	Silylation	**Organic acids in cultured maize embryos**	Akg, Cit, Fum, Icit, Mal, Oaa, Suc	[42]
	-	**Organic acids in foodstuffs**	Ace, But, Cit, Dod, Fum, Hda, Hex, Lac, Lev, Mal, 2-Mbut, Non, Oct, Pen, Pro, Sor, Suc	[43]
	Silylation	Organic acids in human plasma, urine and rat brain tissue	Aaa, Gluy, Oaa, Pyr	[44]
	Silylation	Metabolomic profiling of human urine in hepatocellular carcinoma	Ace, But, Male, Pro, Tar, Xyl	[45]
	Silylation	**Metabolite profiling of a fermented soybean paste during fermentation**	Cit, Fum, Gal, Glu, Ita, Lac, Mal, Malo, Oxa, Suc, Tar	[46]

[a] Bioprocess monitoring applications are in **bold.**

Aaa acetoacetate, Ace acetate, Akg α-ketoglutarate, But butyrate, Buta butanoic acid, Cap caproate, Cit citrate, Dec decanoic acid, Dod dodecanoic acid, For formate, Fum fumarate, Fur 2-furoic acid, Gal galactarate, Gla glycolate, Glu gluconate, Gly glyceric acid, Glyo glyoxylate, Had heptadecanoic acid, Hep heptanoic acid, Hex hexanoic acid, Icit isocitrate, Ita itaconate, Lac lactate, Lev levulinic acid, Mal malate, Male maleinate, Malo malonate, Non nonanoic acid, Oaa oxaloacetate, Oct octanoic acid, Oxa oxalate, Pen pentanoate, Pro propionate, Pyr pyruvate, Sor sorbic acid, Suc succinate, Tar tartrate, Tet tetradecanoic acid, Val valeric acid

Liquid chromatography (LC) has been used extensively for the analysis of carboxylic acids from various matrices and applications. The most common LC sub-technique used is ion chromatography (IC), but reversed phase liquid chromatography (RPLC) has also been used. IC is an analysis technique to separate ionic compounds, such as inorganic cations and anions, and low-molecular-weight (LMW) organic acids and bases. The separation can be based on ion-exclusion, ion-exchange and/or ion-pair phenomenon. [47] In ion-exclusion chromatography, the electric charges of the dissociated functional groups in the stationary phase of the column are the same as that of the ionic compounds to be separated. Thus,

when analyzing anionic compounds, such as carboxylates, cation-exchange resin functionalized with anionic groups (e.g. sulfonate, carboxylate) is used in the column. The dissociation equilibrium that is formed between the neutral, undissociated form and the corresponding anionic form of the acidic solute is vitally important. Amongst other things, this equilibrium is dependent on the acidity and activity of the analyte, and on the proton activity, electrolyte content and dielectric constant of the mobile phase. The more dissociated the analytes are, the less interaction there is with the stationary phase because of electrostatic repulsion, and the faster they reach the detector. The undissociated analytes have an interaction with the stationary phase, which causes retardation related to the mobile phase flow. [48] The separation principle of ion-exchange chromatography is the opposite to that of ion-exclusion chromatography: with anionic analytes, anion-exchange resin is used in the column. Hence the more dissociated analytes interact with the stationary phase longer than undissociated ones that elute with the mobile phase flow. The retention is mainly influenced by the counter-ion type, temperature, and the ion strength, pH and modifier content of the mobile phase. When using ion-pair chromatography, a lipophilic ionic compound is added to the stationary phase of the column to enhance the formation of ion-pairs between stationary phase and analyte. [47]

RPLC is probably the most common LC technique in general. It is well suited to the analysis of polar and ionogenic analytes. The stationary phase is nonpolar, chemically modified silica or other nonpolar packing material, and the mobile phase is a mixture of organic solvent and aqueous buffer or water. The retention is based on the interactions between analyte and solvent because the interaction between analyte and stationary phase is relatively weak. The retention decreases with increasing polarity of the analyte and the most important parameter affecting the retention of nonionic analytes is the concentration and type of the organic modifier. A buffer chemical (phosphate, ammonium acetate, formate or carbonate) is often used in RPLC to reduce the protolysis of ionogenic analytes because the retention of ionic compounds is low. [47]

Carboxylic acids are usually monitored by refractive index (RI) or ultraviolet (UV) detectors but electrochemical (ED), conductivity (CD) and mass spectrometer (MS) detectors have also been used. In bioprocess monitoring, IC has been used to analyze carboxylic acids in cultivation media [50], different cultivations [52, 53, 61], biohydrogen production [54], wine [55], autohydrolyzed bagasse [57], cultured maize embryos [42], pretreated lignocellulosic biomass [59], and milk fermentation [60]. Other applications include kraft pulp liquors [49, 58] and different juices [51, 56]. RPLC has been used to study carboxylates in lignocellulosic biomass [59], milk fermentation [60] and *Escherichia coli* cultivation [61]. Examples of LC methods for the analysis of carboxylic acids are presented inTable 4.

Table 4. LC methods for carboxylic acid analysis.

LC mode	Detection	Application [a]	Analytes	Ref.
IC	CD, MS	Analysis of the acid fraction from kraft pulp liquors	Ace, Caa, 3,4-Ddp, For, Gis, Gly, 2-Hba, 2-Hpa, Lac, Mal, Msuc, Oxa, Suc, Xis	[49]
	CD	**Organic profiles of four different cultivation media**	Ace, Aco, Akg, But, Cca, Cit, For, Fum, Glu, Icit, Lac, Mal, Male, Oxa, Pro, Pyr, Suc, Tar	[50]
	ED	Organic acids in grape juice	Akg, Cit, Fum, Mal, Oxa, Suc, Tar	[51]
	RI	***Lactobacillus buchneri* cultivation**	Ace, Lac	[52]
	UV	***Fibrobacter succinogenes* and *Clostridium coccoides* cultivations**	Ace, Akg, But, Cit, For, Fum, Iba, Lac, Mal, Pro, Pyr, Suc	[53]
		Fermentative biohydrogen production	Ace, But, Iba, Pro	[54]
		Organic acids in wine	Ace, Cit, For, Lac, Mal, Suc, Tar	[55]
		Organic acids in orange juice	Cit, Mal, Suc	[56]
		Organic acid composition of auto-hydrolyzed bagasse	Ace, For, Lev	[57]
	MS	Analysis of the acid fraction from kraft pulp liquors	Adi, Fum, Gis, Glu, Gly, Lac, Mal, Male, Msuc, Oxa, Suc	[58]
		Organic acids in cultured maize embryos	Akg, Cit, Fum, Icit, Mal, Oaa, Suc	[42]
RPLC	UV	**Organic profile of pretreated ligno-cellulosic biomass**	Ace, Adi, For, Fum, Ita, Lac, Lev, Mal, Male, Mmal, Pro, Suc	[59]
		Organic acids in milk fermentation process	Ace, Cit, For, Lac	[60]
	MS	***Escherichia coli* cultivation**	Ace, Cit, Fum, 3-Hpr, Lac, Mal, Malo, Pro, Pyr, Suc	[61]

[a] Bioprocess monitoring applications are in **bold**

Ace acetate, Aco aconitate, Adi adipinate, Akg α-ketoglutarate, But butyrate, Caa chloroacetate, Cca citraconate, Cit citrate, 3,4-Ddp 3,4-dideoxy-pentonate, For formate, Fum fumarate, Gis glucoisosaccharinate, Glu glutarate, Gly glycolate, 2-Hba 2-hydroxybutanoate, 2-Hpa 2-hydroxy-4-pentenoate, 3-Hpr 3-hydroxypropionate, Iba isobutyrate, Icit isocitrate, Ita itaconate, Lac lactate, Lev levulinate, Mal malate, Male maleinate, Malo malonate, Mmal methylmalonate, Msuc methylsuccinate, Oaa oxaloacetate, Oxa oxalate, Pro propionate, Pyr pyruvate, Suc succinate, Tar tartrate, Xis xyloisosaccharinate

Capillary electrophoresis (CE) has become an increasingly important analysis method in the determination of carboxylic acid composition. The separation principles of CE are presented in Section 1.3. The use of capillary electrophoresis in the monitoring of bioprocesses has been extensively reviewed by Alhusban et al. [62]

For the analysis of carboxylates, UV detection, especially indirect UV detection, is the most common detection method. The principle of indirect UV detection is illustrated in Section 1.3.3. In addition, CD and MS have been used as detection methods. In bioprocess monitoring, carboxylates have been studied in beverages [55, 63, 64, 69, 70, 76], cell extracts [73, 87, 88], milk fermentation [75], and cultivations of white-rot fungi [78] and *Catharanthus roseus* cells [85]. Other applications include soil and plant extracts [65], coffee [66], juices [67, 86] drugs [68, 79], alfalfa roots [71], Bayer liquor [74], aerosol particles [77, 84], amine solutions [80], honey [81], biodiesel [82] and cellulose processing effluents [83]. Examples of CE analyses of carboxylic acids are summarized in Table 5. All the examples used CZE.

Table 5. CE methods for carboxylic acid content measurement.

Detection	Application [a]	Analytes	Ref.
UV	**Organic acids in beverages**	Ace, But, Cit, For, Glu, Gluc, Lac, Mal, Male, Oxa, Pyr, Suc, Tar	[63]
	Organic acids in grape-derived products	Ace, Cit, For, Fum, Lac, Mal, Oxa, Suc, Tar	[64]
	Organic acids in soil and plant extracts	Ace, Cit, Fum, Mal, Male, Malo, Oxa, Tar	[65]
	Short-chain organic acids in coffee	Ace, Cit, Citr, For, Fum, Gly, Icit, Lac, Mal, Male, Mes, Oxa, Pro, Suc	[66]
	Adulteration markers in orange juice	Cit, Icit, Mal, Tar	[67]
	Organic acids in traditional Chinese medicine	Fum, Lau, Lin, Suc	[68]
	Organic acids in wines	Ace, Cit, Fum, Lac, Mal, Oxa, Suc, Tar	[69]
	Organic acids in beer	Akg, Fum, Mal, Mes, Oxa, Pyr	[70]
	Organic acids in Plateau alfalfa roots	Aco, Cit, Mal	[71]
indirect UV	**Organic acids in port wine**	Ace, Glyo, Lac, Mal, Suc, Tar	[72]
	Carboxylic acid metabolites from the tricarboxylic acid cycle in *Bacillus subtilis* cell extract	Ace, Akg, Cit, For, Fum, Icit, Lac, Mal, Pyr, Suc	[73]
	Organic acids in Bayer liquor	Ace, For, Malo, Oxa, Suc	[74]
	Carboxylates in milk fermentation using *Lactobacillus delbruecki* and *Streptococcus thermophilus*	Ace, Cit, For, Lac	[75]
	Organic acids in beverages	Ace, Cit, Lac, Mal, Suc, Tar	[76]
	Dicarboxylic acids in atmospheric aerosol particles	Adi, Aze, Glu, Malo, Oxa, Pim, Seb, Sub, Suc	[77]
indirect UV	**Production of organic acids by different white-rot fungi**	Mal, Malo, Oxa, Tar	[78]
	Organic acids in pharmaceutical drug substances	Ace, For, Msa, Piv, Suc, Tfa	[79]
	Organic acids in amine solutions for sour gas treatment	Ace, But, For, Gly, Mal, Oxa, Pro, Tar	[80]
	Organic acids in honey	Cit, For, Gluc, Mal, Oxa, Cit	[81]
	Organic acids in wines	Ace, Cit, For, Lac, Mal, Suc, Tar	[55]
CD	Carboxylic acids in biodiesel	Ace, For, Pro	[82]
MS	Carbohydrate- and lignin-derived components in complex effluents from cellulose processing	Aze, Dec, Glc, Glr, 8-Hoa, Mal, Suc, Thr, Xyl	[83]
	Functionalized carboxylic acids from atmospheric particles	Adi, Aze, Cma, Glu, 8-Hoa, 3-Hmg, 5-Oaa, 6-Oha, 7-Ooa, 4-Opa, 4-Opim, 4-Osa, Pim, Seb, Sub	[84]
	Anionic metabolites for *Catharanthus roseus* (a flower) cultured cells	Akg, Cit, Fum, Icit, Mal, Suc	[85]
	Carboxylic acids in apple juice	Cit, Mal, Male, Suc, Tar	[86]
	Organic acids in *Bacillus subtilis* extracts	Akg, Cit, Fum, Lac, Mal, Pyr, Suc	[87]
	Carboxylic acids in *Escherichia coli* extracts	Akg, Cit, Mal, Suc	[88]

[a] Bioprocess monitoring applications are in **bold**

Ace acetate, Aco aconitate, Adi adipinate, Akg α-ketoglutarate, Ara arabonic acid, Aze azelaic acid, But butyrate, Cit citrate, Citr citraconate, Cma citramalate, Dec decanoic acid, For formate, Fum fumarate, Gal galacturonic acid, Gala galactaric acid, Glc glycerate, Glr glucoronate, Glu glutarate, Gluc gluconate, Gly glycolate, Glyo glyoxylate, 3-Hmg 3-hydroxy-3-methylglutarate, 8-Hoa 8-hydroxyoctanoic acid, Iba isobutyrate, Icit isocitrate, 2-Ipa 2-isopropylmalate, Ita itaconate, Lac lactate, Lau lauric acid, Lev levulinate, Lin linolenic acid, Mal malate, Male maleinate, Malo malonate, Mes mesaconic acid, Mmal methylmalonate, Msa methanesulfonic acid, Msuc methylsuccinate, 5-Oaa 5-oxoazelaic acid, 6-Oha 6-oxoheptanoic acid, 7-Ooa 7-oxooctanoic acid, 4-Opa 4-oxopentanoic acid, 4-Opim 4-oxopimelic acid, 4-Osa 4-oxosebabic acid, Oxa oxalate, Pim pimelic acid, Piv pivalic acid, Pro propionate, Pyr pyruvate, Seb sebacic acid, Sor sorbate, Sub suberic acid, Suc succinate, Tar tartrate, Tfa trifluoroacetate, Th threonic acid, Xyl xylonate

1.2.1.2 Phenolic acids/compounds

In the IUPAC Gold Book it is defined that phenols are compounds having one or more hydroxyl groups attached to a benzene or other arene ring. [9] For example in winemaking technology, phenolic compounds are responsible for wine sensory properties such as color, flavor, astringency and bitterness. [89] In addition, phenolic compounds are present in vegetables, fruits, chocolate, honey, herbs, beverages, oil and cereals as antioxidants. [90] In bioprocesses, phenolic compounds are produced by some fungi and bacteria as a part of their secondary metabolism [91] or they originate from hydrolyzed lignocellulosic material [92]. The use of GC for the analysis of phenolic compounds is a time-consuming process because of the necessary purification of the sample and silylation before analysis. In bioprocess monitoring, GC has been used to study phenolic compounds in beer, wine and soy sauce [43]. In addition, it has been used to analyse phenolic compounds in tobacco [40], rye grass samples [41], clinical samples [93, 94], leaf extracts [95], yerba mate [96] and beverages [97, 98]. GC methods for the analysis of phenolic compound are presented in Table 6.

Table 6. GC methods for phenolic compound analysis.

Detection	Derivatization	Application [a]	Analytes	Ref.
MS	Silylation	Volatile organic acids in tobacco	Ben	[40]
	Silylation	Organic acids in the metabolites in rye grass samples	Asc, Ben, 4-Hba, 6-Hba, Nic, Qui	[41]
	-	**Organic acids in foodstuffs**	Ben, Paa, Pht, Tol	[43]
	Silylation	Metabolic profiling of cerebrospinal fluid	Ben, Hip, 4-Nba	[93]
	Silylation	Metabolic profiling of human urine	Asc, Ura	[94]
	Silylation	Metabolic profiling of *Arabidopsis thaliana* leaf extracts	Ben	[95]
	-	Phenolics in yerba mate	Ben, Fur, Gua, Phe, Van	[96]
FID	-	Organic acids in beverage samples	Ben, Sor	[97]
	Methylation	Phenolics in soft drink, juice, food dressing, and cough syrup	Ben, Sor	[98]

[a] Bioprocess monitoring application are in **bold**

Asc ascorbate, Ben benzoic acid, Fur furfural, Gua guaiacol, 4-Hba 4-hydroxybenzoate, 6-Hba 6-hydroxybenzoate, Hip hippuric acid, 4-Nba 4-nitrobenzoic acid, Nic nicotinic acid, Paa phenylacetate, Phe phenol, Pht phthalate, Sor sorbic acid, Qui quinic acid, Tol toluic acid, Ura uric acid, Van vanillin.

In the analysis of phenolic compounds, RPLC with UV detection is the most common LC technique. In bioprocess monitoring, RPLC has been used to study phenolic compounds in wines [99–101] and lignocellulosec biomass [59]. In addition, it has been used in the analysis of orange juice [56] and in retention modeling [102]. The methods for the analysis of phenolic compounds by RPLC are presented in Table 7.

Table 7. LC methods for phenolic acid analysis.

Detection	Application [a]	Analytes	Ref.
UV	**Phenolic composition of grape extracts and wines**	Cat, Cou, Gal, Epi, Res	[99]
	Organic acids in orange juice	Asc, Caf, Chl, Cou, Fer, Gal, 4-Hba, Pro, Sin, Vaa	[56]
	Organic profile of pretreated lignocellulosic biomass	Ben, Fua, Fur, Gal, 3,4-Hba, 3,5-Hba, 4-Hba, 3,4-Hbd, 4-Hbd, 4-Hc, 3-Mca, 4-Mca, Phe, Sya, Syr, Tol, Vaa, Van	[59]
	Phenolic compounds in red wine	Caf, Cat, Cou, Fer, Mor, Que	[100]
	Phenolic compounds in Brazilian fortified wines	Caf, Cat, Cou, Fer, Gal, Que, Res	[101]
	Retention modeling	Ben, Pht, Sal	[102]

[a] Bioprocess monitoring application are in **bold**

Asc ascorbate, Ben benzoate, Caf caffeic acid, Cat catechin, Chl chlorogenic acid, Cou p-coumarate, Epi epicatechin, Fer ferulate, Fua 2-furoic acid, Fur furfural, Gal gallic acid, 3,4-Hba 3,4-dihydroxybenzoate, 3,5-Hba 3,5-dihydroxybenzoate, 4-Hba 4-hydroxybenzoate, 3,4-Hbd 3,4-hydroxybenzaldehyde, 4-Hbd 4-hydroxybenzaldehyde, 4-Hc 4-hydroxycoumarin, 3-Mca 3-hydroxy-4-methoxycinnamate, 4-Mca 4-hydroxy-3-methoxycinnamate, Mor morin, Phe phenol, Pht phtalic acid, Pro protocatechuic acid, Que quercitin, Res resveratrol, Sal salicylic acid, Sin sinapic acid, Sya syringic acid, Syr syringaldehyde, Tol toluic acid, Vaa vanillate, Van vanillin.

Capillary electrophoresis has been used to study phenolic compounds from various matrices. CZE is the most common CE technique, but MEKC has also been used. The most common detection method is UV because of the good UV absorbance of the benzene ring in the structure. In addition, MS has been used as a detection method. In bioprocess monitoring, CE has been used for the determination of phenolic compounds in different beverages [63, 70, 104–106] and in cultured cells of *Catharanthus roseus* [85]. It has also been used for the analysis of phenolic compounds in soil and plant extracts [65], coffee [66], Chinese medicine [68], oils [90, 103], plants [71, 107], cellulose processing effluents [83], and from athmospheric particles [84] and aerosols [108]. CE analysis methods of phenolic compounds are presented in Table 8.

Table 8. CE for the analysis of phenolic compounds.

Detection	Application [a]	Analytes	Ref.
UV	**Organic acids in beverages**	Asc, Ben, Sor	[63]
	Organic acids in soil and plant extracts	Ben, Cou, Fer, 4-Hba, Pht, Sal, Sin	[65]
	Short-chain organic acids in coffee	Fur, Pyro, Qui	[66]
	Organic acids in traditional Chinese medicine	Asc, Ben, Caf, Cou	[68]
	Phenolic acids in vegetable oils	Ben, Caf, Cin, Cou, Fer, Gal, Hba, 2,4-Hba, Syr, Vaa	[90]
	Phenolics in virgin olive oil	Caf, Cou, Fer, 3,4-Hpa, 4-Hpa, Vaa	[103]
	Organic acids in beer	4-Aba, 4-Hba, Ben, Fer, Gal, Pht, Pro, Pyro, Sin, Sor, Syr	[70]
	Phenolic compounds in wines	Caf, Cat, Cin, Cou, Epi, Fer, Gal, 3,4-Hba, Kae, Myr, Nar, Que, Res, Rut, Vaa	[104]
	Organic acids in Plateau alfalfa roots	Gal	[71]
	Phenolic compounds in red wine	Caf, Cat, Cou, Epi, Fer, Gal, Kae, Pro, Que, Rut, Syr, Vaa	[105]
	Polyphenols in Spanish wines	Caf, Cin, Cou, Epi, Fer, Fis, Gal, 3,4-Hba, 4-Hba, Hga, Hva, Que, Res, Sin, Syr, Van, Ver	[106]
	Phenolic acids in *Brassica oleracea* (broccoli)	Caf, Cou, Fer, Sin	[107]
MS	Carbohydrate- and lignin-derived components in complex effluents from cellulose processing	Ava, Fer, 4-Hap, 4-Hba, 4-Hbd, Vaa, Van	[83]
	Functionalized carboxylic acids from atmospheric particles	Ben, Cin, 2-Hba, 4-Hba, 2-H3n, 2-H4n, Hpht, 4-Mpa, Pht, Tpa	[84]
	Anionic metabolites for *Catharanthus roseus* cultured cells	Shi	[85]
	Organic acids in atmospheric aero-sols	Ben, 4-Mpa, Pht, Pyr, Tol, Tere, Tri, Trim	[108]

[a] Bioprocess monitoring applications are in **bold**

4-Aba 4-aminobenzoate, Ace acetoguaiacone, Asc ascorbate, Ava acetovanillone, Ben benzoate, Caf caffeic acid, Cat catechin, Cin trans-cinnamate, Con coniferyl aldehyde, Cou p-coumarate, Epi epicatechin, Fer ferulate, Fis fisetin, Fur furanoate, Gal gallic acid, 4-Hap 4-hydroxyacetophenone, Hba p-hydroxybenzoate, 2-Hba 2-hydroxybenzoate, 3,4-Hba 3,4-dihydroxybenzoate, 4-Hba 4- hydroxybenzoate, 4-Hbd 4-hydroxybenzaldehyde, Hga homogentistic acid, 2-H3n 2-hydroxy-4-nitrobenzoate, 2-H4n 2-hydroxy-4-nitrobenzoate, 3,4-Hpa 3,4-dihydroxyphenylacetate, 4-Hpa 4-hydroxyphenylacetate, Hpht homophthalate, Hva homovanillate, Kae kaempferol, 4-Mpa 4-methylphtalate, Myr myricetin, Nar narirutin, Phe phenol, Pht phthalate, Pro protocatechuic acid, Pyr pyromellitic acid, Pyro pyroglutamate, Que quercitin, Qui quinate, Res resveratrol, Rut rutin, Sal salicylic acid, Shi shikimate, Sin sinapinate, Sor sorbate, Sya syringaldehyde, Syr syringic acid, Tere terephthalate, Tol toluic acid, Tpa terephthalate, Tri trimesic acid, Trim trimellitic acid, Vaa vanillic acid, Van vanillin, Ver veratric acid

1.2.1.3 Amino acids

Amino acids are important metabolites of the cell. They are the building blocks of proteins and peptides, which makes them one of the most important groups of small molecules in living organisms. Usually they contain one or two primary ami-

no groups, one or two carboxyl groups, and a side chain with properties varying between different amino acids. [109] Because of the high polarity, low volatility and zwitterionic character of amino acids, their simultaneous separation and detection can be difficult. When using GC as an analysis method, amino acids must be derivatized to increase their volatility. FID and MS are the most common detectors in GC analysis of amino acids. In bioprocess monitoring, amino acids have been studied by GC in fermented soy bean paste [46] and beer [110]. It has also been used in the analysis of amino acids in rye grass [41], clinical samples [45, 93, 94], leaf extracts [95], and in quince fruit and jam [111]. GC methods for the analysis of amino acids from different applications are summarized in Table 9.

Table 9. GC for the analysis of amino acids.

Detection	Derivatization	Application [a]	Analytes	Ref.
MS	Silylation	Amino acids in the metabolites in rye grass samples	Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	[41]
	Silylation	Metabolomic profiling of human urine in hepatocellular carcinoma	Ala, Asp, Gly, Leu, Lys, Pro, Ser, Thr, Trp, Val	[45]
	Silylation	**Metabolite profiling of a fermented soybean paste during fermentation**	Ala, Arg, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	[46]
	Silylation	Metabolic profiling of cerebro-spinal fluid	Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val	[93]
	Silylation	Metabolic profiling of human urine	Ala, Asp, Gly, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, Val	[94]
	Silylation	Metabolic profiling of *Arabidopsis thaliana* leaf extracts	Ala, Asp, Glu, Leu, Val	[95]
FID	BHT	**Amino acids in beer and raw materials**	Ala, Asp, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val	[110]
	Chloroformate	Free amino acids in quince fruit and jam	Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	[111]

[a] Bioprocess applications are in **bold**

Ala alanine, Arg arginine, Asn asparagine, Asp aspartate, Cys cysteine, Gln glutamine, Glu glutamate, Gly glycine, His histidine, Ile isoleucine, Leu leucine, Lys lysine, Met methionine, Phe phenylalanine, Pro proline, Ser serine, Thr threonine, Tyr tyramine, Trp tryptophan, Val valine

HPLC has been used to study amino acids in various matrices. The most common technique is RPLC because amino acids are polar compounds. When using UV or LIF detection, amino acids are usually derivatized pre- or post-column. Underivatized amino acids can be detected with MS. RPLC has been used to study amino acids in fermentations [115, 117]. In addition, it has been used for the determination of amino acids in clinical samples [112–114, 116]. IC has been used to study amino acids in urine. [118] Applications for amino acid analyses are presented in Table 10.

Table 10. HPLC for the analysis of amino acids.

LC mode	Detection	Application [a]	Analytes	Ref.
RPLC	UV	Amino acids in rat serum	Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Hyp, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Tau, Thr, Trp, Tyr, Val	[112]
		Amino acids of hydrolyzed proteins	Ala, Arg, Asn, Asp, Gln, Glu, Leu, Lys, Phe	[113]
		Amino acids in patient blood samples	Ile, Leu, Met, Phe, Tyo, Val	[114]
	LIF	**Non-canonical amino acids in fermentation process of *Escherichia coli***	Leu, Nleu, Nval, Val	[115]
		Amino acids in biological fluids	Ala, Arg, Asn, Asp, Cys, Glu, Gly, Hcys, His, Ile, Leu, Lys, Met, Orn, Phe, Ser, Tau, Thr, Trp, Tyr, Val	[116]
	MS	**Amino acid metabolism monitoring during fermentation**	Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	[117]
IC	UV	Amino acids in urine	Cre, Crn, His, Met, Phe, Trp, Tyr	[118]

[a]Bioprocess monitoring applications are in **bold**

Ala alanine, Arg arginine, Asn asparagine, Asp aspartate, Cre creatinine, Crn creatine, Cys cysteine, Gln glutamine, Glu glutamate, Gly glycine, Hcys homocysteine, His histidine, Hyp hydroxyproline, Ile isoleucine, Leu leucine, Lys lysine, Met methionine, Nleu norleucine, Nval norvaline, Orn ornithine, Phe phenylalanine, Pro proline, Ser serine, Tau taurine, Thr threonine, Trp tryptophan, Tyo tyrosine, Tyr tyramine, Val valine

Amino acid analysis is very common with CE. The lack of a strong chromophore in the aliphatic amino acid structure sets challenges for the detection. With UV detection, the amino acids must be derivatized or else indirect UV detection must be employed. LIF detection also requires derivatization but the sensitivity is far better than with UV detection. MS is also a versatile detection method for the analysis of amino acids. In addition, CD has been used. CZE has been used to study amino acids in a bacterial cultivation [119] and in beer [120]. In bioprocess monitoring, MEKC has been used to study *Bacillus* subtilis extracts. [87] It has also been used to analyze amino acid concentrations in juice [121], pineapple leafs [122], dietary supplements [123], human serum [124] and soy sauce [125]. CE methods for the amino acid analysis are summarized in Table 11.

Table 11. CE for the analysis of amino acids.

CE mode	Detection	Application [a]	Analytes	Ref.
CZE	UV	**Amino acids in *Bacillus subtilis* cultivation**	Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	[119]
	CD	**Amino acids in Swiss lager beer**	Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	[120]
MEKC	LIF	Amino acids in pomegranate juice and for the detection of adulteration with apple juices	Ala, Arg, Asn, Asp, Glu, Leu, Pro, Ser, Trp	[121]
	MS	**Organic acids in *Bacillus subtilis* extracts**	Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val	[87]
		Amino acids in pineapple leaf extract	Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	[122]
		Amino acids in dietary supplements containing royal jelly	Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val	[123]
		Amino acids in human serum	Ala, Arg, Ile, Leu, Lys, Met, Phe, Pro, Thr, Tyr, Val	[124]
		Amino acids in soy sauce	Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	[125]

[a] Bioprocess application are in **bold**

Ala alanine, Arg arginine, Asn asparagine, Asp aspartate, Cys cysteine, Gln glutamine, Glu glutamate, Gly glycine, His histidine, Ile isoleucine, Leu leucine, Lys lysine, Met methionine, Phe phenylalanine, Pro proline, Ser serine, Thr threonine, Tyr tyramine, Trp tryptophan, Val valine

1.2.2 Online monitoring

The United States Food and Drug Administration (FDA) has launched a Process Analytical Technology (PAT) initiative that encourages the use of real-time process monitoring and controlling tools to enhance the feasibility, productivity and quality control of manufacturing processes. [126] Better monitoring of the production process allows verifying and tracing back the integrity and quality of the products. The greatest advantage of automated measurements is the absence of manual steps of the analysis procedure such as sample pretreatment, which is usually the most laborious and error-prone part of the analysis.

1.2.2.1 *In situ* probes

Most common online monitoring tools in biotechnology are *in situ* probes that are inserted into the bioreactor and provide real-time measurement information. There are several general requirements for a reliable probe in bioprocess monitoring: the possibility of calibration, linear dependency, precision at both low and high cell densities, they should be sterilizable, they should withstand high temperature and

pressure and they should be resistant to corrosion and biologically inactive. For industrial applications, the probe calibration should be repeatable after several cultivations, sterilizations and cleaning cycles. The most common *in situ* probes are based on electrochemical, temperature, direct optical or electrical measurement principles and in most cases are specific for a certain parameter or analyte. [127]

Electrochemical and temperature probes are the prevailing sensor technology in process control. They are used to measure temperature, pH and dissolved oxygen. The temperature analysis is performed by a platinum resistance temperature detector. The electrical resistance of the platinum wire is measured and the result is proportional to temperature. Traditional pH analysis is carried out with a standard potentiometric ion selective glass electrode, and a Clark amperometric electrode is used to measure dissolved oxygen that is a substrate in many bioprocesses. [128]

Optical-density (OD) probes are the most common *in situ* probes for online bioprocess monitoring in bioprocesses using unicellular microorganisms. They are used for estimating cell growth and biomass concentration by measuring light absorption or scattering continuously in the visible (390–700 nm) and near-infrared (750–1400 nm) wavelengths. The wavelength required is dependent on the size of the measured object: smaller objects require lower wavelengths. However, this approach cannot analyze cell viability, and any particles or bubbles that might be present in cultivation media can interfere with the analysis. [129]

Infrared (IR) spectroscopy is becoming more important in bioprocess monitoring. The analysis is based on molecular vibrations of organic compounds in the IR region that are characteristic for certain molecule structures. Sensors using near-IR and mid-IR spectra can simultaneously detect several organic compounds including glucose, fructose, lactate, glutamate, glutamine, proline, ammonia and CO_2. As a drawback, the calibration of this method can be complex and the peaks in IR spectra are usually broad due to overlapping which necessitates multivariate data analysis methods. [126, 130]

Fluorescence spectroscopy is a technique specific for compounds that possess fluorescent properties. The principle of fluorescence is presented in Section 1.3.3 in more detail. It is more sensitive than light absorption measurements because the measured signal has, in principle, zero background. Many biological compounds have fluorescent properties, e.g. proteins, enzymes, coenzymes, amino acids, vitamins, and primary or secondary metabolites of microbial growth. The applications are sensitive but not specific. The most common applications are protein and NAD(P)H sensors to study protein content and for biomass estimation, respectively. Simultaneous measurement of multiple analytes can be achieved with 2D fluorescence, in which the fluorometer uses several excitation and emission wavelengths. However, two compounds with same fluorescent properties cannot be separated. [131, 132]

Dielectric spectroscopy utilizes the complex electrical properties of viable cells through capacitance and conductance measurements. It can provide information on the total and viable cell volume, since only cells with intact membranes have electrical properties. The analysis of viable cells is important as a measure of

growth of the microorganism. In addition, some growth-related products can be indirectly monitored with dielectric spectroscopy. [133]

In general, the issue with spectroscopic techniques is the complexity of the spectrum. In addition, cultivation conditions have an effect on the spectrum. Because of this, the determination of blank and linearity calibration of the measurement range of spectroscopic sensors for the analysis of specific compounds often requires an extensive experimental approach.

1.2.2.2 Separation techniques

Liquid chromatography, gas chromatography and capillary electrophoresis are the most common offline analysis techniques that are used in biotechnology. These methods are also applicable to online monitoring. The advantage of chromatographic online techniques is that there is a wide variety of methods available and several compounds and/or compound groups can be analyzed simultaneously. However, the analysis takes more time than with the *in situ* probes and thus is not performed in real-time mode. In addition, the automated sampling and sample treatment, the most difficult and important parts of the online analyses, involve challenges. The sampling must be safe for the process without any risk of contamination, and the sample must be representative of the cultivation. Furthermore, the sample treatment must be designed for the analysis purposes: cells must be removed from the sample for the analysis of extracellular compounds or cells must be lysed for the analysis of intracellular compounds. A sample port has been designed, constructed and patented for the liquid sampling of bioprocess samples at VTT Technical Research Centre of Finland. [134] Other automated sampling devices are for example bioPROBE (BBI-Biotech, Germany), GPA 1000 (Groton Biosystems, USA), Seg-Flow (Flownamics, USA), and MultiTRACE (Trace Analytics, Denmark).

Liquid chromatography is the most common online chromatographic analysis method. Assembling the device is much simpler than with other chromatographic techniques because the sample is in liquid form. There are at least three manufacturers worldwide that sell online LC devices: Waters® (USA) has developed the PATROL™ UPLC™ Process Analyzer that uses ultraperformance liquid chromatography (UPLC), Dionex (USA) has a DX-800 Process Analyzer that is based on both ion chromatography (IC) and high performance liquid chromatography (HPLC), and Bayer (Germany) has developed the BaychroMAT® process analysis system that combines HPLC, gel permeation chromatography and gas chromatography. In addition, Groton Biosystems has developed a sampling system interface called Automated Reactor Sampling System (ARS™) that is marketed together with Agilent 1200 Series LC system (USA). Furthermore, several research laboratories have made their own online LC devices that have been used in several biotechnological applications. Online HPLC has been used for the monitoring of sugar consumption and carboxylic acid production of *Escherichia coli* [135] and to monitor and control ethanol fermentation by

Zymomonas mobilis [136]. It has also been used to monitor extracellular metabolite concentrations during a batch cultivation of *S. cerevisiae* [137], anions and carboxylic acids in wastewater treatment processes [138], degradation products of dyes in textile industry process waters [139, 140], for carbohydrates and volatile fatty acids in fermentative biohydrogen production [141], and for glucose and ethanol in *Escherichia coli* cultivation [142].

Gas chromatography is the most common chromatographic analysis technique for gaseous and volatile sample compounds whereas for online analysis of aqueous samples it is probably the least popular because of the necessary derivatization procedure. However, online GC has been used to analyze dihydroxyacetone and glycerol from fermentation broths by pyrolytic methylation GC with a vertical microfurnace pyrolyzer. In this system, the analytes were converted into their corresponding methyl ethers and analyzed by GC with a flame ionization detector (FID). [143] An online GC-MS system has also been used to monitor crude wastewater in an automated manner. A two-stage injector was assembled in-house to enable the analysis of 140 volatile and semi-volatile compounds. [144]

Capillary electrophoresis is a new approach for online monitoring. Only one commercial approach has been established. Fixion (Capilix, Netherlands) is an online measurement tool for industrial applications that exploits microchip CE technology and is designed to analyze inorganic cations and anions, and volatile fatty acids. Other applications are performed with reconstructed offline or in-house built CE devices. Online CE (UCE) with an in-house constructed sample flow cell has been developed at VTT Technical Research Centre of Finland. The UCE device has been used for process monitoring in the pulp and paper industry to analyze dissolvable inorganic ions and carboxylic acids from circulation waters of pulp and paper machines. [145, 146] In online bioprocess monitoring, CE has been used to analyze carboxylic acids [147], adenosine triphosphate (ATP) and adenosine diphosphate (ADP) [148], and heavy metals [149]. In addition, glutamate and γ-aminobutyric acid (GABA) levels in rat brain have been studied with *in vivo* microdialysis coupled online to CE-LIF. [150]

1.3 Capillary electromigration techniques

Capillary electrophoresis is a family of related separation techniques that use narrow-bore fused silica capillaries to separate large and small molecules in a high electric field. According to their separation principles, the most common capillary electromigration techniques are divided into the following categories: capillary zone electrophoresis, micellar electrokinetic chromatography, capillary gel electrophoresis, capillary isoelectric focusing and capillary electrochromatography. [151] The uniqueness of capillary electrophoretic techniques is that there are no limitations regarding polar solvents, analytes or samples. Resolution and efficiency are high in optimized methods, creating a great potential for rapid detection and quantification. [152] The techniques used in this study are reviewed briefly in the following.

1.3.1 Capillary zone electrophoresis

In capillary electrophoresis, the separation of analytes is based on their differences in velocity in an electric field. The velocity of an ion (v) can be presented as:

$$v = \mu_{ep} \cdot E \quad (1)$$

where μ_{ep} is the electrophoretic mobility of an ion and E is the electric field strength applied during analysis (in V/cm). The electrophoretic mobility is strongly influenced by the composition of the background electrolyte (BGE) including pH, ionic strength, concentration of organic cosolvents, and electrolyte additives. In addition, the μ_{ep} is proportional to the size (radius, r) and charge (q) of the molecule:

$$\mu_{ep} = \frac{q}{6\pi\eta r} \quad (2)$$

where η is the viscosity of the BGE solution. Therefore, for a given ion and solution medium, the electrophoretic mobility is a constant which is characteristic for that specific ion. From this equation it can be noticed that small, highly charged molecules have high mobility whereas large molecules with low charge number have low mobility. [153, 154]

In addition to the electrophoretic mobility, electroosmotic flow (EOF) also has an influence on the separation of analytes. EOF is a bulk property of the BGE. It is a consequence of the surface charge of the interior wall of fused silica capillary i.e. the anionic silanol groups (SiO^-). Counterions originating from BGE (usually cations) build up an oppositely charged counterionic layer near the capillary surface to maintain charge balance. A diffuse double-layer is formed and a potential difference (zeta-potential) is created very close to the capillary wall. When voltage is applied across the capillary, a double-layer wall consisting of cations starts to move towards the cathode, creating the bulk flow that is called EOF. The magnitude of EOF can be calculated from:

$$v_{eo} = \frac{\varepsilon\zeta}{\eta} \cdot E \quad (3)$$

or

$$\mu_{eo} = \frac{\varepsilon\zeta}{\eta} \quad (4)$$

where v_{eo} is the electroosmotic velocity, ε the dielectric constant of BGE solution, ζ zeta-potential, η viscosity of the BGE solution, E electric field strength and μ_{eo} electroosmotic mobility. Because zeta-potential is determined by the surface

charge on the capillary wall, it is strongly pH dependent. This results in higher EOF with higher pH. Furthermore, zeta-potential is dependent on ionic strength of the BGE as described by double-layer theory. When ionic strength is increased, the double-layer is compressed, which decreases zeta-potential and thus EOF is reduced. As long as the temperature is kept constant, the applied electric field is proportional to the electroosmotic velocity. [150, 155]

Figure 6 presents the effect of electrophoretic mobility (μ_{ep}) and electroosmotic mobility (μ_{eo}) on the total mobility (μ_{tot}) of an analyte that is neutral (N), positively charged (+) or negatively charged (−), and is influenced by positive electric field. For a neutral compound, the total mobility is equal to electroosmotic mobility, for cations the total mobility is the sum of electrophoretic and electroosmotic mobility, and for anions the total mobility is the difference between these mobilities. [156] Because of the electroosmotic flow, all the analytes will migrate to the detector eventually.

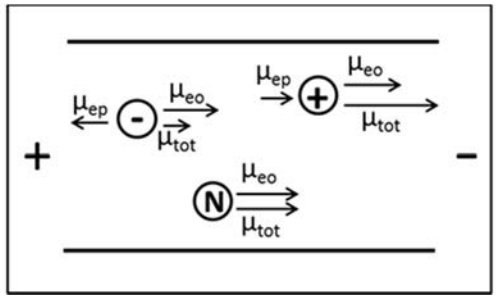

Figure 6. Schematic presentation of CZE separation principle. Adapted from [153]

Overall, the electroosmotic flow (EOF) is one of the most important factors affecting the separation efficiency in capillary electrophoresis. In CZE, the most rapid separation is achieved when the electrophoretic mobility of the analyte and EOF are in the same direction. This phenomenon is called co-EOF, and it decreases resolution because in that case the analytes mainly move to the detector too fast. In the other case, when the electrophoretic mobility and EOF are in different directions (counter-EOF), the resolution is better but the analysis time is increased. Thus, the control of EOF is of high importance in either case. Methods for controlling EOF are summarized in Table 12. [157]

Table 12. Methods for controlling EOF. [150]

Variable	Effect
Electric field	Directly proportional to the electroosmotic velocity.
pH of BGE	EOF increases when pH increases.
Ionic strength of BGE	EOF decreases when ion strength increases.
Temperature during analysis	The viscosity of BGE changes approximately 2–3% per °C, thus EOF increases when temperature increases.
Organic modifier in the BGE	Changes the zeta-potential and the viscosity of BGE.
BGE additives (e.g. surfactants)	Change the magnitude and direction of EOF. Anionic surfactants increase EOF and cationic surfactants decrease EOF.
Capillary coating	Increases, decreases or stops EOF
Covalent bonded surface coating	EOF changes depending on the charge and polarity of the coating.

When analyzing anionic compounds with CZE using normal polarity, the analytes reach the detector at the late part of the analysis with the aid of EOF as presented in Figure 6. When the polarity is changed to negative, anions have electrophoretic mobility towards the anode, but the direction of EOF is towards the cathode as shown in Figure 7A which causes peak broadening. To overcome this problem, the fused silica capillary needs to be coated with cationic surfactant that changes the direction of the EOF. [158] Furthermore, capillary surface modification suppresses interactions of sample components with the surface. [159] The capillary coating can be achieved by dynamic or covalent and non-covalent coating.

Dynamic coating of a capillary is achieved by using modifiers in BGE. Because the modifier is a chemical in the BGE, the coating is regenerated continuously and permanent stability is not needed. When cationic surfactant is added to the electrolyte, EOF is reversed because of the formed double layer and positive charge on the capillary surface making the EOF move in the same direction as negatively charged anions (Figure 7C). This results in good peak shapes. [160] The generation of dynamic coating is easy, and normal fused silica capillary can be used. The modification chemicals are usually quite cheap, and the coating is applicable over a wide range of buffer concentrations and pH values. However, the dynamically coated capillaries need equilibrium time to obtain reproducible surface and constant EOF, and post-column analyses (MS, enzymatic assays) are usually not possible because they are sensitive to additives. The modifiers used for dynamic coating are surfactants, and thus the concentration must be below the critical micelle concentration (CMC) or the mechanism of the CE separation will change to micellar electrokinetic chromatography (MEKC). Usually the concentration is around 0.5 mM. Some cationic modifiers are listed in Table 13. [156]

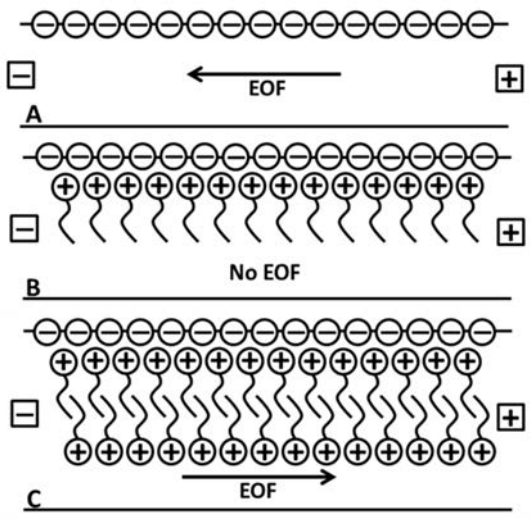

Figure 7. Elimination and reversal of electroosmotic flow with a cationic surfactant. Adapted from [156]

Covalent and non-covalent coating of a capillary is typically achieved by attaching covalently hydrophilic polymers such as polyethyleneimine or polyacrylamide to the silanol groups on the capillary wall. In most cases, the capillary is first deactivated by silanization reagent and then coated with a functional group. [161] Even though these modifications are meant to be permanent and they should require little or no maintenance, regeneration of the capillary might be necessary even between analyses depending on the sample matrix. Furthermore, the pH range of the permanently coated capillaries is not as wide as with the dynamically coated capillaries and they are significantly more expensive. [156] However, in our study, the repeatability of migration times with a permanently coated capillary was better than with a dynamically coated capillary because analysis time is shorter and the coating is more robust. [Publication III]

1.3.2 Micellar electrokinetic chromatography

Micellar electrokinetic chromatography (MEKC) was originally developed for the analysis of neutral compounds that CZE is not able to separate because neutral compounds migrate with EOF. Takeshi Nakawaga introduced an idea combining the micellar solubilization phenomenon known in chromatography with CZE separation. Instead of a chromatographic approach in which micelles act as stationary phase, in CE it is referred to as pseudostationary phase because the micelles are moving freely in the BGE solution. [162]

In MEKC a pseudostationary phase is formed in the capillary from the BGE and a surfactant above its critical micelle concentration (CMC) that is measured in

water. In addition to the separation based on charge-to-mass ratio, the migration of an analyte is also dependent on its partition coefficient between the micelles and aqueous phase of the BGE. [163]

Detergents are water-soluble, surface-active agents that consist of a hydrophilic head and hydrophobic or lipophilic tail. Because of their amphiphilic character, detergent molecules aggregate in solution to form micelles. They can also align at aqueous/non-aqueous interfaces, thus reducing surface tension, increasing miscibility and stabilizing emulsions. Detergents can be divided into four categories according to the nature of the hydrophilic head group: non-ionic, anionic, cationic and zwitterionic. [164] Common detergents in MEKC and their properties are summarized in Table 13.

Table 13. Detergents used in MEKC and their properties. [161]

Chemical	MW (g/mol)	CMC (mM)	Average micellar weight (g/mol)
Nonionic			
Brij®35	1200	0.05–0.1	48 000
Triton® X-100	625	0.2–0.9	80 000
Tween® 20	1228	0.06	*
Anionic			
Sodium cholate	431	9–15	900–1300
Sodium deoxycholate	415	2–6	1200–5000
Lithium dodecyl sulfate	272	7–10	*
Sodium dodecyl sulfate (SDS)	289	7–10	18 000
Cationic			
Hexadecyltrimethylammonium bromide (CTAB)	365	1	62 000
Myristyltrimethylammonium hydroxide (MTAH)	239	*	*
Trimethyl(tetradecyl)ammonium bromide (TTAB)	337	4–5	27 000
Zwitterionic			
3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)	615	6	6150
3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO)	631	8	7000

MW molecular weight
CMC critical micelle concentration in water at 20–25 °C
*Data not available

The separation principle of MEKC is presented in Figure 8. When an electric field is applied to the capillary, the ionic micelles start to migrate towards the electrode having a polarity opposite to the charge of the micelle. However, the EOF is usually stronger than the electrophoretic mobility of the micelles and micelles will migrate in the same direction as the analytes but at retarded velocity. [159] When an analyte is injected into the micellar BGE, it interacts with the micelles. The separation depends on the individual partitioning equilibrium of the analytes

between the micellar and aqueous phase. Analytes with higher interactions with the micelles have lower mobilities than analytes interacting mainly with the bulk solution. When using anionic micelles and normal polarity, the migration order is opposite to that in CZE. Anions will migrate first as they remain mostly in bulk solution due to the electrostatic repulsion of micelles; neutral compounds migrate second according to their hydrophobicity; and cations migrate last due to strong electrostatic attraction with the micelles. However, this generalization does not apply if strong hydrophobic interactions between analyte and micelles occur since they overcome repulsion and attractions. Furthermore, electrophoretic mobilities of analytes can also change the migration order. [148]

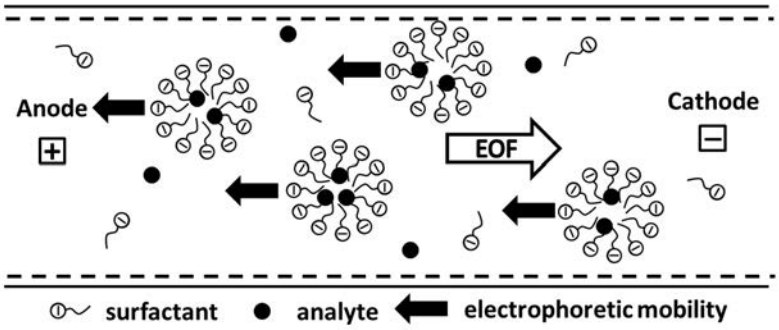

Figure 8. Schematic presentation of MEKC separation principle. Adapted from [148].

1.3.3 Detection methods

The narrow-bore capillary that is used in CE makes the detection of analytes challenging. Low analyte concentrations in a sample with high detection sensitivity and narrow analyte zone widths require innovative methods to detect low concentrations from nanoliter (or even smaller) sample volumes. Careful choice of the detector enables separations of analytes in low concentrations from many different and complex matrices. [165] The most common detection methods used in CE are summarized in Table 14 along with their detection limits. The detection methods used in this study are presented later on.

Table 14. Most common detection methods for CE. [166]

Method	Mass detection limit (mol)	Concentration detection limit (mol/L)*	Notes
UV/Vis absorption (UV/Vis)	10^{-12}–10^{-15}	10^{-5}–10^{-7}	Universal and most common detection method, diode array detection (DAD) provides spectral information.
Laser induced fluorescence (LIF)	10^{-18}–10^{-20}	10^{-9}–10^{-12}	The most sensitive detection method, usually requires derivatization.
Amperometry (AD)	10^{-18}–10^{19}	10^{-10}–10^{-11}	Sensitive and selective but only for electroactive analytes. Not robust.
Conductivity (CD)	10^{-15}–10^{-16}	10^{-6}–10^{-7}	Universal.
Mass spectrometry (MS)	10^{-16}–10^{-17}	10^{-8}–10^{-9}	Sensitive and gives structural information. Interfacing CE and MS is complicated.
Indirect UV, indirect fluorescence, indirect amperometry	10–100 times less sensitive than the direct method		Universal but with lower sensitivity than that of the direct method.

*Assume 10 nL injection

UV/Vis detection. UV/Vis detection is the most common detection method in CE and is compatible with all the modes of CE. However, some restrictions in the BGE composition exist due to the optical properties of the BGE itself. With UV/Vis detection, any molecule that possesses a UV chromophore in its structure can be detected. A chromophore is a functional group that absorbs light in the UV/Vis region of the electromagnetic spectrum. If the analyte does not contain a chromophore or it absorbs weakly, the analyte can either be derivatized or detected with indirect UV detection. In indirect UV detection, a highly UV absorbing chemical is added to the BGE to produce a high and constant background signal. When an analyte with no UV absorbance reaches the detector, the analyte is seen in the electropherogram as a negative peak. The detection with UV/Vis is based on the Beer-Lambert law which indicates that absorbance is proportional to the concentration of the analyte. The magnitude of the signal is also dependent on the analyte; analytes with high molar absorptivities of 10^4 to 10^5 are strong absorbers and analytes with molar absorptivities of $\leq 10^3$ are weak absorbers. Furthermore, the pH and composition of the BGE, and the degree of ionization have an effect on the molar absorptivity value and on the maximum wavelength of absorption. [162] The Beer-Lambert law is presented as:

$$A = \alpha l c, \tag{5}$$

where A is absorbance, α is molar absorption coefficient (in $cm^{-1}M^{-1}$), *l* is optical path length and c is concentration.

Fluorescence detection. Fluorescence is the most sensitive and second most common detection method in CE. The detection limits are below 10^{-21} mol, making it almost 1000 times more sensitive than UV detection. [162] Fluorescence can be divided into three stages: 1) excitation, 2) excited-state lifetime, and 3) fluorescence emission. These stages can be illustrated by the Jablonski diagram (Figure 9A) which represents the electronic states of each stage. In stage 1, the fluorophore absorbs a photon of energy $h\nu_{EX}$ that is delivered by a light source such as a laser, and moves to the excited electronic single state (S_1'). The excited state exists for a limited time, typically 1–10 ns (stage 2). First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) which is the origin of fluorescence emission. Second, the molecules that were not excited by the absorption in stage 1 return to the ground state S_0. After this, stage 3 occurs when electronically excited fluorophore emits a photon of energy $h\nu_{EM}$ and returns to the ground state. Because of the energy dissipation during stage 2, the energy of this photon is lower than in stage 1. The difference between these photon energies ($h\nu_{EX} - h\nu_{EM}$) is called Stokes shift (Figure 9B). [162, 167]

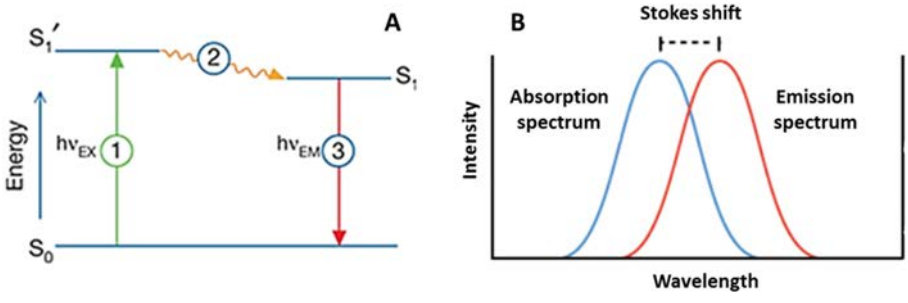

Figure 9. A) Jablonski diagram, and B) Stokes shift. [164]

The intensity of fluorescence detection is also defined by the Beer-Lambert law. The most important parameters of a fluorophore are molar absorption coefficient (α), fluorescence quantum yield and photostability of the molecule. Fluorescence quantum yield illustrates the number of fluorescence photons emitted per excitation photo absorbed, thus accounting for the efficiency of fluorescence detection with certain fluorophores. Theoretical maximum of the quantum yield is 1.0.

However, most of the analytes do not have native fluorescence and therefore the use of this detection method involves attaching fluorescent probe molecules to the analyte. Most fluorescent probes can be designed to contain a reactive moiety capable of coupling to a specific functional group of a biomolecule. With this derivatization procedure it is possible to enhance the selectivity of the detection. [162] Common fluorescent labels in capillary electrophoresis and their properties and applications are listed in Table 15.

Table 15. Common fluorescent labels used in CE.

Label	MW (g/mol)	Excitation/ emission maximum wavelength (nm)	Quantum yield	α [164]	Reactivity	Ref.
8-Aminonaphtalene-1,3,6-trisulfonic acid (ANTS)	427.34	356/512	na	7 200	carbohydrates	[168]
8-Aminopyrene-1,3,6-trisulfonic acid (APTS)	523.40	420/500	na	19 000	carbohydrates	[165]
Fluorescamine	278.26	391/464	0.11	7 800	primary aliphatic amines	[165]
Fluorescein	332.31	490/514	0.93	93 000	amines	
Fluorescein isothiocyanate (FITC)	389.38	492/518	na	77 000	amines	[165, 169]
Oregon green® succinimidyl ester 5-isomer	509.38	495/521	na	76 000	primary and secondary aliphatic amines	
Oregon green® succinimidyl ester 6-isomer	509.38	496/516	na	82 000	primary and secondary aliphatic amines	
1-Pyrenyldiazomethane (PDAM)	242.27	340/375	na	41 000	carboxylic acid	[165]

MW molecular weight

na not available

α molar absorption coefficient (in $cm^{-1}M^{-1}$)

Oregon green™ 488 succinimidyl ester (OG-SE) used in the Publication IV is the fluorinated analogue of fluorescein. The molecular structure of OG-SE is presented in Figure 10A. Conjugates of Oregon green fluorophores are more photostable than those of fluorescein and are not as pH sensitive. The excitation and emission maximums wavelengths of the label are at 496 nm and 524 nm, respectively, (Figure 10B) and are well suited for detection with argon-ion laser. Reaction of OG-SE with primary and secondary amines creates stable amide and imide linkages, respectively, and the reaction occurs rapidly in mild conditions. [170]

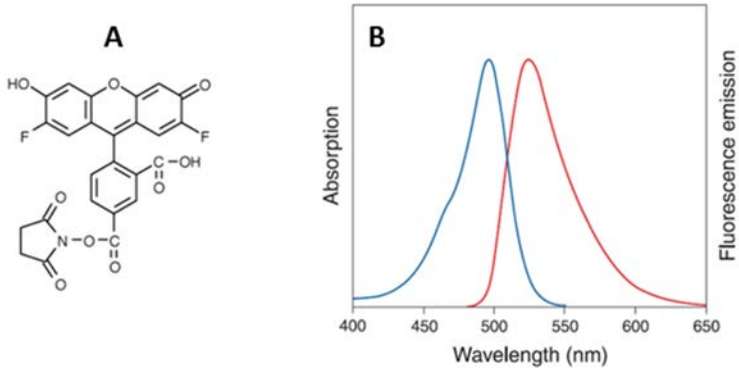

Figure 10. A) Structure of Oregon green® 488 succinimidyl ester [167], and B) Absorption and fluorescence emission spectra of Oregon Green® 488 [164]. The blue line is absorption and the red line is emission.

2. Aims of the study

The overall object of this study was to develop novel analytical methods to measure and control the productivity of microorganisms in bioreactors during cultivations. For this purpose, capillary electrophoresis with UV- and LIF-detection was used to assist research of industrial biotechnology and validation of biotechnological manufacturing processes. In addition, an online CE system was assembled from modified commercial capillary electrophoresis device and an in-house constructed flow-through sample vial.

Specifically the aims were:

- to develop CE methodologies and technologies to study carboxylic acids that are present in bioprocesses in both offline (I) and online (III) mode
- to develop CE methodologies to study phenolic compounds with inhibitory effects on bioprocesses (II)
- to develop CE methodologies to study the consumption of amino acids by yeast during beer fermentation (IV).

3. Materials and methods

3.1 Chemicals and materials

All the materials and chemicals that were used in this study are summarized in Table 16.

Table 16. Chemicals and materials used in this study.

Compound	Manufacturer/ supplier	Comments	Publ.
18-crown-6-ether (18C6)	Sigma-Aldrich	BGE additive, surfactant	II, IV
2,3-Pyridinecarboxylic acid (2,3-PDC)	Sigma-Aldrich	BGE additive, UV absorbing reagent for indirect UV detection	I
3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO)	Sigma-Aldrich	BGE additive, surfactant	II, IV
4-Hydroxybenzoic acid	Sigma-Aldrich	Standard	II
Acetic acid	Fluka	Standard	I
Acetoguaiacone	Sigma-Aldrich	Standard	II
Acrodisc® GHP syringe filter	Pall Life Science	0.45 µm, hydrophilic polypropane membrane, for filtration of BGE	I–IV
L-Alanine	Merck	Standard	IV
Ammonia	Merck	25%, pH adjustment	I
Ammonium acetate	Sigma-Aldrich	BGE salt	II
Arabonic acid	VTT	Standard	I
L-Arginine	Fluka	Standard	IV
L-Asparagine	Fluka	Standard	IV
L-Aspartic acid	Fluka	Standard	IV
Benzoic acid	Fluka	Standard	II
Calcium chloride dihydrate	Riedel-de Haen	BGE additive, complex forming agent	I
Cinnamic acid	Sigma-Aldrich	Standard	II
Citric acid (monohydrate)	Merck	Standard	I
Coniferyl aldehyde	Sigma-Aldrich	Standard	II
p-Coumaric acid	Sigma-Aldrich	Standard	II

Compound	Manufacturer/ supplier	Comments	Publ.
β-Cyclodextrin	Sigma-Aldrich	BGE additive, surfactant	II, IV
L-Cysteine	Fluka	Standard	IV
Dimethylsulfoxide (DMSO)	Sigma-Aldrich	Solvent for fluorescent label	IV
eCAP amine capillary	Beckmann Coulter	Permanently coated amine capillary, i.d. 50 µm, o.d. 360 µm	III
Ethanol	Altia	99.5%, solvent for standards	II
Formic acid	Merck	Standard	I
Fused silica capillary	Teknolab	i.d. 50 µm, o.d. 360 µm	I, II, IV
Galactaric acid	Fluka	Standard	I
Galacturonic acid	Fluka	Standard	I
Gluconic acid (lactone)	Fluka	Standard	I
L-Glutamic acid	Fluka	Standard	IV
L-Glutamine	Fluka	Standard	IV
L-Glycine	Merck	Standard	IV
Glycolic acid	Fluka	Standard	I
L-Histidine	Fluka	Standard	IV
Isocitric acid	Sigma-Aldrich	Standard	I
L-Isoleucine	Fluka	Standard	IV
α-Ketoglutaric acid	Sigma-Aldrich	Standard	I
Lactic acid (lithium salt)	Fluka	Standard	I
L-Leucine	Fluka	Standard	IV
L-Lysine	Fluka	Standard	IV
Magnesium chloride hexahydrate	Riedel-de Haen	BGE additive, complex forming agent	I
Maleic acid	Sigma-Aldrich	Standard	I
Malic acid	Sigma-Aldrich	Standard	I
Malonic acid	Merck	Standard	I
Methanol	Fluka	LC-MS grade, solvent for standards	II
Methanol	Rathburn	BGE additive	I
L-Methionine	Fluka	Standard	IV
Myristyltrimethyl-ammoniumhydroxide (MTAH)	Waters	BGE additive, surfactant and EOF modifier	I
Oregon green® 488 succinimidyl ester	Molecular probes Inc.	Fluorescent label	IV
Oxalic acid	Sigma-Aldrich	Standard	I
Phenol	Sigma-Aldrich	Standard	II
L-Phenylalanine	Merck	Standard	IV
1,4-Piperazinediethane sulfonic acid (PIPES)	Sigma-Aldrich	Standard	III
L-Proline	Fluka	Standard	IV
Propionic acid	Fluka	Standard	I
L-Serine	Fluka	Standard	IV

Compound	Manufacturer/ supplier	Comments	Publ.
Sodium acetate	Sigma-Aldrich	BGE salt	II
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	BGE additive, surfactant	II, IV
Sodium hydroxide, 0.1M solution	FF-Chemicals Ab	Capillary conditioning	I–IV
Sodium tetraborate decahydrate	Sigma-Aldrich	BGE salt	II, IV
Succinic acid	Sigma-Aldrich	Standard	I
Syringaldehyde	SAFC	Standard	II
L-Threonine	Fluka	Standard	IV
L-Tryptophan	Fluka	Standard	IV
L-Tyrosine	Merck	Standard	IV
DL-Valine	Sigma-Aldrich	Standard	IV
Vanillic acid	Fluka	Standard	II
Vanillin	Sigma-Aldrich	Standard	II
Water	Millipore	Milli-Q purified water for solvent and capillary conditioning	I–IV
Xylonic acid (calcium salt)	VTT	Standard	I

3.2 Instruments

3.2.1 Capillary electrophoresis

All capillary electrophoretic analyses in Publications I–IV were made with the P/ACE MDQ capillary electrophoresis system (Beckmann Coulter Inc., Fullerton, CA, USA). The data were collected and processed with 32Karat Software (Beckmann Coulter Inc.). The CE device was equipped with a photodiode array detector (Beckmann Coulter Inc.) with UV wavelength range 190–600 nm in Publications I–III, and with laser-induced fluorescence detection (Beckmann Coulter Inc.) in Publication IV. The LIF detector had an argon-ion laser with 488 nm excitation and 520 nm emission wavelengths.

3.2.2 Online analysis system

In Publication III, an online capillary electrophoresis system (Figure 11) was assembled from a peristaltic pump with an 8-channel pump head (MCP, Ismatec Labortechnik Analytik, Zurich, Switzerland), a 3-way solenoid-operated pinch valve (Bio-Chem valve, Boonton, NJ, USA), a cross-flow filter (A-Sep, Applikon, Schiedam, the Netherlands) with 45 mm diameter filter membranes (GVWP, Millipore) of 0.22 µm pore size, a 500-µL flow-through sample vial made in-house and a single-channel peristaltic pump (Watson Marlow 101U/R, Falmouth, England).

Silicone tubing (Saint-Gobain, Charny, France) with 0.8 mm inner diameter and 2.4 mm outer diameter was used for fluid lines. Pharmed tubing (Saint-Gobain) with 1.6 mm inner diameter and 3.2 mm outer diameter was used in the pumps. A National Instruments USB-6009 data acquisition device (National Instruments, Austin, TX, USA) was used for sending analog voltage signals to the 8-channel peristaltic pump, for sending digital output signals to an additional relay supplying 24 V to the pinch valve, and for receiving digital input signals from the CE instrument. National Instruments LabVIEW software (version 8.5) was used to develop control software for the automated measurement sequence. Timing of the sampling was controlled by the CE software and the sampling and filtration sequence was started on the basis of the digital output signal sent from the CE instrument.

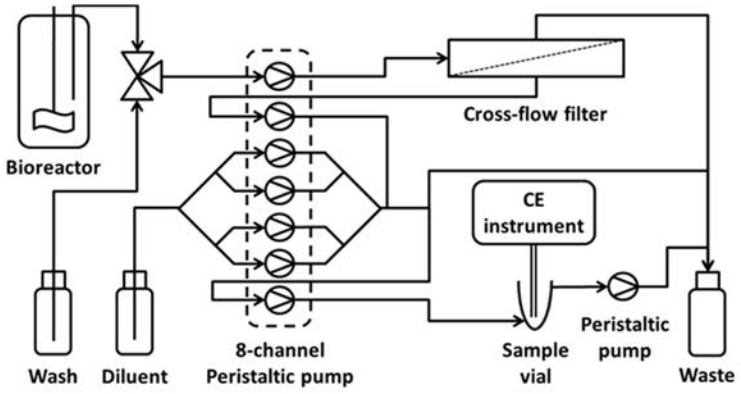

Figure 11. Schematic view of online capillary electrophoresis system. [III]

In order to enable online capillary electrophoretic analysis of carboxylic acids, a commercial capillary electrophoresis device was modified and a flow-through vial (Figure 12A) of 500 µL volume was in-house engineered for this project. The vial was made from a solid PEEK block by drilling, and inserted inside a plastic vial holder (Beckmann Coulter, Figure 12B and C). The flow-through vial was placed to the inlet side buffer tray inside the CE device from where the injections were performed. It was essential to make the fluid flow from the bottom of the vial upwards because this decreased the possibility of air bubbles to become trapped in the vial, which in turn would not be favorable in sample injection to capillary electrophoresis. Furthermore, with this approach the surface height of the liquid sample remains constant during the analysis.

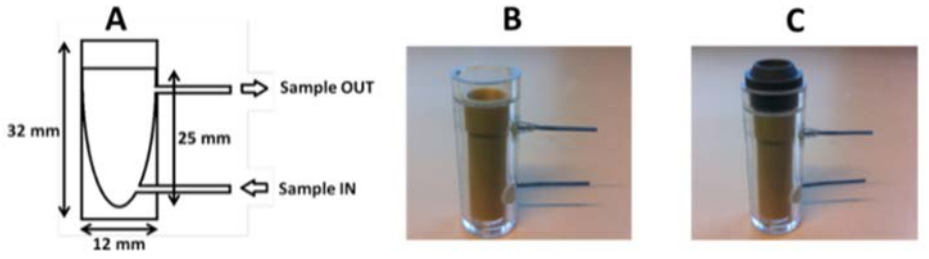

Figure 12. A) Schematic figure of the flow-through sample vial with dimensions. Picture of the sample vial B) without and C) with cap. [III]

The sampling sequence of the online CE system is presented in Table 17. The sampling duration was determined by measuring the time needed for the sample to reach the flow-through vial, and the liquid volume of vial was changed at least 5 times. The sampling started when the Labview software received a trigger signal from the CE device.

Table 17. Sampling sequence.

Task	Duration (s)	Notes
Start	1	Start of sequence
Wait	1	Sequence is halted and waits for a trigger signal from CE device
Sampling	200	Valve is opened and pump is turned on, liquid flow is from bioreactor toward flow-through vial through the cross-flow filter
Break	400	Pump off, sample injection to the capillary is executed during this task according to the CE method
Wash	300	Valve is closed and pump is turned on, liquid flow is from wash bottle toward flow-through vial through the cross-flow filter; tubing, filter and flow-through vial are cleaned
Pump off	1	Pump is turned off
End	1	Sequence ends and next run is started

3.2.3 Other instrumentation

All pH measurements were performed with a Knick 766 Laboratory pH meter (Berlin, Germany) equipped with a Mettler Toledo InLab® Routine pH electrode. The calibration was performed with commercial buffers of pH 4.00, 7.00 and 10.00 (FF-Chemicals Ab, Haukipudas, Finland).

3.3 Methods

Before all CE analyses the BGE was degassed by ultrasound for 10 minutes and filtered through a 0.45 µm Acrodisc GHP syringe filter (Pall Life Science, Ann Arbor, USA). Detailed descriptions of method development and capillary conditioning are presented in Publications I–IV.

3.3.1 CZE with indirect UV detection for studying carboxylic acids

In Publication I, the 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 25% ammonia. Fused silica capillary with total length 80 cm (70 cm to the detector) was used. Standards and samples were injected by using 0.5 psi pressure for 15 s. Separation was carried out at -20 kV (reversed polarity) at a constant capillary temperature of 25 °C. Indirect UV detection was at 254 nm wavelength. Samples were centrifuged at 10 000 rpm for 5 minutes and diluted with Milli-Q purified water 5-fold, 100-fold and/or 250-fold.

3.3.2 CZE with direct UV detection for studying phenolic acids

In the optimized method of Publication II, the BGE was prepared with 40 mM sodium tetraborate and 40 mM 18-crown-6-ether in milli-Q purified water. The pH of the solution was 9.0. Standards and samples were injected by using 0.5 psi pressure for 5 s. Separation was carried out at +30 kV at a constant capillary temperature of +20 °C. Fused silica capillary with total length 50 cm (40 cm to the detector) was used. Sample cartridge temperature was set at +10 °C. Direct UV detection was at 200 and 254 nm. For the determination and quantification of phenols, vanillin, coumaric acid, vanillic acid, benzoic acid and cinnamic acid, 200 nm was used. For the determination and quantification of coniferyl aldehyde, acetoguaiacone, syringaldehyde and 4-hydroxybenzoic acid, the detection wavelength was 254 nm. The samples were centrifuged at 10 000 rpm for 5 minutes and analyzed without dilution or diluted 2-fold with Milli-Q purified water.

3.3.3 Online CE analysis of carboxylic acids

The online and offline CE analyses of Publication III were performed with a BGE that consisted of 20 mM 2,3-PDC with 80 mg/L Ca^{2+}, 40 mg/L Mg^{2+} and 10% (v/v) IPA (pH 9 adjusted with 25% ammonia). Negative voltage was set to –20 kV, capillary temperature was 25 °C, and detection wavelength was 254 nm. Amine coated eCAP silica capillary with total length 60 cm (50 cm to the detector) was used. Injection was performed with 0.5 psi vacuum for 5 s.

The sampling was performed both online and offline. In the *K. lactis* cultivation, the online sampling was performed once an hour until 73 hours and thereafter

every two hours until the cultivation was ended at 137 hours. In the *S. cerevisiae* cultivation, the online sampling was performed once an hour until the cultivation was ended at 64 hours. The samples were drawn from the bioreactor and filtered with a cross-flow filter using a 0.22 µm pore size filter membrane to provide cell-free sample. The sample was diluted 5-fold with sterile Milli-Q purified water online. Then the sample was injected to the CE device from the flow-through sample vial that was placed in the inlet side of the CE buffer tray. During the analysis, all of the lines including the sample vial were flushed with sterile milli-Q purified water. The sample was injected about 8 minutes after the sampling was started. The total sample volume removed from the reactor was approximately 9 mL per sample. In order to correct errors of sampling, dilution and analysis, results were corrected with internal standards that were added to the cultivation media (1,4-Piperazinediethane sulfonic acid, PIPES) and to diluent (gluconic acid). Offline samples were drawn manually from the bioreactor, centrifuged 10 min at 3 000 rpm (Biofuge Stratos, Heraeus Instruments, Osterode, Germany), diluted 5-fold (v/v) with Milli-Q purified water and frozen. The volume of the samples was 3 mL. The samples were analyzed with CE for carboxylic acids and with HPLC for sugars and alcohols. A total of 97 online and 41 offline samples were collected from the *K. lactis* cultivation, and 62 online and 23 offline samples from the *S. cerevisiae* cultivation.

3.3.4 MEKC with LIF detection for studying amino acids

In the optimized analysis method of Publication IV the BGE consisted of 50 mM tetraborate, 30 mM SDS and 20 mM 18C6, pH 9.0. Standards and samples were injected by using 0.5 psi pressure for 10 s. The analysis voltage and temperature were +30 kV and 15 °C, respectively. Total capillary length was 60 cm with 50 cm to the detector. Detection with LIF was set to 488 nm excitation and 520 nm emission wavelengths.

The samples were centrifuged at 10 000 rpm for 5 minutes and diluted prior to analysis. The OG-SE derivatization procedure was performed by adding 10 µL of 2 mM OG-SE solution, 5 µL of 5-fold diluted sample and 35 µL of 30 mM tetraborate buffer (pH 9.0) to an Eppendorf tube. The reaction mixture was vortexed for 10 s and microcentrifuged for 20 s before leaving it at room temperature for the reaction to proceed. After 15 minutes, 5 µL of the sample was diluted with 395 µL of 30 mM tetraborate buffer (pH 9.0) and analyzed with the optimized analysis method. The dilution was 400-fold.

3.3.5 Bioreactor cultivations

All cultivations were batch cultivations using wheat straw hydrolysate (Publication I), synthetic spruce hydrolysate (Publication II), spruce hydrolysate (Publication II), YNB-medium (Publication III), YDB-medium (Publication III) or wort (Publication IV) as cultivation media. Microorganisms were Gluconobacter oxydans (Publication I),

Saccharomyces cerevisiae (Publications II–IV) and *Kluyveromyces lactis* (Publication III). Detailed descriptions of the bioreactor cultivations are presented in Publications I–IV.

3.3.6 Other analysis techniques

In Publications III and IV the bioreactor samples were also analyzed by HPLC in order to determine the concentrations of carbohydrates and alcohols. A Waters Alliance 2690 HPLC system (Waters, Milford, MA, USA) with Waters 2487 dual wavelength UV (210 nm) detector and Waters 2414 differential refractometer were used with an injection volume of 20 µL in Publication III and 10 µL in Publication IV. In Publication III, the columns used were an Aminex HPX-87H Organic Acid Column (300 mm x 7.8 mm, Bio-Rad, Hercules, CA, USA) connected to a Fast Acid Analysis Column (100 mm x 7.8 mm, Bio-Rad). In Publication IV, the columns were an ICSep ION-310 Fast Analysis Column (150 mm x 6.5 mm, Transgenomic, Inc., Omaha, NE, USA) connected to an ICSep ICE-ORH-801 Column (300 mm x 6.5 mm, Transgenomic). The eluent was 5 mM H_2SO_4 (Titrisol, Merck), flow rate 0.5 mL/min and analysis temperature +55 °C. In Publication IV, cell density and cell dry weight were determined. The cell density was determined as the optical density of the sample and it was measured with a Shimadzu UV-1201 spectrometer (Kyoto, Japan) at 600 nm (= OD_{600}). The cell dry weight (CDW) was determined by filtering 20 mL of sample through a tared filter paper, washing it with distilled water and drying it in an oven (105 °C) for at least 16 hours. After that the cells were weighed with the filtration paper and the CDW was calculated.

4. Results and discussion

4.1 Determination of carboxylic acids by CZE with indirect UV detection

The aim of Publication I was to develop a CE method for the simultaneous separation, identification and quantification of carboxylic acids that are potentially present in microbial cultivations. The optimized analysis method was used to monitor carboxylic acid production in *Gluconobacter oxydans* fermentation of wheat straw hydrolysate.

4.1.1 Method development

To improve the separation efficiency of anionic analytes in CE, it is essential to apply a negative voltage during analysis to shorten the analysis times. In addition, EOF modifier must be used to invert the direction of EOF towards the detector. [64] There are a wide variety of EOF modifiers available and myristyltrimethylammonium hydroxide (MTAH) used in this study is a common cationic surfactant in the analysis of carboxylic acids. [64, 77] Other possible cationic surfactants include tetradecyltrimethylammonium bromide [65] and cetyltrimethylammonium bromide [55, 71, 72, 74, 78, 79, 81]. The surfactants are added to the BGE in low concentrations in order to prevent the conversion of CZE to MEKC.

Aliphatic carboxylic acids have no or very low UV absorbance because they lack a chromophore in their structure. The use of indirect UV detection by addition of UV absorbing chemical to the BGE enables the analysis without the derivatization procedure. As a result of the UV absorbing chemical, the detector is able to monitor high UV background signal during analysis and the analytes without UV absorption are seen as negative peaks in the electropherogram. There are several chemicals available for this purpose such as potassium hydrogen phthalate [78, 79], 1,3,5-benzenetricarboxylic acid [76], 2,3-pyridinedicarboxylic acid (2,3-PDC) [77], 2,6-pyridinecarboxylic acid [55, 72, 81] and trimellitic acid [63]. 2,3-PDC was chosen as UV absorbing chemical on the basis of previous studies of the research group. [77] As presented in Figure 13, 20 mM concentration resulted in the best separation efficiency and the most stable baseline.

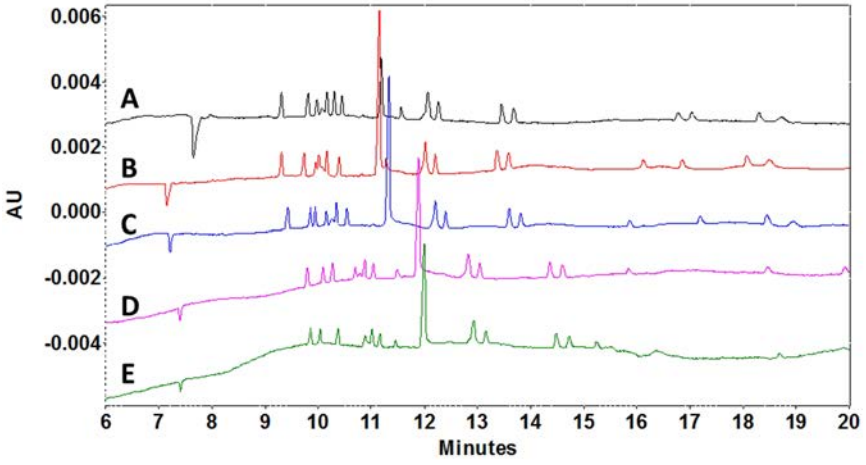

Figure 13. Effect of 2,3-PDC concentration on the separation. BGE 0.3 mM MTAH, 30 mg/L Ca^{2+}, 30 mg/L Mg^{2+}, 10% MeOH (v/v), and A) 20 mM 2,3-PDC, B) 25 mM, C) 30 mM, D) 40 mM, and E) 50 mM, pH 9.0. Other conditions: negative voltage –20 kV, capillary temperature 25 °C, sample injection 0.5 psi 15 s, and indirect UV detection at 254 nm. Unpublished data.

Cations of alkali earth metals have strong tendencies to form partially dissociated complexes in solution with anions of carboxylic acids and other weak acids. The affinity of this association tends to increase with the number of carboxylic acid groups of the acid anion, and the tendency is higher with aliphatic acids than with aromatic acids. [171] 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 BGE. [172]

It has been noted that the use of metals in BGE decreases the efficiency and reproducibility of the analysis. Metals can absorb to the silanol groups of the capillary wall, thus lowering zeta potential and decreasing electroosmotic flow. In addition, the complex formation of the analytes with the metals attached to silanol groups causes peak broadening and decrease in efficiency. The use of EOF modifier in BGE helps to prevent this undesirable phenomenon. [169]

The addition of methanol, up to 20%, to the BGE reduces EOF which results in increased migration times and resolution. In addition, due to its lower dielectric constant than that of water, it promotes complex formation reactions and the formation of highly complex compounds. This results in additional possibilities for separation improvement. [169]

The optimized method was able to resolve 18 different carboxylic acids that are potentially present in bioprocess cultivations. Different microorganisms have different metabolic routes and various substrates are used cultivations, and

therefore the number of products is high. In addition, many side-products may also be formed. Therefore, it was essential that the method was able to resolve as many carboxylates as possible even though they are not present in the samples simultaneously. After Publication I was published, 4 carboxylic acids were added to the method. The separation of 22 carboxylates is presented in Figure 14.

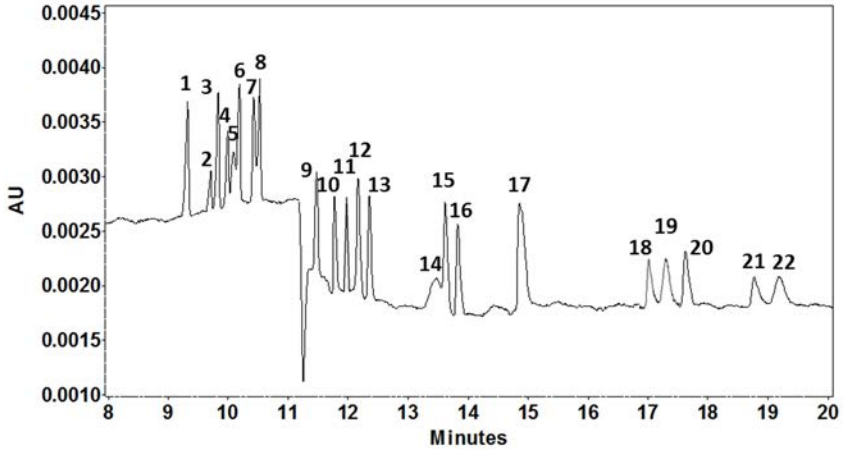

Figure 14. Electrophoregram of the optimized analysis method. BGE 20 mM 2,3-PDC, 0.3 mM MTAH, 30 mg/L Ca^{2+}, 30 mg/L Mg^{2+}, and 10% MeOH (v/v), pH 9.0. Other conditions: negative voltage –20 kV, capillary temperature 25 °C, sample injection 0.5 psi 15 s, and indirect UV detection at 254 nm. Peak assignments: 1) formate, 2) fumarate, 3) malonate, 4) maleinate, 5) α-ketoglutarate, 6) succinate, 7) oxalate, 8) malate, 9) adipic acid, 10) isocitrate, 11) galactarate, 12) acetate, 13) glycolate, 14) glyoxylate, 15) propionate, 16) lactate, 17) levulinate, 18) citrate, 19) xylonate, 20) arabonate, 21) gluconate, and 22) galacturonate. Unpublished data.

4.1.2 Analysis of cultivation samples

The optimized analysis method was used for the analysis of cultivation samples from *Gluconobacter oxydans* culture on wheat straw hydrolysate. Wheat straw hydrolysate is rich in xylose (39 g/L) but also contains some glucose (5.3 g/L). In addition, high concentrations of formic, malic and acetic acids were analyzed from the hydrolysate but they did not have an inhibitory effect on the cultivation. Electrophoregrams of samples are presented in Figure 15.

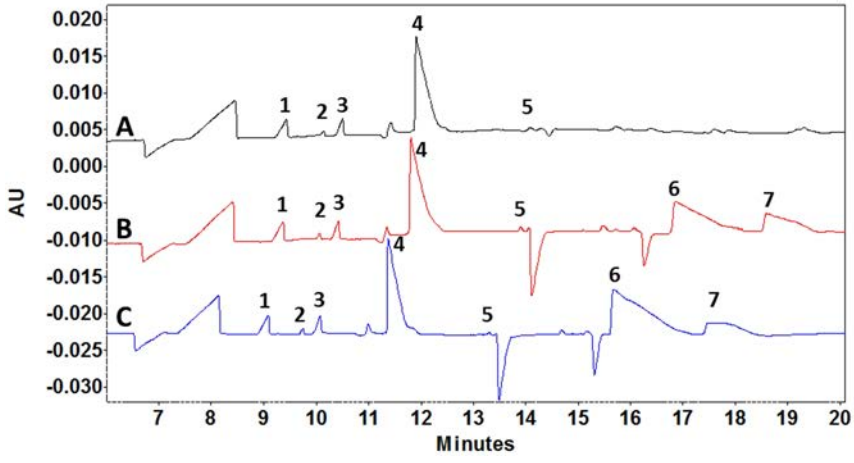

Figure 15. Electrophoretic separation of carboxylic acids in a *G. oxydans* cultivation. Fermentation time: (A) 0h, (B) 19.8 h, and (C) 119.1 h. Peak assignments: 1. formate, 2. succinate, 3. malate, 4. acetate, 5. lactate, 6. xylonate, and 7. gluconate. Analysis conditions as described in Figure 14. [Publication I]

The production yield of xylonic acid from xylose was 45%, and that of gluconic acid from glucose was 96%. As presented in Figure 16, the production of gluconate was completed within 20 hours but the production of xylonate continued for over twice as long.

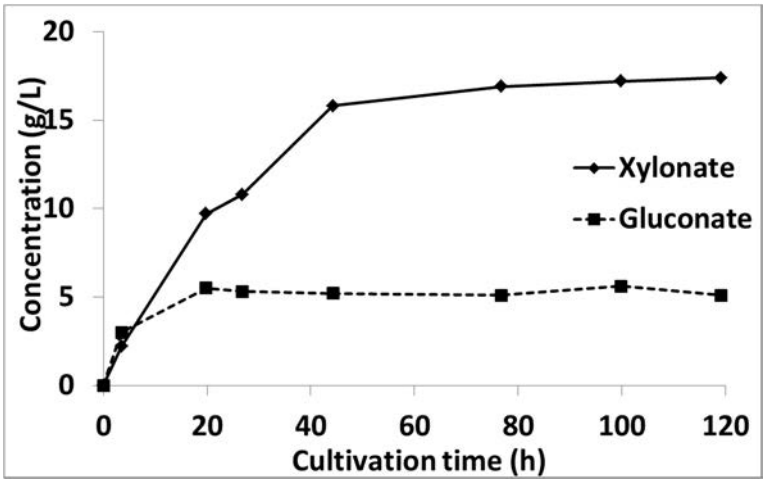

Figure 16. Product formation rate of xylonate and gluconate during *G. oxydans* cultivation on wheat straw hydrolysate. [Publication I]

4.2 Determination of phenolic compounds by CZE-UV

In Publication II, a CE method for the analysis of phenolic compounds in bioprocesses was developed. The optimized method was used to study the concentrations of phenolic compounds during *Saccharomyces cerevisiae* cultivations in synthetic inhibitor mix media and in spruce hydrolysate. The composition of synthetic inhibitor mix is presented in Table 18. The concentrations together amount to a 100% inhibitor mix.

Table 18. Synthetic inhibitor composition of spruce hydrolysate. [92]

Compound	Concentration (mg/L)
5-Hydroxymethylfurfural (5-HMF)	3400
Furfural	1100
Acetic acid	6300
Formic acid	1200
Levulinic acid	2400
Hydroquinone	20
Vanillin	140
Syringaldehyde	120
Coniferyl aldehyde	50
Cinnamic acid	1
4-Hydroxybenzoic acid	20
Acetoguaiacone	20

4.2.1 Method development

Sodium acetate, ammonium acetate and sodium tetraborate were studied as background electrolyte chemicals, and sodium tetraborate was found to be the most suitable one. Tetraborate ion is able to form complexes with certain analytes containing hydroxyl groups and to produce negatively charged borate complexes which enhance the separation efficiency. [173]

The analysis of phenolic compounds present in hydrolysates is very difficult with UV detection. The concentrations of analytes are low and the matrix consists of a wide variety of UV-absorbing compounds. The separation efficiency was enhanced with the use of MEKC. Several surfactants were studied to find a suitable one for this application. The effects of sodium dodecyl sulfate (SDS), β-cyclodextrin, 3-[3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane-sulfonate (CHAPSO) and 18-crown-6-ether (18C6) additions were tested. SDS and CHAPSO had some effect on the separation and β-cyclodextrin hampered the analysis but 18C6 improved the separation efficiency significantly. The effect of 18C6 addition to the BGE is presented in Figure 17. The addition of 18C6 does not provide MEKC, and so the analysis method was CZE.

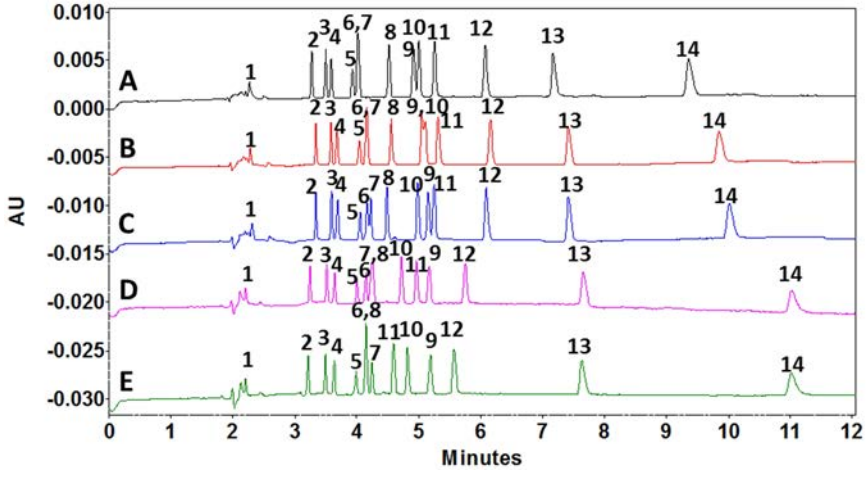

Figure 17. Effect of 18C6 addition to BGE. BGE 40 mM tetraborate, pH 9 with A) 10 mM, B) 20 mM C) 30 mM, D) 40 mM and E) 50 mM 18C6. Other conditions: voltage +14 kV, capillary temperature 20 °C, total capillary length 30 cm (20 cm to the detector), sample injection 0.5 psi 5 s, and UV detection at 200 nm. Peak assignments: 1) phenols, 2) coniferyl aldehyde, 3) acetoguaiacone, 4) syringaldehyde, 5) vanillin, 6) ferulic acid, 7) cinnamic acid, 8) catechol, 9) benzoic acid, 10) p-coumaric acid, 11) vanillic acid, 12) 4-hydroxybenzoic acid, 13) caffeic acid, and 14) protocatechuic acid. Unpublished data.

After the most potential electrolyte solutions were found, the capillary length was tested to enhance the separation efficiency. Different separation voltages were also tested. The optimized separation of 14 different phenolic compounds is presented in Figure 18.

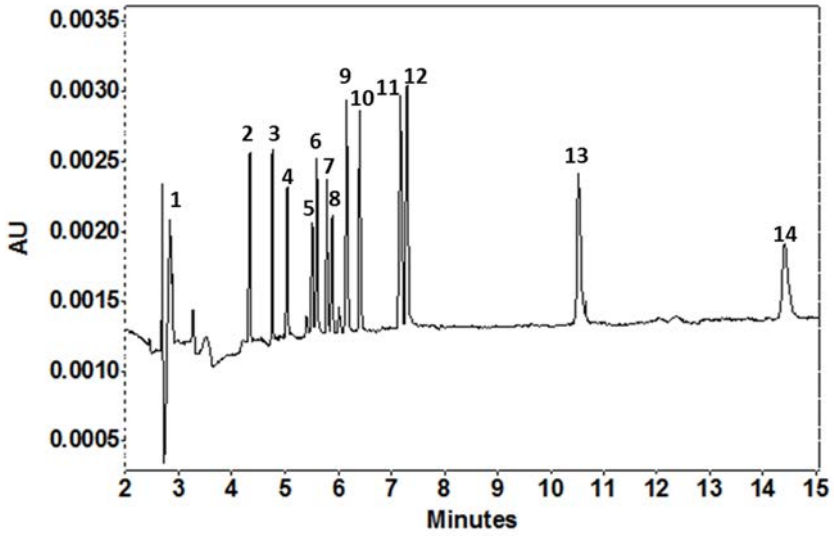

Figure 18. Separation of 14 phenolic compounds. BGE 40 mM tetraborate with 40 mM 18C6, pH 9.0. Other analytical conditions: voltage +30 kV, temperature 20 °C, capillary length 50 cm (40 cm to the detector), injection 0.5 psi 5 s and detection at 200 and 254 nm wavelengths. Peak assignments (and concentrations): 1) phenols (20 mg/L), 2) coniferyl aldehyde (20), 3) acetoguaiacone (20), 4) syringaldehyde (20), 5) vanillin (10), 6) ferulic acid (20), 7) cinnamic acid (10), 8) catechol (5), 9) p-coumaric acid (20), 10) vanillic acid (15), 11) benzoic acid (10), 12) 4-hydroxybenzoic acid (10), 13) caffeic acid (20), and 14) protocatechuic acid (10). Unpublished data.

4.2.2 Analysis of cultivation samples

Four cultivations were performed and the samples were analyzed with the optimized method. Three of the cultivations were made with synthetic spruce hydrolysate medium in which the inhibitory compounds were added to the media in concentrations simulating real spruce hydrolysate. Synthetic medium is used when individual inhibitor conditions need to be regulated, and their effects on the organism and on the overall bioprocess are studied. [174] An electropherogram of the cultivation #1 in which the final concentration of the inhibitor mix was 50% is presented in Figure 19: the concentrations of the 100% inhibitor mix were presented in Table 18 above.

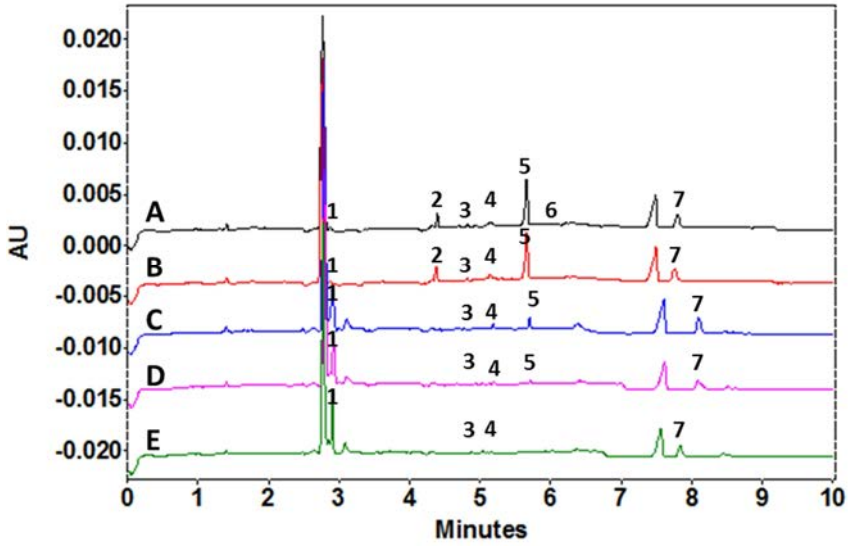

Figure 19. Electrophoretic separation of phenolic compounds present in cultivation 1 with synthetic medium at A) 0h, B) 2h, C) 19h, D) 23h, and E) 48h. Peak assignments: (1) phenols, (2) coniferyl aldehyde, (3) acetoguaiacone, (4) syringaldehyde, (5) vanillin, (6) cinnamic acid, and (7) 4-hydroxybenzoic acid. Analysis conditions as described in Figure 18. [Publication II]

In cultivation #1 the inhibitor concentration was 50% and the initial biomass was approximately 0.15 g/L. As illustrated in Figure 20, the concentrations of vanillin and coniferyl aldehyde decreased during the cultivation whereas the concentration of phenols increased until the concentration of vanillin was below the detection limit. Furthermore, the glucose was consumed in less than 20 hours. However, the yeast was able to consume the xylose only slightly during the cultivation. The concentration of syringaldehyde remained approximately constant throughout the cultivation.

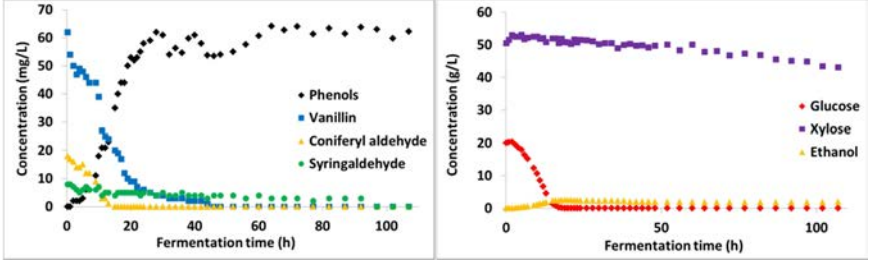

Figure 20. Diagram of cultivation #1 results. [Publication II] HPLC results are unpublished data.

In cultivation #2 the inhibitor concentration was decreased to 20%, but the initial biomass was same as in cultivation #1, 0.15 g/L. Figure 21 shows that the concentrations of vanillin, coniferyl aldehyde and acetoguaiacone decreased below the detection limit. The concentration of these phenols increased until other phenolic compounds were not detectable. The glucose was consumed in *ca*. 15 hours which was faster than in cultivation #1. The yeast was able to utilize xylose until the end of cultivation, although the consumption rate was not high. The concentration of syringaldehyde remained unchanged throughout the cultivation.

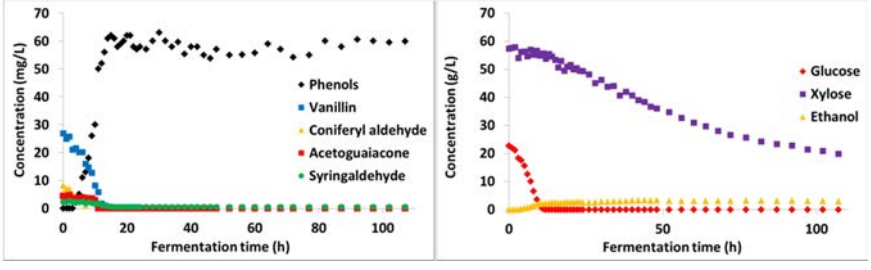

Figure 21. Diagram of cultivation #2 results. [Publication II] HPLC results are unpublished data.

The inhibitor mix concentration in cultivation #3 was the same as in cultivation #2, but the initial biomass concentration was increased 10-fold to 1.5 g/L. The glucose utilization rate was about 5 hours and the xylose was consumed in 20 hours. Figure 22 presents that the concentrations of vanillin and coniferyl aldehyde decreased below their detection limits as in other cultivations and the concentration of phenols increased, although the concentration was two times lower and it started to decrease after 15 hours. The concentration of syringaldehyde remained unchanged.

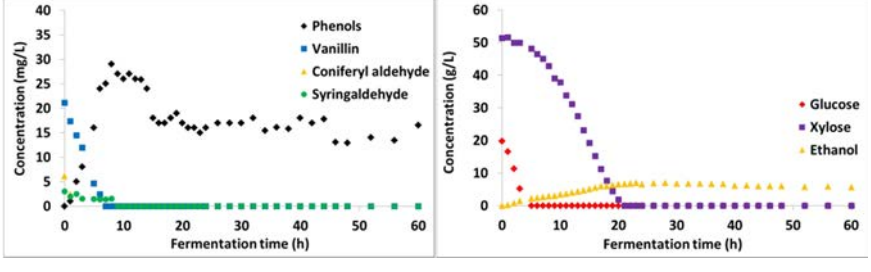

Figure 22. Diagram of cultivation #3 results. [Publication II] HPLC results are unpublished data.

It can be concluded from cultivations #1–3 that the concentrations of inhibitors and of yeast biomass both affected the xylose utilization rate of the yeast. In addition, the ethanol production rate was also affected by the inhibitors and yeast biomass.

Cultivation #4 was performed in spruce hydrolysate which is a harsher medium for the cultivation than synthetic hydrolysate because it contains more compounds with possible synergistic inhibitory effects on microorganisms. Electropherograms of 4 time points of the cultivation are presented in Figure 23, in which a more complex background than in the cultivations on synthetic hydrolysate can be seen.

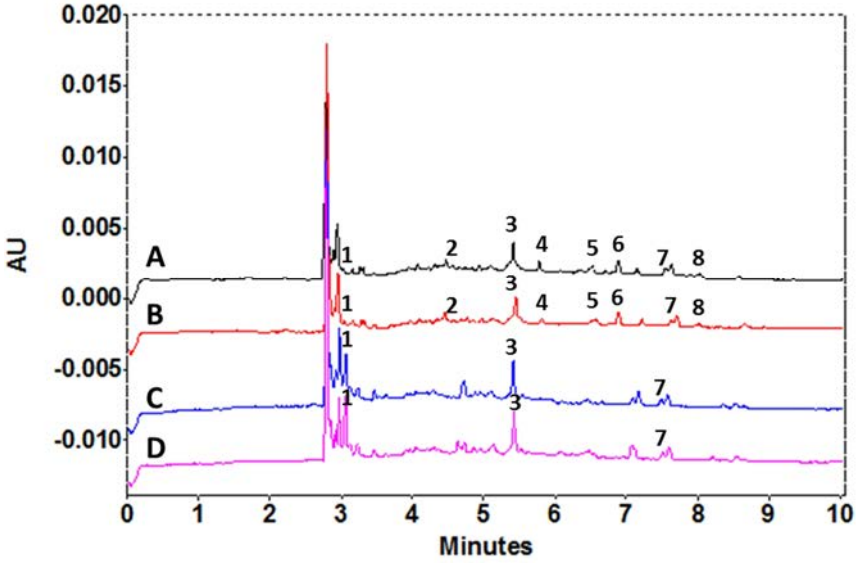

Figure 23. Electropherogram of the phenolic compounds present in cultivation 4 with spruce hydrolysate at A) 0.3h, B) 4h, C) 49h, and D) 88h. Peak assignments: (1) phenols, (2) coniferyl aldehyde, (3) syringaldehyde, (4) vanillin, (5) p-coumaric acid, (6) vanillin acid, (7) benzoic acid, and (8) 4-hydroxybenzoic acid. Analysis conditions as described in Figure 18. [Publication II]

More phenolic compounds were observed in spruce hydrolysate, and probably because of the harshness of the cultivation media, the results differed from those of the synthetic inhibitor mix cultivations. The concentrations of coniferyl aldehyde and vanillin were decreased, and, surprisingly, in addition to the concentration of phenols, the concentration of syringaldehyde was also increased. The concentrations of p-coumaric, vanillic, benzoic and 4-hydroxybenzoic acids were approximately constant. A diagram of the cultivation #4 results is presented in Figure 24.

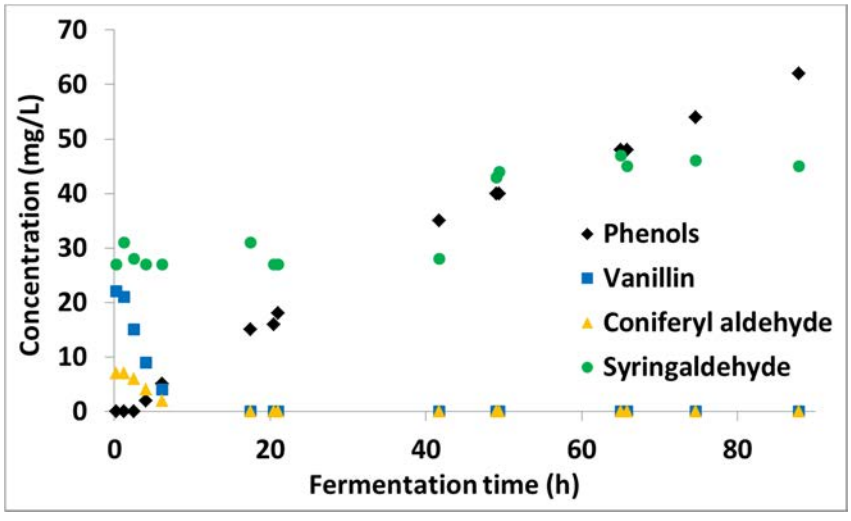

Figure 24. Diagram of cultivation #4 results. [Publication II]

4.3 Online analysis of carboxylic acids by CZE with indirect UV detection

In Publication III, the aim was to assemble an online CE system for the monitoring of carboxylic acid production by *Kluyveromyces lactis* and *Saccharomyces cerevisiae* during bioreactor cultivation. A flow-through vial was designed and constructed to enable the online analysis with commercial CE device.

4.3.1 Method development

As in Publication I, 2,3-PDC at the optimized concentration was used as background electrolyte and UV absorbing agent in this study.

When using online CE, it is essential that the analyses remain repeatable and reliable for several days. Drawbacks related to the dynamic coating of the capillary include potential interferences between dynamic coating agent and analytes, and removal of the coating agent from the capillary. [175] During routine analysis of carboxylic acids with the analysis method described in Publication I, occasional irreproducibility of the dynamic capillary coating was observed. Furthermore, drifting of migration times occurred especially when the same capillary was used for some time. Therefore, in order to reach the performance needed for online analysis of carboxylic acids, an amine-coated eCAP capillary was used to obtain higher analysis precision. The capillary utilizes polyamine-modified surface and because of the strong cationic charge that is created on the capillary wall, the EOF is reversed, and negative voltage must be applied. Furthermore, the analysis time is shorter than with untreated capillary. [158] As a result, the repeatability of

migration was improved and the analyte zones were narrow which led to lower quantification limits than in Publication I.

Because of the permanently coated capillary, the concentrations of Ca^{2+} and Mg^{2+} were re-optimized. The effect of Ca^{2+} addition on the BGE is presented in Figure 25, where it can be observed that the addition enhanced the separation of succinate and malate, and of acetate and glycolate. It can be noted from the electropherogram that the greatest complex formation effect of Ca^{2+} was with malate, glycolate and gluconate because retardation of migration times can be seen. Furthermore, the addition resulted in better peak shapes.

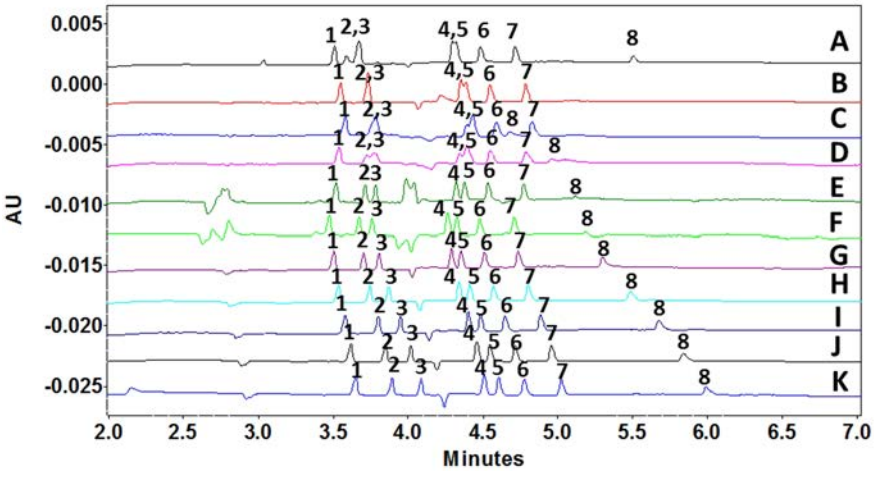

Figure 25. The effect of Ca^{2+} addition on the BGE performance. BGE 20 mM 2,3-PDC (pH 9) and A) 0 mg/L, B) 10 mg/L, C) 20 mg/L, D) 30 mg/L, E) 40 mg/L, F) 50 mg/L, G) 60 mg/L, H) 70 mg/L, I) 80 mg/L, J) 90 mg/L, and K) 100 mg/L Ca^{2+}. Peak assignments: 1. formate; 2. succinate; 3. malate; 4. acetate; 5. glycolate; 6. glyoxylate; 7. lactate; and 8. gluconate. The concentration of the analytes was 50 mg/L. Other analysis conditions: amine coated eCAP silica capillary with 50 µm I.D. and 50/60 cm length, separation voltage –20 kV and temperature 25 °C, injection with 0.5 psi vacuum for 5 s. [Publication III]

After Ca^{2+} was optimized, the concentration of Mg^{2+} was studied. It was noted that Mg^{2+} improved the separation especially between acetate and glycolate, and made the method slightly faster as presented in Figure 26. However, in concentrations above 50 mg/L the complex formation with malate became a drawback and the peak started to diminish. Furthermore, the gluconate peak started to become smaller with higher Mg^{2+} concentration.

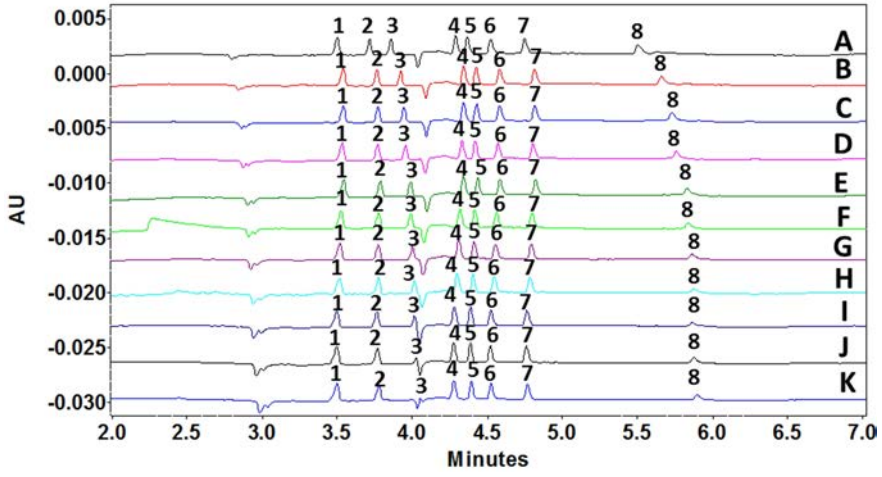

Figure 26. The effect of Mg^{2+} addition on the BGE performance. BGE 20 mM 2,3-PDC with 80 mg/L Ca^{2+} (pH 9) and A) 0 mg/L, B) 10 mg/L, C) 20 mg/L, D) 30 mg/L, E) 40 mg/L, F) 50 mg/L, G) 60 mg/L, H) 70 mg/L, I) 80 mg/L, J) 90 mg/L, and K) 100 mg/L Mg^{2+}. Peak assignments: 1. formate; 2. succinate; 3. malate; 4. acetate; 5. glycolate; 6. glyoxylate; 7. lactate; and 8. gluconate. The concentration of the analytes was 50 mg/L. Other analysis conditions as described in Figure 25. [Publication III]

Methanol was added to the BGE but it was noted (Figure 27) that in the electropherogram the glyoxylate peak started to broaden and made the identification and data processing difficult especially in low concentrations. The addition of alcohol had a significant effect on the separation because of its retardation effect on the analytes, and therefore the addition of isopropanol was tested (Figure 28). Addition of isopropanol in 10% (v/v) quantity enabled better resolution for the separation.

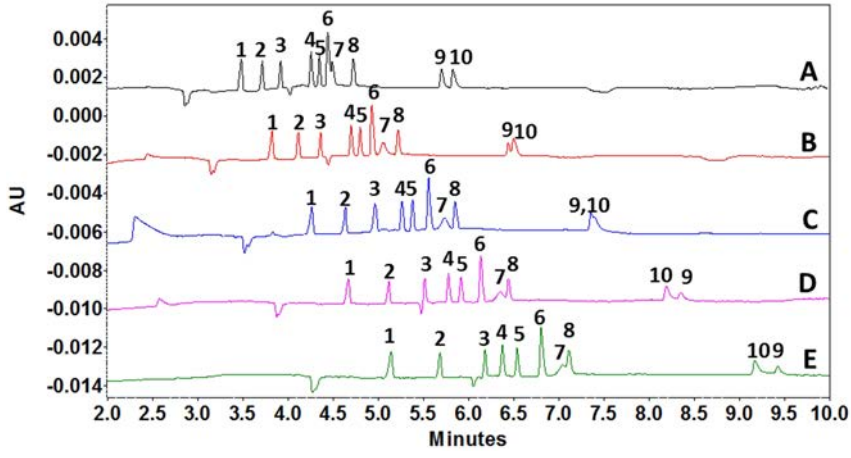

Figure 27. The effect of methanol addition on the separation efficiency. BGE 20 mM 2,3-PDC, 80 mg/L Ca^{2+} and 40 mg/L Mg^{2+} (pH 9) with A) 0%, B) 5%, C) 10%, D) 15%, and E) 20% methanol (v/v). Peak assignments: 1. formate; 2. succinate; 3. malate; 4. acetate; 5. glycolate; 6. PIPES; 7. glyoxylate; 8. lactate; 9. gluconate; and 10. xylonate. The concentration of the analytes was 20 mg/L, except PIPES, gluconate and xylonate 50 mg/L. Other analysis conditions as described in Figure 25 [Publication III]

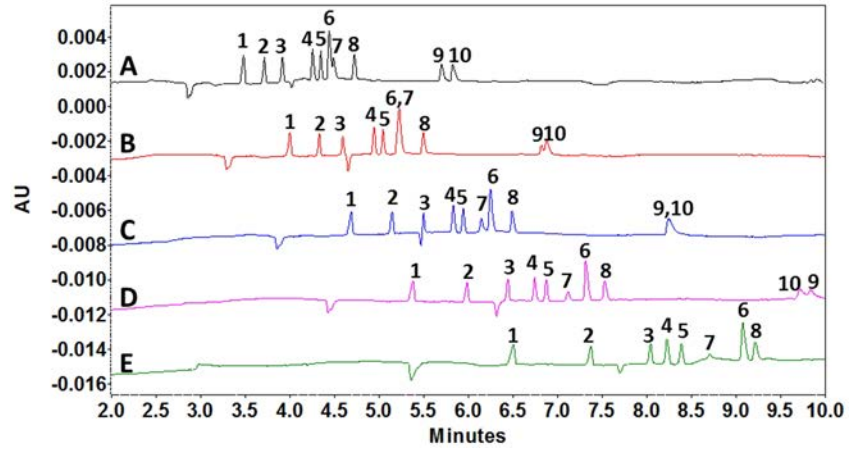

Figure 28. The effect of isopropanol addition on the separation efficiency. BGE 20 mM 2,3-PDC, 80 mg/L Ca^{2+} and 40 mg/L Mg^{2+} (pH 9) with A) 0%, B) 5%, C) 10%, D) 15%, and E) 20% isopropanol (v/v). Peak assignments: 1. formate; 2. succinate; 3. malate; 4. acetate; 5. glycolate; 6. glyoxylate; 7. PIPES; 8. lactate; 9. gluconate; and 10. xylonate. The concentration of the analytes was 20 mg/L, except PIPES, gluconate and xylonate 50 mg/L. Other analysis conditions as described in Figure 25. [Publication III]

4.3.2 Online analysis of cultivations

The optimized analysis method was used to analyze carboxylic acid production online during *K. lactis* and *S. cerevisiae* cultivations. The *K. lactis* cultivation lasted for almost 140 hours, and a total of 97 online and 41 offline samples were collected. The separation of carboxylates during the cultivation is presented in Figure 29.

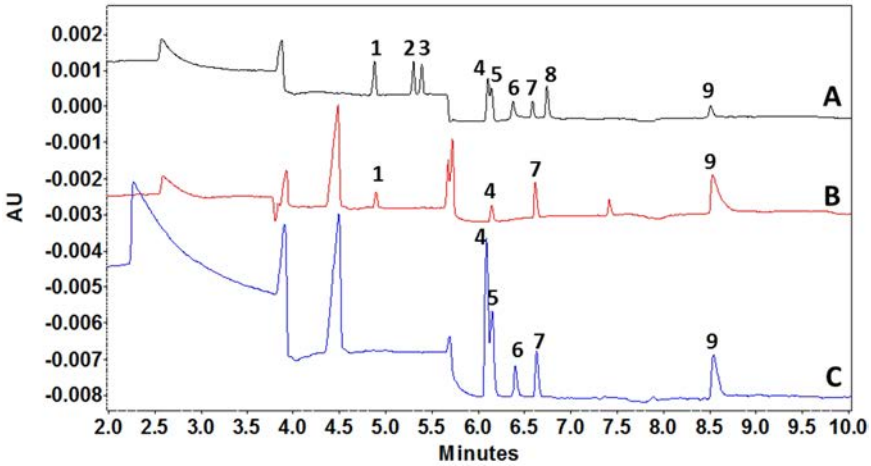

Figure 29. Online analysis of carboxylic acids during a bioreactor cultivation of *K. lactis*. A) Separation of standards at 50 mg/L; B) bioreactor sample at 0 h; and C) bioreactor sample at 60 h. Peak assignments: 1. formate; 2. malate; 3. succinate; 4. acetate; 5. glycolate; 6. glyoxylate; 7. PIPES; 8. lactate; and 9. gluconate. Analysis conditions: BGE 20 mM 2,3-PDC with 80 mg/L Ca^{2+}, 40 mg/L Mg^{2+} and 10% IPA (pH 9), amine coated eCAP silica capillary with 50 µm I.D. and 50/60 cm length, separation voltage –20 kV and temperature 25 °C, injection with 0.5 psi vacuum for 5 s. [Publication III]

The purpose of the *K. lactis* cultivation was to monitor the production of glycolate from ethanol in aerobic conditions. The online analysis results were compared to offline analysis results as presented in Figure 30 where the HPLC analysis results are also summarized. The concentration of acetate increased to about 1 g/L (Figure 30A) but it began to decrease after 90 hours of cultivation. At that point, the yeast had consumed all the ethanol (Figure 30D) provided as substrate and it started to use acetate as carbon source. From Figure 30 B and C it can be noted that the concentrations of glycolic and glyoxylic acids increased throughout the cultivation. The concentrations of acetate and glyoxylate were slightly higher in online analysis compared to offline analyses, which was probably due to losses during manual sampling. In the manually taken sample, the conditions during sample preparation are not as oxidizing as in the cultivation which enhances the formation of glycolate from glyoxylate. For this reason, and because the amount of

yeast cells increases during cultivation, the offline concentration of glycolate is higher especially at the end of the cultivation. In addition, the change in the conditions during sample preparation compared to those in the cultivation might enable the formation of acetaldehyde or ethanol from acetate. In the online analysis system the cells are removed from the sample faster than during manual sampling, and the balanced cultivation conditions are not altered by the sampling event. Furthermore, in this particular cultivation, the online analysis of the product glycolate and the by-products acetate and glyoxylate could be used to optimize the aeration conditions for maximizing the production of glycolate.

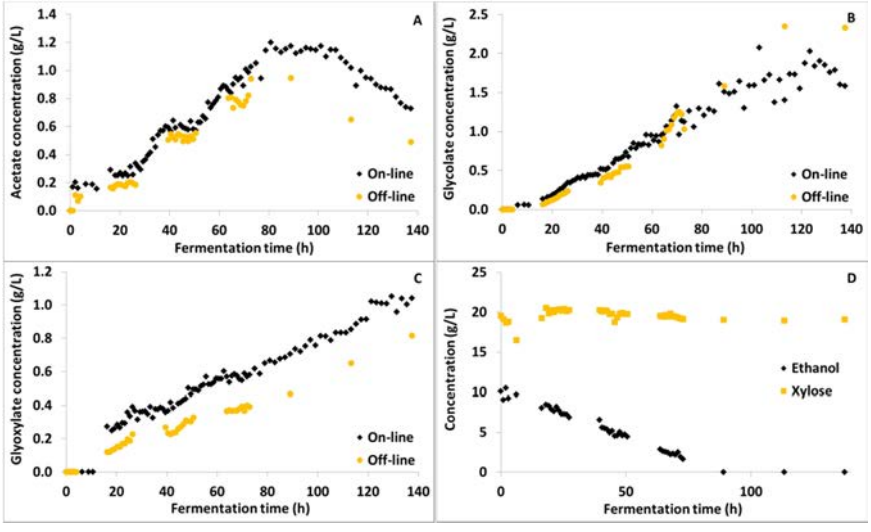

Figure 30. Comparison of online and offline CE analysis results of a *K. lactis* cultivation, and the offline HPLC results. A) Acetate; B) glycolate; C) glyoxylate; and D) HPLC results of ethanol and xylose. [Publication III]

The *S. cerevisiae* cultivation lasted for almost 70 hours, and in total 62 online and 23 offline samples were collected. The separation of carboxylates during the cultivation is presented in Figure 31.

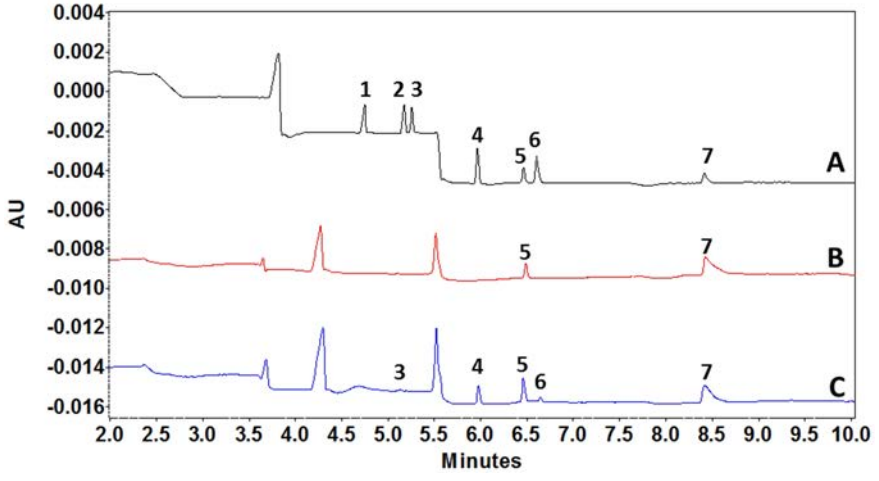

Figure 31. Online analysis of carboxylic acids during a bioreactor cultivation of *S. cerevisiae*. A) Separation of standards at 50 mg/L; B) bioreactor sample at 0 h; and C) bioreactor sample at 22 h. Peak assignments: 1. formate; 2. malate; 3. succinate; 4. acetate; 5. PIPES; 6. lactate; and 7. gluconate. Analysis conditions as described in Figure 29. [Publication III]

The objective in the *S. cerevisiae* cultivation was to study the production of ethanol from xylose under anaerobic conditions in which the carboxylates are by-products of the cultivation. As presented in Figure 32, the concentrations of malate and acetate (Figure 32A and B, respectively) increased during cultivation but the production of lactate (Figure 32C) ended already after 20 hours. At that point, glucose had been consumed almost completely (Figure 32D). When the cultivation was ended at 64 hours, xylose was still not completely utilized by the yeast. As the HPLC results depict, alcohols were also formed during the cultivation, especially ethanol and xylitol but also small amounts of glycerol. Online measurements provided detailed information about the dynamics of the production of the by-products acetate, malate and lactate when comparing the metabolism of glucose and xylose.

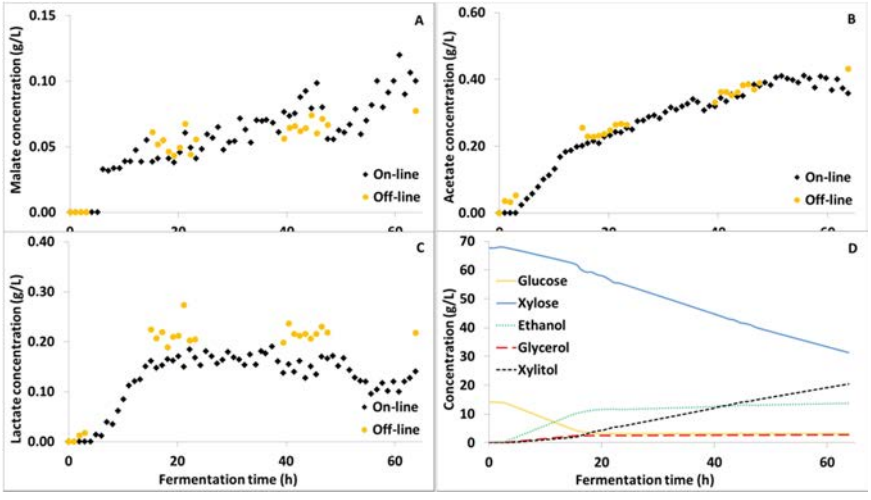

Figure 32. Comparison of online and offline CE analysis results of a *S. cerevisiae* cultivation, and the offline HPLC results. A) Malate; B) acetate; C) lactate; and D) HPLC results of glucose, xylose, ethanol, glycerol, and xylitol. [Publication III]

During both cultivations, concentrations of internal standards were monitored, and during data processing they were used to correct the results. 1,4-piperazinedi-ethane sulfonic acid (PIPES) was added to the cultivation media in order to study the sampling and filtration, and gluconic acid was added to the diluent in order to study the online dilution process. The data collected from the internal standard peak areas during the cultivation is presented in Figure 33. It can be seen that in both cultivations, the concentration of gluconic acid remained approximately the same throughout the cultivation but the concentration of PIPES began to decrease slowly. This was primarily caused by the online filtration because in the *S. cerevisiae* cultivation (Figure 33B) the filter paper was changed at 45 hours and the peak area was normalized. However, the concentration started to decrease quite rapidly, probably due to the high cell concentration in the bioreactor.

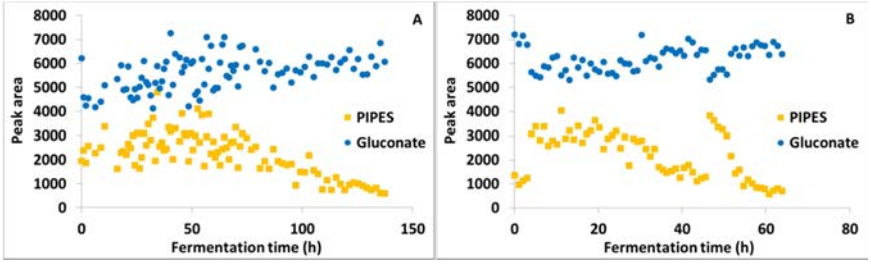

Figure 33. Comparison of internal standard peak areas. A) K. lactis, B) *S. cerevisiae*. PIPES was added to the cultivation media and gluconate to the diluent. [Publication III]

4.4 Determination of amino acids by MEKC-LIF

In Publication IV, CE with LIF detection was used to develop an analysis method for studying amino acids. The optimized method was used to monitor amino acid consumption of *S. cerevisiae* during beer fermentation.

4.4.1 Method development

For the analysis of amino acids, an extensive study was made to find a suitable BGE composition. The tested electrolyte chemicals were tetraborate [166, 176–179], phosphate [180], tetraborate–phosphate, lithium carbonate [181], ammonium acetate [182] and ammonium formate [120, 183–185] which are all common electrolytes in amino acid analyses. Tetraborate was found to be the most suitable electrolyte chemical for the method. The separation efficiency of tetraborate is presented in Figure 34.

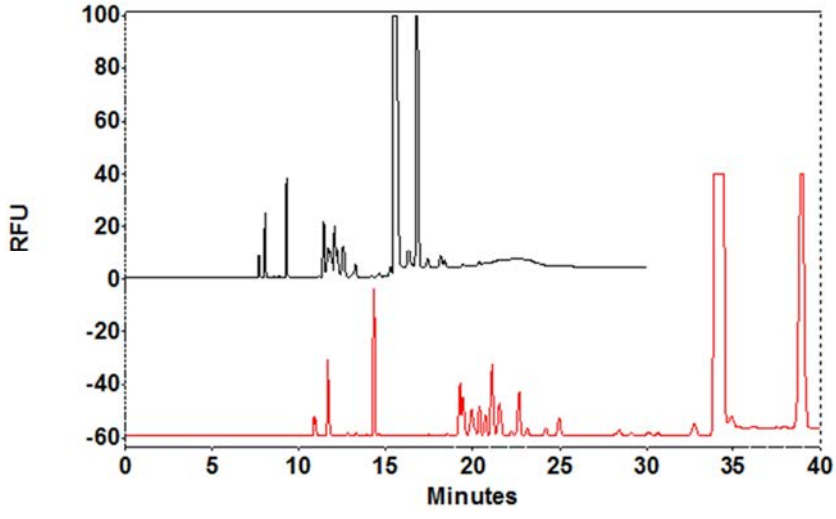

Figure 34. The effect of tetraborate concentration on the separation of amino acids. A) 30 mM, and B) 50 mM. Other analysis conditions: voltage +25 kV, temperature 20 °C, capillary length 60 cm (50 cm to the detector), injection 0.5 psi 10 s and LIF excitation 488 nm and emission 520 nm. Unpublished data.

To enhance the separation between amino acids sodium dodecyl sulfate (SDS) [162, 166, 176, 186], 3-[3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) [178], 18-crown-6-ether (18C6) [187] and β-cyclodextrin [166,174,188] were tested. It was noted that the addition of CHAPSO in 10 mM concentration gave the best separation efficiency above all other BGE solutions tested but there were precipitation problems with the BGE that could not be re-

solved. The addition of the second best surfactant SDS to the BGE is presented in Figure 35.

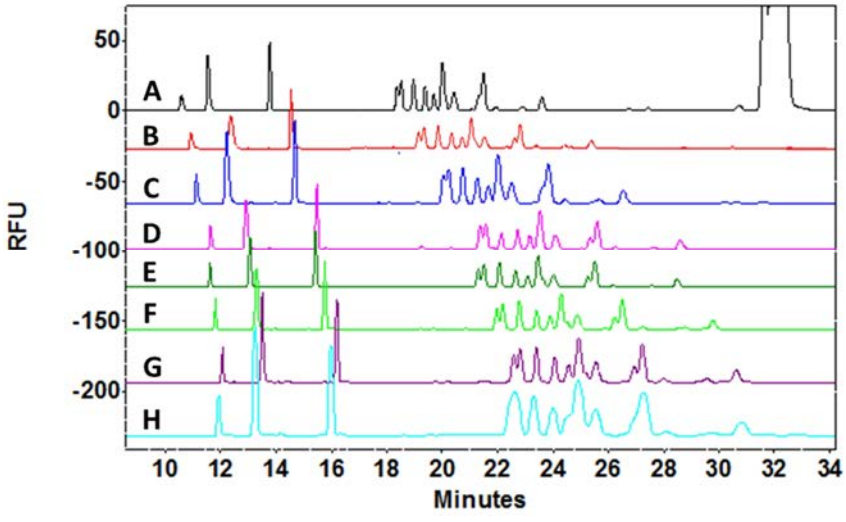

Figure 35. SDS addition to the BGE. BGE: 50 mM tetraborate with A) 20 mM, B) 30 mM, C) 40 mM, and D) 50 mM SDS, pH 9.0. Other conditions as in Figure 34. Unpublished data.

The separation efficiency was not as good with the SDS compared to CHAPSO, and therefore further modification was needed. β-Cyclodextrin and 18C6 were added to the BGE consisting of tetraborate and SDS. 18C6 is classified as a class I organic modifier in SDS-mediated MEKC and it is used to tune selectivity and resolution. Class I modifiers have an effect on the pseudostationary phase through direct interaction with the micelles. [184] The effect of 18C6 addition to the BGE is presented in Figure 36.

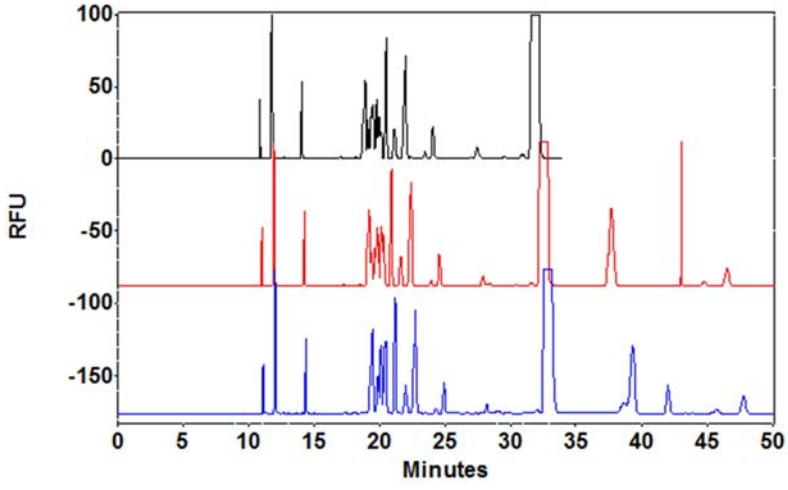

Figure 36. The effect of 18C6 addition to the BGE. BGE: 50 mM tetraborate with 30 mM SDS and A) 10 mM, B) 20 mM and C) 30 mM 18C6, pH 9.0. Other conditions as in Figure 34. [Publication IV]

After optimization of the BGE, other analysis conditions were tested. The separation of amino acids with the optimized analysis method is presented in Figure 37. Leucine and isoleucine migrated as one peak because no chiral selector was used in this study.

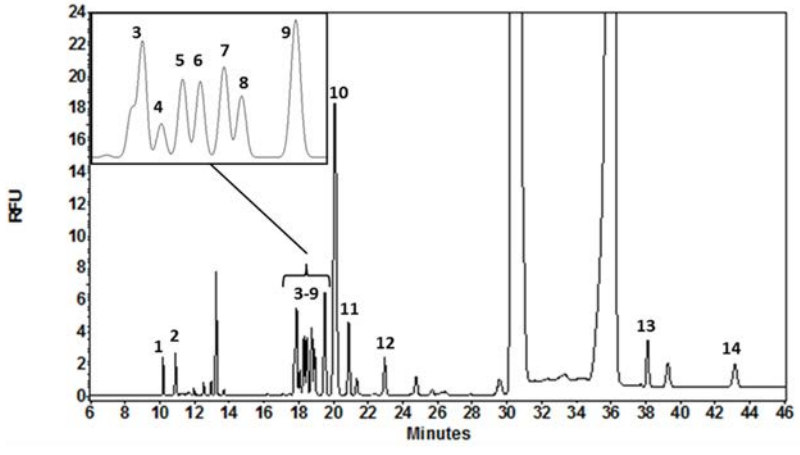

Figure 37. Separation of amino acids. BGE: 50 mM tetraborate, 30 mM SDS and 20 mM 18C6, pH 9.0. Other analysis conditions: voltage +30 kV, temperature 15 °C, capillary length 60 cm (50 cm to the detector), injection 0.5 psi 10 s and LIF excitation 488 nm and emission 520 nm. The current during analysis was ~62 µA. Peak assignments: 1) arginine, 2) lysine, 3) leucine/isoleucine, 4) tyramine, 5) histidine, 6) glutamine, 7) valine, 8) phenylalanine, 9) threonine/asparagine, 10) proline, 11) serine, 12) glycine, 13) glutamic acid, and 14) aspartic acid. [Publication IV]

4.4.2 Labeling chemistry

Succinimidyl esters react with nucleophiles with release of N-hydroxysuccinimide (Figure 38). Reaction with primary and secondary amines creates stable amide and imide linkages, respectively. With succinimidyl esters, the hydrolysis and amine reactivity both increase with increasing pH. [189] For our purpose, it was important that the derivatization reaction occurs in mild conditions and relatively rapidly. As presented in Figure 39, the use of 6-isomer resulted in less peaks originated from the label in the electropherogram. The resulting reaction products are very stable at room temperature.

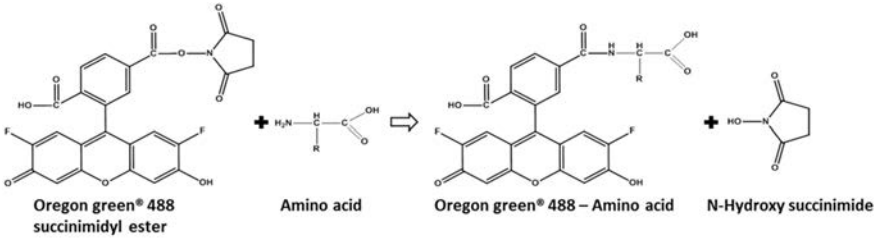

Figure 38. Labeling reaction of Oregon green 488® succinimidyl ester (6-isomer) with an amino acid. [Publication IV]

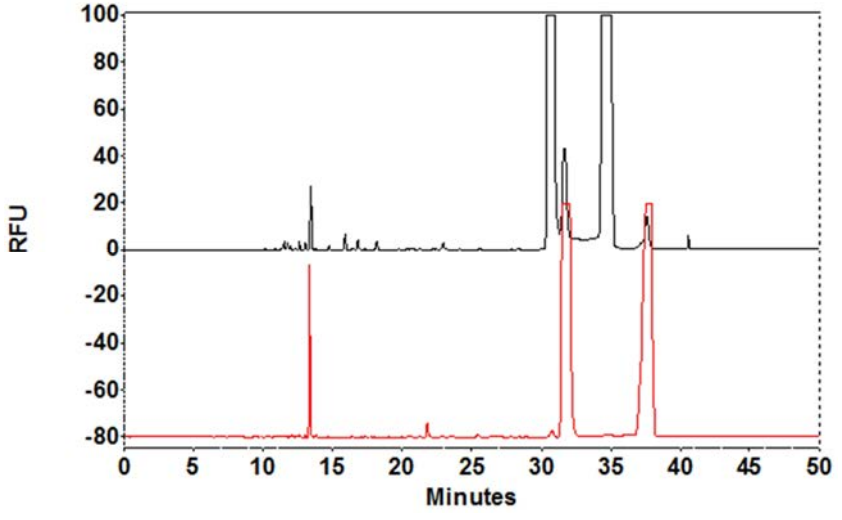

Figure 39. Comparison of 5- and 6-isomers of Oregon green 488® succinimidyl ester. A) 5-isomer and B) 6-isomer. Analysis conditions as in Figure 37. [Publication IV]

4.4.3 Analysis of beer fermentation samples

Beer fermentation samples were analyzed with the optimized analysis method for the determination of amino acids. An electropherogram of standard separation and a cultivation sample is presented in Figure 40.

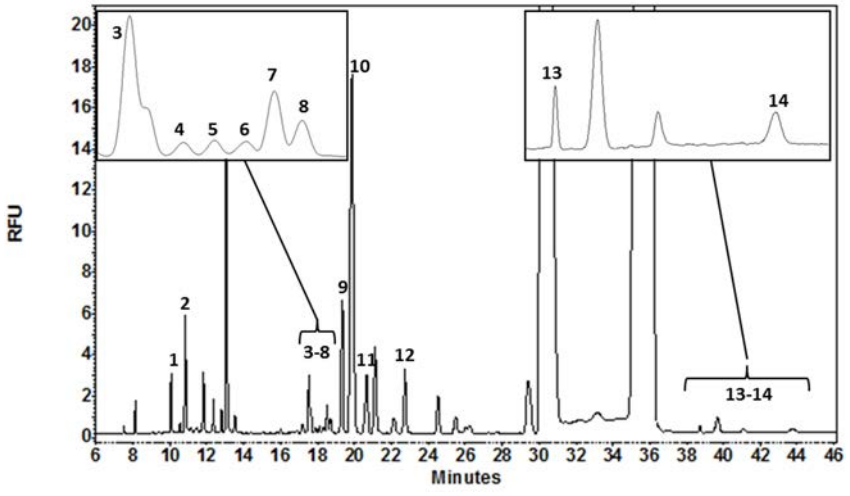

Figure 40. Electrophoretic separation of a cultivation sample. Analysis conditions as in Figure 37. Peak assignments: 1) arginine, 2) lysine, 3) leucine/isoleucine, 4) tyramine, 5) histidine, 6) glutamine, 7) valine, 8) phenylalanine, 9) threonine/ asparagine, 10) proline, 11) serine, 12) glycine, 13) glutamic acid, and 14) aspartic acid. [Publication IV]

The results of the fermentation are presented in Figure 41, where the concentration changes of amino acids are presented as a function of fermentation time. It can be seen that the concentrations of most of the amino acids decreased during cultivation, as expected. The most rapid decrease in amino acid concentrations occurred during the first 30 hours of the cultivation when the glucose was also consumed (Figure 42A). In addition, the fastest increase in biomass concentration (Figure 42B) occurred at the same time, indicating that glucose and most of the amino acids are used for the growth of the biomass. The increase in some amino acid concentrations might have been caused by stress experienced by the yeast cells during the fermentation. When yeast cells are stressed they may release proteolytic enzymes, in particular protease A, which has the effect of liberating free amino acids from peptides or proteins.

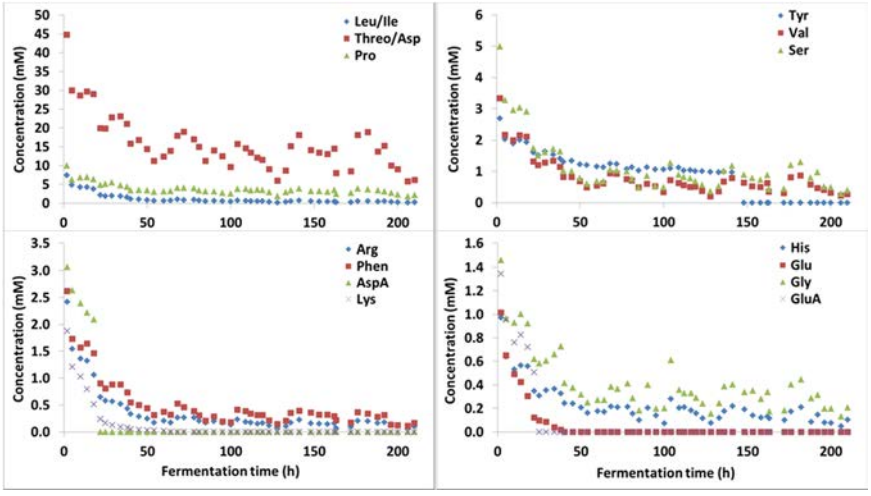

Figure 41. The consumption of amino acids by *Saccharomyces cerevisiae* during beer fermentation. [Publication IV]

The results in Figure 42A show that the concentrations of maltose and maltotriose decreased during the cultivation and the concentration of ethanol increased. In the beginning of the cultivation some glycerol was formed but the concentration remained low. The results in Figure 42B illustrate that the biomass concentration remained approximately the same between 70 h and 120 h but started to decrease after maltose was consumed from the fermentation broth.

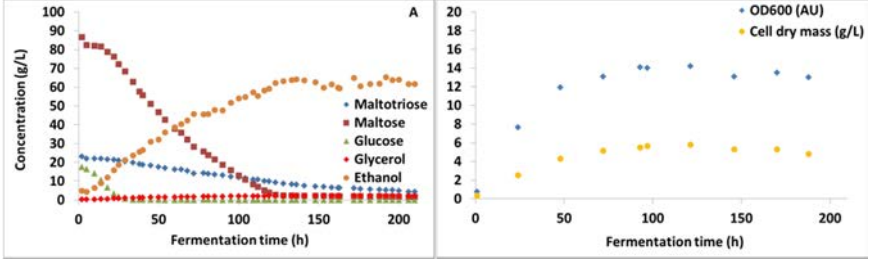

Figure 42. A) HPLC and B) biomass results of the beer fermentation. [Publication IV]

5. Conclusions and future prospects

New capillary electrophoretic analysis methods with UV- and LIF-detections were developed for the monitoring of bioprocesses in both offline and online mode. Offline CE analyses were performed for the analysis of bioreactor cultivations containing carboxylic, phenolic and amino acids. An online CE set-up was modified from a commercial CE device and a flow-through sample vial was designed and constructed to enable sampling to the CE instrument. The online analysis system was used to monitor carboxylic acid production in two different bioreactor cultivations.

The offline analysis of carboxylic acid enabled the simultaneous analysis and quantification of 22 carboxylic acids. The analysis method was used for monitoring *Gluconobacter oxydans* cultivation in wheat straw hydrolysate, which has a complex composition. The *G. oxydans* was able to convert xylose to xylonic acid and glucose to gluconic acid with 45% and 96% yield, respectively.

Carboxylic acids were also monitored online in *Kluyveromyces lactis* and *Saccharomyces cerevisiae* cultivations. In genetically engineered *K. lactis* cultivation the production of glycolic acid from ethanol in aerobic conditions was studied, and in *S. cerevisiae* cultivation the production of ethanol from xylose was monitored under anaerobic conditions in which carboxylates are by-products. The online and offline analysis results correlated rather well in both cultivations.

Phenolic compounds were studied in cultivations that were performed in a synthetic inhibitor mix and in spruce hydrolysate medium. The optimized analysis method was able to separate and quantitate 14 phenolic compounds. It was noted that the *Saccharomyces cerevisiae* strains used in this study were able to decrease the concentrations of some of the phenolic compounds. In addition, the concentration of inhibitors and biomass had an effect on the productivity of ethanol and on the consumption of glucose and xylose that were used as substrates.

Amino acid consumption of *Saccharomyces cerevisiae* during beer fermentation was studied with CE using LIF detection. The amino acids were labelled with Oregon Green 488 succinimidyl ester-label, which is a fluorinated analogue of fluorescein. Reaction of OG-SE with primary and secondary amines creates stable amide and imide linkages, respectively, and the reaction occurs rapidly in mild conditions. The most rapid consumption of amino acids occurred during the first 30 hours when glucose was also utilized. This indicates that most of the growth of

the yeast occurred during that time, and the OD_{600} and CDW results supported this observation.

Capillary electrophoresis is widely known for its use in academia and in the analysis of macromolecules such as DNA, RNA and proteins. This thesis demonstrates the use of capillary electrophoresis in industrial applications illustrating its versatility for the analysis of small molecules that are present in bioprocesses. With small adjustments, a commercial CE device was used as an automated bioprocess monitoring device that produced high quality data from real processes in a robust and repeatable manner. Overall, the CE was proven to be a diverse and feasible routine analysis device for several different cultivations using even hydrolysate as cultivation medium. With CE, it is possible to develop analysis methods for a wide variety of compounds that are present in bioprocesses and other processes. In addition, depending on the sampling and sample treatment, CE has potential for the online and offline analysis of cultivations using other microorganisms than yeast and bacteria, such as filamentous organisms, and mammalian and plant cells.

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PUBLICATION I

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

In: Journal of Chromatography A 1217,
pp. 1537–1542.

Journal of Chromatography A, 1217 (2010) 1537–1542

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

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

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ARTICLE INFO

Article history:
Received 1 September 2009
Received in revised form
22 December 2009
Accepted 24 December 2009
Available online 4 January 2010

Keywords:
Capillary electrophoresis
Organic acids
Complex forming agents in electrolyte solution
Bioprocess monitoring

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 estimated 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

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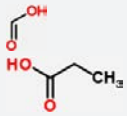
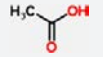
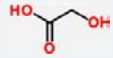
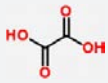
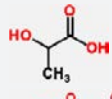
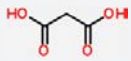
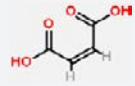
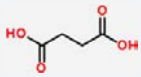
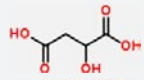
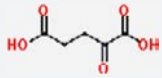
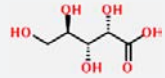
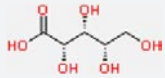
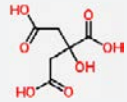
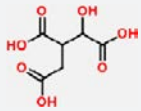
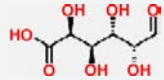
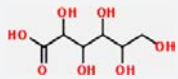
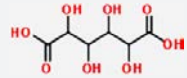
doi:10.1016/j.chroma.2009.12.075

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 Ca^{2+} and Mg^{2+} 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

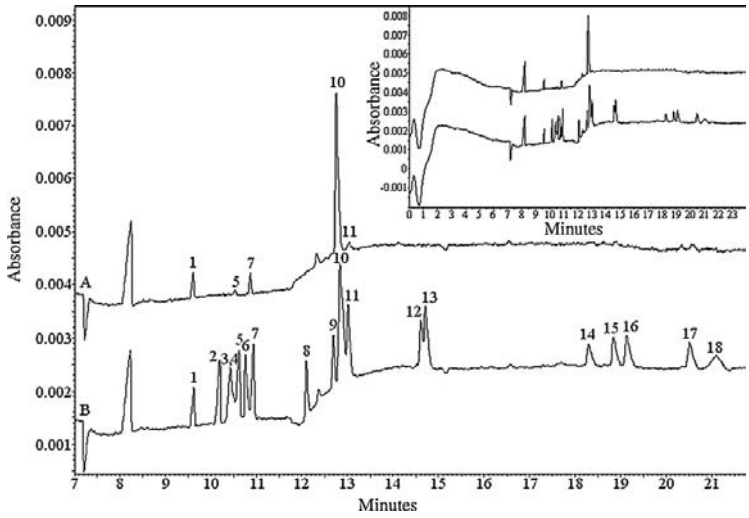


Fig. 1. (A) Blank cultivation medium; and (B) cultivation medium where the studied 18 organic acids have been added in concentration of 50 mg/l. Peak assignments: 1, formic acid; 2, malonic acid; 3, maleic acid; 4, α-ketoglutaric acid; 5, succinic acid; 6, oxalic acid; 7, malic acid; 8, isocitric acid; 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, capillaries 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. Complexation 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

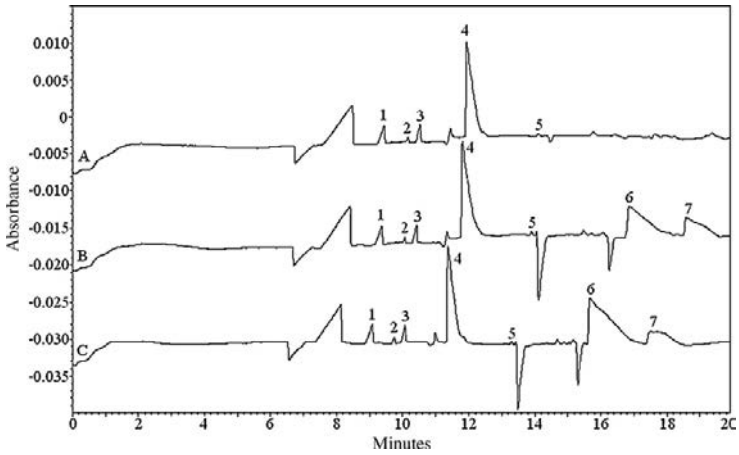


Fig. 2. Electrophoretic separation of carboxylic acids in wheat straw hydrolyzate. Fermentation time: (A) 0 h; (B) 19.8 h; and (C) 119.1 h. Peak assignments: 1, formic acid; 2, succinic acid; 3, malic acid; 4, acetic acid; 5, lactic acid; 6, xylonic acid; and 7, gluconic acid. Analysis conditions as 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-

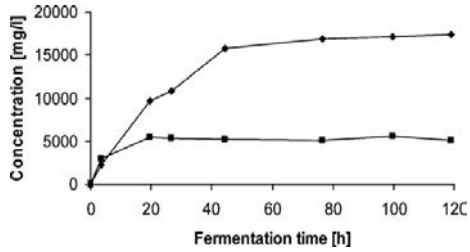


Fig. 3. Production of xylonic (♦) and gluconic (■) acids from wheat straw hydrolyzate by *Gluconobacter oxydans*.

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

This work was carried out within the Finnish Centre of Excellence in Green Chemistry and White Biotechnology Research granted by Academy of Finland (grant number 118573). Cultivations were performed within the EU project BioSynergy (038994-SES6). We also thank Tarja Laakso for technical assistance with the fermentations.

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PUBLICATION II

Capillary electrophoresis for the monitoring of phenolic compounds in bioprocesses

In: Journal of Chromatography A 1278,
pp. 175–180.

Journal of Chromatography A, 1278 (2013) 175–180

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Capillary electrophoresis for the monitoring of phenolic compounds in bioprocesses

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ARTICLE INFO

Article history:
Received 12 October 2012
Received in revised form
19 December 2012
Accepted 3 January 2013
Available online 10 January 2013

Keywords:
Bioprocess monitoring
Capillary electrophoresis
Phenolic compounds

ABSTRACT

Hydrolysates of lignocellulosic biomass, used as substrates for the sustainable production of fuels and chemicals often contain high amounts of phenolic compounds inhibiting the production microbiota. Quantification of these inhibitor compounds may help to understand possible difficulties in bioprocessing and further the development of more efficient, robust and tolerable processes. A separation method based on capillary electrophoresis with UV detection was developed for the simultaneous quantification of 10 phenolic compounds that may have inhibitor properties. Intraday relative standard deviations were less than 0.7% for migration times and between 2.6% and 6.4% for peak areas. Interday relative standard deviations were less than 3.0% for migration times and between 5.0% and 7.2% for peak areas. The method was applied to demonstrate that *Saccharomyces cerevisiae* was able to decrease the concentrations of vanillin, coniferyl aldehyde, syringaldehyde, acetoguaiacone and cinnamic acid during the cultivation, whereas the concentrations of phenols increased.

1. Introduction

The use of lignocellulosic materials as a renewable source for the production of fuels and chemicals has increased during recent years. Lignocellulose is a natural, abundant and cheap material that consisting of three main components: cellulose, hemicellulose, and lignin [1]. In order to obtain monomeric sugars that can be metabolized by microorganisms, cellulose and hemicellulose must be hydrolyzed. Particularly during acid hydrolysis, several byproducts are also formed in addition to sugars. Some of these byproducts can be toxic to the production organism. Therefore in high concentrations they decrease the product yield by inhibiting the metabolism [2].

Inhibitors originating from lignocellulosic material can broadly be divided into three groups: aliphatic acids, furan derivatives, and phenolic compounds. The number and amount of these inhibiting byproducts may vary significantly between different raw materials and pretreatments and hydrolysis conditions [3]. Analysis of the inhibitors may provide additional information for the optimization of bioprocesses and may represent a valuable tool in the production of tolerant production hosts [4].

The inhibition mechanisms of phenolic compounds have been studied, but the mechanisms are still not completely understood. It has been found that biomass yields, growth rates and ethanol productivities are decreased. Phenolic compounds may also act on cell membranes. In general, aldehydes and ketones are stronger inhibitors than alcohols [4–7].

Two approaches can be used to study the effects of phenolic compounds in cultivation; either the use of hydrolysates derived from lignocellulosic materials or synthetic media in which all the necessary compounds have been artificially added to the cultivation medium. When using synthetic inhibitors, it is easier to regulate individual inhibitor conditions and to study their effects on the organism and on the overall bioprocess [8]. The inhibitor composition of synthetic spruce hydrolysate is presented in Table 1 [9].

Phenolic compounds have been analyzed from different hydrolysates by gas chromatography (GC) [3,10] and liquid chromatography (LC) [2,11]. The use of GC for the analysis of phenolic compounds is rather time-consuming process because of the necessity of purification of the sample and silylation before analysis. LC is a versatile analysis technique, but it is time-consuming and it lacks sensitivity. Capillary electrophoresis (CE) has also been used for the analysis of phenolic compounds in green tea [12], olive oil [13], wines and beverages [14–16], propolis ("bee glue") [17], and berries [18]. Phenolic compounds of lignocellulosic origin have been analyzed by CE with UV and mass spectrometric (MS) detection from aged papers [19,20] and with CE–MS from complex effluents from cellulose processing [21] and from aerosol samples from biomass burning [22]. To the best knowledge of the authors, CE has not hitherto been used for the analyses of phenolic compounds in lignocellulosic hydrolysates.

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0021-9673/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2013.01.004

Table 1
Synthetic inhibitor composition of spruce hydrolysate.

Compound	Concentration (mg/L)
5-HMF	3400
Furfural	1100
Acetic acid	6300
Formic acid	1200
Levulinic acid	2400
Hydroquinone	20
Vanillin	140
Syringaldehyde	120
Coniferyl aldehyde	50
Cinnamic acid	1
4-Hydroxybenzoic acid	20
Acetoguaiacone	20

In this study a CE method was developed for the simultaneous separation, identification, and quantification of 10 phenolic compounds. This method was applied for bioprocess monitoring of yeast cultivations using either synthetic media or spruce hydrolysate as cultivation media. Using the method, identification and quantification of the phenolic inhibitors were acheived. This study enables a monitoring system with great importance for the optimization of bioprocesses and the development of tolerant hosts for biofuels production. The phenolic compounds investigated in this study are presented in Table 2.

2. Experimental

2.1. Chemicals

Standards were prepared in LC–MS grade methanol from Fluka (Buchs, Switzerland), in 99.5% ethanol from Altia (Rajamaki, Finland) or in 18 mΩ deionized water (Millipore, Bedford, MA, USA) at 1000 mg/L quantity according to their solubility presented in Table 2. Phenol (≥99.5%), coniferyl aldehyde (98%), *p*-coumaric acid (≥98%), 4-hydroxybenzoic acid (99%), acetoguaiacone (98%), vanillin (99%), and cinnamic acid (≥99%) were purchased from Sigma–Aldrich (Steinheim, Germany). Benzoic acid (≥99.5%) and vanillic acid (≥97%) were from Fluka, and syringaldehyde (≥98%) was from SAFC (Arklow, Ireland).

Background electrolyte solutions (BGE) were prepared from sodium tetraborate decahydrate (≥99.5%), sodium acetate (≥99%), ammonium acetate (≥98%), sodium dodecyl sulphate (SDS) (≥99%), 18-crown-6-ether (99%), β-cyclodextrin (≥97%), and CHAPSO (≥98%), which were purchased from Sigma–Aldrich. A 0.1 M sodium hydroxide solution for the capillary conditioning was from FF-Chemicals Ab (Haukipudas, Finland).

2.2. Instrumentation

All the measurements were made with the P/ACE MDQ capillary electrophoresis system (Beckmann Coulter Inc., Fullerton, CA, USA) equipped with a PDA detector. Untreated fused-silica capillaries (Teknolab, Trollasen, Norway) with inner diameter 50 μm, outer diameter 365 μm, and total length 50 cm (40 cm to the detector) were used. Capillaries were conditioned before use with 0.1 M NaOH, Milli-Q purified water, and BGE for 10 min each. Voltage (+30 kV) was then applied for 5 min. Between analyses, capillaries were rinsed with 0.1 M NaOH and 18 mΩ deionized water for 1 min and with BGE for 1.5 min. Data were collected and processed with 32Karat software (Beckmann Coulter Inc., Fullerton, CA, USA).

2.3. Analysis conditions

BGE was prepared with 40 mM sodium tetraborate and 40 mM 18-crown-6-ether in Milli-Q purified water. The pH of the solution was 9.0. Before analysis, the BGE was degassed by ultrasound and filtered through a 0.45 μm Acrodisc GHP syringe filter (Pall Life Science, Ann Arbor, MI, USA).

Standards and samples were injected by using 0.5 psi pressure for 5 s, giving rise to an injection volume of 5 nL. Separation was carried out at +30 kV at constant capillary temperature +20 °C. Sample cartridge temperature was set at +10 °C. Direct UV detection was at 200 and 254 nm. For the determination and quantification of phenols, vanillin, coumaric acid, vanillic acid, benzoic acid and cinnamic acid 200 nm were used. For the determination and quantification of coniferyl aldehyde, acetoguaiacone, syringaldehyde and 4-hydroxybenzoic acid the detection wavelength were 254 nm.

2.4. Cultivation conditions

2.4.1. Cultivation 1

Saccharomyces cerevisiae strain HDY·GUF6 (provided by NEMO EU-project partner Prof. Eckhard Boles at Goethe University of Frankfurt in Germany) was maintained in 1.5 L culture in Biostat B bioreactor (Sartorius, Germany) at pH 5.0, temperature +30 °C, agitation 200 rpm, under anaerobic conditions with 0.5 L/min nitrogen. Cultures were inoculated with cells grown for 24 h in YPD-medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose) at 30 °C and 200 rpm to give an initial biomass concentration of approximately 0.15 g/L. Cultivation was performed in synthetic medium simulating spruce hydrolysate supplemented with minimum mineral medium [23] and with 20 g/L glucose and 50 g/L xylose as the main carbon sources. The final concentration of the inhibitor mix was 50%. The inhibitor mix in 100% concentration is presented in Table 1.

2.4.2. Cultivation 2

Same as cultivation 1, but with 20% inhibitor mix.

2.4.3. Cultivation 3

Same as cultivation 2, but the initial biomass concentration was 1.5 g/L.

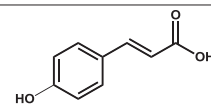
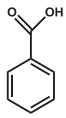
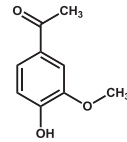
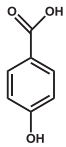
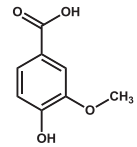
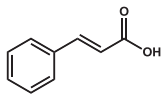
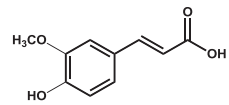
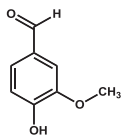
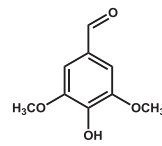
2.4.4. Cultivation 4

S. cerevisiae (ethanol red) was maintained in 1.5 L culture in a Biostat CT-DCU bioreactor (Sartorius, Germany) at pH 4.9, temperature +32 °C, agitation 300 rpm, and in anaerobic conditions with 1 L/min nitrogen. The cells were grown for 24 h in medium containing 16.6 g/L glucose, 7.5 g/L $(NH_4)_2SO_4$, 3.5 g/L KH_2PO_4, 0.75 g/L $MgSO_4$, 10 mL/L trace metal solution [24], and 1 mL/L vitamin solution [24] (sterile filtered) in pH 5.0, temperature +30 °C and agitation 180 rpm. After this, cells were transferred to the bioreactor where they were grown for 20 h in medium containing 20 g/L glucose, 20 g/L $(NH_4)_2SO_4$, 10 g/L KH_2PO_4, 2 g/L $MgSO_4$, 27 mL/L trace metal solution, and 2.7 mL/L vitamin solution (sterile filtered) in pH 5.0, +30 °C, agitation 800 rpm and air flow rate 1.2 L/min to give an initial biomass concentration of approximately 3.5 g/L. Cultures were maintained in medium derived from spruce hydrolysate (heat exploded, pH adjusted to 4.8; SEKAB, Örnsköldsvik, Sweden) supplemented with 0.5 g/L $(NH_4)_2HPO_4$, 1 g/L yeast extract and 0.025 g/L $MgSO_4{\cdot}7H_2O$.

2.5. Sample preparation

For calibration, five concentration levels of standard mixtures were prepared for the 10 phenolic compounds. The concentration range in the mixtures was 1–100 mg/L. Before analyses both the cultivation and the hydrolysate samples were centrifuged at

Table 2
Phenolic compounds and their structures.

Compound	Molecular weight (g/mol)	Structure	Solubility	Compound	Molecular weight (g/mol)	Structure	Solubility
Phenol	94.11		Water	*p*-Coumaric acid	164.16		Ethanol
Benzoic acid	122.12		Water	Acetoguaiacone	166.17		Methanol
4-Hydroxybenzoic acid	138.12		Water	Vanillic acid	168.15		Methanol
Cinnamic acid	148.17		Methanol	Coniferyl aldehyde	178.18		Methanol
Vanillin	152.15		Water	Syringaldehyde	182.17		Methanol

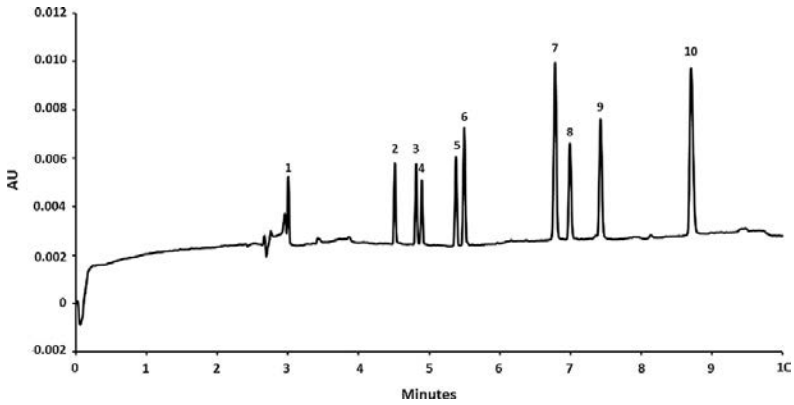


Fig. 1. Electrophoretic separation of 10 phenolic compounds. Peak assignments: (1) phenol; (2) coniferyl aldehyde; (3) acetoguaiacone; (4) syringaldehyde; (5) vanillin; (6) cinnamic acid; (7) *p*-coumaric acid; (8) vanillic acid; (9) benzoic acid; and (10) 4-hydroxybenzoic acid. The concentrations of the analytes were 20 mg/L. Analysis conditions as described in the text.

10,000 rpm for 5 min. In addition, the hydrolysate samples were diluted to 1:2 (v/v) with Milli-Q water.

3. Results and discussion

3.1. Method validation

For the development of a method for quantification of phenolic compound using capillary electrophoresis, sodium acetate, ammonium acetate, and sodium tetraborate were studied in the optimization of BGE in different concentrations and pH values. Sodium tetraborate was found to be the most suitable buffer chemical for this application. The phenolic compounds present in hydrolysates are difficult to analyze with direct UV detection, because of the low concentrations of the analytes and the complex media containing large number of UV-absorbing compounds. In order to enhance separation of the studied compounds, the use of micellar electokinetic chromatography (MEKC) was studied. In MEKC a pseudostationary phase is formed in the capillary from the buffer and a surfactant above its critical micelle concentration (cmc). In addition to the separation based on charge-to-mass ratio, the migration of an analyte is also dependent on its partition coefficient between the micelles and aqueous phase of the BGE [25]. Several surfactants were studied in order to identify a suitable one for this application. Additions of sodium dodecyl sulphate (SDS) and β-cyclodextrin were found to be ineffective. 3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) had some effect on the separation, but the addition of 18-crown-6-ether improved the separation significantly. pH between 8 and 11 was tested for the BGE. An electropherogram of the optimized analysis is presented in Fig. 1.

Different analytical conditions were also examined. Voltage, analysis temperature, capillary length, and detection wavelength were optimized in the ranges of 5–30 kV, 15–40 °C, 30–80 cm (total length), and 200–300 nm, respectively.

In the optimized method 40 mM sodium tetraborate decahydrate with 40 mM 18-crown-6-ether, pH 9 was used for the analysis. Voltage was set to +30 kV, capillary temperature +20 °C, total capillary length was 50 cm, and detection wavelengths were 200 and 254 nm.

Fig. 1 illustrates an electropherogram of standard solution where the 10 phenolic compounds presented in Table 2 are separated.

3.2. Quantification

Concentration calibration between 1 mg/L and 100 mg/L was used for each phenolic compound in the cultivation samples. Intraday relative standard deviations (RSD%) for migration times that

Table 3
Performance of the method under optimized conditions.

	Phenol	Coniferyl aldehyde	Acetoguaiacone	Syringaldehyde	Vanillin
Migration time (intraday)[a]	0.1	0.1	0.1	0.1	0.1
Migration time (interday)[b]	0.8	1.4	1.6	1.6	1.8
Peak area (intraday)[a]	6.4	2.8	3.6	3.2	3.7
Peak area (interday)[b]	7.2	5.0	5.8	5.9	5.9
Calibration correlation coefficient	0.9712	0.9973	0.9980	0.9987	0.9985
Detection limit (mg/L)	0.2	0.1	0.2	0.3	0.3

	Cinnamic acid	*p*-Coumaric acid	Vanillic acid	Benzoic acid	4-Hydroxybenzoic acid
Migration time (intraday)[a]	0.1	0.1	0.5	0.5	0.7
Migration time (interday)[b]	1.8	2.3	2.3	2.4	3.0
Peak area (intraday)[a]	2.6	2.8	2.9	3.1	2.9
Peak area (interday)[b]	6.9	6.4	6.2	7.2	6.4
Calibration correlation coefficient	0.9942	0.9876	0.9990	0.9915	0.9949
Detection limit (mg/L)	0.1	0.1	0.2	0.3	0.3

[a] Percentage of relative standard deviation, $n = 10$.
[b] Percentage of relative standard deviation, $n = 5$.

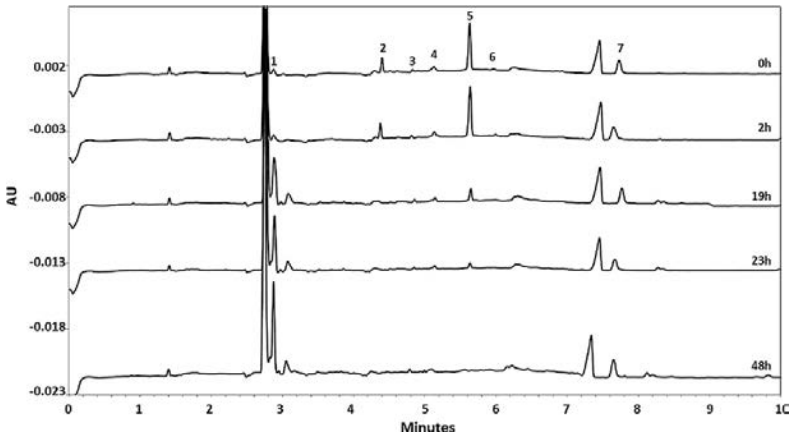


Fig. 2. Electrophoretic separation of phenolic compounds present in cultivation 1 with synthetic media at 5 different time points. Peak assignments: (1) phenol; (2) coniferyl aldehyde; (3) acetoguaiacone; (4) syringaldehyde; (5) vanillin; (6) cinnamic acid; and (7) 4-hydroxybenzoic acid. Analysis conditions as described in the text.

were less than 0.7% and those for peak areas that were between 2.6% and 6.4%. Interday RSD% for migration times of the studied compounds were less than 3.0% and those for peak areas were between 5.0% and 7.2%. The detection limits for the phenolic compound standards were between 0.1 and 0.3 mg/L. Correlation coefficients for the calibration curves varied between 0.9712 and 0.9987. The correlation coefficients, repeatability, and reproducibility of the analysis method are summarized in Table 3.

3.3. Analysis of cultivation samples

Electropherograms of the samples are presented in Figs. 2 (cultivation 1) and 3 (cultivation 4). Significant results of the cultivations are summarized in Fig. 4.

The optimized method was applied to the analyses of phenolic compounds in four different cultivations. The purpose of this study was to compare the concentration levels of these compounds during different cultivations. In cultivations 1–3 a synthetic medium was used to study the effect of inhibitors presented in Table 1 on the *S. cerevisiae* strain HDY.GUF6. Cultivations 1 and 2 are comparable, since they both had the same initial biomass concentration (0.15 g/L), but with different inhibitor mix concentrations (50% vs. 20%, respectively). Cultivations 2 and 3 had the same inhibitor mix concentration (20%), but the initial biomass concentrations were 0.15 g/L and 1.5 g/L, respectively. During these cultivations concentrations of vanillin, coniferyl aldehyde, and syringaldehyde decreased below their detection limit and at first the concentration of phenols increased. Furthermore in cultivation 2 the acetoguaiacone and in cultivation 1 the cinnamic acid concentrations decreased below their detection limit. Thereafter the concentration increase of phenols halted or even started to decrease. This implies that *S. cerevisiae* is able to metabolize these phenolic compounds. The concentrations of acetoguaiacone and 4-hydroxybenzoic acid remained at the same level throughout the cultivation in cultivations 1 and 3. In cultivation 2, the concentration of 4-hydroxybenzoic acid remained constant. Higher inhibitor concentration (Fig. 4A) in cultivation 1 slowed down the conversion of phenolic compounds when compared to cultivation 2 (Fig. 4B). Higher initial biomass concentration in cultivation 3 (Fig. 4C) enabled faster conversion compared to cultivation 2.

Spruce hydrolysate is a more hostile medium for the cultivation than the synthetic hydrolysate and contains more compounds

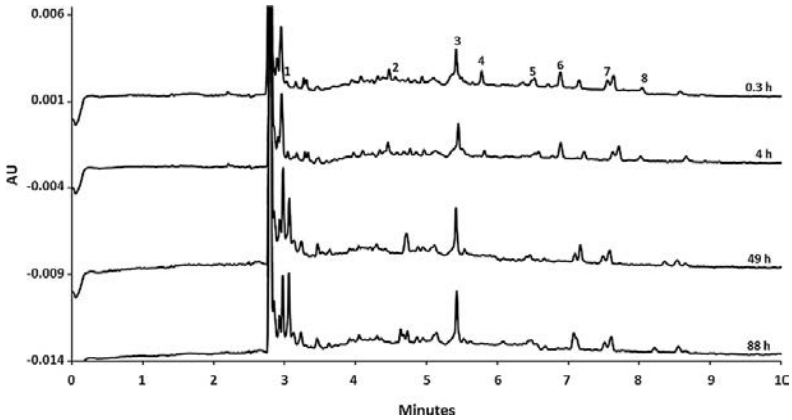


Fig. 3. Electrophoretic separation of phenolic compound present in cultivation 4 with spruce hydrolysate at 4 different time points. Peak assignments: (1) phenol; (2) coniferyl aldehyde; (3) syringaldehyde; (4) vanillin; (5) *p*-coumaric acid; (6) vanillic acid; (7) benzoic acid; and (8) 4-hydroxybenzoic acid. Analysis conditions as described in the text.

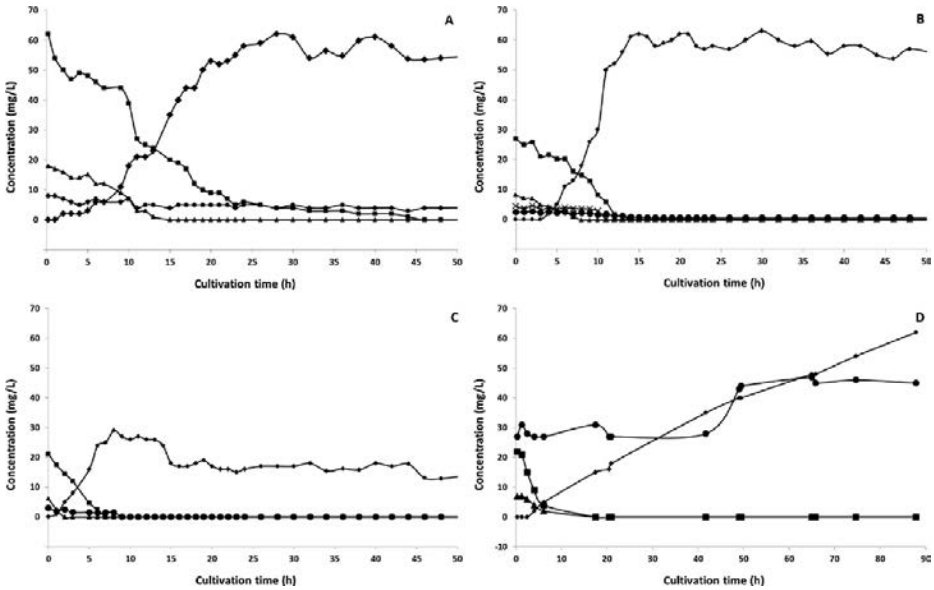


Fig. 4. Diagram of the cultivation results. (A) Cultivation 1; (B) cultivation 2; (C) cultivation 3; and (D) cultivation 4. Symbols: phenol (♦); syringaldehyde (●); vanillin (■); acetoguaiacone (×); and coniferyl aldehyde (▲).

with possible synergistic inhibitory effects on microorganisms. Cultivation 4 was performed in spruce hydrolysate, and phenolic compounds were analyzed in order to test the resolving power of the analysis method in more complex media. Although the repeatability of the migration times was very good even with the spruce hydrolysate, the migration of the analytes was tested with standard addition methods for compounds characterization. It was noted that in more phenolic compounds were observed this cultivation medium (Fig. 4D). Furthermore, probably due to the harshness of the media, results differed from the synthetic media used in cultivations 1–3. The concentrations of coniferyl aldehyde and vanillin were decreased. Surprisingly, in addition to the concentration of phenols, the concentration of syringaldehyde was also increased. The concentrations of *p*-coumaric, vanillic, benzoic and 4-hydroxybenzoic acids were approximately constant.

4. Conclusions

An analysis method for the identification and quantification of 10 phenolic compounds was developed for capillary electrophoresis. This method was successfully used in the monitoring of four different *S. cerevisiae* cultivations. The yeast strains used in this study were able to tolerate synthetic media with inhibitor mix or real spruce hydrolysate and were able to metabolize some of the phenolic compounds. Metabolic routes for the degradation of phenolic compounds are not generally known for yeast.

Acknowledgements

This work was carried out within the Finnish Center of Excellence in White Biotechnology – Green Chemistry Research granted by the Academy of Finland (grant number 118573). Heidi Turkia was also supported by Graduate School for Chemical Sensors and Microanalytical Systems (CHEMSEM). Cultivations were performed within the EU project NEMO, which received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) (grant agreement no. 222699). We also thank Stefan Bruder and Eila Leino for their technical assistance in performing the cultivations.

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PUBLICATION III

Online Capillary Electrophoresis for Monitoring Carboxylic Acid Production by Yeast during Bioreactor Cultivations

In: Analytical Chemistry 85, pp. 9705–9712.

analytical chemistry

Article

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Online Capillary Electrophoresis for Monitoring Carboxylic Acid Production by Yeast during Bioreactor Cultivations

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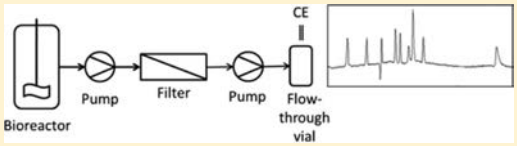
Supporting Information

ABSTRACT: Bioprocess monitoring can improve the understanding and control of biotechnological processes. When analyses are carried out as automated online measurements, manual steps of the analysis procedures are avoided, thus decreasing both the time required for analyses and systematic errors. In this study, an online capillary electrophoresis (CE) system with flow-through sample vial made in-house and action control programming was assembled to monitor carboxylic acid production by *Kluyveromyces lactis* and *Saccharomyces cerevisiae* during two different bioreactor cultivations. The relative standard deviations were less than 0.6% for intraday migration times and the total analysis time was less than 20 min. The system operated continuously and automatically up to 6 days and produced data concerning carboxylic acid production during the cultivations. The successful test runs demonstrated that this system has potential for the monitoring of biotechnological processes.



The analysis of products, byproducts, and other physicochemical parameters is of great importance in bioprocess technology. When optimizing bioprocesses for the production of fuels and chemicals, the lack of accurate real-time monitoring of different physical, chemical, and biological cultivation parameters can represent a significant bottleneck. The US Food and Drug Administration (FDA) has issued an initiative for process analytical technology (PAT) with the goal of improving understanding and control of manufacturing processes as a result of real-time monitoring.[1] The greatest advantage of automated measurements is the avoidance of manual steps during the analysis procedure. In addition to the analysis of pH and temperature, the most common online analyses in bioprocess monitoring are performed in situ with different kinds of probes utilizing infrared or fluorescence spectroscopy. The biggest drawback of these approaches is the limited number of analytes which can be determined and the difficulty of calibration, but the main advantage is that these analyses are conducted in real-time.[2] When using chromatographic techniques in online bioprocess monitoring, the analysis of several compounds is possible simultaneously. Liquid chromatography (LC) is probably the most common technique in chromatographic online analyses. There are at least three companies worldwide manufacturing online LC devices, including Waters, Dionex and Bayer. Online HPLC has been used for the monitoring of sugar consumption and carboxylic acid production during *Escherichia coli* fermentations,[3] and for online monitoring and control of ethanol fermentation by *Zymomonas mobiliz*.[4] Extracellular metabolite concentrations have been monitored during a batch cultivation of *Saccharomyces cerevisiae*.[2] Online HPLC has also been used for monitoring anions and carboxylic acids in wastewater treatment processes,[5] degradation products of dyes in textile industry process waters,[6,7] for carbohydrates and volatile fatty acids in fermentative biohydrogen production,[8] and for glucose and ethanol in *Escherichia coli* cultivation.[9]

For the analysis of gaseous and volatile samples, GC is the most common chromatographic technique. However, the online GC analysis of aqueous samples can be difficult because of the necessary derivatization procedure. Dihydroxyacetone and glycerol have been analyzed from fermentation broths by pyrolytic methylation GC with a vertical microfurnace pyrolyzer in which the analytes were converted into their corresponding methyl ethers and analyzed by GC with a flame ionization detector (FID).[10] An online GC-MS system has been used for automated monitoring of crude wastewater. A two-stage injector made in-house enabled the analysis of 140 volatile and semivolatile compounds.[11]

Capillary electrophoresis (CE) is a relatively new approach for online monitoring. Hitherto, commercial devices are not available so all the published work has been carried out with reconstructed offline or in-house built CE devices. Online CE has been used for process monitoring in the pulp and paper industry. A device that used an in-house constructed sample flow cell was developed for the analysis of dissolvable inorganic ions as well as some carboxylic acids from circulation waters of pulp and paper machines.[12,13] Portable CE instruments have been developed for the analysis of inorganic anions and cations,

Received: July 11, 2013
Accepted: September 23, 2013
Published: September 23, 2013

dx.doi.org/10.1021/ac402113x | *Anal. Chem.* 2013, 85, 9705−9712

and organic acids.[14,15] In online bioprocess monitoring, CE has been used for the analysis of carboxylic acids,[16] adenosine triphosphate (ATP) and adenosine diphosphate (ADP),[17] and heavy metals.[18] Furthermore, in vivo microdialysis has been coupled online to CE-LIF in order to study glutamate and GABA levels in rat brain.[19]

Carboxylic acids are important metabolites of several biochemical pathways in microorganisms. Because they are frequently either the main products or significant byproducts of bioprocesses, their monitoring is often of considerable importance.[20]

In this study, a commercial capillary electrophoresis device was modified, a flow-through sample vial was designed and constructed, and a sampling and filtration system was assembled to enable online analysis of carboxylic acid production by two yeast species, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*.

EXPERIMENTAL SECTION

Reagents and Materials. All chemicals used for standards were of analytical reagent grade and were prepared in 18 mΩ deionized water (Millipore, Bedford, MA, USA) at 10 g/L. Acetic acid, D-lactic acid (lithium salt), and glycolic acid were purchased from Fluka (Buchs, Switzerland). Formic acid was from Merck (Darmstadt, Germany). Gluconic acid (sodium salt), glyoxylic acid, (monohydrate), malic acid, succinic acid, and PIPES (1,4-piperazinediethane sulfonic acid, disodium salt) were from Sigma-Aldrich (Steinheim, Germany).

Chemicals used for the background electrolyte solution (BGE) were 2,3-pyridinedicarboxylic acid (2,3-PDC) (Sigma-Aldrich), calcium chloride dihydrate and magnesium chloride hexahydrate (Riedel-de Haen, Seelze, Germany), methanol (Merck), and isopropanol (Merck). A 0.1 M sodium hydroxide solution for the capillary conditioning was from FF-Chemicals AB (Haukipudas, Finland). Amine regeneration solution for capillary conditioning was purchased from Beckman Coulter (Fullerton, CA, U.S.A.).

Online Setup. The automated sampling system presented in Figure 1 consisted of a peristaltic pump with an 8-channel

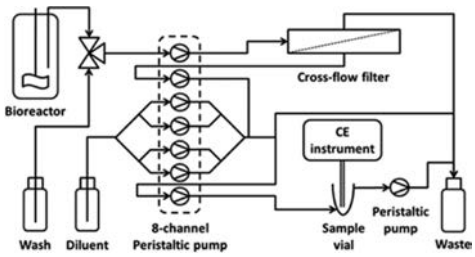


Figure 1. Schematic figure of the online analysis system.

pump head (MCP, Ismatec Labortechnik Analytik, Zurich, Switzerland) for withdrawing the sample from the bioreactor and delivering it forward to filtration and to analysis, and also for pumping the diluent; a 3-way solenoid-operated pinch valve (Bio-Chem valve, Boonton, NJ, U.S.A.) for selecting between the sampling line from the bioreactor and a line for washing liquid; a cross-flow filter (A-Sep, Applikon, Schiedam, the Netherlands) with 45 mm diameter filter membranes (GVWP, Millipore) of 0.22 μm pore size for providing a cell-free sample; a 500-μL flow-through sample vial made in-house with an inlet at the bottom and outlet at the top (Figure 2A); and a single-channel peristaltic pump (Watson Marlow 101U/R, Falmouth, England) for withdrawing the sample from the sample vial. The flow-through sample vial was made from solid PEEK block by drilling, and inserted inside plastic vial holder (Beckmann Coulter, Figures 2B and C). Holes were drilled to fit the PEEK tubing for liquid flow. The sample vial was placed in the inlet side of the buffer tray in the CE instrument, from where the sample was injected. As presented in Figure 2A, the sample flow was from the bottom of the vial to the top to maintain the sample liquid surface constant. Furthermore, this configuration prevents problems caused by air bubbles. To insert the tubing from pump to the sample vial, a hole was drilled to the covers of CE device. From that same hole the tubing from the sample vial to the waste via pump was run. Silicone tubing (Saint-Gobain, Charny, France) with 0.8 mm inner diameter and 2.4 mm outer diameter was used for fluid lines. Pharmed tubing (Saint-Gobain) with 1.6 mm inner diameter and 3.2 mm outer diameter was used in the pumps. Sample was diluted 5-fold (v/v) by mixing the filtered sample pumped through one pump channel to the diluent pumped through four pump channels. A National Instruments USB-6009 data acquisition device (National Instruments, Austin, TX, U.S.A.) was used for sending analog voltage signals to the 8-channel peristaltic pump, for sending digital output signals to an additional relay supplying 24 V to the pinch valve, and for receiving digital input signals from the CE instrument. National Instruments LabVIEW software (version 8.5) was used to develop control software for the automated measurement sequence. Timing of the sampling was controlled by the CE software and the sampling and filtration sequence (Table 1) was started on the basis of the digital output signal sent from the CE instrument.

Capillary Electrophoresis. All the measurements were made with the P/ACE MDQ capillary electrophoresis system (Beckman Coulter) equipped with a PDA detector. Amine-coated eCAP silica capillaries (Beckman Coulter) with inner diameter 50 μm, outer diameter 365 μm, and total length 60 cm (50 cm to the detector) were used. Capillaries were conditioned before use with 0.1 M NaOH, Milli-Q purified water, amine regeneration solution and BGE for 4, 2, 4, and 4 min, respectively. Voltage (−20 kV) was then applied for 1 min. Between analyses, capillaries were rinsed with 0.1 M NaOH, Milli-Q purified water, amine regeneration solution and with BGE for 2, 1, 2, and 2 min, respectively. The BGE vials were replaced every 25 analyses. Data was collected and processed with 32Karat software version 8.0 (Beckman Coulter).

Internal Standards. Two internal standards were used to correct and monitor possible defects in the automated sampling, filtration, dilution and analysis steps. PIPES was added to the cultivation broth at 1 g/L and gluconic acid to the diluent at 250 mg/L. Thus in CE analysis both internal standards were at 200 mg/L.

Bioreactor Cultivations. Batch bioreactor cultivations were carried out in a 1.6 L bioreactor (KLF+ solo, Bioengineering, Wald, Switzerland) The preculture of genetically modified *Kluyveromyces lactis* (strain H3985)[21] was cultivated for 24 h in shake-flasks containing synthetic complete medium lacking uracil (SC-ura) with 6.7 g/L yeast nitrogen base (YNB) and 20 g/L glucose. The bioreactor cultivation medium contained SC-ura with 6.7 g/L YNB, 20 g/L xylose, 15 g/L ethanol, and 250 μL/L silicone antifoam agent. The bioreactor was inoculated with cells from a preculture in 20 mL

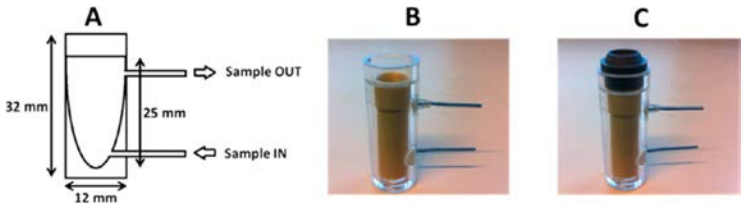


Figure 2. (A) Schematic figure of the flow-through sample vial with dimensions. Picture of the sample vial (B) without and (C) with cap.

Table 1. Sampling Sequence

task	duration (s)	notes
start	1	start of sequence
wait	1	sequence is halted and waits for a trigger signal from CE device
sampling	200	valve is opened and pump is turned on, liquid flow is from bioreactor toward flow-through vial through the filter
break	400	pump off, waiting for injection
wash	300	valve is closed and pump is turned on, liquid flow is from wash bottle toward flow-through vial through the filter
pump off	1	pump is turned off
end		sequence ends and next run is started

of 0.9% NaCl to start the cultivation at an optical density at 600 nm (OD600) of 0.5. Cultivation conditions were: pH 5.0, temperature +30 °C, agitation 500 rpm and aeration 0.33 L/min air.

The preculture of genetically modified *Saccharomyces cerevisiae* (strain VTT C-10883)[22] was grown for 24 h in shake flasks on Yeast Peptone Dextrose (YPD)-medium containing 10 g/L yeast extract, 20 g/L peptone and 20 g/L D-glucose. The medium used in the bioreactor contained 20 g/L glucose, 50 g/L xylose, 5 g/L (NH4)2SO4, 3 g/L KH2PO4, 0.5 g/L MgSO4·7H2O, 1 mL/L trace mineral solution,[23] 1 mL/L vitamin solution,[23] and 250 μL/L silicone antifoam agent. The cells were added to the bioreactor in 20 mL of 0.9% NaCl so that the initial OD600 was 0.5 in the cultivation broth. The cultivation conditions were: temperature +30 °C, pH 5.0, agitation 500 rpm and anaerobic conditions were maintained with a stream of 0.33 L/min nitrogen to the bioreactor headspace.

Sampling. In the *K. lactis* cultivation, the online sampling was performed once an hour until 73 h and thereafter every two hours until the cultivation was ended at 137 h. In the *S. cerevisiae* cultivation, the online sampling was performed once an hour until the cultivation was ended at 64 h. In both cultivations offline sampling was also performed at some of the sampling points in order to enable comparison between online and offline analyses. Offline samples were drawn manually from the bioreactor, centrifuged 10 min at 3 000 rpm (Biofuge Stratos, Heraeus Instruments, Osterode, Germany) and diluted 5-fold (v/v) with Milli-Q purified water. A total of 97 online and 41 offline samples were collected from the *K. lactis* cultivation, and 62 online and 23 offline samples from the *S. cerevisiae* cultivation.

Offline Analyses. The concentrations of xylose and ethanol in the *K. lactis* cultivation and the concentrations of glucose, xylose, xylitol, glycerol and ethanol in the *S. cerevisiae*

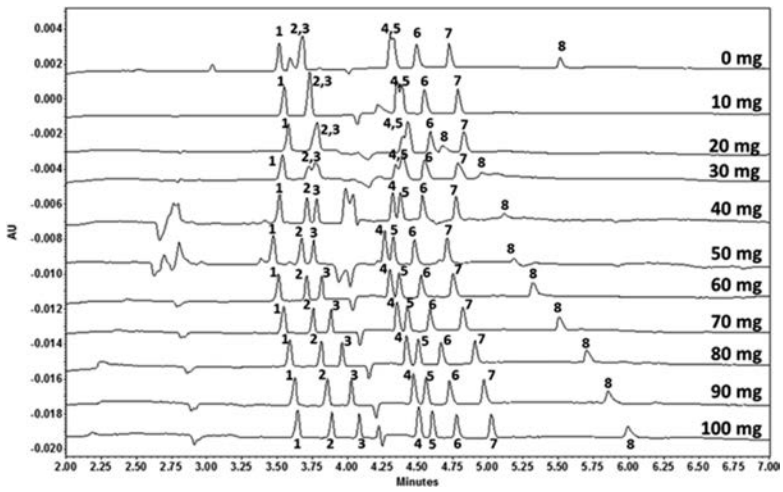


Figure 3. Effect of the addition of Ca^{2+} as complex-forming agent to the BGE. Peak assignments: 1, formate; 2, succinate; 3, malate; 4, acetate; 5, glycolate; 6, glyoxylate; 7, lactate; and 8, gluconate. The concentration of the analytes was 50 mg/L. Other analysis conditions: BGE 20 mM 2,3-PDC (pH 9), amine coated eCAP silica capillary with 50 μm I.D. and 50/60 cm length, separation voltage −20 kV, temperature 25 °C, and injection with 0.5 psi vacuum for 5 s.

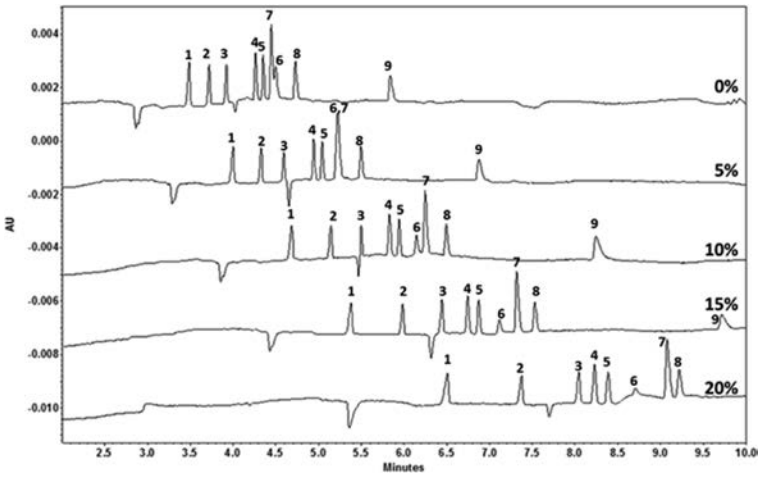


Figure 4. Effect of isopropanol addition to the BGE. Peak assignments: 1, formate; 2, succinate; 3, malate; 4, acetate; 5, glycolate; 6, glyoxylate; 7, PIPES; 8, lactate; 9, gluconate. The concentration of the analytes was 20 mg/L, except PIPES and gluconate 50 mg/L. Other analysis conditions: BGE 20 mM 2,3-PDC with 80 mg/L Ca^{2+} and 40 mg/L Mg^{2+} (pH 9), amine coated eCAP silica capillary with 50 μm I.D. and 50/60 cm length, separation voltage −20 kV, temperature 25 °C, and injection with 0.5 psi vacuum for 5 s.

cultivation were analyzed offline using HPLC. A Waters Alliance 2690 HPLC system (Waters, Milford, MA, U.S.A.) with Waters 2487 dual wavelength UV (210 nm) detector and Waters 2414 differential refractometer were used with an injection volume of 20 μL. The columns used in this study were an Aminex HPX-87H Organic Acid Column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA, U.S.A.) connected to a Fast Acid Analysis Column (100 mm × 7.8 mm, Bio-Rad). The eluent was 5 mM H_2SO_4 (Titrisol, Merck), flow rate 0.5 mL/min and analysis temperature +55 °C.

RESULTS AND DISCUSSION

Method Development. In our previous work, a CE analysis method using uncoated fused silica capillaries was developed for the simultaneous quantification of 18 carboxylic acids potentially present in bioreactor samples with indirect UV detection.[24] In this study, it was noted that the dynamic coating developed on the capillary was not as robust as it should be in online bioprocess monitoring in which the analyses cannot be made with many replicates. Furthermore, in the future possible automated control of a cultivation based on analysis data requires very repeatable migration times. A permanently coated amine capillary was found to fulfill both requirements. In our previous work, intraday relative standard deviations (RSD%) for migration times varied between 0.74% and 1.61% whereas with permanently coated capillary the RSD% were between 0.3% and 0.6%. Furthermore, the analysis time was much shorter improving from 20 min to less than 10 min. The amine coated capillary is positively charged, thus it changes the direction of electroosmotic flow. Hence, negative voltage was applied during the analysis.

Because of this fundamental change in the analysis method, other factors also had to be re-examined. The concentration of the UV absorbing agent and the pH of the BGE were the same but the concentrations of complex forming-agents Ca^{2+} and Mg^{2+}, and the addition of alcohol were revalidated. The concentrations of both complex-forming agents were studied between 0 and 100 mg/L individually and in different mixtures. The effect of Ca^{2+} addition to the BGE is presented in Figure 3. The most notable effects on the separation were observed with succinate, malate, acetate, glycolate, and gluconate. The optimal concentration of Ca^{2+} was chosen to be 80 mg/L. Using this concentration, the separation enhancement properties of Mg^{2+} were studied and is presented in Supporting Information Figure S-1. Mg^{2+} improved the separation especially between acetate and glycolate, and made the method slightly faster. The optimal concentration of Mg^{2+} was 40 mg/L.

Table 2. Performance of the Method under Optimized Conditions

	formate	succinate	malate	acetate	glycolate
RSD% of migration time (intraday)[a]	0.3	0.3	0.3	0.4	0.4
RSD% of migration time (interday)[b]	1.0	1.0	1.1	1.1	1.1
RSD% of peak area (intraday)[a]	14.5	13.7	15.1	18.3	12.2
RSD% of peak area (interday)[b]	16.3	13.9	16.0	20.7	13.3
detection limit (mg/L)	0.5	0.5	1	1	1
calibration correlation coefficient	0.9996	0.9980	0.9973	0.9996	0.9982
		glyoxylate	PIPES	lactate	gluconate
RSD% of migration time (intraday)[a]		0.5	0.5	0.5	0.6
RSD% of migration time (interday)[b]		1.1	1.2	1.2	1.4
RSD% of peak area (intraday)[a]		13.1	12.3	12.6	9.7
RSD% of peak area (interday)[b]		16.4	13.6	13.7	10.3
detection limit (mg/L)		2	2	1	3
calibration correlation coefficient		0.9985	0.9988	0.9988	0.9996

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

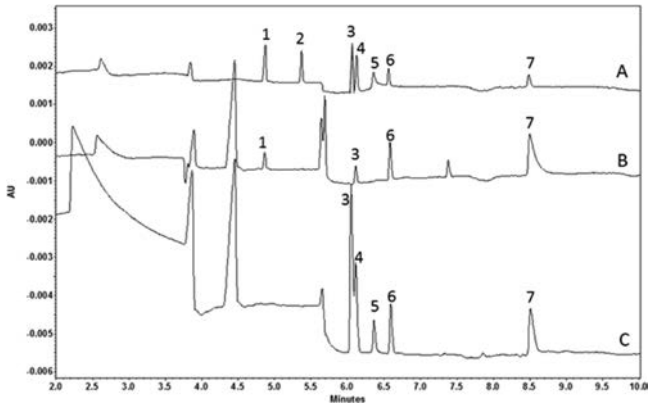


Figure 5. Online analysis of carboxylic acids during a bioreactor cultivation of *K. lactis*. (A) Separation of standards at 50 mg/L, (B) bioreactor sample at 0 h, and (C) bioreactor sample at 60 h. Peak assignments: 1, formate; 2, malate; 3, acetate; 4, glycolate; 5, glyoxylate; 6, PIPES; and 7, gluconate. Analysis conditions: BGE 20 mM 2,3-PDC with 80 mg/L Ca^{2+}, 40 mg/L Mg^{2+}, and 10% IPA (pH 9), amine coated eCAP silica capillary with 50 μm I.D. and 50/60 cm length, separation voltage −20 kV and temperature 25 °C, injection with 0.5 psi vacuum for 5 s.

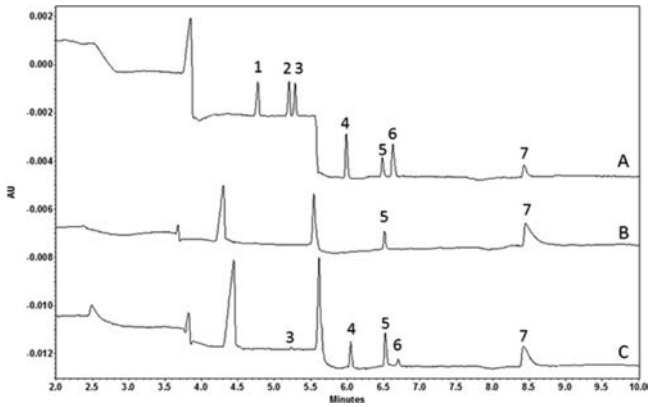


Figure 6. Online analysis of carboxylic acids during a bioreactor cultivation of *S. cerevisiae*. (A) Separation of standards at 50 mg/L, (B) bioreactor sample at 0 h, and (C) bioreactor sample at 22 h. Peak assignments: 1, formate; 2, malate; 3, acetate; 4, PIPES; 5, lactate; 6, gluconate. Analysis conditions as described in Figure 5

Table 3. Correlation of the Online (x) and Offline (y) CE Analyses

K. lactis			
	acetate	glycolate	glyoxylate
equation	$y = 0.8758x - 0.0396$	$y = 1.2193x - 0.1573$	$y = 0.7493x - 0.0416$
correlation coefficient	0.9530	0.8870	0.9703
S. cerevisiae			
	acetate	malate	lactate
equation	$y = 0.9578x + 0.0312$	$y = 0.6967x + 0.0121$	$y = 1.2767x + 0.0165$
correlation coefficient	0.9768	0.7927	0.9061

When reassessing the concentration of methanol in BGE, it was noted that the addition of methanol made the glyoxylic acid peak broader, which made the separation significantly poorer especially at low concentrations (Supporting Information Figure S-2). Because of this phenomenon, the effect of isopropanol on the separation was tested (Figure 4). Isopropanol at a concentration of 10% (v/v) was found to be the most suitable auxiliary substance for this purpose. In general, the addition of alcohol slows down the separation and thus increases the resolution.[25] The addition of either of the alcohols had the greatest effect on the separation of PIPES and glyoxylic acid, which were not separated without the addition.

Other analysis conditions were also revised. Voltage and analysis temperature were optimized in the ranges from −15 to −30 kV and 20 to 30 °C, respectively. Indirect UV detection was set to 254 nm. Because of the flow-through vial that was used for the online analysis of the cultivation samples, it was not possible to use normal pressure injection. Instead, a vacuum was applied to the outlet vial during the injection. However, this approach led to poorer repeatability of the peak area. The intraday RSD% varied between 5.6% and 8.5% for pressure injection, whereas for vacuum injection the repeatability was

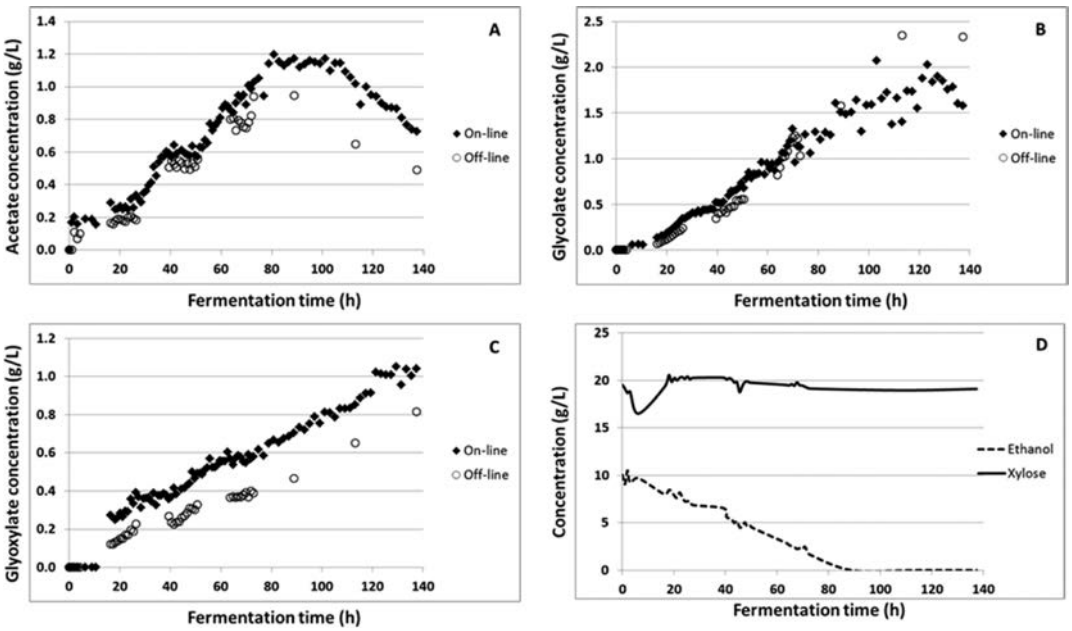


Figure 7. Comparison of online and offline CE analysis results of a *K. lactis* cultivation, and the offline HPLC results: (A) acetate, (B) glycolate, (C) glyoxylate, and (D) HPLC results of ethanol and xylose.

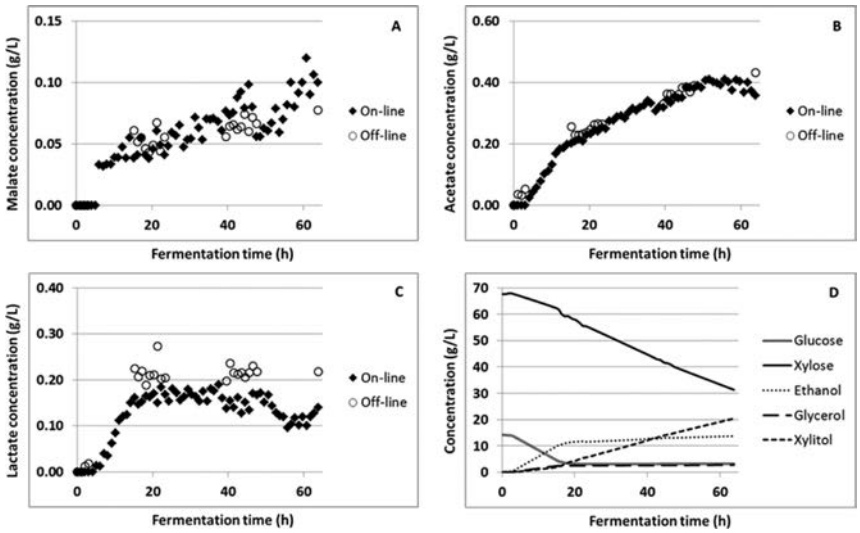


Figure 8. Comparison of online and offline CE analysis results of a *S. cerevisiae* cultivation, and the offline HPLC results: (A) malate, (B) acetate, (C) lactate, and (D) HPLC results of glucose, xylose, ethanol, glycerol, and xylitol.

between 9.7% and 18.3%, but it was still suitable for our purposes. The injection time was tested between 3 and 5 s. The performance of the optimized analysis method was studied in offline analysis mode and the summary is presented in Table 2.

In the optimized method 20 mM 2,3-PDC with 80 mg/L Ca^{2+}, 40 mg/L Mg^{2+}, and 10% (v/v) IPA (pH 9 adjusted with 25% ammonia) was used for the analysis. Negative voltage was set to −20 kV, capillary temperature was 25 °C, total capillary length was 50 cm, and detection wavelength was 254 nm.

Analysis of Cultivation Samples. The online analysis of carboxylic acids during bioreactor cultivations was performed automatically with the optimized CE method. The sampling

interval was either once an hour or once per two hours. Under optimized conditions the separation could be performed at least every 20 min to facilitate accurate monitoring of faster processes. The sample was injected about 8 min after the sampling was started. The total sample volume removed from the reactor was approximately 9 mL per sample. To correct errors of sampling, dilution and analysis, results were corrected with internal standards that were added to the cultivation media (PIPES) and to diluent (gluconic acid). It was noted that in both cultivations, the concentration of gluconic acid remained the same throughout the cultivation, but the concentration of PIPES started to decrease slowly. This was primarily caused by the online filtration because in *S.* cerevisiae cultivation the filter paper was changed at 45 h and the concentration normalized. Data collected from the internal standards is presented in Supporting Information Figures S-3 and S-4. Offline samples were taken and treated manually directly after the sampling. The volume of the samples was 3 mL. The separation of carboxylic acids during cultivations of *K. lactis* and *S. cerevisiae* is presented in Figures 5 and 6, respectively.

In *K. lactis* cultivation, in which the purpose was to study the production of glycolate from ethanol in aerobic conditions, the online and offline CE analysis results correlated rather well, as illustrated in Table 3 in which offline results are presented as a function of online results. The concentration of acetate increased to about 1 g/L (Figure 7A), but it began to decrease after 90 h of cultivation. At that point, the yeast had consumed all the ethanol (Figure 7D) provided as substrate and it started to consume acetate as a substrate. As presented in Figures 7B and C, the concentrations of glycolic and glyoxylic acids increased throughout the cultivation. The concentrations of acetate and glyoxylate were higher in online analysis compared to offline analysis. This was probably due to losses during manual sampling. In the manually taken sample, the conditions during sample preparation (centrifugation) are not as oxidizing as in the cultivation, thus enhancing the formation of glycolate from glyoxylate. For this reason, and because the level of yeast cells increases during cultivation, the offline concentration of glycolate is higher especially at the end of the cultivation. On the other hand, the change in the conditions during sample preparation might enable the formation of acetaldehyde or ethanol from acetate. In the online analysis system, cells are removed from the sample faster than in manual sampling and the balanced cultivation conditions are not altered by the sampling event. Furthermore in the case of this particular cultivation, the online analysis of the product glycolate and the byproducts acetate and glyoxylate could be used to optimize the aeration conditions for maximizing the production of glycolate.

The correlation between online and offline analyses of the *S. cerevisiae* cultivation was also rather good, as presented in Table 3. In this study the purpose was to study the production of ethanol from xylose under anaerobic conditions in which carboxylic acids are byproducts of the cultivation. The concentrations of malate (Figure 8A) and acetate (Figure 8B) increased throughout the cultivation, but the production of lactate (Figure 8C) ended already after 20 h. At that point, as presented in the HPLC results (Figure 8D), glucose had been consumed almost completely. When the cultivation was ended at 64 h, xylose was still not completely utilized by the microorganism. As presented in Figure 8D, alcohols were also formed during the cultivation, particularly ethanol and xylitol but also small amounts of glycerol. Online measurements provided detailed information about the dynamics of the production of the byproducts acetate, malate and lactate when comparing the metabolism of glucose and xylose.

CONCLUSIONS

An online capillary electrophoresis system with flow-through sample vial was constructed for monitoring bioreactor cultivations. The procedure was used to monitor carboxylic acid production by two yeasts, *K. lactis* and *S. cerevisiae*. The online system ran independently for almost 6 days, indicating its potential as a continuous monitoring tool for feed-back control of bioreactor cultivations.

ASSOCIATED CONTENT

Supporting Information

Effect of Mg^{2+} addition to the BGE, the effect of methanol addition the BGE, and variations of internal standard peak areas during cultivations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was carried out within the Finnish Centre of Excellence in White Biotechnology—Green Chemistry Research granted by the Academy of Finland (grant number 118573) and partly within the EU-project Nanobe, which received funding from the European Community's Seventh Framework Programme (FP7/2007−2013) under grant agreement no. 227243. H.T. was also supported by Graduate School for Chemical Sensors and Microanalytical Systems (CHEMSEM). Outi Koivistoinen and Laura Salusjärvi are acknowledged for providing the *K. lactis* and *S. cerevisiae* strains, respectively, for the bioreactor cultivations. We would also like to thank Ingmar Stuns for constructing the flow-through sample vial.

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PUBLICATION IV

Capillary electrophoresis with laser-induced fluorescence detection for studying amino acid uptake by yeast during beer fermentation

Submitted to Journal of Chromatography B.

Capillary electrophoresis with laser-induced fluorescence detection for studying amino acid uptake by yeast during beer fermentation

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ABSTRACT

The amino acid composition of cultivation broth is known to affect the biomass accumulation, productivity, and vitality of yeast during cultivation. In addition, it is known to affect the concentration of flavor-active higher alcohols, vicinal diketones, and esters in beer. The total concentration and composition of amino acids is also important because the use of sugar supplements in cultivation has the effect of reducing the nitrogen/carbon ratio, potentially limiting yeast growth and necessitating the addition of nitrogen supplements. In this study capillary electrophoresis with laser-induced fluorescence (LIF) detection was used for the determination of amino acid consumption by *Saccharomyces cerevisiae* during beer fermentation. Intraday relative standard deviations were less than 2.1% for migration times and between 2.9% and 9.9% for peak areas. Interday relative standard deviations were less than 2.5% for migration times and between 4.4% and 18.9% for peak areas. The detection limit was even as low as 62.5 pM which equals to below attomole level detection. The method was applied to study the rate of amino acid utilization during fermentation.

Keywords: Bioprocess monitoring; Capillary electrophoresis; Amino acids; Laser-induced fluorescence detection; Beer fermentation

1. Introduction

Nitrogen is one of the main elements in macromolecules of all living organisms. It plays a central role in structure and function, and most organisms have detailed control mechanisms to maintain a constant supply of nitrogen. Yeast is able to use a wide variety of compounds as nitrogen sources, but it prefers ammonia, asparagine, glutamine or glutamate. In the absence of these primary nitrogen sources or if they are present in concentrations low enough to limit growth, other nitrogen sources such as nitrite, nitrate, amides, amino acids and peptides are used. [1]

Amino acid composition of cultivation broth is known to affect the biomass accumulation, productivity, and vitality of yeast during cultivation. In addition, it is known to affect the concentration of flavor-active higher alcohols, vicinal diketones, and esters in beer. The total concentration and composition of amino acids is also important because the use of sugar supplements in cultivation has the effect of reducing the nitrogen/carbon ratio, potentially limiting yeast growth and necessitating the addition of nitrogen supplements. [2] Thus, it is essential for the optimization of process parameters and cultivation performance to monitor amino acid uptake and consumption.

All-malt brewery wort contains a wide variety of natural nitrogen sources, of which amino acids are the most abundant. It contains all the physiologically active amino acids, but there can be differences in individual amino acid concentrations between wort types. However, the overall proportions of amino acids tend to be relatively constant. Amino acids are taken up

sequentially, although the exact order of uptake is strain specific. [2]

Capillary electrophoresis (CE) is an excellent analysis tool for bioprocess monitoring. It has high resolution, strong separation efficiency, fast analysis times and low consumption of sample and reagents. [3] CE has been used extensively for the analysis of amino acids in different matrices and with different detectors. Amino acids have been analyzed with indirect UV [4], mass spectrometric (MS) [5–8], conductivity [9] and laser-induced fluorescence (LIF) [10–15] detection.

Amino acid separation and identification by direct UV detection is very difficult, because they do not have a chromophore in their structure. It is possible to use indirect UV detection, in which chemicals containing chromophores are added to the background electrolyte (BGE) solution, but the sensitivity is even lower than with direct UV detection. [13] Laser-induced fluorescence (LIF) detection is used widely to improve the sensitivity of CE. It is the most sensitive detection method available with detection limits below 10^{-21} mol, which makes it almost 1000 times more sensitive than UV detection. Most of the analytes do not have native fluorescence and therefore the use of this detection method involves attaching fluorescent probe molecules to the analyte. Most fluorescent probes can be designed to contain a reactive moiety capable of coupling to a specific functional group of a biomolecule. With this derivatization procedure it is possible to enhance the selectivity of the detection. [16]

Oregon green™ 488 carboxylic acid, succinimidyl ester (OG-SE) is a fluorinated analogue of fluorescein. Conjugates of Oregon green fluorophores are more photostable than those of fluorescein and are less pH sensitive. The excitation and emission maxima of the label are at 496 nm and 524 nm, respectively, which are suitable for use with an argon-ion laser. Reaction of OG-SE with primary and secondary amines creates stable amide and imide linkages, respectively, and the reaction occurs rapidly in mild conditions.

In this study a derivatization technique coupled with capillary electrophoresis separation was developed for the simultaneous monitoring, identification and quantification of amino acids. This method was applied for monitoring the amino acid uptake of yeast during beer fermentation. The results were compared to the consumption of sugars present in the wort and to the production of ethanol during the bioprocess. This study provides a monitoring system with great importance for the optimization of process parameters and cultivation performance.

2. Experimental

2.1 Chemicals

Stock solutions of amino acid standards were prepared in 18 mΩ deionized water (Millipore, Bedford, MA, USA) at 10 mM concentration. Oregon green® 488 carboxylic acid, succinimidyl ester 6-isomer (Molecular Probes Inc., Eugene, OR, USA) was prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) as a 2 mM solution.

L-isoleucine (≥99.5%), L-serine (≥99%), L-leucine (≥99%), L-histidine (≥99.5%), L-threonine (≥99%), L-tryptophan (≥99%), L-lysine (≥98%), L-methionine (≥99%), L-arginine (≥99%), L-glutamic acid (≥99%), L-cysteine (≥99.5%), L-asparagine (≥99%), L-aspartic acid (≥99%), L-proline (≥99%), and L-glutamine (≥99%) were purchased from Fluka (Buchs, Switzerland). L-tyrosine (≥99%), L-phenylalanine (≥99%), L-alanine (≥99%) and L-glycine (≥99.7%) were from Merck (Darmstadt, Germany). DL-valine (≥97%) was from Sigma-Aldrich.

Background electrolyte (BGE) solutions were prepared from sodium tetraborate decahydrate (≥99.5%), sodium dodecyl sulfate (SDS) (≥99%), 18-crown-6-ether (99%), β-cyclodextrin (≥97%), and CHAPSO (≥98%), which were purchased from Sigma-Aldrich. A 0.1 M sodium hydroxide solution for the capillary conditioning was from FF-Chemicals Ab (Haukipudas, Finland).

2.2 Instrumentation

All the measurements were made with a P/ACE MDQ capillary electrophoresis system equipped with an LIF detector (Beckmann Coulter Inc., Fullerton, CA, USA). The LIF detector had an argon-ion laser with 488 nm excitation and 520 nm emission wavelengths. Bare fused-silica capillaries (Teknolab, Trollasen, Norway) with inner diameter 50 µm, outer diameter 365 µm, and total length 60 cm (50 cm to the detector) were used. The capillaries were conditioned before use with 0.1 M NaOH, Milli-Q purified water, and BGE for 10 minutes each. Voltage (+30 kV) was then applied for 5 minutes. Between analyses, capillaries were rinsed with 0.1 M NaOH, 18 mΩ deionized water and with BGE for 2, 1 and 2 minutes, respectively. Data was collected and processed with 32Karat software (Beckmann Coulter Inc., Fullerton, CA, USA).

2.3 Analysis conditions

BGE was prepared with 50 mM sodium tetraborate, 30 mM SDS and 20 mM 18-crown-6-ether in milli-Q purified water. The pH of the solution was 9.0. Before analysis, the BGE was degassed by ultrasound and filtered through a 0.45 µm Acrodisc GHP syringe filter (Pall Life Science, Ann Arbor, MI, USA). Fused silica capillaries with inner diameter 50 µm, outer diameter 360 µm and total lengths of 60 cm (50 cm to the detector) were used.

Standards and samples were injected by using 0.5 psi pressure for 10 s, giving rise to an injection volume of 8 nL. Separation was carried out at +30 kV at constant capillary temperature +15 °C. Sample cartridge temperature was set at +15 °C. The analytes were detected with the LIF detector using an excitation wavelength of 488 nm and emission wavelength of 520 nm. Concentrations of sugars and alcohols were analyzed by a Waters Alliance 2690 HPLC system (Milford, MA, USA) with Waters 2487 dual wavelength UV (210 nm) detector and Waters 2414 differential refractometer with an injection volume of 10 µL. The columns were an ICSep ION-310 Fast Analysis Column (150 mm x 6.5 mm, Transgenomic, Inc., Omaha, NE, USA) connected to an ICSep ICE-ORH-801 Column (300 mm x 6.5 mm, Transgenomic). The eluent was 5 mM H_2SO_4 (Titrisol, Merck), flow rate 0.5 mL/min and analysis temperature +55 °C. The optical density of the sample was measured by a Shimadzu UV-1201 spectrometer (Kyoto, Japan) at wavelength 600 nm (=OD_{600}). OD_{600} is a measure of cell density. The cell dry weight (CDW) was determined by filtering 20 mL of sample through a measured filter paper, washing it with distilled water and drying it in an oven (105 °C) for at least 16 hours. After that the cells were weighed with the filtration paper and the CDW was calculated.

2.4 Cultivation conditions and sampling

To obtain a pre-culture, *Saccharomyces cerevisiae* strain VTT A-6035 (VTT, Finland) was propagated as a 2 L culture in a Biostat C-DCU bioreactor (B. Braun Biotech International, Germany) at temperature +24 °C, agitation 400 rpm, under aerobic conditions with 0.4 L/min air. The bioreactor was inoculated with cells grown for 16 hours in YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose) at 28 °C and 200 rpm. Cultivation was performed in wort (Sinebrychoff, Finland) for 26 hours. After this the yeast was allowed to sink to the bottom of the bioreactor without agitation for 22 hours at 10 °C. Excess fermentation broth was removed and the cells were transferred to a 10 L Biostat C-DCU bioreactor (B. Braun Biotech International, Germany). The cultivation was maintained in wort at temperature +20 °C, agitation 200 rpm and nitrogen flow rate 0.4 L/min for 210 hours. Samples were collected automatically from the bioreactor with a Jipster sampling carousel (Medicel, Espoo, Finland) every hour and stored at -30 °C. During sampling the samples were filtered through 0.22 µm GV filtration paper (Millipore) with an automated A-SEP cross flow filtration module (Applikon, Delft, the Netherlands).

2.5 Sample preparation

The OG-SE derivatization procedure was performed by adding 10 µL of 2 mM OG-SE solution, 5 µL of sample and 35 µL of 30 mM tetraborate buffer (pH 9.0) to an Eppendorf tube. The reaction mixture was vortexed for 10 s and microcentrifuged for 20 s to bring possible

droplets to the bottom of the tube before leaving it at room temperature for the reaction to proceed. After 15 minutes, the sample was diluted by adding 5 µL of labeled sample with 395 µL of 30 mM tetraborate buffer (pH 9.0) and analyzed with the optimized analysis method.

For calibration, six concentration levels of standard mixtures were prepared for the studied amino acids present in the cultivation samples. Before analyses, cultivation samples were centrifuged at 10 000 rpm for 5 minutes and diluted 100-fold with milli-Q purified water.

3. Results and discussion

3.1 Fluorescence labeling chemistry

Succinimidyl esters react with nucleophiles, releasing N-hydroxysuccinimide (Figure 1). Reaction with primary and secondary amines creates stable amide and imide linkages, respectively. In the case of succinimidyl esters, the hydrolysis and amine reactivity both increase with increasing pH. [17] For our purpose, it was important that the derivatization reaction should occur in mild conditions and relatively fast. In this study, the reaction was tested at room temperature in tetraborate buffer concentrations between 10 and 150 mM, and pH values between 2 and 12. Derivatization reaction time was tested between 5 and 60 minutes. The optimal reaction occurred in 30 mM tetraborate buffer at pH 9.0, and the derivatization reached completion in approximately 15 minutes. The resulting reaction products were very stable at room temperature.

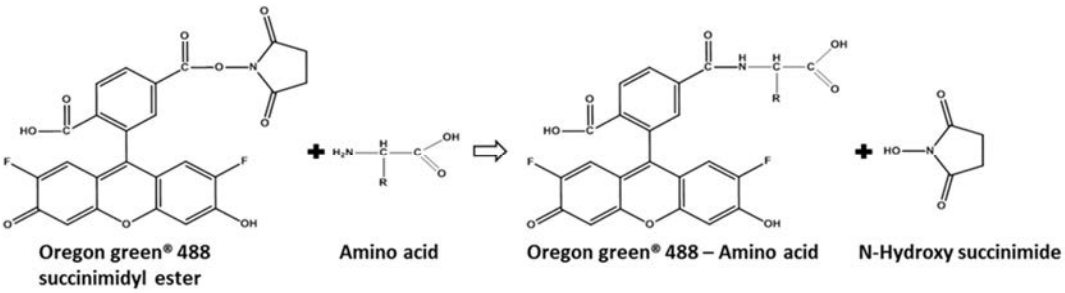

Figure 1. Labeling reaction of Oregon green 488® succinimidyl ester (6-isomer) with an amino acid.

3.2 Method validation

For the analysis of amino acids, an extensive study was made to determine the best BGE composition. The tested electrolyte chemicals were tetraborate [10, 12–14, 18], phosphate [15], tetraborate–phosphate, lithium carbonate [19], ammonium acetate [9] and ammonium formate [5–8] which are common electrolytes in amino acid analyses. The pH values of BGE solutions varied between 5 and 10. Tetraborate was found to be the most suitable electrolyte chemical for the method. Because the relative difference of molecular weights between labeled amino acids is small, the separation is not as straightforward as without labeling. To enhance the separation between amino acids, sodium dodecyl sulphate (SDS) [3,10,16,18], 3-[3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO) [19], 18-crown-6-ether (18C6) [20] and β-cyclodextrin [10,13,21] were tested. It was noted that the addition of CHAPSO in 10 mM concentration gave the best separation efficiency of all the BGE solutions tested, but there were precipitation problems with the BGE that could not be resolved. The separation efficiency was not as good with SDS compared to CHAPSO, and therefore further modification was needed. B-Cyclodextrin and 18C6 were added to the

BGE consisting of tetraborate and SDS. 18C6 is classified as a class I organic modifier in SDS-mediated MEKC and it is used to tune selectivity and resolution. Class I modifiers have an effect on the pseudostationary phase through direct interaction with the micelles. [20] The optimum pH of the optimized BGE was tested at values between 8 and 10.

Different analytical conditions were also examined. Voltage, analysis temperature, capillary length, and detection wavelength were optimized in the ranges of 20–30 kV, 15–30 °C, and 60–80 cm (total length), respectively.

In the optimized method 50 mM sodium tetraborate decahydrate with 30 mM SDS and 20 mM 18-crown-6-ether, pH 9 was used for the analysis. Voltage was set to +30 kV, capillary temperature +15 °C and total capillary length was 60 cm. The sample was injected to the capillary with 0.5 psi pressure for 10 s. An electropherogram of standard solution containing amino acids of this study are analyzed is presented in Figure 2.

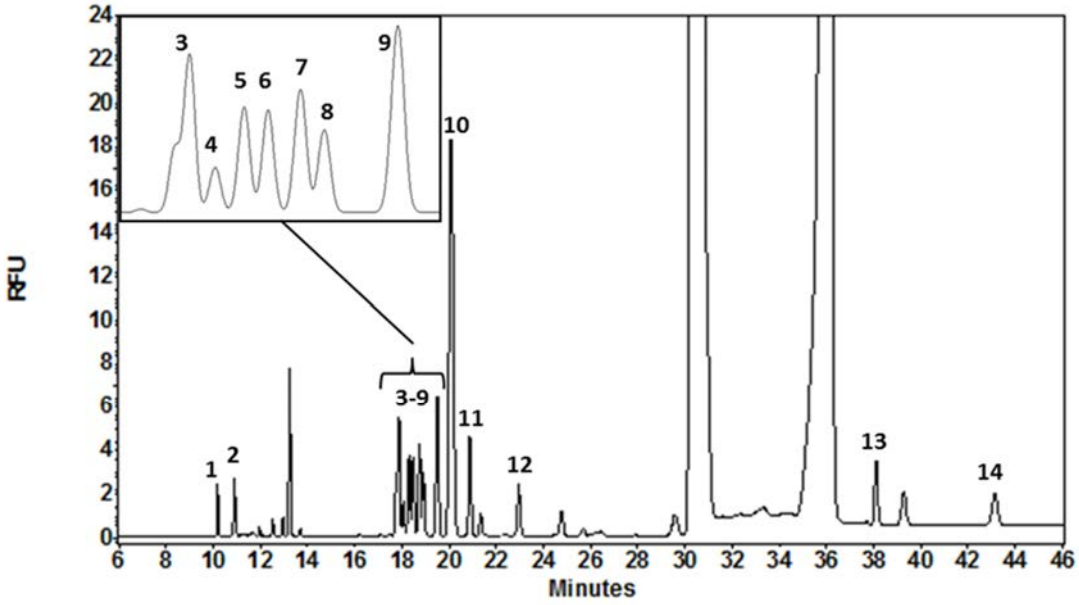

Figure 2. Electrophoretic separation of the studied amino acids. BGE: 50 mM tetraborate, 30 mM SDS and 20 mM 18C6, pH 9.0. Other analysis conditions: voltage +30 kV, temperature 15 °C, capillary length 60 cm (50 cm to the detector), injection 0.5 psi 10 s and LIF excitation 488 nm and emission 520 nm. The current during analysis is ~62 µA. Peak assignments: 1) arginine, 2) lysine, 3) leucine/isoleucine, 4) tyramine, 5) histidine, 6) glutamine, 7) valine, 8) phenylalanine, 9) threonine/asparagine, 10) proline, 11) serine, 12) glycine, 13) glutamic acid, and 14) aspartic acid.

3.3 Method performance

Intraday relative standard deviations (RSD%, n=8) for migration times varied between 0.1% and 2.1% and those for peak areas between 2.9% and 9.9%. Interday RSD% for migration times of the studied compounds were less than 2.5% and those for peak areas were between 4.4% and 18.9%. The detection limits for the amino acid standards were between 62.5 pM and 31.25 nM. Correlation coefficients for the calibration curves varied between 0.9944 and 0.9997. The method performance parameters are summarized in Table 1.

3.4 Analysis of cultivation samples

The optimized method was applied to the analysis of amino acids in beer fermentation broth samples. The purpose of this study was to monitor amino acid consumption of yeast during cultivation. An electropherogram of a cultivation sample is presented in Figure 3.

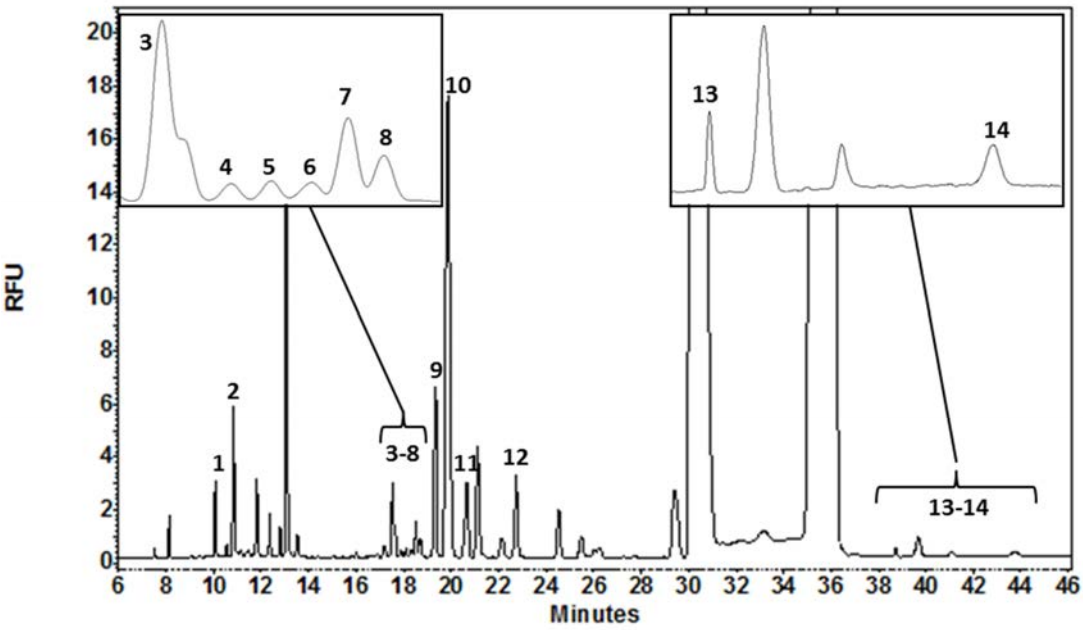

Figure 3. Electrophoretic separation of a cultivation sample. Analysis conditions as in Figure 2. Peak assignments: 1) arginine, 2) lysine, 3) leucine/isoleucine, 4) tyramine, 5) histidine, 6) glutamine, 7) valine, 8) phenylalanine, 9) threonine/asparagine, 10) proline, 11) serine, 12) glycine, 13) glutamic acid, and 14) aspartic acid.

Results of the cultivation are presented in Figure 4, from which it may be noted that the most rapid amino acid consumption occurred during the first 30 hours of cultivation when glucose was present in the cultivation broth as depicted in Figure 5A. After glucose was consumed, the rate of amino acid consumption decreased significantly. The glucose and amino acids are used for growth of the yeast, which is supported by the optical data and cell dry mass measurements presented in Figure 5B. Aspartic acid, glutamine and glutamic acid were consumed most efficiently to a concentration below the quantification limit in less than 40 hours. In addition, lysine, leucine and isoleucine were also utilized, although at a lower rate. The concentrations of proline and tyramine decreased only slightly. Other amino acids were utilized to some extent. The production of ethanol from other carbohydrate sources halted at the same time as maltose was consumed. The increase in some amino acid concentrations might have been caused by stress experienced by the yeast cells during the fermentation. When yeast cells are stressed they may release proteolytic enzymes, in particular protease A, which has the effect of liberating free amino acids from peptides or proteins.

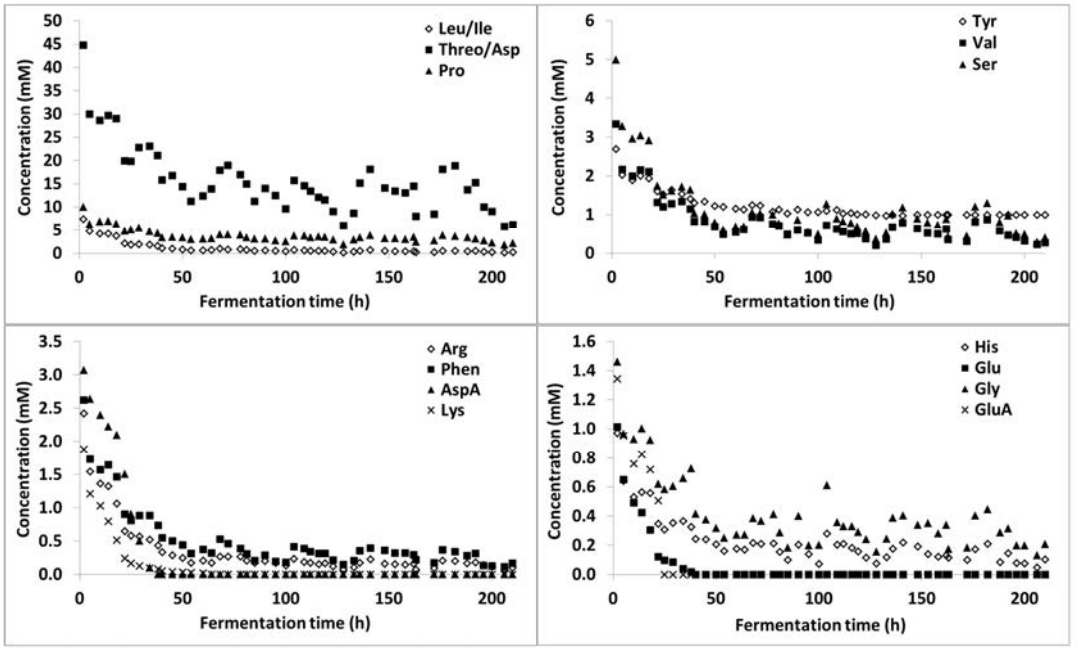

Figure 4. The consumption of amino acids by *Saccharomyces cerevisiae* during beer fermentation.

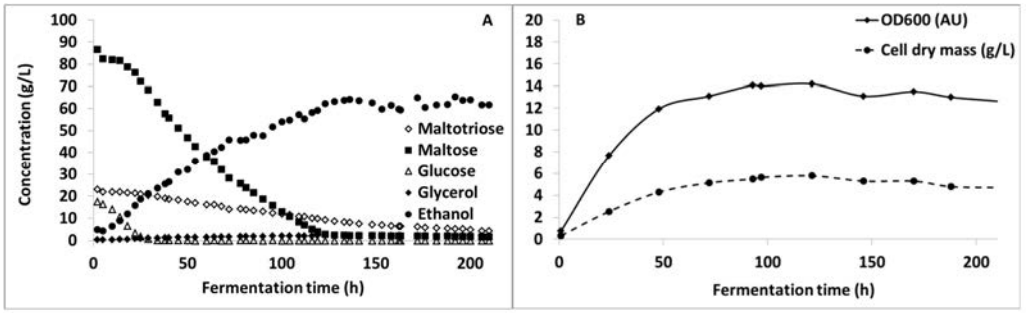

Figure 5. A) HPLC and B) biomass results of the beer fermentation.

4. Conclusions

An analysis method for the measurement of amino acids was developed for capillary electrophoresis. This method was used in the monitoring of amino acid consumption of *Saccharomyces cerevisiae* during beer fermentation. The yeast utilized all amino acids to some extent, and was able to produce ethanol from maltose and maltotriose in addition to glucose. This analysis method can be used for optimization of cultivation conditions and for the indication of cell stress during fermentation. The use of LIF as detection method enabled the analysis of amino acids even below the attomole level. The results correlated well with the results that can be found from the literature.

Acknowledgements

This work was carried out within the Finnish Centre of Excellence in White Biotechnology – Green Chemistry Research granted by the Academy of Finland (grant number 118573) and in the Centre of Separation Technology (CST) of Lappeenranta University of Technology (LUT). Heidi Turkia was also supported by Graduate School for Chemical Sensors and Microanalytical Systems (CHEMSEM) and LUT Booze project. Cultivations were performed within the project Biocontrol (project number 1050/31/07), which received funding from the National Technology Agency of Finland (TEKES). We also thank Toni Paasikallio for performing the cultivations, and Dr. Brian Gibson and Dr. John Londesborough for brewing discussions. We are very grateful to Sinebrychoff for providing the wort for the experiments.

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Series title and number
VTT Science 47

Title	**Capillary electrophoresis for monitoring of carboxylic, phenolic and amino acids in bioprocesses**
Author(s)	Heidi Turkia
Abstract	Bioprocess technology is a multidisciplinary industry that combines knowledge of biology and chemistry with process engineering. It is a growing industry because its applications have an important role in the food, pharmaceutical, diagnostics and chemical industries. In addition, the current pressure to decrease our dependence on fossil fuels motivates new, innovative research in the replacement of petrochemical products. Bioprocesses are processes that utilize cells and/or their components in the production of desired products. Bioprocesses are already used to produce fuels and chemicals, especially ethanol and building-block chemicals such as carboxylic acids. In order to enable more efficient, sustainable and economically feasible bioprocesses, the raw materials must be cheap and the bioprocesses must be operated at optimal conditions. It is essential to measure different parameters that provide information about the process conditions and the main critical process parameters including cell density, substrate concentrations and products. In addition to offline analysis methods, online monitoring tools are becoming increasingly important in the optimization of bioprocesses. Capillary electrophoresis (CE) is a versatile analysis technique with no limitations concerning polar solvents, analytes or samples. Its resolution and efficiency are high in optimized methods creating a great potential for rapid detection and quantification. This work demonstrates the potential and possibilities of CE as a versatile bioprocess monitoring tool. As a part of this study a commercial CE device was modified for use as an online analysis tool for automated monitoring. The work describes three offline CE analysis methods for the determination of carboxylic, phenolic and amino acids that are present in bioprocesses, and an online CE analysis method for the monitoring of carboxylic acid production during bioprocesses. The detection methods were indirect and direct UV, and laser-induced frescence. The results of this work can be used for the optimization of bioprocess conditions, for the development of more robust and tolerant microorganisms, and to study the dynamics of bioprocesses.
ISBN, ISSN	ISBN 978-951-38-8107-8 (Soft back ed.) ISBN 978-951-38-8108-5 (URL: ttp://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online)
Date	December 2013
Language	English, Finnish abstract
Pages	103 p. + app. 36 p.
Name of the project	
Commissioned by	
Keywords	Bioprocess monitoring, capillary electrophoresis, online capillary electrophoresis, carboxylic acids, phenolic acids, amino acids
Publisher	VTT Technical Research Centre of Finland P.O. Box 1000, FI-02044 VTT, Finland, Tel. +358 20 722 111

Julkaisun sarja ja numero
VTT Science 47

Nimeke	**Kapillaarielektroforeesin käyttö karboksyyli-, fenoli- ja aminohappojen monitorointiin bioprosesseissa**
Tekijä(t)	Heidi Turkia
Tiivistelmä	Bioprosessitekniikka on monitieteellinen teollisuudenala, joka yhdistää biologian ja kemian tietämyksen kemiantekniikkaan. Se on kasvava teollisuudenala, koska sen sovellukset ovat hyvin tärkeitä ruoka-, farmasia-, diagnostiikka- ja kemianteollisuudelle. Tämän lisäksi riippuvuutta öljystä tulee vähentää merkittävästi, mikä mahdollistaa uuden ja innovatiivisen tutkimuksen petrokemikaalituotteiden korvaamiseksi. Bioprosessit ovat prosesseja, jotka käyttävät soluja ja/tai niiden osia tuottamaan haluttuja tuotteita. Bioprosesseja on jo käytetty polttoaineiden ja kemikaalien tuottamiseen, erityisesti etanolin ja ns. building-block kemikaalien, kuten karboksyylihappojen, tuottamiseen. Jotta bioprosesseista saadaan tehokkaampia ja taloudellisesti kannattavampia, raaka-aineiden on oltava halpoja ja bioprosessien on toimittava optimaalisissa olosuhteissa. Erilaisten parametrien mittaaminen on välttämätöntä, sillä ne antavat tietoja prosessin tilasta ja kriittisistä prosessiparametreista, joita ovat biomassa, lähtöaineet ja tuotteet. Offline-mittausten lisäksi online-määritykset kasvattavat merkitystään bioprosessien optimoinnissa. Kapillaarielektroforeesi (CE) on monipuolinen analyysitekniikka, jolla ei ole rajoituksia polaaristen liuottimien, analyyttien tai näytteiden kanssa. Optimoitujen menetelmien korkea resoluutio ja tehokkuus mahdollistavat nopean detektoinnin ja kvantifioinnin. Tämä työ osoittaa CE:n potentiaalin monipuolisena bioprosessimonitorointityökaluna. Osana tätä tutkimusta kaupallinen CE-laitteisto muutettiin toimimaan online-analysointilaitteistona prosessien automaattista monitorointia varten. Tämä työ kuvaa kolme offline-CE analyysimenetelmää karboksyyli-, fenoli- ja aminohappojen analysointiin sekä yhden online-CE analyysimenetelmän karboksyylihappojen monitorointiin bioprosessien aikana. Käytetyt detektointimenetelmät olivat epäsuora ja suora UV sekä laser-indusoitu fluoresenssi. Työn tuloksia voidaan käyttää bioprosessiolosuhteiden optimoinnissa, elinvoimaisempien ja kestävämpien mikro-organismien kehittämisessä sekä bioprosessien dynamiikan tutkimisessa.
ISBN, ISSN	ISBN 978-951-38-8107-8 (nid.) ISBN 978-951-38-8108-5 (URL: http://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (painettu) ISSN 2242-1203 (verkkojulkaisu)
Julkaisuaika	Joulukuu 2013
Kieli	Englanti, suomenkielinen tiivistelmä
Sivumäärä	103 s. + liitt. 36 s.
Projektin nimi	
Toimeksiantajat	
Avainsanat	Bioprocess monitoring, capillary electrophoresis, online capillary electrophoresis, carboxylic acids, phenolic acids, amino acids
Julkaisija	VTT PL 1000, 02044 VTT, puh. 020 722 111

Capillary electrophoresis for monitoring carboxylic, phenolic and amino acids in bioprocesses

Bioprocess technology is a multidisciplinary industry that combines the knowledge of biology and chemistry with process engineering. The applications have an important part in the food, pharmaceutical, diagnostics and chemical industries. The current pressure for decreasing the dependence on fossil fuels introduces new and innovative research opportunities in the replacement of petrochemical products. Bioprocesses utilize cells or their components in the production of desired products, such as fuels and chemicals. In order to enable more efficient, sustainable and economically viable bioprocess-based production processes, the raw materials must be cheap and the bioprocesses must be operated at optimal conditions. Bioprocess monitoring technologies are valuable tools both during the development of the bioprocesses and while operating them.

Capillary electrophoresis (CE) is a versatile analysis method for the determination of a variety of compounds from different matrices. It is an electrodriven separation technique that separates molecules according to their charge-to-mass ratio. This thesis presents three offline-methods and one online-CE-method for the determination of carboxylic, phenolic and amino acids in bioprocesses. These analysis methods were successfully used for bioprocess monitoring.

ISBN 978-951-38-8107-8 (Soft back ed.)
ISBN 978-951-38-[8108-5 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)