TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

Universität für Bodenkultur Wien

Optimization of a medium composition for a *Clostridium sp.* and the effect on its metabolism

Diplomarbeit

zur Erlangung des akademischen Grades

Diplom-Ingenieur (Dipl.-Ing.)

auf der

Universität für Bodenkultur Wien

durchgeführt auf der technischen Universität Wien
in der Abteilung “Biochemical Engineering”
am Institut “Bioprocess Technology”

unter der Aufsicht und Unterstützung von

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Matrikelnummer: 0640981
Wien, Jänner 2014

Abstract

The future depletion of fossil fuels has increased the interest of searching for alternative energy sources. Between them, biogas production from biomass sources seems to be the optimal candidate. To increase the efficiency of this biological process the biomass has to be hydrolysed beforehand. Among the different ways to go on with this step, the biological degradation of biomass results to be the most promising one, regarding ecologically beneficial strategies. There is a wide range of microorganisms that have provided positive results in this area, specially the strains belonging to the genus *Clostridium*. Therefore, bioprocess strategies have to be developed in the near future, in order to increase biomass productivities of these strains.

The goal of this project was to determine a specific and economically more feasible medium composition to increase the biomass productivity of a specific *Clostridium sp*, named *Fritz*. In a continuous culture mode, the effects of different medium compounds on biomass productivity were described and quantified via pulses. Here we were able to identify yeast extract as the best suitable complex compound, as well as to settle the optimal substrate concentration at 3.5 g/L sucrose. Additionally, we established that our specific *Clostridium sp.* was not able to grow on organic acids or alcohols, when sugar was missing. We also identified a memory effect of this strain while running a continuous culture for a long time, thereby further increasing volumetric biomass productivity.

Furthermore, different screenings with the *Clostridium sp*. were done in serum flasks in different carbon sources (sugars), and at decreasing ammonia and phosphate salts concentrations for the reference and the preselected substrate. We established sucrose as the best substrate and reduced the salt concentration in close batch and continuous mode up to 15% and 25%, respectively, thereby increasing biomass concentration and feasibility of the medium. We also showed that this specific *Clostridium sp*. was not able to grow on previously described defined media for other selected *Clostridium* strains. The results obtained from both, the continuous culture and the serum flask experiments, were used to determine the optimal medium composition for increasing the biomass productivity of our *Clostridium sp.*

Keywords: renewable energy, biogas, biological degradation, *Clostridium*, biomass productivity, pulse, screening, continuous culture, carbon source

Kurzfassung

Die zukünftige Erschöpfung fossiler Brennstoffe hat das Interesse an alternativen Energiequellen stark erhöht. Unter ihnen scheint die Verarbeitung von Biomasse zu Biogas mit Hilfe von Mikroorganismen der aussichtsreichste Kandidat zu sein. Damit es auch so bleibt, muss der Prozess, bei dem Biomasse zu verwertbaren Substraten hydrolysiert wird, noch weiter optimiert werden. Von den vielen Möglichkeiten dies zu erreichen, ist der biologische Abbau von Biomasse durch Mikroorganismen ökologisch am sinnvollsten. Es gibt eine große Anzahl an Mikroorganismen, die positive Resultate in diesem Bereich erzielt haben. Dabei haben sich die zum Genus *Clostridia* zugehörigen Mikroorganismen als besonders effizient hervorgetan. Dementsprechend müssen in naher Zukunft Strategien in der Bioprozesstechnik entwickelt werden, die die Biomasseproduktivität dieser Stämme erhöht. Das primäre Ziel dieses Projektes war es für einen bestimmten *Clostridium* Stamm ein bestimmtes und kostengünstiges Medium zu entwickeln. Das Medium sollte dabei die Biomasseproduktivität dieses Stammes erhöhen. In einem kontinuierlichen Fermentationsprozess wurden die Effekte von verschiedenen Medienkomponenten auf die Biomasseproduktivität mit Hilfe der „Pulstechnik“ untersucht und quantifiziert. Dabei wurde Hefeextrakt als bester Komplexstoff identifiziert, sowie 3.5 g/L Sucrose als optimale Zuckerkonzenration. Zusätzlich haben wir festgestellt, dass unser bestimmter Clostridienstamm bei Abwesenheit von Zucker als Substrat nicht auf organischen Säuren und Alkoholen wächst. Wir haben auch festgestellt, dass es einen sogenannten „memory effect“ in kontinuierlichen Prozessen gibt, der zu einer höheren volumetrischen Biomasseproduktivität führt. Zusätzlich wurden Batchexperimente in kleinen Serumfläschen mit unterschiedlichen Kohlenstoffquellen (Zuckern) durchgeführt. Außerdem haben wir Experimente mit Ammonium- bzw. Phosphatsalzen in Maltose (Referenz) und in Sucrose in absteigenden Konzentrationen durchgeführt. Dabei haben wir nachgewiesen, dass Sucrose das geeignetste Substrat ist und dass die Salzkonzentration im Batch auf 15% und im kontinuierlichen Prozess auf 25% reduziert werden kann. Es wurde auch gezeigt, dass unser Clostridienstamm nicht auf für andere Clostridienstämme definierte Medien wächst. Die Resultate beider Prozesse wurden herangezogen, um ein bestimmtes und kostengünstiges Medium herzustellen, um die Biomasseproduktivität zu erhöhen.

Schlagwörter: alternative Energiequellen, Biogas, biologischer Abbau, *Clostridium*, Biomasseproduktivität, Puls, Screening, kontinuierlicher Prozess, Kohlenstoffquelle

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List of abbrevations

BM	Basal Medium
CBP	Consolidated Bioprocessing
CEM	Cellulose-Enzyme-Microbe Complex
CDW	Cell Dry Weight
CER	Carbon Evolution Rate
C_i	concentration of specific metabolite (i) in g/L
CM	Complex Medium
CMC	Carboxymethyl Cellulose
CO_2	Carbon Dioxide
CSL	Corn steep liquor
GHG	Greenhouse gases
H_2	Hydrogen
HER	Hydrogen Evolution Rate
HPLC	High Performance Liquid Chromatography
ICP-OES	Inductively coupled plasma optical emission spectrometry
M_i	Molecular Weight [g/mol]
N_2	Nitrogen
N	Number of carbon atoms in a molecule
OD_{580}	Optical Density at 580 nanometre
PFD	Process Flow Diagram
PM	Peptone from meat pepsin-digested
PSM	Pepton from soymeal papin-digested
q_i	specific production rate (metabolites and biomass) or specific uptake rate (substrate) in C-mmol/g/h
r_i	volumetric productivity (metabolites and biomass) or volumetric inflow rate (substrate) in C-mmol/L/h
SP	Soy peptone
TE	Trace Elements
TP	Tryptone/Peptone ex casein

TS	Tryptic soy broth
YE	Yeast extract
YNB	Yeast nitrogen base without amino acids and ammonium sulphate
$Y_{i/s}$	Yield of a metabolite or biomass (i) over substrate
$Y_{i/x}$	Yield of a metabolite (i) over biomass

Preface

The here presented master thesis was done at the Vienna University of Technology in the research division biochemical engineering, subdivision bioprocess technology from April to October 2013.

I originally came from the University of Natural Resources and Applied Sciences in Vienna, where I studied biotechnology. My home university was mainly focused on biochemical and microbiological engineering. Nevertheless, I did not want to focus on either the biochemistry or microbiology branch but on the technological aspects and industrial applications of my related studies. Here the department of biochemical engineering at the Vienna University of Technology proved to be a great place, where I could fulfil my wish and work on industry-related projects.

I found an interesting topic for me, which was related to renewable energies. It was about environmentally clean biogas production. I was eager to contribute to a future, which would rely less on fossil fuels and more in biomass and wastes, to preserve the environment. Besides I would work with anaerobic microorganism, which I found quite interesting and challenging. I also wanted to work on a project, whose results would actually be applied in the “real world”.

So, at this point I would like to thank my examiner and main advisor Christoph Herwig for his contextual support, his calmness and positive feedback. It always motivated me to meet his standards.

Additionally, I want to thank my invaluable second advisor Ester Martinez Porqueras. She calmly stayed with me and always gave constructive critics. I learned a great deal from her in regard to organisation, efficiency and writing skills. I did not even know I lacked the ladder until I met her. Her work ethic and especially her working pace were unmatched. At times I had real difficulties to keep up (and she always looked so fresh, despite these our working hours).

At last but surely not the least, I want to thank my parents for their unwavering support throughout these years. I doubt that I would have made it so far without them, although they always deny it.

1 Overview

The worldwide demand for energy is growing rapidly. About 88% is met at present time by fossil fuels. Scenarios have shown that the energy demand will increase during this century by a factor of two. At the same time, concentrations of greenhouse gases (GHGs) in the atmosphere are raising rapidly, with fossil fuel-derived carbon dioxide (CO_2) emissions being the most important contributor. In order to minimize related global warming and to preserve the environment, alternative energy sources must be developed. Another important worldwide challenge is the security of energy supply, as most of the fossil fuel reserves are concentrated in politically unstable countries. In this regard, biogas production from high molecular biomass like wastes and energy crops will play an important role (1).

Biogas is a versatile renewable energy source, which can replace fossil fuels in power and heat production. Further, it can be used as a gaseous fuel for vehicles. Methane-rich biogas can also replace natural gas as a feedstock for producing chemicals (1). The production of biogas through anaerobic digestion has great advantages over other forms of bioenergy production. It has been evaluated as one of the most energy efficient and environmentally friendly technologies due to low, or even no emission of potentially harmful greenhouse gases (2).

The European energy production from biogas reached 6 million tons of oil equivalents in 2007 with an annual increase of more than 20%. Germany has become the biggest biogas producing country in the world due to the strong development of agricultural biogas plants. At the end of 2008, approximately 4000 agricultural biogas production units were operated on German farms (1). In comparison, 294 agricultural biogas production units were operated in Austria at that time (3).

1.1 Digestion process

Biogas plants produce methane by anaerobic digestion of lignocellulosic biomass, further called substrate. This process is complex and can be divided up into four phases: hydrolysis, acidogenesis, acetogenesis and methanation (1). These steps are carried out by different microorganisms, but not necessarily in strict order from hydrolysis to methanation. They can also run parallel and interchangeably.

Hydrolyzing microorganisms are responsible for the initial attack at high molecular substrate (e.g. polymers) to produce mainly acetate and hydrogen, as well as varying

amounts of fatty acids, such as butyrate and formiate, or alcohols like butanol or ethanol. They excrete hydrolytic enzymes (e.g. cellulase, cellobiase, xylanase, amylase, lipase, and protease) to degrade the high molecular substrate. Most of the hydrolytic bacteria are strict anaerobes such as *Bacteriocides, Clostridia*, and *Bifidobacteria* (1). The microorganism used in this project belonged to the genus *Clostridium.* It was a spore-forming, gram-positive, rod-shaped obligate anaerobe bacterium.

The degraded substrate is further processed by acetogenic microorganisms to acetate and hydrogen. At last, acetate and hydrogen are converted to methane by methanogenic microorganisms. Only few species are able to degrade acetate into methane and carbon dioxide, whereas all methogenic bacteria are able to convert hydrogen and carbon dioxide to methane (1).

1.2 Biomass hydrolysis

Biogas and biofuels can be produced from several lignocellulosic biomass feedstocks like sugar-cane (sucrose rich), corn grain (starch rich) and grass (lignocellulose) (4). The focus on biological lignocellulosic biomass hydrolysis is based on the large availability, low cost and environmentally friendly production of lignocellulose. Additionally, the greenhouse gas emission with lignocellulosic biomass is almost zero. The main disadvantage, however, is the expensive technology for production of liquid or gaseous biofuels from lignocellulose (5). Lignocellulosic resources include corn stover, wheat straw and woody residues from forests. These resources are widely available and do not compete with resources of the food industry, which is also a great advantage (4).

The conversion of lignocellulose comprises two steps: hydrolysis of cellulose to sugars, and fermentation of sugars to the corresponding biofuel. Hydrolysis is a crucial step and the factors that have been identified to affect hydrolysis are porosity, cellulose fiber crystallinity and lignin and hemicellulose amount. The increase in porosity, reduction of cellulose crystallinity, and removal of lignin and hemicellulose in pretreatment processes can improve the hydrolysis significantly (6).

1.2.1 Pretreatment of biomass

The pretreatment of lignocellulose needs to fulfill the following requirements: (I) improve the formation of sugars; (II) avoid degradation of carbohydrates; (III) avoid formation of inhibitory byproducts to subsequent fermentation processes; and (IV) be cost-effective (6). Pretreatment is generally done through chemical, physical or biological methods (4). The main advantage of chemical methods is that acids penetrate lignin without any pretreatment of biomass, cleaving the cellulose and hemicellulose that

are protected by lignin to form sugar molecules (4). There can be used concentrated or diluted acids, like sulphurous, sulphuric, hydrochloric, phosphoric, nitric or formic acid. Sulphuric and hydrochloric acids are the most commonly used for chemical pretreatment of biomass. In concentrated acid treatment the concentration is between 10-30%. The process is conducted at low temperatures, producing high yields of cellulose. But this process requires large amounts of acids causing corrosion to equipment. On the other hand, diluted acid treatment lies between 2-5% acid concentration and is carried out at high temperatures (4).

Physical pretreatment is mainly done by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The power requirement for the machines depends on the desired final particle size and the biomass characteristics. Woody residues from forest generally require more energy than straw or corn stover biomass (6).

Biological or enzymatic pretreatment (which is highly specific) is carried out by cellulase enzymes coming from aerobic or anaerobic microorganism. Filamentous fungi are the main source of cellulase enzymes (4). Although many anaerobic cellulolytic microorganisms like *Clostridia* produce highly specific cellulases, they do not do it at high concentrations. This fact and the very low growth rate of *Clostridia* were the reasons why research in the past had focused on cellulase production from fungi. The use of enzymes in the hydrolysis of lignocellulose is more effective than the use of inorganic catalysts, like acids or physical treatment, because of the high substrate specificity and the ability to work at mild process conditions (4). However, the use of enzymes is still limited for several reasons. They are unstable at high temperatures. This is the reason why research shifted towards thermophilic cellulase producing microorganisms. Furthermore, the costs of enzyme isolation and purification are still very high and it is very difficult to recover them from reaction mixtures. Additionally, preparations of cellulases from one organism are not efficient for hydrolysis of different feedstocks (4). Another major problem is the inhibition of cellulase activity by cellobiose and glucose. To circumvent this issue, several methods were developed, such as the use of high concentrations of enzymes. However, this increases the costs immensely.

Another method was the combination of hydrolysis and fermentation in one step, called simultaneous saccharification and fermentation (SSF). This SSF method has several advantages. It increases the hydrolysis rate by conversion of sugars that would otherwise inhibit the cellulase activity, requires lower enzyme concentrations, has a shorter process time, requires less reactor volume because a single reactor is used instead two like in the two-stage process. However, disadvantages might be the incompatibility of temperatures of hydrolysis and fermentation, end-product tolerance of microorganism and possible inhibition by end-products (6).

1.2.2 Consolidated bioprocessing

There also exists a potential new variety of the SSF method, called consolidated bioprocessing (CBP). Basically, it also tries to combine the two process steps in one, but with the crucial difference that it uses natural cellulolytic or recombinant microorganism instead of pure enzymes for the hydrolysis of biomass. The main goal with natural microorganism is to increase the product yields and concentrations of microorganism and product (5). Anaerobic bacteria like *Clostridia* are of particular interest but a lot of research needs still to be done in this area. On the other hand, the main goal with recombinant microorganism is to genetically engineer a cellulase system into high growing and producing non-cellulolytic microorganisms in order to increase the hydrolysis rate (5).

The idea is that such hydrolysing microorganisms contain complex cellulase systems, called cellulose-enzyme-microbe (CEM) complexes, which interact with cellulose in a highly specific fashion, thus successfully competing for biomass with other microorganism, including contaminants while keeping the advantages of the SSF method. This further increases the stability of industrial processes. In comparison to that, processes with pure cellulase enzymes are carried out by cellulose-enzyme (CE) complexes that do not involve living, cellulolytic microorganism (5).

Although the cost of producing pure cellulase enzymes was reduced significantly over the last decade, the potential of CBP as a lower cost and high efficient process is not diminished. Another huge advantage with living microorganism is the fact that they do not discriminate between different feedstocks and thus can be used for woody residues, straw and corn grain without negative effect on efficiency (5).

1.3 Theory behind the metabolic pathways of *Clostridia sp.*

In this section findings from various studies on how to influence the metabolism of *Clostridia sp.* with process parameters in batch and continuous mode are summarized. The results of our project are compared to those findings, to see if our *Clostridium* strain *Fritz* aligned with the general view of the metabolic pattern of *Clostridia* or showed a different response to these parameters.

1.3.1 Batch

The metabolic pathway of C*lostridia* is generally divided into two distinct pathways, solventogenesis and acidogenesis (Figure 1).

In a batch mode the first phase would be the acidogenesis, occurring in the exponential growth phase. Here the bacterium utilizes the main carbon source and converts it into

organic acids (e.g. acetate, butyrate) with the release of energy (7). In some cases, lactate, formiate, propionate and/or succinate are produced additionally. This varies from species to species.

The electron flow in this phase is directed towards formation of molecular hydrogen with the help of an important enzyme called ferredoxin. This enzyme can either transfer electrons via hydrogenase to generate molecular hydrogen or transfer electrons via NADH-ferredoxin oxidoreductase and NADPH-ferredoxin oxidoreductase to generate NADH or NADPH (8).

In the second step, occurring during the stationary phase, the bacterium shifts from the acidogenesis phase to the solventogenesis phase, where it utilizes acetate and butyrate additionally to the main carbon source. Presumably, an important factor for the change of phases is a specific threshold concentration of undissociated butyric acid. A high initial butyric acid concentration also leads to solventogenesis (9). The theory behind this is that the low pH value stresses the bacterium. In order to reveal itself from the stress, it either sporulates or shifts its metabolism to solventogenesis, reducing the amount of organic acids and increasing the amount of solvents, thereby increasing pH to a favourable value (7). The electron flow is redirected to the generation of NADH, an electron carrier, resulting in a decrease of molecular hydrogen levels. This is very important, because the solvent pathways depend on a big pool of NADH to be initiated.

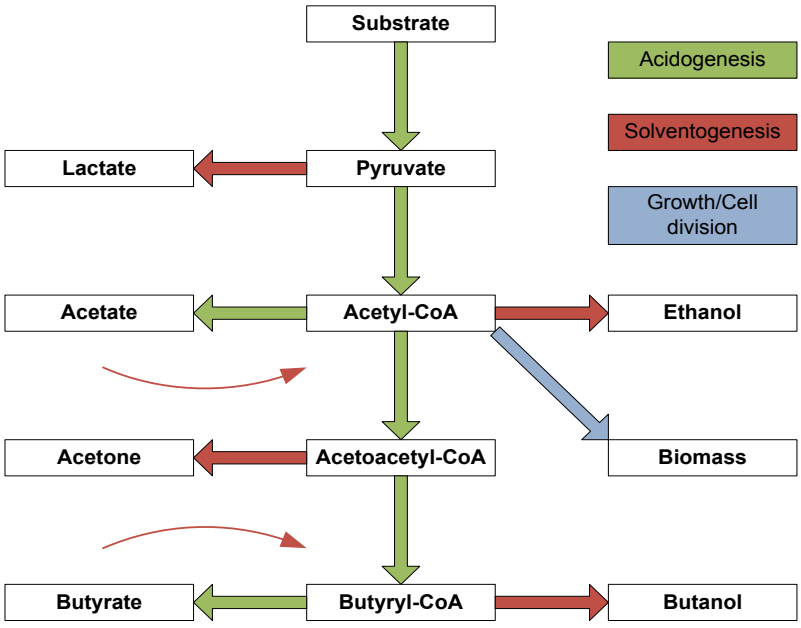

Figure 1 Schematic diagram of the general metabolism of Clostridia. It varies slightly from species to species. The narrow red arrows indicate a utilization of organic acids as additional carbon sources besides the main substrate in the solventogenesis phase

1.3.2 Continuous mode

Regarding the continuous mode, the bacterium is expected to stay in the first phase, acidogenesis, because the process starts in the exponential phase of a batch process and is kept in steady state while running the continuous mode. But this is only the case, if there are no phosphate or nitrogen limiting conditions and/or excess of substrate (7,10,11).

In the case of excess substrate supply, meaning no-carbon limiting conditions, growth is at a maximum with a maximum substrate uptake rate. To reoxidize the resulting high amount of NADH back to NAD^+, the carbon flow is shifted towards solventogenesis. Because of the excess of substrate, enough ATP can be generated through glycolysis alone, resulting in a low additional ATP demand. Consequently, the production of acetate and butyrate is low (12).

The previously mentioned threshold concentration of undissociated butyric acid as another factor for the metabolic shift in a batch mode is not relevant in the continuous process. This threshold is never reached, because of the constant volume exchange.

Furthermore, if a more reduced substrate than glucose, like glycerol, is used additionally as the main carbon source, the metabolism shifts partially to solventogenesis, depending on the ratio of the substrates (10). This is the case, because additional reducing power from glycerol is being oxidized through NAD^+/NADH. As known, the disruption of the NAD^+/NADH ratio towards NADH induces solventogenesis and the formation of e.g. butanol, ethanol or acetone (13).

It seems possible to control the metabolism of *Clostridia* in a continuous mode with parameters like pH, substrate feeding concentration, quality of substrate, initial metabolite concentrations and initial salt concentrations.

2 Goal

In biogas/biofuel production the degradation of high molecular substrates like lignocellulose depends on the concentration of the hydrolysing microorganism. This very first step to production is a rate-determining step, which needs to be improved. Therefore, the primary goal of this project was to maximize the biomass concentration and volumetric biomass productivity of *Fritz*, a specific *Clostridium sp.* used in anaerobe lignocellulosic biomass hydrolysis systems, by optimizing the medium composition used for its cultivation. For the growth and survival of microorganisms like *Clostridia,* several nutrients like carbon, phosphor, and sulphur are necessary. Trace elements like iron are important for the growth rate of microorganisms and must be added (14). The optimal amounts of macronutrients for high *Fritz* biomass productivities were determined in this project. Additionally, process parameters like dilution rate (D) or process time were evaluated to increase volumetric biomass productivities.

The secondary goal was to study the metabolism of *Fritz* under different sets of parameters and to monitor its metabolic shifts when changing working conditions (substrate feeding concentration, type of substrate, dilution rate, complex compounds or salt concentration), trying to connect the process parameters with the underlying metabolic patterns. The result of this study gave us more insight into *Fritz* metabolism. With this knowledge, it might be possible to control a continuous running process with *Fritz* to the desired outcome (be it biomass or metabolites) by setting the right values of the working parameters.

3 Summary

Figure 2 summarizes the most important parameters related to volumetric biomass productivity. The approach was to optimize one component at a time and find out which type of component and which concentration were the optimal ones to cultivate *Fritz* at the highest volumetric biomass rates. Regarding substrate (the main carbon and energy source) we proved that organic acids and alcohol were not a viable option. After a series of screenings in closed batches and continuous cultures in a bioreactor, we established sucrose as the best substrate at a concentration of 3.5 g/L. Being able to feed low concentrations of substrate and still yield high volumetric biomass productivity reduces operational costs and makes the process economically more viable.

The next component to analyse was the complex compound. Here, yeast extract proved to be the best choice at a concentration of 2 g/L after a series of screenings with different complex compounds in closed batches and pulses in continuous experiments.

Trace elements and vitamins in a defined medium were poor substitutes to complex compounds as they did not yield any growth.

Furthermore, it was proved that reducing the N-P concentration in the basal medium down to 25% had no negative impact on biomass concentration and productivity in a continuous culture, while it further reduced operational costs. Additionally, it reduced the amount of salts polluting the environment, thus damaging it less.

As far as the continuous process is concerned we established that a dilution rate (D) of 0.2 h^{-1} was optimal for biomass productivity. Additionally, the process was far more stable and more resistant to contamination at this high D. Generally, increasing D will lead to higher volumetric biomass productivities, higher biomass yields but lower biomass concentrations. Also, time is favourable for *Fritz* to effectively and efficiently increase volumetric biomass productivity, because subsequent generations of *Fritz* get better accustomed to the working conditions.

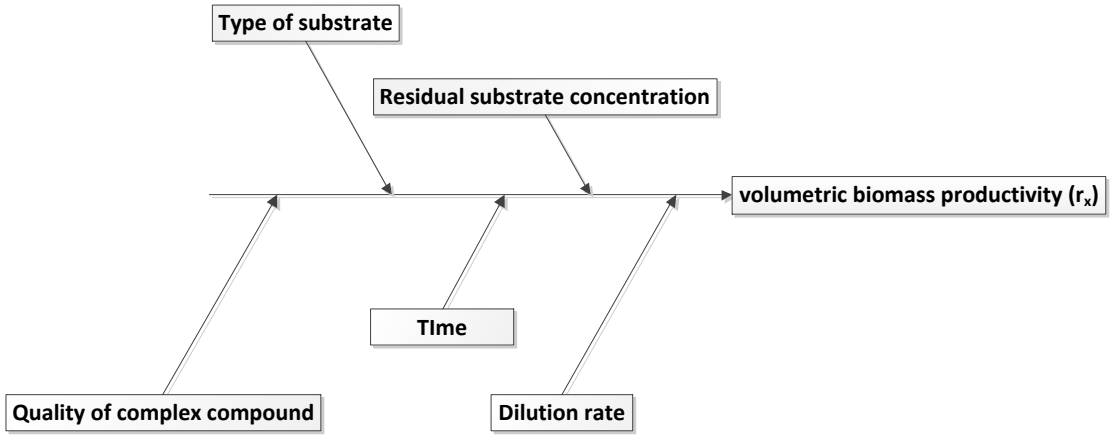

Figure 2 Parameters affecting the volumetric biomass productivity of Fritz.

In our study with *Fritz* we found out that the metabolism of this strain could be divided generally in the solventogenesis and the acidogenesis phases. In solventogenesis more ethanol and lactate were produced and in the acidogenesis more acetate and butyrate. Changing parameters like substrate supply (carbon-limitation, non-carbon limitation), quality of substrate, type of sugar or feeding N-P concentration had an impact on the metabolism of *Fritz* towards one of those phases. It was concluded that biomass productivity is generally linked to the acidogenesis metabolism phase in all experiments, except for the experiment at different Ds. However, in this case the resulting solventogenesis did not occur because of the higher dilution rate, but because of the higher residual substrate concentration.

4 Material and methods

4.1 Chemicals and gases

Deionized purified water was used for preparation of media and solutions. Nitrogen (N_2) was of 99.999% purity (Air Liquide, Schwechat, Austria). Pre-cultures preparation was performed in an anaerobic glove box (Coy Laboratory Products, Grass Lake, USA) under a H_2/N_2 (0.05 m^3/m^3) atmosphere.

4.2 Microorganism

The *Clostridium sp.* strain named as *Fritz* was handed over suspended in anaerobe flasks by the company *Schmack*/*Microbenergy*. From a fresh culture, cryoculture vials were prepared and kept at -80 ºC until further use.

4.3 Medium composition

4.3.1 Closed batches in serum flasks

A complex medium described by *Schmack* was used to carry out the screenings with *Fritz*. This medium contained, if no other specification was done (per L): $(NH_4)H_2PO_4$ 0.3125 g, $(NH_4)2HPO_4$ 0.5208 g, $MgCl_2.6H_2O$ 0.0521 g, $CaCl_2.2H_2O$ 0.0167 g, $FeCl_2.4H_2O$ 0.0005 g. The corresponding substrate and the preselected complex compound were added to a final concentration of 5 g/L and 2 g/L, respectively. The medium was supplemented with 5 g/L $NaHCO_3$ to keep the pH value around 7.0 during the cultivation.

The basal medium for the closed batch cultures was prepared in an anaerobic glove box, disposed in an inert atmosphere by flushing pure N_2 and sterilized at 121°C for 20 min. Sterile, anaerobic solutions of substrate, complex compound (when required) and $NaHCO_3$ were added separately before the inoculation.

4.3.2 Continuous cultures in a bioreactor

The standard medium described by Schmack was used as reference to carry out the experiments with *Fritz* on continuous mode. The reference basal medium contained, if no other specification was done (per L): $(NH_4)H_2PO_4$ 0.3125 g, $(NH_4)_2HPO_4$ 0.5208 g, $MgCl_2.6H_2O$ 0.0521 g, $CaCl_2.2H_2O$ 0.0167 g, $FeCl_2.4H_2O$ 0.0005 g, yeast extract 2 g. This reference basal medium contained the corresponding substrate in the amounts specified in section Results.

Medium for batch cultures was sterilized at 121°C for 20 min and made anaerobic by flushing with N_2. Sterile, anaerobic solution of the corresponding substrate was added separately after the sterilization.

Medium for continuous cultures was sterile filtered, after adjusting the pH to the desired value, in a previous autoclaved 20 L bottle.

4.4 Experimental Set-Up

4.4.1 Closed batches in serum flasks

Cultures of *Fritz*, to be used anaerobic for closed batch experiments, were grown at 40°C in a working volume of 50 mL in pressure-resistant 100 mL bottles sealed with natural gum stoppers.

4.4.2 Continuous culture in a bioreactor

Pre-cultures of *Fritz*, to be used anaerobic for inoculation, were grown at 40°C in a working volume of 50 mL in pressure-resistant 100 mL bottles sealed with natural gum stoppers. For inoculation, 50 to 100 mL of culture suspension was transferred into the bioreactor. Cultivations were carried out in a jacketed 2 L table-top bioreactor (Applikon B.V., The Netherlands) at a working volume of 0.8 L. Prior to inoculation the bioreactor system, as well as tubing and solutions were made anaerobic by flushing with N_2 for five minutes. Afterwards, cultures were continuously sparged with N_2 at 7 L/h and stirred at 150 rpm. The pH was measured with a pH-electrode (Mettler-Toledo GmbH, Vienna, Austria) and automatically maintained at 7.0±0.2 by applying anaerobic 2.0 M NaOH using a peristaltic pump (Ismatec SA, Glattburg, Switzerland). The temperature of the media was kept thermostatically at 40.5±0.5°C by using an external water bath (LAUDA Proline P5, BARTELT, Vienna, Austria), which was connected to the outer heating jacket of the bioreactor.

In a continuous mode the medium was applied using a peristaltic pump (Preciflow, Lambda Laboratory Instruments, Zürich, Switzerland) operated on controlled set-points for designated dilution rates (D). The medium inflow rate was recorded gravimetrically, as also the product and the bioreactor weight had been (Sartorius AG, Göttingen, Germany). The fermenter broth volume was kept constant by withdrawal of suspension using a metallic tube disposed at the level of working volume, which was connected to a peristaltic pump (Ismatec SA, Glattburg, Switzerland) by silicon tubing. Vessels for medium and base were pressurized in regular intervals by using N_2 to guarantee anaerobic conditions throughout the process.

4.5 Sample measurements

30 to 50 ml samples were taken from the bioreactor at each step/change during the process. Following analysis experiments were performed with these samples:

- OD_{580} measurements
- CDW measurements
- Cell fixation
- HPLC measurements

In the screenings carried out on closed batch mode, 1.5 mL samples were taken from each serum flask at defined time intervals. Following analysis were performed with these samples:

- OD_{580} measurements
- HPLC measurements

4.5.1 OD_{580} measurements

For a continuous tracking of the culture growth in closed batch experiments, optical density (OD) measurements at 580 nm were done. To correlate the OD_{580} measurement to the biomass concentration for each single sample, a linear regression with five different dilutions was performed. The relation between OD_{580} and CDW was found to be the following: $CDW\ [g/L] = 0.5252 \cdot OD_{580} + 0.0219$

To compare *Fritz* cellular growth between the different experiments in each screening OD_{580} measurements were carried out until a decrease in this parameter was observed. Tracking the OD_{580} values continuously, instead of just considering one point overnight, was important to compare and contrast the effects of the medium composi-

tion in the cellular growth of *Fritz*. Furthermore, for each of the experiments tested in the screening, a triplicate run and a control were done.

4.5.2 Analysis of biomass

Cell dry weight (CDW) was determined in quadruplicate by transferring 5 or 10 mL of fermenter broth in pre-weight reaction tubes. The reaction tubes were centrifuged at 5100 rpm for 10 min, or at 4100 rpm for 21 min at the higher sample volumes (centrifuge Signum 4K15, rotor 11156). 4 ml of supernatant was kept for further analysis and the rest was discarded, while the cell pellet was resuspended in 5 ml of distilled water. Then the tubes were centrifuged at 5100 rpm for 10 min. Again the supernatant was discarded and the cell pellet was dried for 72 h at 105°C. Cell pellet dry mass was determined gravimetrically (Sartorius Acculab, Sartorius AG, Göttingen, Germany).

Average elementary composition [carbon (C), hydrogen (H), nitrogen (N), oxygen (O), phosphorus (P) and sulfur (S)] of *Fritz* was determined by analysing steady state conditions of a standard continuous culture at D=0.1 h^{-1} (Mikroanalytisches Laboratorium, University of Vienna, Vienna, Austria) and found to be $CH_{1.804}O_{0.512}N_{0.210}S_{0.003}P_{0.029}$. The ash content was assumed to correlate to 2.8% of the total CDW. That corresponds to a final molecular weight of 26.67 g/C-mol and to a degree of reduction of 4.37 mol-e-/C-mol.

4.5.3 Cell fixation

Cell Fixation Solution was prepared according to the protocol provided by *Schmack*/*Microbenergy* and stored at 4°C for maximum of 8 days. For cell fixation 900 µl of sample were transferred into an Eppendorf tube and gently mixed with 100 µl of Cell Fixation Solution. The fixed samples were stored at 4°C.

4.5.4 Analytical methods

Off-gas components, H_2 and CO_2, were detected individually via serially applied gas analyzer systems (BlueSens gas sensor GmbH, Herten, Germany). H_2 measurements were corrected in respect to off-gas composition, according to manufacturer's information.

N_2 inflow rate was controlled by using a mass flow controller (Brooks Instrument, Matfield, USA).

All fermentation parameters and variable pump set-points were controlled by using the process information management system Lucullus 3.1 (SecureCell AG, Schlieren, Switzerland).

The qualitative analysis of sugars, alcohols and organic acids in the culture supernatant were done by high performance liquid chromatography (HPLC) (Agilent 1100 Series, USA) using a SUPELCOGEL C-610H column (9 µm particle size, 300.7.8 mm, Sigma Aldrich, USA) at 30°C, with 0.1% (v/v) H3PO4 in distilled water (traces of NaN_3) as mobile phase (0.5 ml/min), followed by a refractive index detector (RID-Detector).

The residual ammonium and phosphate concentrations were quantified via enzymatic analysis with photometric principle using an enzymatic robot system (CuBiAn XC, Innovatis, Germany).

4.6 Process Flow Diagram

The Process Flow Diagram (PFD) in Figure 3 shows the different sensors and control strategies used in the process for *Fritz* biomass productivity optimization.

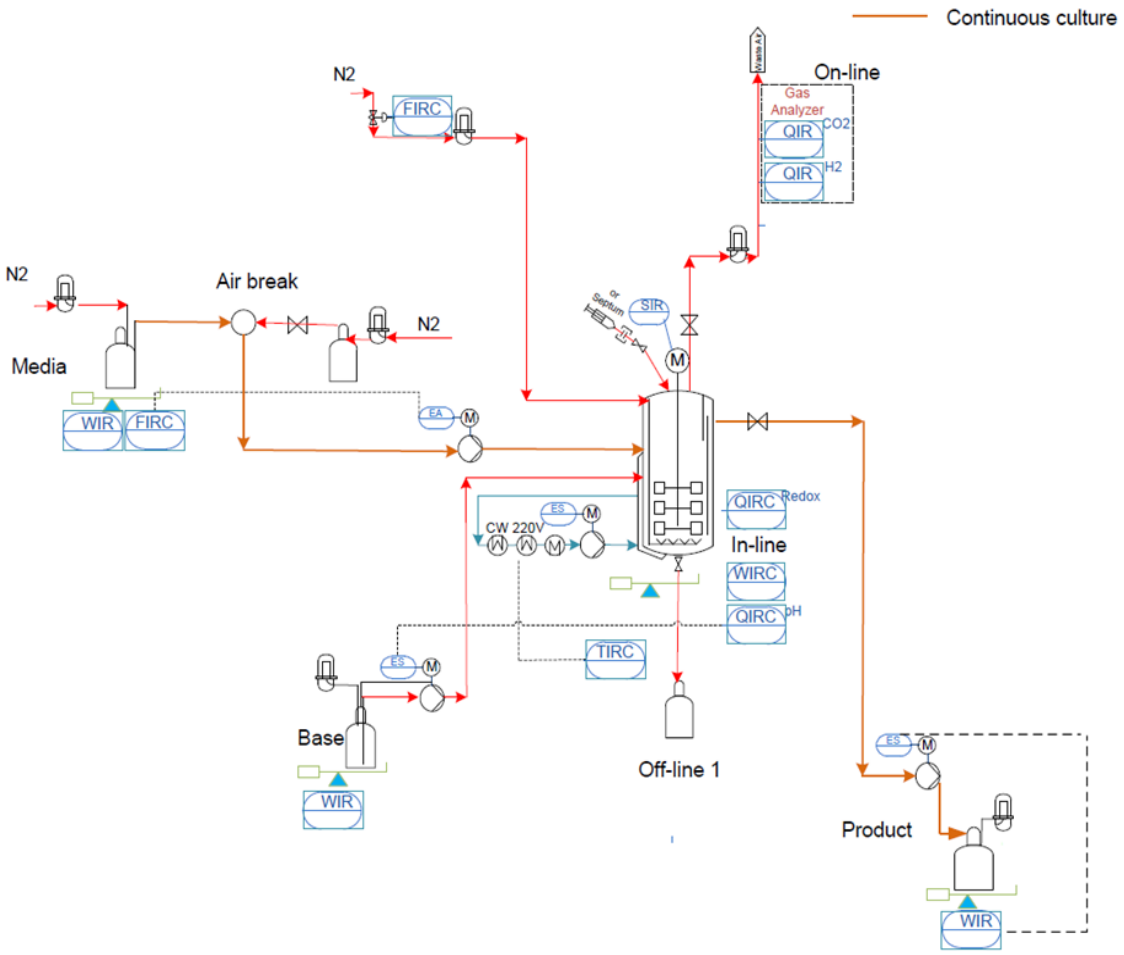

Figure 3 PFD developed in lab scale to cultivate Fritz and increase its biomass productivity in a bioreactor operated on continuous mode.

4.7 Formulas for calculation

To calculate the biomass productivity in the system, a mass balance at each step of the process was calculated. Regarding the biomass productivity as r_x, the mass balance to be considered at each pseudo-steady-state was Eq [1]:

$$r_x[Cmmol/L/h] = D_{in} \times C_{x,out} = \frac{F_{in}}{V_R} \times C_{x,out} \quad [1]$$

Furthermore, other physiological parameters related to *Fritz* metabolism were analysed to compare the responses of the system at changes in the working conditions. These parameters were substrate uptake concentration (Eq. [2]), product yields (Eq. [3]), volumetric substrate uptake rate for steady state conditions (Eq. [4]), volumetric production rate for steady state conditions (Eq. [5]) as well as specific production/uptake rates (Eq. [6]) for steady state. Additionally, for the calculation of the pulses Eq. 7 for volumetric substrate uptake rate and Eq. 8 for volumetric production rate were used.

$$\Delta C_s[g/L] = C_{s,in} - C_{s,out} \quad [2]$$

$$Y_{i/s}\ [Cmol/Cmol] = \frac{r_i}{r_s} = \frac{D \times C_{i,out}}{D \times (C_{s,in} - C_{s,out})} \quad [3]$$

$$\mathrm{r_s}\ [Cmmol/L/h] = [D_{in} \times (c_{s,0} - c_{s,1})] \times \frac{N}{M_i} \times 1000 \quad [4]$$

$$\mathrm{r_i}\ [Cmmol/L/h] = [D_{in} \times (c_{i,1} - c_{i,0})] \times \frac{N}{M_i} \times 1000 \quad [5]$$

$$q_i\ [Cmmol/g/h] = \frac{r_i}{x} = \frac{D \times C_{i,out}}{x} \quad [6]$$

$$\mathrm{r_s}\ [Cmmol/L/h] = [D_{in} \times (c_{s,0} - c_{s,2}) + \frac{C_{s,2} - C_{s,1}}{t_2 - t_1}] \times \frac{N}{M_i} \times 1000 \quad [7]$$

$$\mathrm{r_i}\ [Cmmol/L/h] = [D_{in} \times (c_{i,2} - c_{i,0}) + \frac{C_{i,2} - C_{i,1}}{t_2 - t_1}] \times \frac{N}{M_i} \times 1000 \quad [8]$$

In Equation 7 and Equation 8 the letter *N* stands for the number of carbon atoms in a molecule, M_i is the molecular weight of a molecule in [g/mol].

5 Results

5.1 Continuous culture in the reference medium

The aim of this first experiment was to test if *Fritz* was able to grow using products of its metabolism as carbon sources, concretely the metabolites (organic acids and alcohols) produced by itself during its growth in continuous mode at D=0.1 h^{-1}.

From previous experiments in continuous mode, it was known that carbon limiting conditions were achieved in the fermenter broth at D=0.1 h^{-1} with maltose at 5 g/L as main carbon and energy source. Based on that, and once the steady state was achieved, the feed pump was stopped and the strain was cultivated on a batch mode on the metabolites present in the fermenter broth. As shown in Figure 4, H_2 and CO_2 signals decreased immediately, as no maltose was being added into the medium, and they did not recover throughout the experiment. That was a signal of no bacterial growth, what was confirmed by a decreased biomass concentration in the off-line OD_{580} measurements. As the growth of *Fritz* did not recover after 24 h, the experiment was stopped.

With this experiment it could be concluded that *Fritz* was not able to grow using organic acids and/or alcohols as substrates; at least if no sugar was present in the broth.

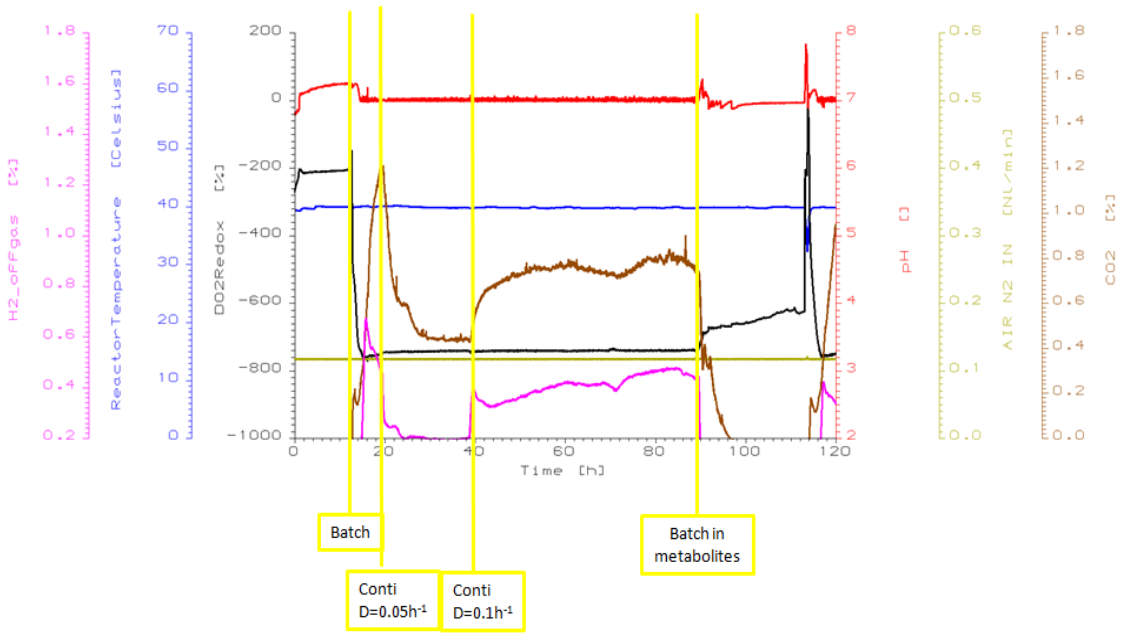

Figure 4 Continuous culture in standard medium with 5 g/L maltose in feed and subsequent batch culture in the metabolites produced during the continuous culture and present in the fermenter broth.

5.1.1 Pulses on continuous mode using the reference medium

In order to perform the analysis of the best medium composition, no carbon limiting conditions at D=0.1 h^{-1} had to be guaranteed. Under these conditions, pulses of different compounds at different concentrations could be done to study the effects in the cellular metabolism. Therewith, metabolic changes in *Fritz* could be identified and quantified.

Firstly, *Fritz* was cultivated on continuous mode in the standard medium with maltose at 5 g/L feeding concentration. Once the steady state at D=0.1 h^{-1} was established, pulses of maltose at different concentrations were done. It was necessary to wait until the steady state was achieved, before proceeding to pulse. Otherwise one would not be able to tell if a change in signal resulted from the pulse or a change in the system itself. The maltose concentration pulsed into the fermenter broth was 5 g/L and 10 g/L, for the first and the second pulse, respectively. These concentrations corresponded to a final feeding concentration of 10 g/L and 15 g/L, respectively, as 5 g/L were already present in the feed. For a period of 17 h after pulsing, samples were taken hourly in order to quantify biomass, substrate and metabolite concentrations. Biomass was calculated based on the OD_{580} values, while the substrate and metabolite concentrations were measured with HPLC.

The response of *Fritz* to a substrate (maltose) pulse involved a decrease of H_2 and a slight increase of CO_2 (Figure 5), what was bound to changes in the cellular metabolism. It involved a redirection of the metabolic pathways in favour to acetate, pyruvate, lactate and biomass, respect to butyrate (Figure 6 and Figure 7). A better understanding of these metabolic changes is possible if looking at Figure 8 and Figure 9, where the yields of the main products found in the fermenter broth were quantified.

Once both of the pulses were quantified, it had to be decided on the maltose concentration that guaranteed no carbon limiting conditions in the fermenter broth. For this study, the specific maltose uptake rates (q_s) were calculated (Figure 10). If the substrate could have been taken up completely by the cells, the tendency in the curve of pulse 2 (10 g/L) should have been the same as in pulse 1 (5 g/L). Regarding Figure 10, there was a representative increase in q_s at a maltose concentration of 5 g/L in pulse 2, comparing with pulse 1. That means, that at least one component present in the medium, rather than maltose, was limited in the culture at 15 g/L maltose. Therefore, 15 g/L maltose initial concentration had to be added in further cultivations, if no C-limiting conditions in the fermenter broth were desired.

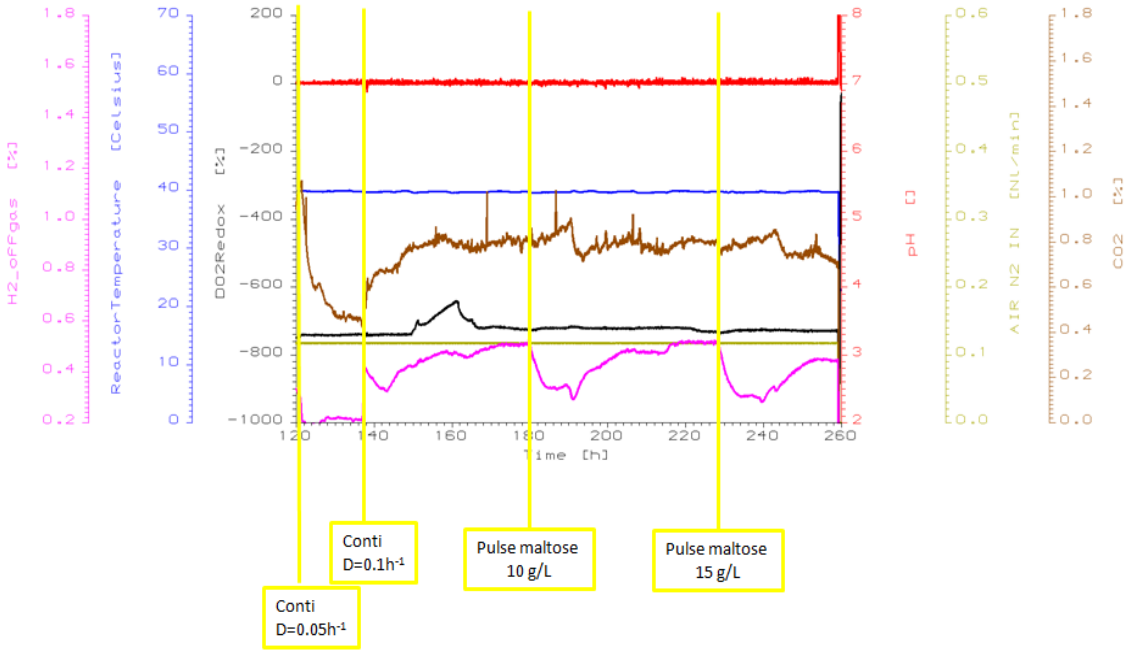

Figure 5 Continuous culture in the reference medium with 5 g/L maltose in feed and subsequent pulses of maltose up to a final feeding concentration of 10 g/L and 15 g/L (pulses of 5 g/L and 10 g/L maltose into the fermenter broth, respectively).

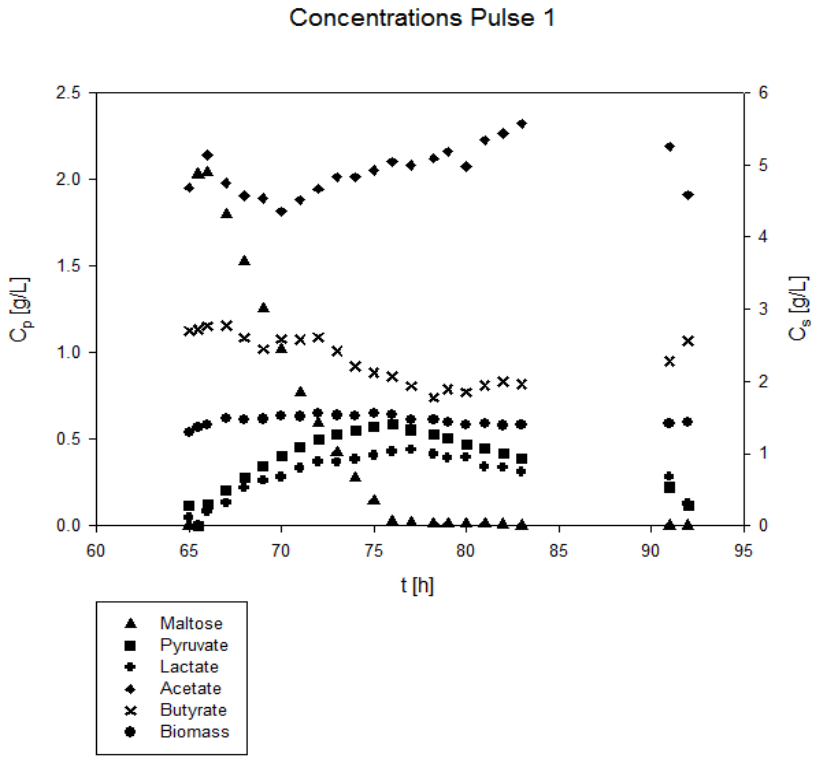

Figure 6 Substrate and product concentrations during the wash-out and uptake of the maltose pulses done up to a final feeding maltose concentration of 10 g/L (pulse of 5 g/L into the fermenter broth).

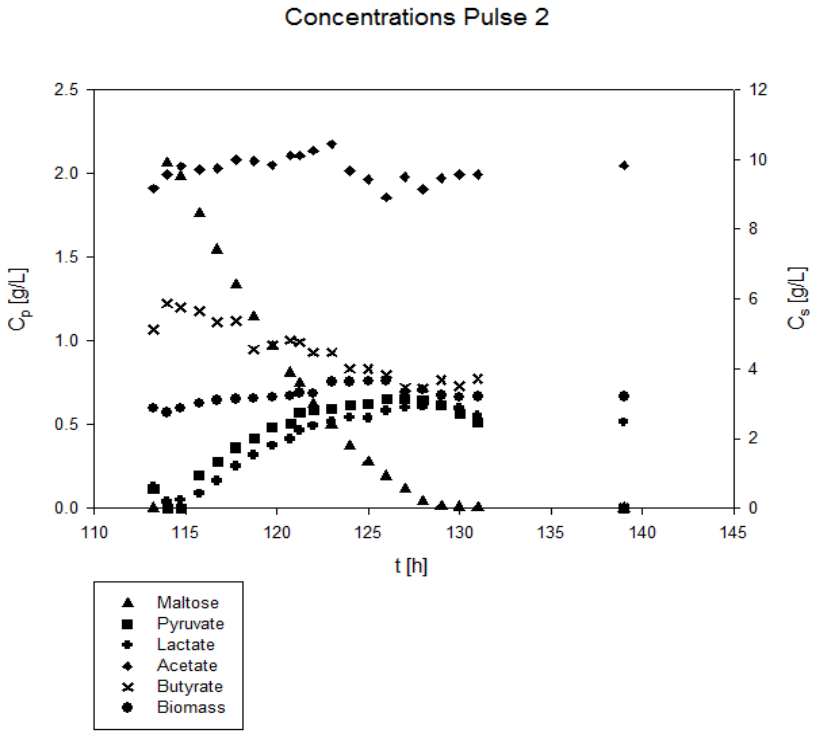

Figure 7 Substrate and product concentrations during the wash-out and uptake of the maltose pulses done up to a final feeding maltose concentration of 15 g/L (pulse of 10 g/L maltose into the fermenter broth).

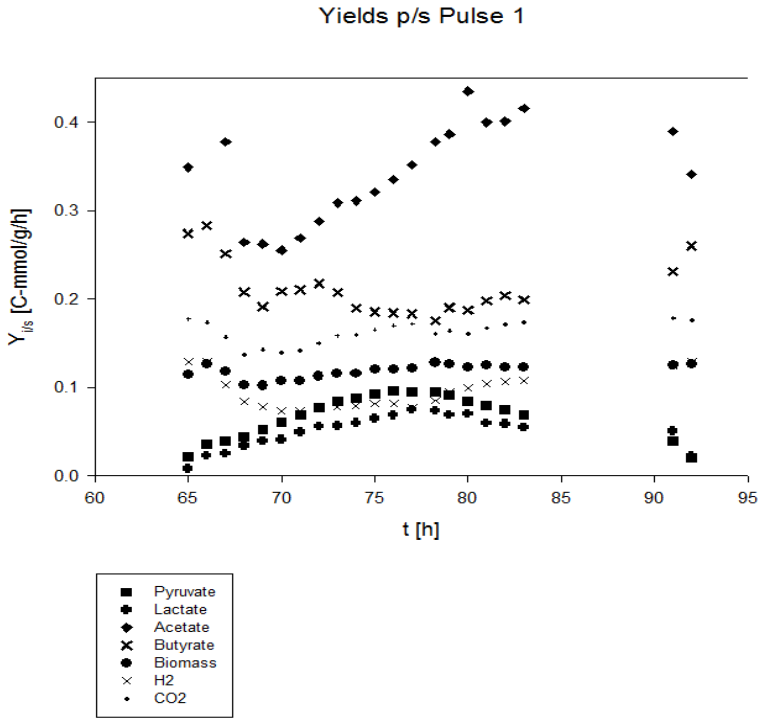

Figure 8 Product yields during the wash-out and uptake of the maltose pulses done up to a final feeding maltose concentration of 10 g/L (pulses of 5 g/L maltose into the fermenter broth).

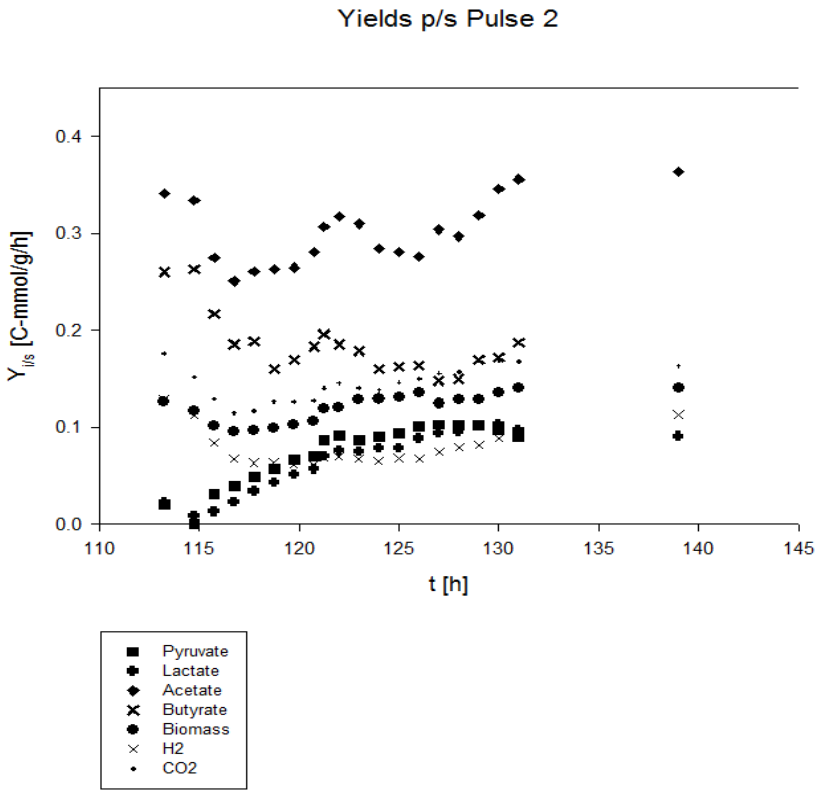

Figure 9 Product yields during the wash-out and uptake of the maltose pulses done up to a final feeding maltose concentration of 15 g/L (pulses of 10 g/L maltose into the fermenter broth).

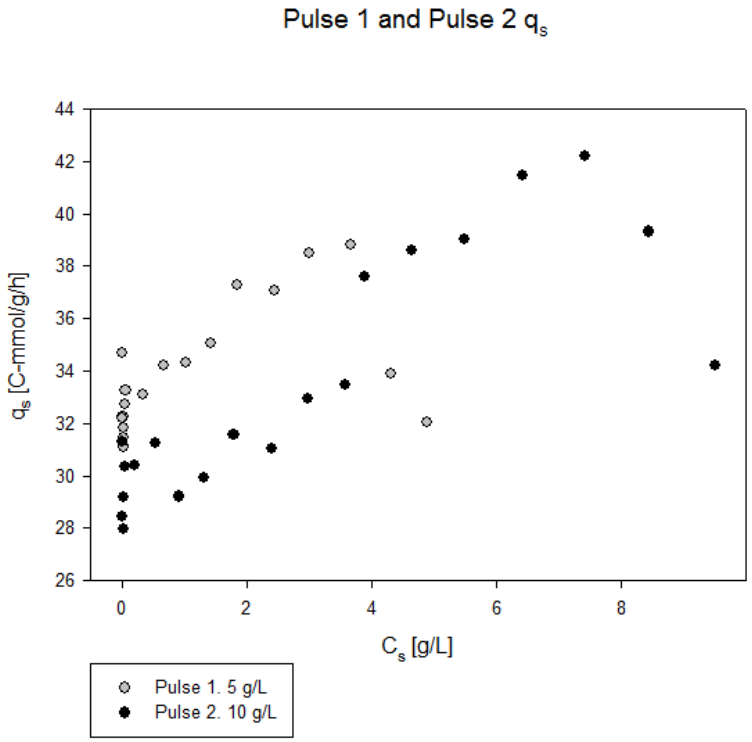

Figure 10 Specific substrate uptake rates (qs) during the wash-out and uptake of the maltose pulses done up to a final feeding maltose concentration of 10 g/L (pulse 1) and 15 g/L (pulse 2) (pulses of 5 g/L and 10 g/L maltose into the fermenter broth, respectively.

5.1.2 Verification with an alternative medium

When no C-limiting conditions are found in the fermenter broth, different components have to be tested to identify the other limiting component/s in the medium. Therefore, dynamic experiments with single pulses of different substances are carried out. These substances could encompass trace elements, vitamins, macro molecules and/or growth factors that are added into a standard (non-defined) medium with the complex compound/s. The identification of limiting components in a fermenter broth would allow the addition of the required amounts of those ones into the feeding medium, in order to maximize the carbon consumption and, therewith, the biomass productivity of *Fritz.*

In order to identify those limiting components in the reference medium composition, no carbon limiting conditions at D=0.1 h^{-1} had to be guaranteed. With this aim, *Fritz* was cultivated in the described standard medium with maltose as main carbon and energy source at 16.6 g/L feed concentration. Once the "pseudo-steady state" at D=0.1 h^{-1} was quantified, a pulse of 1 mL trace elements (TE, trace element solution SL-10) was done. For a period of 16 h after pulsing, samples were taken hourly in order to quantify biomass and metabolite concentrations. Biomass was calculated based on the OD_{580} values, while the substrate and metabolite concentrations were measured with HPLC.

Before pulsing 1 mL TE, the maltose concentration in the fermenter broth was 6.8 g/L at 0.79 g/L biomass. These concentrations supported the conclusion made previously in section 5.1.1, as *Fritz* was able to grow up to a maximal substrate uptake rate of 10 g/L maltose. After the pulse, no change was observed (Figure 11). It was 10 h later, when a significant increase in H_2 productivity took place, which also correlated to the increase in acetate production (Figure 12). Nevertheless, no explanation for this fact could be found, except for a possible contamination; that could have explained the cells found in the feed tubing.

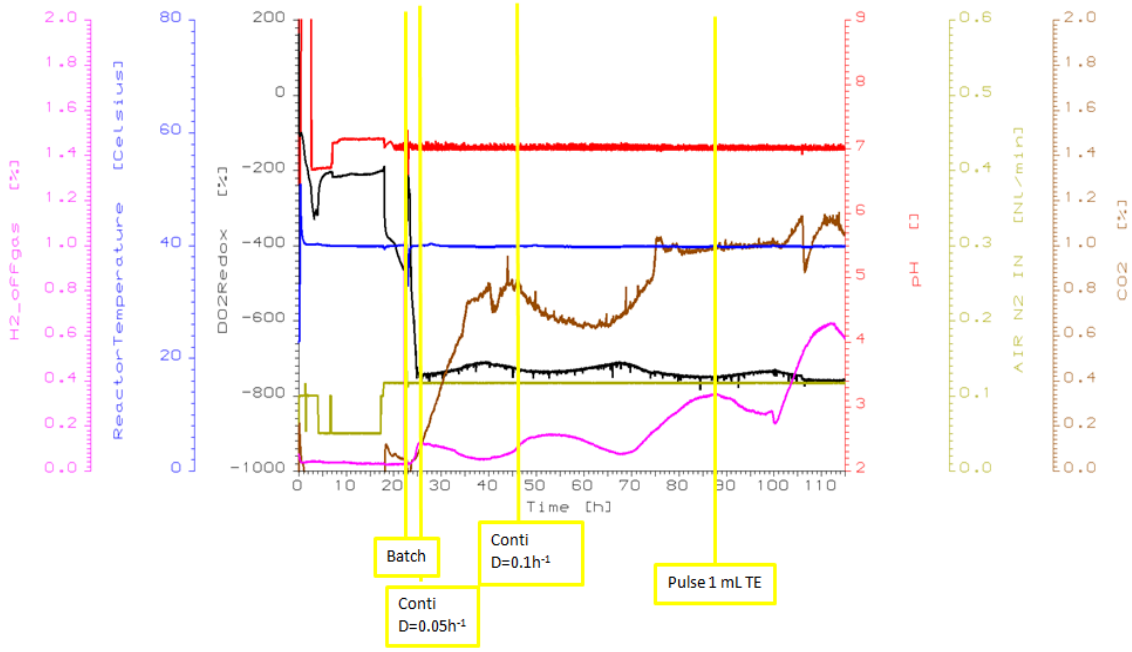

Figure 11 Continuous cultures in standard medium with 16.6 g/L maltose in feed and subsequent pulse of 1 mL TE.

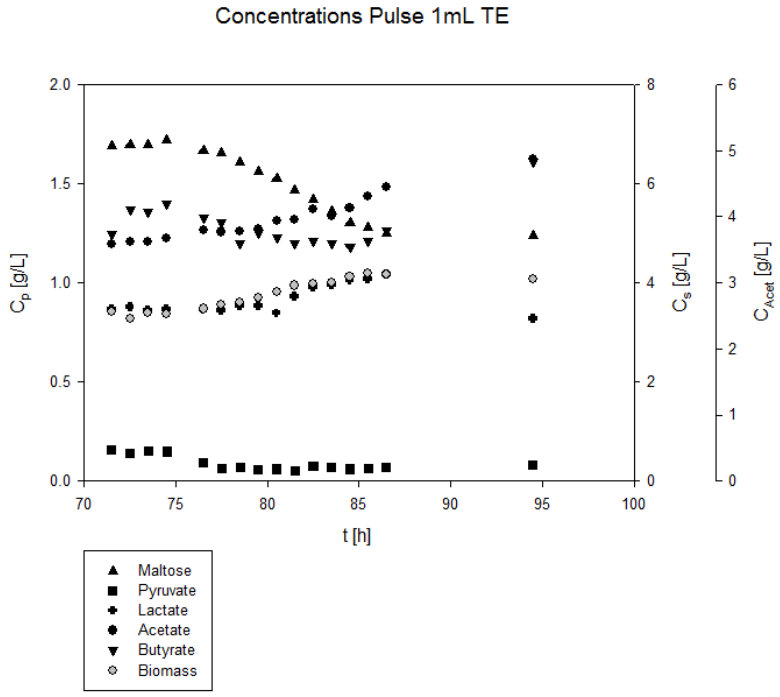

Figure 12 Substrate and product concentrations during the wash-out of 1 mL TE pulse in continuous culture in standard medium with 16.6 g/L maltose in feed.

5.1.3 Conclusion

- No growth in batch mode was observed, if cultivating Fritz in organic acids (butyric acid and acetic acid) and alcohols (ethanol), when no sugar was present in the broth.
- Pulsing maltose in a continuous culture in the reference medium induced metabolic changes in Fritz.
  - Increase of acetate, pyruvate, lactate and biomass concentrations.
  - Decrease of butyrate concentrations.
- Carbon limiting conditions were found up to 10 g/L maltose in the reference medium.
- The biomass concentration in a continuous culture at D=0.1 h^{-1} increased from 0.53 g/L to 0.79 g/L if cultivating Fritz in 5 g/L or 16 g/L maltose, respectively; at the expenses of C-limiting conditions in the culture.

5.2 Screening with different carbon sources

Aiming to find other carbon and energy sources rather than maltose in order to increase biomass productivity of *Fritz*, different substrates, related to *Fritz* in the literature, were tested.

5.2.1 Results

The following screening allowed us to study the growth of *Fritz* in different substrates at different initial concentrations. The substrates chosen for the screening were monosaccharides (fructose, glucose, xylose) and disaccharides (lactose, maltose, sucrose). In this case 1 mL maltose cryoculture vial stored at -80°C was used as inoculum.

Firstly, the six mentioned substrates were tested at an initial concentration of 5 g/L (Figure 13). The maximal OD_{580}, and therefore maximal biomass concentration, was obtained working with fructose, glucose, maltose and sucrose. Even when the growth in sucrose took a longer adaptation phase, the maximal specific growth rate of Fritz was almost the same for those four substrates. The growth in lactose did not achieve OD_{580} values higher than 0.6, even when the substrate was completely taken by the cells (Table 1). No growth was observed cultivating the strain in xylose.

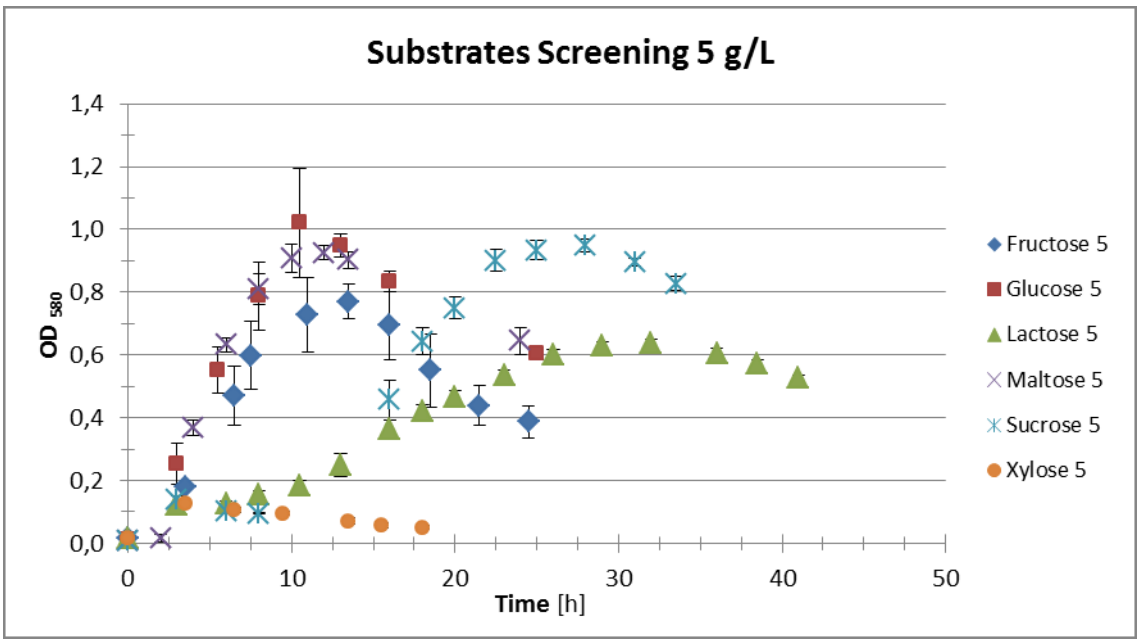

Figure 13 Screening with different substrates at an initial concentration of 5 g/L.

Secondly, *Fritz* was grown in an initial concentration of 10 g/L in each of the four preselected substrates (Figure 14). Maximal OD_{580} values were achieved if cultivating the strain in fructose, glucose or sucrose. No difference in the maximal OD_{580} compared to the results at 5 g/L (Figure 13) was observed if using maltose at 10 g/L (Figure 14) as substrate.

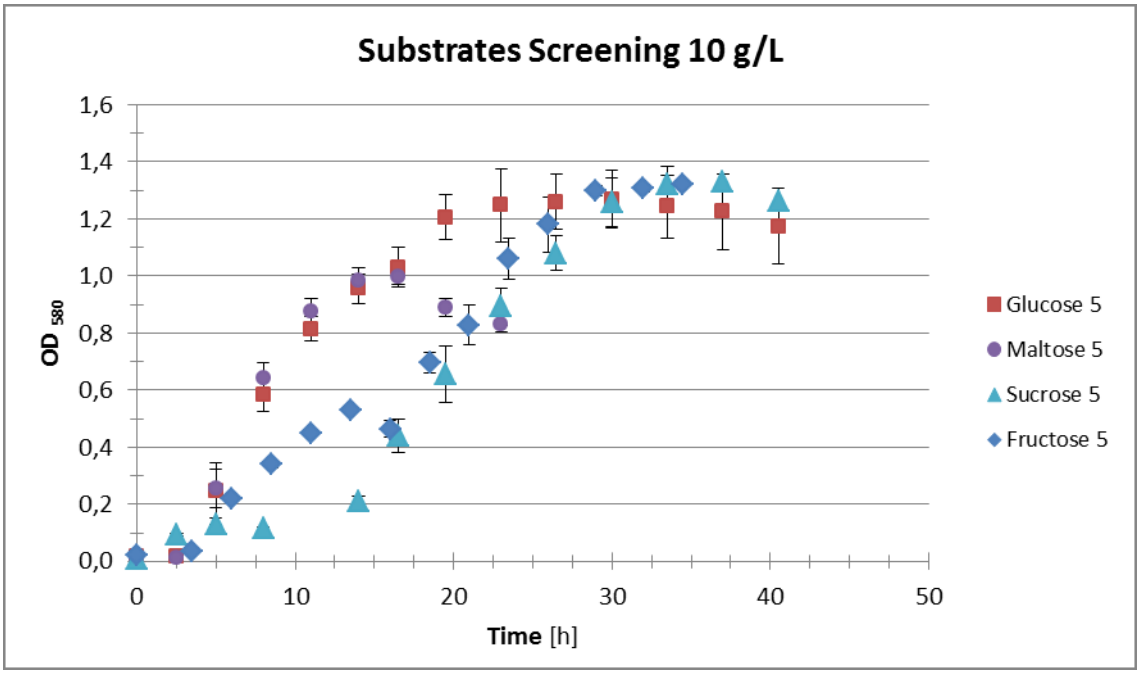

Figure 14 Screening with different substrates at an initial concentration of 10 g/L.

A better comparison of the growth of Fritz at 5 g/L and 10 g/L for each of the four pre-selected substrates can be observed in Figure 15.

Furthermore, trying to get a better understanding of the close batches in the different substrates at both initial concentrations, high performance liquid chromatography (HPLC) and inductively coupled plasma optical emission spectrometry (ICP-OES spectrometry) were performed. The samples measured with these techniques corresponded to the samples at the highest OD_{580} values before the stationary growth phase was detected. HPLC was used to do a quantitative analysis of the metabolites produced during the cell growth. That could have helped to detect possible metabolite inhibitions growing *Fritz* at 5 g/L (Table 1) or 10 g/L (Table 2) initial substrate concentrations.

Table 1 HPLC results at the highest OD580-values after growing Fritz in different substrates at an initial concentration of 5 g/L.

	Fructose 5 g/L	Glucose 5 g/L	Lactose 5 g/L	Maltose 5 g/L	Sucrose 5 g/L	Xylose 5g/L
	Concentration [g/L]					
Substrate	0.57	0.00	0.06	0.36	0.77	5.04
Pyruvate	1.04	0.80	0.37	1.03	0.49	0.00
Lactate	1.38	1.58	1.06	2.02	1.60	1.42
Formate	0.52	0.55	0.31	0.85	0.55	0.00
Acetate	1.14	1.30	2.12	1.61	1.68	0.00
Ethanol	2.33	2.55	2.01	2.85	2.55	1.23
Butyrate	0.29	0.21	0.92	0.41	0.50	0.00

Table 2 HPLC results at the highest OD580-values after growing Fritz in different substrates at an initial concentration of 10 g/L.

	Fructose 10 g/L	Glucose 10 g/L	Maltose 10 g/L	Sucrose 10 g/L
	Concentration [g/L]			
Substrate	2.33	1.39	5.11	3.13
Pyruvate	0.53	0.93	0.81	0.21
Lactate	1.46	2.37	1.72	1.19
Formate	0.46	0.52	0.58	1.74
Acetate	1.52	1.30	1.61	2.66
Ethanol	2.33	2.74	2.18	2.25
Butyrate	0.43	0.25	0.61	1.57

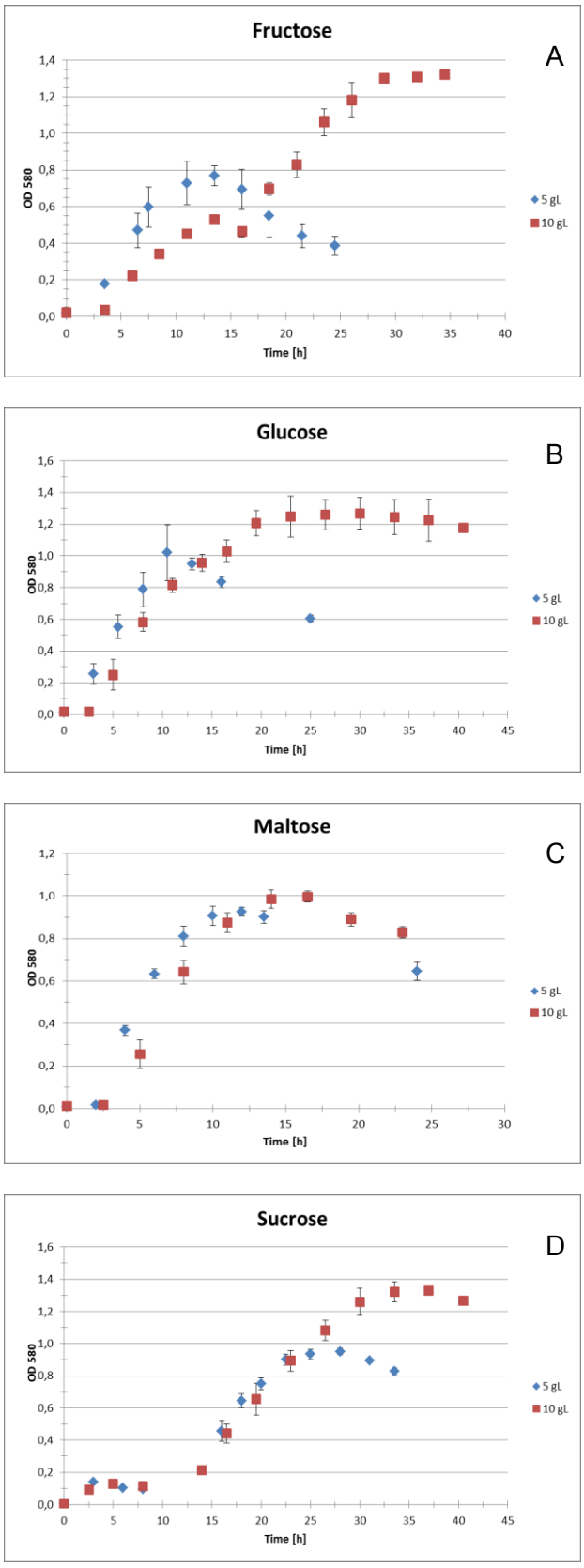

Figure 15 Growth of Fritz at 5 g/L and 10 g/L initial concentrations on fructose (A), glucose (B), maltose (C) and sucrose (D).

Regarding Table 1 and Table 2, the maximal concentration of each of the metabolites at the end of the exponential growth phase could be settled at 1.00±0.06 g/L pyruvate, 2.20±0.25 g/L lactate, 1.74 g/L formiate, 2.39±0.38 g/L acetate, 2.80±0.08 g/L ethanol, and 1.25±0.46 g/L butyrate. Nevertheless, it was not possible to guarantee if there was an inhibitory effect of one or more of the metabolites produced during the bacterial growth. The ICP-OES spectrometry was done to study possible depletion of trace components during bacterial growth. Therefore, the sample at the end of the exponential growth phase was compared with the sample of the control run, for each of the substrates tested. Nevertheless, the results got from this technique were not concluding, as the amounts of some of the measured compounds were higher in the sample that in the control. This could be explained considering cell lysis during the experiment. Regarding Figure 13 and Figure 14, fructose, glucose and sucrose were the final preselected substrates. Considering the HPLC results in Table 2, no carbon limiting conditions could be found at the highest OD_{580} values at 10 g/L feeding substrate concentration. Nevertheless it could not be established if it was due to metabolite/s inhibition and/or other component/s depletion. With the aim of working under C-limiting conditions at higher feeding substrate concentrations in continuous cultures carried out in a bioreactor, the complex reference basal medium used in the close batch experiments had to be improved. For this purpose, and considering the economic aspects of the whole process, sucrose was the selected substrate to be used in further experiments. Sucrose had the lower price among the preselected substrates (Table 3).

Table 3 Price comparison between different carbon sources

	Company	Nr.	Amount	Price	Price
			[g]	[€]	[€/100g]
CMC	Roth	6190.2	1000	60.9	**6.09**
Cellobiose	Roth	5840.2	100	115.5	**115.5**
Fructose	Roth	4981.2	2500	40.2	**1.61**
Glucose · H_2O	Roth	X997.3	1000	24.5	**2.45**
Glutamin	Roth	3772.2	500	75.5	**15.1**
Maltose · H_2O	Roth	8951.3	500	36.35	**7.27**
Raffinose · $5H_2O$	Roth	5241.3	100	111.9	**111.9**
Starch (wheat)	VWR	1.11685.1000	1000	54.3	**5.43**
Sucrose	Roth	4661.1	1000	15.55	**1.56**
Whey (Lactose)	Roth	8921.1	1000	16.9	**1.69**
Xylose	SigmaAld	W360600	5000	200	**4**

5.2.2 Conclusions

- The cultivation of *Fritz* in fructose, glucose, maltose or sucrose at 5 g/L performed at the same maximal specific growth rate. The maximal OD_{580} values were [0.7, 1.1].
- No growth was observed if cultivating *Fritz* in xylose at 5 g/L.
- Cultivating *Fritz* in lactose at 5 g/L ended up in a maximal OD_{580} value of 0.64±0.02.
- The cultivation of *Fritz* in fructose, glucose, maltose or sucrose at 10 g/L performed at the same maximal specific growth rate.
- No difference in the maximal OD_{580} values was observed cultivating *Fritz* in maltose at 5 g/L or 10 g/L.
- Higher OD_{580} values [1.1, 1.4] were achieved at 10 g/L than at 5 g/L if growing *Fritz* in fructose, glucose or sucrose.
- Pyruvate, lactate, acetate and ethanol and butyrate were the main metabolites found in the culture broth.
- Possible metabolite inhibition could have taken place during the growth of *Fritz*, but it could not be settled.
- The depletion of trace components in the culture broth could not be determined using ICP-OES spectrometry due to the release of cell compounds in the culture broth by cell lysis.
- Sucrose was the selected substrate for further experiments in continuous cultures in a bioreactor due to its lower price and the resulting high biomass concentration if cultivating *Fritz* on it.

5.3 Screening in defined media with maltose

Once the new substrate to be tested was selected, it was also important to determine if *Fritz* was able to grow in defined media, using predefined vitamin and/or trace element solutions instead of yeast extract.

5.3.1 Medium composition

The complex medium described by Schmack was used as starting point to generate different defined media to cultivate *Fritz*. From this reference complex medium (CM) on, four different defined media were tested. Trace elements solution consisted of (per L): $FeCl_2.4H_2O$ 1.5 g, $ZnCl_2$ 0.7 g, $MnCl_2.4H_2O$ 0.1 g, H_3BO_3 6 mg, $CoCl_2 \cdot 6H_2O$ 0.19 g, $CuCl_2 \cdot 2H_2O$ 2 mg, $NiCl_2 \cdot 6H_2O$ 24 mg, $Na_2MoO_4 \cdot 2H_2O$ 36 mg, Na_2WO 15 mg and $Na_2SeO_3 \cdot 5H_2O$ 15 mg. Vitamin solution contained (per L): biotin 20 mg, folic acid 20 mg, pyridoxine-HCl 100 mg, riboflavin 50 mg, thiamin-HCl 50 mg, nicotinamide 50 mg, cobalamin 50 mg, p-aminobenzoic acid 50 mg, lipoic acid 50 mg and pantothenic acid 50 mg.

- Medium 1 (M1): the 2 g/L yeast extract present in CM was replaced by 1mL and 2 mL trace elements and vitamin solutions, respectively.
- Medium 2 (M2): just 1 g/L yeast extract was replaced by 1mL of both, trace elements and vitamin solutions.
- Medium 3 (M3): the yeast extract present in CM was replaced by 1mL and 10 mL trace elements and vitamin solutions, respectively. Furthermore, $FeCl_2 \cdot 4H_2O$ was replaced by 0.625 g/L $FeSO_4 \cdot 7H_2O$.
- Medium 4 (M3): M3 was supplemented with 0.5 g/L cysteine-$HCl \cdot H_2O$.

Furthermore, defined media from other *Clostridium sp.* were tested. These media were found in the literature and worked positively with their corresponding strains.

- Medium for *C. cellulolyticum* contained (per L): $(NH_4)_2SO_4$ 1 g, KH_2PO_4 1.4 g, K_2HPO_4 2.21 g, $MgCl_2 \cdot 6H_2O$ 0.1 g, $CaCl_2 \cdot 2H_2O$ 0.02 g, 1 mL trace elements solution and 10 mL vitamin solution. This medium was supplemented with 5 g/L maltose, 0.5 g/L cysteine-$HCl \cdot H_2O$ and 5 g/L $NaHCO_3$.
  Trace elements solution consisted of (per L): 50 mL 10M HCl, $FeSO_4 \cdot 7 \cdot H_2O$ 5 g, $ZnSO_4 \cdot 7 \cdot H_2O$ 1.44 g, $MnSO_4 \cdot 7 \cdot H_2O$ 1.12 g, H_3BO_3 0.03 g, $CoCl_2 \cdot 6H_2O$ 0.02 g, $CoSO_4 \cdot 7H_2O$ 0.25 g, $NiCl_2$ 0.04 g, $Mo_7(NH_4)_6O_{24} \cdot 4H_2O$ 1 g, and $Na_2SeO_3 \cdot 5H_2O$

0.03 g. Vitamin solution contained (per L): biotin 0.1 g, riboflavin 0.25 g, thiamin 0.25 g, nicotinamide 0.15 g, cobalamin 0.1 g, p-aminobenzoic acid 0.25 g, and pantothenic acid 0.25 g.

- Medium for *C. thermoaceticum* contained (per L): $(NH_4)_2SO_4$ 2 g, KH_2PO_4 5 g, K_2HPO_4 7 g, $MgSO_4$ 0.3 g, $CaCl_2 \cdot 2H_2O$ 0.025 g, 10 mL trace elements solution and 10 mL vitamin solution. This medium was supplemented with 5 g/L maltose, 0.5 g/L cysteine-$HCl \cdot H_2O$ and 5 g/L $NaHCO_3$.

  Trace elements solution consisted of (per L): $ZnCl_2$ 0.015 g, H_3BO_3 0.015 g, $CoCl_2 \cdot 6H_2O$ 1.7 g, $CuCl_2 \cdot 2H_2O$ 0.015 g, $NiCl_2$ 0.27 g, Na_2SeO_3 1 g, EDTA 0.5 g, $Na_2MoO_4 \cdot 2H_2O$ 6g, and $Na_2WO_4 \cdot 2H_2O$ 1g. Vitamin solution contained (per L): nicotinamide 0.2 g.

5.3.2 Results: defined media derived from reference complex medium (CM)

This screening allowed us to study the growth of *Fritz* in different defined media with maltose at 5 g/L as main carbon and energy source. Maltose was the reference substrate, as it was the one used by *Schmack* in the production plant.

Firstly, M1, directly derived from the complex standard medium (CM) and supplemented with 5 g/L maltose, was tested (Figure 16). In this case 1 mL cryoculture vial stored at -80°C was used as inoculum (_C). As no growth was observed, *Fritz* was cultivated in 5 g/L complex medium and 1mL of this culture was used as inoculum in M1 (_I) (Figure 16). The goal was to study if the cells could only grow in a defined medium just after having grown in the standard complex one (CM), as a response to an adaptation phase.

As no positive response was obtained, a next experiment was done using M2 (Figure 16). What we realised was that *Fritz* could grow in this medium, but up to really low OD_{580} values and with a lower maximal specific growth rate, comparing with its growth in the CM (Figure 17). This behaviour could have been related to the fact that some components present in the yeast extract were limiting the growth of the strain in M2.

Still thinking about a defined medium, M3 and M4 were prepared. These media contained an S-source ($FeSO_4$) and, in case of M4, also a reduction agent (Cysteine-$HCl \cdot H_2O$). The goal of trying these media was based on the fact that no sulphur was present in CM (except for the undefined concentration in the yeast extract), what could have made it to be the limiting component in the medium. Nevertheless, *Fritz* did not grow in none of the two new media tested (Figure 16).

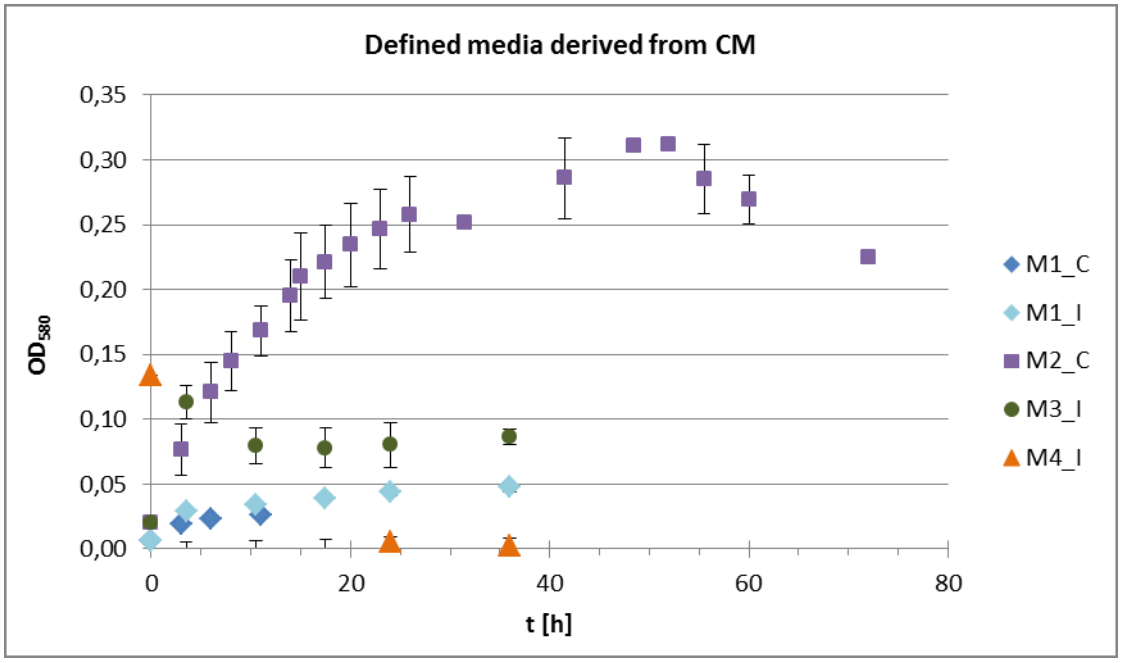

Figure 16 Screening with different defined media at an initial concentration of 5 g/L maltose, using as inoculum a cryoculture (C) or culture broth from a previous complex culture (I).

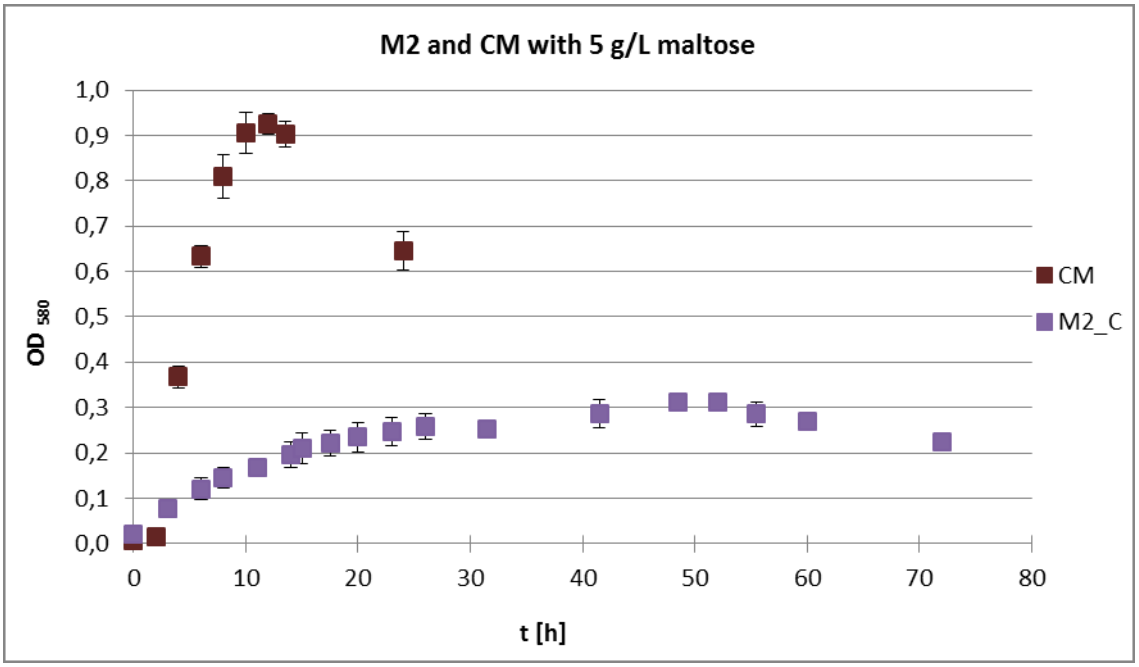

Figure 17 Comparison between M2 and CM in 5 g/L maltose.

5.3.3 Results: defined media from literature

Two *Clostridium sp.*, according to published articles, had been successfully cultivated in defined media. These were *Clostridium cellulolyticum* and *Clostridium thermoaceticum*.

Both media where tested for *Fritz* cultivation using a cryoculture as inoculum (_C) or a previous cultivated culture of *Fritz* in the reference complex medium (_I). Nevertheless, no growth was observed in none of both cases after a cultivation time of 48 h (Figure 18).

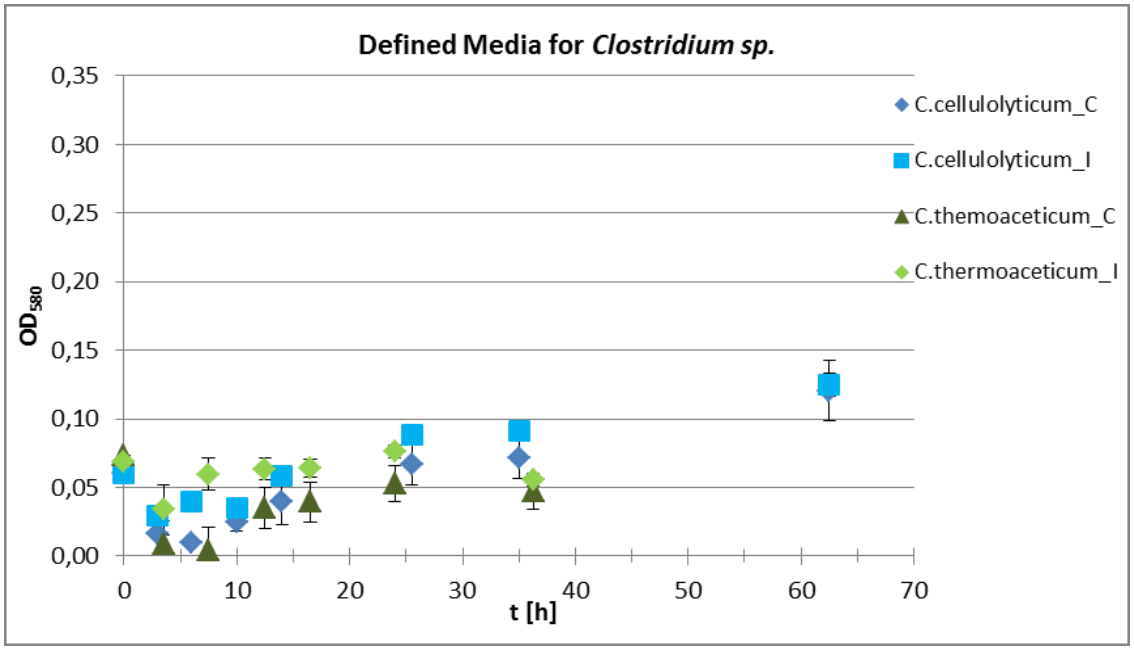

Figure 18 Screening with different defined Clostridium sp. media at an initial concentration of 5 g/L maltose using a cryoculture as inoculum (C) or culture broth from a previous complex culture (I).

5.3.4 Conclusions

- No growth was observed in M1, neither using 1 mL cryoculture vial as inoculum nor using a fresh inoculum grown in complex medium.
- Growth in M2 was observed, but it was much lower than in CM in maltose at an initial substrate concentration of 5 g/L. It could have been because some component/s present in the yeast extract was/were limiting Fritz growth.
- Fritz did not grow in defined media described for *C. cellulolyticum* and *C. thermoaceticum*, using maltose as substrate at 5 g/L.

5.4 Continuous culture using an alternative medium: sucrose instead of maltose as main carbon and energy source

Once it was ensured that complex compounds were necessary for *Fritz* growth, and a new promising substrate (sucrose) was selected to substitute maltose, new experiments in a bioreactor on continuous mode were carried out.

5.4.1 Contamination Issue

5.4.1.1 Sterility Test

Before starting any experiment with sucrose a sterility test was performed. Therewith we could check the sterility of the system and possible contamination sources in the bioreactor, bottles, and/or tubing.

We simulated a batch and a continuous culture at two dilution rates (D), in the same order as it was going to be done in experimental runs. Once the system was autoclaved and the final set up was disposed, the substrate (sucrose) was added into the medium at a final concentration of 12 g/L. After a couple of hours a continuous culture at D=0.05 h^{-1} was started. 24 h later D was increased to D=0.1 h^{-1} and left in this mode for a total of 48 h. After this simulation process no contamination was observed in any part of the system.

5.4.1.2 Backward growth

Another aspect to consider about the system, but once the inoculation was done, was the possibility of *Fritz* growing backwards. This idea came because the “possible contamination” in previous runs was firstly detected at the end of the feed tube instead at the exit of the bottle feed. In order to avoid this possible growth, an air-break was added to the initial set-up (Figure 3).

5.4.2 Continuous culture in sucrose

The first step was to quantify the growth of *Fritz* in the pseudo-steady states at dilution rates of 0.1 h^{-1} and 0.2 h^{-1} using the standard medium with 12 g/L sucrose and 2 g/L YE (Table 4). Two main advantages were observed at the highest dilution rate:

1) Higher biomass productivity, as there was no wash-out of the cells.
2) Lower contamination risk.

Furthermore, higher stability growth conditions at higher dilution rates could be observed, comparing the on-line signals (H_2 and CO_2) at both dilution rates (Figure 19 and Figure 20).

Moreover, the quantification of the pseudo-steady states with the selected substrate (sucrose) could be compared with the one done for the reference substrate (maltose) in previous experiments (Table 5). Comparing Table 4 and Table 5, the positive effects of using sucrose instead of maltose could be remarked. Although no significant differences in r_x, q_x or $Y_{x/s}$ could be detected between both substrates at D=0.1 h^{-1} under no C-limiting conditions, higher cellular activity was observed (higher CER) using sucrose. This, together with a lower price of this selected substrate, makes it more attractive for further use.

Table 4 Pseudo-steady state at dilution rates of 0.1 h^{-1} and 0.2 h^{-1} using the standard medium with 12 g/L sucrose and 2 g/L yeast extract

C_{in}	D_{in}	Sucrose	Lactate	Acetate	Butyrate	Ethanol	Biomass
[g/L]	[1/h]	[g/L]					
12.1	0.10	4.68	0.52	1.50	0.46	1.87	0.60
12.7	0.20	6.66	0.41	0.52	0.50	2.18	0.53
C_{in}	D_{in}	r_x	q_x	$Y_{x/s}$	CER	C-Balance	DoR-Balance
[g/L]	[1/h]	[Cmmol/L/h]	[Cmmol/g/h]	[C-mol/C-mol]	[C-mmol/L/h]	[C-mol]	
12.1	0.10	2.24	3.75	0.09	5.74	0.95	0.95
12.7	0.20	3.94	7.50	0.09	4.76	1.03	1.16

Table 5 Steady-state at D=0.1 h^{-1} using the standard medium with 5 g/L maltose and 2 g/L yeast extract. Pseudo-steady state at D=0.1 h^{-1} using the standard medium with 16.6 g/L maltose and 2 g/L yeast extract.

C_{in}	D_{in}	Maltose	Lactate	Acetate	Butyrate	Ethanol	Biomass
[g/L]	[1/h]	[g/L]					
5.3	0.10	0.00	0.04	1.95	1.12		0.53
16.6	0.10	6.80	0.86	3.61	1.26	1.44	0.79
C_{in}	D_{in}	r_x	q_x	$Y_{x/s}$	CER	C-Balance	DoR-Balance
[g/L]	[1/h]	[Cmmol/L/h]	[Cmmol/g/h]	[C-mol/C-mol]	[C-mmol/L/h]	[C-mol]	
5.3	0.10	1.99	3.78	0.11	3.33	0.94	0.90
16.6	0.10	2.98	3.75	0.09	4.10	1.06	1.07

5.4.3 Pulses in a continuous culture with sucrose as substrate

Firstly, it was important to consider the results obtained from the screening in defined media. Regarding Figure 17, it was possible to realize that some component/s present in the yeast extract (YE) were limiting the growth of Fritz, as lower OD_{580} values and a lower maximal specific growth rate were observed if cultivating Fritz in 1 g/L YE (M2) instead of in 2 g/L YE (CM).

Our first goal was to find the limiting components in the complex medium, if cultivating Fritz in 12 g/L sucrose, as no C-limiting conditions were ensured at that substrate feeding concentration. Trace elements (TEs) from the fermenter broth of a previous continuous culture in maltose and from close batch runs in different substrates were measured using ICP-EOS. As we did not get any clear information about limiting TEs in the fermenter broth using this technique, we proceed to pulse complex compound solutions using sucrose as main carbon and energy source. The aim of this fermentation was to find out which complex compounds, instead of which single component, induced higher r_x and $Y_{x/s}$ during a continuous culture with Fritz.

5.4.3.1 Pulses of yeast extract (YE)

Fritz was grown in continuous culture in sucrose at D=0.1 h^{-1} (Figure 19). Once the pseudo-steady-state was achieved (Table 4), a pulse with 1.2 g/L YE was done. From the on-line signals and the HPLC results it was possible to see a positive response in the uptake of the substrate after the pulse (Figure 21A and Figure 21B). This fact is remarked in Figure 21D at the beginning of the pulse, where higher specific substrate uptake rates (q_s) were registered. Moreover, at this initial point of the pulse, higher specific biomass production rates (q_x) of *Fritz* could be determined. The decrease in both parameters during the pulse due to an uptake of the limiting components present in the adding YE and/or due to the wash-out of these ones with the out stream stands up for the presence of essential growth elements in the complex compound limited in the fermenter broth.

Due to the contamination and stability problems observed at D=0.1 h^{-1}, a new continuous culture at D=0.2 h^{-1} was carried out (Figure 20). At this dilution rate, a new YE-pulse was done. Therewith we wanted to study the new results (Figure 22A to Figure 22C) and compare them with the ones obtained working at D=0.1 h^{-1} (Figure 21A to Figure 21C). The effect of the YE-pulse in the metabolism of *Fritz* is easier to be studied looking at the on-line signals (volumetric rates) of H_2 and CO_2, as well as at the off-line data of the substrate and the biomass, our product of interest, at D=0.1 h^{-1}. The curves at this lower dilution rate are softer and last longer than at D=0.2 h^{-1}, due to a slower wash-out of the pulse. That made possible a better quantification of the samples at D=0.1 h^{-1}, what helped to study the metabolic changes during the pulse. We realized that during the YE-pulses no significant increase in biomass concentration (Figure 21B and Figure 22B), but in pyruvate, lactate and butyrate concentrations (Figure 21C and Figure 22C) took place. Just at the beginning of the YE-pulses, the metabolic shift to biomass production could be observed, what remarked the necessity of the cells for the limiting components present in the yeast extract, which seemed to have been pulsed not in enough quantity due to the relative quick decrease of q_s and q_x.

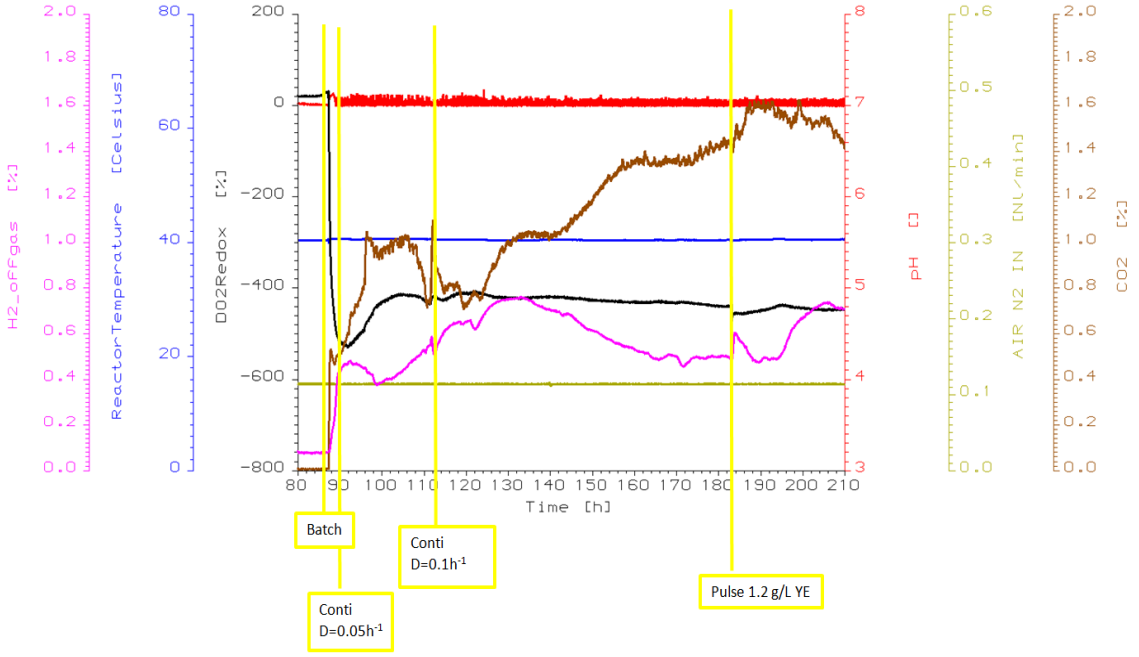

Figure 19 Continuous culture at D = 0.1 h^{-1} in standard medium with 12 g/L sucrose and 2 g/L YE in feed. Pulse of 1.2 g/L YE.

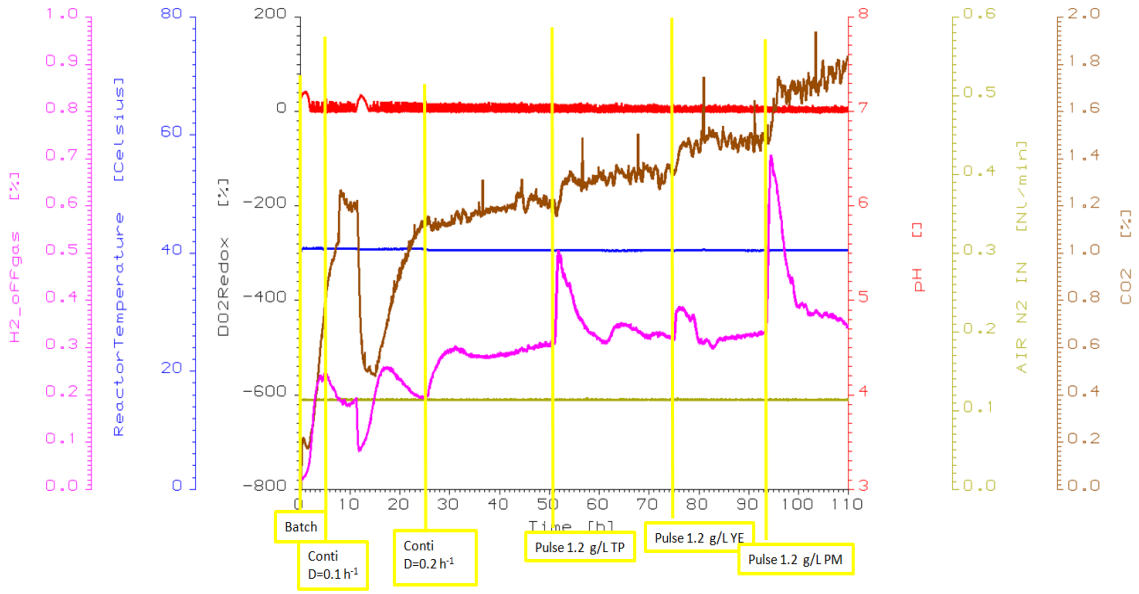

Figure 20 Continuous culture at D = 0.2 h^{-1} in standard medium with 12 g/L sucrose and 2 g/L YE in feed. Pulses of 1.2 g/L TP, YE or PM.

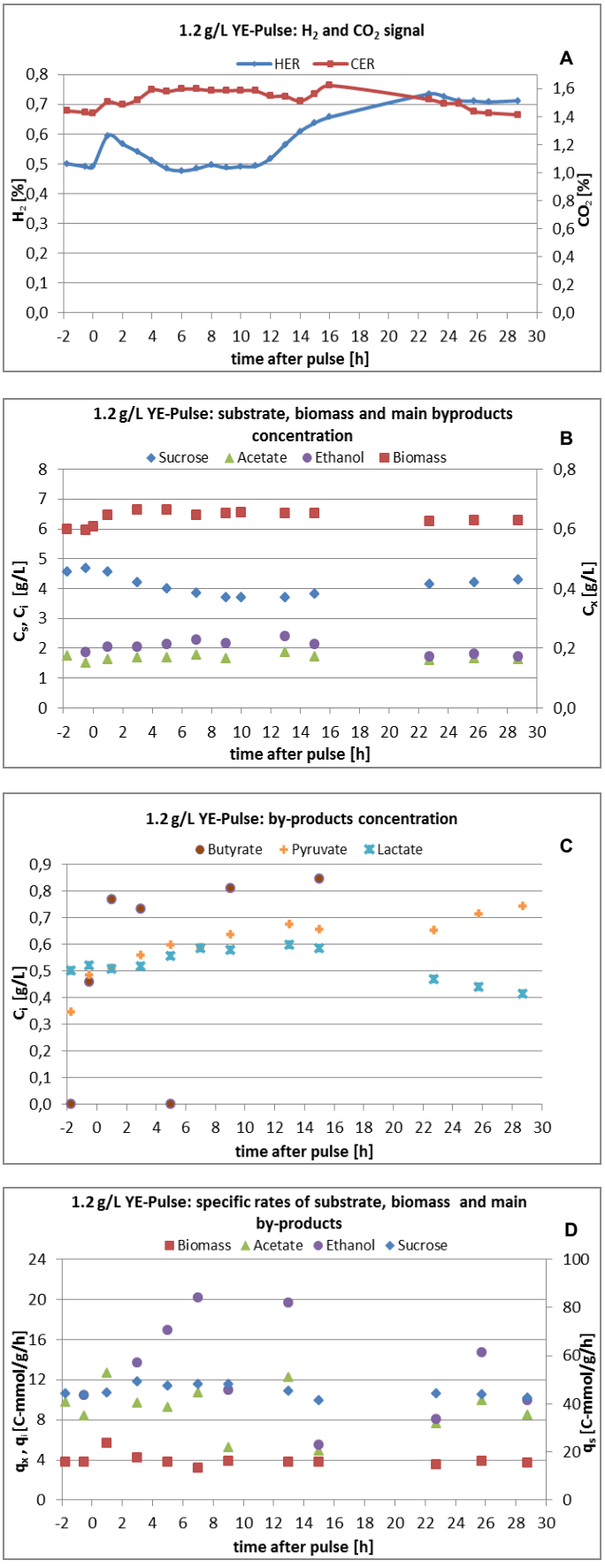

Figure 21 Pulse of 1.2 g/L YE in continuous mode at D=0.1h^{-1} in standard medium with 12 g/L sucrose and 2 g/L YE in feed: On-line data (A), off-line data (B, C) and specific rates (D).

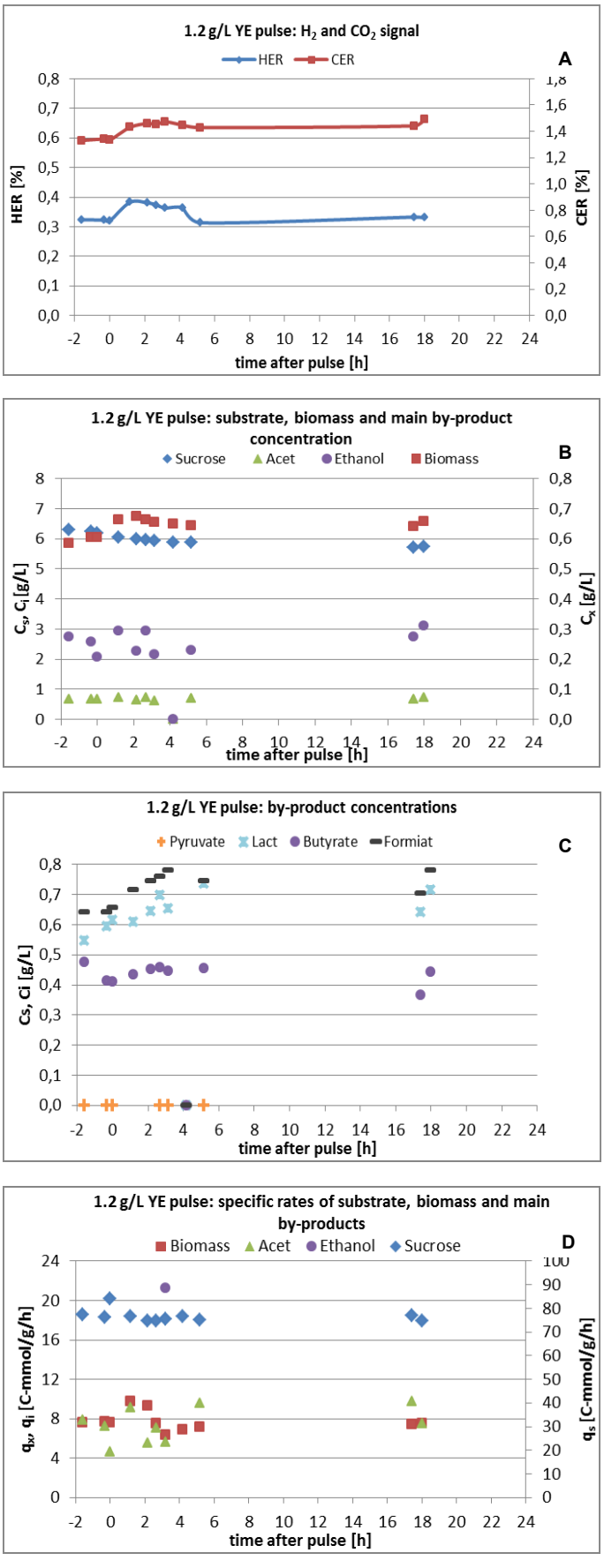

Figure 22 Pulse of 1.2 g/L YE in continuous mode at D=0.2 h^{-1} in standard medium with 12 g/L sucrose and 2 g/L YE in feed: On-line data (A), off-line data (B, C) and specific rates (D).

5.4.3.2 Pulse of tryptone/peptone ex casein (TP)

One of the other pulses carried out at D=0.2 h^{-1} was the one containing the complex compound tryptone/peptone ex casein (TP). Therewith we wanted to test another source for the limiting components in the fermenter broth, to see if the metabolic shift of *Fritz* toward biomass production was higher than with YE, the reference complex compound.

Figure 23A to Figure 23C shows different on-line and off-line data. Regarding on-line data (Figure 23A), the response of *Fritz* to the TP-pulse was higher than to YE-pulse, as higher CER and more significantly higher HER were recorded. Nevertheless, no significant change in biomass concentration (Figure 23B) was observed. Furthermore, q_x increased abruptly at the beginning of the pulse, what correlated with the decreased in q_s at that point (Figure 23D).

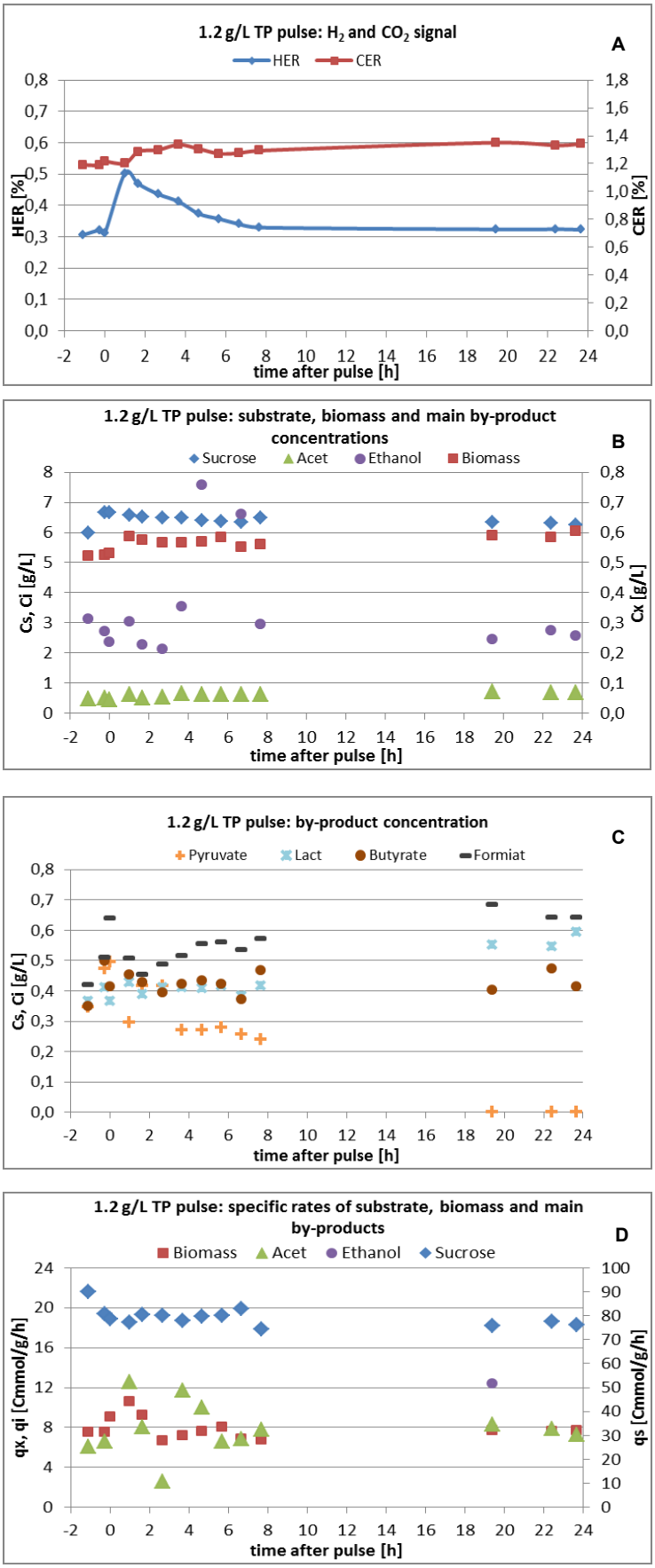

Figure 23 Pulse of 1.2 g/L TP in continuous mode at D=0.2 h-1 in standard medium with 12 g/L sucrose and 2 g/L YE in feed: On-line data (A), off-line data (B, C) and specific rates (D).

5.4.3.3 Pulse of peptone from meat (PM)

The last pulse done in continuous culture at D=0.2 h^{-1} was with peptone from meat pepsin-digested (PM). The use of this complex compound ended up in a higher metabolic response towards H_2 and CO_2 production (Figure 24A). Furthermore, a higher significant biomass increase was detected, which lead in a higher substrate uptake (Figure 24B). On the other hand, no significant increase on q_x and decrease on q_s in respect with the other pulsed components were observed (Figure 24D).

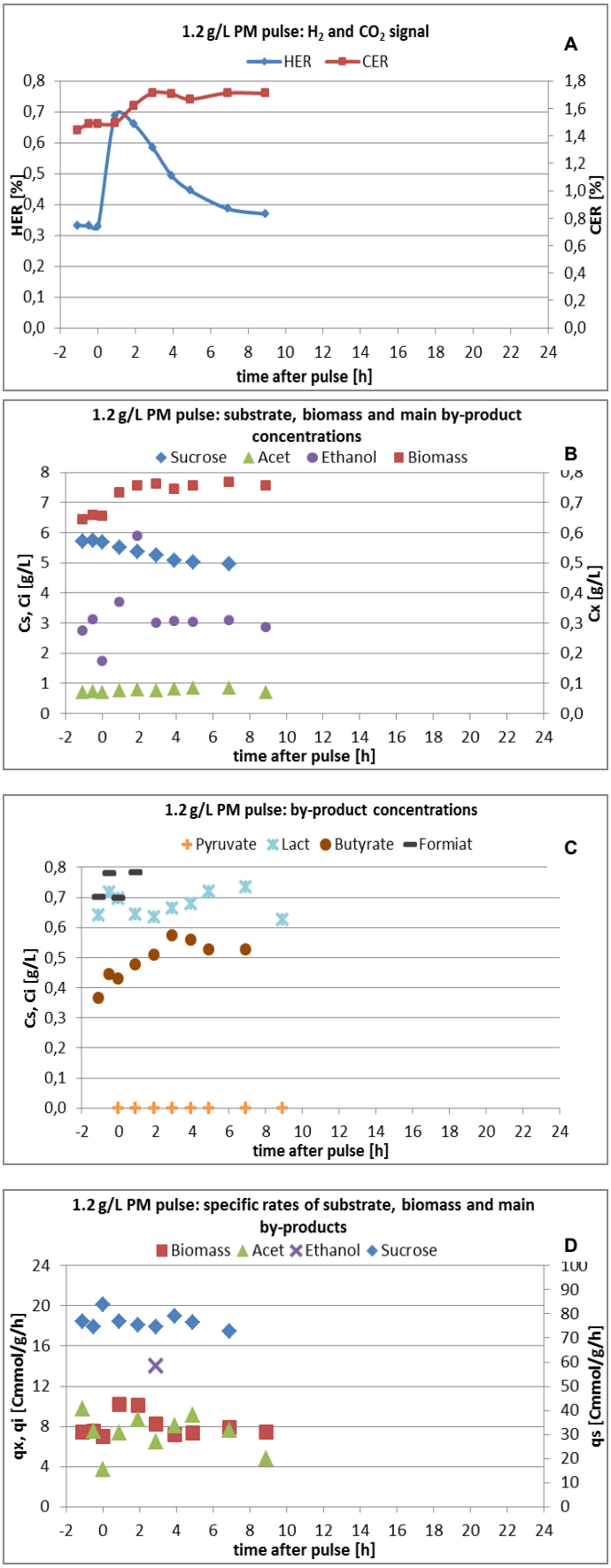

Figure 24 Pulse of 1.2 g/L PM in continuous mode at D=0.2 h^{-1} in standard medium with 12 g/L sucrose and 2 g/L YE in feed: On-line data (A), off-line data (B, C) and specific rates (D).

Comparing *Fritz* biomass productivity, our product of interest, between the three pulses, a higher response was observed if pulsing PM into the cultivation medium (Figure 25).

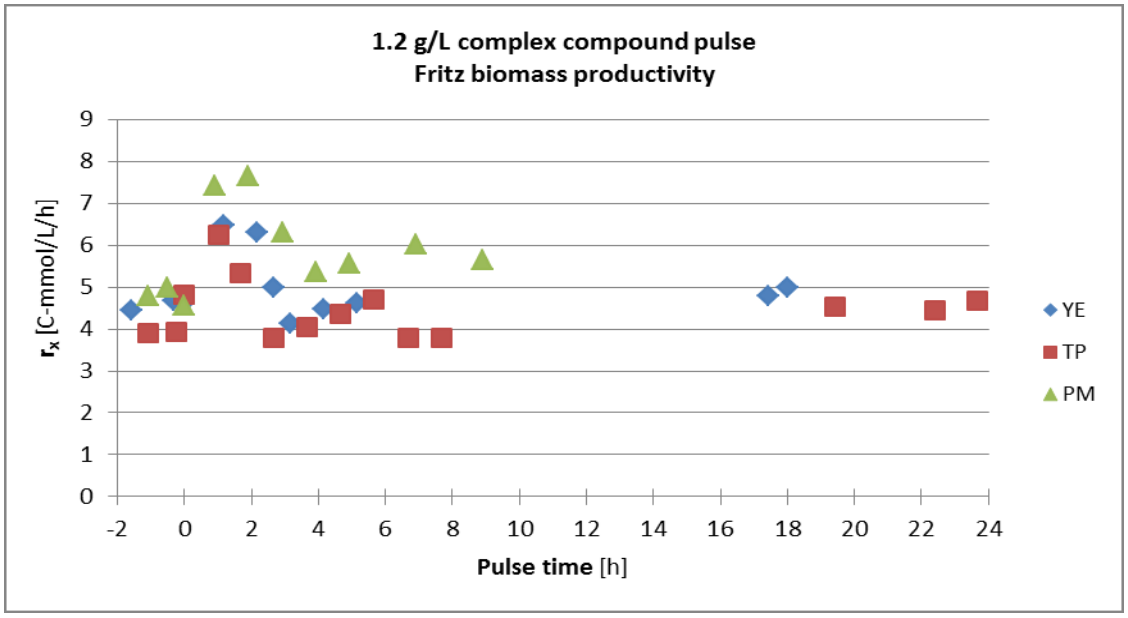

Figure 25 Biomass productivity of Fritz in a response of the cellular metabolism to a pulse 1.2 g/L of YE, TP or PM during a continuous culture at D=0.2 h^{-1}.

5.4.4 Conclusion

- The use of sucrose as substrate in a continuous culture in complex medium with *Fritz* ended up in higher cellular activity responses (CER) than the use of maltose for the same purpose.
- Higher dilution rates ended up in more stable growth conditions.
- Metabolic shifts due to a 1.2 g/L YE-pulse could be better quantified at D=0.1 h^{-1} than at D=0.2 h^{-1}.
- A pulse of a complex compound involved higher biomass concentrations and substrate uptake, as some limiting components of the fermenter broth were present in the complex source.
- Peptone from meat (PM) resulted in the highest significant CER and HER signal, biomass increase and substrate uptake.
- No difference in q_x and q_s could be determined between TP, YE and PM.
- Higher r_x was achieved if 1.2 g/L PM was pulsed into the fermenter broth of a Fritz culture on continuous mode at D=0.2 h^{-1}.

5.5 Screening in standard medium with maltose or sucrose at different initial NH_4^+ and PO_4^{3-} concentrations

With the aim of reducing operating costs and preserving the environment, lower buffer concentrations required for *Fritz* growth without N-P-limiting conditions were tested.

5.5.1 Results

The following screening allowed us to study the growth of *Fritz* at different initial NH_4^+ and PO_4^{3-} concentrations. The substrates chosen for the screening were the disaccharides maltose and sucrose. Firstly, the NH_4^+ and PO_4^{3-} concentrations in the fermenter broth of a *Fritz* culture grown in 12 g/L sucrose at a pseudo-steady state of D=0.1 h^{-1} were measured (Table 6). Therewith the amounts of NH_4^+ and PO_4^{3-} required for cellular growth were calculated.

Table 6 NH_4^+ and PO_4^{3-} concentrations in a Fritz culture grown in 12 g/L sucrose at a pseudo-steady state of D=0.1 h^{-1}.

	Broth		Used	
	[mM]	[%]	[mM]	[%]
PO_4^{3-}	5.91±0.39	[77.7, 86.9]	1.27±0.39	[22.3, 13.1]
NH_4^+	11.71±0.89	[78.5, 90.3]	2.16±0.89	[21.5, 9.7]

Considering the NH_4^+ and PO_4^{3-} percentages-Used by the cells (Table 6), the initial preselected concentrations of those components with respect to the standard complex medium (100%) were 50%, 25%, 15% and 5% (Table 7).

Table 7 Initial NH_4^+ and PO_4^{3-} concentrations for N-P-Screening.

		100%	50%	25%	15%	5%
$(NH_4)H_2PO_4$	[g/L]	0.313	0.156	0.078	0.047	0.016
$(NH_4)_2HPO_4$	[g/L]	0.521	0.260	0.130	0.078	0.026
PO_4^{3-}	[mM]	6.66	3.33	1.67	1.00	0.33
NH_4^+	[mM]	10.60	5.30	2.65	1.59	0.53

Comparing Table 6 and Table 7 it could be settled that no limitation was expected at initial N-P-concentrations higher than 25% with respect to the standard ones. Meanwhile, a possible limitation could be considered at 15% and more probably at 5%.

To carry on the N-P-screening, standard basal media with the corresponding initial N-P-concentrations were prepared. The selected substrates were added prior to inoculation. To avoid long lag-phases, the inoculum used for each of the cultures, and provided as a cryoculture, was previously cultivated in the same corresponding substrate at 5 g/L.

On the one hand, the effect of cultivating *Fritz* in maltose at different initial N-P-concentrations (Figure 26) could not be clearly identified. This fact could be explained considering side reactions of the components present in the medium, and/or a higher instability of the system if using maltose as main carbon and energy source.

On the other hand, there was a remarkable difference in *Fritz* growth if cultivating it on 5 g/L sucrose at initial NH_4^+ and PO_4^{3-} concentrations lower than 15% with respect to the standard ones (Figure 27). Using an N-P-reduction up to 5% involved not only a lower OD_{580}, and therefore a lower biomass concentration of *Fritz* in the culture, but also a lower maximal growth rate (μ_{max}) of the strain. That correlated with an N- and/or P-limitation in the culture at initial NH_4^+ and PO_4^{3-} concentrations of 0.53 mM and 0.33 mM, respectively.

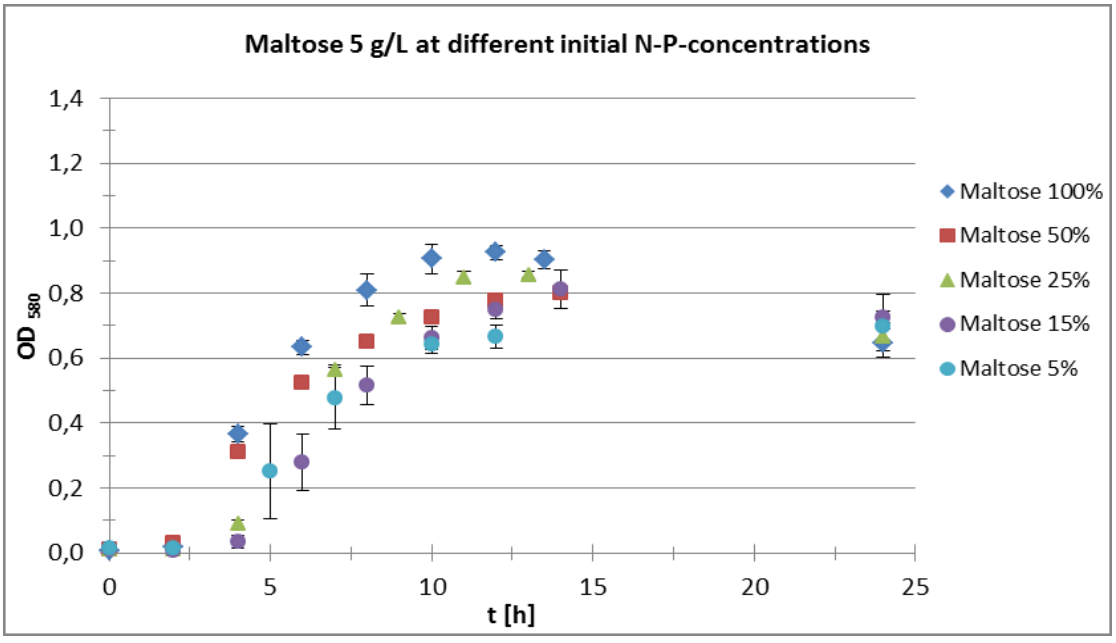

Figure 26 Screening with different initial N-P-concentrations at an initial concentration of 5 g/L maltose.

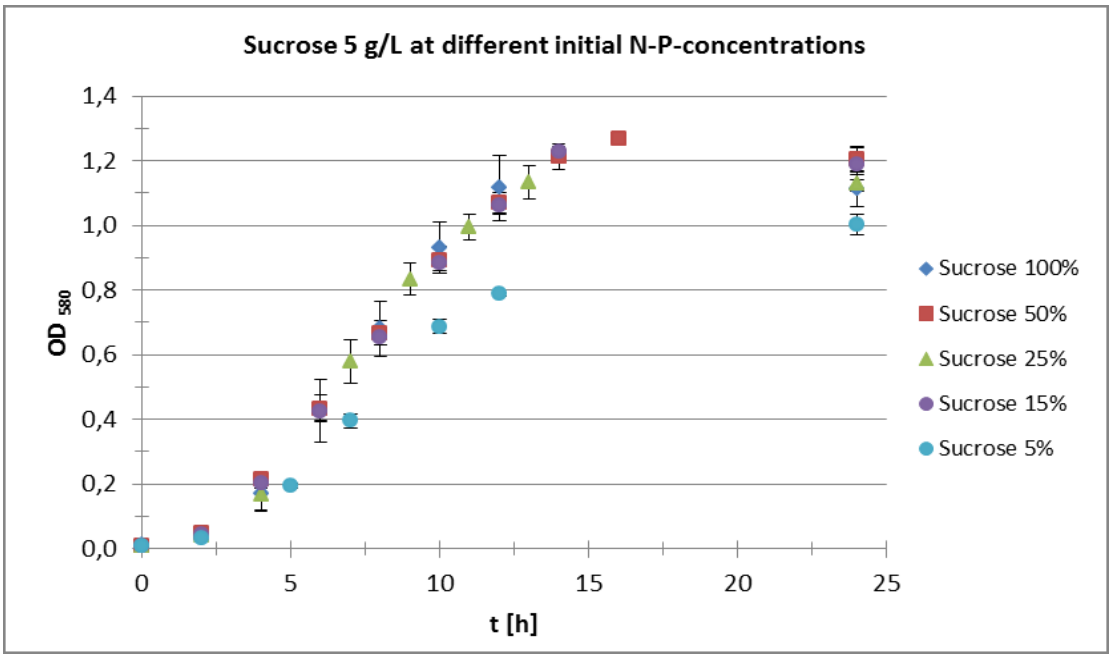

Figure 27 Screening with different initial N-P-concentrations at an initial concentration of 5 g/L sucrose.

5.5.2 Conclusion

- No N-P-limitation could be determined if *Fritz* was grown at 5 g/L maltose.
- Lower biomass concentrations and lower maximal growth rate of *Fritz* on sucrose at 5 g/L was recorded at initial NH_4^+ and PO_4^{3-} concentrations of 0.53 mM and 0.33 mM, respectively.
- Reducing the initial NH_4^+ and PO_4^{3-} concentrations up to 1.59 mM and 1.00 mM respectively, did not induce N-P-limitation in *Fritz* growth on sucrose at 5 g/L.

5.6 Screening in standard basal medium with maltose or sucrose using different complex compounds

Another attempt to increase *Fritz* biomass productivities while reducing operation costs was to look for new complex compounds to substitute the prescribed YE. PM resulted to be the preselected one if considering the results from previous continuous cultures in the reference basal medium, but new experiments were done in close batches in serum flasks to compare all the complex components under the same circumstances.

5.6.1 Screening with different complex compounds autoclaved within BM

This screening allowed us to study the growth of *Fritz* in different complex compounds at an initial concentration of 2 g/L. The substrates chosen for the screening were the disaccharides maltose and sucrose. The complex compounds selected for the screenings in the standard basal medium were:

- Complex compounds:
    - Yeast extract (YE)
    - Trypton/Peptone ex casein (TP)
    - Peptone from meat pepsin-digested (PM)
    - Peptone from soymeal papin-digested (PSM)
    - Soy peptone (SP)
    - Tryptic soy broth (TS)

- Yeast nitrogen base without amino acids and ammonium sulphate (YNB)

To avoid long lag-phases, the inoculum used for each of the cultures, and provided as a cryoculture, was previously cultivated in the same corresponding substrate at 5 g/L.

Firstly, the six mentioned complex compounds at an initial concentration of 2 g/L were tested separately. Therefore, for each screening, a complex compound was autoclaved within the reference basal medium. Previous inoculation, the corresponding substrate (maltose or sucrose) was added up to 5 g/L initial concentration. The maximal OD_{580}, and therefore maximal biomass concentration, was obtained working with YE, PSM and SP if using maltose (Figure 28) as main carbon and energy source (OD_{580} = [0.8, 0.9]). Working with sucrose (Figure 29) instead of maltose provided higher maximal OD values (OD_{580} = [0.9, 1.2]) if using YE or SP as complex compound in the medium.

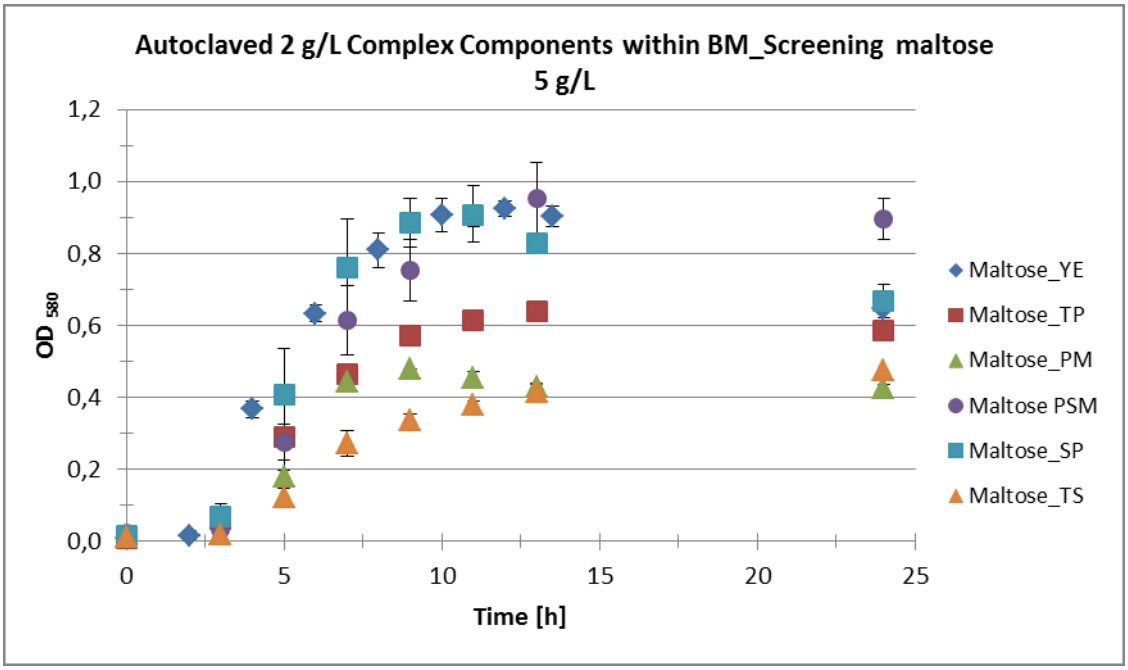

Figure 28 Screening in maltose at 5 g/L with different complex compounds at an initial concentration of 2 g/L. Complex compound autoclaved within the standard basal medium.

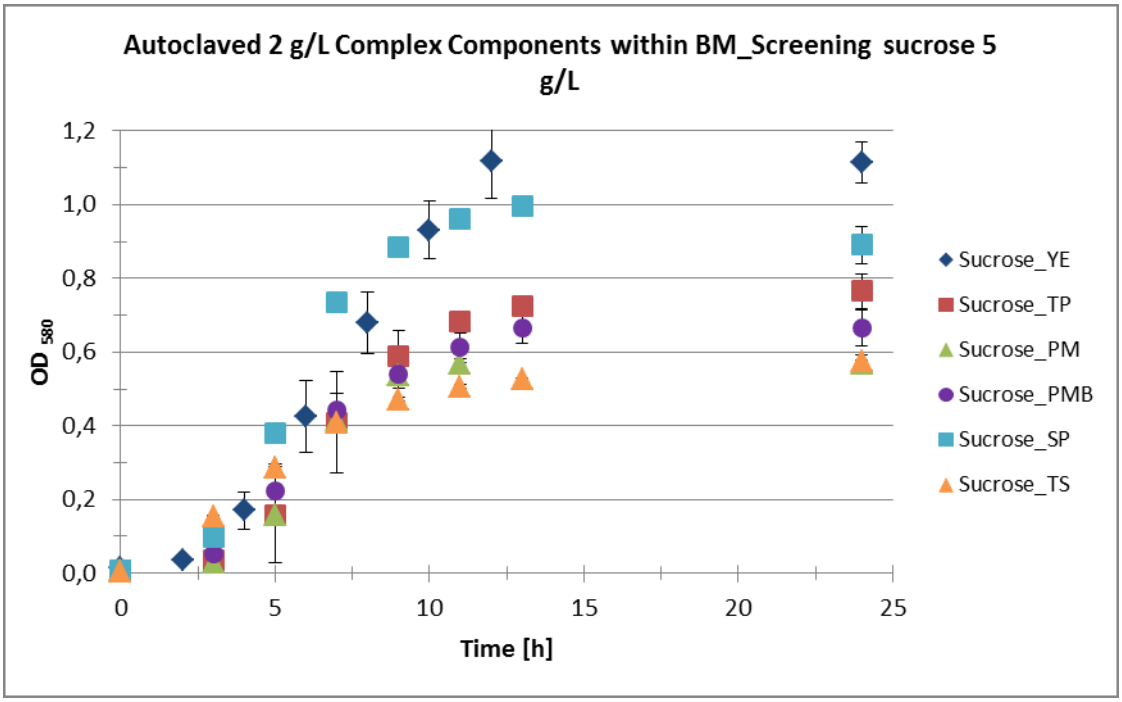

Figure 29 Screening in sucrose at 5 g/L with different complex compounds at an initial concentration of 2 g/L. Complex compound autoclaved within the standard basal medium.

To determine if the lower biomass production using maltose instead of sucrose was due to a substrate limitation at the highest OD values, HPLC analyses were carried out (Table 8 and Table 9). As shown in Table 8 there were no C-limiting conditions when *Fritz* growth slowed if using maltose as main carbon and energy source. On the contrary, growing *Fritz* on sucrose involved a higher substrate uptake up to the stationary

phase if using YE, TP or SP as complex compound (Table 9). The substrate consumption is higher, the higher the biomass concentration is at the beginning of the stationary phase. Nevertheless, the fact that no C-limiting conditions were achieved in every screening means that essential components in *Fritz* growth were present in significantly different amounts in each of the complex compounds.

Table 8 HPLC results at the highest OD580 values after growing Fritz in maltose at 5 g/L with different complex compounds at an initial concentration of 2 g/L. Complex compound autoclaved within the standard medium.

	Maltose					
	YE	**TP**	**PM**	**SP**	**PSM**	**TS**
	P5	P6	P4	P4	P6	P6
	Concentration [g/L]					
Substrate	0.36	1.72	4.27	1.45	1.36	2.93
Pyruvate	1.03	0.60	0.19	0.35	0.34	0.41
Lactate	2.02	4.94	6.23	1.40	4.44	4.56
Formate	0.85	0.47	0.35	0.55	0.71	0.23
Acetate	1.62	1.03	0.53	1.66	1.45	0.47
Ethanol	2.85	2.78	2.86	1.95	1.81	3.33
Butyrate	0.41	0.45	0.31	0.66	0.71	0.29

Table 9 HPLC results at the highest OD580 values after growing Fritz in sucrose at 5 g/L with different complex compounds at an initial concentration of 2 g/L. Complex compound autoclaved within the standard medium.

	Sucrose					
	YE	**TP**	**PM**	**SP**	**PSM**	**TS**
	P6	P6	P5	P6	P6	P6
	Concentration [g/L]					
Substrate	0.47	0.69	3.10	0.72	2.66	2.86
Pyruvate	0.49	0.84	0.39	0.53	0.40	0.44
Lactate	4.22	4.59	4.53	4.82	4.35	4.44
Formate	0.71	0.50	0.34	0.61	0.46	0.42
Acetate	1.39	1.13	0.34	1.81	0.73	0.77
Ethanol	2.18	3.10	2.67	3.69	1.94	4.18
Butyrate	0.45	0.50	0.13	0.81	0.43	0.43

The highest OD_{580} values in sucrose using YE or SP (Figure 29) correlated with the lowest residual substrate concentrations in the medium (Table 9), what did not entirely match if maltose was used in the cultivation medium (Figure 28, Table 8). Nevertheless, even when the growth in sucrose ended up in higher biomass concentrations, the maximal growth rate (μ_{max}) in maltose or sucrose using YE or SP was the same (Figure 30).

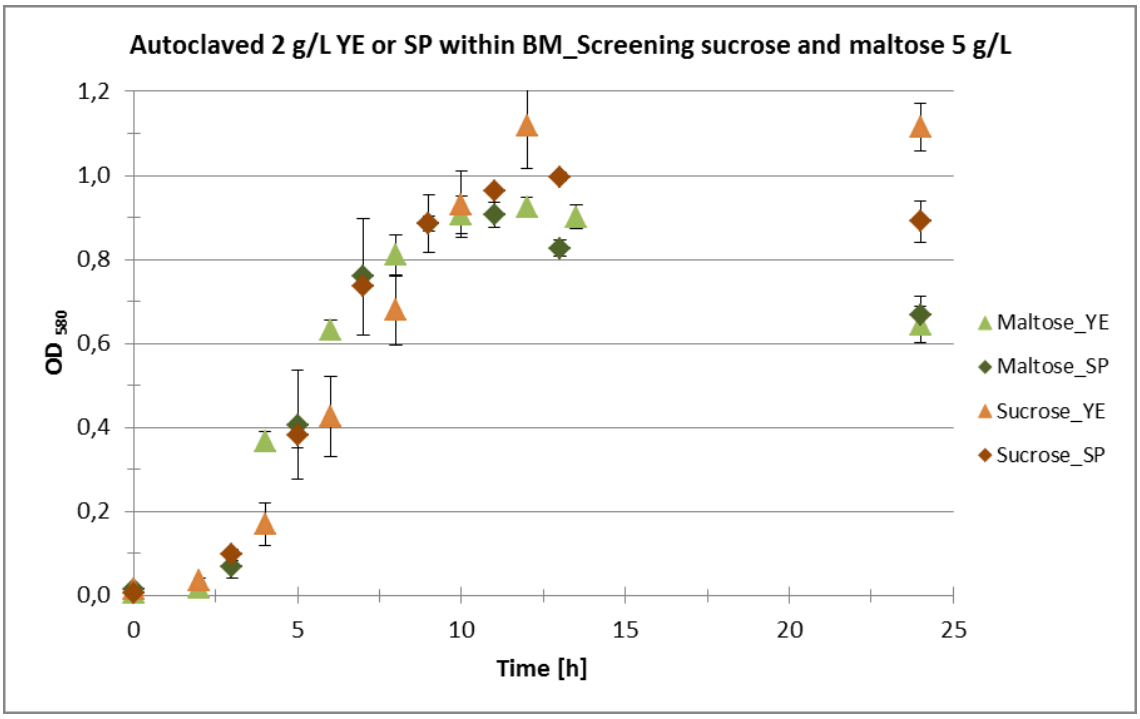

Figure 30 Screening in maltose or sucrose at 5 g/L with YE or SP at an initial concentration of 2 g/L. Complex compound autoclaved within the standard basal medium.

Between the preselected complex compounds (YE and SP) (Figure 30), it had to be considered if changing YE for SP could bring any advantage in the production. On one hand, from the economical point of view, there is just a slight different in price between both compounds (Table 10). On the other hand, as YE and SP allowed a maximal substrate uptake in close batches on sucrose (Table 9), it was necessary to work with *Fritz* on a continuous mode to see if higher biomass concentrations, and therewith higher biomass productivities, could be achieved, if changing SP for YE.

Table 10 Price comparison of complex compounds.

	Company best price	Nr.	Amount [g]	Price [€]	Price [€/100g]
Trypton/Peptone ex casein	Roth	8952.5	5000	346.15	6.9
Peptone from meat pepsin-digested	Roth	2366.4	2500	775.77	31.0
Soy peptone	Roth	2365.4	2500	134.25	5.4
Pepton from soymeal papin-digested	Roth	2832.3	1000	97.15	9.7
Tryptic soy broth	Fluka	22092	500	65	13.0
Yeast Extract	Roth	2363.5	5000	299.25	6.0
YNB	Sigma	Y1251	1000	1340	134.0

5.6.2 Screening with different complex compounds autoclaved separately from BM

The screening in section 5.6.1 allowed a better reproducibility and understanding of the effects of each complex compound on *Fritz* growth, as these compounds were autoclaved together with the basal medium (as it is done in the industrial plant).

Nevertheless, for the pulses done in a continuous culture (section 5.4.3), sterile anaerobic solutions of different complex compounds were autoclaved separately at high concentration (80 g/L) with the aim of pulsing them at each pseudo-steady-state at a final concentration of 1.2 g/L. To determine if the slightly responses to the pulses recorded during the continuous culture were not due to the wash-out and/or utilization of the pulsed compound by the cells, but to the effect of autoclaving a complex compound at such high concentrations (80 g/L), the following screening was done. The reference basal medium was autoclaved just with the corresponding inorganic salts, while anaerobic, separately autoclaved solutions of substrate and complex compound were added (were pulsed) before inoculation. The initial concentration of these complex compounds was 2 g/L, from a standard solution of 80 g/L. In this screening we just took three of the six mentioned complex compounds (YE, PM, TP); the ones pulsed in a previous fermentation (Figure 20). What we realized was that autoclaving the basal medium and the complex compound separately ended up in lower maximal OD_{580} values than if autoclaving them together (Figure 31 and Figure 32). This could be explained considering the effects of the Maillard reaction, which occurs in a larger extend at higher concentrations of sugars and organic nitrogen (both found in complex compounds).

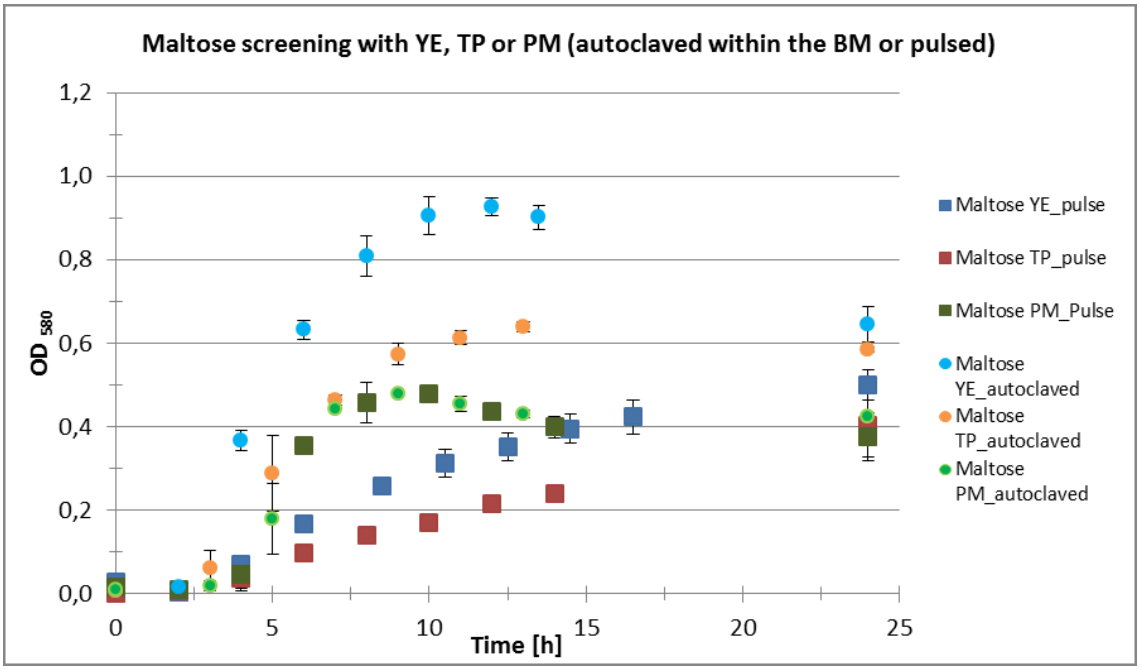

Figure 31 Screening in maltose at 5 g/L with YE, TP or PM at an initial concentration of 2 g/L. Complex compound autoclaved in an 80 g/L solution, separately from the standard medium and pulsed before inoculation.

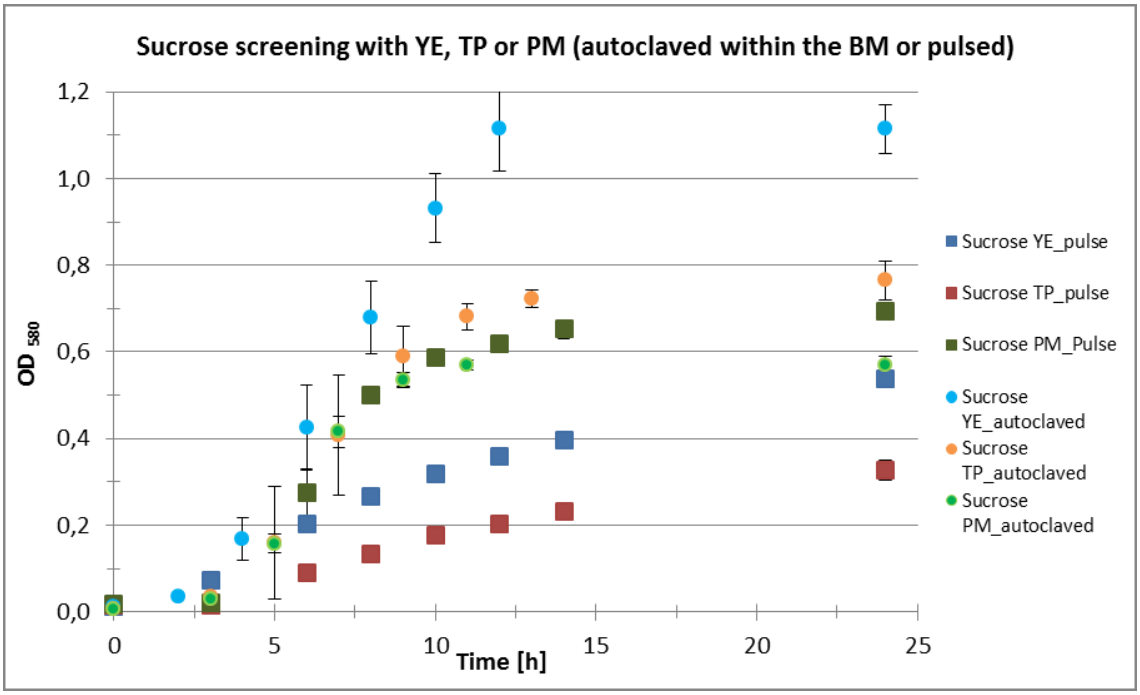

Figure 32 Screening in sucrose at 5 g/L with YE, TP or PM at an initial concentration of 2 g/L. Complex compound autoclaved in an 80 g/L solution, separately from the standard medium, and pulsed before inoculation.

Figure 31 and Figure 32 remark the importance of the reactions within the media components when heating them together at higher concentrations. Therefore, it could be stated, that the results observed for each of the pulses during the fermentation shown in Figure 20 could have been significantly higher if the pulsing complex compound would have been autoclaved separately at lower concentrations. The disadvantage of doing it like this, and the reason why we did not do it in this way from the beginning, was the high volume increase in the reactor if pulsing a solution at low concentrations. Another possibility to avoid this problem would have been to pulse an 80 g/L filtered solution; but that could have assured sterility, not anaerobic conditions.

Another aspect to stand up for this idea, lower growth if pulsing a high concentrated autoclaved complex compound solution, is shown in Table 11. No C-limiting conditions were achieved in neither of the substrates used for the different screenings, even when using YE.

Table 11 HPLC results at the highest OD580 values after growing Fritz in maltose or sucrose at 5 g/L with different complex compounds at an initial concentration of 2 g/L. Complex compound autoclaved separately from the standard medium at 80 g/L and pulsed before.

	Maltose			Sucrose		
	YE	**TP**	**PM**	**YE**	**TP**	**PM**
	P8	P7	P5	P6	P6	P6
	Concentration [g/L]					
Substrate	2.74	3.62	3.03	3.25	4.44	2.57
Pyruvate	0.35	0.32	0.47	0.26	0.17	0.25
Lactate	6.38	4.85	4.71	5.00	4.36	4.50
Formate	0.42	0.17	0.37	0.29	0.13	0.35
Acetate	0.75	0.30	0.65	0.52	0.34	0.47
Ethanol	2.18	1.10	1.01	2.42	2.55	2.67
Butyrate	0.24	0.09	0.24	0.20	0.17	0.24

5.6.3 Screening with yeast nitrogen based without amino acids and ammonium sulphate

As described previously, the presence of a complex compound in the standard medium was necessary to ensure *Fritz*'s growth (5.3). With the aim of studying if the negative effects in the bacterial growth due to a substitution of the YE for a vitamin and/or trace element solution was due to the lack of an essential component present in the YE or due to the elimination of the organic nitrogen (amino acids), a new screening with 2 g/L yeast nitrogen based without amino acids and ammonium sulphate (YNB) was carried out.

For this screening two methodologies were followed. Firstly, the YNB was autoclaved within the BM, due to the better results got from other previously tested complex compounds. Secondly, due to a precipitation of the salts in the medium if autoclaving all components together, the YNB was autoclaved separately from the standard basal medium. Trying to solve the problem of autoclaving high concentrated solutions, not because the risk of a Maillard reaction (as there is no organic nitrogen present in YNB), but because of the risk of further precipitations, the YNB was sterile filtrated at an end concentration of 17 g/L. None of the both methodologies ended up in bacterial growth (Figure 33).

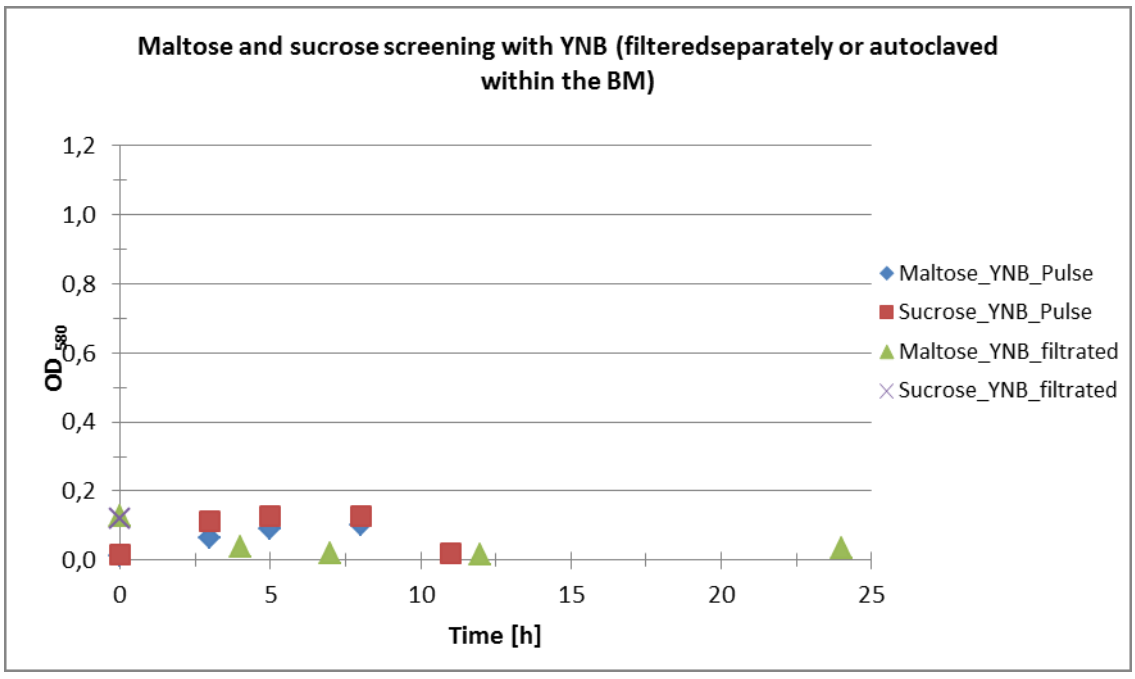

Figure 33 Screening in maltose or sucrose at 5 g/L with YNP at an initial concentration of 2 g/L. YNP autoclaved within the standard medium or filtrated separately at 17 g/L.

5.6.4 Conclusions

- Higher OD_{580} values, and therefore biomass concentrations, were achieved using YE, PSM or SP autoclaved within the basal medium, when cultivating *Fritz* in maltose.
- Higher OD_{580} values, and therefore biomass concentrations, were achieved using YE or SP autoclaved within the basal medium, when cultivating *Fritz* in sucrose.
- C-limiting conditions at the beginning of the stationary phase were found in YE with maltose, and in YE, TP and SP with sucrose; if autoclaving the complex compound within the basal medium. That means, using higher substrate concentrations could lead in higher biomass productivity.
- Autoclaving a stock solution of a complex compound at high concentrations involved lower biomass concentrations at the beginning of the stationary phase. Therefore, the complex compound should be autoclaved or filtrated at the desired concentration (2 g/L) within the standard basal medium, instead of being pulsed at a higher concentration up to the desired one.
- *Fritz* did neither grow in maltose nor in sucrose, if the standard basal medium was supplemented with YNB instead of a complex compound containing amino acids.

5.7 Continuous culture in alternative media with sucrose as main carbon and energy source

Considering the results from previous experiments with *Fritz* on closed batches in serum flasks and in a bioreactor on continuous mode, new attempts were done with a final purpose: to define the alternative medium composition and the operating conditions that ensure maximal *Fritz* biomass productivity.

5.7.1 Looking for substrate-limiting conditions

The aim of this experiment was to test at which substrate feeding concentration, on continuous mode at D= 0.2 h^{-1}, the growth of *Fritz* on sucrose was C-limited. This was an important working parameter regarding a higher stability and a reproducibility of the process.

From previous experiments in continuous mode at D=0.2 h^{-1} and non C-limiting conditions, it was known that *Fritz* was able to consume 6.0 g/L sucrose if feeding 12.7 g/L. Considering this result, the first feed medium to be tested in this experiment contained 6 g/L sucrose. Surprisingly, no C-limiting conditions were achieved at this substrate feeding concentration (Table 12). Therefore, the sucrose feeding concentration was reduced to 5 g/L, but no positive result in C-limitation was observed. As a result, the substrate feeding concentration in further runs was gradually decreased, always considering the substrate consumption from the previous run. Actually, no C-limiting conditions were achieved in any of the runs, even if the substrate feeding concentration was reduced up to 3.5 g/L (Table 12).

Table 12 Residual substrate concentration and substrate consumption at different sucrose feeding concentrations, growing Fritz on continuous mode at D=0.2 h-1.

Substrate	Feed [g/L]	Residual [g/L]	Consumption [g/L]
Feed 1	6.22	1.00	5.00
Feed 2	5.17	0.53	4.47
Feed 3	4.36	0.42	3.58
Feed 4	3.87	0.19	3.31

Considering different physiological parameters of *Fritz* related to biomass (Table 13), it could be stated that there was a clear increase in biomass concentration, and therefore biomass productivity, if the sucrose feeding concentration was reduced. That means that metabolic shifts in *Fritz* occurred, if the feeding conditions were changed, even when no substrate depletion in the cultivation broth was observed. Due to these not C-limiting conditions, the results presented are related to "pseudo-steady-states", calculated as steady-states after the theoretical five volume exchanges once the CO_2-signal was constant at least for one volume exchange.

Table 13 Physiological parameters related to biomass during a continuous culture of Fritz at D=0.2h^{-1} in sucrose at different feeding concentrations.

$C_{s,in}$	ΔC_s	OD_{580}	x	r_x	q_x	$Y_{x/s}$
[g/L]	[g/L]	[-]	[g/L]	[C-mmol/L/h]	[C-mmol/g/h]	[C-mol/C-mol]
6.33	5.35	0.87	0.48	3.59	7.50	0.10
5.17	4.64	0.94	0.52	3.87	7.50	0.12
4.40	3.97	1.08	0.59	4.42	7.50	0.16
3.89	3.62	1.11	0.61	4.55	7.50	0.18

With the aim to validate the results of Table 13, different sucrose feeding concentrations at the beginning of different experiments were tested (Table 14). The goal of these runs was to check if the biomass concentrations got higher values at lower sucrose concentrations because the strain got used to the cultivation conditions with time or because its metabolism was dependent of the feeding sucrose in that way.

Table 14 Physiological parameters related to biomass, growing Fritz on continuous mode at D=0.2h^{-1} in sucrose at different initial feeding concentrations of different experiments.

$C_{s,in}$	ΔC_s	OD_{580}	x	r_x	$Y_{x/s}$
[g/L]	[g/L]	[-]	[g/L]	[C-mmol/L/h]	[C-mol/C-mol]
12.70	6.04	0.96	0.53	3.94	0.09
6.33	5.35	0.87	0.48	3.51	0.10
3.54	3.09	0.74	0.41	3.06	0.14
3.21	2.66	0.71	0.39	2.96	0.16

Regarding Table 14, higher biomass concentrations and productivities were obtained at higher sucrose feeding concentrations, a tendency opposed to the previously described one (Table 13). The fact that the growth tendency of *Fritz* changed depending on the cultivation conditions generated the idea of a memory effect in *Fritz*. Nevertheless, low substrate feeding concentrations were favorable for the biomass yield $Y_{x/s}$, an important physiological parameter considering the economy of the whole process.

5.7.2 Reduction of NH_4^+ and PO_4^{3-} feeding concentrations

Considering previous results on sucrose at different NH_4^+ and PO_4^{3-} initial concentrations in serum flasks, *Fritz* growth could be ensured without N-P-limitation up to a maximal NH_4^+ and PO_4^{3-} reduction of 85% (percentages in respect with the reference basal medium).

Considering previous results (Table 13 and Table 14), the sucrose feeding concentration was settled at 3.5 g/L. After quantifying the pseudo-steady-state of the strain at 100% N-P (Table 15), the NH_4^+ and PO_4^{3-} feeding concentrations were reduced to 15%. In a continuous culture, a decrease of 85% in the N-P-feeding concentrations influenced negatively the culture. That was not only because of the strong pH fluctuations observed due to a lower buffer capacity in the cultivation medium (Figure 34), but also because of the volumetric biomass productivity decrease detected in the fermenter broth (Table 15). A 10% increase in the N-P-feeding concentrations involved an increase in the biomass concentration (Table 15) and other changes in the stoichiometry of the system (Figure 34). Regarding biomass concentration, productivity and yield, a final N-P-feeding concentration of 25% should be considered for further experiments.

Table 15 Physiological parameters related to biomass during a continuous culture of Fritz at $D=0.2h^{-1}$ in sucrose at different N-P-feeding concentrations.

$N\text{-}P_{in}$	$C_{s,in}$	ΔC_s	OD_{580}	x	r_x	$Y_{x/s}$
[%]	[g/L]	[g/L]	[-]	[g/L]	[C-mmol/L/h]	[C-mol/C-mol]
100	3.54	3.09	0.74	0.41	3.06	0.14
15	3.69	3.38	0.72	0.40	2.99	0.13
25	3.41	2.92	0.75	0.41	3.10	0.15

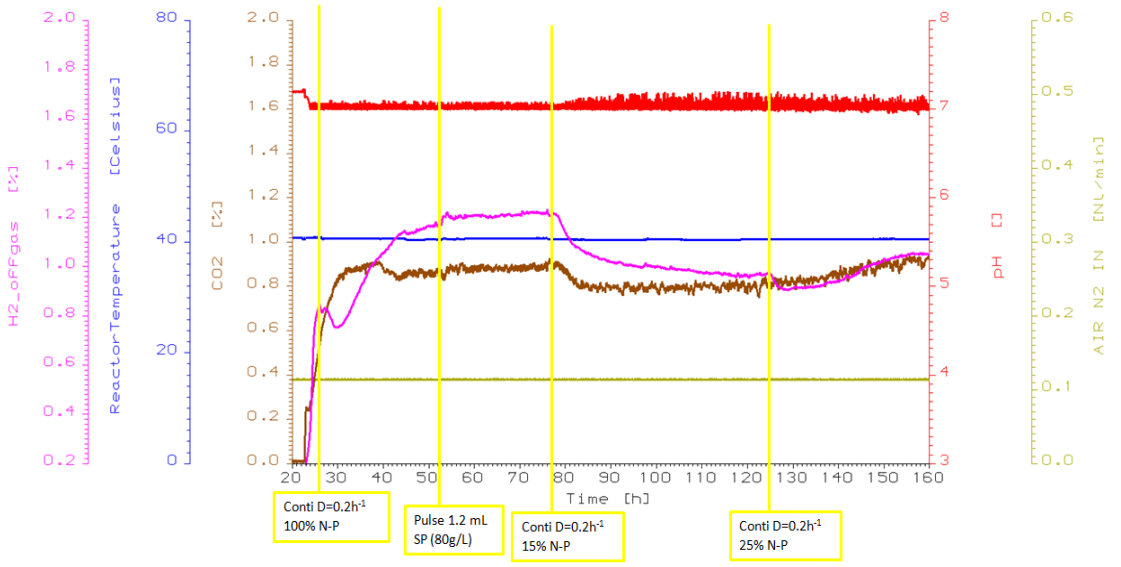

Figure 34 Continuous culture of Fritz on sucrose at 3.5 g/L feeding concentration.

5.7.3 Testing other complex compounds

As shown in Table 15, growing *Fritz* at 3.5 g/L sucrose and 100% NH_4^+ and PO_4^{3-} feeding concentrations did not end up in C-limiting conditions, even when the offgas signals were stable for a long period of time. This effect was related to the limitation of other component/s in the fermenter broth present in the YE considered in the medium composition. Therefore, and following the methodology of previous dynamic experiments, a pulse of soy peptone (SP) was done, in order to provide the limiting components in the culture and therewith to study possible metabolic shifts towards biomass production. It was decided on soy peptone (SP) because it yielded the second best result with sucrose as substrate (Figure 29).

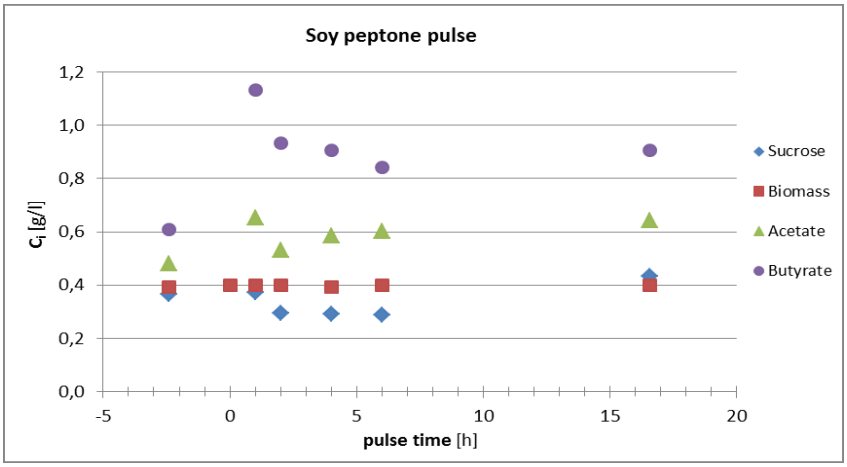

Figure 35 Response of Fritz to a soy peptone pulse during a continuous culture in 3.5 g/L sucrose and 100% N-P at D=0.2 h^{-1}.

As shown in Figure 35, the addition of a complex compound (SP) into the medium affected positively the culture in respect to substrate uptake, although no significant increase in biomass was recorded. Instead butyrate concentration increased. Furthermore, looking at Figure 36, the effects on biomass productivity were much higher if pulsing YE instead of SP. The effect of adding SP into the medium composition was also tested on continuous mode, by replacing the complex compound YE for SP at the same concentration (2 g/L). Nevertheless, no pseudo-steady-state could be established due to problems with the set-up.

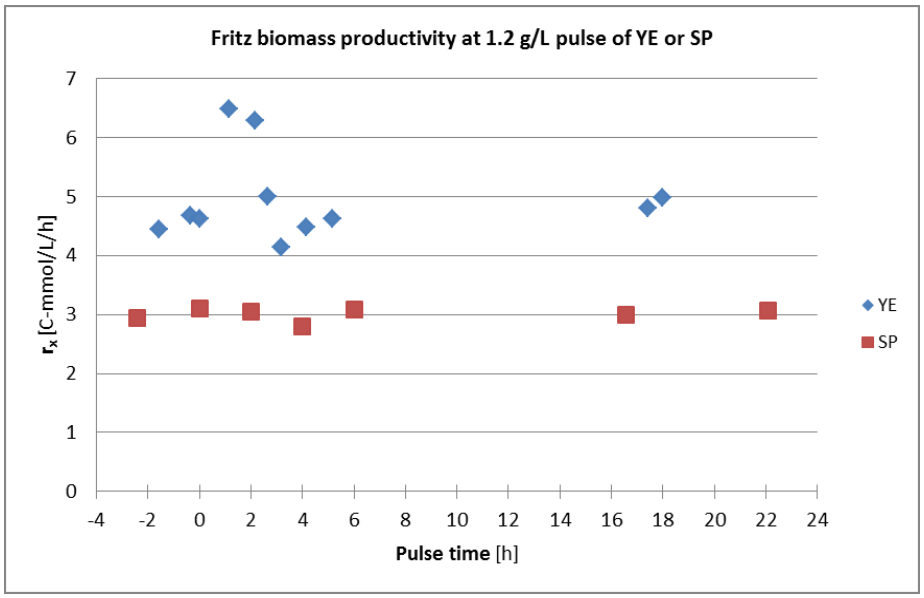

Figure 36 Biomass productivity of Fritz in a response of the cellular metabolism to a pulse 1.2 g/L of YE, or SP during a continuous culture at D=0.2 h^{-1}.

5.7.4 Conclusion

- Reducing sucrose feeding concentrations in *Fritz* cultures involved increasing biomass yields.

- Long-term cultures were favorable for biomass productivities and biomass yields, especially at low substrate concentrations.

- 0.079 g/L and 0.130 g/L $(NH_4)H_2PO_4$ and $(NH_4)_2HPO_4$, respectively, were necessary in the medium composition to ensure that a reduction of the NH_4^+ and PO_4^{3-} feeding concentrations did not impact negatively the culture.

- A soy peptone pulse into the culture affected positively the substrate uptake. Nevertheless, higher responses were previously observed if pulsing yeast extract.

6 Discussion

In this section, the metabolic shifts of *Fritz* due to changes in working parameters (e.g. substrate feeding concentration, dilution rate …) are discussed. The aim was to be able to tell by monitoring online signals like H_2 and CO_2, if the microorganism was currently in the solventogenesis or acidogenesis phase (Figure 1) and which phase was more favourable for biomass yield and productivity, the two key physiological parameters of our project.

Solventogenesis refers to the condition of the metabolism, where *Fritz* is producing more alcohol (ethanol). Additionally, it generally correlates with the production of lactate. Acidogenesis, on the other hand, refers to the condition, where *Fritz* is producing more organic acids (acetate and butyrate).

6.1 Metabolism of *Fritz*

6.1.1 Initial sucrose feeding concentration

The aim of this experiment (section 5.4 and 5.7) was to study the behaviour of the strain by changing only the initial sucrose feeding concentration in different runs from high sucrose concentrations (no carbon limiting conditions) to low sucrose concentrations (pseudo carbon-limiting conditions). These sucrose concentrations were per L: 12.7 g, 6.33 g, 3.54 g and 3.21 g. The N-P and complex compound concentrations were kept constant at 100% N-P and 2 g/L YE, respectively. (Note: process parameters like temperature, stirrer speed, pH and N_2 inflow rate were kept constant for all experiments).

By changing only one parameter at a time, we made sure that a change in the signals, and therefore in the metabolism, was attributed to the changing parameter and not to the whole system. The samples used to quantify the effects of a disruption in the system, in respect with a reference state, were taken in “pseudo-steady states”, when the CO_2 and H_2 signals were constant over at least one volume exchange (after the theoretical five volume exchanges).

As shown in Table 16, biomass concentration (c_x) and biomass productivity (r_x) as well as the specific substrate uptake rate (q_s) (Figure 41) were higher at higher initial sucrose feeding concentration (12.7 and 6.33 g/L) whereas the biomass yield ($Y_{x/s}$) (Figure 40D, Table 16) decreased. Thus, high initial sucrose feeding concentrations

affected the stoichiometry of the culture negatively towards biomass. This suggests a metabolic shift during low sucrose feeding concentrations towards our product of interest. Nevertheless, there is an explanation (next sections) regarding the redirection of the main energy and carbon source in the cases where no biomass was produced when expected.

Although no carbon limiting conditions were achieved in these experiments with any initial sucrose feeding concentration, the data show that cellular distribution of carbon and energy is dependent on this feeding substrate concentration. That would mean, high sucrose concentrations and sucrose uptake rates induce solventogenesis and low sucrose concentrations and uptake rates induce acidogenesis (Figure 37).

Table 16 Physiological parameters related to biomass during a continuous culture of Fritz at D=0.2h^{-1} with 100% N-P and 2 g/L YE in sucrose at different initial feeding concentrations.

$c_{s,in}$	Δc_s	r_s	q_s	c_x	r_x	q_x	$Y_{x/s}$
[g/L]	[g/L]	[C-mol/L/h]	[C-mol/g/h]	[g/L]	[C-mol/L/h]	[C-mmol/g/h]	[C-mol/C-mol]
12.7	6.04	42.35	80.66	0.53	3.94	7.50	0.09
6.33	5.59	39.20	84.07	0.47	3.50	7.50	0.09
3.54	3.09	21.69	53.16	0.41	3.06	7.50	0.14
3.21	2.66	18.68	47.39	0.39	2.96	7.50	0.16

Regarding the metabolites, there was a clear increase in hydrogen production when feeding with low initial sucrose concentrations (3.54 g/L and 3.21 g/L) compared to high initial sucrose feeding concentrations (12.7 and 6.33 g/L). The hydrogen yield and productivities increased with decreasing initial sucrose feeding concentration. The hydrogen evolution rate (HER) and the $Y_{H2/s}$ reached their respective maximum of 4.51 mmol/L/h and 0.21 mol/C-mol with 3.54 g/L sucrose, the second lowest sucrose feeding concentration tested (Figure 43).

Carbon dioxide production and yield showed similar trends as those for biomass when looking at Figure 40D and Figure 42B. Plotting the yield of carbon dioxide against the yield of biomass shows a linear correlation (Figure 38). Considering this correlation, it would be possible to study the expected trend of biomass growth via the CO_2 online signal at the process control.

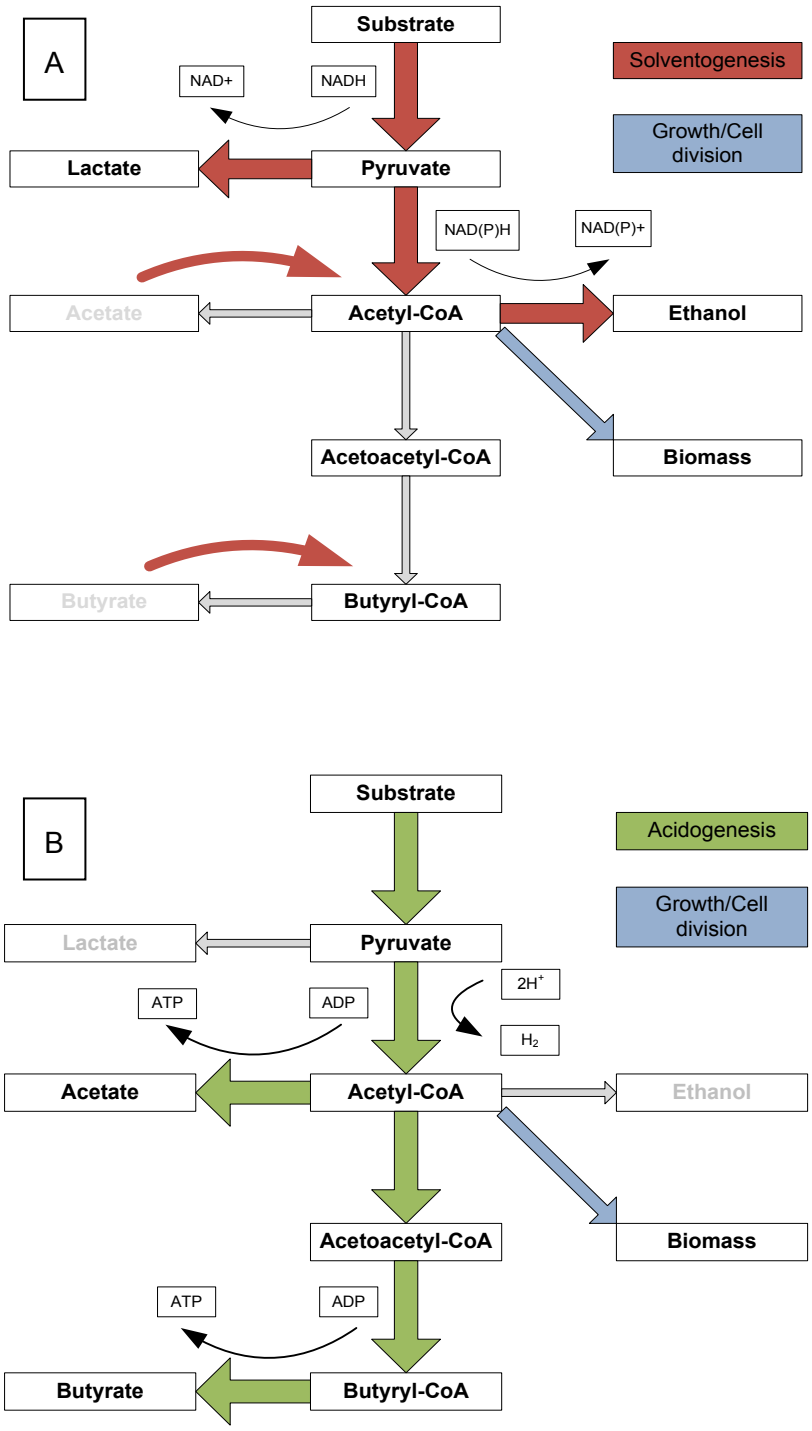

Figure 37 schematic flow diagram of Fritz with solventogenesis and acidogenesis at high sucrose concentrations (A) and at low sucrose concentration (B) at D=0.2 h^{-1}.

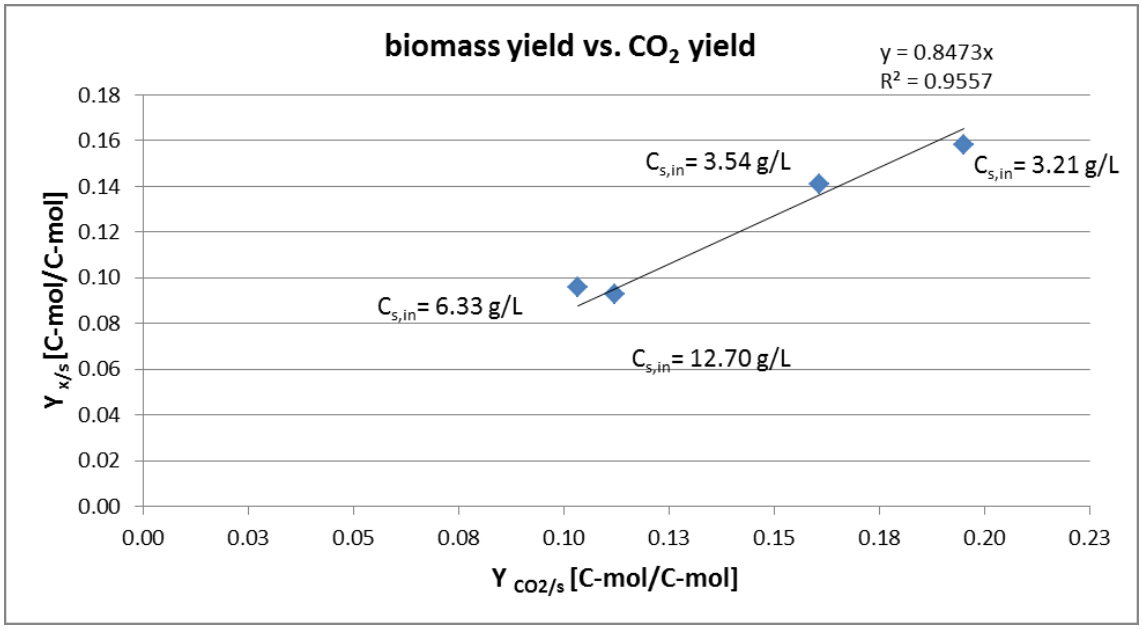

Figure 38 Comparison between biomass yield and carbon dioxide yield at D=0.2 h^{-1} with different initial sucrose concentrations showing high correlation.

Acetate productivity did not show a clear trend. The average value was 3.48±0.38 C-mmol/L/h. However, the lowest sucrose feeding concentration resulted in the highest acetate yield (0.2 C-mol/C-mol), compared to the yield of the highest initial sucrose feeding concentration (0.08 C-mol/C-mol) (Figure 44B). This fact correlates with a shift in the stoichiometry.

Further, butyrate productivities and yields were much higher at the lower feeding concentrations (Figure 45B). The average yield was 0.36±0.02 C-mol/C-mol at the lowest sucrose concentrations, compared to 0.12±0.02 C-mol/C-mol at the highest ones. These results implicate a stoichiometric shift towards butyrate at lowering sucrose feeding concentrations, as it happened with hydrogen (Figure 43) and acetate production (Figure 44).

A study in *C. butyricum* suggested that an increase in electron flow towards H_2 formation using the enzyme ferredoxin, results in an increase of acid production (acetate and butyrate) due to the necessity of reoxidating the NADH present in excess in the media at C-limiting conditions (8). This study also established that the electron flow determines the carbon flow within the strain. These results correlate with our observations, as H_2, acetate and butyrate production levels increased concomitantly at lower initial sucrose feeding concentrations and, therefore, at lower residual substrate concentrations .

Also, when looking at the total “acid yield” (acetate and butyrate) and the total “solvent yield” (ethanol and lactate) compared to the yield of H_2/CO_2 (Figure 39), it clearly

shows an increase of acid production when more hydrogen was produced at lower initial sucrose feeding concentrations. Conversely, a decrease of solvent production was observed. Therefore, the impacts of changing the working parameters in *Fritz* cultivations on acetate and butyrate production might be followed monitoring the H_2 signal of a continuous process. It is important to note, that lactate is not a solvent. For the sake of the argument and for the fact, that lactate is produced in higher amounts during solventogenesis, ethanol and lactate are summarized as “solvent yield”. Lactate is produced in higher amount during solventogenesis because it represents another pathway for NADH reoxidation in *Fritz* metabolism.

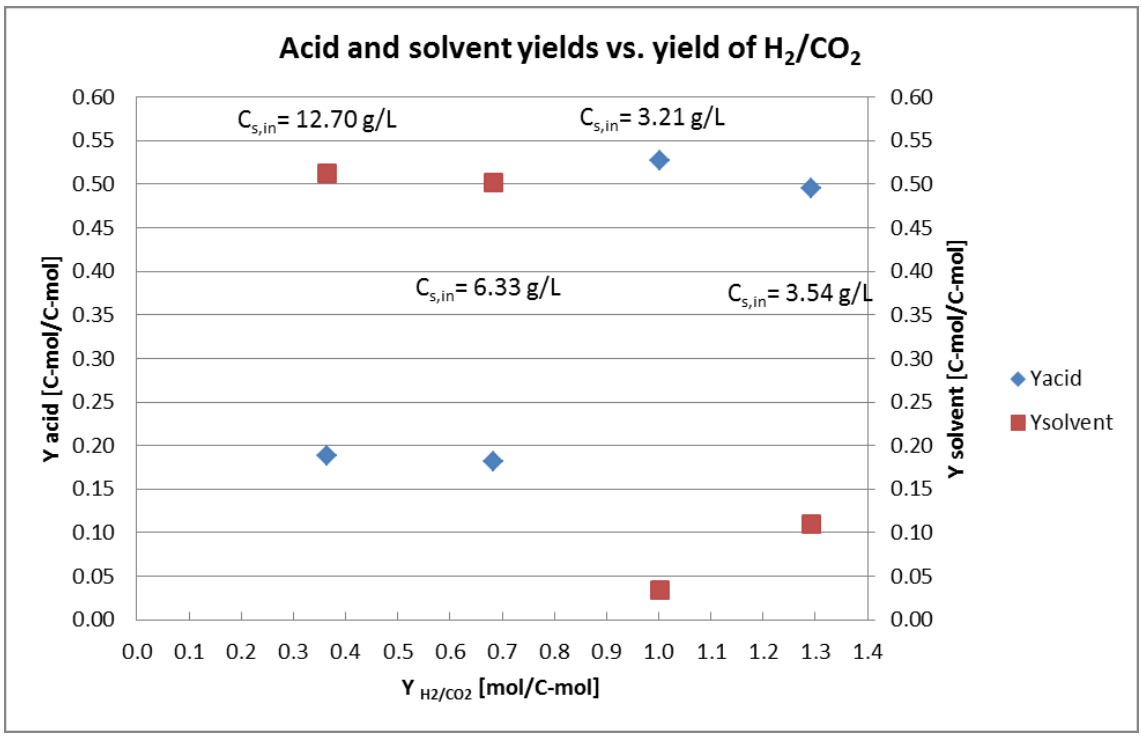

Figure 39 Comparison between the acid yield (acetate and butyrate) and the solvent yield (ethanol and lactate) to the yield of H2/CO2 at different initial sucrose feeding concentrations and D=0.2 h^{-1}.

Moreover, ethanol and lactate production rates and yields resulted in significantly higher values at the highest initial sucrose feeding concentration (Figure 46, Figure 47), corresponding to the highest residual substrate concentration. The yield of ethanol at 6.33 g/L initial sucrose feeding concentration was lower than at 12.70 g/L, resulting 0.37±0.03 and 0.45±0.03 C-mol/C-mol respectively. But the carbon balance at 6.33 g/L did not close perfectly, presumably due to errors in the measurements.. Nevertheless, these errors did not change the overall trend in the shift if comparing high and low initial sucrose feeding concentrations.

In an older study on continuous mode with the strain *C. acetobutylicum* on glucose, an increase in the ethanol, butanol and acetone yield under non-carbon limiting conditions (130 mM glucose compared to 44 mM glucose at limiting conditions) was observed, from 1.8, 2.0, 0.54 to 3.1, 36.6 and 20 mol/mol, respectively (12). Furthermore, the study showed that an imbalance of the NADH/NAD^+ pool towards NADH induced solvent production, as no H_2 formation took place. Solvent production requires high amounts of ATP. With non-carbon limiting conditions at high initial substrate concentrations, enough ATP and NADH can be generated alone through the glycolysis pathway. Therefore, the demand for additional ATP via acetate/butyrate production is low, resulting in low production of both organic acids. The high amount of NADH is reoxidized to NAD^+ over the ethanol and butanol pathways. This theory explains the high ethanol and butanol concentrations got at high residual substrate concentrations in this study (12). However, these statements change when substrate is the limiting component in the culture. Solvent production decreases and the required ATP is then additionally generated via the acetate and butyrate pathways, which resulted in an increase of productivity and yield for both metabolites (12). Our results correlated with these observations. Looking at the yields and productivities of our experiments (Figure 40-47), more substrate was used for acetate, butyrate and H_2 production if feeding at low initial substrate concentrations (3.54 and 3.21 g/L). These sucrose concentrations did not end up in C-limiting conditions in the fermenter broth, but they correlated with very low residual sucrose concentrations (0.45 and 0.55 g/L). On the other hand, more substrate was redirected to ethanol and lactate production at the highest initial sucrose feeding concentrations tested (Figure 46A, Figure 47A). This means, that there is a clear shift in *Fritz* metabolism from solventogenesis towards acidogenesis, if changing from high to low sucrose feeding concentrations.

Interestingly the lactate yield and productivity (Figure 47) were significantly higher at 6.33 g/L than at 12.70 g/L but it could not be established why the discrepancy was so high. However, both values were much higher compared to the lowest initial sucrose feeding concentrations and align the observed trend. At high substrate concentrations reoxidation of NADH through ethanol alone is not enough and additional reoxidation over lactate is activated. Furthermore, a study found out that high concentrations of fructose-1,6-biphosphate, an intermediate of the glycolysis pathway, induces the formation of lactate (15). This finding matches well with our observations as we observed high lactate concentrations and yield at high initial sucrose feeding concentrations (Figure 47).

Considering the observations from the mentioned studies and our own data, the main hypothesis is that *Fritz* is able to adjust the ratio between acid (acetate and butyrate)

and solvent production (ethanol and lactate) (Figure 39) according to the state of the NADH/ NAD^+ pool at almost carbon limiting (3.54 and 3.21 g/L) and non-carbon limiting conditions (12.70 and 6.33 g/L) on continuous mode (D=0.2 h^1) in order to redirect the stoichiometry of its metabolism towards biomass production under these conditions.

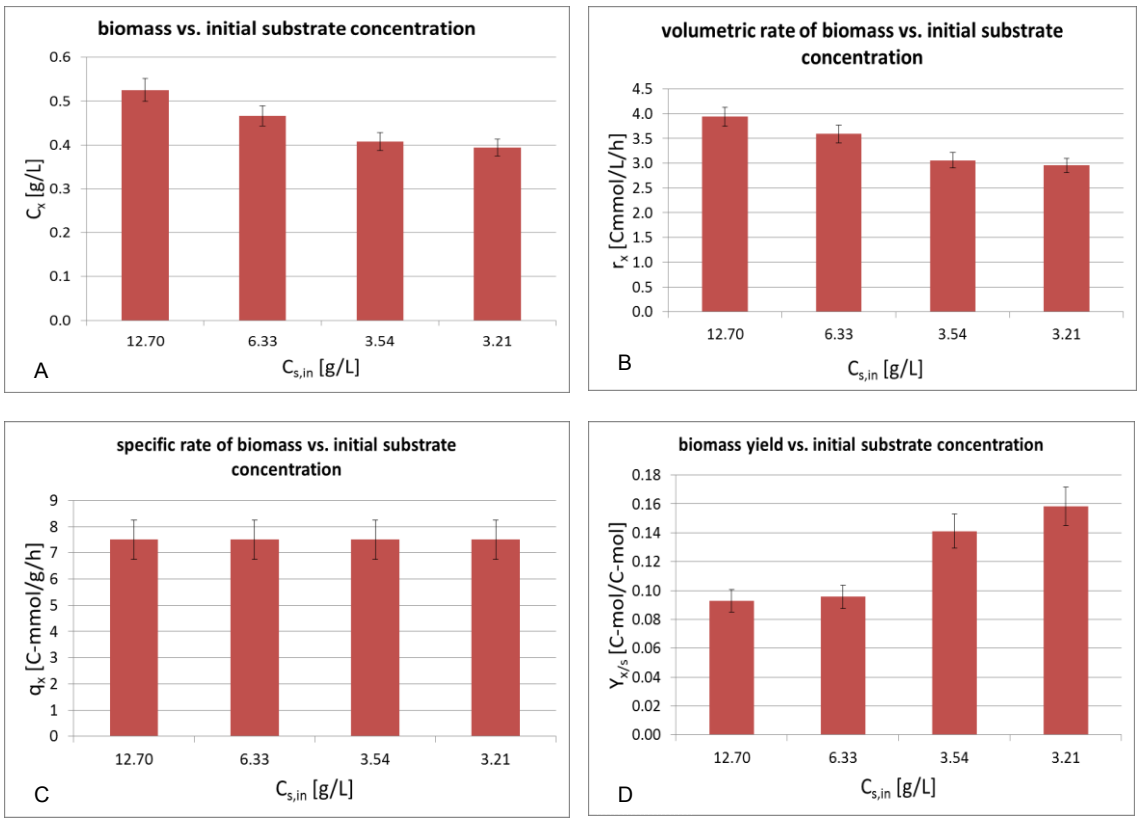

Figure 40 Physiological parameters related to biomass at D=0.2 h^{-1} with different initial sucrose feeding concentration of 12.7, 6.33, 3.54 and 3.21 g/L.

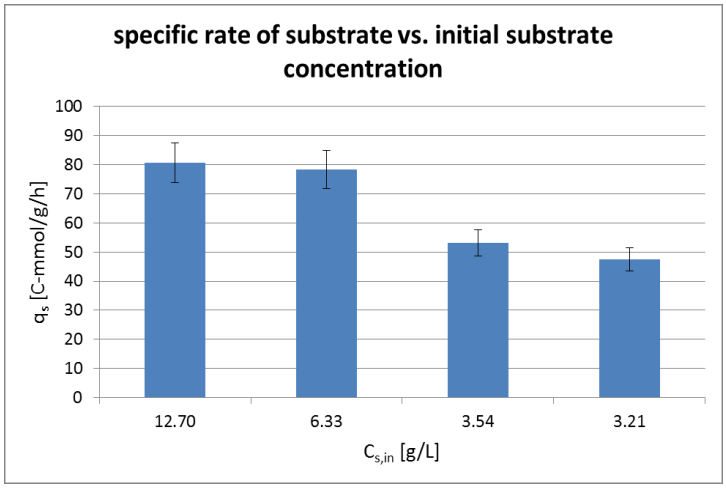

Figure 41 Consumption, volumetric rate and specific uptake rate of sucrose at D=0.2 h^{-1} with different initial sucrose concentrations of 12.7, 6.33, 3.54 and 3.21 g/L.

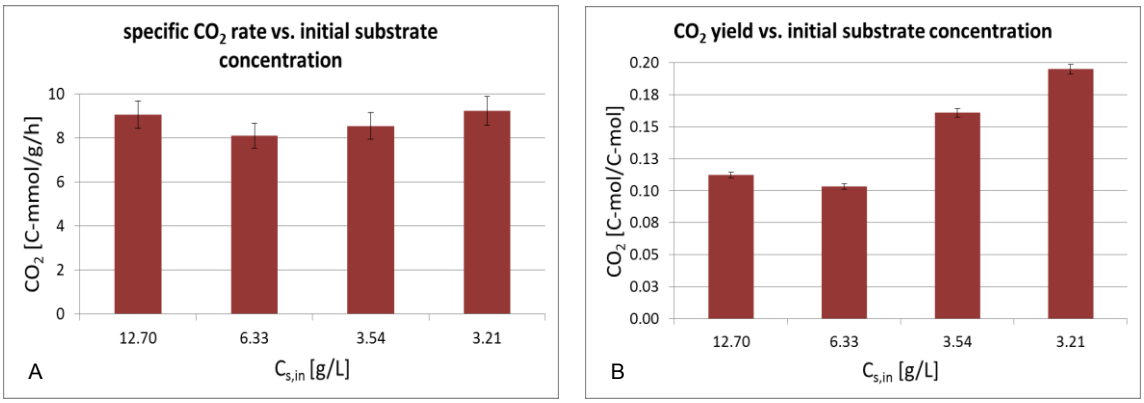

Figure 42 Physiological parameters related to carbon dioxide at D=0.2 h^{-1} with different initial sucrose feeding concentration of 12.7, 6.33, 3.54 and 3.21 g/L.

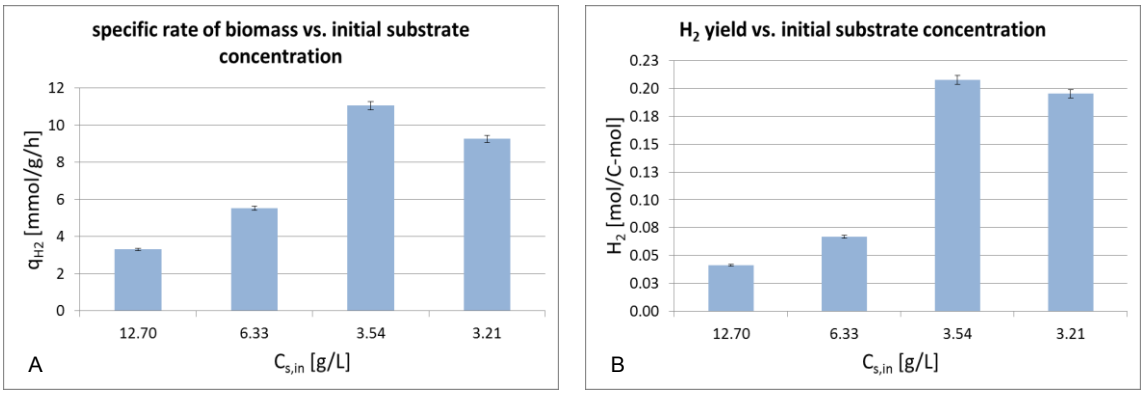

Figure 43 Physiological parameters related to hydrogen at D=0.2 h^{-1} with different initial sucrose feeding concentration of 12.7, 6.33, 3.54 and 3.21 g/L.

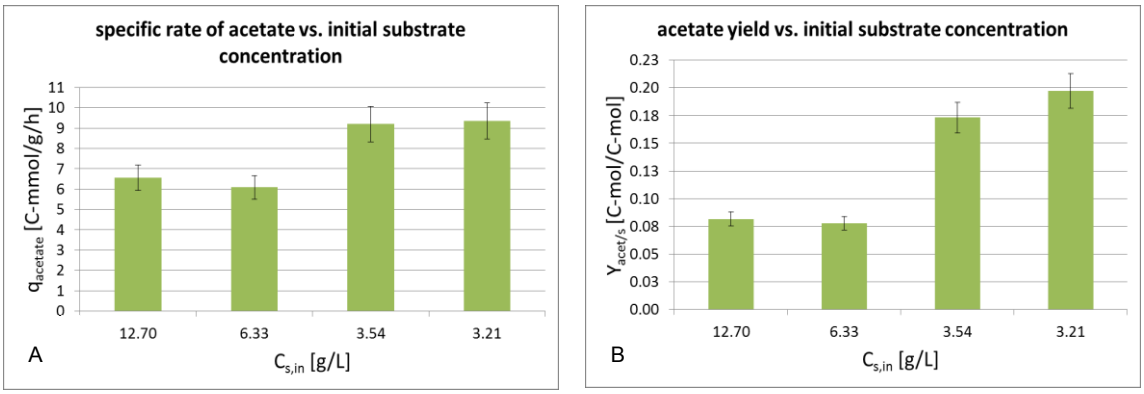

Figure 44 Physiological parameters of acetate with sucrose at D=0.2 h^{-1} with different initial sucrose concentrations of 12.7, 6.33, 3.54 and 3.21 g/L.

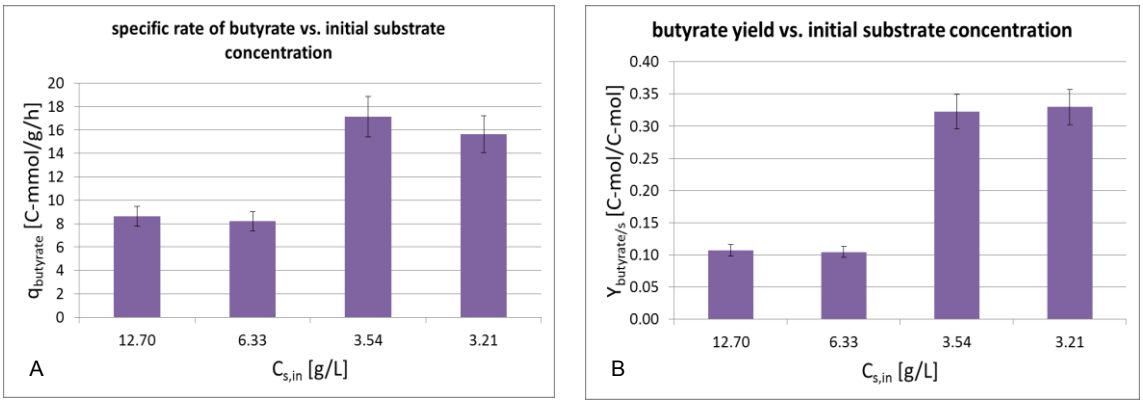

Figure 45 Physiological parameters of butyrate with sucrose at D=0.2 h^{-1} with different initial sucrose concentrations of 12.7, 6.33, 3.54 and 3.21 g/L.

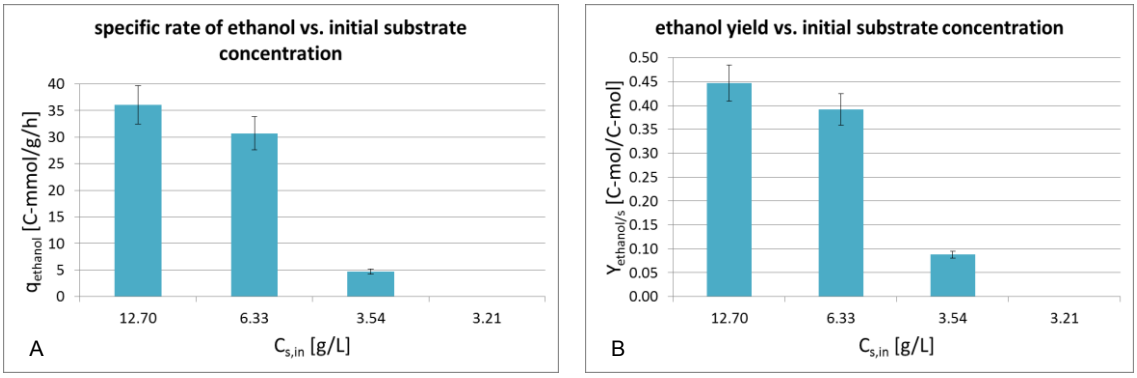

Figure 46 Physiological parameters of ethanol with sucrose at D=0.2 h^{-1} with different initial sucrose concentrations of 12.7, 6.33, 3.54 and 3.21 g/L.

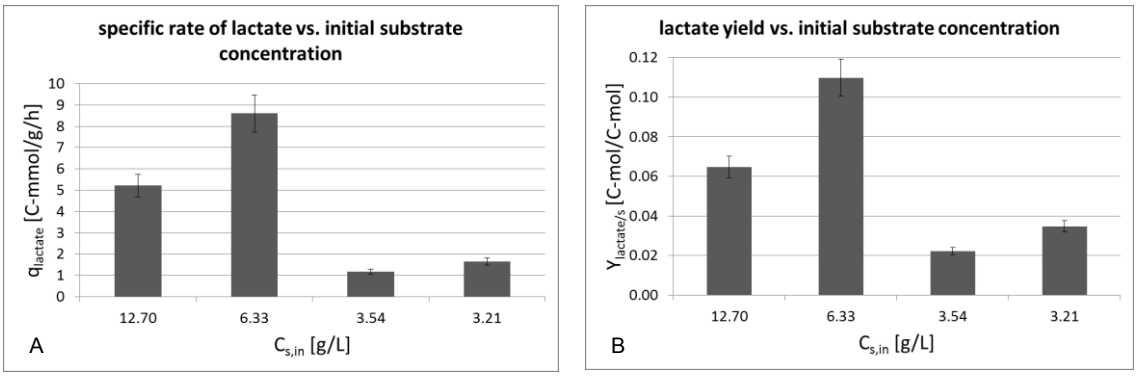

Figure 47 Physiological parameters of lactate with sucrose at D=0.2 h^{-1} with different initial sucrose concentrations of 12.7, 6.33, 3.54 and 3.21 g/L.

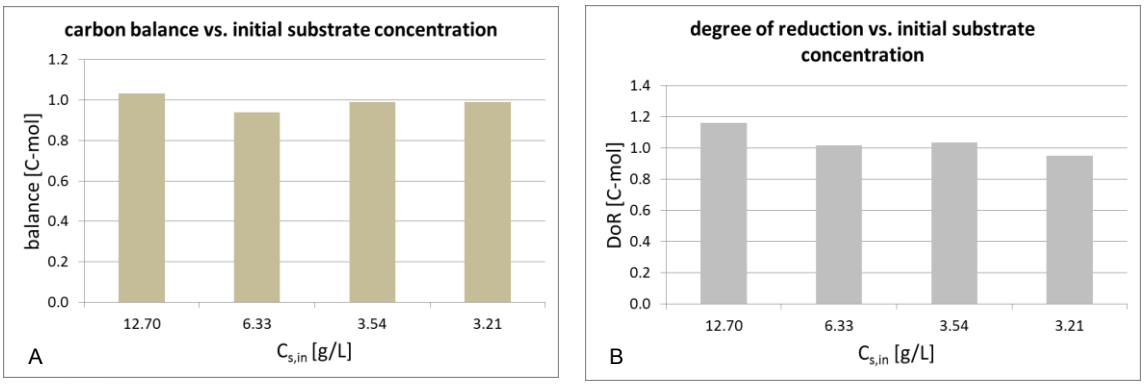

Figure 48 carbon balance and degree of reduction with sucrose at D=0.2 h^{-1} with different initial sucrose concentrations of 12.7, 6.33, 3.54 and 3.21 g/L.

Another interesting observation of this experiment was the high production and yield of butyrate at low initial sucrose feeding concentrations (Figure 45B, Table 16). If this high production at low sucrose concentrations cannot be avoided, operators of anaerobic fermentations might as well take advantage of this by-product of *Fritz* and use it for further processing into economically more valuable products.

Butyric acid has many uses in different industries, and currently there is a great interest in using it as a precursor to biobutanol. Biofuels, like biobutanol offer many advantages like sustainability, a reduction of GHGs and security of supply (16). In addition to its use as a biofuel, butyric acid has also many applications in pharmaceutical and chemical industries. It is well known for its anticancer effects as it induces morphological and biochemical differentiation in a variety of cells leading to concomitant suppression of neoplastic properties (16). Consequently, these studies present on various prodrugs that are derivatives of butyric acid were carried out for their potential use in treatment of cancers and hemoglobinopathies, including leukemia and sickle cell anemia (SCA), and also to protect hair follicles of radio-and chemotherapy-induced alopecia.

In chemical industries, the main application of butyric acid is in the production of cellulose acetate butyrate plastics (CAB) (16). By introducing the butyryl group into cellulose acetate polymers, the resulting polymer exhibits better performance in terms of its solubility in organic solvents due to enhanced hydrophobicity, better flexibility, and light and cold resistance (16).

Furthermore, although butyric acid itself has an unpleasant smell, butyric acid esters such as methyl, ethyl, and amyl butyrate are used as fragrant and flavoring agents in beverages, foods and cosmetic industries (16). Table 17 shows the economic value of a potential harvest of butyrate from a continuously run process.

Table 17 Price comparison between the main metabolites of Fritz

	Company	Nr.	Amount	Preis
			[g]	[€]
Acetate	Sigma	320099	1000	60
Butyrate	Sigma	W22119	1000	80
Ethanol	Sigma	34852	1000	110
Lactate	Sigma	W261114	1000	57

When looking at Figure 46, ethanol production and yield are high as well. But these high values are achieved at non-carbon limiting conditions with an excess supply of substrate. This state of process is not in our desire as we want to run a carbon-limiting process with as little substrate usage as possible in regard of the biomass yield and the economics of the process. Although ethanol is more valuable than butyrate, it is not a by-product of the carbon-limited process we desire. Additionally, a comparison to a real ethanol producer like *Saccharomyces cerevisiae* CBS 8066 shows that *Fritz* would not be near as economically feasible as the yeast strain for ethanol production (Table 18) (17). In this case, the production was carried out in an anaerobic, glucose-limited continuous process, whereas our process was not carbon-limited. Even with glucose limitation, *S. cerevisiae* CBS 8066 reached a higher ethanol yield than Fritz.

Table 18 Comparison between Fritz at high sucrose concentration and a specialized ethanol producer like *S. cerevisiae* CBS 8066

Strain	Y ethanol/s
Fritz	0.45 C-mol/C-mol
S. cerevisiae CBS 8066	0.50 C-mol/C-mol

6.1.2 Decreasing sucrose feeding concentrations over time

Originally, this experiment was performed to find out at which sucrose feeding concentration carbon limiting conditions could be found, if cultivating *Fritz* on continuous mode at D=0.2 h^{-1}. The medium contained 100% N-P and 2 g/L yeast extract. We started with 6.33 g/L of sucrose concentration because this was the maximum uptake under non-carbon limiting conditions (Table 4). But we did not achieve carbon-limiting conditions with 6.33 g/L sucrose feeding concentration. Surprisingly, even when reducing the sucrose feed concentration to 5.17, 4.40 and 3.89 g/L, carbon limitation was never achieved (Figure 49A).

But the experiment showed something else. Biomass concentration, volumetric productivity and yield increased significantly at decreasing sucrose feeding concentrations and specific sucrose uptake rates over time, and reached their respective maxima at the last and lowest sucrose feeding concentration (3.89 g/L). Normally, the biomass concentration and productivity is proportional to the sucrose uptake rate, meaning it increases with higher uptake rates and decreases with lower uptake rates. This was the case in the experiment in section 6.1.1 when independent initial sucrose feeding concentrations were tested, but not in this section, related to decreasing sucrose feeding concentrations over time. These biomass results implicate a memory effect of *Fritz*. The subsequent generations get accustomed to the substrate and the environmental conditions over time, making them more efficient in the utilization of substrate towards production of biomass. Thus, running a continuous process with *Fritz* over a long period of time is favourable for efficient and effective biomass growth.

Regarding the metabolites, carbon dioxide production did not show an upward or downward trend (Figure 53A). However, the specific productivity decreased from the two highest sucrose feeding concentrations (6.33 and 5.17 g/L) to the two lowest ones (4.40 and 3.89 g/L) from 8.51±0.16 to 6.67±0.27 C-mmol/g/h, respectively. The carbon dioxide yield increased from 0.10±0.01 to 0.16±0.01 C-mol/C-mol with lowering sucrose feeding concentration over time (Figure 53C).

Plotting the carbon dioxide yield over the biomass yield did not show a correlation between both physiological parameters (Figure 52) as high as in the previous experiment (Figure 38), but a directly correlation between both parameters could be observed. Initially, the carbon dioxide and biomass yields showed a high correlation but with time this correlation weakened because carbon dioxide was produced as a by-product from additional metabolite production. The carbon dioxide productivity and yield must have resulted additionally from ethanol. Nevertheless, the correlation (Figure 52) was still high enough for carbon dioxide and biomass to follow the same trend. Therefore, when the carbon dioxide signals changes, a change in biomass can be expected over time.

The hydrogen production and yield clearly increased when lowering the sucrose feeding concentration over time. This suggests a shift of the electron flow towards hydrogen. But specific production of hydrogen remained constant after switching sucrose feeding concentration to 5.17 g/L and further reduction to 3.89 g/L (Figure 54). Also, acid production increased and solvent production decreased at increasing hydrogen levels, confirming our previous experiment in section 6.1.1 and the theory that the shift of the electron flow towards hydrogen production induces acidogenesis (production of acetate and butyrate). Thus, monitoring the hydrogen online signal in a continuous pro-

cess would enable to study possible metabolic shifts of *Fritz* due to changes in the working conditions or process parameters.

Further, acetate concentration and yield increased significantly if lowering the sucrose feeding concentration (Figure 55). But there was no significant difference beteween 4.40 and 3.89 g/L sucrose feeding concentration in concentration and yield. Butyrate concentration and yield showed the same trend as acetate and hydrogen. It clearly increased with lowering sucrose feeding concentration over time, which also indicates a stoichiometric shift towards butyrate. Moreover, there was not a significant difference observable between the specific butyrate productivity and yield at the two lowest sucrose concentrations (4.40 and 3.89 g/L) (Figure 56). To see the relationship between acetate, butyrate and hydrogen more clearly the H_2/CO_2 yield was plotted against the "acid yield" (acetate and butyrate) and the "solvent yield" (ethanol and lactate) (Figure 50).

Conversely, the ethanol and lactate results show a clear and significant decreasing trend over time if lowering the sucrose feeding concentration (Figure 57, Figure 58). Here again, our results match with the theory in section 1.3.2 and our previous experiment in section 6.1.1.

To sum up, this experiment and the previous one showed clearly that there is a metabolic shift matching the theory where high substrate concentrations or non-carbon limiting concentrations are favorable for solventogenesis. On the other hand, (pseudo) C-limiting conditions favor acidogenesis and therewith the shift in *Fritz* stoichiometry towards biomass production.

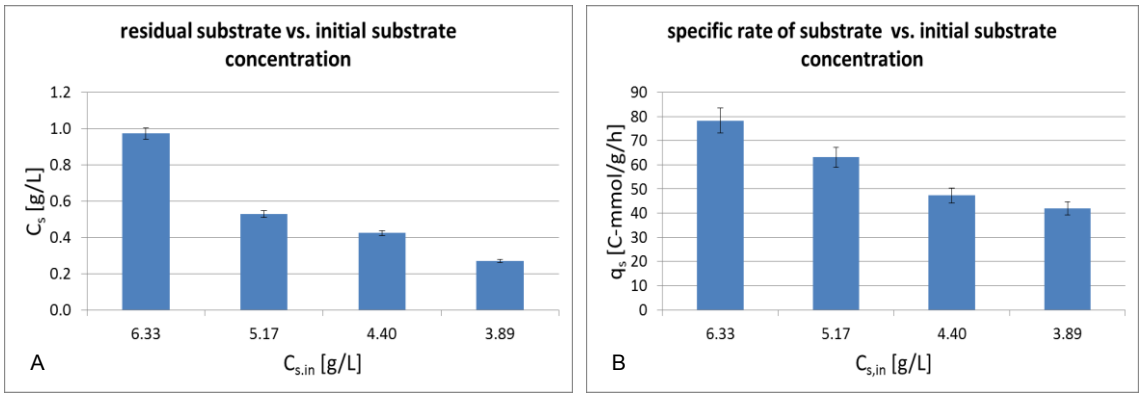

Figure 49 Physiological parameters of Fritz for sucrose at D=0.2 h^{-1}, 100% N-P and 2g/L YE. The feeding concentrations were 6.33, 5.17, 4.40 and 3.89 g/L over time, starting from the highest to the lowest sucrose concentration.

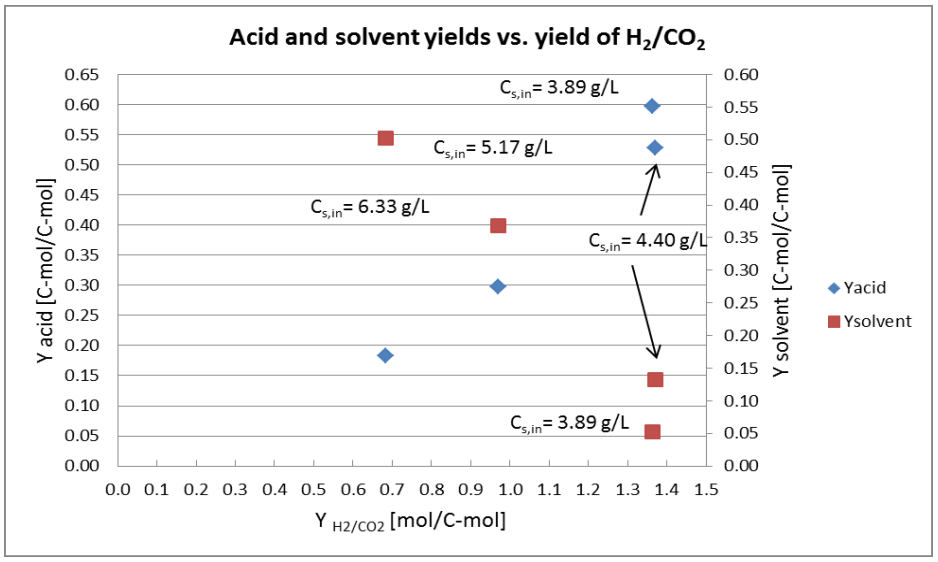

Figure 50 Comparison between the acid yield (acetate and butyrate) and the solvent yield (ethanol and lactate) to the yield of H2/CO2 at different sucrose feeding concentrations over time and D=0.2 h^{-1}.

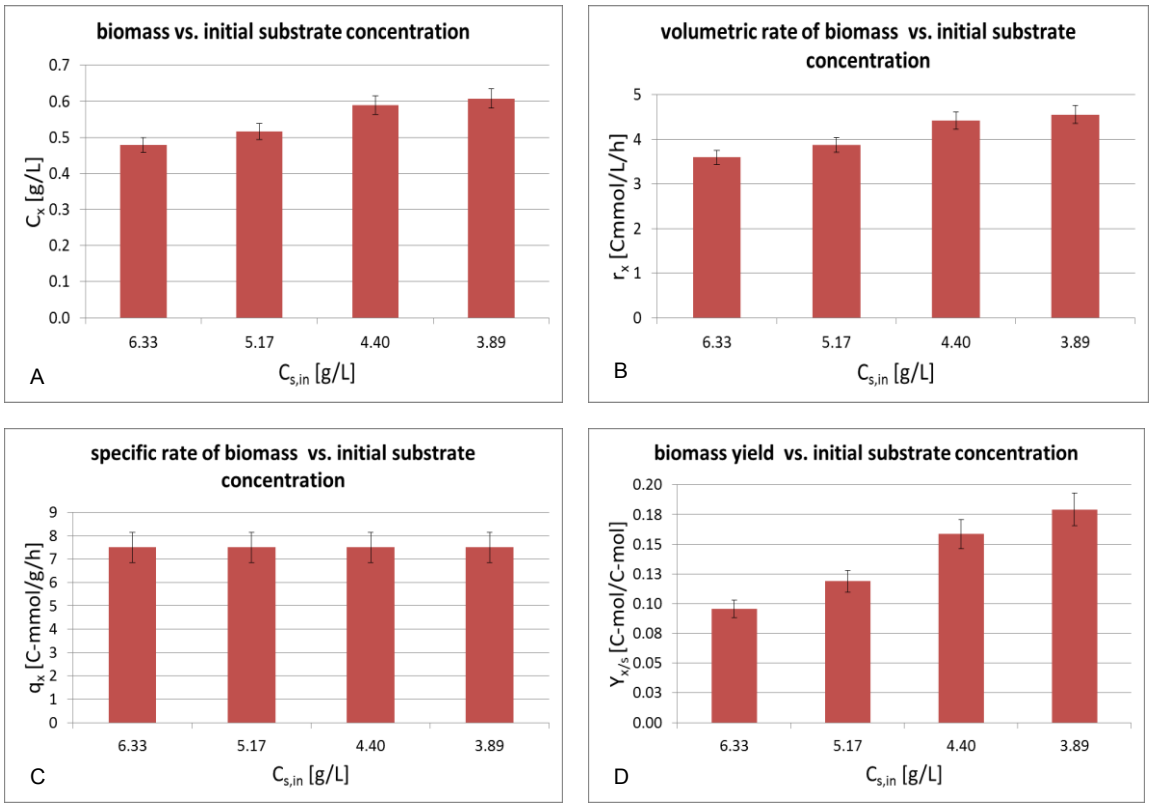

Figure 51 Physiological parameters of Fritz for biomass at D=0.2 h^{-1}, 100% N-P and 2g/L YE. The feeding concentrations were 6.33, 5.17, 4.40 and 3.89 g/L over time, starting from the highest to the lowest sucrose concentration.

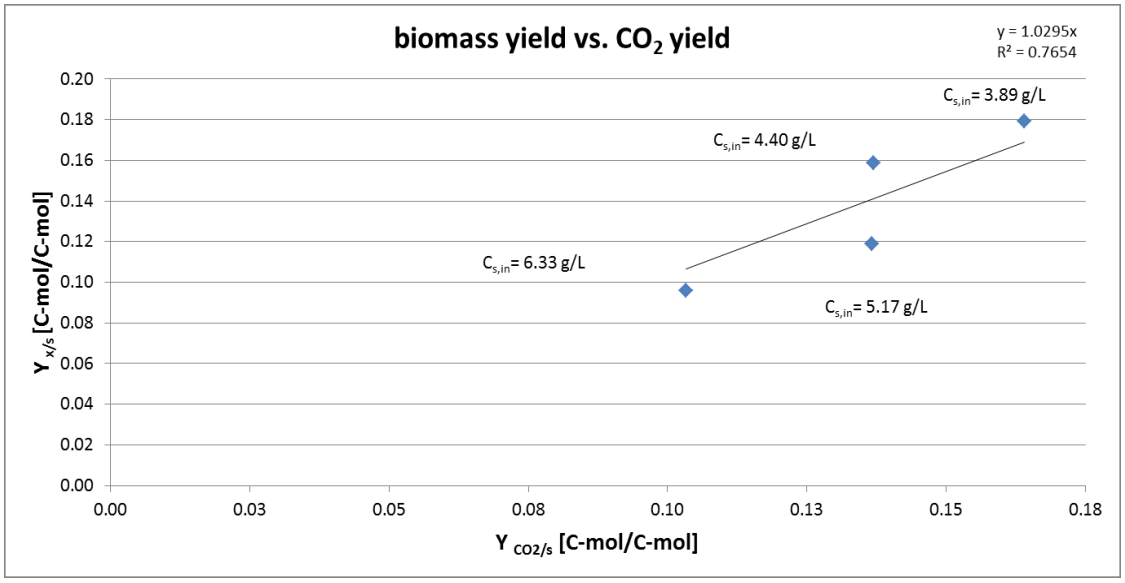

Figure 52 Comparison between biomass yield and carbon dioxide yield at D=0.2 h^{-1} with sucrose feeding concentrations of 6.33, 5.17, 4.40 and 3.89 g/L over time, showing insufficient correlation.

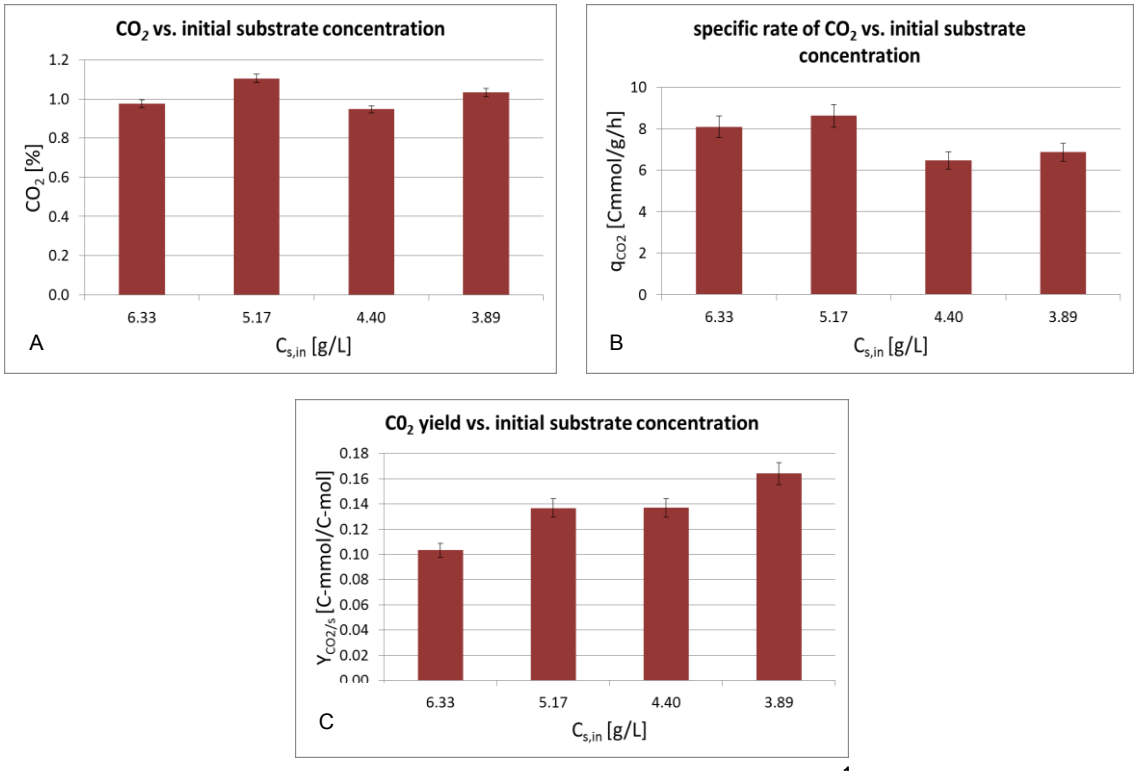

Figure 53 Physiological parameters of Fritz for carbon dioxide at D=0.2 h^{-1}, 100% N-P and 2g/L YE. The feeding concentrations were 6.33, 5.17, 4.40 and 3.89 g/L over time, starting from the highest to the lowest sucrose concentration.

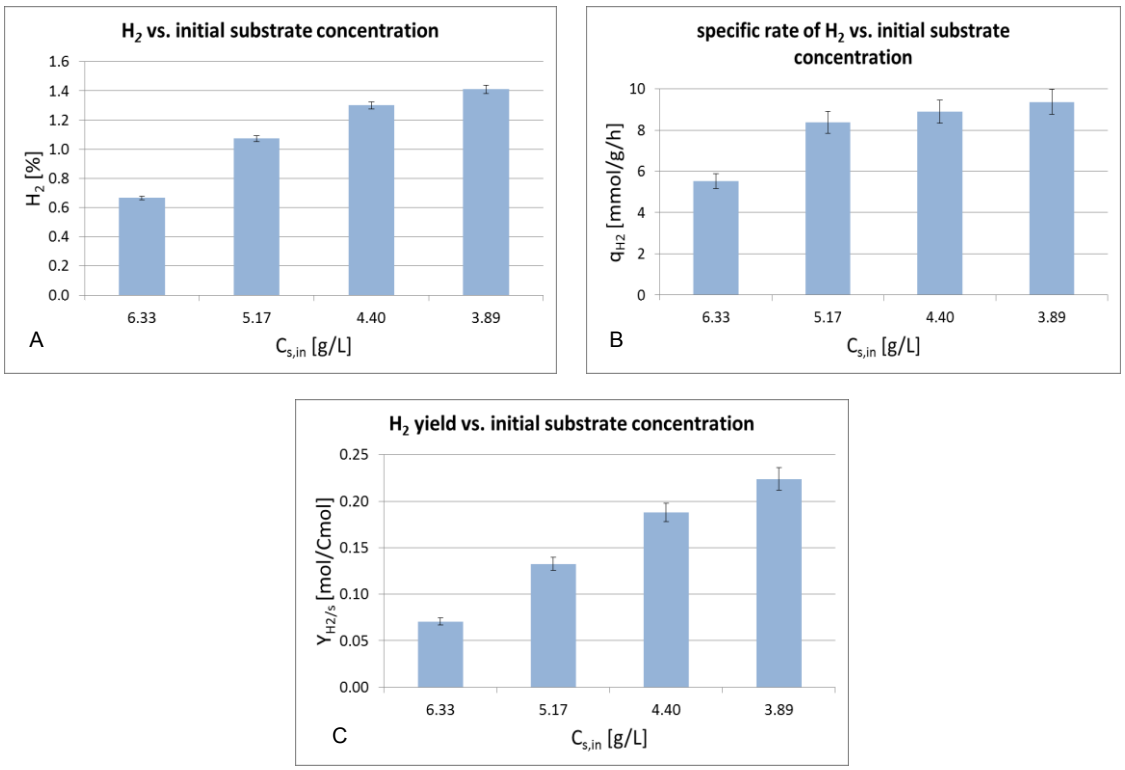

Figure 54 Physiological parameters of Fritz for hydrogen at D=0.2 h^{-1}, 100% N-P and 2g/L YE. The feeding concentrations were 6.33, 5.17, 4.40 and 3.89 g/L over time, starting from the highest to the lowest sucrose concentration.

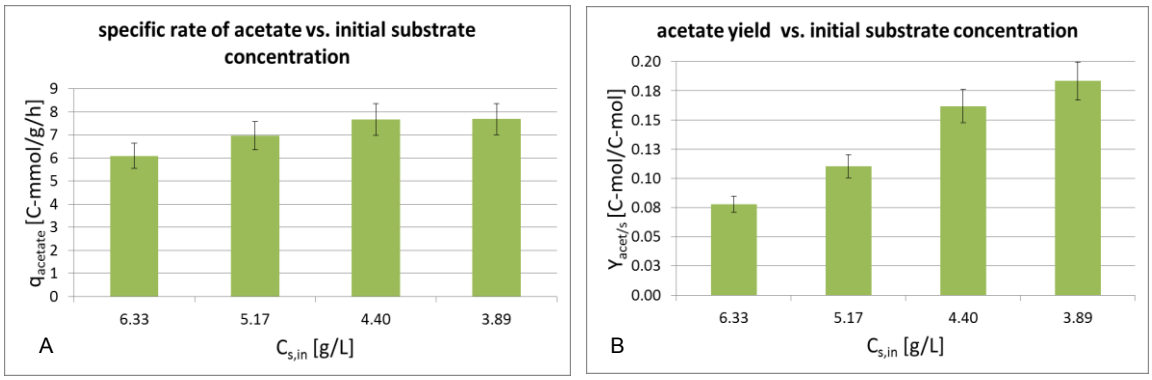

Figure 55 Physiological parameters of Fritz for acetate at D=0.2 h^{-1}, 100% N-P and 2g/L YE. The feeding concentrations were 6.33, 5.17, 4.40 and 3.89 g/L over time, starting from the highest to the lowest sucrose concentration.

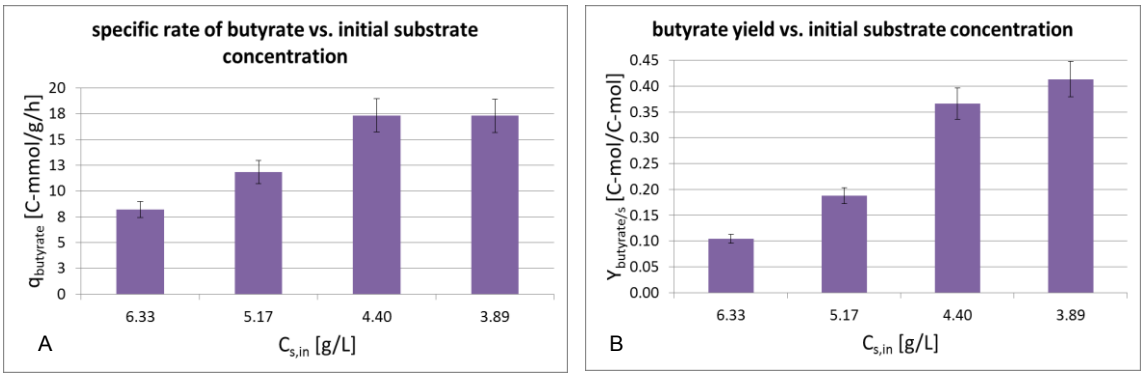

Figure 56 Physiological parameters of Fritz for butyrate at D=0.2 h^{-1}, 100% N-P and 2g/L YE. The feeding concentrations were 6.33, 5.17, 4.40 and 3.89 g/L over time, starting from the highest to the lowest sucrose concentration.

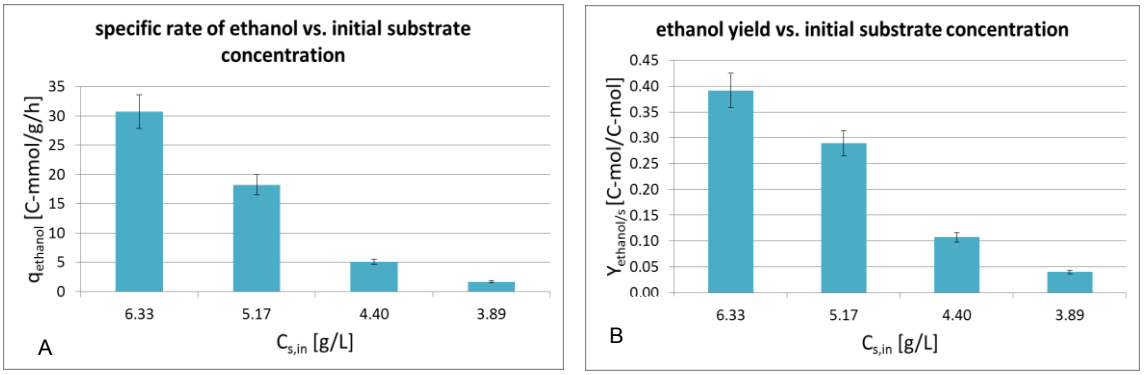

Figure 57 Physiological parameters of Fritz for ethanol at D=0.2 h^{-1}, 100% N-P and 2g/L YE. The feeding concentrations were 6.33, 5.17, 4.40 and 3.89 g/L over time, starting from the highest to the lowest sucrose concentration.

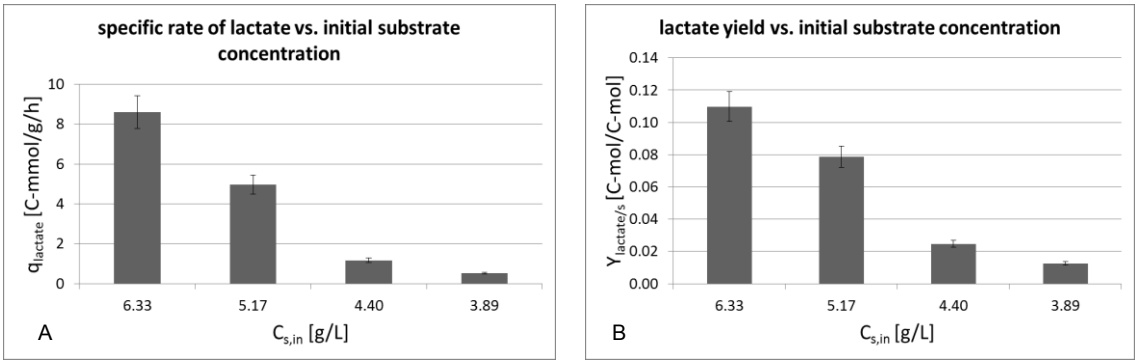

Figure 58 Physiological parameters of Fritz for lactate at D=0.2 h^{-1}, 100% N-P and 2g/L YE. The feeding concentrations were 6.33, 5.17, 4.40 and 3.89 g/L over time, starting from the highest to the lowest sucrose concentration.

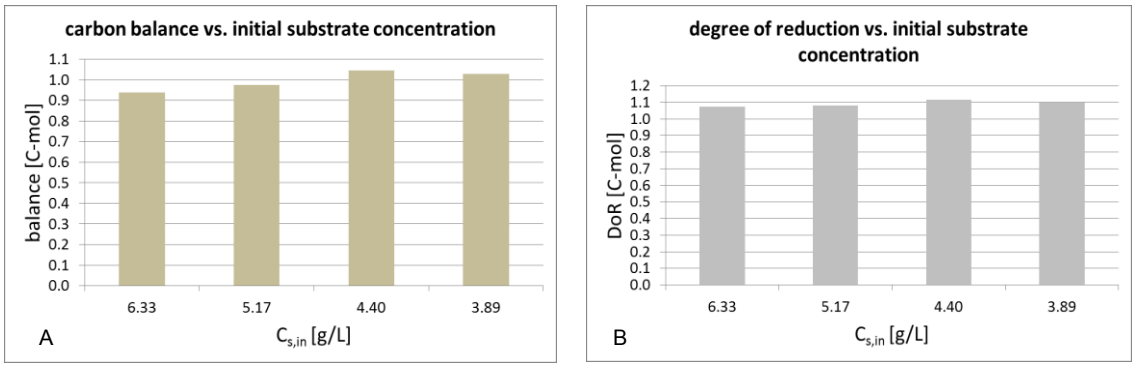

Figure 59 carbon balance and degree of reduction with sucrose at D=0.2 h^{-1} with sucrose concentrations of 6.33, 5.17, 4.40 and 3.89 g/L over time.

6.1.3 Different nitrogen and phosphate concentration

The goal of this experiment was to observe if changes in the metabolic activity of *Fritz* occurred when reducing the nitrogen and phosphate concentration (N-P) in the reference feed medium from 100 % to 25% and 15%. Would the reduction of N-P have any impact on biomass productivity and on the stoichiometry of *Fritz* metabolism? The medium contained 2 g/L YE and 3.5±0.14 g/L sucrose for all the N-P concentrations tested.

The lowest sucrose uptake rate (49.52 C-mmol/L/h) was achieved at 25% N-P and the highest one (59.39 C-mmol/L/h) at 15% N-P (Figure 61B). But biomass productivity (2.99 C-mmol/L/h) at 15% N-P was not higher compared to 25% N-P. The biomass yield decreased at decreasing N-P concentrations from 0.15 C-mol/C-mol (25% N-P) to 0.13 C-mol/C-mol (15% N-P) (Figure 60). This means that utilization of sucrose towards biomass production was less efficient at 15% N-P compared to 25% and 100%, while reducing the N-P concentration from 100% to 25% showed no significant effect on the biomass productivities or yield. Considering the key physiological parameters r_x and $Y_{x/s}$, the amount of nitrogen and phosphate in the medium could be reduced to 25% N-P without affecting them negatively.

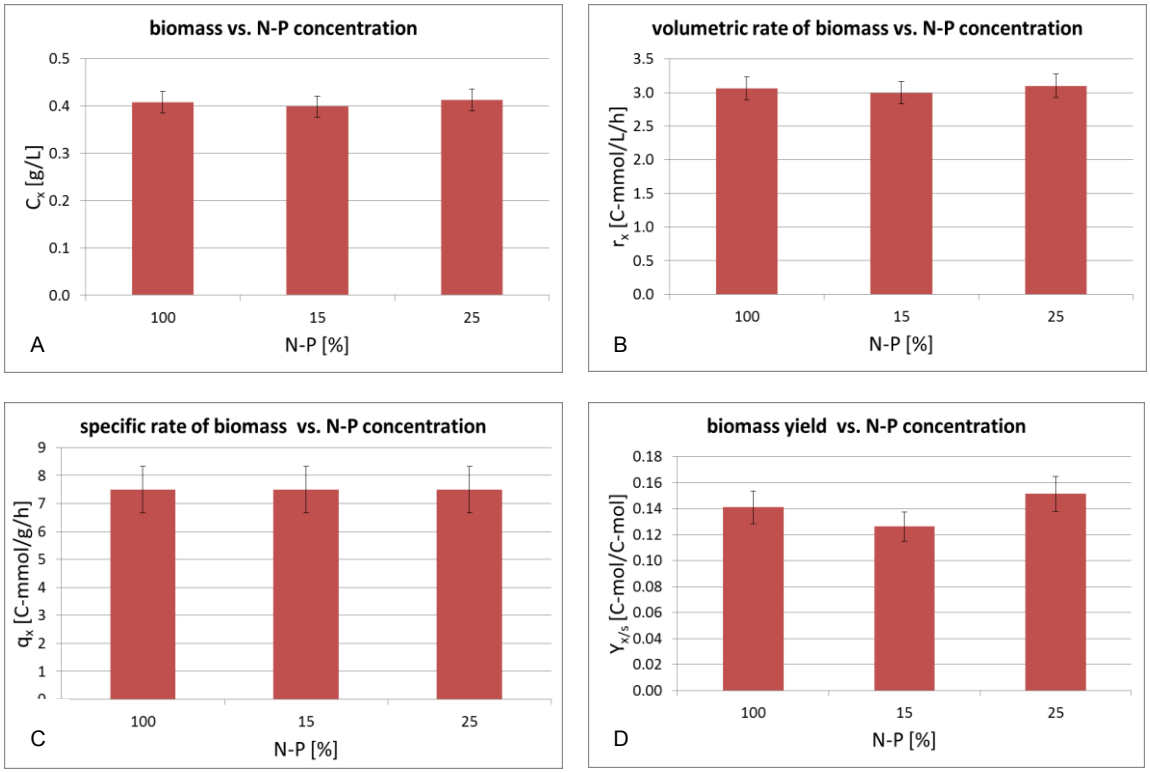

Figure 60 Physiological parameters of Fritz related to biomass at D=0.2 h^{-1} with 3.51, 3.69 and 3.41 g/L sucrose as well as 100, 15 and 25% N-P, respectively.

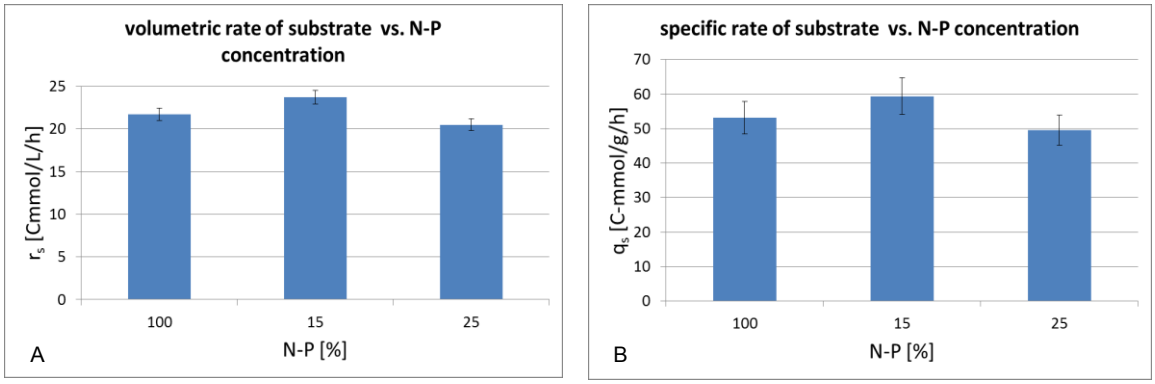

Figure 61 Physiological parameters of Fritz related to sucrose at D=0.2 h^{-1} with 3.51, 3.69 and 3.41 g/L sucrose as well as 100, 15 and 25% N-P, respectively.

Reducing the N-P concentration from 100% to 15% resulted in a significant decrease on carbon dioxide concentrations, productivities and yields. Increasing it to 25% N-P concentration did not change the carbon dioxide concentration and specific productivity (Figure 62A, Figure 62B), although the yield increased slightly (Figure 62C). The lower carbon dioxide yield at 15% N,P resulted from a higher sucrose uptake rate as Figure 61 demonstrates.

The hydrogen concentration, productivities and yield decreased significantly when reducing from 100% to 15%, but it slightly recovered when increasing to 25% N-P (Figure 63). The lowest hydrogen yield and concentration were achieved at 15% N-P. Thus, a decrease in the N-P concentration results in lower hydrogen levels.

The acetate specific productivity did not change significantly when reducing to 15% or 25% N-P. But the yield decreased at 15% N-P, indicating a stoichiometric shift where less acetate is produced at lower N-P concentrations (Figure 64B). The butyrate concentration and yield changed significantly when reducing from 100% and 25% to 15% N-P, but specific productivity remained constant. There was no change in the butyrate parameters after reducing from 100% to 25% N-P (Figure 65). There was a stoichiometric shift towards less butyrate at 15% N-P, as it happened with hydrogen and acetate. As shown in the previous experiments, these three metabolites correlated well with each other.

On the other hand, ethanol concentrations, specific productivity and yield increased significantly when reducing N-P from 100% to 15% or 25%. But there was no significant difference in ethanol parameters between 15% and 25% N-P concentrations (Figure 66). Lactate showed a similar trend as ethanol. The concentrations, specific productivity and yield all increased significantly from 100% N-P to 15% or 25%. However, unlike in ethanol, there was a significant increase in concentration and yield when comparing 15% and 25% N-P (Figure 67).

So, when looking at the data, reduction of N-P from 100% to 15% or 25% resulted in a decrease of hydrogen, acetate and butyrate as well as an increase in ethanol and increase in lactate production. As shown in previous experiments, an increase in acid production (acetate and butyrate) aligns with acidogenesis while solvent production or solventogenesis (ethanol) decreases, and vice versa. These observations illustrate that the change from solventogenesis to acidogenesis and vice versa does not happen abruptly. The change is rather happening gradually, depending on the number of parameters favorable either for solventogenesis or acidogenesis (e.g. pH, substrate concentration, phosphate concentration). Although we never reached phosphate limitation, our observations of the data showed a trend when reducing N-P concentration to a near-limitation of 15% N-P, as *Fritz* gradually shifted from acid to solvent production.

Regarding the ammonium concentration, a study concluded that limiting ammonium concentrations enhanced acid production (11). In our case acid production did not increase, and it even slightly decreased if reducing the feeding N-P concentrations (Figure 64B, Figure 65B). The yields of acetate and butyrate decreased as well at 15% N-P concentration, together with the biomass yield. This indicates a connection be-

tween the acidogenesis and optimal biomass productivity. Presumable, not enough ATP is being generated with the drop in acid yields, thus not enough energy is available for growth.

Varying the salt concentration down to at least 15% is yet another possibility to induce a metabolic shift in *Fritz* towards specific metabolites if desired. In the case of phosphate the shift would occur towards solvent production. Ammonium, on the other hand, would induce acidogenesis. As our goal is not a specific metabolite, but the biomass productivity itself, such steep decrease is not necessary.

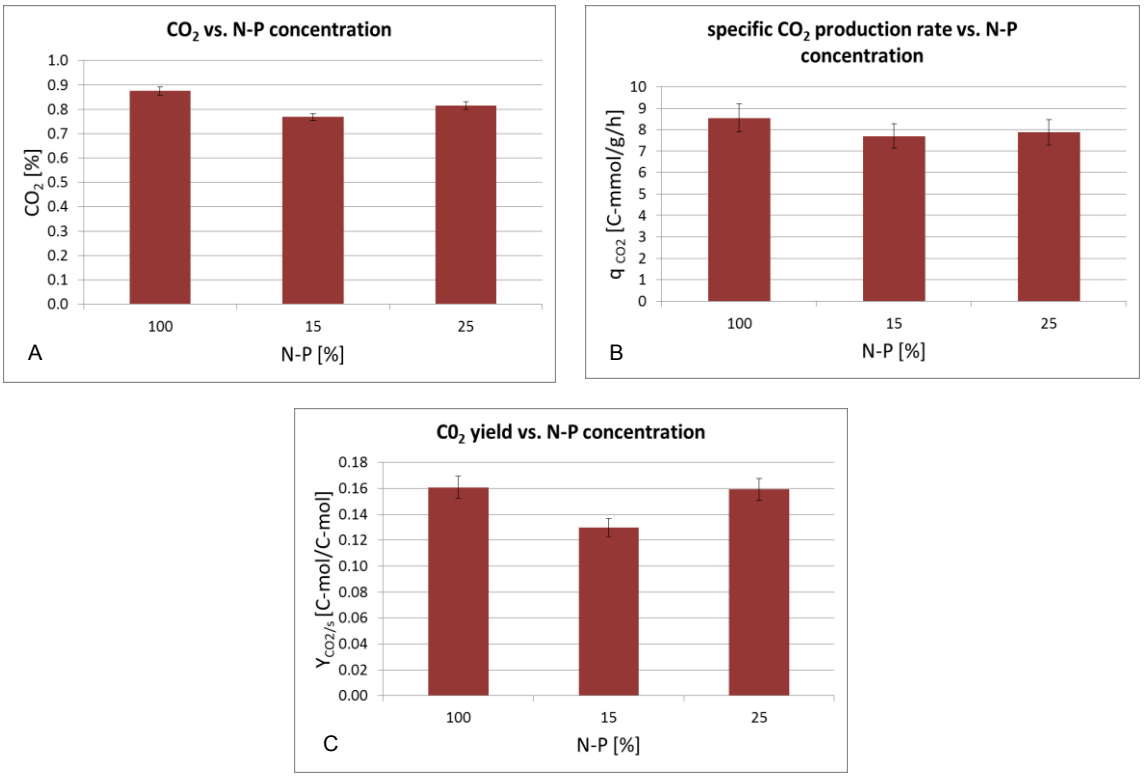

Figure 62 Physiological parameters of Fritz related to carbon dioxide at D=0.2 h^{-1} with 3.51, 3.69 and 3.41 g/L sucrose as well as 100, 15 and 25% N-P, respectively.

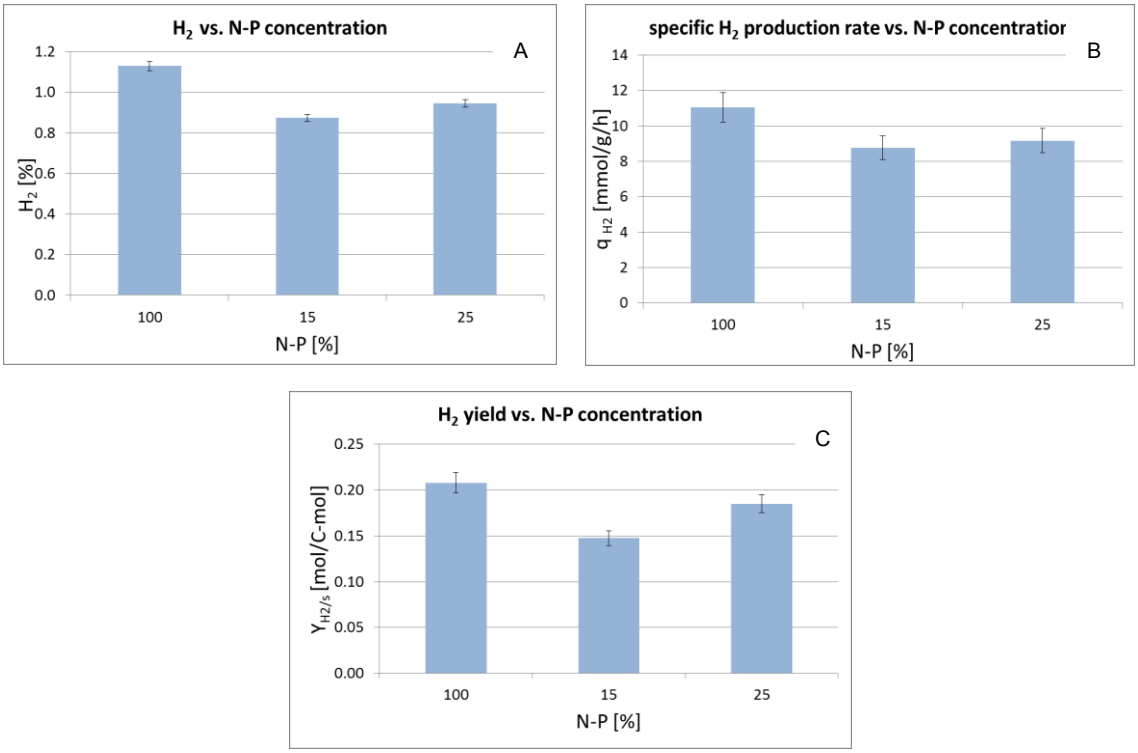

Figure 63 Physiological parameters of Fritz related to hydrogen at D=0.2 h^{-1} with 3.51, 3.69 and 3.41 g/L sucrose as well as 100, 15 and 25% N-P, respectively.

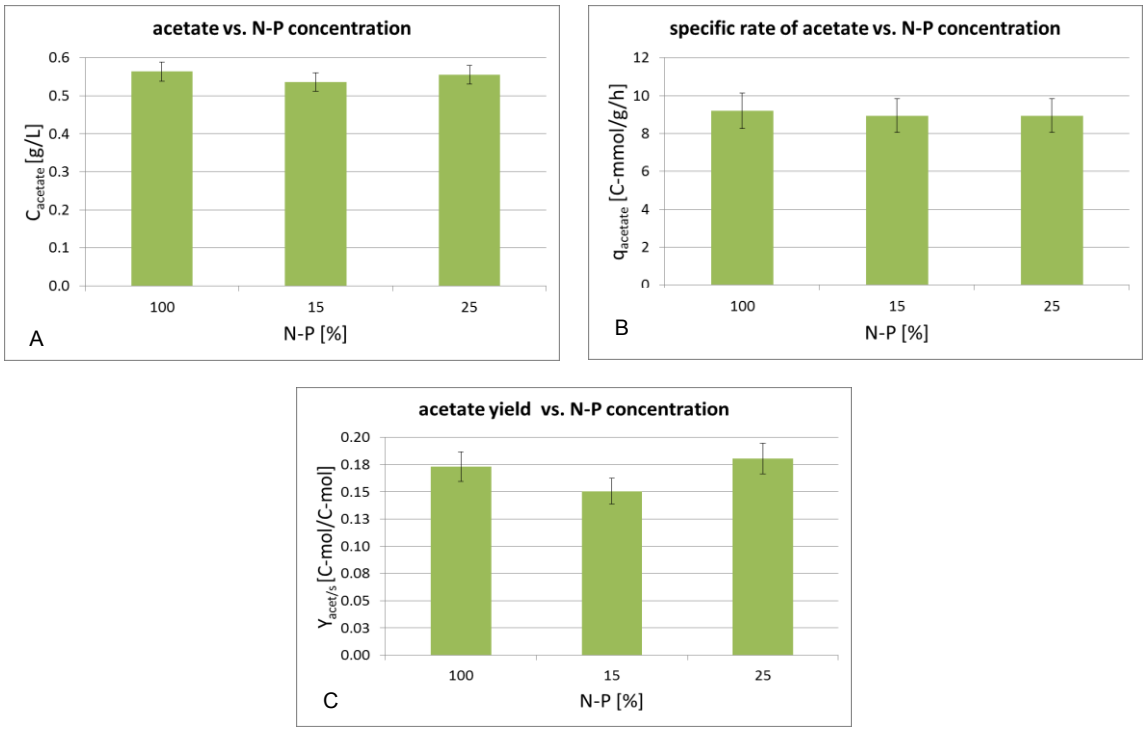

Figure 64 Physiological parameters of Fritz related to acetate at D=0.2 h^{-1} with 3.51, 3.69 and 3.41 g/L sucrose as well as 100, 15 and 25% N-P, respectively.

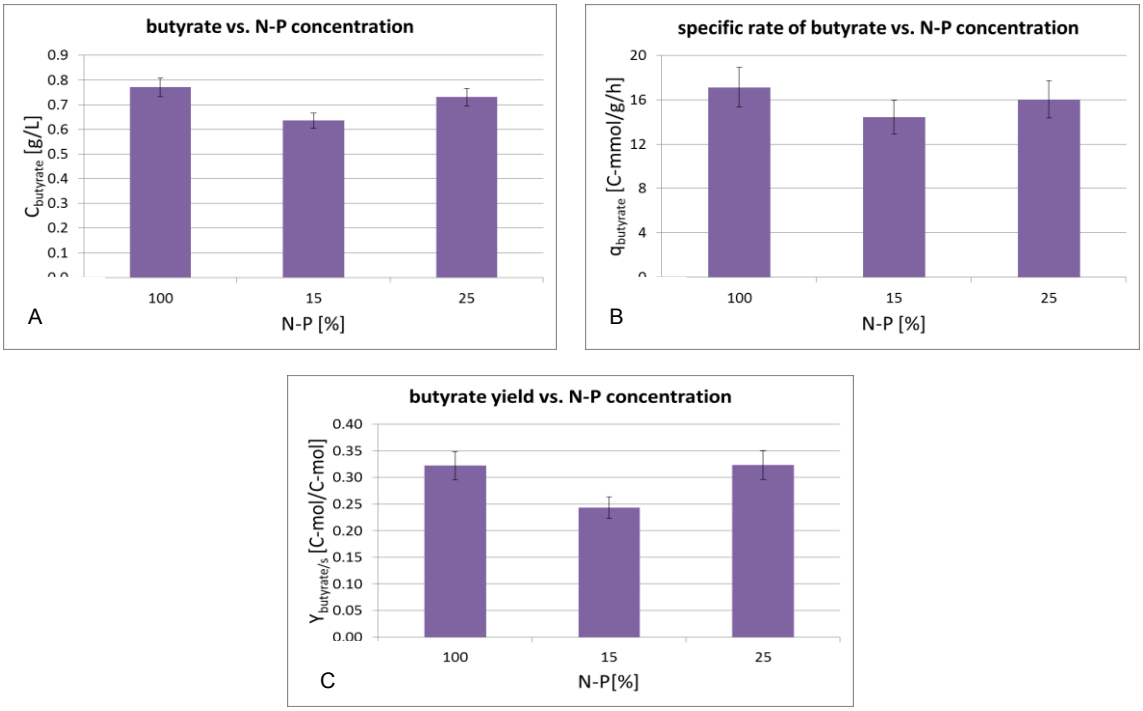

Figure 65 Physiological parameters of Fritz related to butyrate at D=0.2 h^{-1} with 3.51, 3.69 and 3.41 g/L sucrose as well as 100, 15 and 25% N-P, respectively.

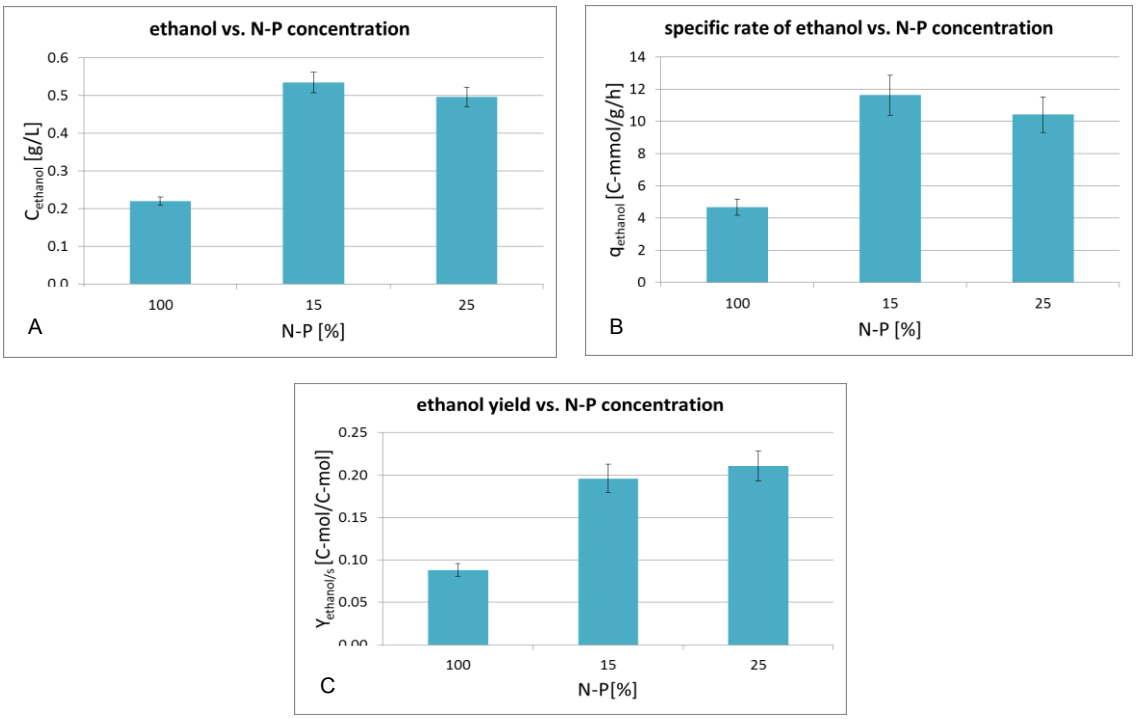

Figure 66 Physiological parameters of Fritz related to ethanol at D=0.2 h^{-1} with 3.51, 3.69 and 3.41 g/L sucrose as well as 100, 15 and 25% N-P, respectively.

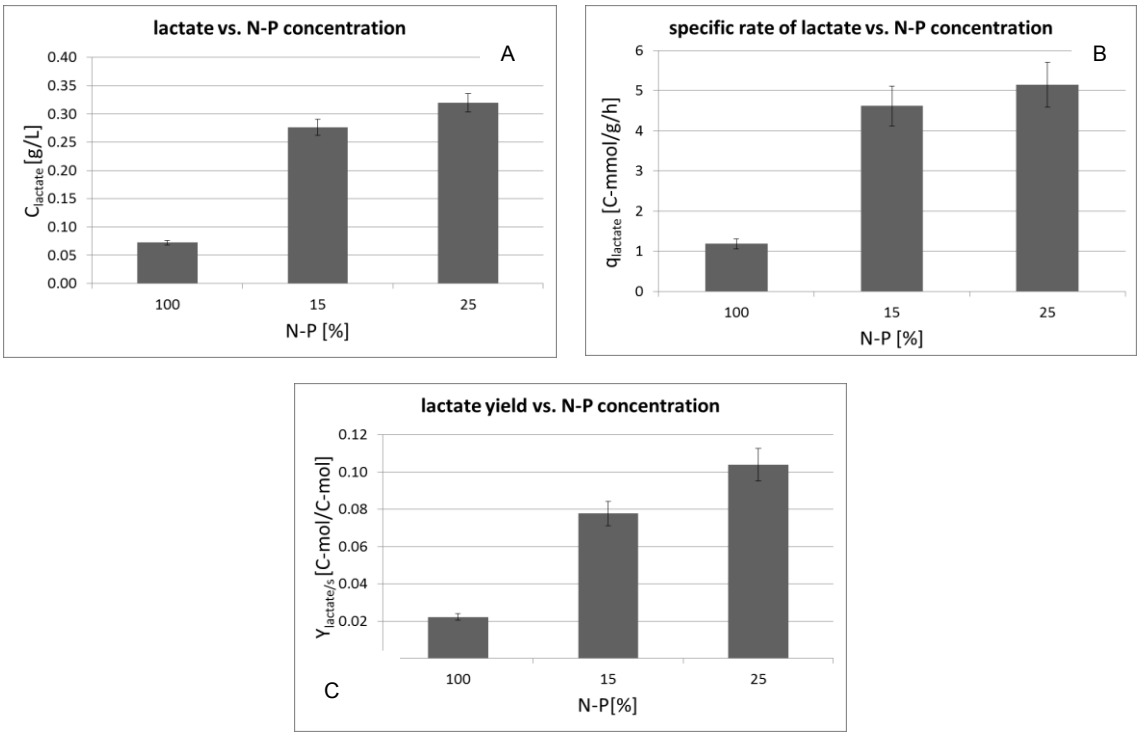

Figure 67 Physiological parameters of Fritz related to lactate at D=0.2 h^{-1} with 3.51, 3.69 and 3.41 g/L sucrose as well as 100, 15 and 25% N-P, respectively.

6.1.4 Continuous cultures under non-carbon limiting conditions with sucrose and maltose at D=0.1 h^{-1}

The aim of this experiment was to observe and analyze the effects of growing *Fritz* at 12.11 g/L sucrose or 16.61 g/L maltose under non-carbon limiting conditions at D=0.1 h^{-1}. Both feed media contained 2 g/L YE and 100% N-P.

Surprisingly, our strain displayed completely different metabolic patterns when growing under maltose at D=0.1 h^{-1} compared to sucrose. The pattern with sucrose at D=0.1 h^{-1} was consistent with the results from previous experiments (section 6.1.1 and section 6.1.2), where we established high solvent production under non-carbon limitations and high acid production at carbon-limiting or low residual substrate concentrations.

The biomass concentration and volumetric productivity under high residual substrate concentrations were lower with sucrose (0.60 g/L and 2.24 C-mmol/L/h respectively) than with maltose (0.79 g/L and 2.98 C-mmol/L/h). However, the biomass yield was the same (0.09 C-mol/C-mol)(Figure 68), even when the substrate uptake was different (Figure 69A). This fact and the lower sucrose consumption of 7.43 g/L compared to 9.80 g/L of maltose suggest that *Fritz* experienced a higher affinity towards sucrose.

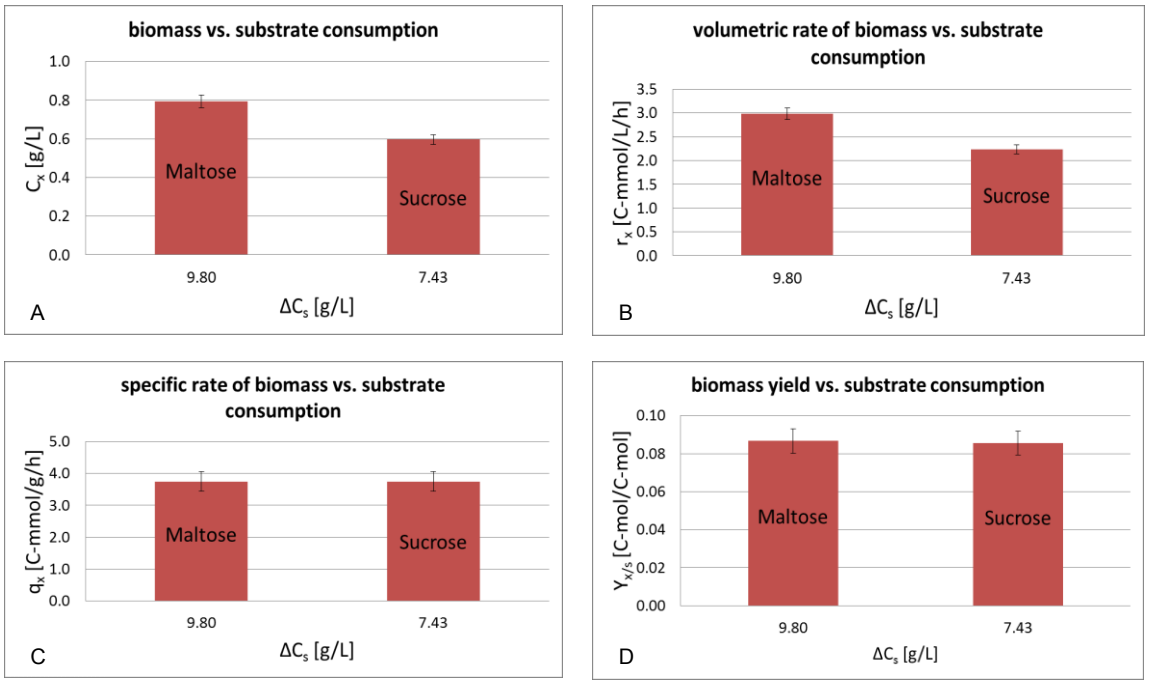

Figure 68 Physiological parameters of Fritz related to the biomass at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

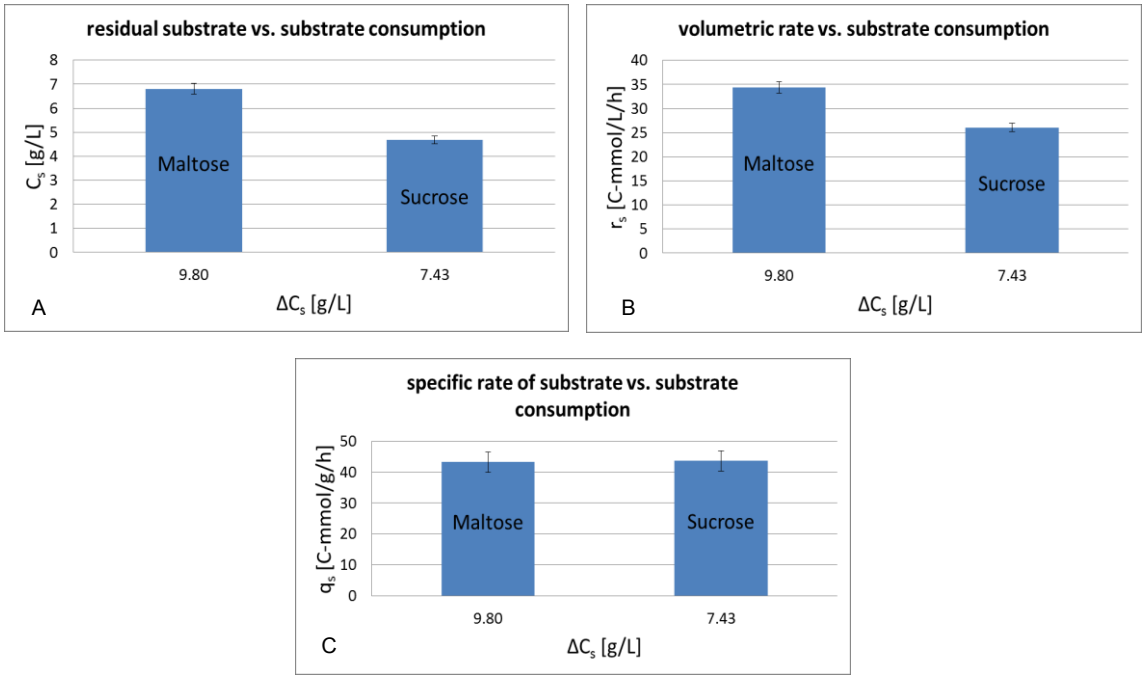

Figure 69 Physiological parameters of Fritz related to the substrates at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

Feeding with sucrose resulted in significant higher values of concentration, specific productivity and yield for carbon dioxide and hydrogen compared to maltose (Figure 71, Figure 72). Interestingly though, acetate and butyrate production were significantly higher with maltose instead of with sucrose (Figure 73, Figure 74). On the other hand, ethanol was higher with sucrose (Figure 75) while lactate was lower (Figure 76).

So, why does the carbon and energy distribution change when using a different substrate? The answer lies in the different assimilation of sucrose and maltose. We propose that *Fritz* takes up maltose over the ABC transporter (ATP-binding cassette) and sucrose over the more energy efficient phosphoenolpyruvate-dependent phosphate transfer system (PTS) pathway; based on a study in which researchers described factors affecting the utilization of carbohydrates by *Clostridia* (18).

Table 19 Metabolite yields related to biomass of Fritz at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

Substrate	$c_{s, in}$	D	$Y_{H2/x}$	$Y_{CO2/x}$	$Y_{pyr/x}$	$Y_{lact/x}$	$Y_{acet/x}$	$Y_{butyr/x}$	$Y_{EtOH/x}$
	[g/L]	[h^{-1}]	[mol/C-mol]	[C-mol/C-mol]					
Maltose	16.61	0.1	0.57	1.34	0.14	0.94	3.92	1.85	2.03
Sucrose	12.11	0.1	1.13	2.57	0.74	0.77	2.23	0.93	2.78

Table 20 Metabolite yields related to substrate of Fritz at D=0.1h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

Substrate	$c_{s, in}$	D	$Y_{H2/s}$	$Y_{CO2/s}$	$Y_{pyr/s}$	$Y_{lact/s}$	$Y_{acet/s}$	$Y_{butyr/s}$	$Y_{EtOH/s}$
	[g/L]	[h^{-1}]	[mol/C-mol]	[C-mol/C-mol]					
Maltose	16.61	0.1	0.05	0.12	0.01	0.08	0.35	0.17	0.18
Sucrose	12.11	0.1	0.10	0.22	0.06	0.07	0.19	0.08	0.24

High molecular biomass (polymers) is one of many important sources of substrates for anaerobe Clostridia, which can be degraded by exoenzymes (extracellular enzymes). This catalytic reaction then releases low molecular substrates which then can be utilized by the cells. In most cases mono- or disaccharides are referred to as the important low molecular substrates for *Clostridia sp*. They can result from the degradation of high molecular substrates or be initially available as free sugars (18).

Now, the transport mechanisms for these oligosaccharides are classified as active or passive. The classification depends on whether the bacteria use energy in form of ATP or not. Passive transport (also called diffusion) like ion gradients systems are used for equilibration of the substrate across the cell membrane and are *uncommon* in bacteria. On the other hand, transport systems, like the ABC Transporter or the PTS way are classified as active transport systems because they use up energy in form of ATP to translocate a substrate over the cell membrane (18). In general, both active systems are common in bacteria. However, among strict and facultative anaerobic bacteria, the *widespread* transport mechanism is the phosphoenolpyruvate-dependent phosphotransferase mechanism. In the PTS mechanism the substrate is transported

through the membrane with concomitantly phosphorylated. It is energetically, a more efficient way of substrate translocation than the ABC transporter (18). The PTS system is composed of two cytosolic proteins, called Enzyme I and His-Protein (HPr), as well as a substrate-specific enzyme complex (Enzyme II) (19). Enzyme I catalyzes the transfer of the phosphate group from phosphoenolpyruvate (PEP) to the His-Protein (HPr). The Enzyme II complex then catalyzes the transfer of phosphate from HPr to the specific substrate while transporting the substrate over the cell membrane (Figure 70) (19). This is the mechanism proposed for the uptake of sucrose by *Fritz* as it has been found in other clostridia like *C. acetobutylicum* or *C. tyrobutyricum* using sucrose.

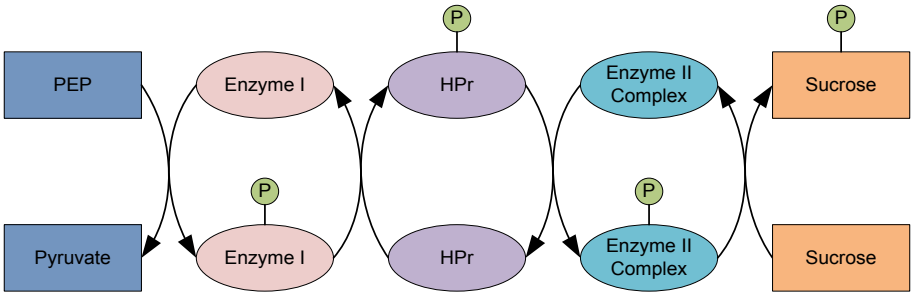

Figure 70 schematic flow diagram of the active transport of sucrose in Fritz through the cell membrane via the phosphoenolpyruvate-dependent phosphate transfer system.

Considering the proposed mechanism for sucrose uptake (Figure 70) this would then result on transformation of sucrose to sucrose-6-phosphate, which needs to be further modified before it can enter the glycolysis pathway within the cell. This further modification is done by two enzymes. The first, sucrose-6-phosphate hydrolase (S6PH) cleaves sucrose-6-phosphate to glucose-6-phopshate and fructose. Then the second enzyme, an ATP-dependent fructokinase (FK) catalyzes the phosporlyation of fructose to fructose-6-phosphate (19), which can enter the glycolysis pathway.

The less common active transport mechanism is the ABC transporter, which utilizes ATP to translocate substrates across the membrane. The transportable substrates include ions, amino acids, peptides, sugars and other molecules that are mostly hydrophilic (20). In bacteria, the majority of ABC transporters consist of a substrate binding protein, two integral membrane components and two membrane-associated ATP-binding cassettes (21). The translocation starts when the substrate binds to the outer binding proteins. This binding triggers the hydrolysis of ATP via the two inner ATP-binding cassettes. The resulting energy from the hydrolysis of ATP is used for a conformational change of the two integral membrane components and concomitant translocation of the substrate into the cell. After translocation, the transporter returns to the initial state through dissociation of ADP and an inorganic phosphate (22).

Looking at the energy balance of the transporters, 1 mol ATP was used to translocate sucrose into the cell and prepare it for the further processing through the glycolysis. To see why it is just 1 mol ATP we have to look at the individual steps of the PTS way (Figure 70) and determine the required ATP for each step. As described above, no ATP is used in the PTS for translocation and phosphorylation. The necessary energy was harnessed from the high-energy intermediate PEP coming from the glycolysis. Also, cleaving the resulting sucrose-6-phosphate into glucose-6-phosphate and fructose required no additional energy in from of ATP. The only step in which ATP was used is during the last step of the PTS way, where fructose is phosphorylated to fructose-6-phosphate via the fructokinase enzyme (FK).

In comparison, the ABC transporter utilizes already 1 mol ATP to translocate substrates across the membrane. In our case the transportable substrate is maltose. But for *Fritz* to use maltose after uptake into the cell, the substrate first needs to be cleaved into monosaccharides. One maltose is cleaved to 2 glucose monosaccharides with no use of ATP. These two glucose molecules then need to be phosphorylated by using additional 2 mol ATP in order to enter the glycolysis pathway. So, at the end of the transport chain 3 mol ATP had to be used to translocate maltose into the cell and prepare it for the glycolysis pathway.

Considering the amount of ATP which is used in both transport ways and the fact that anaerobic bacteria generally produce low levels of energy and therefore need to use their energy extremely efficient, it is clear why the PTS way is widespread among them. This theory of the two transport mechanism explains why in our experiments pyruvate was more accumulated in sucrose than in maltose (Figure 77B). It resulted from the conversion of PEP, which was produced in high amounts to keep the PTS way running. This high phosphoenolpyruvate concentration was then converted to pyruvate. But *Fritz* could not ferment more pyruvate than it already had been fermenting under these set conditions, presumably because of other limiting or inhibiting metabolic factors and thus accumulated the excess pyruvate. Contrariwise, the low pyruvate concentration when growing on maltose supports our proposition that maltose is assimilated with the ABC transporter and not the PTS way. Accordingly, the high acid production in non-carbon limiting conditions (which should not be the case as we established) can be explained with high additional demand of ATP resulting from the ABC transporter besides biomass growth and reoxidation of NAD(P)H via the ethanol pathway. The higher lactate concentration also supports this view. In order to save some ATP and to keep up with the NADH production, *Fritz* reoxidizes more NADH through the lactate pathway, which is not ATP dependent. To make it clear, ethanol production does not use

ATP per se, but a high NAD(P)H and ATP concentration are a prerequisite to initiate ethanol production.

To sum up, the two completely different metabolic patterns result from different assimilation of sucrose (PTS pathway) and maltose (ABC transporter).

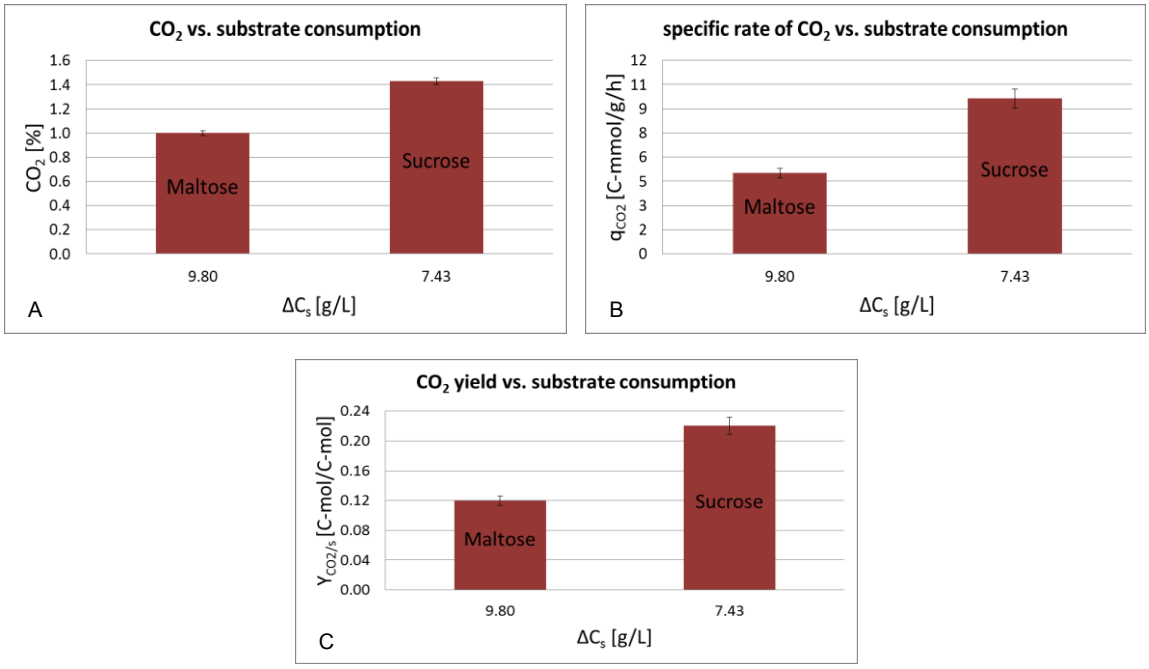

Figure 71 Physiological parameters of Fritz related to the carbon dioxide at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

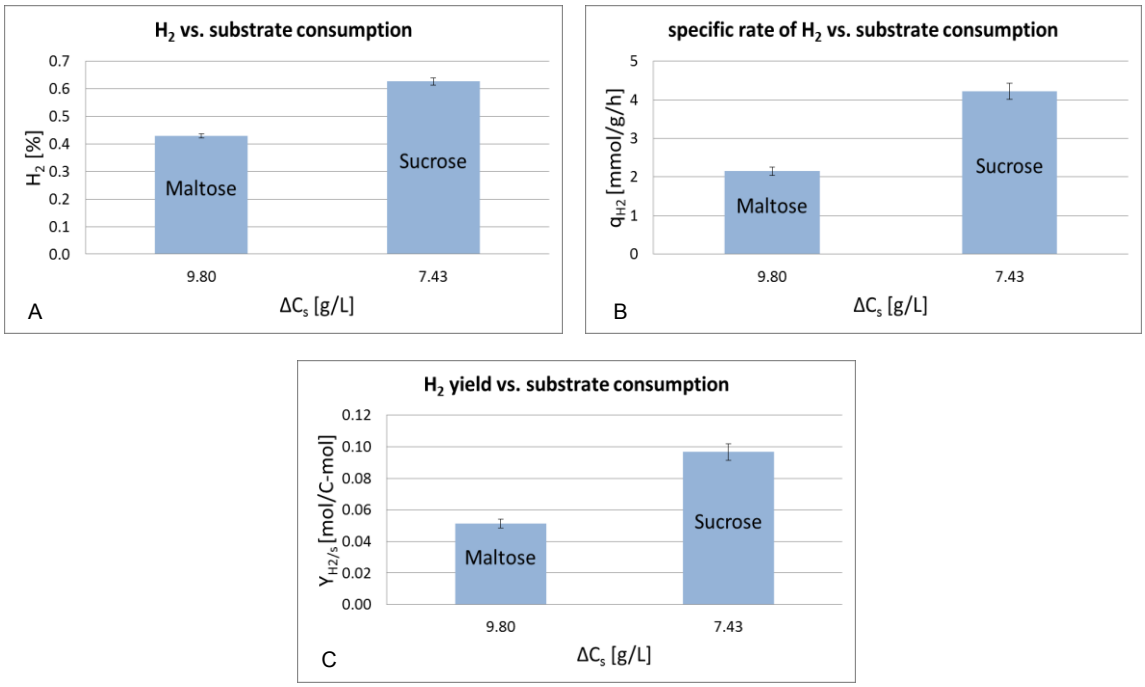

Figure 72 Physiological parameters of Fritz related to the hydrogen at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

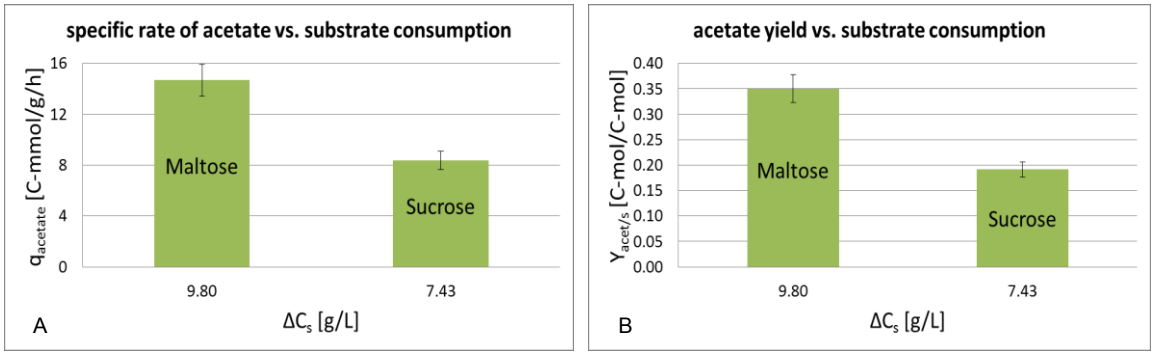

Figure 73 Physiological parameters of Fritz related to acetate at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

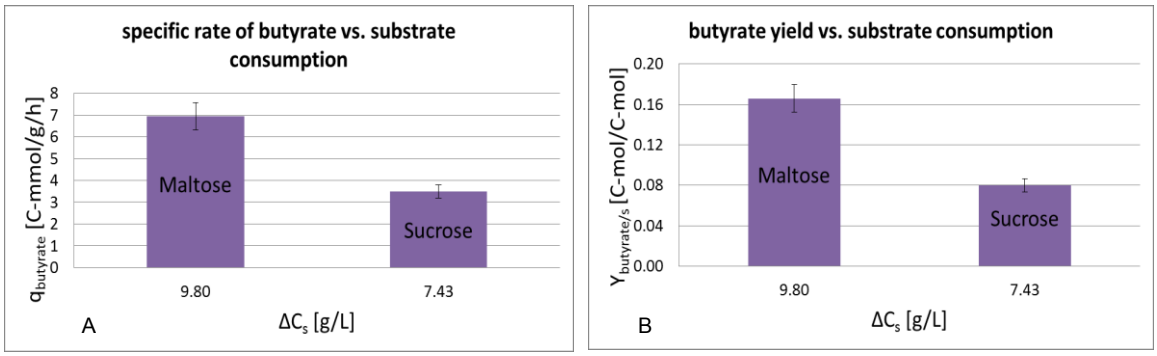

Figure 74 Physiological parameters of Fritz related to butyrate at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

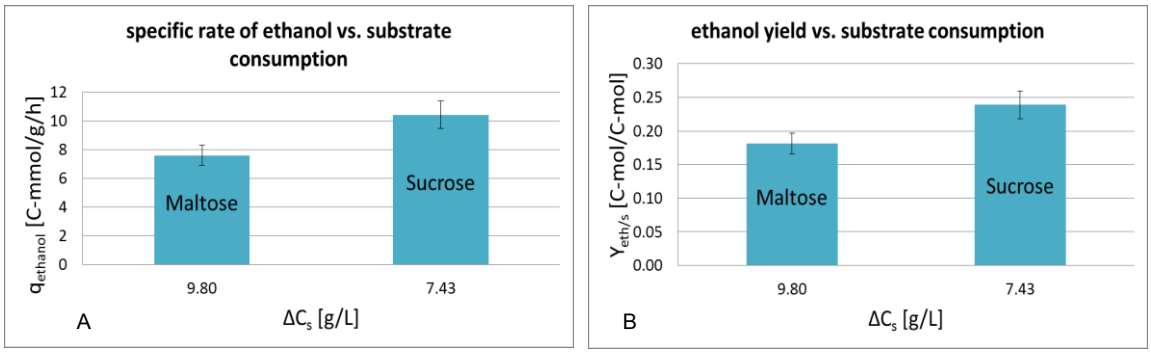

Figure 75 Physiological parameters of Fritz related to ethanol at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

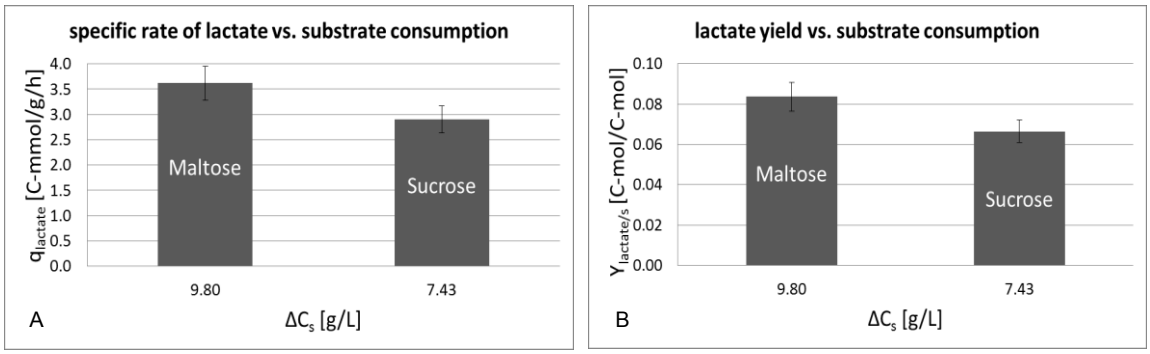

Figure 76 Physiological parameters of Fritz related to lactate at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

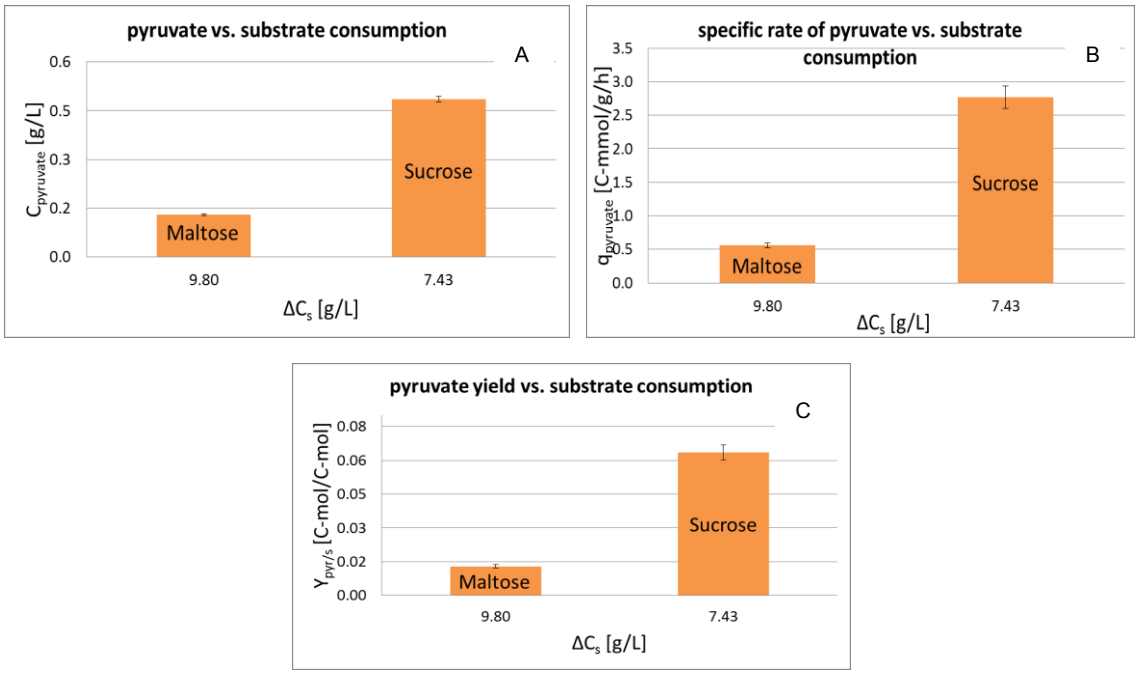

Figure 77 Physiological parameters of Fritz related to pyruvate at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

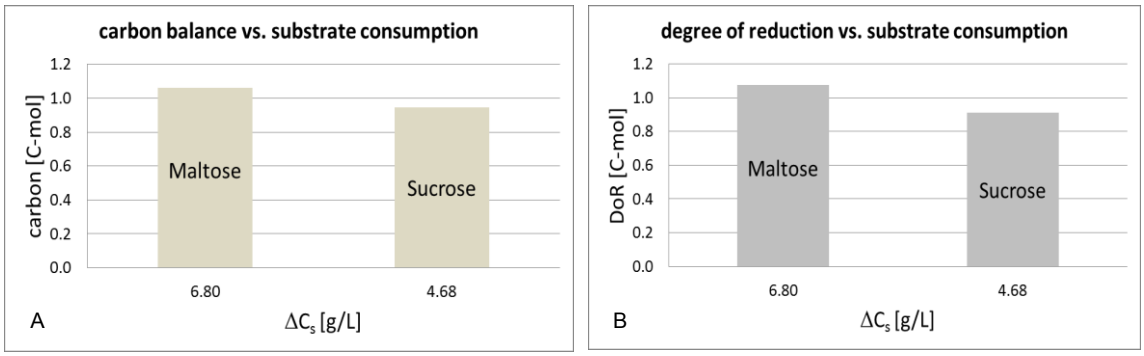

Figure 78 carbon balance and degree of reduction (DoR) of Fritz at D=0.1 h^{-1} with 16.61 g/L and 12.11 g/L maltose and sucrose, respectively.

6.1.5 Continuous culture with sucrose at D=0.1 h^{-1} and D=0.2 h^{-1}

The aim of this experiment was to see if the metabolism of *Fritz* would change when increasing the dilution rate from 0.1 to 0.2 h^{-1}. Like with all experiments the reference medium was used, containing 100% N-P and 2 g/L YE. The concentration of sucrose in the feed was 12.70 g/L. Thus, the continuous culture was set up under non-carbon limiting conditions. Because in this experiment the focus was on the effect of the dilution rates, volumetric productivity and specific productivity were not taken into consideration, when analyzing the metabolic pattern.

Increasing the dilution rate from 0.1 h^{-1} to 0.2 h^{-1} resulted in a significantly lower biomass concentration (0.53±0.02 g/L) compared to 0.60±0.02 g/L at the dilution rate of 0.1 h^{-1} (Figure 79A). While the residual concentration of sucrose (Figure 80A) increased, the biomass yield remained constant at 0.89±0.01 C-mol/C-mol. So, utilization of substrate towards biomass did not change at the higher dilution rate, although the sucrose uptake rate almost doubled from 43.64±3.25 to 80.66±6.01 C-mmol/g/h (Figure 80B). Changing the dilution rate from 0.1 h^{-1} to 0.2 h^{-1} had no effect on biomass yield.

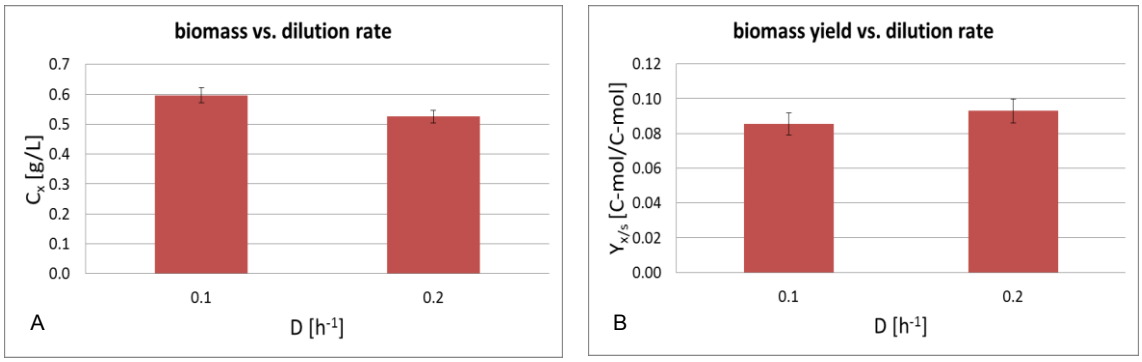

Figure 79 Physiological parameters of Fritz related to biomass with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

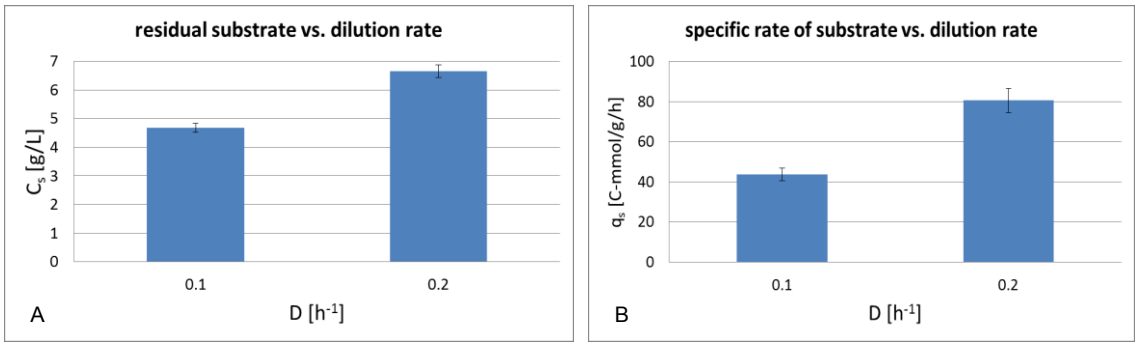

Figure 80 Physiological parameters of Fritz related to substrate with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

Comparing the yields of the metabolites it can be observed that the carbon distribution towards ethanol was the highest at both dilution rates of 0.1 and 0.2 h^{-1}. This is because *Fritz* is in the solventogenesis phase under non-carbon limiting conditions as we established in previous experiments. The increase in pyruvate yield (Figure 86B) and sucrose uptake rate (Figure 80B) supported our idea of the previous section (6.1.4), where the assimilation of sucrose by *Fritz* over the PTS system was introduced.

The acetate yield decreased at a dilution rate of D=0.2 h^{-1} from 0.19±0.01 to 0.08±0.01 C-mol/C-mol and the concentration from 1.50±0.07 to 0.52±0.02 g/L (Figure 83). Considering the findings of our previous experiments, this means that an increase of substrate supply per time in an already non-carbon limiting condition further reduced the demand of additional ATP, thus reducing production of acetate and carbon distribution towards acetate substantially. Additional support to the fact that ATP demand was lowered at increasing the dilution rates was the fact that the butyrate concentration increased insignificantly from 0.46±0.03 to 0.50±0.03 g/L (Figure 84A). Looking at the concentrations, acetate fell on a similar level as butyrate. Even the yields for both organic acids were not far away from each other, with 0.08 C-mol/C-mol and 0.11 C-mol/C-mol for acetate and butyrate, respectively. Furthermore, the decrease on hydrogen production and yield proved this statement. The H_2 offgas values fell from 0.63±0.01 to 0.43±0.01% and the H_2 yields from 0.10±0.01 to 0.04±0.00 mol/C-mol when increasing the dilution rate to 0.2 h^{-1} (Figure 82). As shown by a previous study, the electron flow towards hydrogen production results in an increase of acetate and butyrate (8). The same holds true, when hydrogen levels decrease, as our experiment and this study demonstrated.

On the other hand, the concentration and yield of ethanol increased at higher dilution rates. The concentration rose from 1.87±0.08 to 2.18±0.11 g/L and the yield from 0.24±0.02 to 0.45±0.04 C-mol/C-mol when switching from a dilution rate of 0.1 to 0.2 h^{-1} (Figure 85). A large part of the increased carbon flow (Figure 80B) was redirected towards ethanol to counterbalance the high NAD(P)H concentrations. These findings match well with observations of another study, in which solvent production in *C. acetobutylicum* was identified in glucose-sufficient cultures as a function of the specific growth rate μ, which equals the dilution rate in a continuous process. They also realized that acid production under these circumstances was low, resulting from the low demand of ATP (12).

Lactate concentration decreased when switching the dilution rate to 0.2 h^{-1} from 0.52±0.03 to 0.41±0.02 g/L but the yield remained constant at 0.07±0.00 C-mol/C-mol. The yield being constant means there was no further need for reoxidation of NADH.

The rise of the ethanol yield was enough to rebalance the NAD^+/NADH ratio after the increase in the dilution rate.

To sum up, increasing the dilution rate from 0.1 to 0.2 h^{-1} did not induce a metabolic shift in the fermentation phase of *Fritz*, which was the solventogenesis. The fact that *Fritz* was under non-carbon limitation was a more dominant factor than the dilution rate. But increasing dilution rate resulted in increasingly lower acid production and even higher solvent production than with 0.1 h^{-1}. Moreover, a higher dilution rate had no significant effect on biomass yield. As mentioned previously, only the acidogenesis phase has a positive influence towards biomass in *Fritz* stoichiometry.

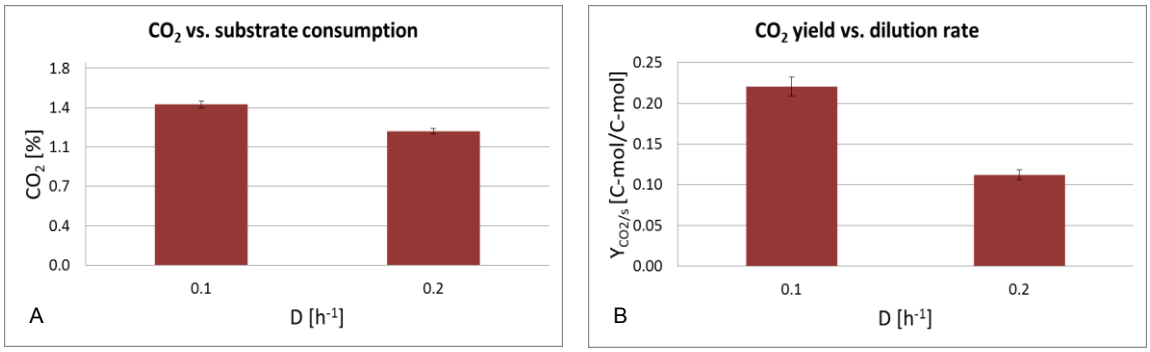

Figure 81 Physiological parameters of Fritz related to carbon dioxide with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

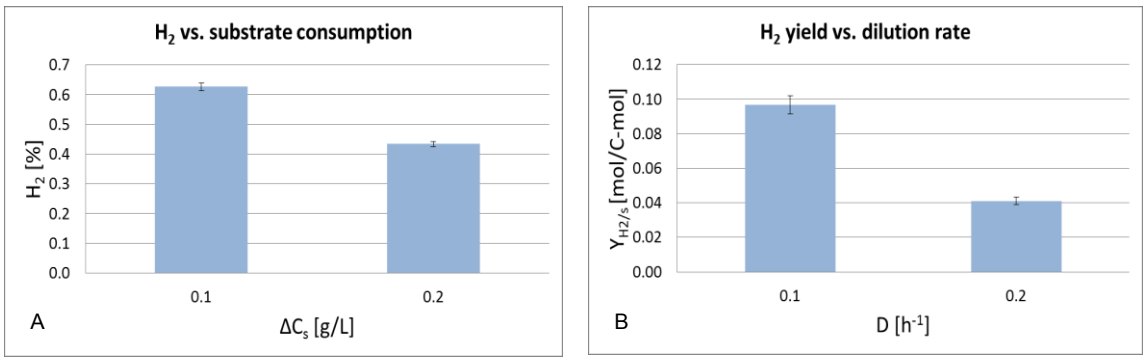

Figure 82 Physiological parameters of Fritz related to hydrogen with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

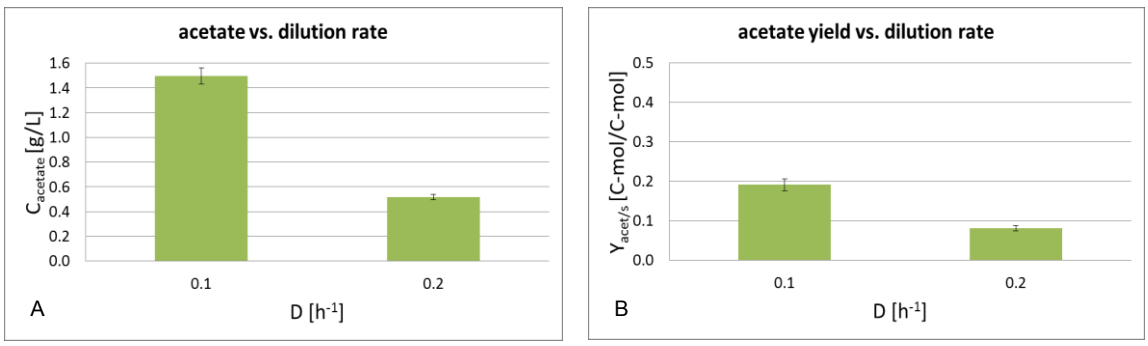

Figure 83 Physiological parameters of Fritz related to acetate with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

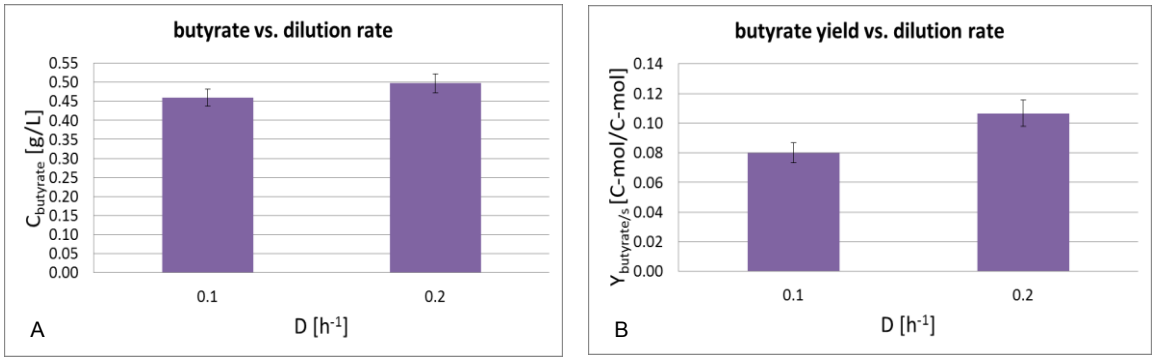

Figure 84 Physiological parameters of Fritz related to butyrate with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

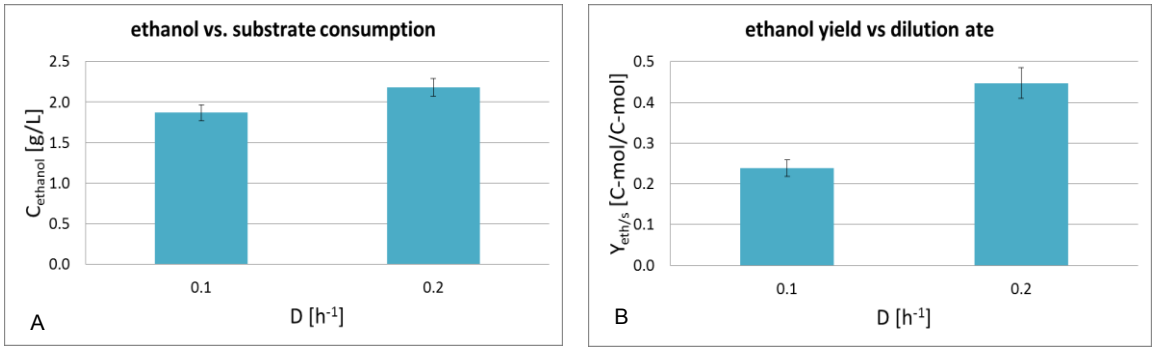

Figure 85 Physiological parameters of Fritz related to ethanol with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

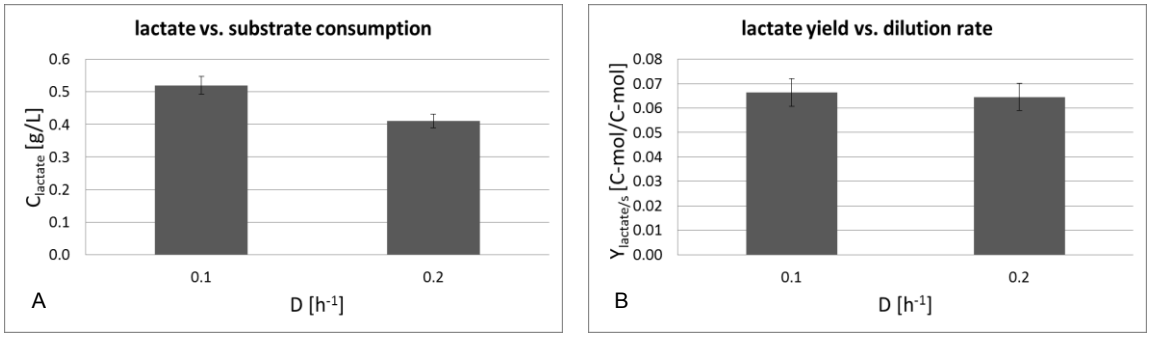

Figure 86 Physiological parameters of Fritz related to lactate with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

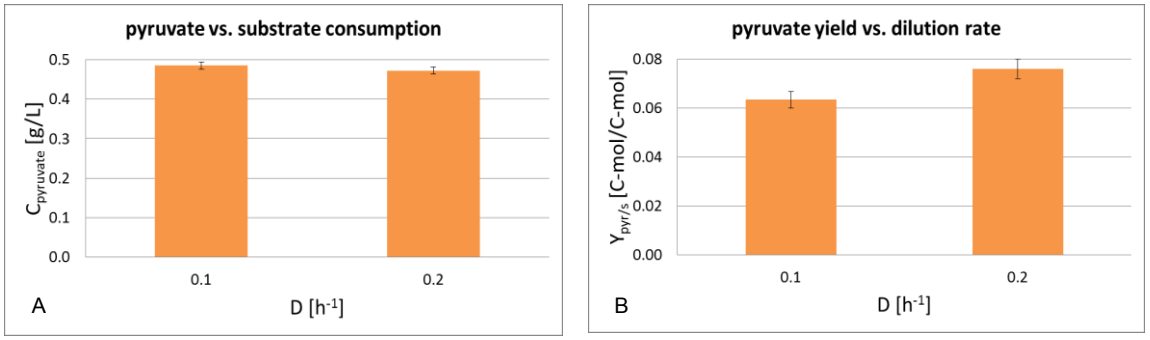

Figure 87 Physiological parameters of Fritz related to biomass with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

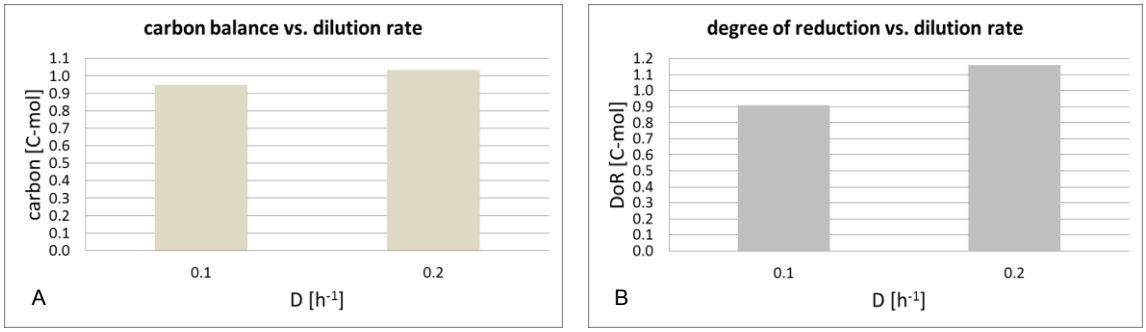

Figure 88 carbon balance and degree of reduction of Fritz related with 12.70 g/L sucrose at dilution rates of 0.1 and 0.2 h^{-1}.

Conclusion

- No growth was observed in batch mode, if cultivating *Fritz* in organic acids (butyric acid and acetic acid) and alcohols (ethanol) when no sugar was present in the broth.
- A maximum substrate uptake of 10 g/L was found if cultivating *Fritz* in 16.6 g/L maltose. Therefore, carbon limiting conditions would have been expected on continuous mode at D=0.1 h^{-1} up to 10 g/L maltose feeding concentration.
- The cultivation of *Fritz* in closed batches in fructose, glucose, maltose or sucrose at 5 g/L performed at the same maximal specific growth rate, achieving maximal OD_{580} values of [0.7, 1.1]. Fritz did grow in 5 g/L lactose, but up to a maximal OD_{580} of 0.64±0.02. No growth was observed if cultivating Fritz in xylose at 5 g/L.

  Higher OD_{580} values ([1.1, 1.4]) were achieved at 10 g/L than at 5 g/L if growing Fritz in fructose, glucose or sucrose in closed batches.

  Sucrose was the selected substrate for further experiments in continuous cultures in a bioreactor due to its lower price and the higher biomass concentrations got if cultivating *Fritz* with it in closed batch mode.
- *Fritz* did not grow in defined media, neither in those derived from the reference complex medium nor in those described for other *Clostridium sp*.
- Metabolic shifts, favorable for biomass production, were observed if pulsing complex compounds (1.2 g/L) into the fermenter broth, growing *Fritz* on a continuous mode at D=0.2 h^{-1}. Higher r_x were recorded if pulsing PM instead of YE or TP into the culture.
- A better quantification of the effects of the different tested complex compounds in closed batch mode (YE, TP, PM, PSM, SP, TS) were observed if autoclaving the corresponding compound within the basal medium instead of adding it separately into the culture.

  SP and YE resulted in the best complex compounds to be used to get high biomass productivities in *Fritz* cultivations in sucrose at 5 g/L in closed batch mode. Nevertheless, working in a bioreactor on continuous mode showed up that YE provided higher *Fritz* biomass productivities than SP.

- Fritz did not grow if the reference basal medium was supplemented with YNB instead of with a complex compound, due to the absence of organic nitrogen (amino acids).
- On one hand, no N-P-limitation could be quantified if *Fritz* was cultivated at 5 g/L maltose. On the other hand, the initial NH_4^+ and PO_4^{3-} concentrations could be reduced up to 15% (compared to the reference basal medium), if growing *Fritz* on sucrose at 5 g/L. But, if working on continuous mode at 3.5 g/L sucrose, 25% was required in order to maintain *Fritz* biomass productivity and yield at the desired values.
- Long-term continuous cultures of *Fritz* were favorable for biomass productivities and yields, especially at low substrate concentrations. Decreasing substrate concentration in a *Fritz* long-term continuous culture over time induced a shift of metabolism towards acid production and favored a stoichiometric shift towards biomass.
- High initial sucrose feeding concentrations resulted in solvent and lactate production, whereas low initial sucrose feeding concentrations resulted in acid production.
- Cultivating *Fritz* on either sucrose or maltose resulted in a completely different metabolic pattern. This pattern was due to different assimilation of both substrates. Sucrose is accumulated via the PTS system, whereas maltose is accumulated via the ABC transport system.
- Increasing the dilution rate from 0.1 to 0.2 h^{-1} had no impact on biomass yield. It also did not affect the corresponding metabolic phase, the solventogenesis. Nevertheless, increasing the dilution rate under non-carbon limitation resulted in even higher solvent and lower acid production.
- Considering all the results got during the different experiments on closed batch and continuous mode, the preselected alternative medium should include sucrose at 3.5 g/L, $(NH_4)H_2PO_4$ at 0.079 g/L, $(NH_4)_2HPO_4$ at 0.130 g/L and yeast extract at 2 g/L.

Outlook

Regarding biomass productivity, a continuous process with an external cell retention or recycle system should be tested using this new proposed alternative medium containing 3.5 g/L sucrose, 2 g/L YE , 0.079 g/L $(NH_4)H_2PO_4$ and 0.130 g/L $(NH_4)_2HPO_4$. In such a system, the bioreactor is connected with a membrane and a part of the bioreactor broth is continuously flowed through this membrane in an external loop. The retentate, consisting on concentrated cells, is recycled back into the bioreactor; while the permeate, a cell-free stream, is discarded. In this way, biomass concentration and productivity can be increased in the fermenter broth. By turning on the bleed at a desired biomass concentration, the cell retention system can be stabilized at this specific biomass concentration while increasing the biomass productivities. Combining the optimized alternative medium with such a process may lead to unmatched high biomass productivities.

The goal in this new project would be to compare the alternative medium with the reference medium running a cell retention system to study if both methodologies, medium optimization and cell retention, have an additive effect on biomass productivity.

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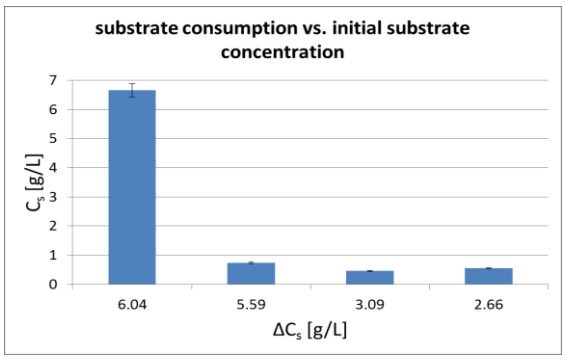
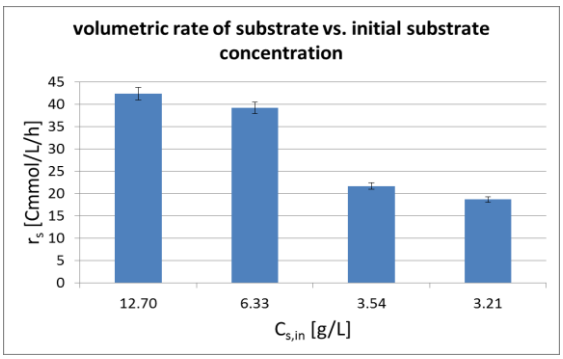
Appendices

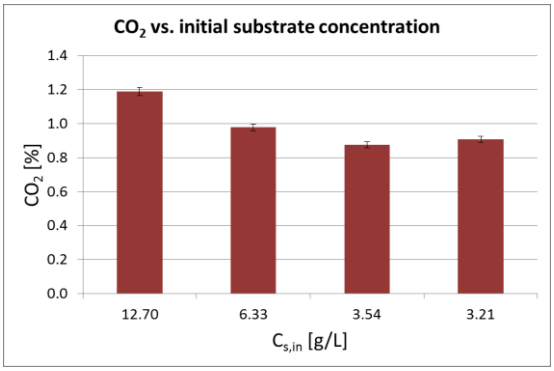
6.2 Initial sucrose concentration

6.2.1 Substrate

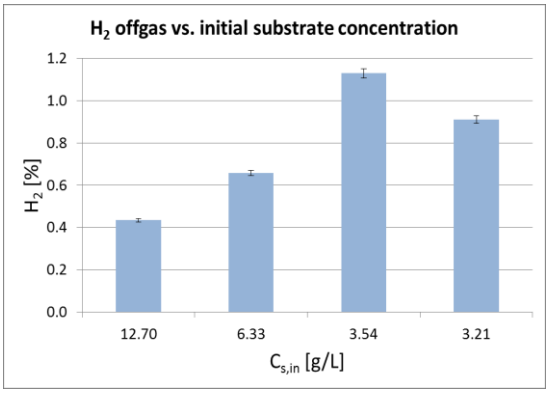




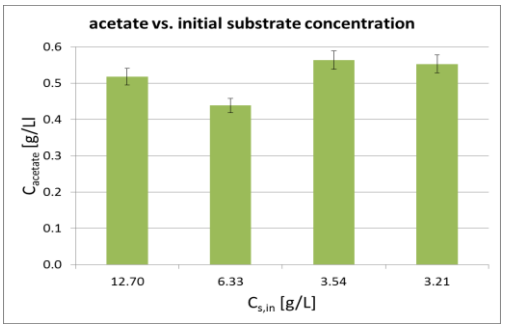
6.2.2 Carbon dioxide



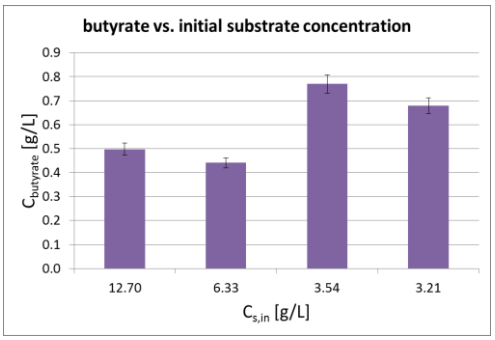
6.2.3 Hydrogen



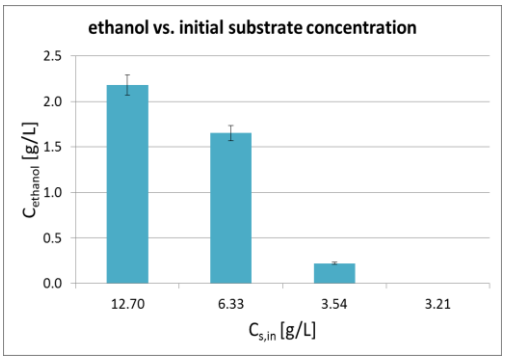
6.2.4 Acetate



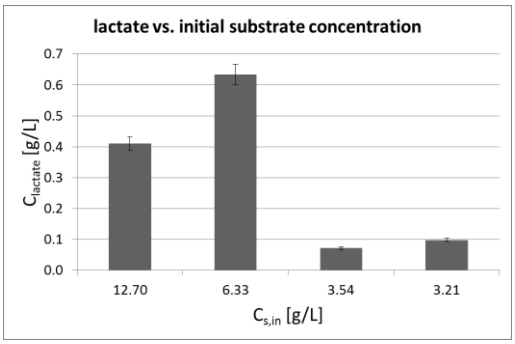
6.2.5 Butyrate



6.2.6 Ethanol

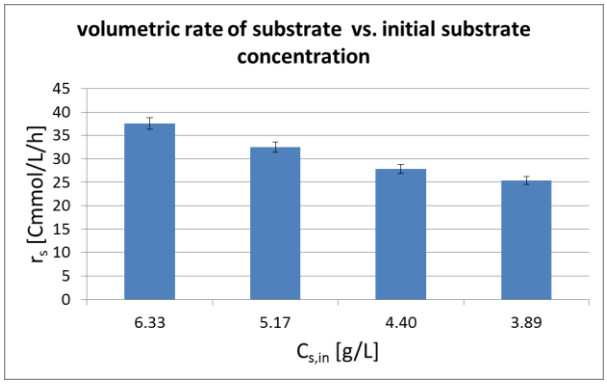


6.2.7 Lactate

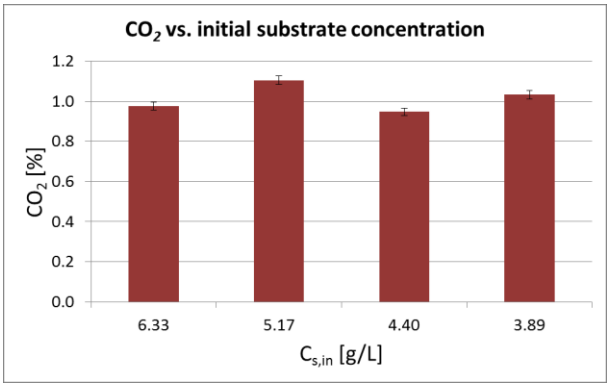


6.3 Different sucrose feed concentrations in one run over time

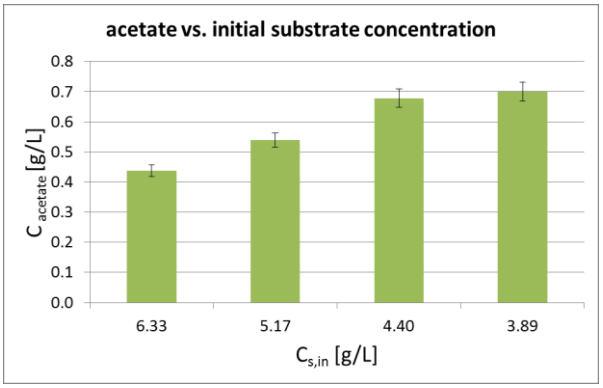
6.3.1 Substrate



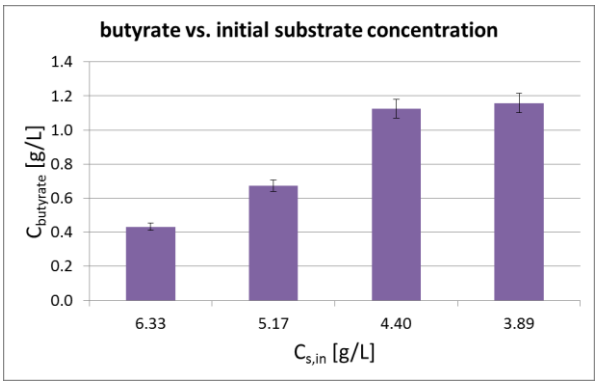
6.3.2 Carbon dioxide



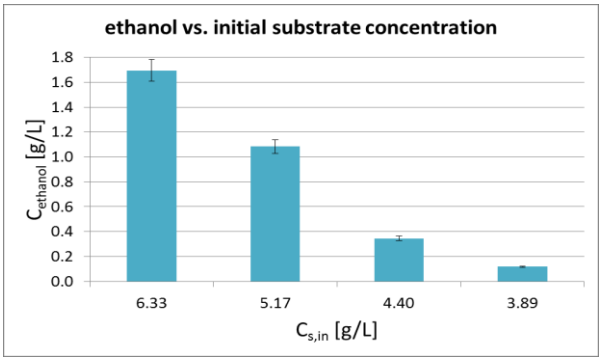
6.3.3 Acetate



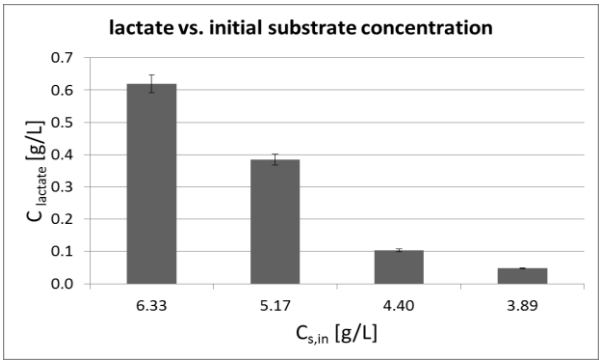
6.3.4 Butyrate



6.3.5 Ethanol

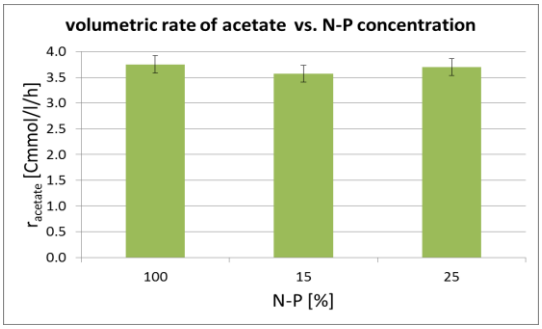


6.3.6 Lactate



6.4 Different nitrogen and phosphate concentration

6.4.1 Acetate



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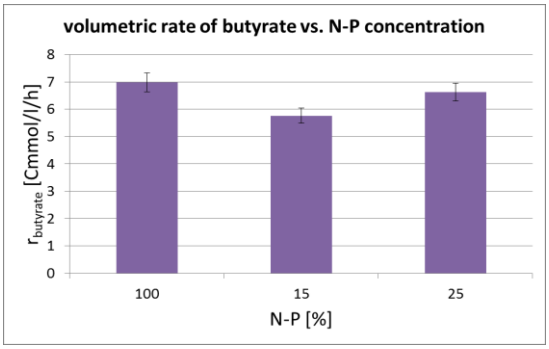

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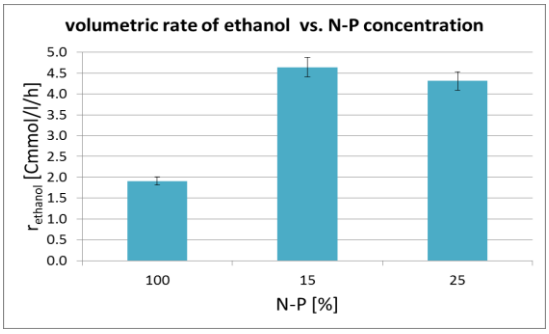
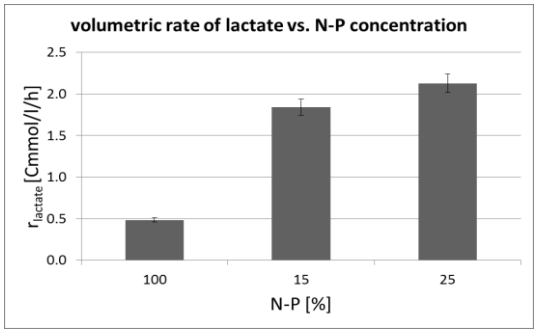
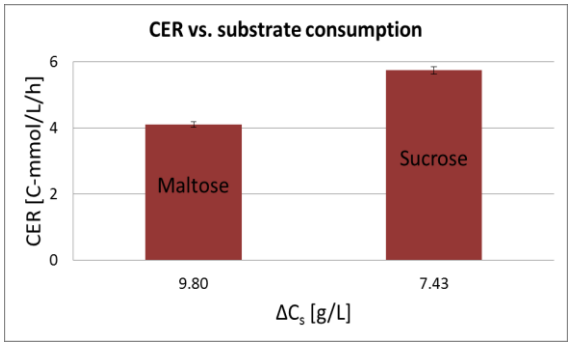


6.4.4 Lactate

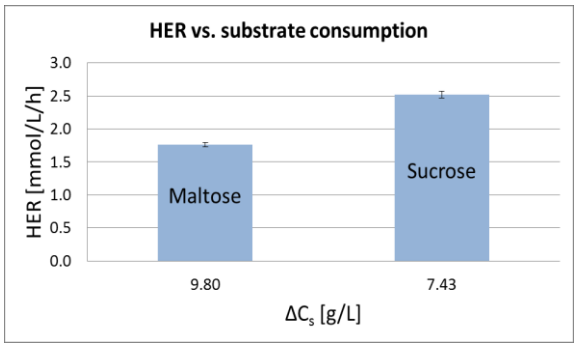


6.5 Continuous cultures under non-carbon limiting conditions with sucrose and maltose at D=0.1 h^{-1}

6.5.1 Carbon dioxide



6.5.2 Hydrogen



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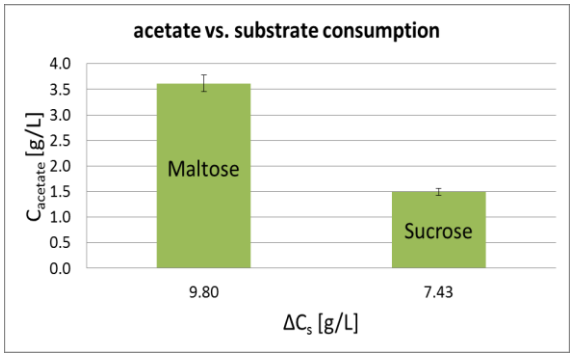
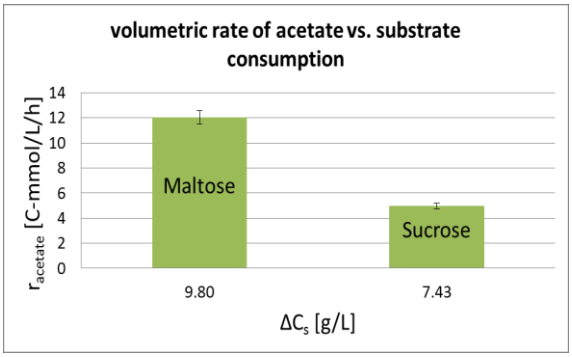
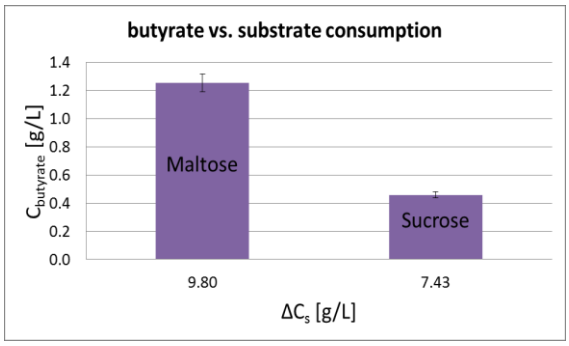
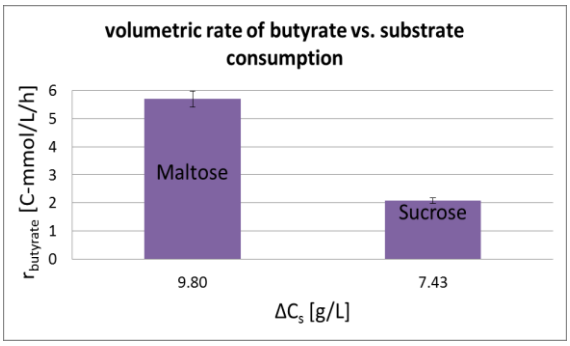


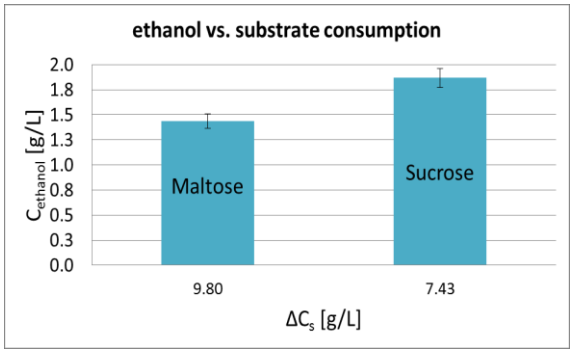


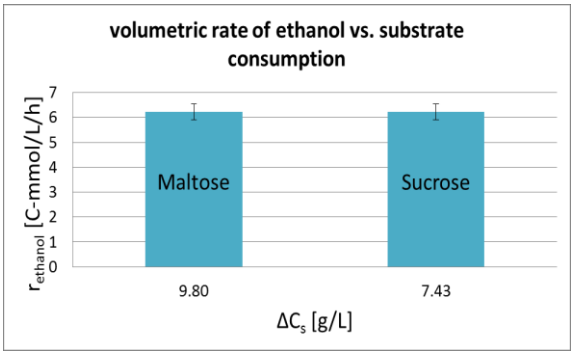
6.5.4 Butyrate



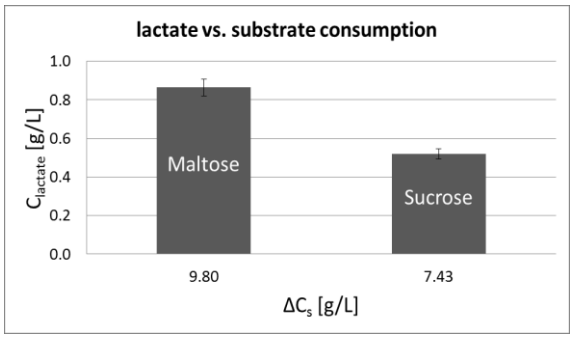


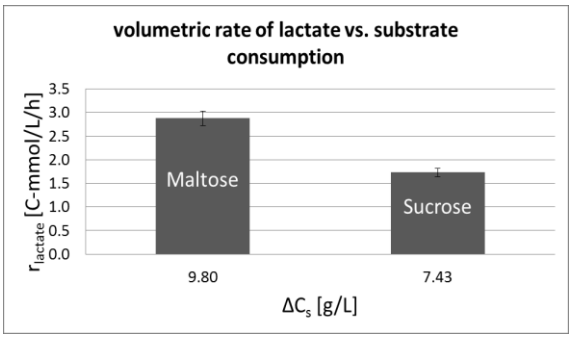
6.5.5 Ethanol





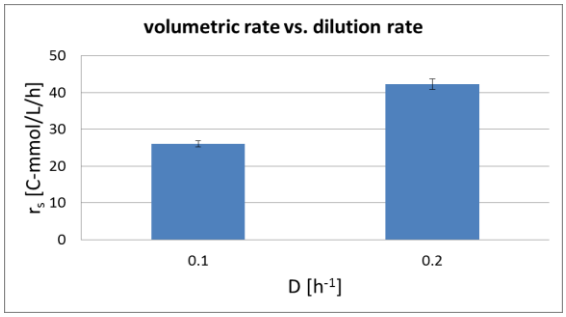
6.5.6 Lactate

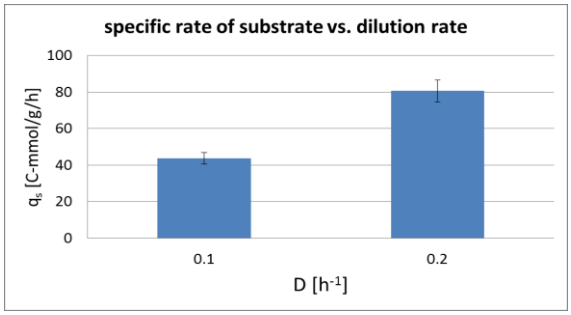




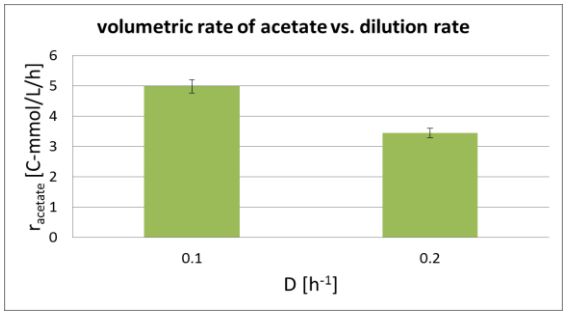
6.6 Continuous culture with sucrose at D=0.1 h^{-1} and D=0.2 h^{-1}

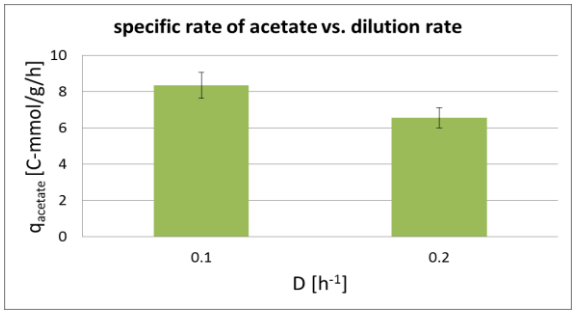
6.6.1 Substrate



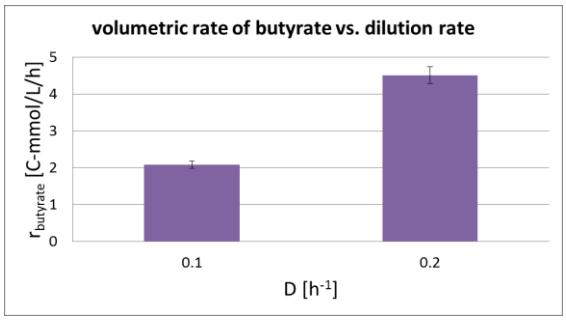


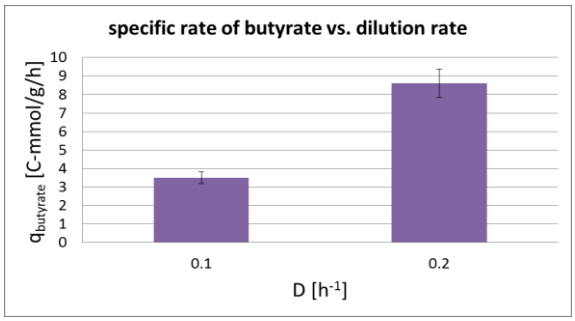
6.6.2 Acetate



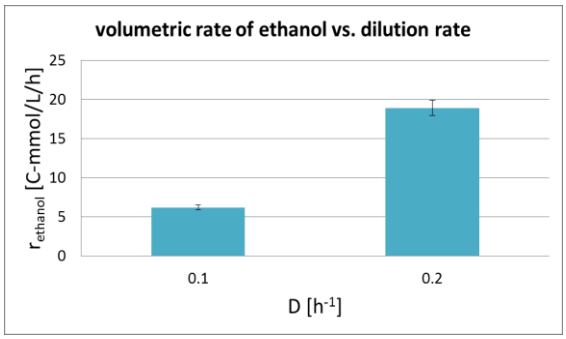


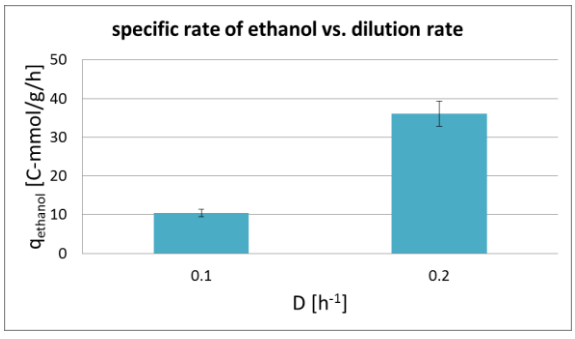
6.6.3 Butyrate



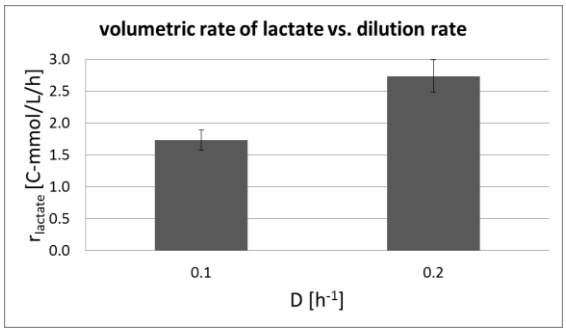


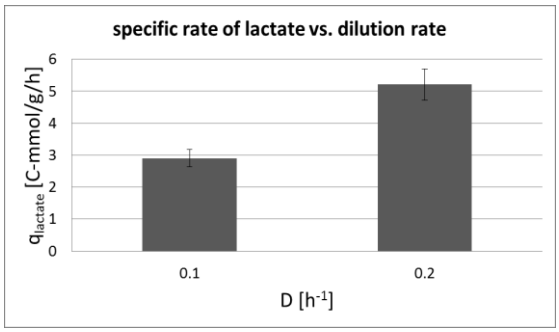
6.6.4 Ethanol





6.6.5 Lactate


volumetric rate of lactate vs. dilution rate
$r_{lactate}$ [C-mmol/L/h]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.1
0.2
D [h^{-1}]


specific rate of lactate vs. dilution rate
$q_{lactate}$ [C-mmol/g/h]
6
5
4
3
2
1
0
0.1
0.2
D [h^{-1}]

Declaration

I herewith declare that I have completed the present thesis independently making use only of the specified literature and aids. Sentences or parts of sentences quoted literally are marked as quotations; identification of other references with regard to the statement and scope of the work is quoted.

Place, Date

Signature

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Masterarbeit selbständig angefertigt habe. Es wurden nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt. Wörtlich oder sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.

Ort, Datum

Unterschrift