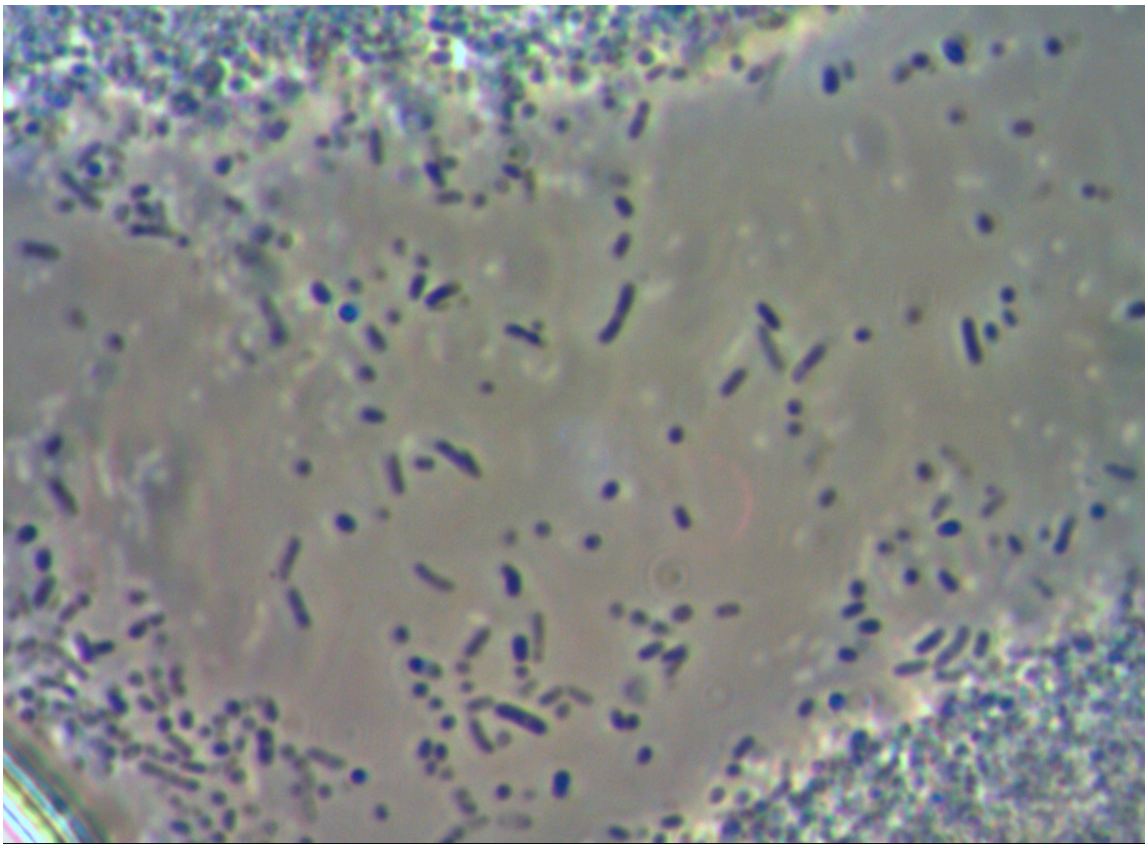
Characterization of Succinate Producing Bacteria



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Characterization of Succinate Producing Bacteria

by

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Picture on front page: *A.succinogenes*. Photo by Sofia Nyström

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Preface including acknowledgements

This master thesis has been performed and written at the Department of Chemical Engineering. All experiments and analyses were conducted at the department, except the flow cytometry analysis which was performed at Applied Microbiology. Special appreciation goes out to Magnus Carlquist for making it possible to perform the analysis and for his guidance throughout the experiment. The master thesis was performed during the period January 2015 to May 2015 and includes 30 hp (= 30 ECTS).

I would like to thank my supervisor Henrik Almqvist for all the support in the laboratory and all the patients with my questions and wonderings. I would also like to thank my examiner Gunnar Lidén for all positive comments and the time he put into my project. A special

Finally, I would like to thank my family and friends for supporting and encouraging me during the whole process.

Abstract

The aim of this project was to study two bacterial species, *Actinobacillus succinogenes* and *Basfia succiniciproducens* for production of succinic acid.

Succinic acid is a dicarboxylic acid and a metabolite in the citric acid cycle. It can be used as a food additive, to produce bulk chemicals and to make bio-based plastic (PBS). The two bacteria examined in this work need carbon dioxide to produce succinic acid and 100 % saving in greenhouse gas emissions have been reported for bio-based industrial production compared to fossil-based production. Since downstream processing is expensive, it is important to achieve high titers of succinic acid. Common purification methods are precipitation, extraction, electrodialysis and crystallization.

Shake flask, batch and chemostat cultivations have been made in order to evaluate the impact of 1) carbon source, 2) yeast extract concentration and 3) nitrogen source on yield and productivity. Regarding the carbon source, the focus has been on the five carbon sugar xylose. Xylose is available in waste streams from pulp mills, which could make it a cheaper feedstock compared to glucose, which is used in the industrial production today. Also, it is essentially a non-food carbon source.

The goal was to maintain a chemostat without biofilm formation, which allows a proper characterization, and this was successfully performed. The conditions were 10 g/l xylose and 3.5 g/l yeast extract. The cultivation gave a succinic acid yield of 0.56 g/g and a productivity of 0.50 g/l·h. The reason behind the successful cultivations was probably that the ratio between the carbon source and yeast extract concentration was suitable.

Cultivations on xylose show promising results. However, the productivity needs to be improved and somewhat higher product yields are possible. Metabolic engineering like deletion of byproduct forming pathways could be one alternative for improving these.

Sammanfattning

Målet med detta projekt var att studera två sorters bakterier, *Actinobacillus succinogenes* och *Basfia succiniciproducens*, och deras produktion av bärnstenssyra.

Bärnstenssyra är en dikarboxylsyra som även är en metabolit i citronsyracykeln. Den kan användas som surhetsreglerare i mat, för att producera andra bulk-kemikalier och för att producera bio-plaster. De två bakteriearter som undersöktes i detta arbete behöver koldioxid för att kunna producera bärnstenssyra. Det har rapporterats om 100 % minskning i utsläpp av växthusgaser för den biologiska produktionen jämfört med bärnstenssyra producerad från fossila källor. Eftersom uppreningsprocessen är kostsam är det viktigt att nå höga koncentrationer av bärnstenssyra. Vanliga metoder för upprening är utfällning, extraktion, elektrodialys och kristallisation.

Odlingar har genomförts i skakflaskor, men även som satsvis och kontinuerliga odlingar i fermentorer för att utvärdera effekten av kolkälla, jästextrakt och kvävekälla på utbyte och produktivitet. Vad gäller kolkällan så har fokus varit på xylos, ett socker med fem kol. Xylos finns i avloppsströmmar från vissa pappersmassabruk vilket gör att det är ett billigare råmaterial jämfört med glukos, som i nuläget används i den industriella produktionen. Xylos har även fördelen att vara en kolkälla som inte används till mat.

Ett delmål med experimenten var att genomföra en chemostat odling utan bildning av biofilm, vilket ger möjlighet för en ordentlig karakterisering. Chemostatodlingen genomfördes med 10 g/l xylos och 3.5 g/l jästextrakt och ingen biofilm bildades. Utbytet av bärnstenssyra var 0.56 g/g och produktiviteten var 0.50 0.50 g/l·h. Anledningen till att odlingen blev framgångsrik var troligen att förhållande mellan kolkällan och jästextraktet var passande.

Odlingar med xylos som kolkälla visar på lovande resultat. Dock måste produktiviteten ökas och även något högre utbyten är möjligt att nå. Ett sätt att förbättra dessa kan vara genom att deletera reaktionsvägar för biprodukter.

Table of Contents

1 Introduction and aim

Succinic acid is a dicarboxylic acid which used in production of, for example, food, pharmaceuticals and in the biodegradable plastic industry. Since the amount of petroleum is decreasing there is a considerable industrial interest for biological production of succinic acid from renewable resources and the production of bio-based succinic acid is almost as big as the petrochemical-based production. By using lignocellulosic feedstock as sugar source the sugar costs can be reduced and the competition with food is avoided. Some lignocellulosic feedstocks contain a lot of sugars with five carbon atoms, like xylose.

There are a number of potential microbial production hosts, both natural producers and genetically modified organisms. In this project, two of the best natural succinate producers will be studied; *Actinobacillus succinogenes and Basfia succiniciproducens*.

In this study, two bacterial species will be investigated, *A. succinogenes and B. succiniciproducens*. Of specific interest is the comparison between xylose and glucose utilization. Most previous studies so far have concerned succinate production from (pure) glucose.

For this purpose, shake flask, batch and chemostat cultivations will be made for these strains. This allows a careful determination of yields and productivities on the two carbon sources. The focus will however be on xylose as carbon source.

Since the project only is 20 weeks, the main constraint is time. The amount of experiments which are possible to conduct are limited. Moreover, two reactors for batch and chemostat cultivations were available. Since the experiments should be performed in duplicate, one experiment at a time was made.

2 Background

The background section serves as an introduction to the project, presenting information about the chemical succinic acid and the organisms studied. Some previous studies are also summarized.

2.1 Succinic acid

This section will present information about succinic acid as a molecule and its commercial use. Furthermore, the industrial production and some purification methods for succinic acid will be described.

2.1.1 The molecule

Succinic acid is a metabolite in the tricarboxylic acid cycle (TCA), where it is an electron donor for the production of FADH2 and fumaric acid. It is odorless and colorless. Succinic acid is a dicarboxylic acid, which means that it contains two –COOH groups (Figure 2.1). Because of these groups it can form salts and esters. It is the two carboxylic groups that mainly decide the chemical behavior. Succinic acid has a boiling point of 235 ºC and pK_{a1}=4.21 and pK_{a2}=5.64. When succinic acid reacts with monoalcohols it forms esters that are important lubricants and plasticizers. When reacting with dialcohols, such as glycols, it forms polyesters (Cornils & Lappe 2012).

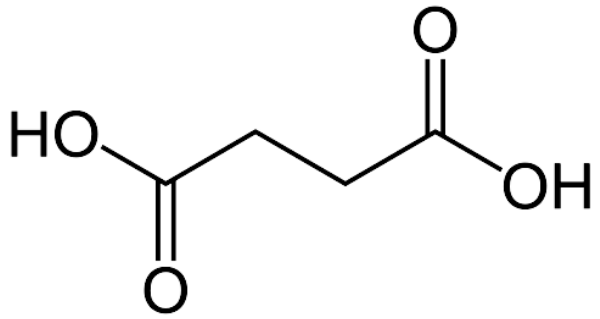

Figure 2.1. Chemical structure of succinic acid.

2.1.2 Commercial use

Succinic acid is used as food and pharmaceutical additive, ion chelator and as acidity regulator in the food industry. An overview of these and other applications for succinic acid are displayed in Figure 2.2.

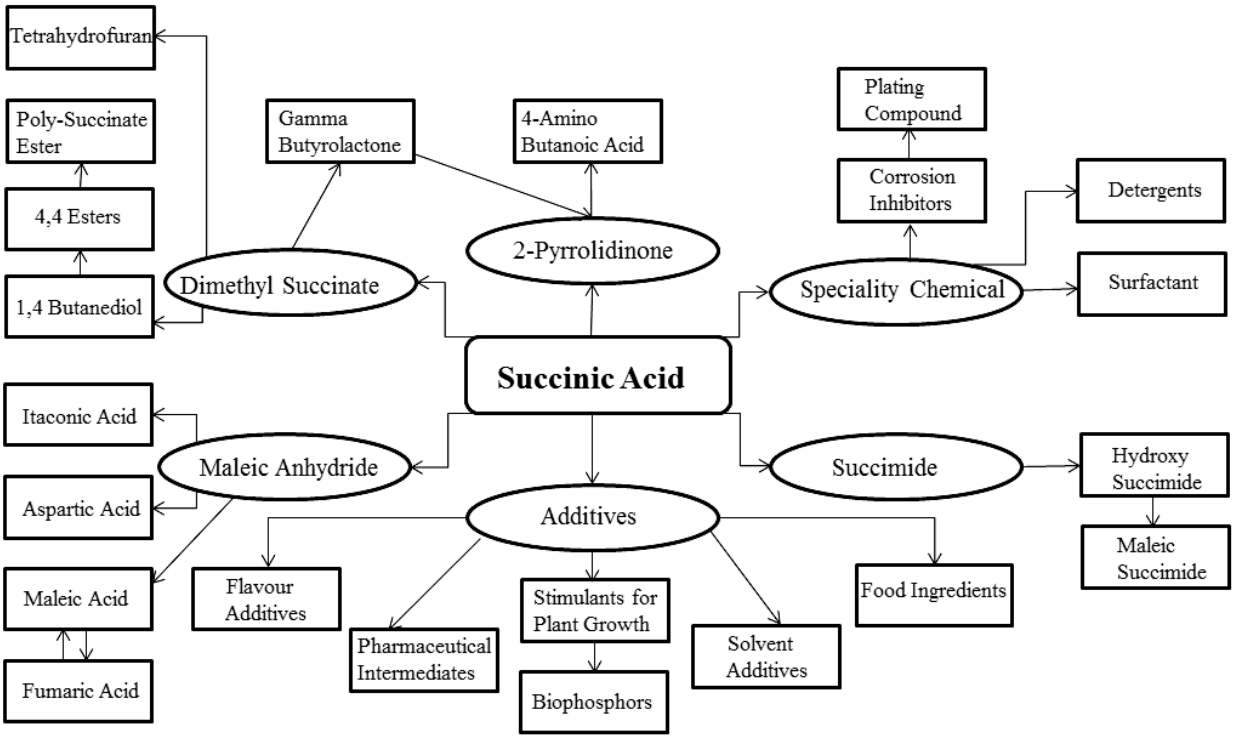

Figure 2.2. Overview of what succinic acid can be used for (Song & Lee 2006).

Previously, succinic acid was mainly produced from petroleum but since the amount of petroleum is decreasing and the environmental impact has to be taken into consideration, other methods for producing chemicals such as succinic acid are desirable. Succinic acid is a metabolite in the tricarboxylic acid cycle (TCA) and can therefore be produced by fermentation. Fermentatively produced succinic acid can thereafter be chemically converted to other bulk chemicals, like 1,4-butanediol (BDO) and adipic acid. Polybutylene succinate (PBS) can also be derived from succinic acid. PBS is the polyester of succinate and BDO and it is an important polymer for the production of bio-based plastics (McKinlay, Vieille, et al. 2007).

For the bio-based succinic acid to be able to get on the market, the production must be cost effective. The bacteria that are used must be able to utilize cheap feed stock and not produce a lot of by-products. Byproducts will make the purification more difficult and hence, more expensive. One way to decrease the costs is to build the production plant next to a process that produces the feedstock, for example a pulp mill. This will minimize the transportation costs (McKinlay, Vieille, et al. 2007).

2.1.3 Industrial production

The advantages of a bio-based production of succinic acid are lower environmental impact and price competitiveness. If the cost of the raw materials can be decreased, the production costs will be significantly reduced.

During 2013 about 38 000 tons of bio-based succinic acid was produced globally. This can be compared to 40 000 tons of fossil-based succinic acid. There are currently four companies working with the production of bio-based succinic acid; Reverdia, Succinity, BioAmber and Myriant, all with glucose as carbon source. Succinity involves the company BASF, and is working with *B.succiniciproducens* at a 10 ktpa plant in Spain. Succinity, BioAmber and Myriant are all planning to start up new production plants with capacities up to 200 ktpa in the coming years (E4tech, RE-CORD and WUR 2015).

The costs for producing bio-based succinic acid compared to petroleum-derived have been almost equal since 2013. It is therefore believed that decreasing the costs for the production of bio-based succinic acid will make it even more competitive on the market.

Besides that the feedstock for bio-based production comes from renewable resources, there is an additional positive environmental impact. BioAmber has reported that production of petro-chemical succinic acid emits 7.1 kg CO_2e/kg which can be compared -0.18 CO_2e/kg for bio-based succinic acid. This is equal to 100 % GHG savings. The energy use for bio-based succinic acid is 2.8 times less than the energy use for fossil-based (E4tech, RE-CORD and WUR 2015).

2.1.4 Purification

To purify succinic acid is a costly process and requires a combination of different purification methods. Therefore it is necessary that the fermentation gives a high titer and if possible, gives conditions that can simplify the purification process. Figure 2.3 shows some options to purify succinic acid. The first step is to remove microbial cells. This can be done with, for example, centrifugation or membrane filtration. The next step in all cases is to remove impurities and to make a primary separation of succinic acid from the fermentation broth. This can be done with electrodialysis, extraction, adsorption or precipitation. The final step to purify succinic acid is often crystallization, but vacuum evaporation can also be used (Cheng *et al* 2012).

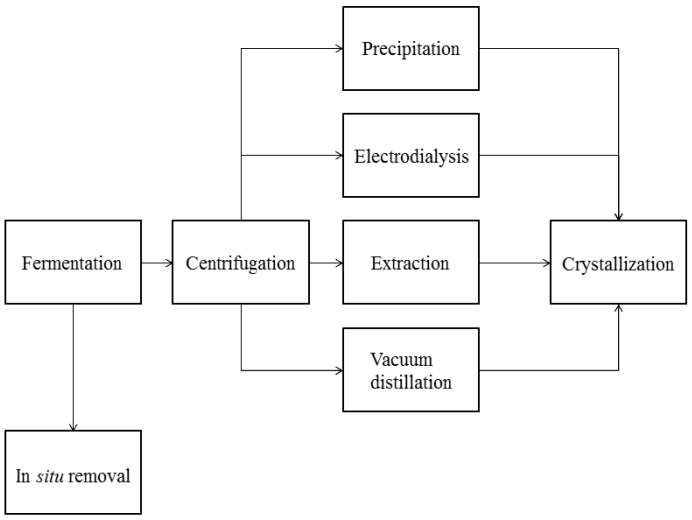

Figure 2.3. Some purification options from fermentation to purified succinic acid.

Precipitation. If $Ca(OH)_2$ or CaO is added to the fermentation broth, succinic acid can be precipitated. $Ca(OH)_2$ and succinic acid forms calcium succinate which can be removed as a solid. The is then reacted with concentrated sulfuric acid so that the succinic acid is solubilized again. However, to precipitate succinate requires a lot of $Ca(OH)_2$ and H_2SO_4 which leads to high operation costs. Furthermore, a lot of gypsum is generated (Cheng et al. 2012).

Electrodialysis. By applying an electrical potential to an ion exchange membrane, succinic acid can be purified from an aqueous solution. In the first step of electrodialysis, the acids are separated from uncharged compounds like sugars and proteins. Since the acids are charged they pass through the membrane, but about 20 % of the succinic acid is lost during the process and the equipment is expensive (Cheng et al. 2012).

Liquid-liquid extraction. This method is based on the difference in solubility for the acids between two liquid phases. It can be used both for purification and concentration. It requires low energy input and gives a high recovery yield. The main drawback is that it requires large amounts of extraction agent. This problem can be solved with reactive extraction where the carboxylic groups in succinic acid are removed, then the succinic acid is recovered by liquid-liquid extraction. Aliphatic amines are good for this purpose and the efficiency depends on, for example, the extractant and the pH of the aqueous phase (Cheng et al. 2012).

Distillation. For example, vacuum distillation at 60 ºC can be used to remove volatile carboxylic acids, like formic acid and acetic acid. The solution with succinic acid is then further purified with crystallization (Cheng et al. 2012).

Crystallization. After vacuum distillation, the temperature can be lowered to 4 ºC and succinic acid can be crystallized. However, the yield and purity for *A.succinogenes* is quite low for this method, only about 45 % purity is obtained. Alternatively, direct crystallization can be used. The principle behind is that the carboxylic acids vary in distribution of undissociated and dissociated forms depending on the pH. The undissociated acids have a different solubility compared to the dissociated. At pH 2 and 4 ºC, succinic acid has only 3 % solubility while the other acids (lactic, formic, acetic) are completely soluble in water. Succinic acid can therefore be selectively crystallized with a purity of 90 %. Some product is, however, lost since some succinic acid still is water soluble (Cheng et al. 2012).

***In situ* separation.** Succinic acid at high concentrations has been known to obstruct the cultivation process by inhibiting substrate consumption, further production of succinic acid and cell growth. By removing succinic acid continuously from the cultivation broth, by for example extraction, the inhibition of growth and production could be prevented. Additionally, there would be minimal need for adding other chemicals. Although, there is a risk for a toxic solvent (Cheng et al. 2012).

The pros and cons of the purification methods are summarized in Table 2.1.

Table 2.1. Summary of advantages and disadvantages for the presented purification methods.

Method	Advantages	Disadvantages
Precipitation	Few technological risks Viable for commercial purification	Large doses of chemicals High operational costs
Electrodialysis	Minimal use of chemicals High recovery yield	Expensive equipment Loss of product
Extraction	Low energy consumption	No efficient extractant found so far Hard to scale-up
Distillation	Effective for removal of volatile compounds	Requires further purification steps
Direct Crystallization	Requires few steps	Low purity and yield Not for commercial purification
***In situ* separation**	Energy-saving Preventing inhibition of biomass growth	Complicated process Not ready for commercial use

2.2 *Actinobacillus succinogenes*

Actinobacillus succinogenes is a natural producer of succinic acid. It is gram-negative bacterium which is pleomorphic and can occur as either rod or coccoid. It has been isolated from the bovine rumen and no signs of toxicity have so far been found (McKinlay et al. 2010).*A. succinogenes* can utilize several different sugars and it can accumulate high concentrations of succinate. The bacteria does not form spores (Guettler et al. 1999).

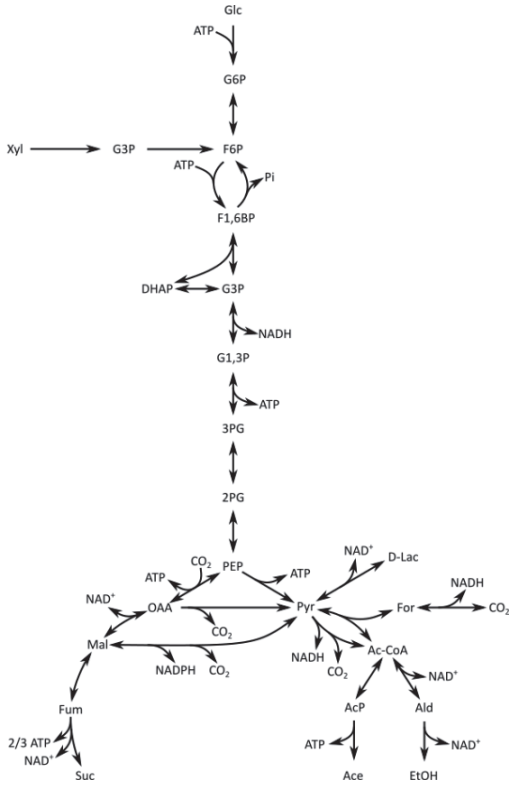

Figure 2.4. Metabolic map for A.succinogenes (McKinlay, Vieille, et al. 2007).

The main by-products are formate and acetate. At the PEP-node, phosphoenolpyruvate splits in two pathways, the C_3 and the C_4 pathway of the tricarboxylic acid cycle (TCA). The TCA occurs in all living cells and it is a series of biochemical reactions which convert a six carbon compound, citric acid, into a four carbon compound, oxaloacetate together with two CO_2 and reducing equivalents in the form of NADH (FADH). These latter compounds are under aerobic conditions oxidized, giving ATP in the process. The C_4 pathway is also called the reductive branch of the TCA cycle and it starts with that the enzyme phosphoenolpyruvate carboxylase which carboxylates phosphoenolpyruvate into oxaloacetate. Oxaloacetate is then converted to succinic acid via the reductive TCA. As can be seen in Figure 2.4, the C_4 pathway requires CO_2 to produce succinic acid. Because of this CO_2 demand, no succinic acid is formed during aerobic conditions. From the C_3 pathway ethanol, lactic acid, formic acid and acetic acid can be produced (McKinlay, Shachar-Hill, et al. 2007). *A.succinogenes* requires three essential amino acids; glutamate, methionine and cysteine. It can utilize different nitrogen sources, for example, ammonium chloride (Mckinlay et al. 2005). Previous studies have shown that succinic acid (>104 g/l), formic acid (>16 g/l) and acetic acid (>46 g/l) have a toxic effect on *A.succinogenes* and inhibits growth (Lin et al. 2008). In order to adjust the succinic acid production to industrial scale, metabolic engineering will probably be needed to divert the flux away from possible byproducts (McKinlay, Vieille, et al. 2007).

The maximum theoretical yield of succinic acid is 1.12 g/g when a bacteria is grown on glucose (Beauprez et al. 2010). One previous study of a chemostat cultivation made on glucose and at a dilution rate of 0.10 h^{-1} ,resulted in a succinic acid yield of 0.61 g/g and a productivity of 0.71 g/l·h (Brink & Nicol 2014). For batch cultivations on glucose, succinic acid yields between 0.46-0.82 g/g have been reported. When grown on glucose and yeast

extract, the yield was 0.62 g/g with a succinic acid productivity of 1.35g/l·h. Logically, the byproduct forming pathways should be deleted in order to enhance production of succinic acid. This has been done for formic acid and the result was that the engineered *A.succinogenes* produced more pyruvate instead of more succinate (Beauprez et al. 2010).

2.3 *Basfia succiniciproducens*

Basfia succiniciproducens is a coccoid, gram-negative bacteria that has been isolated from the bovine rumen. So far, no investigations imply that the bacterium have any toxicity towards human, fish or bovine cells. Like *A.succinogenes*, the bacterium belongs to the family Pasteurallaceae. Under anaerobic conditions it naturally produces succinic acid (Kuhnert et al. 2010). The metabolism of *B.succiniciproducens* is equal to the one for *A.succinogenes* (Figure 2.4) (Becker et al. 2013). Previous studies have shown that formate and acetate are the main by-products (Becker et al. 2013). When performing continuous cultivation for fermenting succinic acid it has been shown that the process is stable and since *B.succiniciproducens* can ferment for example glycerol it is possible for the production process to be cost competitive (Scholten, Renz, & Thomas, 2009).

For *B.succiniciproducens* in batch cultivations grown on glucose, a succinic acid yield of 0.49 g/g was reported. In order to improve the succinic acid yield, lactate dehydrogenase and pyruvate-formate lyase was deleted. This resulted in a 1.44 times higher succinic acid yield (Becker et al. 2013).

2.4 Other organisms

Other microorganisms, which do not produce succinic acid naturally, can be engineered in order to increase the yield. Organisms like *Escherichia coli* and *Saccharomyces cerevisiae* are common to use, since their metabolic pathways are well known.

One way to increase the yield of succinic acid in *E.coli* is to delete succinate dehydrogenase, the enzyme which converts succinate into fumarate in the oxidative TCA (Figure 2.5). This disrupts the oxidative TCA cycle and allows succinic acid to be an end product. This had a succinic acid yield of 0.22 mol/mol, but a lot of alfa-ketoglutarate was accumulated. It was therefore examined what would happen if the alfa-ketoglutarate dehydrogenase complex (sucAB) was overexpressed in order to get more of it converted into succinic acid. The native promoter of alfa-ketoglutarate dehydrogenase (sucAB) was replaced with a strong promoter. This resulted in a 20% higher succinate yield (0.28 g/g) compared to were only sucCD was deleted and 55.8 % higher compared to the control strain. The level of alfa-ketoglutarate decreased to 0.27 mM, compared to 5.5 mM for the strain where alfa-ketoglutarate dehydrogenase was not overexpressed (20% decrease) (Li et al. 2013).

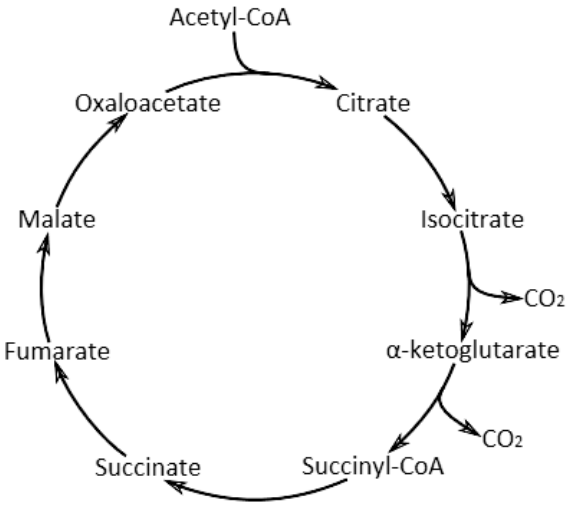

Figure 2.5. The tricarboxylic acid cycle.

Another possibility for production of succinic acid is to use *Lactobacillus plantarum*. This strain has an incomplete TCA cycle and produces succinic acid in small amounts. In order to improve the yield of succinic acid pyruvate carboxylase (PC), phosphoenolpyruvate carboxylase (PEPCK) and malic enzyme (ME) was overexpressed in order to increase the metabolic flux from the glycolysis. The enzymes were overexpressed in one strain each and all strains showed an increased activity of the enzyme, suggesting that these three enzymes are active and that they could increase the metabolic flux from the glycolysis to the TCA cycle. All strains gave a higher yield of succinic acid compared to the control strain. The strain with PC gave the highest yield, it converted 23.8 % of glucose to succinic acid. There were no signs of succinic acid being accumulated intracellular. One final approach was to overexpress two enzymes at the same time. The combination PC/PEPCK gave a slightly higher conversion of glucose to succinic acid; 25.3 %. The reason behind this is that PC gives a higher succinate yield while PEPCK enhances biomass formation and the combination of both gave a successful result, a yield of 0.34 g/g (Tsuji et al. 2013).

3 Material and methods

The material and methods- section will describe the procedure of the cultivations and analyzes. Recipes for all stock solutions can be found in Appendix A.

Before starting the pre-culture or cultivation, all equipment to be in contact with the bacteria was autoclaved. *A.succinogenes* (CCUG-43843) ordered from CCUG in Gothenburg, Sweden and *B.succiniciproducens* (DSM-22022) ordered from DSMZ in Braunschweig, Germany were used.

Pre-culture. The pre-cultures were cultivated in 100 ml shake flask with 50 ml TSB medium for the shake flask cultivations and in 250 ml shake flasks with 50 ml TSB medium for the batch cultivations. The pre cultures were put in a 37 ºC water bath (1092, GFL, Hanover, Germany) for ten to twelve hours before the inoculation. Before inoculation the OD value was measured to confirm it was around1-1.5.

Sampling. Samples for OD and HPLC were taken continuously throughout the cultivation. A "dead volume" of 2 ml was taken out before each sampling in order to remove residues from previous sampling.

The experimental plan for the experiments is summarized in Appendix D. All cultivations contained glucose or xylose as carbon source, yeast extract, deionized water, pre-culture and mineral medium. The volume of pre-culture and mineral medium (Appendix A) was each 10 % of the cultivation volume.

Shake Flask cultivation. The cultivation was performed in 300 ml shake flasks at 37 ºC (Gyromax^{TM} 929, Amerex Instruments Inc, Lafayette, USA). The cultivation volume was 100 ml. Additionally to what is mentioned above, the shake flask also contained 0.8 µl antifoam and magnesium carbonate as buffer. Carbon dioxide was supplied to the shake flasks as extra carbon source and in order to maintain the pH. Shakeflask cultivations were performed with both *B.succiniciproducens* and *A.succinogenes* and the experiments can be found in Appendix D.

Batch cultivation. The batch cultivations were performed with both bacteria and in two 1.0 L bioreactors (SARA, Belach, Stockholm, Sweden). The experiments are summarized in Appendix D. They were performed in duplicate at the same time. The working volume was 500 ml and 2 ml of antifoam was added throughout the cultivation. The same medium was used as for the shake flask cultivations. The reactors were connected to a computer where the sparging rate of carbon dioxide could be controlled. The reactors were set to have a temperature of 37 ºC, stirring at 300 rpm and a pH at 6.6. pH was controlled with 5 M NaOH solution. The amount of added NaOH was monitored with the help of a scale, together with the volume of waste and sample that were taken out of the reactors. This was done to correlate the accurate volume to each sample when calculating the yields. The setup of the batch experiment is shown in Figure 3.1.

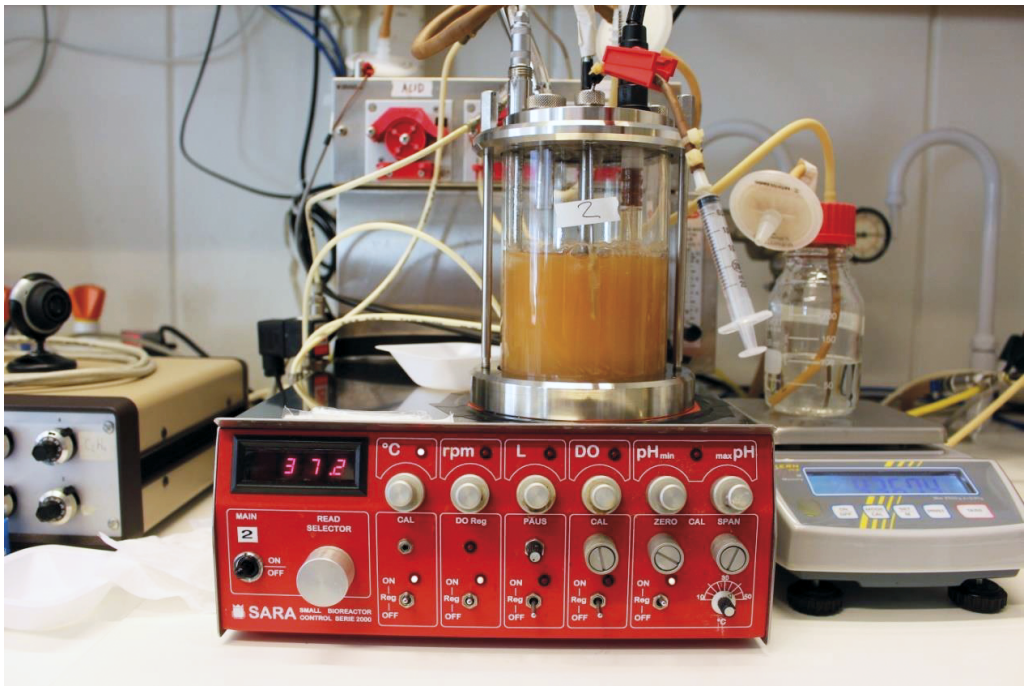

Figure 3.1. Set up of the batch bioreactor.

Chemostat cultivation. Chemostat experiments were made with *A.succinogenes*. The setup (Figure 3.2) is equal to the one for the batch cultivation, but with 10 L of feed in 25 L bottles, one for each reactor. The feed contained 10 g/l xylose, 10 % mineral medium (Appendix A), 3.5 g/l yeast extract, 7 l deionized water and 2 % antifoam. There was also an outlet connected to the reactor, with a pump running continuously. The outlet tube was positioned at the liquid surface. That way the reactor volume was constant at 500 mL. Three dilution rates were tested; 0.05, 0.10 and 0.15 h^{-1}. Samples were taken after the volume in the reactor had been exchanged three times.

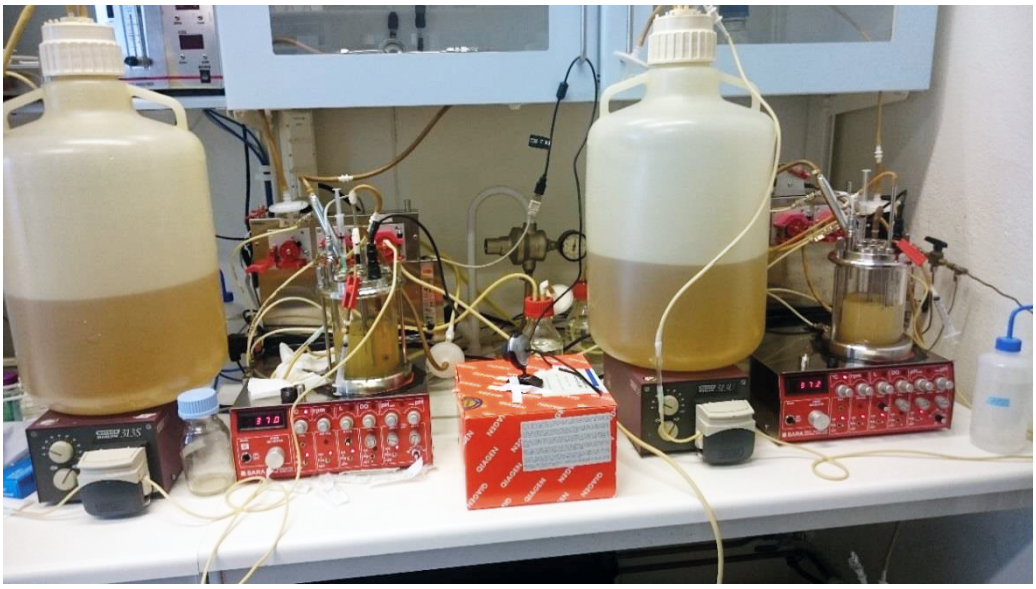

Figure 3.2. Set up of the chemostat.

Microscopy. The pre-cultures were checked for contamination with a microscope (CH40, Olympus, Tokyo, Japan). In the end of the batch and shake flask cultivations, a contamination check was performed again. Throughout the chemostat cultivation, microscopy check was made for each dilution rate. Pictures taken with microscopy (Optiphot, Nikon, Tokyo, Japan) used the camera VisiCam®1.3 (VWR, Leuven, Belgium).

Optical density (OD). 2 mL of each culture was sampled into Eppendorf tubes. The samples were diluted to achieve an OD between 0.1-0.7, depending on how far the growth had come. The samples were measured in a spectrophotometer (V-1200, VWR, Radnor, Pennsylvania) with a wavelength set at 600 nm. For the shakeflask experiments, the samples were diluted in 7.4 % HCl in order to remove magnesium carbonate. Water was used as a blank.

HPLC analysis. After the OD measurements, the samples were centrifuged (Mini, Scanspeed, Randburg, South Africa) for five minutes at 13.5 rpm. The supernatants were then poured into a syringe and filtered into new Eppendorf tubes through a 0.2 μm membrane filter. The samples were stored in the freezer until the time of the HPLC (Waters, Millford, MA, USA) analysis. Standards were prepared for the HPLC analysis according to Table 1 in Appendix B. The samples were analyzed for sugars and organic acids. The column used was HPX-87H at 60 ºC. The mobile phase consisted of 5mM sulfuric acid and was set at a flow rate of 0.60 ml/min. The samples were analyzed by both a RI-detector and an UV-detector.

Dry Weight determination. 10 mL of the culture was sampled into five Eppendorf tubes, two mL in each. The Eppendorf tubes were centrifuged for five minutes at 13.5 rpm. Then, the supernatants were removed and 1 mL of DI-water were added to each tube, which were then vortexed until the cell pellet were completely dissolved. The process was then repeated once again. The samples were then transferred to glass sample tubes, which had been dried in the oven at 105 ºC for a few hours, cooled down in a desiccator and then weighed. Once the sample had been transferred to the sample tube they were put in the oven to dry for 16-24 hours and then cooled in the desiccator for an hour, then weighed again. The difference was the dry weight. The dry weight was converted to concentration and plotted against the OD-value for each point and a straight line was fitted between the data points. The equation that was generated was used to convert OD values to g/L. The optical density values used in the plot was measured at different dilutions, but for the same sample. The experiment was done both for *B. Succiniciproducens* and *A. succinogenes*. For *A.succinogenes* the equation is $C=0.430 \cdot OD-0.00187$ and $R^2=0.9999$. For *B.succiniciproducens* the equation is $C=0.421 \cdot OD-0.00113$ and $R^2=0.9899$. The plots can be found in Appendix E.

Free amino acid analysis. A color reagent and a dilution reagent were prepared according to Appendix C. In the color reagent it is ninhydrin that colors the sample by reacting with primary and secondary amines, but also with ammonia. A calibration curve with glycine ranging from 0.2-2 mg/l was prepared. When preparing the samples the first step was to dilute them so they fit into the calibration curve. Then, 0.5 ml of color reagent was added to 1 ml of the diluted sample. After vortexing, the sample was put in a water bath at 100 ºC for 16 minutes and then cooled in water with ice for 20 minutes. In the next step, 2.5 ml of dilution

reagent was added and the sample was vortexed again. The sample was then measured in a photometer at 570 nm with water as a blank (Lie 1973).

Flow cytometry. The samples were diluted with phosphate buffer to have an OD value around one, and a volume of 2 ml. The dye solutions thiazole orange (TO) and propidium iodide (PI) were used. TO colors all cells while PI only colors dead or injured cells with wounded membranes. 5 µl of each solution were added to each sample and the samples were thereafter vortexed and incubated in room temperature for ten minutes. The samples were then put in the flow cytometer (Accuri C6,BectonDickinson,Franklin Lakes, NJ, USA) and the cells were quantified.

4 Results

In order to fulfill the goal with this work, shake flask, batch and chemostat cultivations were conducted. The experiments were performed with the intention of investigate the impact of yeast extract and sugar concentration on the final concentration of biomass and products. All experiments were performed in duplicates, as mentioned earlier.

4.1 Questions at issue

Previous work has shown that *A.succinogenes* is prone to immobilization at certain conditions, but the exact conditions have not been confirmed. The immobilization causes problems in the industrial scale because it will cause biofouling in the reactors which thereby will be harder to clean. Also, if there is more than one layer of biofilm, it is only the outermost layer who can consume the carbon source and produce succinic acid. In that case, some carbon source would have been wasted on biomass production. A third reason is that in a chemostat yield and productivity are constant. With biofilm formation being a non-controlled process these conditions changes and the exact behavior cannot be studied.

With this work, we want to find out why the bacteria forms biofilm, as in Figure 4.1. The following questions will be investigated through a series of experiments (Appendix C).

- If the type of carbon source (glucose/xylose) influence the yield of succinic acid
- The impact of yeast extract concentration
- If lack of nitrogen source causes biofilm formation
- If there are a shortage of free amino acids which cause the clustering
- Is there a change in physiology
- If the bacteria is dead or alive in the biofilm

Figure 4.1. A. succinogenes forming biofilm, after 72 hours of batch cultivation.

Solving the immobilization is also key to being able to make proper chemostat experiments.

4.2 Shake flask

The first experiments were shake flask cultivations at a yeast extract concentration of 5 g/l and a xylose concentration of 20 g/l. Figure 4.2 shows the cultivations of *A.succinogenes* (left) and *B.succiniciproducens* (right). The figures display a typical cultivation behavior.

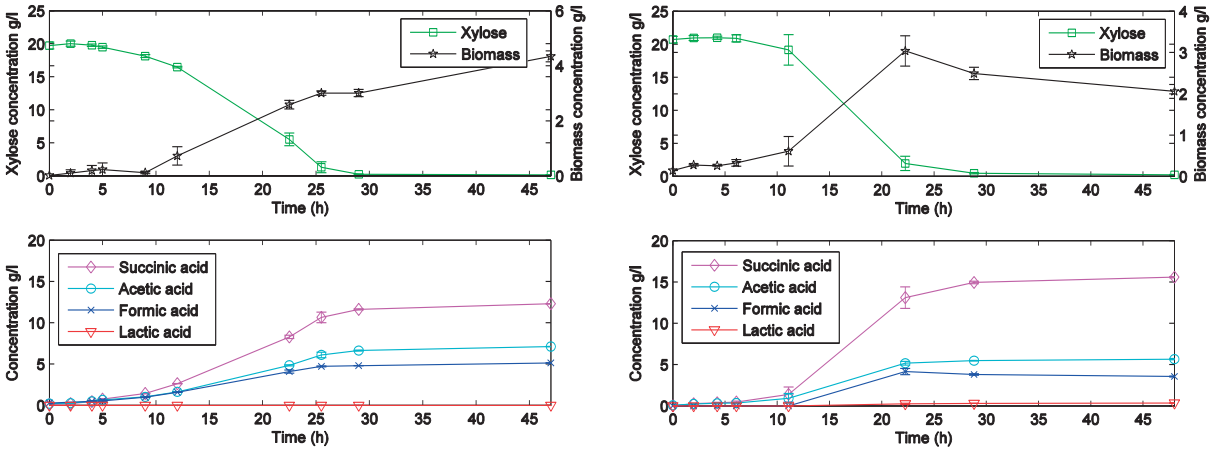

Figure 4.2. Shake flask cultivation of A.succinogenes (left) and B.succiniciproducens (right) on 20 g/l xylose and 5 g/l yeast extract

The impact on succinate yield and biofilm formation depending of the concentration of yeast extract and carbon source was investigated with two approaches. In the first approach, *A.succinogenes* was cultivated in 12.5 g/l yeast extract and with 0, 5 and 10 g/l of xylose. The yeast extract not only contains nitrogen and amino acids, but can also serve as a carbon source. This experiment was also done in order to study how well the bacteria grew on yeast extract alone (0 g/l xylose). The result is shown in Figure 4.3. As can be seen, the bacteria can grow and produce small amounts of acid without addition of xylose.

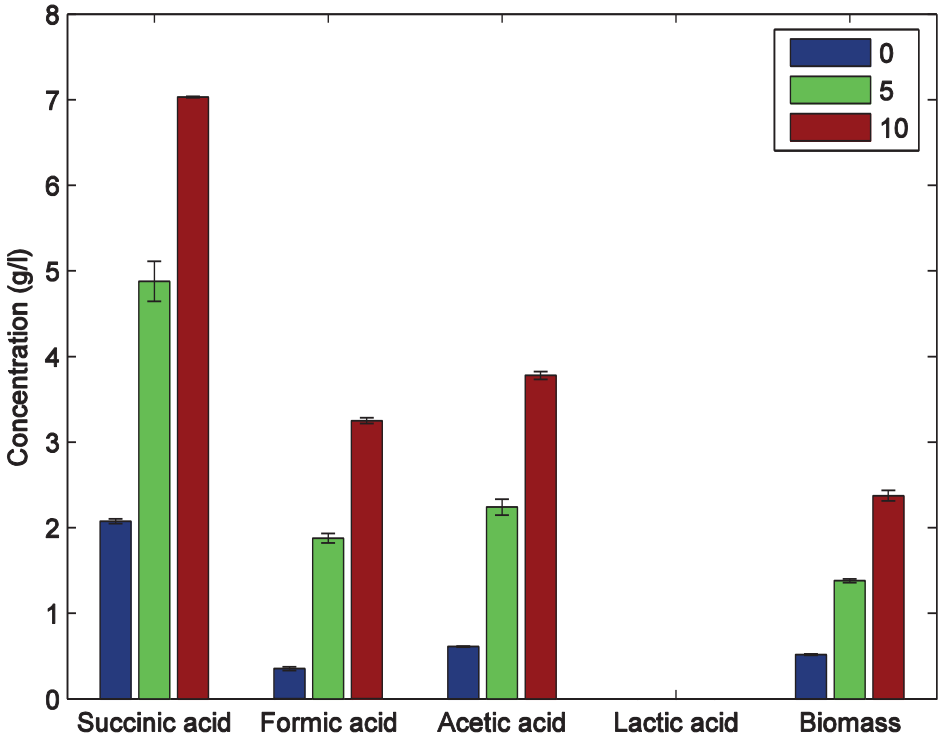

Figure 4.3. Compilation of the result from the cultivation of A.succinogenes with 12.5 g/l yeast extract and 0, 5 and 10 g/l xylose.

The second approach was to keep the sugar concentrations constant while varying the yeast extract concentration. Three different yeast extract concentrations were investigated; 3.125, 6.25 and 12.5 g/l together with 25 g/l xylose. A cultivation with 6.25 g/l yeast extract and 25 g/l glucose was also made. Figure 4.4 shows the result for *A. succinogenes* and Figure 4.5 shows the result for *B.succinicproducens*. By looking at the figures it can be observed that the yield of succinic acid for *A.succinogenes* increased with increasing yeast extract concentration and that glucose gave a better succinate yield. The opposite was observed for the cultivation with *B.succiniciproducens* where glucose gave the lowest yield of succinic acid and with similar succinate yield for the different yeast extract concentrations.

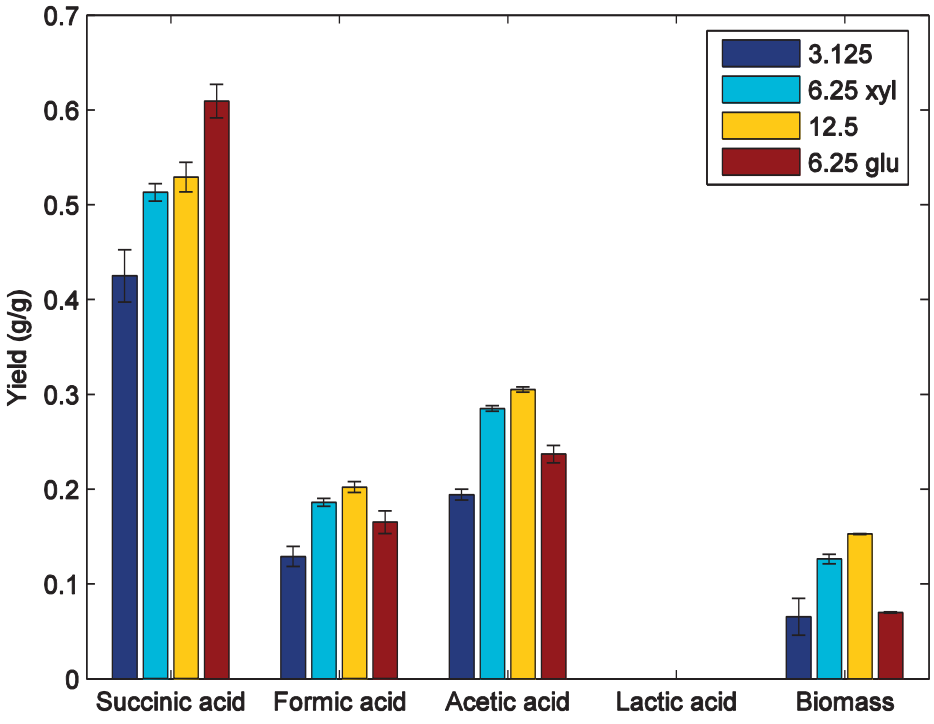

Figure 4.4. Result for varying the yeast extract concentration for A.succinogenes at a constant concentration of carbon source.

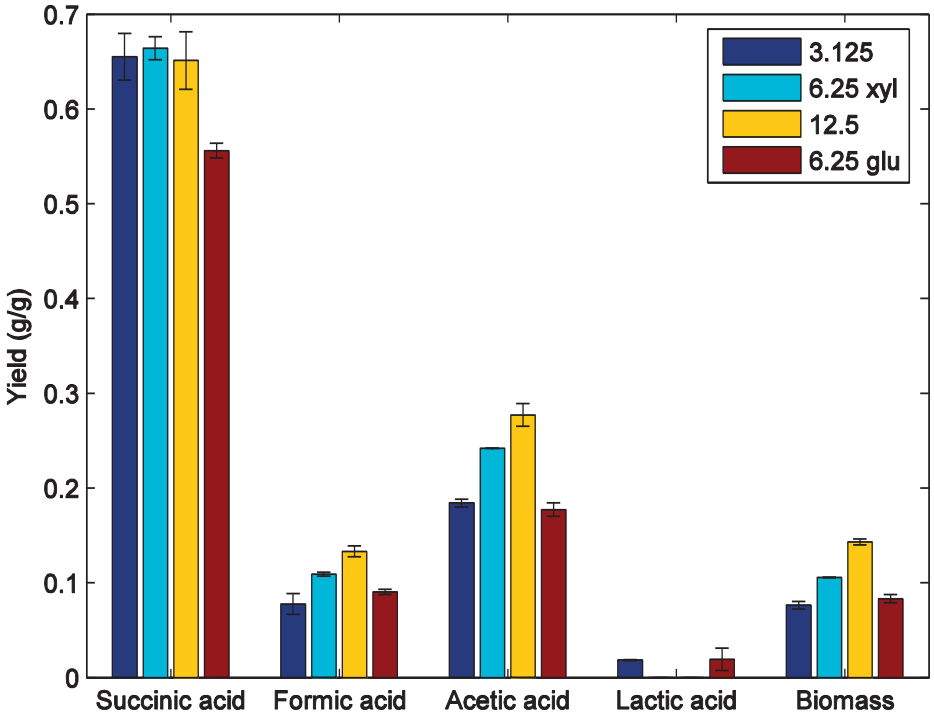

Figure 4.5. Result for varying the yeast extract concentration for B.succiniciproducens at a constant concentration of carbon source.

Since the bacteria (especially *A.succinogenes*) forms biofilm, the measured OD decreases after it has reached its maximum value because the cells stick to the wall, as in Figure 4.1. Since this would affect the yield the maximum OD is considered to be constant throughout the cultivation and that is the OD the Y_{sx} is based on.

One theory was that a depletion of the nitrogen source led to a stopped growth and caused the bacteria to form biofilm. This theory was investigated by adding different concentrations (0, 1, 2 and 4 g/L) of ammonium chloride, acting as a nitrogen source, to shakeflasks with 25 g/l xylose and 3.12 g/l yeast extract. Figure 4.6 shows the result of biofilm formation after 72 hours of cultivation.

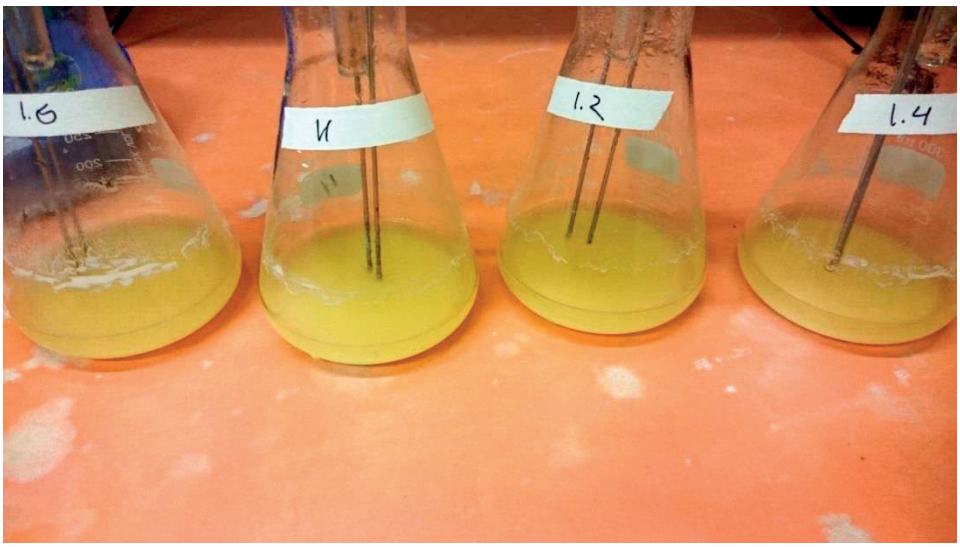

Figure 4.6. Shakeflask cultivation with addition of ammonium chloride.

Even though a difference in biofilm formation can be seen between the shakeflasks, no significant difference was found in xylose consumption, biomass formation or production of succinic acid.

4.3 Batch mode cultivation in bioreactors

Based on the results from the shakeflask cultivations four conditions for the batch cultivations, in bioreactors were chosen.

- In the first experiment, *A. succinogenes* was cultivated in a yeast extract concentration of 6.25 g/l and 50 g/l of xylose.
- The second batch experiment was performed with *A. succinogenes* in 12.5 g/l yeast extract and 50 g/l of xylose.
- The third batch cultivation was *B.succiniciproducens* with 12.5 g/l yeast extract and 50 g/l xylose.
- The fourth batch was performed with *B.succiniciproducens* in 6.25 g/l yeast extract and 50 g/l of xylose.

As mentioned, the biomass yield are based on the highest measured concentration. This might result in an underestimation of the biomass concentration which could have affected the carbon balances. The conditions and result for the batch experiments are summarized in Table 4.1. All cultivations started with 50 g/l xylose.

Table 4.1. The conditions and result from the batch experiments with 50 g/l xylose.

	Batch 1	**Batch 2***	**Batch 3**	**Batch 4**
Organism	*A.succinogenes*	*A.succinogenes*	*B.succinici-producens*	*B.succinici-producens*
YE(g/l)	6.25	12.5	6.25	12.5
Y_{ss} (g/g)	0.60 ± 0.035	0.53	0.56 ± 0.035	0.61 ± 0.038
Y_{sf} (g/g)	0.11 ± 0.0049	0.15	0.16 ± 0.014	0.15 ± 0.017
Y_{sa} (g/g)	0.15 ± 0.0028	0.22	0.18 ± 0.014	0.19 ± 0.017
Y_{sl} (g/g)	0.011 ± 0.011	0.013	0.071 ± 0.0018	0.060 ± 0.0027
Y_{sx} (g/g)	0.090 ± 0.0018	0.081	0.081 ± 0.038	0.058 ± 0.0018
q_s (g/l·h)	0.55 ± 0.0037	0.58	0.19 ± 0.0069	0.65 ± 0.020
q_{suc} (g/l·h)	0.33 ± 0.021	0.29	0.20 ± 0.0049	0.36 ± 0.029
$Xylose_{end}$ (g/l)	9.8 ± 0.94	11	18 ± 0.97	2.4 ± 0.20
C-balance (%)	90.4	96.1	102.7	103.7

The carbon balances are calculated based on c-moles and the general biomass formula $CH_{1.2}O_{0.5}N_{0.2}$ was used for the biomass. The carbon source contribution from carbon dioxide is not included.
** This experiment was not performed in duplicate*

4.4 Chemostat

The conditions were chosen based on the results from the shakeflask and batch experiments to achieve a chemostat without biofilm formation. The chemostat experiment were carried out with *A.succinogenes* at 10 g/l of xylose and 3.5 g/l of yeast extract. The maximum growth rate (μ_{max}) for *A.succinogenes* was calculated to 0.18 h^{-1}, based on the batch cultivations. Three dilution rates were therefore used; 0.1, 0.05 and 0.15 h^{-1}. The result is shown in Table 4.2. Again, the carbon dioxide is not taken into account.

Table 4.2. Results from the chemostat cultivation.

Dilution rate (h^{-1})	**0.05**	**0.10**	**0.15**
Y_{ss} (g/g)	0.57 ± 0.0019	0.56 ± 0.0014	0.58 ± 0.0042
Y_{sf} (g/g)	0.24 ± 0.018	0.25 ± 0.0099	0.25 ± 0.051
Y_{sa} (g/g)	0.28 ± 0.0057	0.28 ± 0.0057	0.29 ± 0.0028
Y_{sl} (g/g)	0	0	0
Y_{sx} (g/g)	0.14 ± 0.020	0.14 ± 0.026	0.10 ± 0.0035
q_s (g/l·h)	0.48 ± 0.011	0.89 ± 0.014	1.2 ± 0.19
q_{suc} (g/l·h)	0.27 ± 0.0057	0.50 ± 0.0064	0.72 ± 0.14
$Xylose_{end}$ (g/l)	0	0.73 ± 0.023	1.5 ± 1.4
C-balance (%)	118.3	117.9	115.8

In Figure 4.7, the chemostat has been running for 245 hours and there is almost no biofilm formation, compared to Figure 4.1.

Figure 4.7. Chemostat after 245 hours cultivation.

When increasing the dilution rate, the productivity increases. When the dilution rate exceeds the maximum growth rate, the rate of formation of biomass will be less than the removal rate and the biomass concentration will gradually decrease. This phenomenon is called washout. In Figure 4.8 a small wash out experiment is shown. It can be seen that the concentration of xylose increases while the biomass concentration decreases. The time zero in the figure symbolizes the time when the dilution rate was increased from 0.15 to 0.20 h^{-1}

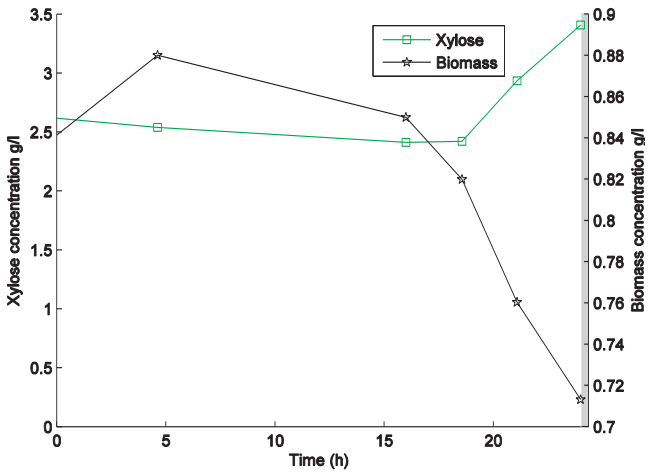

Figure 4.8. An attempt of wash out.

6.5 Free amino acid analysis

One theory that was investigated as the reason for biofilm formation was that the amount of free amino acids decreases during a cultivation. Free amino acids analysis was performed as described in the material and method. The calibration curve with glycine can be found in Appendix C. Samples from the beginning, middle and end of a cultivation were analyzed for Batch 1 and 2. The result of this analysis is shown in Figure 4.9 where it is clear that the amount of free amino acids decrease during the cultivation.

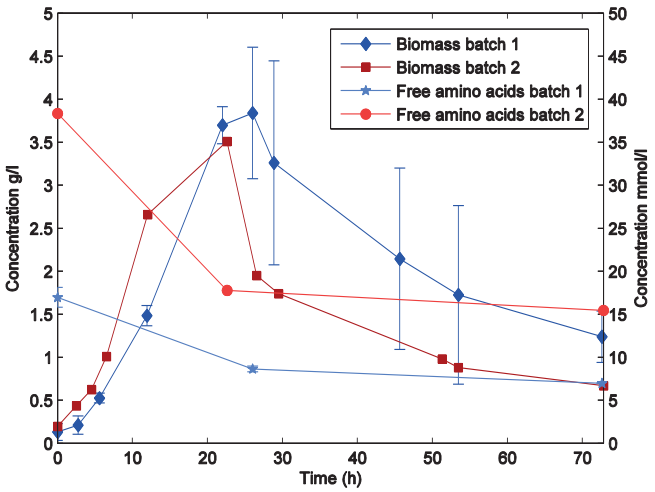

Figure 4.9. Concentration of biomass and free amino acids for batch cultivations of A.succinogenes. As seen, there are big standard deviations for batch 1. The most likely reason for these are uncontrolled biofilm formation.

Four samples were analyzed for the chemostat, one from the start of the batch cultivation and one for each dilution rate. Table 4.3 shows the results which correspond to the results displayed in Figure 4.9. The dilution rate 0.20 h^{-1} was not made in duplicate.

Table 4.3. Concentration of free amino acids in the chemostat.

Dilution rate (h^{-1})	Batch	0.05	0.10	0.15
Concentration (mmol/l)	16 ± 1.6	3.9 ± 0.36	5.0 ± 0.71	9.4 ± 0.66

6.6 Flow cytometry

During the chemostat cultivations, an opportunity to analyze samples in a flow cytometer arose. With flow cytometry, physical and chemical properties of the bacteria can be measured. The cells pass a detector individually and can be quantified while properties such as size, shape and viability can be studied. During the analysis, six samples were analyzed in the flow cytometer; one from a shake flask cultivation in TSB, one with cells which had been killed with ethanol and samples from four different dilution rates in the chemostat. It would of course have been interesting to analyze samples from the batch cultivations as well, but this was not possible for reasons of time and resources. In the TSB-cultivation, the cells were young and healthy. This served as a reference for viable cells. The cells killed with ethanol symbolized dead cells. These two samples were in order to be able to evaluate the viability of the cells in the chemostat samples.

In Figure 4.10, the difference in FL1 for the analyzed samples are shown.

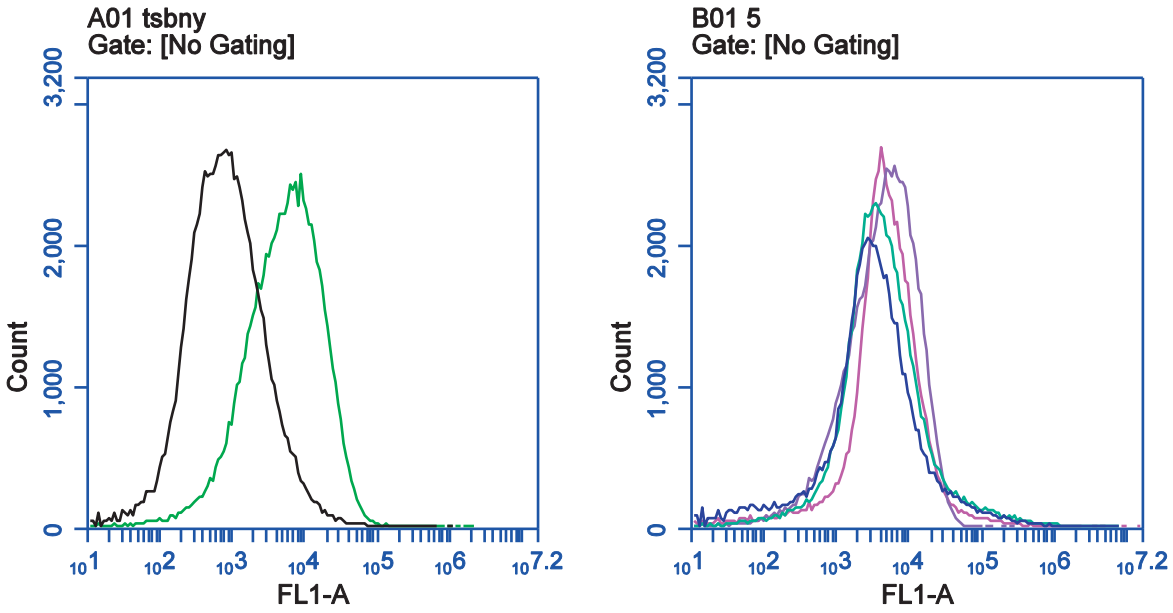

Figure 4.10. Difference in FL1. Dead cells (black) and the TSB sample (green) are shown to the left while the different dilution rates are shown to the right. 0.10- purple, 0.05-pink, 0.15-green, 0.20- blue.

This can be compared to Figure 4.11, where the samples have been plotted against FL3.

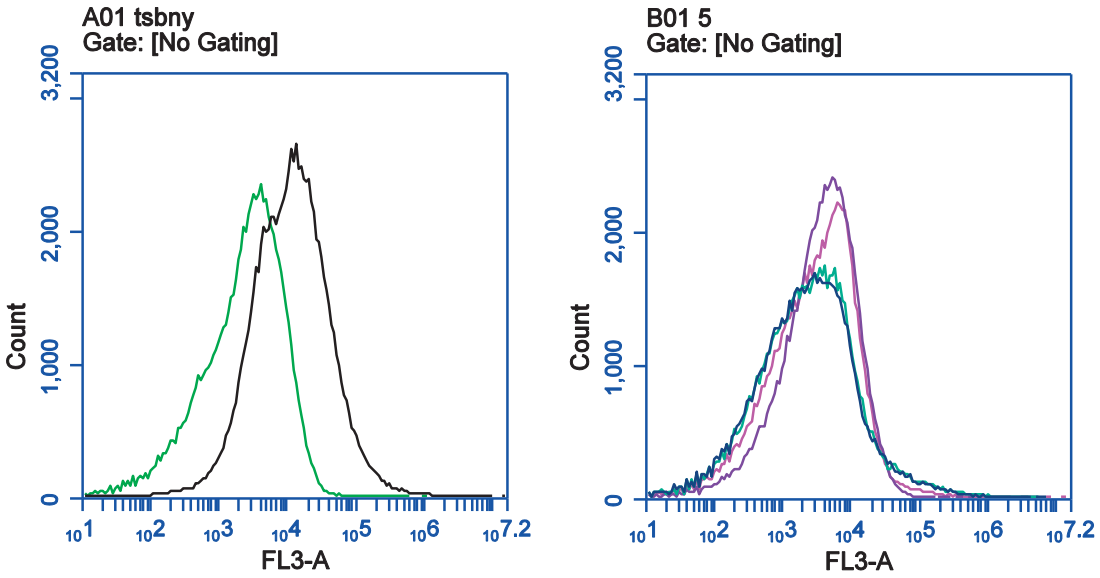

Figure 4.11. Difference in FL3. Dead cells (black) and the TSB sample (green) are shown to the left while the different dilution rates are shown to the right. 0.10-purple, 0.05-pink, 0.15-green, 0.20- blue.

In Figure 4.12, FL3 have been plotted against FL1. This gives information about the viability of the cells, because dead cells have higher FL1and lower FL3 compared to living cells. This is because FL3 shows the cells which have been colored with TO and FL1 shows the cells that have been colored with PI.

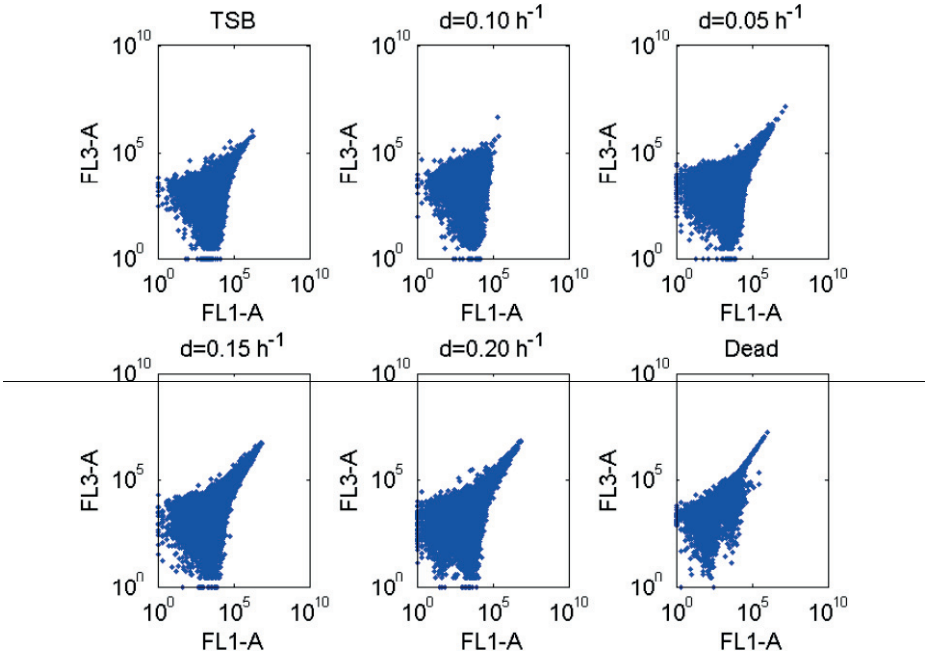

Figure 4.12. FL3 (TO) plotted against FL1 (PI).

4.7 Visual inspection of morphology

As mentioned in the material and method section, the samples from the shake flask cultivations were diluted with HCl when measuring the optical density. This was done to remove the carbonate so it would not affect the OD measurement. For batch cultivations, where no $MgCO_3$ was added, water was normally used. When HCl was used for batch cultivations, however, the sample did not dissolve. This was more apparent when the batch had been going for a few hours. Figure 4.13 shows this difference. It can be seen that the batch sample in HCl (left) did not dissolve, while the batch sample in water (right) dissolved.

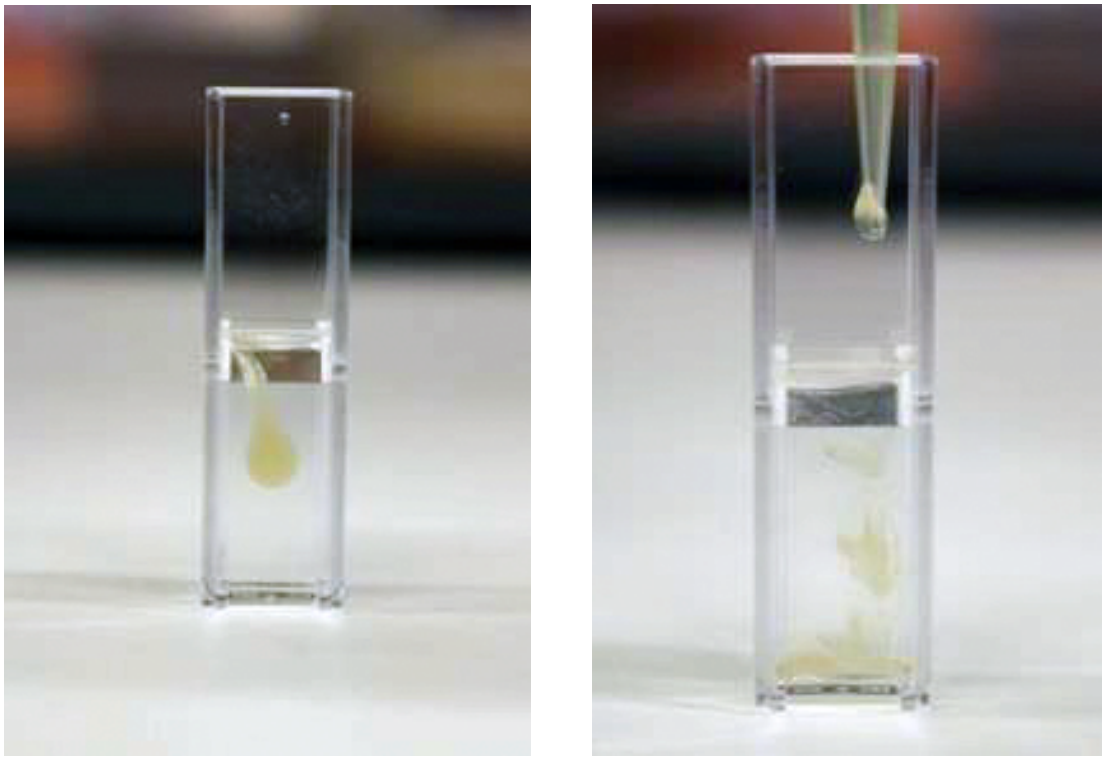

Figure 4.13 Difference when a batch sample is dissolved in HCl (left) and water (right).

In the beginning of a cultivation, when the bacteria is fresh and has access to a lot of nutrients, it does not form biofilm. A sample from a pre culture, *A.succinogenes* in TSB medium, is shown to the left in Figure 4.14. To the right in the figure on the other hand, the cultivation has been going on for a couple of hours and the bacteria have formed biofilm. When comparing the pictures it can be interpreted as that *A.succinogenes* has become more rod like throughout the cultivation.

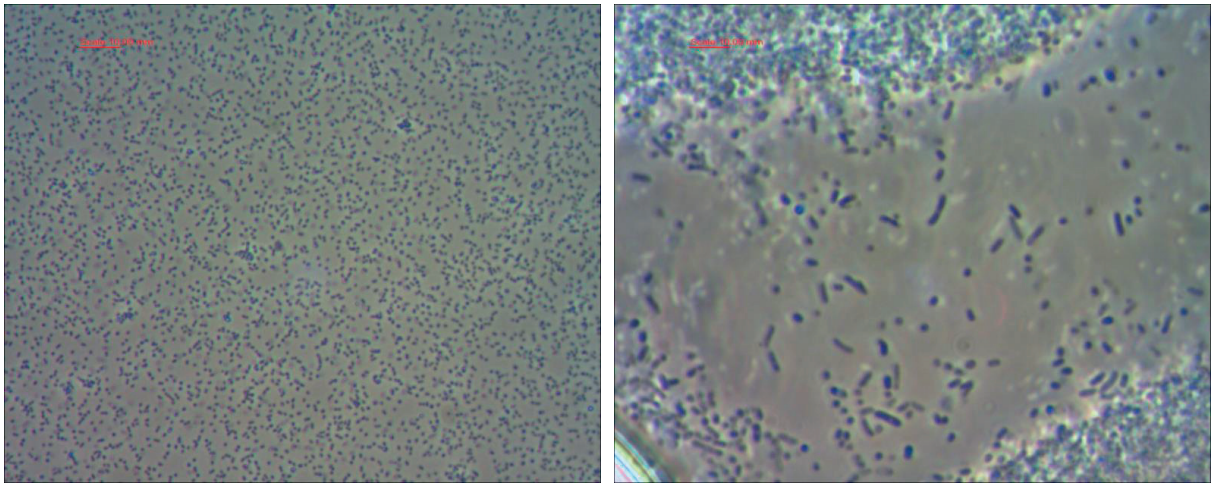

Figure 4.14. A.succinogenes in TSB medium (left) and when it has formed biofilm (right). Magnification 40 respectively 100.

When comparing the cell pellet from the preparations of HPLC samples from all cultivations, it could be seen that the cell pellet changed in color throughout the cultivation. In the beginning, the cell pellet was white but as the time went, more and more cells turned red, see Figure 4.15. This phenomenon was observed for both *A.succinogenes* and *B.succiniciproducens*. When both red and white cells were present they were separated, indicating a difference in density.

Figure 4.15. Beginning of the cultivation to the left and towards the end of the cultivation to the right.

In Figure 4.16 microscopic picture of *B.succiniciproducens* from white cell pellet (left) respectively red cell pellet (right) can be seen.

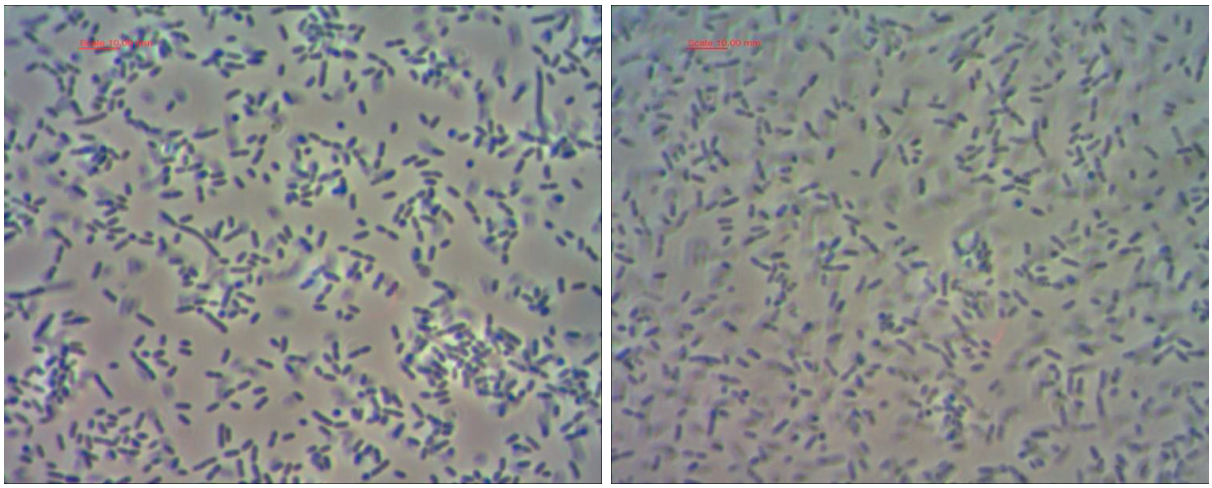

Figure 4.16. B.succiniciproducens from white cell pellet (left) and from red cell pellet (right). Magnification 100.

Even though the cell pellet with *B.succiniciproducens* also showed a difference in color, no difference in shape was observed.

5 Discussion

Shake flask

In the experiment with addition of 0, 5 and 10 g/l xylose (Figure 4.3) it can be seen that the bacteria grew and produced acids without the addition of xylose as carbon source, meaning there was something in the yeast extract it could use as carbon source. However, the amount of succinic acid formed from yeast extract was limited.

For *B.succiniciproducens* (Figure 4.5), the succinic acid yield was almost the same for different concentrations of yeast extract. However, the byproducts and biomass concentration increased somewhat when the yeast extract concentration was increased. One explanation for this could be that in order to reach a higher concentration of succinic acid, the concentration of carbon source must be increased. For A.succinogenes (Figure 4.4), the succinic acid yield on glucose was higher than on xylose, independently on the yeast extract concentration. However, the biomass yield on glucose was similar to the biomass yield on xylose with 3.12 g/l yeast extract.

From the shakeflask cultivation with addition of ammonium chloride to shakeflask with *A.succinogenes*, no noticeable difference between the shake flasks was observed. Since A.succinogenes can utilize NH_4Cl as nitrogen source (Mckinlay et al. 2005) This indicated that it was not a depletion in nitrogen source that causes the bacteria to form biofilm.

Batch bioreactor

The batch cultivation (Table 4.1) for *B.succiniciproducen*s with 12.5 g/l yeast extract is slightly better compared to the one with 6.25 g/l. The yield of succinic acid is a bit higher, but mostly it is the productivity and xylose consumption rate which increased. The xylose consumption in Batch 3 was quite low (63 %). One possible explanation could be that *B.succiniciproducens* needs more nutrients from the yeast extract then what was present, which is consistent with the higher productivities in Batch 4. For *A.succinogenes* on the other hand, the difference was not as apparent. However, since batch 2 was not performed in duplicate a significance test cannot be made. Both bacteria reached their highest biomass concentration faster when the yeast extract concentration is increased. The final biomass concentrations are quite similar with the high and low yeast extract concentration. This applies for both bacteria.

Compared to previous studies with batch mode cultivations on glucose the succinic acid yield on xylose was similar for *A.succinogenes* (Brink & Nicol 2014) the succinic acid yield for *B.succiniciproducens* on xylose was about 24 % higher compared to when grown on glucose (Becker et al. 2013), consistent to the shake flask cultivations. For *A.succinogenes* on the other hand, the shakeflask cultivations comparing xylose and glucose showed a slightly higher yield of succinic acid for glucose cultivations. The productivities for growth on xylose were lower compared to growth on glucose from previous studies for *A.succinogenes* (Brink & Nicol 2014). This indicates that even though the same or higher succinic acid yields can be achieved, there is still work to do to make the xylose cultivations competitive. On the other

hand, since xylose is cheaper than glucose, maybe the two processes will be equally costly in the end. In addition, xylose has the advantage of being derived from a non-food feedstock.

In none of the batch mode cultivations all sugar was consumed. In these cases, higher succinic acid titers could probably have been reached. Why not all sugar was consumed is difficult to say. One reason could be that when the acids reach a high concentration they become toxic for the bacteria and that is why the growth stops. This is, however, not very likely since the concentrations of acids are far beyond the limits of what previous studies have shown to be toxic (Lin et al. 2008). It is more likely that there is something in the yeast extract that the both bacteria needs, for example an essential amino acid, which runs out. The ratio between carbon source and yeast extract concentration would need to be optimized.

Chemostat

When it comes to the chemostat cultivations, the yields of the acids were similar at different dilution rates (Table 4.2). The biomass concentration decreased a little bit for 0.15 h^{-1}. This was probably due to that 0.15 h^{-1} approach the maximum growth rate of *A.succinogenes* (0.18 h^{-1}) and therefore the bacteria started to get washed out. The wash-out experiment (Figure 4.8) proved that the dilution rate 0.20 d^{-1} was very close the maximal growth rate of *A.succinogenes* since it can be seen in Figure 4.8 that the concentration of biomass decreased while the concentration of xylose increased. The decrease in biomass goes quite slow which means that the maximal growth rate has not been exceeded, since the biomass concentration would be almost zero in that case.

For the dilution rate 0.05 h^{-1} about 99 % of the xylose was utilized, for 0.10 h^{-1} 92 % and for 0.15 h^{-1} 85 % of the xylose was utilized. The yields of the acids are similar, but the productivity and consumption rate increases with increased dilution rate. To have an efficient production, both yield and productivity must be sufficient. For that reason, as high dilution rate as possible should be used. However, it is also important to not waste any feed by letting it just pass through the reactor and therefore it is necessary to compromise and choose a lower dilution rate. With this in mind, 0.10 d^{-1} appears to be a suitable dilution rate. Another way to increase the productivity is to increase the concentration of xylose and yeast extract in the feed. Then, higher succinic acid yield and thereby productivity would be obtained even at a lower dilution rate like 0.05 h^{-1}. The xylose and yeast extract concentration should be increased in ratio, lowering the risk for biofilm formation. Compared to previous studies on glucose (Brink & Nicol 2014) the succinic acid yield on xylose is quite similar but the productivity is lower and the same reasoning as for the batch experiments can be concluded.

Biofilm formation

When comparing biofilm formation in a batch cultivation (Figure 4.1) and the chemostat (Figure 4.7), significantly less biofilm has been produced in the chemostat. In the chemostat it did not affect the optical density, indicating that keeping the bacteria in low concentration so that it will not get crowded will prevent biofilm formation. An even more likely explanation, is that the continuous supply of fresh nutrition gives the bacteria all it needs to grow. Also, the ratio between the concentrations in carbon source and yeast extract are probably in a good range.

As mentioned, it seems like the bacteria are not equally prone to form biofilm if the concentration of biomass is relatively low. This could correspond to the fact that the bacteria comes from the bovine rumen. Because when there is a lot of nutrient the bacteria will float around, proliferate and try to find a new host. On the other hand, when the nutrient is decreasing (when the concentration of bacteria is high because they have consumed it), they are more prone to adhere to the wall (or stay in the rumen) to assure its own survival.

Carbon balances

The carbon balances for the batch mode cultivations do not close because the carbon dioxide is not taken into account. Since the bacteria forms biofilm the biomass yield is estimated from the highest measured OD-value. This might not be correct, because the biomass concentration could be underestimated when the cells stick to the walls in the reactor. Also, from the sampling of the highest OD-value there was usually ten hours until the next sampling which means that the OD-value could have increased further before it started to decrease. *B.succiniciproducens* usually grew faster than *A.succinogenes* and therefore the biomass yields for the batch mode cultivations of *B.succiniciproducens* are more reliable. This explains why the carbon balances are lower for *A.succinogenes*. During the chemostat cultivation, there was no biofilm formation so the biomass yield was not underestimated. However, as mentioned the carbon dioxide was not taken into account which resulted in higher carbon balances. Another, not so likely, possibility is that an additional byproduct is formed, for example, ethanol. Ethanol was not analyzed for in the HPLC but there were no unidentified peaks formed during the analyzes, meaning unknown byproducts where not the reason for deviating carbon balances. The variation in the HPLC analysis itself must also be kept in mind.

Free amino acid analysis

The amino acid analysis showed that the amount of free amino acids decreased during a cultivation (Figure 4.9). Contrary to expectations they did not run out. Although, the calibration curve was made with glycine which is the smallest of the amino acids which means that even though the curve was recalculated to mol/l it might not be representable to all of the amino acids. Also, other amino acids might not give the same value in absorbance. Since ninhydrin reacts with amine groups, maybe amino acids like lysine would give twice as high absorbance because it has two amine groups. For future work that could be investigated. Moreover, even if the amount of free amino acids did not run out according to the analysis it could be that one of the essential amino acids for the bacteria has run out. That could explain why the bacteria stops growing. For future work it could be investigated for each amino acid, by HPLC, if the amount is decreasing. If one of the essential amino acids is limited it could explain why the bacteria stops growing even if there is still sugar. The analysis is a spectrophotometric method which yields some uncertainties. Also, the reaction is very quick and it is a matter of seconds to perform the measurement in order to get a reliable result.

Flow cytometry

From the flow cytometry experiment (Figure 4.12) it can be seen that throughout the cultivation the bacteria approaches the plot of the dead cells. This indicates that the dilution

rate does not affect the state of the bacteria, but how long time the cultivation has been going on does.

The chemostat samples analyzed in the flow cytometer had very little (or non) biofilm formation and therefore they were not ideal to analyze in order to evaluate the state of the bacteria in the biofilm. It would have been more interesting to analyze samples from a batch cultivation with a lot of biofilm, like the one in Figure 4.1. Still, the conclusion is that the bacteria probably feels quite alright during the whole cultivation. This is based on that the FL1 and FL3 plots is merely located between the TSB and dead cells in values (Figure 4.10 and 4.11).

Visual changes in morphology

One reason for why the cell pellet has a different color could be because the bacteria secreted a protein with a pigment or that the bacteria changed shape. This could explain why the differently colored cells get separated during centrifugation, due to differences in density. When looking at Figure 4.14, it could be interpreted as that *A.succinogenes* has become more rod-like when it forms the biofilm. However, this was not possible to conclude for *B.succiniciproducens*.

In Figure 4.13, the difference for when a sample from a batch cultivation was dropped into HCl and water can be seen. A sample from the beginning of a batch cultivation dissolves completely in HCl. One reason why the sample behaves in this way could be that the bacteria have produced proteins that denatures in the contact with HCl and therefore the sample cannot be dissolved. Maybe that protein also changes the color of the cells or it is connected to the change in shape discussed earlier. Another explanation is that after a couple of hours of cultivation the cell wall is damaged and the hydrochloric acid can reach into the cell and destroy the proteins. This shows that something happens to the cells throughout the cultivation and maybe it could be connected to the course of biofilm formation.

As carbon source, glucose is the most common and gives the highest yield of succinic acid, at least for *A.succinogenes*. Though, in the industrial scale it is important to use a cheaper sugar source so that the process can be cost effective. *B.succiniciproducens* on the other hand seems to work better with xylose irrespective of the concentration of yeast extract. Instead, yield of the byproducts increase while for *A.succinogenes* both byproducts and the product increase with increased yeast extract concentration.

6 Conclusions

- It was possible to produce succinic acid from xylose with similar yields of succinic acid as for cultivations on glucose.
- The productivities for production on xylose were lower compared to glucose for both bacteria.
- The yield of succinic acid, biomass and by-products increased with increasing concentration of yeast extract for *A.succinogenes*.
- For *B.succiniciproducens*, however, the succinic acid yield was unaffected of increasing yeast extract concentration, while the yield of by-products and biomass increased.
- Shortage of nitrogen source was not the reason for biofilm formation.
- Even though the amount of free amino acids are decreasing during a cultivation this alone cannot explain the standstill in growth.
- The flow cytometry analysis showed that the bacteria maintain similar state during the whole cultivation process, probably due to fresh supply of nutrition.
- The exact state of the bacteria in the biofilm is yet to be investigated.
- The results showed that it was possible to have a chemostat cultivation without biofilm formation, even though the yield of succinic acid needs to be improved.

7 Future work

It would be of interest to do more cultivations, especially chemostat cultivations. For example, chemostat cultivations were the concentration of carbon source and yeast extract is increased in ratio. For example, 30 g/l xylose and 10.5 g/l yeast extract and compare the result to the chemostat with 10 g/l xylose and 3.5 g/l yeast extract. It is also necessary to increase the concentration of carbon source to reach high titers of succinic acid in order to be competitive on the market.

It would be interesting to repeat the experiments with glucose or a combination of glucose/xylose to see if the bacteria prefers one of the carbon sources over the other. But also to compare yields and productivities.

Most of the focus in this report has been on *A.succinogenes*, therefore it would be interesting to repeat the chemostat and the experiment with addition of 0, 5 10 g/l of xylose with *B.succiniciproducens*. In addition, also the amino acid analysis and flow cytometry with *B.succiniciproducens* would be interesting to investigate.

For the amino acid analysis, all samples during a cultivation could be analyzed to get a more exact behavior. This, in order to investigate if it is a shortage of amino acids that cause the biofilm formation, or a high concentration of succinic acid which causes the decrease in growth. A method for analyzing individual amino acid in the HPLC would necessary to see of any of the essential amino acids is missing when the growth stops. Also, flow cytometry for batch experiments with a lot of biofilm formation could be of interest to really see in which condition the bacteria is.

Metabolic engineering of the bacteria could be considered. For example, overexpression of PEPCK could increase the flow to succinic acid. Deletions of the pathways forming the byproducts, mainly acetic and formic acid, could also be one way to go. Although, deletion of these pathways could result in an increase in other byproducts, such as lactic acid or ethanol. Also, the engineered bacteria may grow slower than the wild type.

Further research on the possibilities of *in situ* separation is of great interest since it would solve important problems.

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9 Table of abbreviations

Symbol	Meaning	Unit
Y_{ss}	Yield (g succinic acid/g xylose)	(g/g)
Y_{sf}	Yield (g formic acid/g xylose)	(g/g)
Y_{sa}	Yield (g acetic acid/g xylose)	(g/g)
Y_{sl}	Yield (g lactic acid/g xylose)	(g/g)
Y_{sx}	Yield (g biomass acid/g xylose)	(g/g)
q_s	Consumption rate of xylose	(g/l·h)
q_{suc}	Production rate of succinic acid	(g/l·h)

10 Appendices

Appendix A: Medium

Tryptone soy broth (TSB)

Peptone from caseine	17.9 g
Peptone from soy	3.0 g
Glucose	2.5 g
NaCl	5.0 g
K_2HPO_4	2.5 g

Dissolve in 1000 ml water.

Succinate growth medium

NaH_2PO_4	11.6 g
Na_2HPO_4	3.1 g
NaCl	10.0 g
$MgCl_2 \cdot 6H_2O$	2.0 g
$CaCl_2 \cdot 2H_2O$	2.0 g

Dissolve in 1000 ml water.

Appendix B: HPLC standard

	Factor	Glucose	Xylose	Succinate	Lactate	Formate	Acetate
Level 1	1.00	10.00	10.00	10.00	5.00	5.00	5.00
Level 2	0.75	7.50	7.50	7.50	3.75	3.75	3.75
Level 3	0.50	5.00	5.00	5.00	2.50	2.50	2.50
Level 4	0.20	2.00	2.00	2.00	1.00	1.00	1.00
Level 5	0.10	1.00	1.00	1.00	0.50	0.50	0.50
Level 6	0.05	0.50	0.50	0.50	0.25	0.25	0.25
Level 7	0.02	0.20	0.20	0.20	0.10	0.10	0.10

Appendix C: Free amino acid analysis

Color reagent

$Na_2HPO_4 \cdot 2H_2O$	49.71 g
Ninhydrin	5.0 g
Fructose	3.0 g
KH_2HPO_4	40 g

Dissolve in 1000 ml water.

Dilution reagent

KIO_3	2.0 g
Pure ethanol	384 mL
H_2O	616 mL

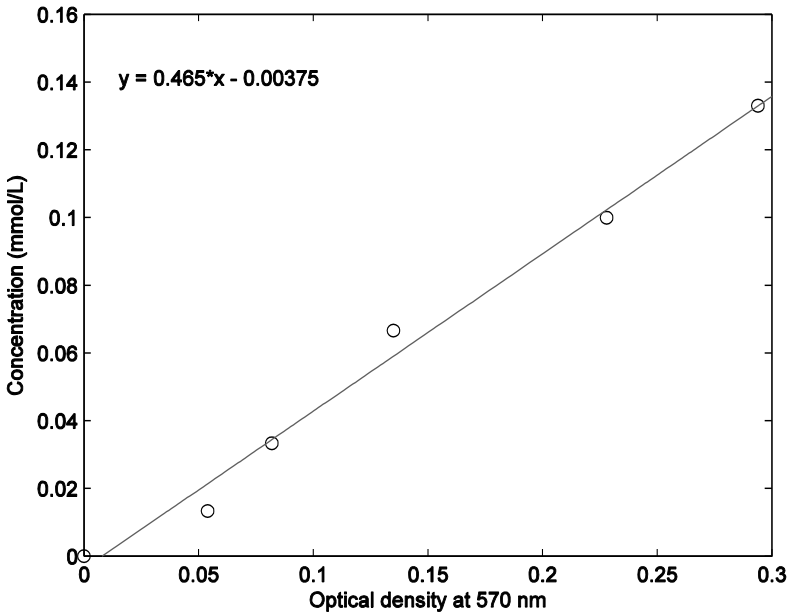

Figure 10.1. Calibration curve with glycine, R^2=0.9876.

Appendix D: Experimental plan

An overview of the planned experiments and the time plan for when the experiments should be carried out is shown in Table 10.1. The main purpose with the shake flask experiments is to see if the growth is affected by the concentration of yeast extract. Then from the shake flask experiments, the conditions for the batch cultivations will be chosen. Thereafter, one chemostat cultivation for each bacteria will be done based on the result from the batch cultivations.

The carbon source will be either glucose (G) or xylose (X) and the organism *A. succinogenes* (A.S) or *B. succiniciproducens* (B.S). The cultivations will be in shake flasks (SF), batch (B) or chemostat (C). All experiments will be performed in duplicates in order to confirm reproducibility. The literature study, result evaluation and report writing will be done continuously during the project and parallel to the experiments.

Table 10.1. Overview of when which experiment will take place.

Week	Experiment (Duplicate)	Number of tries
3	YE:5, X20, A.S, SF	2x2
	YE:5, X20, B.S, SF	
4-5	Literature study	
6	YE:3.12, X25, A.S, SF	4x2
	YE:6.25, X25, A.S, SF	
	YE:3.12, X25, B.S, SF	
	YE:6.25, X25, B.S, SF	
7	YE:12.5, X25, A.S, SF	4x2
	YE:6.25, G25, A.S, SF	
	YE:12.5, X25, B.S, SF	
	YE:6.25, G25, B.S, SF	
8	YE:6.25, X50, A.S, B	1x2
9	YE:12.5, X0, A.S, SF	3x2
	YE:12.5, X5, A.S, SF	
	YE:12.5, X10, A.S, SF	
	Dry weight determination, A.S and B.S	
10	YE:6.25, X50, A.S, B	1x2
11	YE:12.5, X50, A.S, B	1x2
12	YE: 3.12, X25, A.S, SF	1x2
	NH_4Cl: 0,1,2,4 g/l	
13	YE:12.5, X50, B.S, B	1x2
14	YE:6.25, X50, B.S, B	1x2
15-16	YE:3.5, X10, A.S, C	1x2
17-18	YE:3.5 X10, A.S, C	1x2
19	Free amino acid analysis, flow cytometry	
20-22	Report	

X=Xylose, G=Glucose, A.S= *Actinobacillus succinogenes*, B.S=*Basfia Succiniciproducens*, SF=Shake Flask, B=Batch, C=Chemostat

Appendix E: Dry weight determination

Dry weight determination was done for both organisms, the linear regression for *A.succinogenes* is shown in Figure 10.2 and in Figure 10.3 for *B.succiniciproducens*.

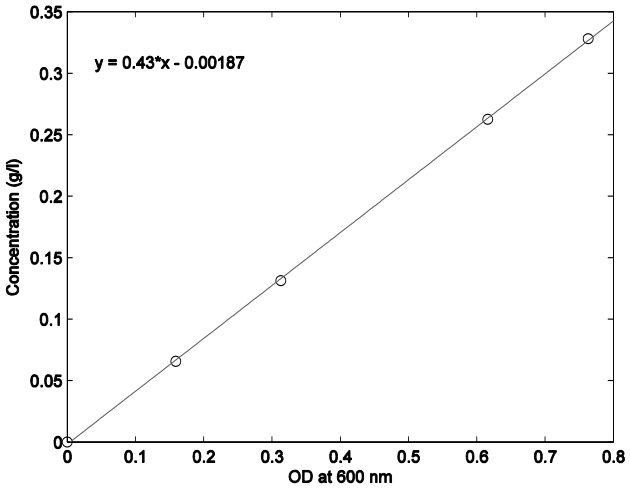

Figure 10.2. Dry weight determination for A.succinogenes. R^2=0.9999.

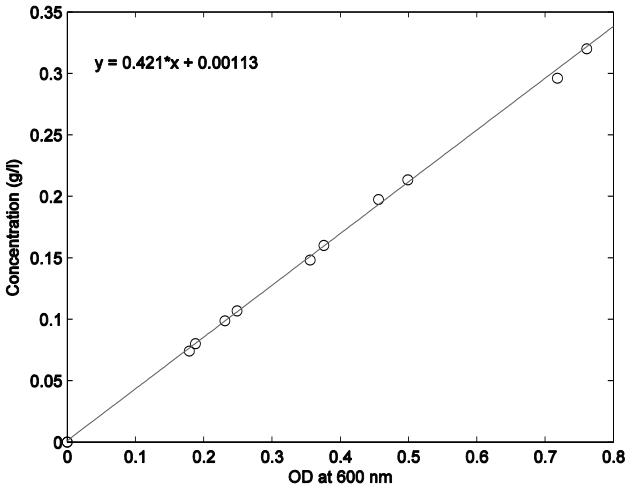

Figure 10.3. Dry weight determination for B.succiniciproducens. R^2=0.9899.

Appendix F: Populärvetenskaplig sammanfattning

Biologisk produktion av bärnstenssyra från sockret xylos

Bärnstenssyra är en kemisk förening med många användningsområden. Bland annat kan den användas för tillverkning av bioplaster. Jag har i mitt exjobb undersökt hur bärnstenssyra kan produceras med bakterier som finns naturligt i komagar.

Bärnstenssyra är en svag syra som har flera användningsområden, bland annat som surhetsreglerare i mat och i tillverkningen av bioplaster. Bärnstenssyra har även pekats ut som en viktig baskemikalie i flera undersökningar.

Bärnstenssyra tillverkas idag både från olja och på biologisk väg. Den ingår nämligen i vår ämnesomsättning och produceras naturligt i de flesta celler. Då det är viktigt att hitta alternativ till att använda fossila råvaror är biologisk produktion av bärnstenssyra från förnybara råvaror av stort intresse. I dagsläget produceras ungefär lika mycket bärnstenssyra på biologisk väg som från olja. Det finns för närvarande fyra företag som producerar bärnstenssyra på biologisk väg; två i Europa och två i USA. Alla företagen använder glukos, den vanligaste enkla sockerarten, som råmaterial. I den biologiska processen har det rapporterats om så gott som total avsaknad av nettoutsläpp av växthusgaser (såsom koldioxid) jämfört med tillverkningen av bärnstenssyra från fossila råvaror. Intressant är att koldioxid faktiskt kan förbrukas vid tillverkningen av bärnstenssyra. De bakterier som har studerats i det här projektet konsumerar koldioxid för att kunna producera syran.

Ett annat alternativ till glukos är att använda sig av sockret xylos. Xylos är ett socker med fem kolatomer som till stor del finns i avfallsströmmar från vissa pappersmassabruk. Detta gör att det dels blir billigare som råmaterial, men ett ytterligare plus är att det fås ur råvara som inte används som mat.

Detta projekt har studerat om det är möjligt att producera bärnstenssyra lika bra från xylos som från glukos. Två av de bästa kända bakterierna på att producera bärnstenssyra har studerats. Båda dessa bakterier kommer från komagar, som är en koldioxidrik miljö. Bakterierna har odlats både satsvis och kontinuerligt. I en satsvis odling tillsätts allt socker från början och sen väntar man tills sockret förbrukats. I en kontinuerlig odling däremot tillsätts sockret och näringsämnen kontinuerligt genom att en vätskeström pumpas in i reaktorn. Bakterierna växer och producerar syra på en jämn koncentration hela tiden. Både bakterierna och syrorna förs ut ur reaktorn i ett utflöde i samma takt som sockret pumpas in. Processens utbyte och effektivitet har utvärderats genom att odla dessa bakterier under olika betingelser och med xylos som kolkälla.

Efter dessa försök har jag kommit fram till att xylos är en nästan lika bra kolkälla som glukos. En intressant observation var att bakterierna klibbade fast på ytorna i reaktorn, så kallad immobilisering. Denna immobilisering verkade inträffa då bakterierna får brist på näring.

Även om processen behöver förbättras, är xylos ett lovande substrat för framtida bärnstenssyraproduktion. Som nämnts tidigare så finns xylos i flera avfallsströmmar som inte utnyttjas till fullo idag. Användning av dessa bidrar till mindre avfall samtidigt som processen minskar miljöpåverkan av växthusgaser.