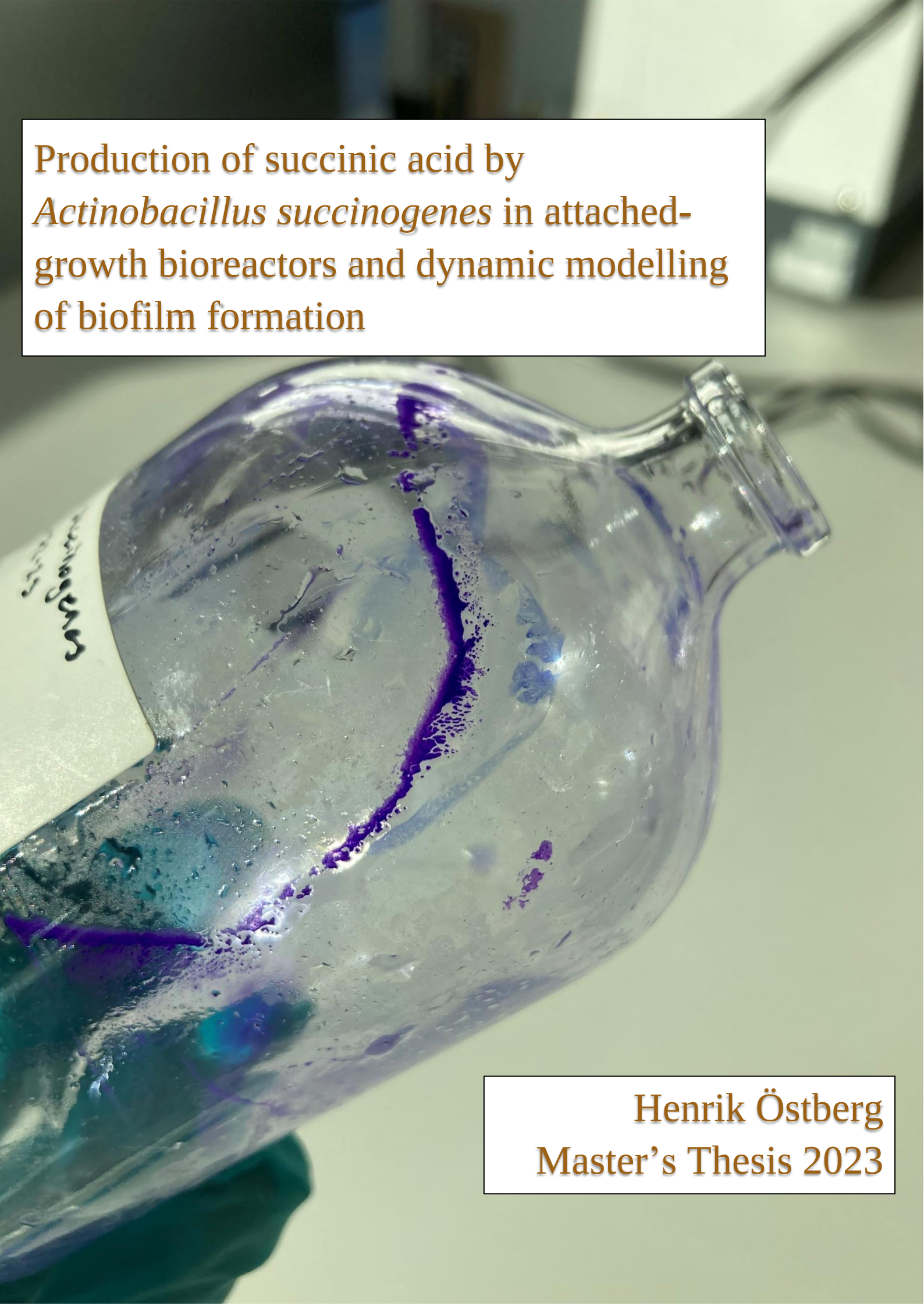
# Production of succinic acid by *Actinobacillus succinogenes* in attached-growth bioreactors and dynamic modelling of biofilm formation



Henrik Östberg

Master's Thesis 2023

# Production of succinic acid by *Actinobacillus succinogenes* in attached-growth bioreactors and dynamic modelling of biofilm formation

Master's Thesis

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Front page picture: Biofilm of *Actinobacillus succinogenes* stained with crystal violet.

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# Abstract

*Actinobacillus succinogenes* is a microorganism is a very efficient producer of succinic acid, a chemical with a wide range of industrial applications. The bacterium is prone to form biofilm, a flocculation of cells attached to a surface. In this study, the bioproduction of succinic acid using *Actinobacillus succinigenes 130Z* was performed in microplate, bottle, and bioreactor systems to gain new insight regarding the biofilm formation process. From microplate experiments the growth kinetics of both suspended and attached cells were characterized at different initial pH and concentration of yeast extract, indicating that biofilm formation is enhanced at lower pH. The maximum specific growth rate was determined to be between 0.178 $h^{-1}$ and 0.413 $h^{-1}$ for suspended cells and 0.063 $h^{-1}$ and 0.089 $h^{-1}$ for attached cells, depending on the initial conditions. During batch fermentation in glass bottles, Kaldnes K1 packing material was used as biofilm support material for comparison between fermentation without and with packing material. No significant difference was found regarding suspended cell growth, substrate consumption, and product formation. A difference was observed regarding the formation of biofilm as measurements indicated more rapid biofilm formation and elevated levels of biofilm on packing material compared to glass surfaces. The maximum specific growth rates when no packing material was present were 0.224 $h^{-1}$ and 0.413 $h^{-1}$ for 20 ml bottles and 100 ml bottles, respectively. With packing material these values were 0.209 $h^{-1}$ and 0.427 $h^{-1}$. Kinetic parameters calculated from the experiments were used to calibrate a kinetic model which well represented the experimental results. A bioreactor setup using a bubble column reactor was constructed and fermentations were performed in both batch and continuous operation. The bioreactor experiments were all contaminated at some time during the fermentation processes with limited results. Using data from a CSTR fermentation the model was validated which showed difficulties in predicting suspended and attached cell growth.

# Preface and acknowledgements

This master's thesis has been performed as a collaboration between the Department of Chemical Engineering at the Faculty of Engineering at Lund University and the Department of Chemical Engineering at Danmarks Tekniske Universitet. All experiments have been performed at the BIOCON group at Danmarks Tekniske Universitet. A special thanks to my supervisors at DTU, Francesco Vigato and Antonio Grimalt Alemany for all the help during the project. A thank you also goes out to my supervisor at LTH, Ed van Niel, as well as my examinator, Magnus Carlquist.

This project was performed from January 2023 until June 2023 and includes 30 hp or HCTS.

# Popular science summary

During this project a bacteria called *Actinobacillus succinogenes* was used production of succinic acid, a chemical that has several applications in the industry. The bacteria have the ability to form biofilm, a cluster of cells that have attached to a surface. This is interesting as the biofilm allow for higher cell concentration and can survive and produce succinic acid under hasher conditions. The process of biofilm formation is complex and is difficult to predict due to their unpredictability, slow growth, and difficult measurement techniques. This can cause problems during fermentations as good predictions are necessary to gain better control over the system. More research is therefore necessary regarding the kinetics of biofilm formation which is the focus of this study.

During the project different cultivation methods were utilized for characterization of cell growth, substrate consumption, and product formation. Biofilm was quantified using crystal violet, a dye commonly used for cell quantification. Biofilm formation was compared on both plastic packing material and glass surfaces in bottles to see if increased formation and better production of succinic acid is possible. A bioreactor setup was constructed to perform fermentations on a larger scale which is important before moving up to even larger scale to finally end with industrial production.

From the results, a mathematical model was produced for prediction of suspended cell growth, attached cell growth, substrate consumption, and product formation. The predictions of the model were compared to the experimental results which showed that it represented the data quite well for batch operation when no changes are made to the system.

The most valuable results from this study were the biofilm formation results which indicated that:

- Biofilm formation is dependent on the pH of the fermentation medium.
- Crystal violet staining can be used as a method for biofilm measurement.
- Attached cell growth occurs more slowly than suspended cell growth.
- Cells attach more rapid to surfaces on plastic packing material than glass surfaces.

More research can be done regarding the biofilm formation process, especially the influence of pH and more refined quantification methods. This could provide more insight in the phenomenon which could be used to improve the accuracy of the prediction model.

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# 1 Introduction and aim

There is an increased interest in bio-based production of chemicals from renewable sources instead of using fossil-based production. One of the many compounds that is currently being researched for its potential to be produced by microorganisms is succinic acid, a chemical used in a wide range of industrial applications.

One specific microorganism that has gained the interest from researchers is *Actinobacillus succinogenes 130Z*, showing promising results in producing succinic acid at high titre, productivity, and yield. *A. succinogenes* was isolated from bovine rumen and has great market potential as it can utilize a variety of substrates as feedstock. Using waste biomass from a variety of industries can greatly reduce the cost of the carbon source which then could be converted to valuable product.

One of the morphological features of *A. succinogenes* is its tendency to form biofilm, an aggregation of microorganisms attached to a surface. This creates an opportunity for fermentation in bioreactors as systems with attached-growth tend to outcompete suspended cell growth systems as a higher cell concentration is achievable. Since bacteria can catalyse biochemical reactions, higher cell concentrations correlate with an increase in product formation.

The purpose of this study is to investigate the production of succinic acid using *A. succinogenes* and to gain insight into the biofilm formation process for increased control during fermentation. Experiments will be performed by cultivation in microplate, bottle, and bioreactor operated both in batch and continuous mode. A prediction model will be calibrated and validated for the fermentation process.

The project stretches over 20 weeks meaning the time is highly constrained. This will limit the number of possible experiments as fermentation times can be rather long and the bioreactor setup will be mostly performed from scratch.

# 2 Background

## 2.1 Succinic acid

Succinic acid is a dicarboxylic acid with the molecular formula $C_4H_6O_4$ with a wide range of industrial applications. The linear and saturated structure of the molecule facilitates the conversion to different bulk chemicals and high value products, making succinic acid highly interesting within many different fields (Cheng et al., 2012). It can be produced chemically using fossil-based raw material but a shift towards more sustainable bio-based production methods is gaining traction in the scientific community. There are several different microorganisms known to produce succinic acid such as *Actinobacillus succinogenes, Basfia succiniciproducens, Mannheimia succiniciproducens, Corynebacterium glutamicum, Escherichia coli, Saccharomyces cerevisiae, Yarrowia lipolytica, etc.* (Dessie et al., 2018; Pateraki, Patsalou, et al., 2016). The reason microorganisms are viable is that succinic acid is an intermediate in the tricarboxylic acid cycle (TCA) and for some bacterial strains, the end product. The TCA is a part of the central carbon pathways which utilize carbon substrates for biomass and product formation. There are three main routes in which substrates can be converted to succinic acid; i) the reductive branch of the TCA, this route can also be called the fermentative pathway as it is active under anaerobic conditions ii) the oxidative branch of the TCA, this route is active under aerobic conditions iii) the glyoxylate pathway, this pathway is active under aerobic conditions when the microorganism is grown on acetate (Cheng et al., 2012). Currently the industrial production of bio-based succinic acid is limited by production costs which are too high in order to be competitive with the fossil-based approach. The costs are related to the entire process with factors such as raw material, the fermentation stage, and downstream processing playing a major role in what needs to become more optimized.

## 2.2 Actinobacillus succinogenes 130Z

One bacterium that is considered very promising due to high titres, yields, and productivities is *Actinobacillus succinogenes 130Z* (Herselman et al., 2017). *A. succinogenes* is a gram-negative, facultative anaerobe, belonging to the Pasteurellaceae family. The microorganism was isolated from bovine rumen where it acts as an organic acid producer, which is a nutrient source for the rumen (McKinlay et al. 2010). Since the role of the rumen is to facilitate digestion of carbohydrates from feedstocks, the microbial environment inside the rumen is adapted for utilization of several different substrates (Hua et al., 2022). The ability to utilize several carbon sources for cellular growth and the production of organic compounds is highly advantageous from an industrial perspective. This is due to the amount of biomass rich in carbohydrates found in waste streams from many industries. Some of the sources of biomass that have been successfully evaluated for production of succinic acid are sugar cane molasses, cheese whey, glycerol, by-products from the agricultural sector, and the paper/pulp industry (Dessie et al., 2018; Pateraki, Patsalou, et al., 2016). Since biomass is seen as waste and is produced in enormous quantities of over 150 billion metric tons each year, the price is lower compared to using pure substrates (Dessie et al., 2018).

### 2.2.1 Metabolic pathways

The journey from substrate to succinic acid is illustrated in Figure 1. *A. succinogenes* have different uptake mechanisms depending on which sugars that are present. Glucose can enter the cells through a permease or through a phosphoenolpyruvate-dependent phosphotransferase system (PTS). Many other sugars use similar PTS systems. Other mechanisms for sugar uptake are ATP-dependent transporters and secondary active transporters such as $H^+$-symports (McKinlay et al., 2010).

In the case of glucose metabolism, it is first converted thought the glycolysis pathway into phosphoenolpyruvate (PEP). PEP is one of the most important nodes in the metabolic system as it is the branching points for the C4 and C3 pathway and dictates the carbon flux distribution. For production of succinic acid, the C4 pathway is desired as it connects to the TCA. *A. succinogenes* only has one PEP-carboxylating enzyme, PEP carboxykinase, which catalyses the reversible reaction from PEP to oxaloacetate (OOA) with the fixation of one carbon dioxide molecule (McKinlay et al., 2010). Metabolic flux analysis has shown that PEP carboxykinase is highly influential for controlling the flux diversion towards succinic acid along with malate dehydrogenase, fumarase, and fumarate reductase (Pateraki, Patsalou, et al., 2016). Malate dehydrogenase catalyses the reaction from OOA to malate, fumarase the

reaction from malate to fumarate, and finally, fumarate reductase the reaction from fumarate to succinic acid. Together these enzymes form the reductive branch of the TCA. The TCA cycle is incomplete in *A. succinogenes* as it lacks the enzymes citrate synthase and isocitrate dehydrogenase which would allow for the conversion of citrate to isocitrate and isocitrate to α-ketoglutarate (αKG). However, the enzymes αKG-dehydrogenase and citrate lyase are present which converts αKG to succinyl-CoA and citrate to OOA, respeectively. This combination of enzymes both missing and present steers the TCA cycle to the reductive branch where succinic acid will be the end product (McKinlay et al., 2010). The flux distribution between the C4 and C3 pathways are influenced by two enzymes that constitutes the reversible shunts between the two. These enzymes are malic enzyme and OOA decarboxylase controlling the reactions between malate/pyruvate and OOA/pyruvate, respectively.

During genome analysis of *A. succinogenes* it was shown that both the glyoxylate pathway and Entner-Doudoroff pathway are absent thus limiting the metabolic system for substrate conversion and succinic acid production (McKinlay et al., 2010). While the oxidative pentose phosphate pathway (PPP) is present the activity is limited in the metabolism. The PPP is the main source of NADPH production in most cells and to compensate, *A. succinogenes* has a transhydrogenase which facilitates conversion between NADH and NAPDH (Pateraki, Patsalou, et al., 2016).

Theoretically one mole of succinic acid can be produced from one mole carbon dioxide and either 0.5 mole glucose, 0.6 mole xylose, or 1 mole glycerol (Pateraki, Patsalou, et al., 2016). However, the production of one mole succinic acid requires two moles NADH which limits the yield due to a restricted redox potential. From the glycolytic pathway, one mole of glucose produces two moles NADH which restricts the yield to one mole succinic acid/mole glucose or 1.12 g/g. This assumes that all carbon flux goes towards the C4 pathway and is converted into succinic acid (Cheng et al., 2012). The yield is also limited through flux towards the C3 pathway for biomass production and by-product formation which is necessary for regeneration of NAD(P)H and ATP (Pateraki, Patsalou, et al., 2016). In the conversion of pyruvate to formate and acetate, 1 ATP and 1 NADH is formed with an additional NADH that can be formed by oxidizing formate. This regenerative pathway causes production of formate and acetate as common by-products which limits the carbon that is used for production of succinic acid (McKinlay et al., 2010). To maintain a higher yield for succinic acid, by-product formation should be reduced. The immobilization of *A. succinogenes* in the form of biofilm is advantageous for the purpose as it lowers the acetyl-CoA requirements by activation of additional reducing powers thus resulting in lower amount of by-product (Bradfield & Nicol, 2014).

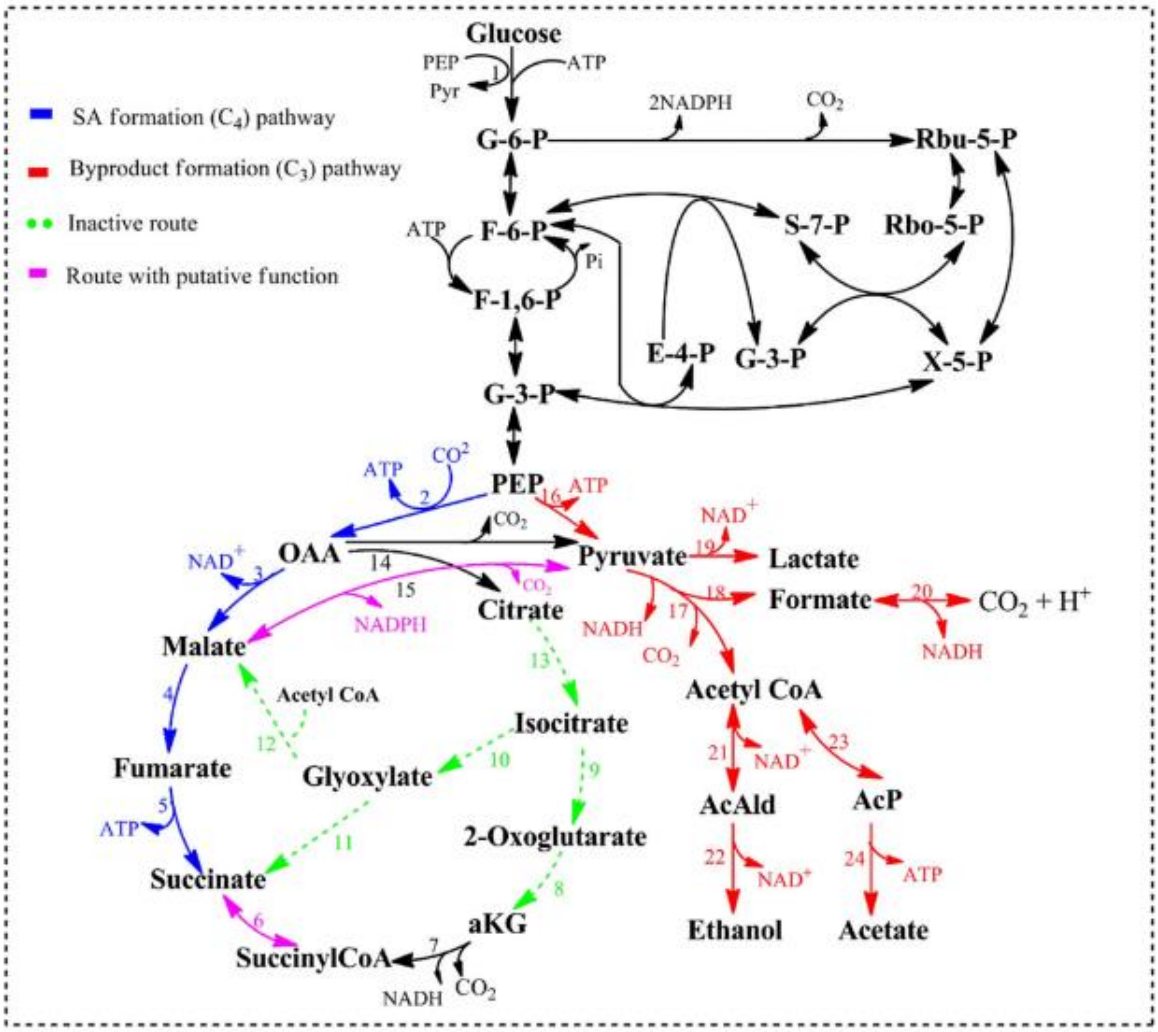


*Figure 1. The central carbon pathways of A. succinogenes for the conversion of glucose. From (Dessie et al., 2018).*

### 2.2.2 Carbon sources

*A. succinogenes* is known to be able to utilize a multitude of different pentoses and hexoses (Pateraki, Patsalou, et al., 2016). This is highly beneficial from an economic standpoint as the substrate is in many cases the most costly factor to consider during the fermentation process (Cheng et al., 2012). By utilizing biomass produced from waste streams from other industrial processes the price of the feedstock can be kept to a minimum. Many different studies have shown promising results when utilizing these feedstocks for production of succinic acid using *A. succinogenes.*

The most abundant renewable feedstock is biomass produced as a by-product in the agricultural sector such as lignocellulosic biomass (Sirohi et al., 2020). The main constituents are cellulose, lignin, and hemicellulose. Through degradation the biomass can be converted to hexoses and pentoses, with the most common types being glucose and xylose. A common method for pre-treatment of biomass is to first remove the lignin though different chemical treatments, followed by enzymatic hydrolysis to produce monomeric sugars (Rashid et al., 2021). However, the degradation process can cause problems in the fermentation stage as some chemicals such as acids and furfurals can still be present in the fermentation medium and act as inhibitors, decreasing the cell concentration (Dessie et al., 2018).

### 2.2.3 $CO_2$ fixation

The production of succinic acid in *A. succinogenes* utilizes carbon dioxide as a secondary substrate. Theoretically 1 mole of carbon dioxide is required to yield 1 mole of succinic acid (Xi et al., 2011). The availability of $CO_2$ for the microorganisms can influence the formation of succinic acid in different ways depending on the saturation level. The metabolic mechanism of $CO_2$ fixation occurs in conversion of PEP to OOA via the enzyme PEP-carboxykinase. This critical node regulates the flux distribution between the C4 and C3 pathway which makes $CO_2$ concentration largely influential in steering the metabolism to succinic acid (McKinlay et al., 2010).

The most suitable concentration range of $CO_2$ is from 8.4 mM (36.8 % saturation) up to 22.8 mM (fully saturated). In this range the concentration of $CO_2$ displays no limiting effect on succinic acid production. In the range of 3.9 mM to 8.4 mM the metabolic flux distribution remains the same but has a negative effect on the productivity. Below 3.9 mM the concentration of $CO_2$ is low enough to affect the metabolic flux distribution causing a shift from the C4 pathway to the C3 pathway producing higher amounts of ethanol (Herselman et al., 2017).

Carbon dioxide permeates through the cellular membrane, causing the intracellular concentration to be dependent on the dissolved $CO_2$ and in turn the partial pressure. At equilibrium in water the balance between dissolved $CO_2$ , bicarbonate and carbonate can be described by Eq 1 and Eq 2 (Xi et al., 2011).

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \tag{1}$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \tag{2}$$

The dissolved $CO_2$ utilized by the cells is dependent on the partial pressure of $CO_2$ and Henry's constant (Xi et al., 2011):

$$CO_2 = \frac{P_{CO_2}}{H} \tag{3}$$

There are two common methods for carbon delivery during fermentation. Either through the addition of carbonate salts in the fermentation broth, or though sparging with carbon dioxide gas (Herselman et al., 2017). One advantage of using carbonate salts is that in addition to the role of supplying $CO_2$, it also acts as a pH buffer necessary during acidification from acid production to maintain a more stable system. Another advantage is that higher carbonate loadings correlate to a better ratio between succinic acid and acetic acid production (Tan et al., 2018). One of the downsides with carbonate loading is that carbonates can be transported into the cell which requires ATP. Some carbonate salts also have solubility issues which is not suitable in an industrial setting (Pateraki, Patsalou, et al., 2016). When operating in larger scale such as in bioreactors and under continuous operation, sparging with $CO_2$ gas is more common. High rates of sparging are linked with lower amounts of by-product formation and better gas-liquid mass transfer. The $CO_2$ gas reacts with the fermentation medium to form $H_2CO_3$ which then deprotonates resulting in lowered pH. Therefore, a pH control system is necessary so that optimal conditions can be maintained (Tan et al., 2018).

### 2.2.4 Growth kinetics

The growth kinetics can be modelled using an extended Monod model considering substrate and product inhibition. The specific growth rate is described by Eq 4.

$$\mu = \mu_{max} \frac{S}{S + Ks + \frac{S^2}{KI}} \prod \left(1 - \frac{P_i}{Pcrit_i}\right)^{n_p} \tag{4}$$

In Eq 4, μ ($h^{-1}$) is the specific growth rate and $\mu_{max}$ ($h^{-1}$) the maximum specific growth rate, S is the substrate concentration (g/L), $K_S$ the substrate affinity constant (g/L), $K_I$ the substrate inhibition constant (g/L), P is the product concentration (g/L), Pcrit the critical product concentration (g/L), $n_p$ the product inhibition exponent. The product inhibition term considers succinic acid, acetic acid, and formic acid as these are the main products for *A. succinogenes*. The production of ethanol and lactic acid is negligible if fermentation is performed under the right conditions.

The growth of the suspended cells is described by Eq 5.

$$\frac{dX^S}{dt} = \mu \cdot X^S - D \cdot X^S \quad (5)$$

$X^S$ refers to the total amount of suspended cells in the system expressed in g-CDW/L (CDW refers to cell dry weight). D is the dilution rate when the fermentation is operated continuously. In batch fermentation the dilution rate is set to 0.

The growth of attached cells or biofilm formation is described by Eq 6.

$$\frac{dX^A}{dt} = \mu_S \cdot X^S \cdot \alpha \cdot A \quad (6)$$

$X^A$ is the total amount of biofilm attached to a surface in the system expressed in g-CDW/L. α is growth associated biofilm formation term based on the amount of biofilm formed per surface area expressed as g-CDW-attached cells/g-CDW suspended cells·cm$^2$. A is the total available surface area for the biofilm to attach to, measured in cm$^2$.

Substrate consumption is described by Eq 7.

$$\frac{dS}{dt} = D\left(S^{in} - S^{out}\right) - \mu_S \cdot X^S \cdot Y_{xs} \quad (7)$$

$Y_{XS}$ is the yield of substrate per suspended biomass expressed as g-substrate/g-CDW biomass.

Product formation is described in Eq 8.

$$\frac{dP}{dt} = \mu_S \cdot X^S \cdot Y_{xp} - D \cdot P \quad (8)$$

$Y_{XP}$ is the yield of product per biomass expressed as g-product/g-CDW biomass. Just as for the specific growth rate the only products that are considered in the system are succinic acid, acetic acid, and formic acid.

### 2.2.5 Model structure

The model is constructed to estimate the six state variables ($X^s$, $X^A$, $S$, $P_{SA}$, $P_{AA}$, $P_{FA}$) through experimental determination of the kinetic parameters ($\mu_{max}$, $\alpha$, $Y_{XS}$, $Y_{XSA}$, $Y_{XAA}$, $Y_{XFA}$,). The kinetic parameters are implemented along with initial values, constants, and kinetic parameters from (Pateraki, Almqvist, et al., 2016) presented in Table 1.

*Table 1. Kinetic parameters for the model. Obtained from Pateraki, Almqvist, et al., 2016.*

| Parameter | Description | Value | Unit |
|---|---|---|---|
| **$K_S$** | Substrate affinty constant | 0.698 | g/L |
| **$K_I$** | Substrate inhibition constant | 55.484 | g/L |
| **$Pcrit_{SA}$** | Critical product concentration of succinic acid | 55 | g/L |
| **$Pcrit_{AA}$** | Critical product concentration of acetic acid | 38 | g/L |
| **$Pcrit_{FA}$** | Critical product concentration of formic acid | 18 | g/L |
| **$n_p$** | Product inhibition exponent | 2.3 | - |

The differential equations and parameters were implemented in Matlab R2022b and solved using an ode45 solver allowing for easy integration.

## 2.3 Biofilm

Many different microorganisms have the morphological ability to flocculate and form structures known as biofilms. Biofilm is a formation of several cells that are attached to a surface on which they can grow. The surface can be both poor or rich in nutrients depending on the species present in the biofilm and their different properties. The microbiome of the biofilm can consist of several different types of microorganisms existing and working in symbiosis. There are also some species that are able to form single-species biofilm under the right conditions where only one type of microorganism is present. The structure of the cell colony is made up of a protective extracellular matrix that consists of polysaccharides secreted from the microorganisms that also allows for cellular communication via transfer of biochemical signals (El Moustaid et al., 2013). The shape of a colony approximately resembles a mushroom. Most cells are located in the top part where they are immobilized with no Brownian movement occurring, whereas in the bottom the cells are sparse. The extracellular polymer matrix (EPS) is densest closest to the cells. The colonies are oriented horizontally in thin films but can also be vertical in thicker colonies. Inside the structure of the biofilm, water channels are present which allow for transportation of solutes from the bulk liquid. The composition of the biofilm is roughly 10-25% cells and 75-90% extracellular polymer matrix with variations in composition depending on the species (Costerton, 1999). Some of the defining features of biofilms are that they are very heterogenous, has a high genetic diversity, the phenotype adapts depending on the cellular growth and transcription of certain genes, and attach to surfaces (El Moustaid et al., 2013). The function of biofilms in nature is to provide protection from shear stress, dehydration, toxic compounds, and to enable protozoan grazing where biomass and nutritional compounds are recycled to the biofilm. The decomposition of organic matter is also facilitated by biofilm due to degradation enzymes being retained inside the biofilm which makes the uptake of nutrients more efficient (Sauer et al., 2022).

Biofilms are not only restricted to cells attached on a surface but also non-surface-attached aggregated bacteria. This also applies within the medical field where surface-associated infections are common for implants and medical devices. Non-surface-associated biofilms are more prone in some lung diseases and in soft tissues. If pathogenic bacteria develop biofilm the disease progresses to become chronic and untreatable, but some studies suggest that this is due to metabolic activity instead of aggregation (Sauer et al., 2022).

### 2.3.1 Biofilm formation

Through staining, Tolker-Nielsen et al. 2000 showed that the physiological structure of biofilm differs between species of bacteria and that movement between microcolonies occurred after critical thickness was reached. They also found that during the early phase of biofilm formation, movement was limited and the bacteria inside the colonies were sessile (Tolker-Nielsen et al., 2000).

The development of biofilm can be divided into five separate stages as shown by Sauer et al. 2002. Through the observation of *Pseudomonas aeruginosa* via microscopy, distinct physiological changes such as biofilm morphology, matrix polymer production, and the activation of quorum sensing-regulatory genes were used to determine transitional behaviour. The conclusion was that the development process could be divided into five stages: i) initial attachment, ii) irreversible attachment, iii) first maturation, iv) second maturation, and v) dispersion (Sauer et al., 2002). The process is depicted in Figure 2.

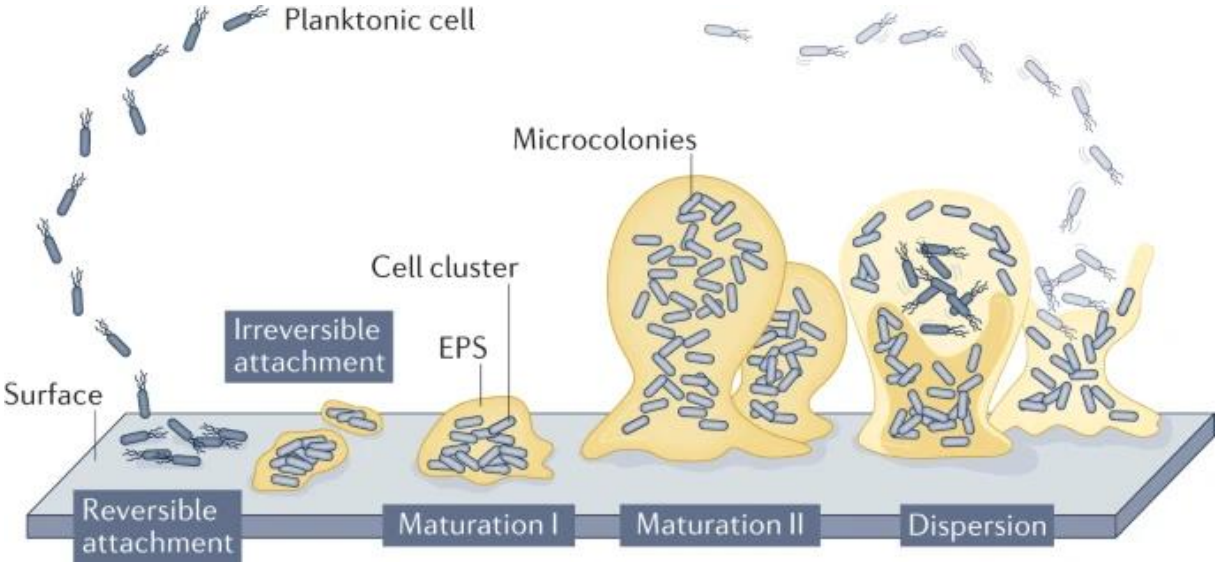


*Figure 2. Biofilm development life cycle. From (Sauer et al. 2022).*

### 2.3.2 Initial attachment

During initial attachment, bacteria make contact with a surface, forming a reversible bond which in some cases detaches over time as the cells are loosely attached. In this process flagella have been shown to be largely influential as cells have been observed to rotate axially at a fixed location on the surface before adhering. When compared to non-motile cells, the flagellated cells displayed significantly greater cell growth (Sauer et al., 2002). The process of attaching to a surface begins by surface sensing of the cells. Signalling mechanisms are present in biofilm forming bacteria that stimulate movement in certain directions depending on the concentration of signalling molecules excreted. Closer to surfaces the concentration is increased, directing the movement towards the surface where the attachment is initiated. Typical signalling pathways in biofilm formation microorganisms are chemotaxis, quorum sensing, and cyclic dimeric guanosine monophosphate (c-di-GMP) (Armbruster & Parsek, 2018; Floyd et al., 2017). C-di-GMP is a intracellular secondary messenger responsible for a number of regulatory processes in the transition from motile to sessile cells. A correlation between increased c-di-GMP concentration and biofilm formation has been proven in several bacterial species (Valentini & Filloux, 2016). The process of surface-signalling is rather complex with many different changes in gene expression and signal cascades.

### 2.3.3 Irreversible attachment

In the second stage of biofilm development the previously reversibly attached cells progress to become irreversibly attached. The cells begin to form clusters of microorganisms. Inside these microcolonies the cells become sessile as their motility stops. Through protein analysis it has been shown that there is an increased activity in the lasB gene which controls the Las quorum sensing system. This signalling system is expected to be involved in forming the architecture of the extracellular matrix. By comparing wild-type bacteria with mutants defective in the Las system it was found that similar quantities of EPS were produced by both strains. However, there was a difference observed regarding the location of the EPS. In the wild type, the EPS was primarily located in gaps between cells, whereas in the mutant the EPS was mostly present on the cell surface. This caused the mutant cells clusters to be more compact as gaps were not filled with EPS allowing for more distance between cells in the wild type strain (Sauer et al., 2002).

### 2.3.4 First maturation

The third stage of development is known as the first maturation. In this stage cell colonies increase in thickness through layering of clusters. The initial point where this stage takes part is when the biofilm thickness is approximately 10 microns. During the first maturation increased activity of the rhlA gene has been shown which leads to activation of the Rhl quorum sensing system (Sauer et al., 2002).

### 2.3.5 Second maturation

During the fourth stage the biofilm reach a maximum thickness of around 100 microns. During this stage, the cells inside of the clusters remain sessile until the maximum thickness is reached at which point clusters start to disperse from the surface (Sauer et al., 2002).

### 2.3.6 Dispersion

The fifth stage follows the last stages of the second maturation. After the maximum thickness has been reached, cells are dispersed from the biofilm by evacuation. The bacteria become motile and swim away from the clusters through openings into the bulk liquid. In these openings a void without any EPS is present in which motile bacteria move around before dispersing (Sauer et al., 2002).

### 2.3.7 Factors affecting biofilm formation

The formation of biofilm is a complex process as the cells undergo drastic physiological changes. There are several factors that influence this transition from motile microbes to attached colonies. The properties of the attachment surface, bulk fluid environment, and the cells themselves are all factors that determine the behaviour of the culture (Alotaibi, 2021).

The surface which the cells can adhere to may influence the rate of attachment and quantity of cells that can attach depending on its properties. Increased hydrophobicity of a surface generally improves the probability of cell attachment. Another property is the structure and material of the surface. Microbes will tend to adhere more readily to surfaces that have a greater roughness compared to smoother surfaces (Alotaibi, 2021).

The environment inside the bulk fluid can heavily affect the formation of biofilm. Factors such as temperature, pH, flow velocity, and nutrient availability play an important role. Cells will transition into biofilm aggregates for survival if the conditions are suboptimal (Alotaibi, 2021). Therefore, the conditions of a culture during fermentation could theoretically be manipulated to favour formation of biofilm.

Cellular properties such as the outside surface, gene expression and production of EPS will also influence the formation (Alotaibi, 2021).

The complex process poses a challenge when it comes to predicting the behaviour of cells prone for attachment, especially in the case of modelling. The many factors make it hard to mathematically describe the many reactions occurring and parameters involved. The easiest factors to manipulate during fermentation for researching biofilm formation are the properties of the bulk liquid which is possible to control and monitor rather accurately.

### 2.3.8 Methods for biofilm growth assessment

The complex structure and growth of biofilms pose some challenges when it comes to measuring the formation process. Various techniques have been investigated and successful results are available. However, there is a lack of standardized protocols and reproducibility in many of the published methods. The heterogeneous morphology, long formation times, and general unpredictability cause difficulties for creating representative measurements of a biofilm system. The methods commonly used can be divided in quantitative or qualitative depending on whether the method produces numerical measurements or must be interpreted through imaging (Wilson et al., 2017).

One of the most common methods in quantification of cells is gram staining using crystal violet. The mechanism behind the method is that crystal violet can form complexes with compounds inside cells. The dye can then be solubilized, and the cells can be quantified by measuring the absorbance using spectroscopy (Wilson et al., 2017).

## 2.4 Fermentation modes

The simplest form of fermentation is batch fermentation where the goal is to maximize the substrate conversion rate. Fermentations are commonly performed at 37-39 degrees Celsius and a pH in the range of 6.8-7.2 (Ferone et al., 2019). For *A. succinogenes,* buffers are used to neutralize the acids produced to maintain a pH of around 6-7.5 (Pateraki, Patsalou, et al., 2016). Fed-Batch can also be used to decrease the inhibitory effect of high substrate concentrations. At glucose concentrations above 100 g/L the yield of succinic acid decreases, and above 158 g/L growth is completely inhibited (Lin et al., 2008). Batch fermentations are commonly performed in microplates or bottles to estimate kinetic parameters for modelling purposes (Pateraki, Patsalou, et al., 2016).

In bioreactors it is not as common to run in batch fermentation due to product inhibition when operating during longer times. The preferred operational mode is continuous. Advantages are higher productivity, high growth rates close to the maximum during an extended period of time, and longer operational times (Ferone et al., 2019).

Since *A. succinogenes* is prone for biofilm formation, free cell continuous bioreactors in which the majority of cells are suspended in the fermentation broth are not commonly used. In immobilized cell reactors, a matrix is available for the cells to adhere to. This allows for retention of the cells inside the reactor, maintaining high biomass concentration. Other advantages are easier separation of cells and products, higher productivities, and more flexibility in reactor design (Ferone et al., 2019). There are three different types of immobilization techniques used, adsorption, entrapment, and covalent bonding. In adsorption, cells naturally attach to surfaces as biofilm aggregates. Entrapment and covalent bonding use chemical additives that retain the cells in the reactor (Qureshi et al., 2005).

Due to the flexibility when utilizing immobilization techniques, there is a variety of reactor configurations available. Continuously Stirred Tank Reactors (CSTRs) use mechanical stirring in combination with constant inflow and outflow to maintain homogenous conditions inside the reactor for optimal control. The mechanical stirring does not allow for the use of adsorbent support as agitation would be limited and mechanical stress could disturb the biofilm. Packed Bed Reactors (PBRs) use different types of support materials that allows for cell adhesion. Depending on the packing density inside the reactor, very high concentrations of biomass are achievable which in some cases can be too excessive, clogging up the reactor and tubing (Qureshi et al., 2005).

## 2.5 Analysis methods

### 2.5.1 Spectrophotometry

Spectrophotometry is a common technique used in microbiology. Simplified it can be described as a method that measures how light interacts with matter which makes it a type of spectroscopy. A spectrophotometer is used which can measure the light intensity in a spectrum of different wavelength. A light beam of known intensity and wavelength is emitted from a source in the spectrophotometer which passes through a sample inside a cuvette. The light intensity after passing through is measured and the difference in intensity describes the absorbance of the sample. In conjunction with Beer-Lamberts law (Eq 9) the absorbance can be used to calculate the concentration of a certain compound inside a sample in a linear relation (Calloway, 1997):

$$A = \epsilon l c \tag{9}$$

$A$ is measured absorbance, $\epsilon$ is the molar extinction coefficient, which is a constant for a specific molecule, $l$ is the path length and therefore width of the cuvette, which is commonly 1 cm, $c$ is the concentration of a molecule in the sample. Using colouring agent such as crystal violet makes spectrophotometry a powerful tool as the concentration of crystal violet in the sample can be determined which can be used to quantify the cells.

To measure biomass formation during a process a certain term is used; optical density (OD). While commonly determined using a spectrophotometer the theory behind it differs (Hills, 2016). Beer-Lamberts law assumes that molecules in a sample are evenly dissolved and not suspended as in a cell culture. Particles in a solution will scatter light rather than absorb it (Mira et al., 2022). However, the sample principle applies that scattering depends on the cell concentration in the sample which makes

OD an estimation of the cell population (Hills, 2016). To increase the accuracy, OD should be converted to actual cell density in cells/volume. This can be done by making a standard curve for calibrating how the OD measurements correlates to cell concentration. Colony forming units/volume (CFU/L) or grams dry weight/volume (CDW/L) are examples of conversions (Mira et al., 2022).

Conversion factors for *A. succinogenes* at three different wavelengths for absorbance to cell dry weight are presented in Table 2.

*Table 2. Factors for converting optical density to cell dry weight.*

| **Wavelength (nm)** | **Conversion factor (1 OD = g CGW/L)** | **Reference** |
|---|---|---|
| **550** | 0.333 | (Jantama et al., 2008) |
| **600** | 0.3138 | (Rhie et al., 2018) |
| **660** | 0.626 | (Zhu et al., 2012) |

# 3 Kinetic growth and biofilm formation in microplates

During the first stage of the project the aim was to investigate the kinetic growth of both suspended cells and biofilm formation. An assay used for quantifying biofilm was evaluated to see if it was feasible to utilize as a tool estimation of biofilm growth over time. After proven to be successful in standalone experiments a procedure was proposed for analysing kinetics for suspended cell growth as well as attached cell growth.

## 3.1 Materials and methods

### 3.1.1 Cell culture

The medium for the cell cultivation was composed of (g/L) yeast extract (5.0), $K_2HPO_4$ (3.0), $MgCl_2$ (0.2), $CaCl_2$ (0.2), NaCl (1), $NaHCO_3$ (5.0), glucose (5.0) mixed with deionized water. First a stock medium containing no added glucose was prepared, 50 ml was added to a bottle and sealed. The bottle was then sparged with nitrogen for 7 minutes to ensure anaerobic conditions. The bottle was sterilized at 121 °C for 20 minutes in an autoclave. 5 ml of a concentrated glucose was added through a sterile filter. 10%(v/v) of inoculum was added to the bottle and placed in an incubator at 37°C overnight.

### 3.1.2 Microplate setup

To measure the growth kinetics of the suspended cells as well as the attached cells, experiments were performed using microplates (Thermo Fisher Scientific 24 wells) made of polystyrene. The microplates were analysed by measuring the absorbance using a microplate reader (BioTek, Synergy H1 microplate reader). For the suspended growth kinetics, the absorbance was measured continuously during four time periods for a total time of almost 70 hours. The protocol was set to keep a constant temperature of 37°C, measurements were taken every 5 minutes and prior to the measurement the plate was orbitally shaken for 10 seconds. The absorbance measurements can be directly correlated to the optical density of the sample.

The medium for the microplates was composed of (g/L) $K_2HPO_4$ (3.0), $MgCl_2$ (0.2), $CaCl_2$ (0.2), NaCl (1), $NaHCO_3$ (5.0), glucose (5.0) and mixed with water. The medium was passed through a sterile filter to reduce the number of particles present in the mixture as it would affect the measurements after staining. The wells were inoculated with 50 µl *A. succinogenes 130Z (DSM 22267)* (10 v/v%) preculture for a total volume of 500 µl. For the biofilm study four different conditions were tested in 5 replicates. The pH was adjusted to 6.8 or 6.3 by addition of 50% phosphoric acid and the concentration of yeast extract added was 5 g/L or 2 g/L. The layout can be seen in Figure 3.

| | | | | | |
|---|---|---|---|---|---|
| pH 6.8<br>YE 5g/L | pH 6.8<br>YE 5g/L | pH 6.8<br>YE 5g/L | pH 6.8<br>YE 5g/L | pH 6.8<br>YE 5g/L | Negative control |
| pH 6.3<br>YE 5g/L | pH 6.3<br>YE 5g/L | pH 6.3<br>YE 5g/L | pH 6.3<br>YE 5g/L | pH 6.3<br>YE 5g/L | Negative control |
| pH 6.8<br>YE 2g/L | pH 6.8<br>YE 2g/L | pH 6.8<br>YE 2g/L | pH 6.8<br>YE 2g/L | pH 6.8<br>YE 2g/L | Negative control |
| pH 6.3<br>YE 2g/L | pH 6.3<br>YE 2g/L | pH 6.3<br>YE 2g/L | pH 6.3<br>YE 2g/L | pH 6.3<br>YE 2g/L | Negative control |

*Figure 3. Microplate setup*

A total of nine plates with 24 wells each were prepared with the conditions described. After inoculum was added one of the plates was placed in the microplate reader to continuously measure the growth of the suspended cells during the entire fermentation process. The other eight were placed in an incubator at 37°C. At each sampling point, one of the microplates were taken out and the biofilm assay was performed according to 3.1.3. Samples were taken at the following time points after incubation was initiated (hours); 4, 21, 25, 29, 45, 49, 53, 69, and 74. Note that the microplate sample taken for the biofilm formation kinetics at 74 hours was the plate placed in the microplate reader in the beginning of

the fermentation for suspended growth measurement. Therefore the conditions for this sample is not entirely the same as for the other samples.

For the second kinetic experiment the protocol was similar with the same conditions, number of plates, and working procedure. The sampling points were narrowed down due to the results from the first kinetic experiment to achieve a better fit. Samples were taken at the following time points after incubation was initiated (hours); 0, 13, 15, 17, 19, 21, 23, 25, 39, and 44.

### 3.1.3 Biofilm assay

The growth kinetics of attached biofilm were measured using a modified protocol for Microtiter Dish Biofilm Formation Assay by O'Toole G.A 2011 (O'Toole, 2011).

1. After incubation, the cells suspended in the bulk media in all wells were poured out, leaving mostly attached cells in the wells.
2. The microplate was gently submerged in a container with tap water as to remove more suspended cells and other components that could interfere with the staining process. This step was performed twice aiming to reduce the background staining.
3. 750 µl of a 0.1% crystal violet solution was added to each well to stain the attached biofilm.
4. The microplate was placed in a shake incubator at 37°C for 15 minutes to cover all the attached cells on the walls of the well.
5. The excess crystal violet was removed by submerging the plate in a container with tap water and blotting on paper. This step was repeated for a total of four rinses.
6. The microplate was left upside down on a paper towel overnight for drying.
7. The next day 750 µl of a 30% acetic acid solution was added to each well to solubilize the attached crystal violet.
8. The microplate was placed in a shake incubator at 37°C for 15 minutes to allow the acetic acid to fully solubilize the crystal violet.
9. 750 µl of the solubilized crystal violet was transferred from each of the wells of the microplate to a new microplate.
10. The absorbance was measured in a microplate reader.

An example of how the microplate looks after staining and drying is shown in Figure 4. The stained culture is very purple and diplay a distinct line in the wells representing the surface level for the liquid-gas interface during the fermentation.

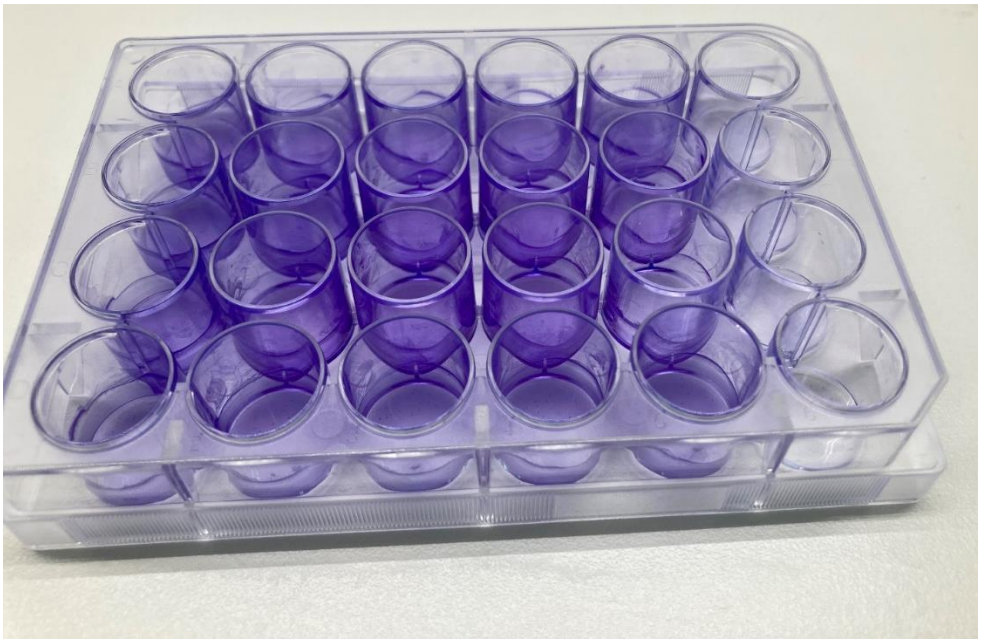

*Figure 4. Microplate after staining with crystal violet and left to dry.*

## 3.2 Results

Results are presented in order of the experiments describing the growth kinetics of suspended cells and biofilm during the fermentations.

### 3.2.1 Experiment 1

Figure 5 shows the growth curves for the suspended cells. A clear difference can be seen depending on the conditions of the fermentation media. The growth in media with 5 g/L of yeast extract reach a higher cell density compared to cells grown in 2 g/L of yeast extract. Comparing the pH conditions, cells starting at an initial pH of 6.8 show higher initial cell concentration compared to the cultures at pH 6.3. Some growth was present in the blanks, either from the inoculum or from contamination. If contaminated, the reliability of the experimental results is decreased.

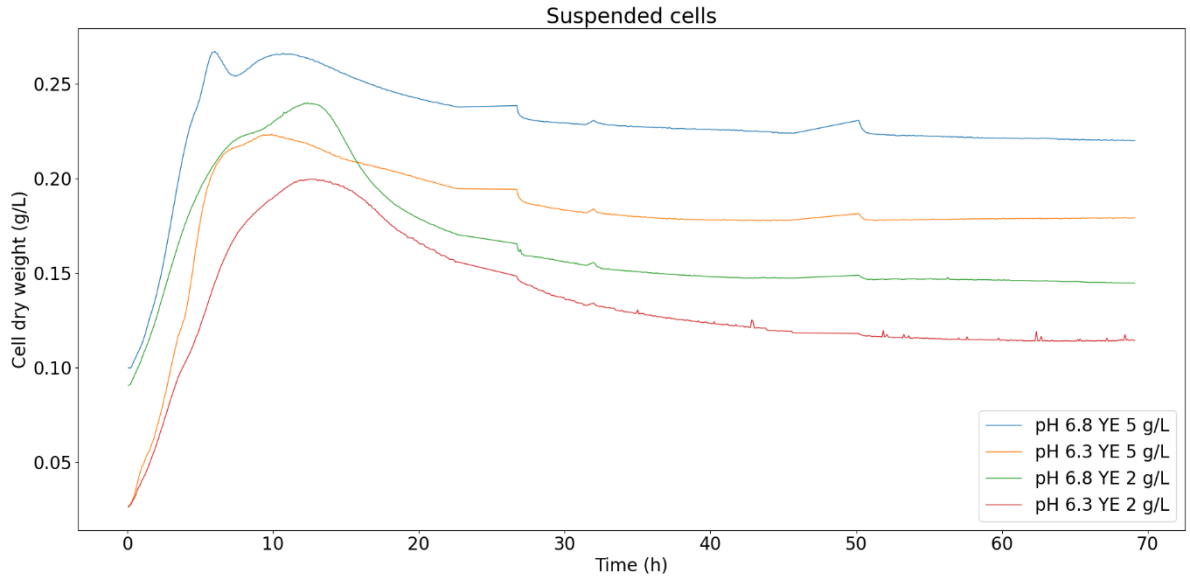


*Figure 5. Suspended cell growth at four different conditions, measured at 660nm.*

Biofilm formation is presented in Figure 6. A significant difference in growth can be seen when comparing the cultures with different pH with a yeast extract concentration of 5 g/L ($P < 0.05$). Those cultures that start with an initial pH of 6.3 form a greater amount of biofilm compared to those starting at pH 6.8 up until the final measurement. At YE 2 g/L a difference between pH 6.8 and 6.3 is seen but cannot be deemed significant ($P > 0.05$). For different yeast extract concentrations, the difference is not as significant ($P > 0.05$). The culture grown at pH 6.3 and YE 5 g/L form more biofilm compared to the culture at pH 6.3 and YE 2 g/L during the first 30 hours before converging. From the curves, it seems that rapid formation occurs sometime before 20 hours until around 25 hours.

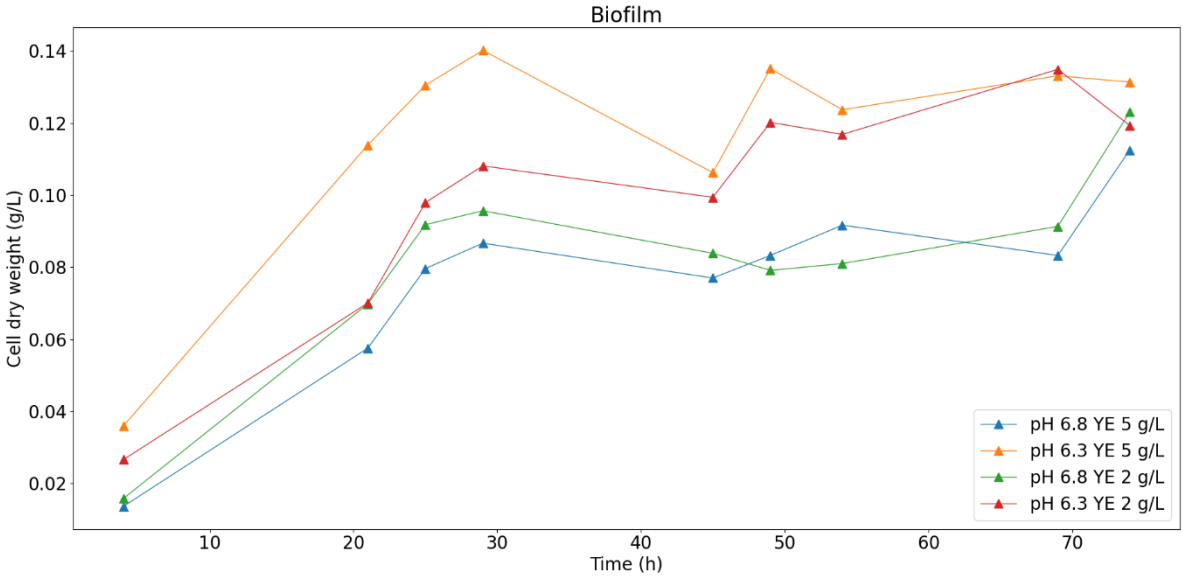


*Figure 6. Biofilm formation at four different conditions, measured at 550 nm.*

The data from the kinetics studies was used to calculate kinetic parameters for both the suspended cells and attached cells. The maximum specific growth rates are presented in

Table 3.

*Table 3. Maximum specific growth rates of suspended cells and attached cells at different initial conditions.*

| **Initial conditions** | **μmax suspended cells (h-1)** | **μmax attached cells (h-1)** |
|---|---|---|
| **pH 6.8 YE 5 g/L** | 0.229 ± 0.017 | 0.084 ± 0.014 |
| **pH 6.3 YE 5 g/L** | 0.353 ± 0.011 | 0.063 ± 0.011 |
| **pH 6.8 YE 2 g/L** | 0.178 ± 0.017 | 0.085 ± 0.008 |
| **pH 6.3 YE 2 g/L** | 0.364 ± 0.033 | 0.067 ± 0.011 |

The maximum specific growth rate for suspended cells is higher for cultures grown at pH 6.3 compared to pH 6.8. For different concentrations of yeast extract, the growth rate is higher for cultures starting at 5 g/L. The maximum specific growth rate of attached cells follows an opposite trend as $\mu_{max}$ for cultures with initial pH 6.8 is higher than pH 6.3. Almost no difference is shown when comparing cultures with different initial yeast extract concentrations.

### 3.2.2 Experiment 2

Due to a defect pH sensor, the stated initial pH is not accurate and higher than desired. Therefore, the comparison between cultures at different pH is difficult to make as the actual initial pH is unknown.

The suspended growth of the cultures presented in Figure 7 show similar characteristics compared to the previous experiment. A small lag phase can be identified during the first 2 hours. For the cultures with a yeast extract concentration of 5 g/L the cells grow exponentially up until around 10 hours after which they reach stationary phase. The cultures with 2 g/L of yeast extract do not grow as rapid and take longer to reach stationarity.

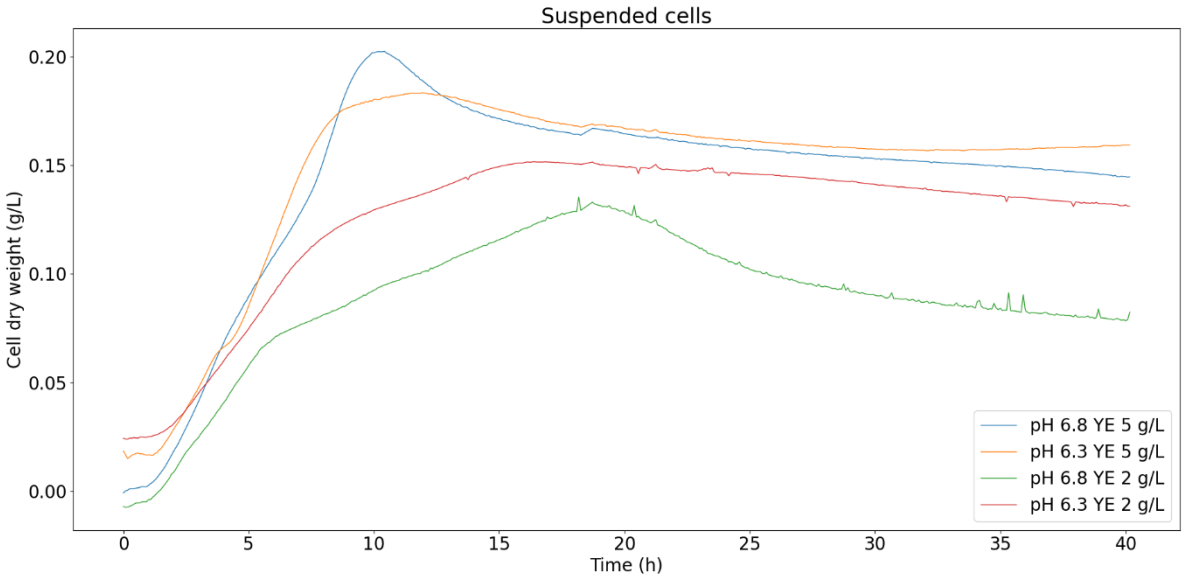


*Figure 7. Suspended cell growth at four different conditions, measured at 550nm.*

Looking at the biofilm formation seen in Figure 8, similar kinetics are seen for all the cultures. After some initial adjustment to the environment, it appears as the biofilm formation grows exponentially after 15 hours up until 21 hours.

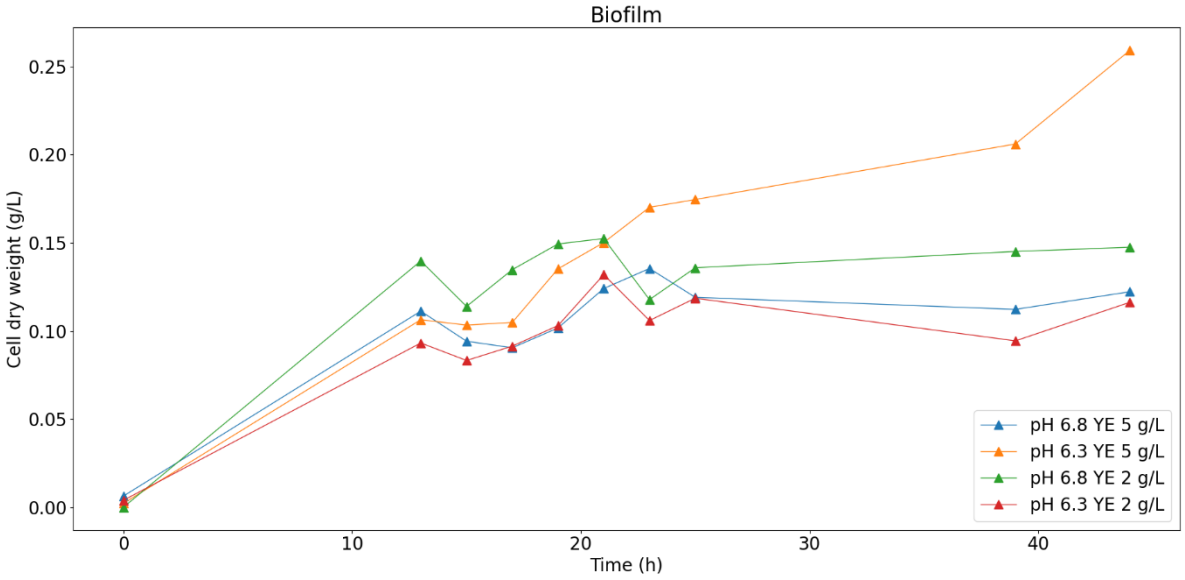


*Figure 8. Biofilm formation at four different conditions, measured at 550 nm.*

The kinetic parameters calculated from the data are presented in Table 4 in the form of maximum specific growth rates for suspended as well as attached cells at the different conditions. However, since the initial pH is unknown, the accuracy of the values is debatable.

*Table 4. Maximum specific growth rates of suspended cells and attached cells at different initial conditions.*

| Initial conditions | $\mu_{max}$ suspended cells | $\mu_{max}$ attached cells |
|---|---|---|
| **pH 6.8 YE 5 g/L** | 0.179 ± 0.031 | 0.084 ± 0.025 |
| **pH 6.3 YE 5 g/L** | 0.213 ± 0.007 | 0.074 ± 0.022 |
| **pH 6.8 YE 2 g/L** | 0.413 ± 0.043 | 0.074 ± 0.008 |
| **pH 6.3 YE 2 g/L** | 0.208 ± 0.002 | 0.089 ± 0.015 |

No observations regarding the specific values or comparison between different initial conditions can be made due to the faulty pH sensor. However, the magnitude of the growth rates lies within the values when compared to the results from experiment 1. This growth rate greatly exceeds all other results and other values found in literature. The accuracy of this value should be questioned. The maximum specific growth rate of attached cells is somewhat higher in the range of 0.074-0.089 $h^{-1}$.

## 3.3 Discussion

Based on the results from the experiments performed in the microplates there are some important factors to consider for further experiments. For suspended cell growth the concentration of yeast extract will have affect the total amount of suspended cells produced. No experiments were conducted where the concentration of yeast extract was further increased. Therefore, it is not known whether the system is limited at 5 g/L and if higher cell concentration is achievable using higher yeast extract concentration. From literature, a concentration of yeast extract above 5 g/L yields higher cell concentration but is economically detrimental (Liu et al., 2008).

The effect of pH on suspended cell growth is correlated with decreased cell growth at lower values. As mentioned, the pH values from experiment 2 are not accurate and no conclusions regarding the pH can be drawn from these results.

The formation of biofilm does not appear to be influenced by the concentration of yeast extract in the fermentation broth in a distinct manner. Although the concentration of biofilm is higher when supplied with 5 g/L instead of 2 g/L for cells growing at pH 6.3 in experiment 1, the difference for cells at pH 6.8 is not significant. No conclusions can be drawn regarding experiment 2.

The initial pH of the fermentation broth seems to affect the biofilm formation quite significantly during the earlier stages of fermentation. Results from experiment 1 suggest both faster attachment and higher biofilm concentration when the initial pH is 6.3 instead of 6.8. The last sample at 74 hours does suggest a rapid increase in biofilm formation for the cultures grown at pH 6.8, but since sampling ceased after this this cannot be validated.

In experiment 2 samples were taken at shorter time intervals during the period of 13-25 hours to investigate the growth kinetics of the biofilm. The results indicate that biofilm grows exponentially during this time, although at a much slower rate than the exponential phase for suspended cells. Comparing curves of the suspended cell growth and biofilm formation the increase in biofilm concentration at around 15-21 hours correlates with a decrease in suspended cell concentration. No noticeable cell death should be present around this time and therefore it can be assumed that suspended cells transition to attached biofilm due to pH decrease from product formation. This could indicate that the growth of attached cells consists of both attachment of suspended cells and simultaneous growth of already attached cells. This theory cannot be proven from the available data.

Looking at the kinetic parameters calculated from the data from the first experiment indicates that the growth rate of suspended cells occurs more rapidly at lower initial pH and at higher initial concentration of yeast extract. The results are reasonable when looking at the growth curve of the cultures in Figure 5. The cultures at lower pH has an initial suspended cell concentration that is lower compared to cultures at higher pH. The reason for this is unknown but as the cells grow exponentially the cell concentration

at lower pH increase more relative to the initial concentration compared to higher pH. The growth rate of the attached cells displays the opposite trend where cultures at higher pH form biofilm at a faster rate during the growth phase, even though lower pH forms a higher total amount of biofilm.

Looking at the values from the two experiments it appears as the maximum specific growth rate for suspended cells of *A. succinogenes 130Z* is between 0.178 $h^{-1}$ and 0.413 $h^{-1}$ depending on the initial conditions. The maximum specific growth rate for attached cells is shown by the results to be somewhere between 0.063 $h^{-1}$ and 0.089 $h^{-1}$, growing at a much slower rate than the suspended cells. However, it should be noted that the surface area, material, and surface roughness should be factors that could influence this value. If more surface area was available, it could be reasoned that the maximum growth rate of the attached cells would seem higher as there is no method for distinguishing between growth of the attached cells and the attachment of suspended cells which would depend on the availability of surface to adhere to.

# 4 Kinetic growth and biofilm formation in batch bottles

In the second phase of the project, the aim was to gain further knowledge regarding the fermentation process by performing experiments on a slightly larger scale in batch conditions. The evaluated factors were suspended cell growth, biofilm formation on both bottle surface and packing material, substrate consumption and product formation. Two different conditions were tested and compared, either cells were grown in bottles containing only fermentation media or grown in bottles containing porous packing material. Two experiments were performed at different volumes, either in 20 ml bottles or 100 ml bottles.

## 4.1 Batch fermentation in 20 ml bottles

During the first experiment the goal was to get initial data and get used to the cultivation technique.

### 4.1.1 Experimental setup

For the kinetic study, batch fermentations were performed in 20 ml glass bottles (ApodanNordic) with or without packing material. In total 30 bottles were prepared, 14 without packing material and 16 with. The two extra bottles with packing material were prepared in case additional techniques for biofilm quantifications were to be tested. The bottles were prepared in duplicates for both packing samples and non-packing samples. At each sampling point four bottles were sampled. Because the method is destructive, the fermentation in the bottles could not be continued as results could not be considered accurate after sampling. To maintain a quantifiable surface area and volume which would allow for biofilm formation, the bottles were kept vertical during the whole fermentation process.

### 4.1.2 Fermentation media

The composition of the cell culture media for the batch fermentations was composed of (g/L) yeast extract (5.0), $K_2HPO_4$ (3.0), $MgCl_2$ (0.2), $CaCl_2$ (0.2), NaCl (1), $NaHCO_3$ (10.0), glucose (10.0) mixed with deionized water. All compounds except glucose were mixed into a stock solution and 7 ml was added to each bottle. Before adding the stock media solution, 16 of the bottles were filled with 10 Kaldnes K1 biofilm carriers. The bottles were then sparged with nitrogen for 5 minutes to achieve anaerobic conditions. The bottles were sealed and autoclaved at 121 °C for 20 minutes. 2 ml of glucose solution was added to the bottles using a sterile filter. The pH was adjusted to 7 by addition of phosphoric acid (50%). 2 ml inoculum were added to the bottles and then placed in a shake incubator at 37 °C.

### 4.1.3 Analysis methods

At each time point the bottles to be sampled were opened thus removing the anaerobic conditions, 2 ml of suspended cell culture were extracted using a pipette and transferred to an Eppendorf tube. The optical density of the samples from the four bottles were measured using a spectrophotometer at 550 nm. If needed the sample were diluted for a final volume of 1 ml in a cuvette.

For analysis of the consumption of glucose and production of succinic acid, formic acid, and acetic acid, the samples collected in the Eppondorf tubes were put in the freezer for preservation before being analysed using High Performance Liquid Chromatography according to 4.1.3.2.

The biofilm formation was analysed for formation of both the packing material as well as on the walls of the bottles. The quantification was performed using a biofilm assay technique similar to the one used in the microplate study but adjusted for staining in batch bottles. The absorbance was measured at 550 nm in a microplate reader.

#### 4.1.3.1 Biofilm formation assay

A similar protocol as for the biofilm formation assay in microplate was used with modifications made to adapt the process for a batch system in bottle fermentation.

1. After extracting a sample of the suspended cell media, the rest of the bulk fluid was poured out.
2. The bottles were washed by filling them with tap water to remove suspended cells still present in the bottle. This was performed twice to reduce the background staining.
3. 5 ml of a 0.1% crystal violet solution was added to each bottle to stain the attached cells on the glass bottle and packing material.

4. The bottles were placed in a shake incubator 37°C for 15 minutes, turning every 5 minutes to cover all surfaces.
5. Excess crystal violet was removed from the bottles by filling them with tap water and blotting on paper. This step was repeated for a total of 4 washes to remove non-attached staining.
6. The packing material in the packed bottles was removed from the bottle and placed on paper to dry.
7. The empty bottles, both the ones which had no packing and where the packing was removed, were placed upside down on a rack to dry.
8. After drying, each packing material was placed in a well in a 24 wells microplate. 1.5 ml of 30% acetic acid was added to the wells to solubilize the crystal violet from the packing material.
9. The plate was placed in a shake incubator for 15 minutes to fully solubilize the crystal violet staining.
10. The packing material was removed from the wells and the plates were read at 550 nm in a microplate reader.
11. After drying, 12 ml acetic acid was added to the bottles with no packing, and 15 ml to the bottles with packing as to compensate for the liquid displacement.
12. The bottles were placed in a shake incubator for 10 minutes to solubilize the crystal violet without solubilizing excessive noise stained above the visible line of fermentation media.
13. 1.5 ml of the solubilized crystal violet solution was extracted and transferred to a 24 well microplate and read at 550 nm in a microplate reader.

Figure 9 shows an extracted packing material before and after washing with acetic acid. The decolourization of the packing material on the right indicates successful dissolution of the crystal violet.

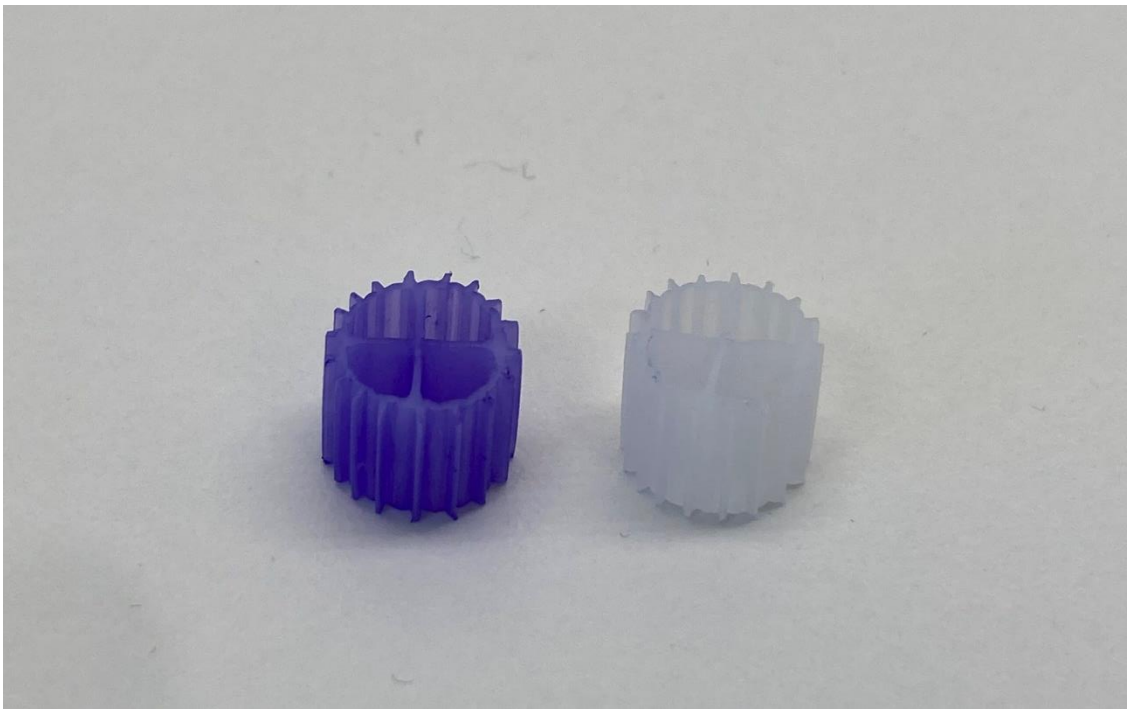

*Figure 9. Packing material extracted from a batch fermentation bottle. On the left a packing material after staining and drying. On the right, a packing material after being washed with acetic acid.*

#### 4.1.3.2 HPLC analysis

The samples were prepared for HPLC analysis through the following procedure:

1. 30 µl of 12 mM sulfuric acid was added to lower the pH to ensure that the acids are in protonated form.
2. The samples were centrifuged at 10 000 rpm for 5 minutes.
3. The supernatant was transferred to HPLC vials through a filter.

### 4.1.4 Results

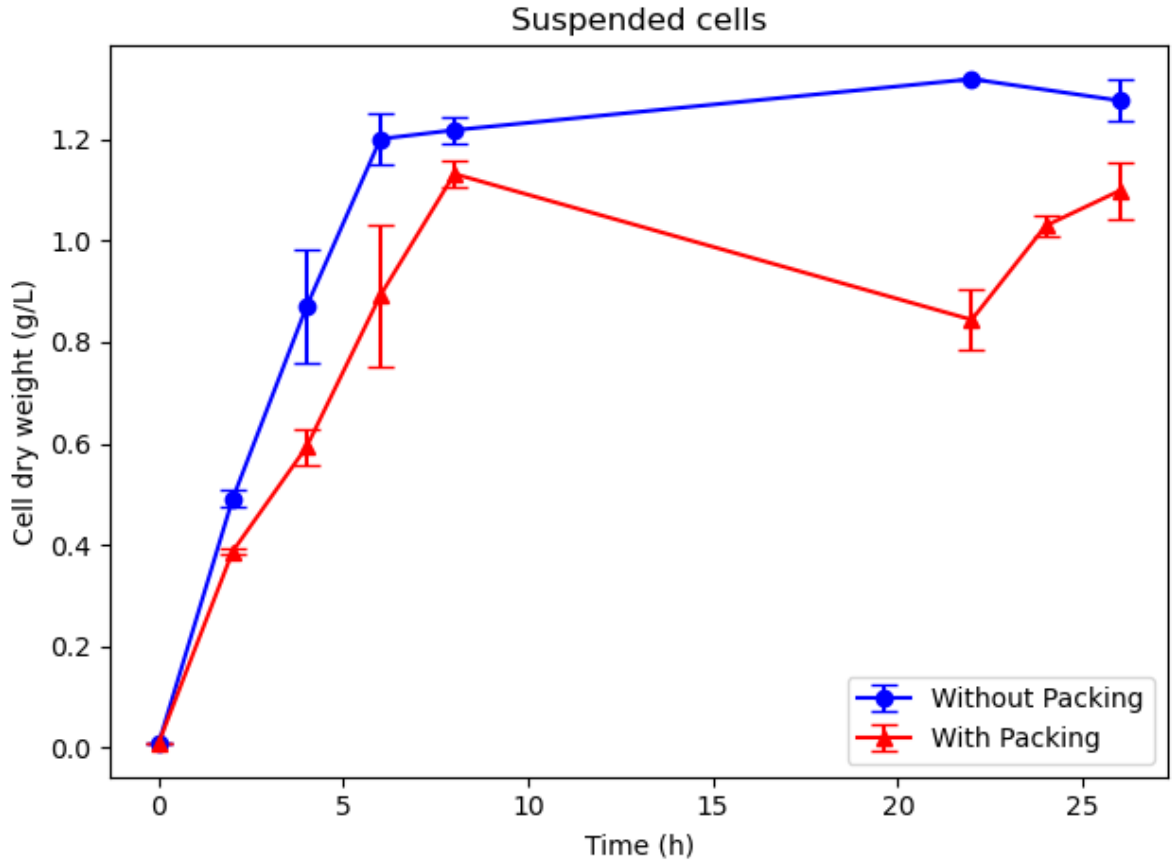


*Figure 10. Growth of suspended cells as an average of duplicates for bottles with and without packing material. OD was measured at 550 nm and converted to CDW/L.*

Looking at Figure 10 for the suspended cell growth there is a difference between the bottle fermentations without packing material and those with packing material. The bottles without packing material reach a maximum concentration of 1.3 g-CDW/L, whereas the bottles with packing material reach a maximum of 1.1 g-CDW/L. The difference is not deemed significant as $P > 0.05$. No lag phase can be identified as the cells appear to growth exponentially from 0 hours to 6 hours without packing and 0 to 8 hours with packing. The maximum specific growth rate calculated from the data was 0.224 $h^{-1}$ for the cultures without packing and 0.209 $h^{-1}$ with packing.

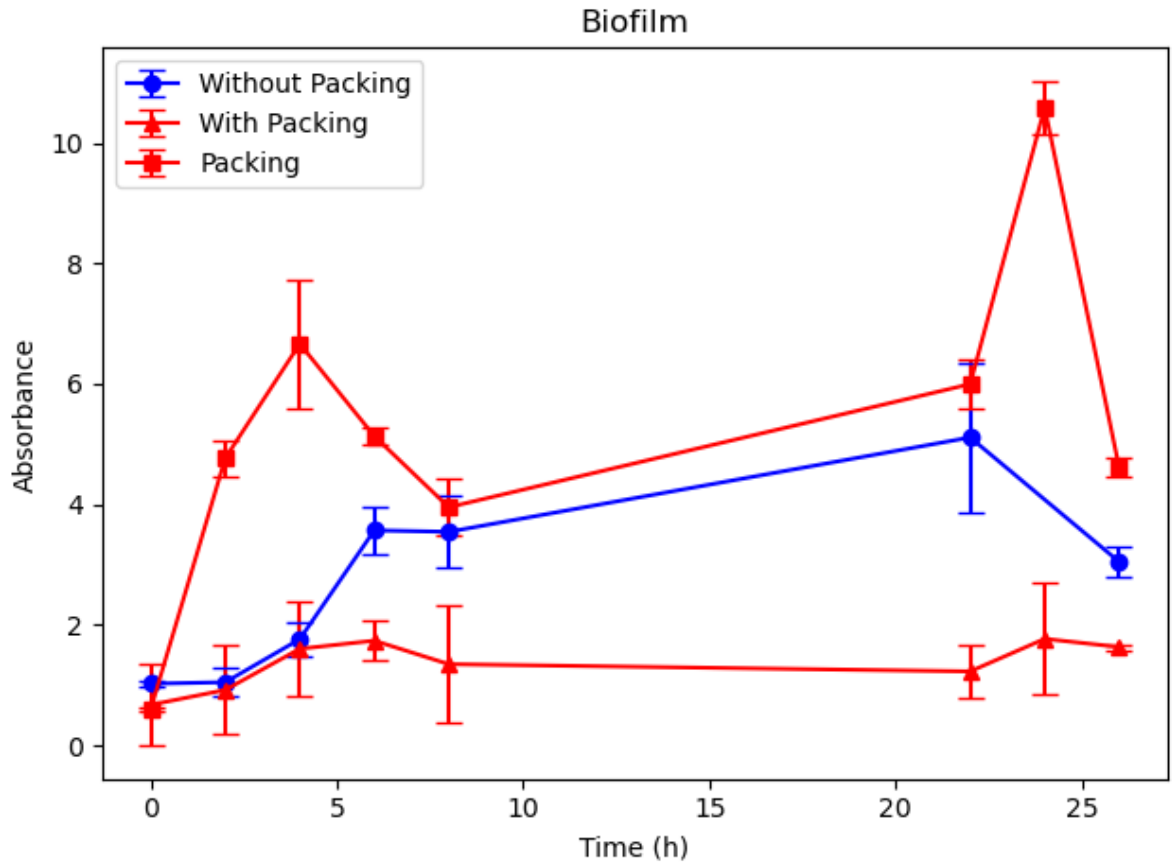


*Figure 11. Biofilm formation on packing material, on the glass of the bottles with packing material, and on bottles without packing material.*

The results from the biofilm assay are presented in Figure 11. The measured absorbance has been normalized for the dilution from the added volume acetic acid. Results are presented as the total amount present in the bottles but with packing material and bottle separated. The biofilm formation in bottles without packing material only occurs on the glass surface of the bottles. From 0 hours to 6 hours the concentration increases exponentially, reaching a maximum value of 3.57. After 6 hours and overnight the absorbance decreases, ending at a final value of 3.05. In bottles with packing material the biofilm formation on the bottle is lower compared to without packing material. The initial growth from 0 hours to 6 hours occurs exponentially reaching 1.76 after 6 hours. The absorbance stays relatively constant overnight but increases again for a final value of 1.64. The growth of biofilm on the packing material is substantially higher than on the glass surface of the bottles. The initial growth is very rapid reaching a value of 6.67 after only 4 hours, after which the absorbance decreases. Some growth occurs overnight but not a substantial amount. However, a maximum absorbance of 10.58 was measured after 24 hours. The final value after 26 hours decreased to 4.61. The difference between biofilm growth on the packing material and the glass surface is significant as $P < 0.05$).

The maximum specific growth rate for attached cell growth calculated during the exponential phase are presented in Table 5. Growth rate on the glass bottle surface was 0.306 $h^{-1}$ for cultures without packing and 0.207 $h^{-1}$ with packing. Since no distinct exponential phase was observed for attached cells on the packing material is makes for high uncertainty when calculating the maximum specific growth rate. The most rapid increase was observed between 0 and 4 hours with a growth rate of 0.599 $h^{-1}$ ,

The available surface on the glass bottles for biofilm formation was roughly approximated using the measurements of the bottles and working volume, including additional surface level height from orbital shaking on the shake incubator. The surface area of the packing material was approximated from available data yielding an area of approximately 2.75 $cm^2$/packing material (Pratiwi et al., 2018). The growth rates normalized for available surface area are seen in Table 5.

*Table 5. Maximum specific growth rate, available surface area, and normalized specific growth rates.*

| Conditions | $\mu_{max}$ ($h^{-1}$) | Surface area ($cm^2$) | $\mu_{max}$/area ($h^{-1}cm^{-2}$) |
|---|---|---|---|
| **Bottle without packing material** | 0.306 | 23.7 | 0.013 |
| **Bottle with packing material** | 0.207 | 27.2 | 0.008 |
| **Packing material** | 0.599 | 27.5 | 0.022 |

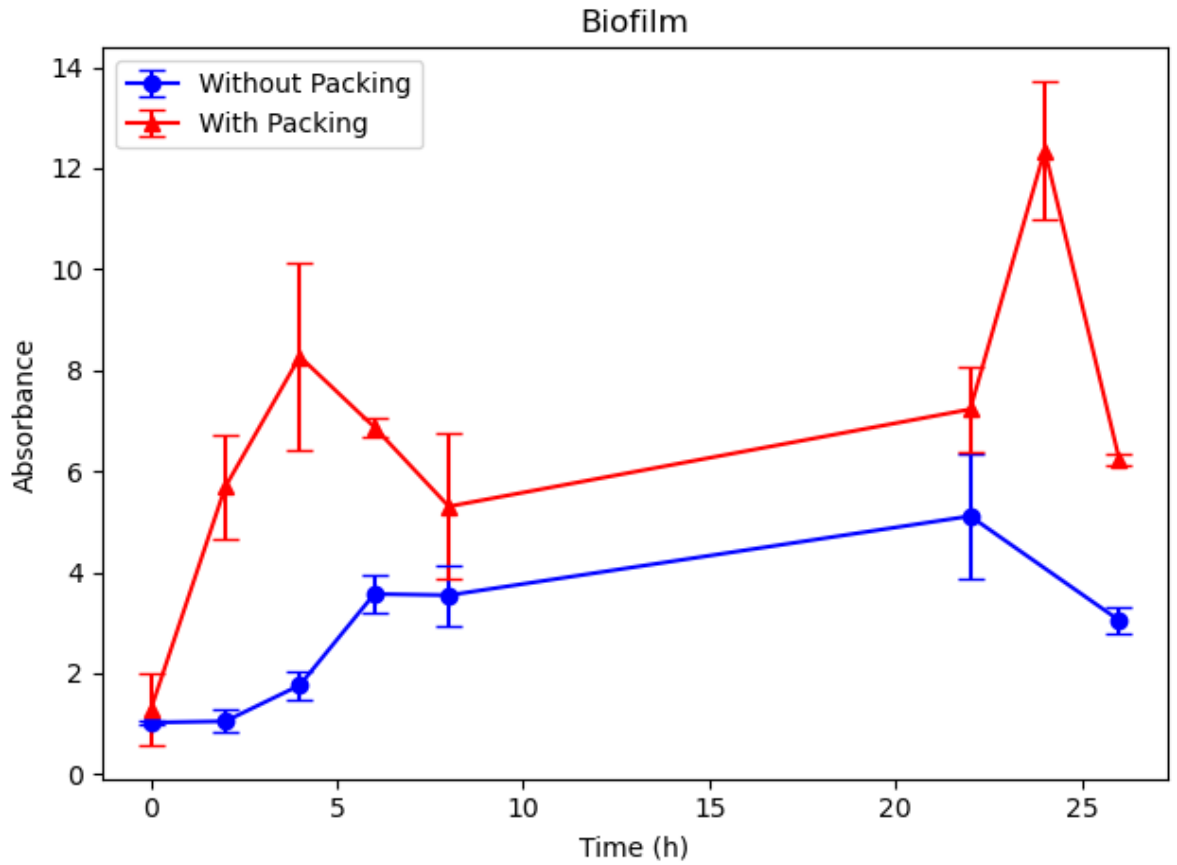


*Figure 12. Total biofilm formation in bottles with packing material and bottles without packing material.*

In Figure 12 the biofilm formation is presented as a total amount in the bottles. Meaning that both the biofilm on the glass walls and the packing material are summed up. The same trends as in Figure 11 can be seen with only increased values in the bottles with packing material.

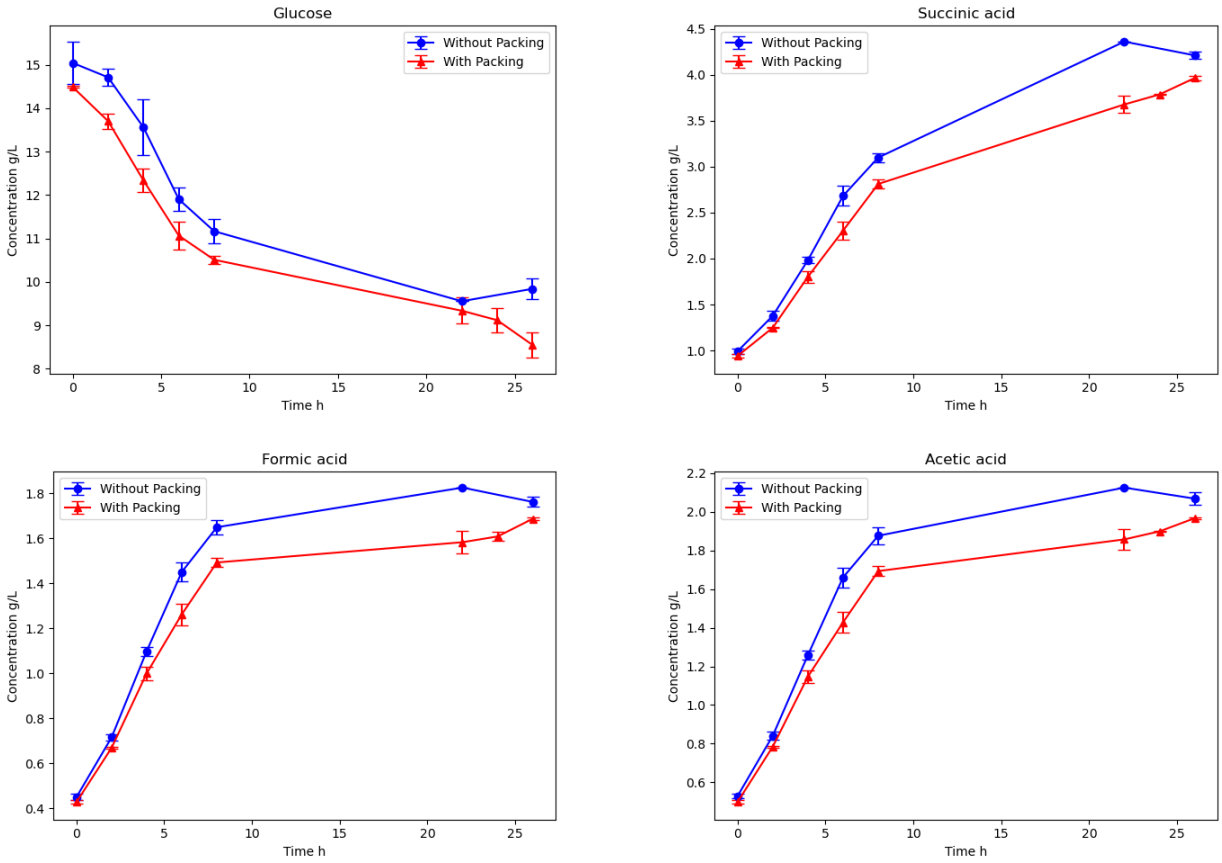


*Figure 13. Glucose consumption and formation of Succinic acid, Formic acid, and Acetic acid.*

In Figure 13 the results from the HPLC analysis are presented in the form of substrate consumption and product formation. The results are presented as averages of the two duplicates for both bottles with no packing and those with. Graphs showing the concentration of lactic acid and ethanol are not present as none was detected in the analysis. The glucose consumption is quite surprising as the initial concentration starts above 14 g/L instead of 10 g/L which was the added amount. The consumption of glucose decreases after 7 hours and effectively causes the final concentration to be 9.84 g/L in bottles with no packing material and 8.55 g/L with packing, leaving a large amount of residual glucose not consumed.

The production of succinic acid as well as formic acid and acetic acid display similar behaviour as seen in Figure 13. The concentration increases linearly up until 8 hours where the curve flattens out. This correlates well with the decreased concentration of glucose. The main product is succinic acid, reaching a final concentration of 4.21 g/L in the non-packing samples and 3.96 g/L with packing. The concentration of formic acid is lower than succinic acid and reaches a final value of 1.76 g/L when no packing is used and 1.69 with packing. The concentration of acetic acid is slightly higher than formic acid with a final concentration of 2.07 g/L without packing and 1.97 g/L with packing.

Titres and yields are presented in

Table 6. In all aspects and for all products, the titres and yields are higher when no packing material is used. The yield for succinic acid is 0.619 g/g without packing and 0.509 g/g with packing. The yields are around half of the theoretical maximum of 1.12 g/g. The carbon balance made over the system resulted in 138.72% without packing material and 112.69% with packing material.

*Table 6. Titres, yields and carbon balance in c-mole from the batch experiment in 20 ml bottles. For the calculations the total amount of attached cells was considered the concentration was estimated using the same conversion factor as the suspended cells. The yields are based on substrate consumption. Attached cells are not included in the carbon balance as the accuracy of the estimation is not validated.*

| Sample type | Product | Titre (g/L) | Yield (g/g) |
|---|---|---|---|
| **Without packing** | Succinic acid | 4.21 ± 0.07 | 0.619 ± 0.062 |
| | Acetic acid | 2.07 ± 0.36 | 0.296 ± 0.036 |
| | Formic acid | 1.76 ± 0.05 | 0.252 ± 0.028 |
| | Suspended cells | 1.28 ± 0.08 | 0.244 ± 0.040 |
| | Attached cells | 1.01 ± 0.04 | 0.129 ± 0.005 |
| **Carbon balance (%)** | | | 138.72 |
| **With packing** | Succinic acid | 3.96 ± 0.01 | 0.509 ± 0.052 |
| | Acetic acid | 1.97 ± 0.00 | 0.247 ± 0.031 |
| | Formic acid | 1.69 ± 0.04 | 0.212 ± 0.024 |
| | Suspended cells | 1.01 ± 0.04 | 0.184 ± 0.000 |
| | Attached cells | 2.08 ± 0.24 | 0.278 ± 0.020 |
| **Carbon balance (%)** | | | 112.69 |

### 4.1.5 Discussion

The results from the suspended cell growth showed a higher concentration in the bottles with no packing material. Even though the samples at the different time points are individual bottles, and therefore, does not entirely represent a growth curve in a complete accurate manner the trend of more suspended cell growth is present throughout the entire fermentation for all bottles. Therefore, it can be reasoned that there is a tendency for higher expected cell density in the fermentation broth when no packing material is present when running a batch fermentation. Due to the higher initial concentration of glucose provided from the HPLC samples this would also explain the elevated cell concentration as more substrate is available.

When comparing the biofilm formed only on the glass bottles the amount is considerably higher in the bottles without packing material, more than twice as much. One aspect that could influence this result is the mechanical stress caused by the packing material during the biofilm assay. As the bottles must be shaken to remove water as well as empty the bottles from packing material it can be assumed that some scraping occurs on the walls which possibly could detach some biofilm. When looking at the biofilm formed on the packing material itself the amount greatly exceeds the biofilm formed on the walls of the bottles for both with and without packing material. Another interesting aspect is the kinetics of the biofilm formation. As seen in the curve the biofilm forms much more rapidly on the packing material and with an earlier onset of attachment. This indicates that the cells more preferably attach to and grow on the plastic biofilm carriers compared to the glass surface on the walls. One explanation for this could be the rougher surface on the packing material. This could also be an aspect that would influence and

explain the large difference in biofilm formation on the glass between the samples. The lower results when packing material is used is caused by the biofilms forming more on the packing itself rather than the bottle. The difference when accounting for the biofilm formation on the bottle surface when packing material is used can be deemed not substantial to consider. In this case only 10 packing materials with a total surface area of 27.5 $cm^2$ are added in each bottle meaning that the additional biofilm formation on the walls of a system would be even less significant as more packing material is used.

The results from the HPLC analysis displayed some unexpected results regarding the initial substrate concentration and substrate consumption. Some additional glucose could be expected from the inoculum. However, not in the amounts showed in the HPLC analysis. There should also not be a difference in the bottles with packing and those without as the same initial conditions should apply for them both. One theory regarding the higher amounts of glucose than expected is peak overlap from other compounds in the HPLC analysis. The measured glucose concentration would display additional glucose originating from other compounds, however, this cannot be proven. The carbon balance made over the systems indicated that the glucose concentrations were not correct as the carbon balance exceeded 100% with quite the margin.

The decrease in product formation most probably is the reason why the cell growth ceases even when more glucose is available. The problem is related to acidification which drops the pH causing cell growth inhibition. Since no pH adjustments are made during the batch fermentation other than the initial adjustment to pH 7, it is expected that the pH would drop below optimal conditions. The buffer present in the fermentation broth is also limited in its effect to counteract the rapid acid production during the first 8 hours. One solution to this problem is to apply an automated pH control system, common in bioreactors. However, for smaller batch experiments a more common method would be to increase the amount of $NaHCO_3$ buffer to neutralize the pH.

The general trend is that more products are formed when no packing material is used, and the cells are mostly in suspended form. This outcome does not correlate to the literature where greater amounts of attached cells in biofilm form should not limit the formation of succinic acid. It should be taken into consideration that the duration of the batch fermentation is limited to 25 hours and the volume to 11 ml. The limitations are factors that might affect the outcome in the favour of fermentation when cells are mostly suspended.

The yields presented in Table 6 show that there indeed is a difference in product formation comparing the cultures without packing material and those with. The yield is higher for all compounds except for the yield of attached cells which would be expected. The theory that more attached cells would be beneficial to produce succinic acid does not correlate with the results from this experiment. However, due to the limitations of the system and clear tendency for pH inhibition this statement can not be concluded from this study alone.

## 4.2 Batch fermentation in 100 ml bottles

Due to the drop in pH inhibiting the growth and product formation in the previous experiment another fermentation was performed in an attempt to achieve higher substrate conversion and less product inhibition. Most conditions and methods remained the same as in the previous fermentation and did not change if not stated.

### 4.2.1 Experimental setup

Fermentations were performed in 100 ml bottles using a total working volume of 45 ml. 6 bottles were prepared containing packing material, 2 without packing material acting as the positive control, and 2 that were not inoculated as the negative control. The bottles containing packing material were set up as the three sampling points for analysing biofilm formation. The total fermentation time was increased to 48 hours.

### 4.2.2 Fermentation media

The initial concentration of glucose was raised to 20 g/L to ensure sufficient supply of substrate during the fermentation. To counteract the rapid decrease in pH the initial concentration of sodium bicarbonate was increased to 30 g/L. This would also give an additional amount of $CO_2$ formation during acid production. The pH was adjusted to 7.3 before inoculating to give additional margins.

### 4.2.3 Analysis methods

Samples of the fermentation broth for OD measurement and HPLC analysis were taken from the positive control and two of the bottles with packing material. Biofilm formation was quantified at 9, 24, and 48 hours by opening the bottles and performing crystal violet staining. The packing material was extracted prior to staining and not stained directly in the bottles as the results from the previous experiment indicated that the inside of the bottles had minor influence on the total amount of biofilm formation when using packing material. At each sample point for biofilm formation the pH was measured.

### 4.2.4 Results

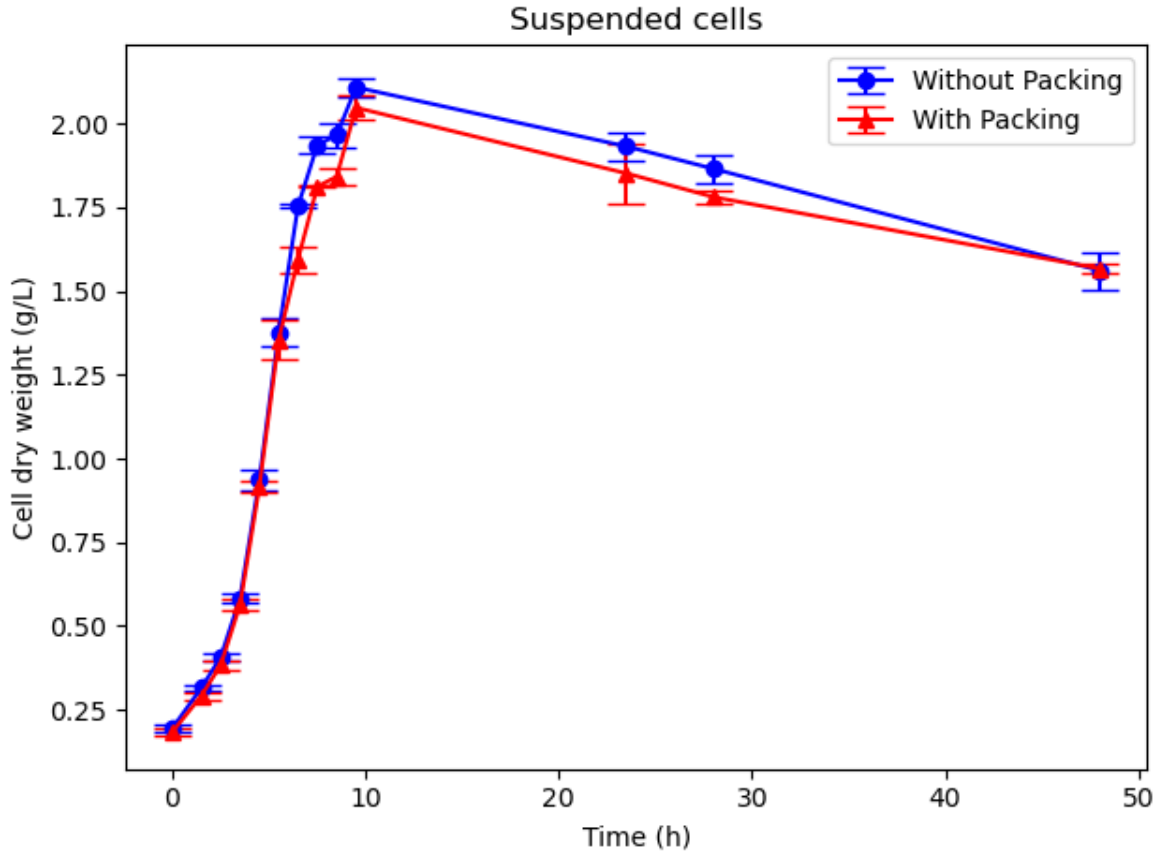


*Figure 14. Growth of suspended cells as an average of duplicates for bottles with and without packing material. OD was measured at 600 nm and converted to CDW/L.*

The measured OD from the suspended cells was converted to g-CDW/L and is presented in Figure 14. A short lag phase can be identified in the beginning but quickly transitions to exponential growth as the cells from the inoculum were already in exponential growth phase. Exponential growth occurred until 9 hours. After this point the cells entered stationary conditions and no more cell growth occurred. Instead, a decrease in cells took place as the fermentation progressed. At sample 10 and 12 at 9.5 hours and 28 hours, respectively, samples were taken from new bottles as the old one was opened for biofilm analysis. This can be seen in the Figure 14 just before 10 hours as sample 10 deviates somewhat from the pattern.

Comparing the cell concentration between the bottles with packing material and those without gives no significant difference ($P < 0.05$). Bottles with no packing material have slightly higher concentration after reaching stationary phase, up until the last sample at 48 hours. It should be considered that all samples taken from the control bottles were from the same bottles during the entire fermentation and the reduction of working volume may have affected the results.

The maximum specific growth rate, $\mu_{max}$, was calculated from the measurements and gave a value of 0.413 $h^{-1}$ without packing and 0.427 $h^{-1}$ with packing, which were higher than the rates obtained using 20 ml bottles.

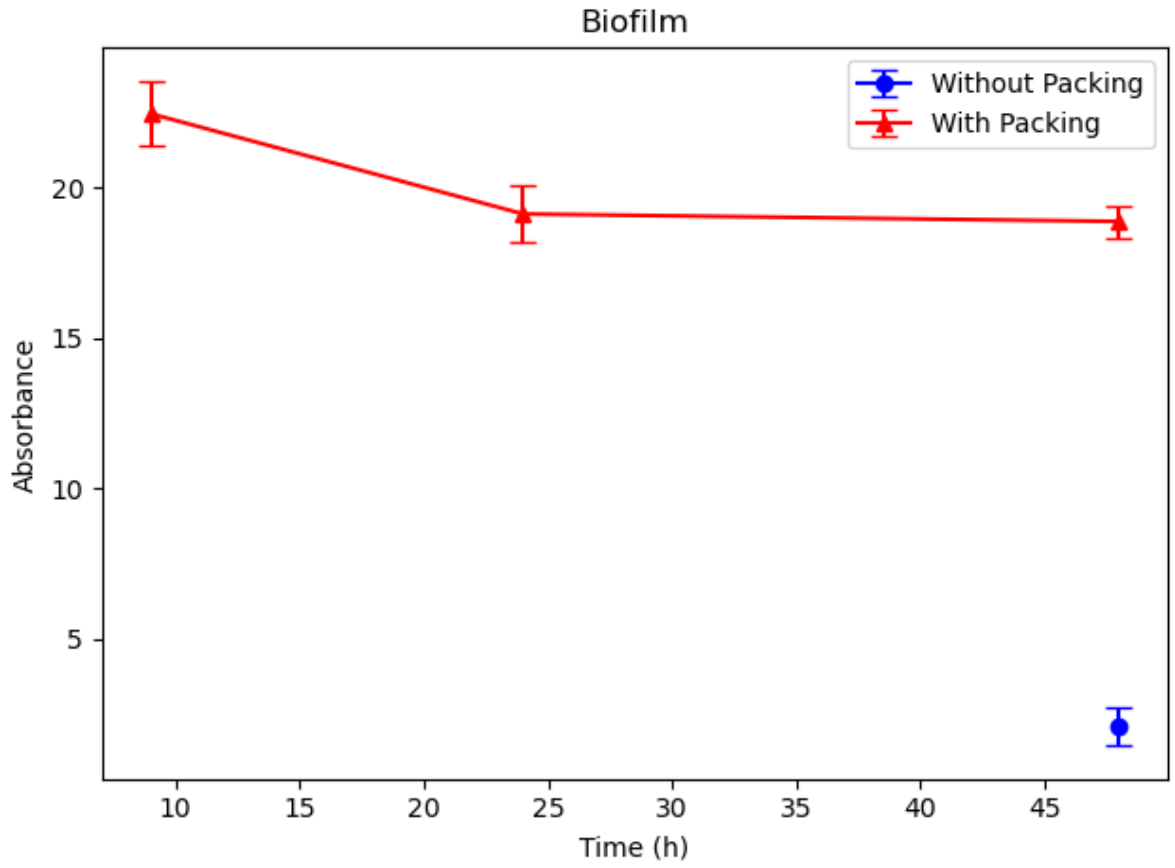


*Figure 15. Biofilm formation on packing material taken at three times during the fermentation, and biofilm formation on bottles without packing material taken at the end of the fermentation.*

Biofilm formation on the packing material was measured at 9, 24, and 48 hours of fermentation. As a comparison the positive control bottles were also stained at the end of the fermentation after 48 hours. The results are presented in Figure 15. The results in the graph are averaged for the two duplicates but the same trend was seen for both. The biofilm formed on the walls of the positive controls was much less than on the packing material. This was expected as the available surface area was lower.

Since the three samples were all from different bottles and only 10 of the total 40 packing materials were used for analysis, the first sample could have had an earlier onset of attachment causing more biofilm to be formed in those bottles. However, since duplicates are used, and both show similar trend in decreased biofilm over time it makes this less credible.

Looking at Figure 11 showing the biofilm formation from the previous experiment, the absorbance reached around 8-10 at maximum. In the new run the absorbance was between 19-22. As expected, it was higher as the duration of the experiment was longer and more packing material is used. However, considering that only 10 packing material were used compared to 40 the expected amount of biofilm should be even higher in the experiment.

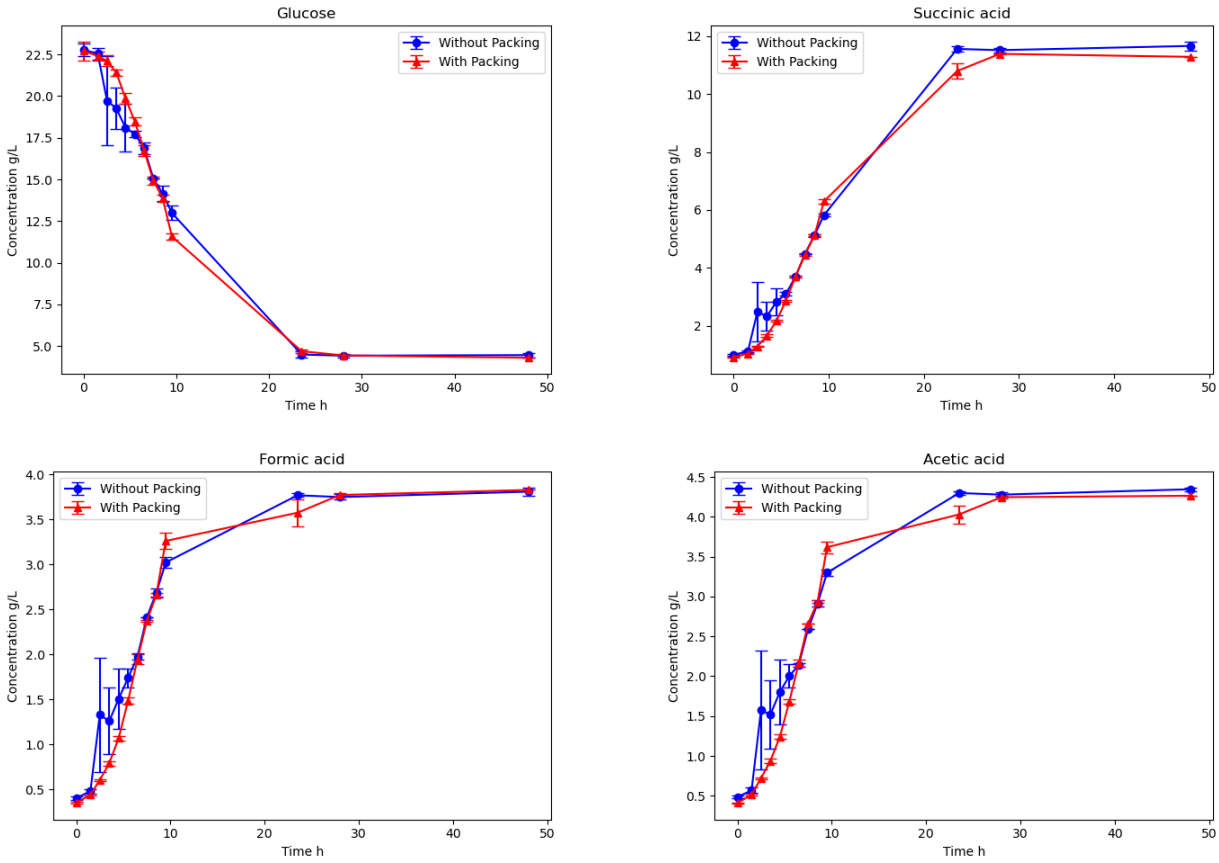


*Figure 16. Glucose consumption and formation of Succinic acid, Formic acid, and Acetic acid.*

In Figure 16, results from the HPLC analysis are presented, showing substrate consumption and product formation. Like the results from the previous experiment, no lactic acid or ethanol was detected and is therefore not presented. The initial glucose concentration was higher than expected just as in the previous run, starting at 22-23 g/L instead of 20 g/L. After 23.5 hours of fermentation the glucose concentration remains stationary around 4.46 ± 0.11 g/L. Product formation, cell growth, and biofilm formation also remain stationary after this point, indicating that no more substrate was consumed.

The main product formed was succinic acid, reaching a maximum concentration of 11.7 g/L for the positive control and 11.4 g/L in bottles with packing material. Formic acid and acetic acid reach concentrations of 3.8 g/L and 4.3 g/L, respectively. Production of formic acid and acetic acid seem highly associated to the growth of the suspended cells. After the suspended cells reach the stationary phase, the concentration of formic acid and acetic acid remain stagnant. Production of succinic acid does not follow this trend as the concentration continued to increase after stationary phase was reached.

*Table 7. pH measurements.*

| Time (h) | pH – Bottles with packing | pH- Bottles without packing |
|---|---|---|
| **9** | 6.74 | - |
| **24** | 5.60 | - |
| **48** | 5.56 | 5.60 |

Table 7 shows the pH measurements that were taken when opening bottles for biofilm analysis. After 9 hours of fermentation the pH was 6.74 and still within the optimal range of 6-7.5. Overnight the pH dropped significantly and remained below 6. The pH for the positive control was only measured after 48 hours resulting in a similar pH. The drop in pH overnight indicated that growth was inhibited from

the acidification from product formation. This correlates to the cell growth and acid production curves. The system was limited by insufficient pH neutralization causing the substrate to not reach full conversion.

*Table 8 Titres, yields and carbon balance in c-mole from the batch experiment in 100 ml bottles. For the calculations the total amount of attached cells was considered the concentration was estimated using the same conversion factor as the suspended cells. The yields are based on the substrate consumption. No error is presented for products with packing as HPLC data from one sample was lost. Attached cells are not included in the carbon balance as the accuracy of the estimation is not validated.*

| Sample type | Product | Titre (g/L) | Yield (g/g) |
|---|---|---|---|
| **Without packing** | Succinic acid | 11.66 ± 0.30 | 0.582 ± 0.002 |
| | Acetic acid | 4.35 ± 0.04 | 0.211 ± 0.006 |
| | Formic acid | 3.81 ± 0.09 | 0.186 ± 0.008 |
| | Suspended cells | 1.56 ± 0.11 | 0.074 ± 0.006 |
| | Attached cells | 0.69 ± 0.42 | 0.113 ± 0.022 |
| **Carbon balance (%)** | | | 101.44 |
| **With packing** | Succinic acid | 11.29 | 0.564 |
| | Acetic acid | 4.26 | 0.209 |
| | Formic acid | 3.83 | 0.189 |
| | Suspended cells | 1.57 ± 0.03 | 0.075 ± 0.008 |
| | Attached cells | 6.29 ± 0.36 | 0.342 ± 0.041 |
| **Carbon balance (%)** | | | 99.76 |

From the data, the titres and yields for the fermentation was calculated and is presented in Table 8. The yield for succinic acid is slightly larger for cultures without packing material at 0.582 g/g compared to 0.564 g/g for cultures with packing material. The difference is not as large as the results from the 20 ml bottle experiment. The yield for acetic acid as well as formic acid are approximately the same when comparing the two cultures. This is also the case for the suspended cells. The yield of attached cells is 0.113 g/g without packing material and 0.342 g/g with packing material, more than three times as much.

### 4.2.5 Discussion

The results from the batch fermentation in 100 ml bottles showed improved results compared to the results obtained in 20 ml bottles. The suspended cell growth was defined more clearly with distinct lag phase, exponential phase, and stationary phase. The difference between cultures grown with packing material and those without is less distinctive as similar cell concentrations were reached. There was a difference observed during the stationary phase where the concentration was slightly higher when no packing material is present. This could be due to more cells attaching to the packing material decreasing the total amount of cells suspended in the broth. The maximum specific growth rate was slightly higher with packing than without but both cultures grew more rapidly compared to the 20 ml bottles as the initial concentration of glucose is higher.

The results from the biofilm analysis gave results that are hard to interpret. The main reason would be the lack of samples since only three are available. The expected results were an increase in attached cell

concentration as growth should occur over time after initial attachment. Results from the microplate and 20 ml bottle experiments indicated that increase should occur between the sample at 9 hours and 48 hours. As the suspended cell concentration decreases after 10 hours it could be speculated that it would from attachment and not cell lysis. However, from the HPLC analysis it is clear that the glucose concentration drops significantly during the growth of the suspended cells leaving small amounts that could be utilized for attached cell growth. Since both the glucose concentration and attached cell concentration remains constant during the stationary phase it does not appear that the attached cells continue to consume glucose even after inhibition of the suspended cell growth. Since the pH decreases during the fermentation the conditions for suspended cell growth becomes suboptimal thus inhibiting further growth. The results from the experiments in microplates indicate that this would be a promoting factor for biofilm formation. The increased scale in bottle fermentation might affect the rate of this transition compared to the smaller volume in microplates. The packing material provide a high amount of available surface area for the suspended cells to adhere to which can explain the large number of attached cells after 9 hours of fermentation. Similar to the results from the 20 ml bottles where the biofilm formed much more rapid on the packing material than the bottle surface. To characterize the kinetics of biofilm formation a larger number of samples would be necessary, especially during the earlier stages of the fermentation process. However, due to the biofilm analysis being rather time consuming this would affect the time intervals of samples making the suspended cell growth as well as substrate consumption and product formation less accurate. The main factor that can be extracted from the attached cell data is the total amount of biofilm formed during the fermentation and the calculated yields.

The results from the HPLC analysis show an increase in production of succinic acid compared to the experiment in 20 ml bottles, although a decrease in yield is observed. The yields of the by-products are also lower indicating that the substrate is not converted to products as efficiently. The attached cell yield is similar to the 20 ml bottle when comparing cultures with no packing material even though the fermentation time is longer. It seems that the attached cell growth is limited by some factor that is not related to the available glucose. The yield for attached cell on the packing material is higher for 100 ml bottles than 20 ml bottles which could simply be due to the increased available surface area.

## 4.3 Model calibration

A first calibration for the model was performed using the results from the batch experiment in 100 ml bottles with packing material. Parameters that could be calculated using the data included maximum specific growth rate, growth associated biofilm formation, growth associated substrate consumption, and growth associated product formations and are presented in Table 9.

*Table 9. Calculated parameters for kinetic modelling.*

| Parameter | Description | Value | Unit |
|---|---|---|---|
| $\mu_{max}$ | Maximum specific growth rate | 0.427 | $h^{-1}$ |
| ***Yxs*** | Growth association constant for substrate | 13.313 | g-S/g-CDW |
| ***Yxsa*** | Growth association constant for succinic acid | 7.505 | g-SA/g-CDW |
| ***Yxaa*** | Growth association constant for acetic acid | 2.789 | g-AA/g-CDW |
| ***Yxfa*** | Growth association constant for formic acid | 2.516 | g-FA/g-CDW |
| ***α*** | Growth association constant for attached cells | 0.0398 | g-XA/g-CDW·$cm^2$ |
| ***A*** | Total amount of available surface area | 110 | $cm^2$ |

The calculated parameters were fed into the model together with the initial conditions of the system. The initial substrate concentration was set to 22.7 g/L. The model prediction and experimental data is presented in Figure 17.

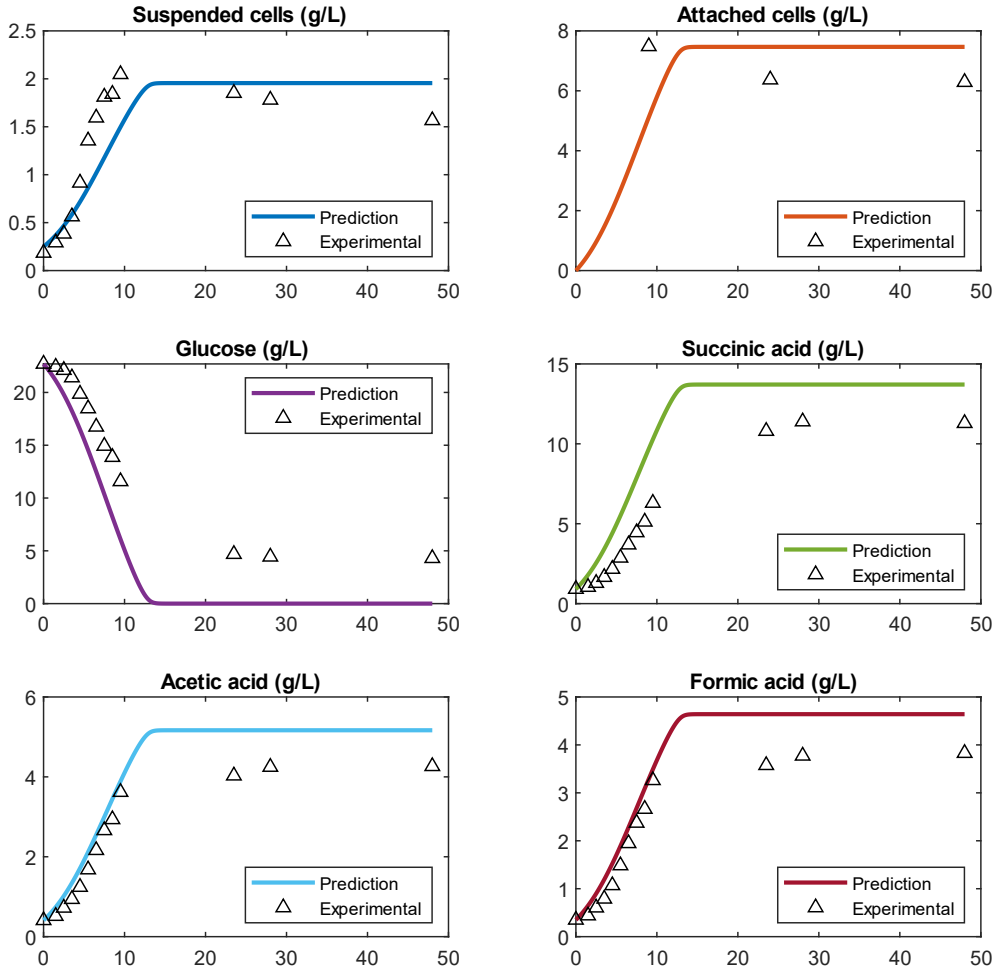


*Figure 17. Experimental results and model prediction using an initial substrate concentration of 22.7 g/L.*

The model prediction well represents the experimental trend. A model refit is needed for the glucose consumption due to its overestimation. This causes a consequent increase in product formation for simulations of batch experiments. The model assumes 100% substrate conversion. This causes a large overshoot in product concentration during the stationary phase. The model seems limited by the inhibition due to decreased pH and product formation. If the pH in the experiment was kept constant at 7 throughout the process the probable outcome would be an increased substrate consumption which would correlate better with the model prediction. For the attached cell concentration, while in a similar range as the model prediction the number of experimental samples prohibits any conclusions to be drawn regarding the fit.

# 5 Bioreactor fermentation

In the third and last phase of the project the aim was to scale up the process even further to fermentation performed in bench-top bioreactors. For more accurate research and what could be expected in an industrial setting the reactors were operated in continuous mode under controlled operational parameters. Experiments were performed both with and without packing material.

## 5.1 Materials and methods

### 5.1.1 Reactor setup

The experiments were performed in duplicate bioreactors. A schematic diagram of the experimental setup is shown in Figure 18 and the real setup in Figure 19. The setup consisted of a bubble column reactor made from polyvinylchloride (PVC) with a working volume of 1.5 litre. The reactor was connected to a tank which delivered fresh medium via a peristaltic pump. The volume in the reactor was controlled by a peristaltic pump continuously pumping fermentation broth out from the reactor to an effluent tank. The outlet was also equipped with a valve to enable sampling from the same outlet. The reactor was supplied with $CO_2$ pumped from a bag using a peristaltic pump and sterilized through a filter (Sartorius Minisart 0.2 µm) before being sparged inside the reactor, acting as both the $CO_2$ source and agitation. At the top of the reactor gas exited and was passed through a filter before being recycled to the gas bag. However, there were difficulties with the recirculation as gas leaks were detected, limiting the amount recycled to the bag. There were also complications with liquid backflows from the reactor into the gas tubing resulting in inefficient supply of $CO_2$. Therefore, adjustments were made to the setup so that the gas was supplied directly from a gas canister to the reactor. This adjustment allowed for continuous sparging of $CO_2$ even overnight as well as preventing problems with backflows. The temperature inside the reactor was controlled by a temperature sensor coupled to a PID system and a heating jacket which held the temperature constant at 38 °C. The pH in the bioreactor was controlled using a pH sensor coupled to a Mettler-Toledo M400 transmitter acting as the PID controller. The transmitter was connected to a peristaltic pump which pumped 8 M NaOH solution to the fermentation broth. A specific lower value was set as the lower pH threshold value. When the sensor registered a pH value lower than the threshold an electric pulse would activate the peristatic pumps to increase the pH. The pH could successfully be controlled at 6.8 in one of the reactors while fluctuations between 6.6 and 7.1 were present in the other due to unknown faults in the PID system which could not be resolved.

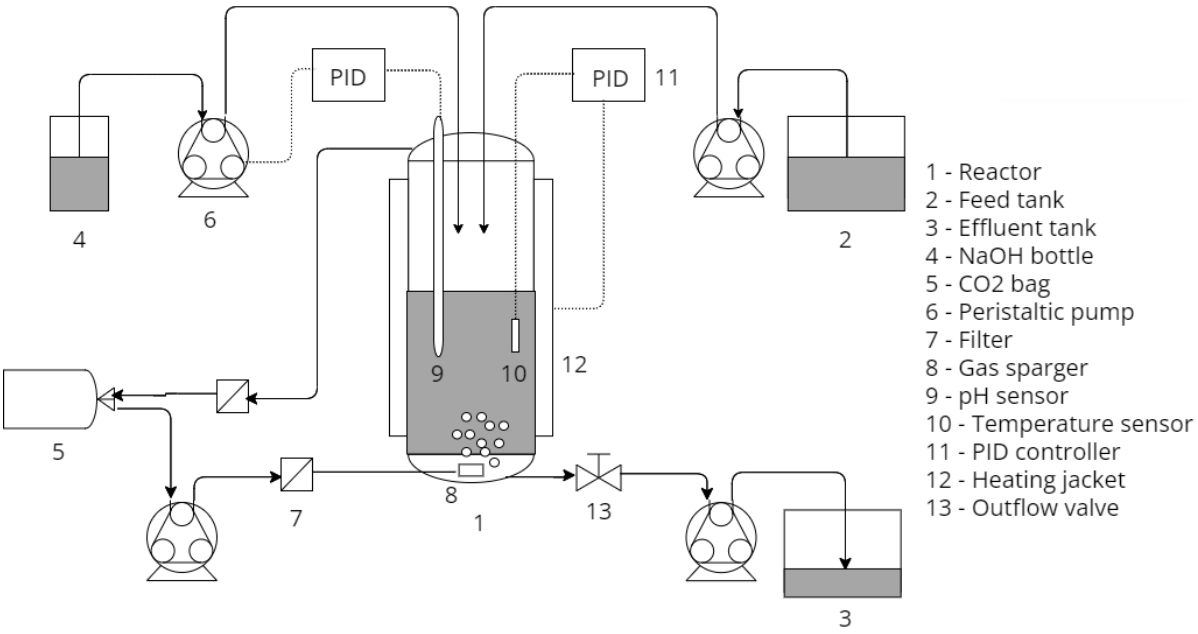


*Figure 18. Schematic diagram of the experimental reactor setup for continuous operation*

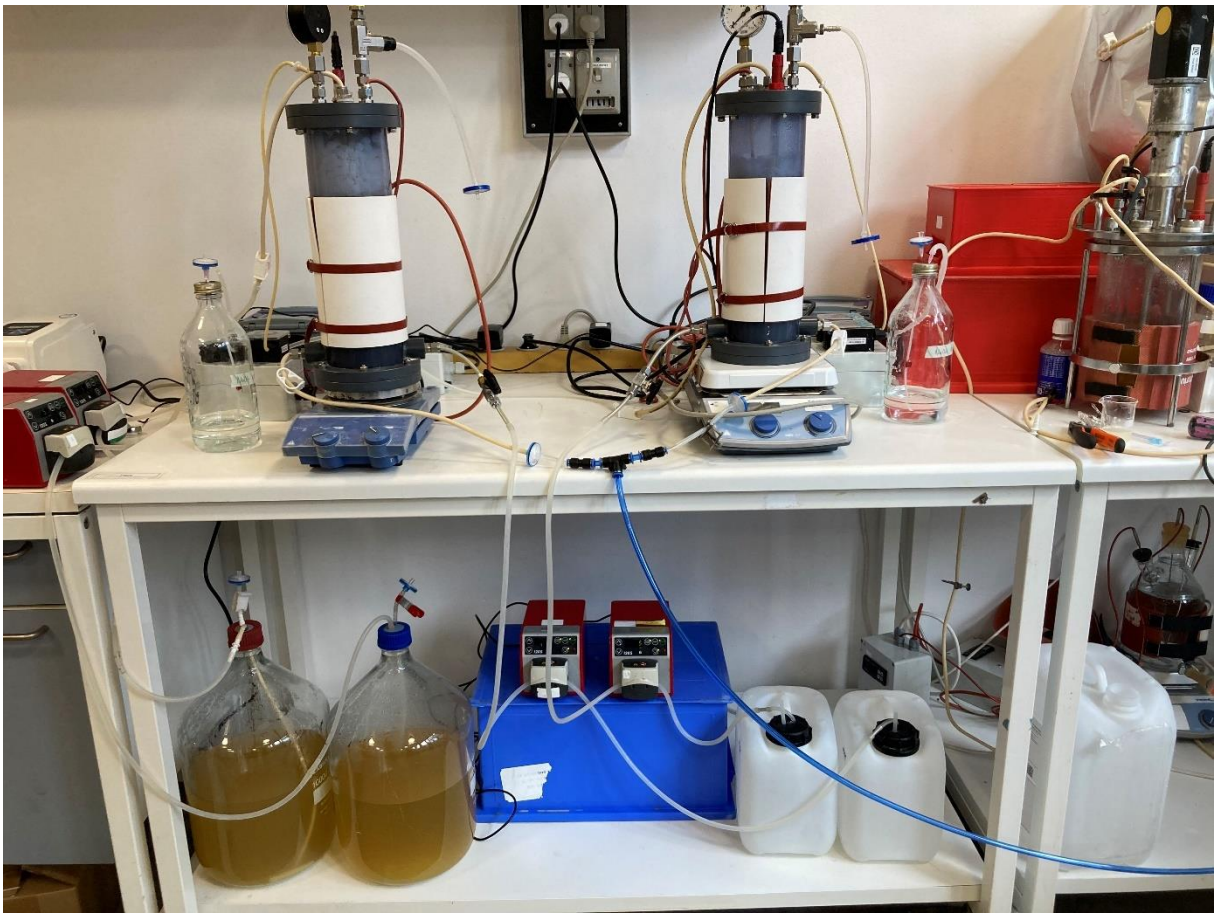

*Figure 19. Real-life reactor setup.*

For experiments using a packed bed reactor the bioreactor was filled with Kaldnes K1 packing material for a set packing density. The packing material were soaked in water for some days before being sterilized together with the fermentation media. One problem with using a reactor made from PVC is that it can not be autoclaved. Therefore, the sterilization was performed using 70% ethanol and UV-radiation.

### 5.1.2 Fermentation media

The composition of the fermentation media for the bioreactor fermentations was composed of (g/L) yeast extract (5.0), $K_2HPO_4$ (3.0), $MgCl_2$ (0.2), $CaCl_2$ (0.2), NaCl (1), glucose (40.0), antifoam (1.0ml/L). All components except yeast extract and glucose were mixed in a bottle with deionized water and autoclaved at 121 °C for 20 minutes. The yeast extract was mixed with deionized water and sterilized by filtration. The glucose was mixed with deionized water together with the antifoam, the solution was sterilized by pasteurization at 90 °C for 8 hours in an oven. Due to results indicating contamination the sterilization method for glucose was changed to filtration.

The same composition was used for the refeed media in the feed tank.

### 5.1.3 Analysis methods

At each time point a sample of 2 ml was collected of an Eppendorf tube from the outlet/sampling port for analysis. The OD was measured in a 1 ml cuvette using a spectrophotometer and diluted if needed. The samples were frozen for preservation until used for further analysis.

Substrate consumption and product formation was analysed through HPLC.

Biofilm formation was analysed through crystal violet staining similar to the ones used during previous experiments with adjustments based on the utilized method.

## 5.2 Batch fermentation with polyurethane tubes

In the first experiment, only one bioreactor was used and set to run as a batch fermentation. A method for biofilm quantification was developed and tested. The method consisted of 7 pieces of tubing made from polyurethane with an inner diameter of 3.2 mm and a length of 40 mm. The pieces were connected to form a longer tube in which the fermentation broth was recycled. In theory this method would allow for sampling and analysation of biofilm formation ex-situ. Without having to open the reactor to pick out packing material causing a high risk for contamination. The recirculation flow was controlled by a peristaltic pump emulating the dilution rate if the setup would be changed to continuous operation. A recirculation flow was set which would represent a dilution rate of 0.1 $h^{-1}$

### 5.2.1 Biofilm formation assay

The biofilm formed in the tubing was analysed through crystal violet staining with adjustments made for the specific application of tubing.

1. Samples were taken by closing the outflow valve and extracting one piece of tubing. After extraction the remaining tubing was merged and connected to the outflow valve which then was opened.
2. The sample tube was washed twice using tap water by submersion and ensuring that water passed through the pipe.
3. The sample tube was submerged in a beaker filled with 0.1% crystal violet ensuring full coverage of the inside for staining.
4. The sample was left in the crystal violet for 10 minutes to avoid excess staining of the outside.
5. The sample was washed 4 times with tap water by submersion.
6. The outside of the sample tube was wiped with ethanol to remove staining on the outside of the tubing as much as possible. The tubing was then left to dry.
7. 1 ml of 30% acetic acid was added to a microplate well.
8. A small piece of silicone tubing was placed on each end of the sample tube. Using a syringe, the acetic acid in the well was sucked up through the tube effectively avoiding dissolving crystal violet present on the outside.
9. The sample tube with was left with acetic acid inside for 15 minutes. Occasionally, the acetic acid was pushed out and sucked back in as to fully dissolve the crystal violet on the inside.
10. The acetic acid was pushed back into the well and the absorbance was read in a microplate reader at 550 nm.

### 5.2.2 Results

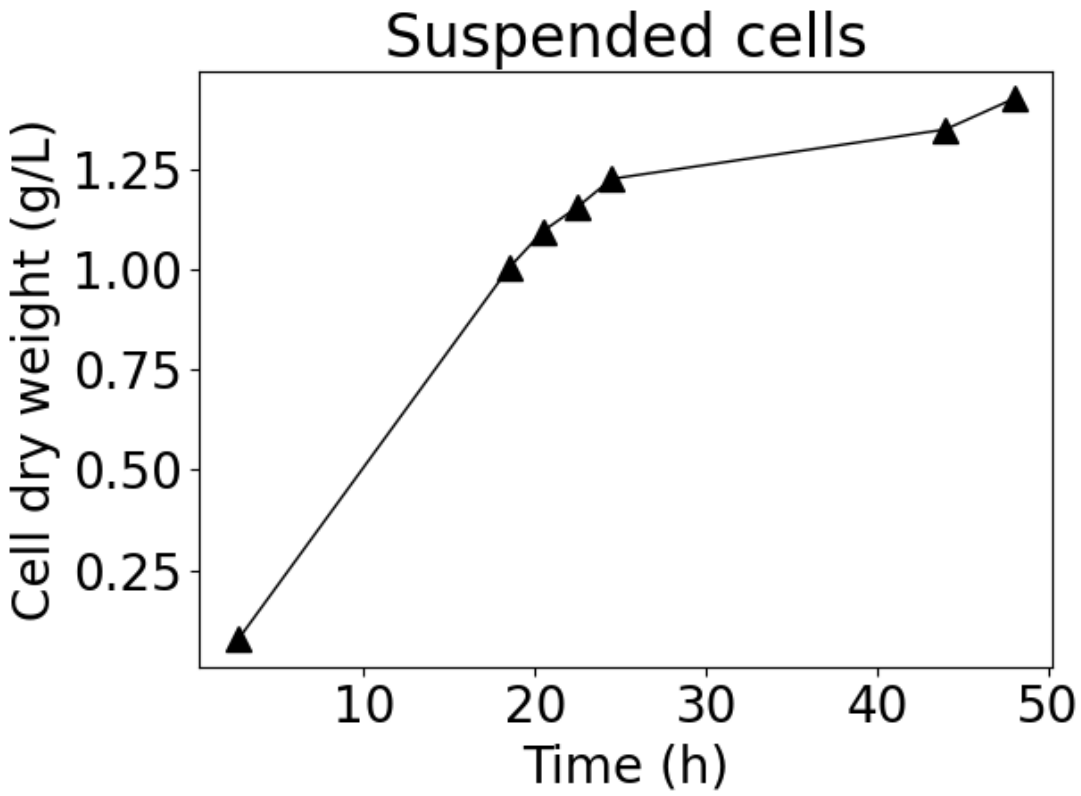


*Figure 20. Suspended cell growth. OD was measured at 550 nm and converted to CDW/L.*

The cell concentration of the fermentation is presented in Figure 20. Only 7 sample points were taken as this reactor run was just a trial. The first sample was taken after 2.67 hours after inoculation with a cell concentration of 0.07 g/L. After 18.2 hours the cell had grown to 1.00 g/L and continued to grow quite steady. The cells reached stationary phase after around 25 hours as the concentration after 44 hours was approximately the same. Since only one sample was taken in the beginning of the fermentation there is no information available regarding a potential lag phase and no characteristic exponential phase.

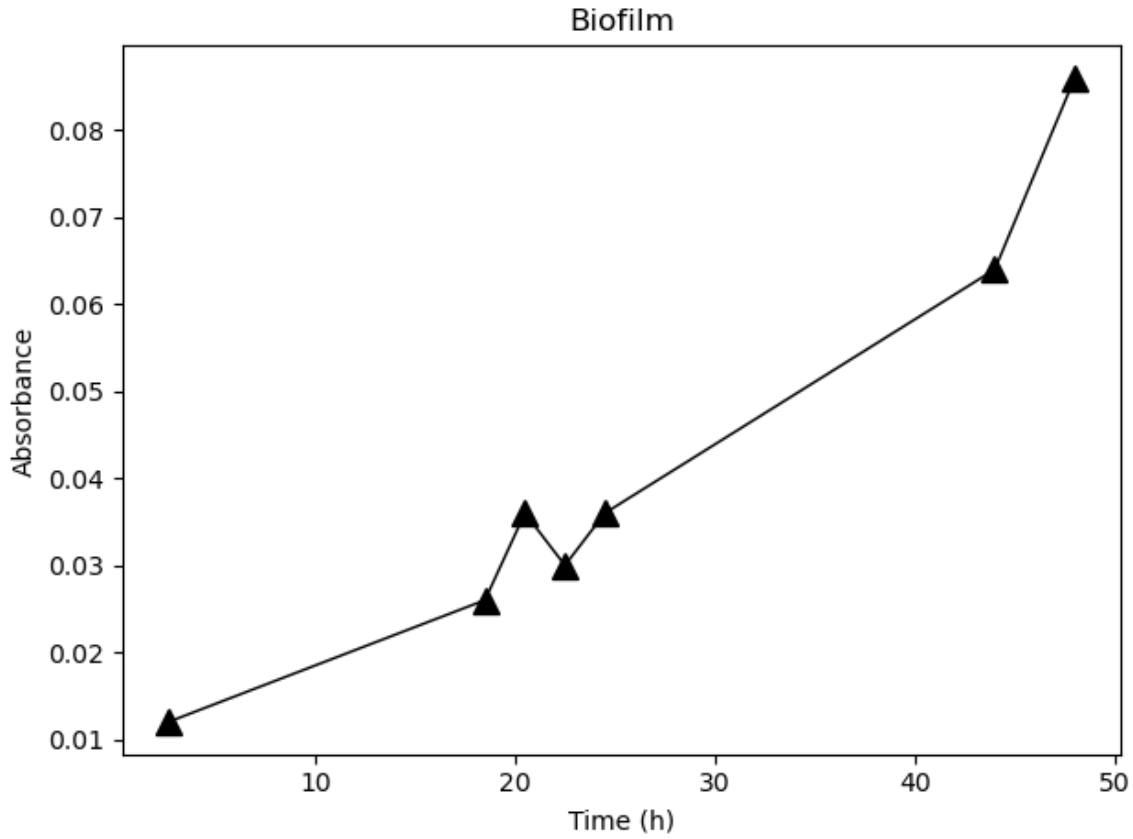


*Figure 21. Biofilm formation on polyurethane tubes. Absorbance was measured at 550 nm after crystal violet staining.*

The biofilm formation from the biofilm assay on the polyurethane tubes is presented in Figure 21. A quite steady linear increase is present from the beginning until the end of the fermentation. The measured absorbance increased from 0.012 at 2.67 hours after inoculation to 0.086 at 48 hours, an increase of more than 7 times.

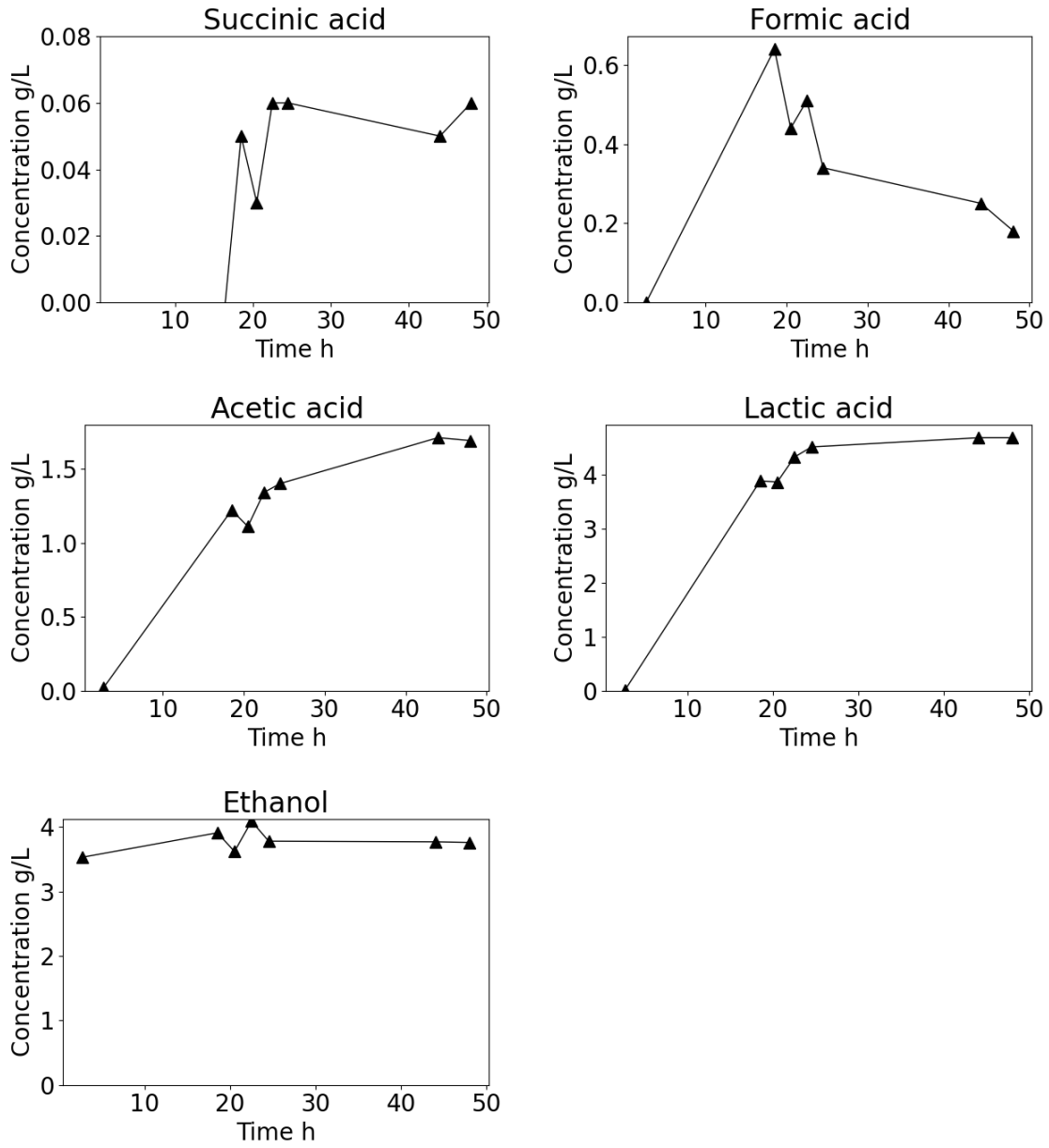


*Figure 22. Product formation.*

The results from the HPLC analysis are presented in Figure 22 where the concentrations of the main metabolites are displayed. Immediately there is a noticeable problem as the amount of succinic acid produced was extremely low compared to the expected amount as it should be the main product. Instead, the main products were lactic acid and ethanol. Looking at the curve for ethanol production the concentration was around 4 g/L even from the start of the fermentation and remains constant throughout the fermentation. These values could most likely be explained by residual ethanol from sterilizing gloves and sample port prior to sampling, contaminating the sample.

### 5.2.3 Discussion

There is not enough data to draw any specific conclusions regarding the suspended cell growth. The cells seemed to grow quite slow as shown as the concentration continued to increase after 18.5 hours of fermentation. This could be due to a longer lag phase but since no samples were taken during the earlier stages of fermentation this can only be speculated.

From the data the biofilm assay seems to be a successful analytical method for quantifying biofilm in tubing as the curve follows a logical increase during the fermentation. It can be hypothesized that using an external recycling of multiple tubes could be used to dynamically quantify biofilm formation. However, it is important to consider that the conditions inside the tubing are not as easily controlled as directly inside the reactor which could affect the accuracy if used as a representation for biofilm formed inside the reactor. Especially if using low recycling rates which would be the case if attempting to emulate low dilution rates in a continuous operation mode. Parameters such as temperature, pH and nutrient concentrations would all be difficult to control.

In the experiments, polyurethane tubing is used as material as it is assumed to have properties closer to HDPE compared to tubing made from rubber or silicone. However, the biofilm formation from the results cannot safely be assumed to be transferrable to the packing material as not control has been performed using tubing made from HDPE. The reason for not using HDPE tubing is due to logistics as delivery would not be possible during the project's timescale.

The results from the HPLC analysis indicate some form of contamination in the reactor. Instead of producing succinic acid as the main product which would be expected if the culture in the reactor was *A. succinogenes.* Instead, the main products were lactic acid and ethanol which in the batch bottle experiments were not present at all. Another reason for this could be problems with backflow in the tubing for $CO_2$ supply. After overnight fermentation the gas bag was completely depleted due to problems with gas recycling. The production of ethanol and lactic acid could be due to lack of $CO_2$ as it is needed for production of succinic acid and deficiency would steer the metabolic flux towards the C3 branch. The production of lactic acid and ethanol require NADH which can be provided by oxidation of formic acid. The results indicate that the concentration of formic acid decrease as the concentration of lactic acid increases which could to supply NADH. However, since this reaction also generates $CO_2$ this would simultaneously allow for production of succinic acid which is barely present. Therefore, the most probable cause is another microorganism growing in the reactor instead of *A. succinogenes.*

## 5.3 Continuous fermentation without packing material

In the second experiment the reactors were operated in continuous mode using no packing material. This would be the positive control to compare results from when packing material was used. The reactors were initially operated in batch to allow sufficient cell growth, avoiding wash out from the beginning. After overnight batch operation the pumps were connected to feed fresh new medium and remove effluent. Dilution rate was set to 0.1 $h^{-1}$ by adjusting the rpm of the peristaltic pumps.

### 5.3.1 Results reactor A

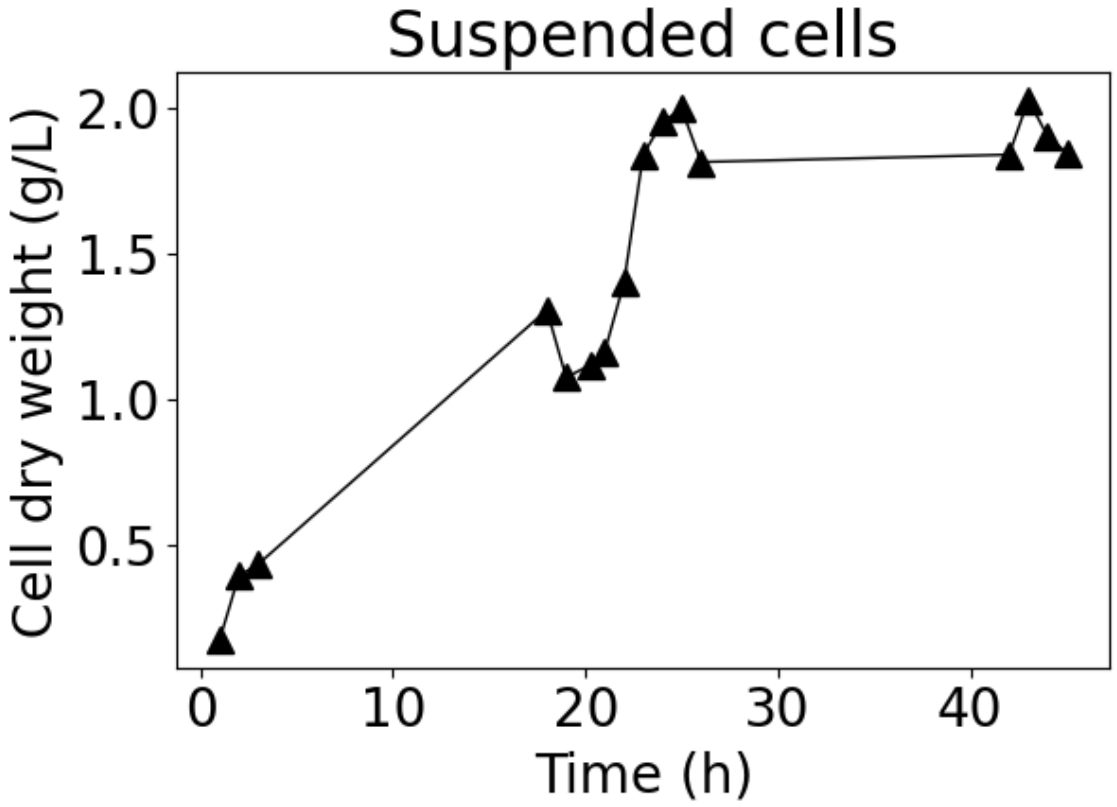


*Figure 23. Suspended cell growth in reactor A. OD was measured at 600 nm and converted to CDW/L.*

The cell concentration of samples taken from reactor A are presented in Figure 23. Fermentation was performed in batch operation overnight after which the cell concentration reached 1.31 g/L. At sample number 6 at 20.25 hours the operational mode was switched to continuous. After the switch to continuous operation a clear increase in growth can be observed with distinct lag phase, exponential phase, and stationary phase. The small lag phase indicates that the batch fermentation reached stationary phase sometime during the overnight fermentation. The reactor was left running in continuous mode overnight and samples were taken again at 42 hours after inoculation. Stationary operation was maintained with no increase in growth or wash-out of cells. The maximum concentration was measured after 43 hours at 2.02 g/L with a final concentration after 45 hours of 1.85 g/L. The maximum specific growth rate reached during the exponential phase was 0.229 $h^{-1}$ when the batch was changed to continuous operation was 0.229 $h^{-1}$.

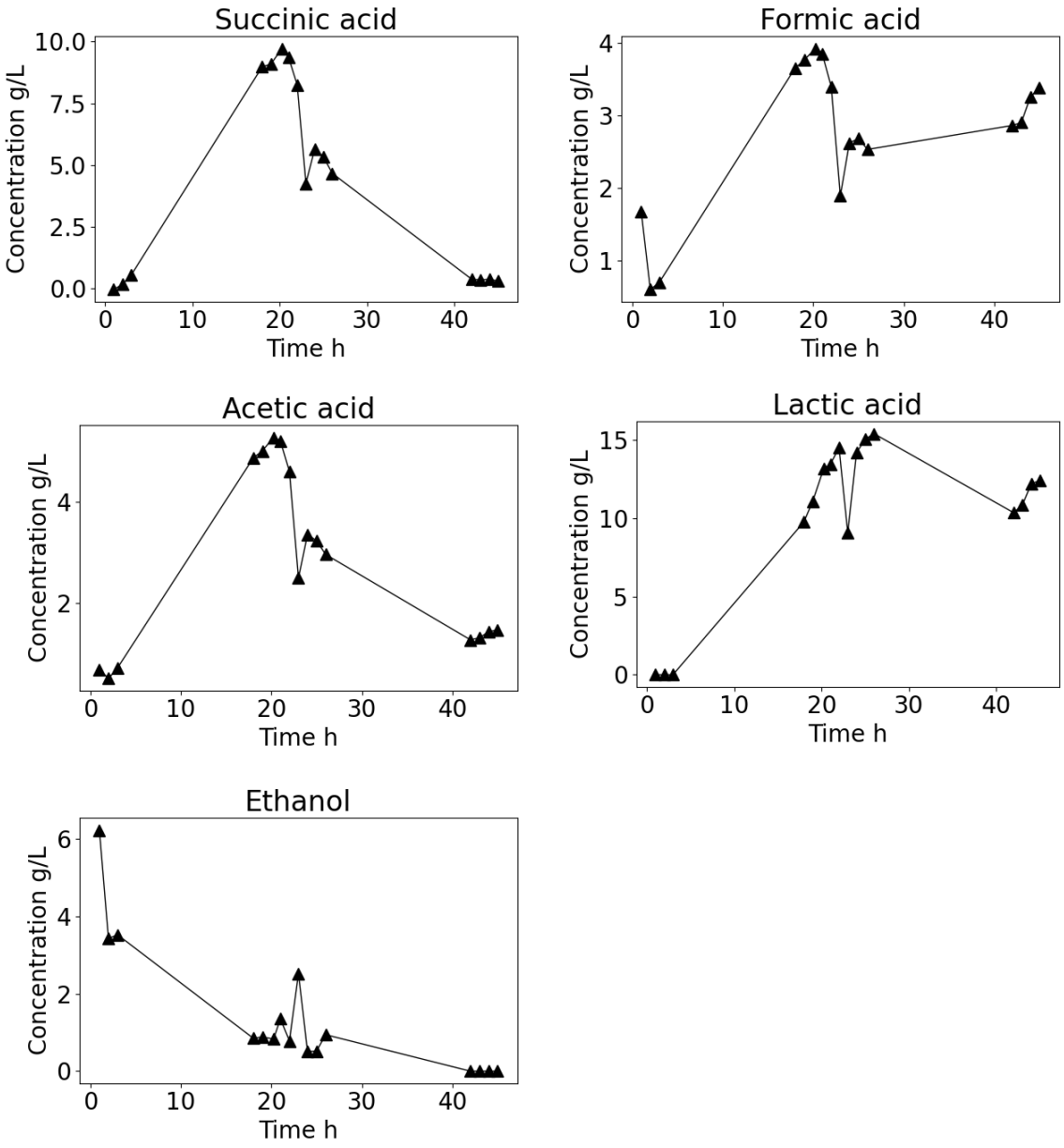


*Figure 24. Product formation in reactor A*

The product concentration analysed using HPLC are seen in Figure 24. Looking at the curve for production of succinic acid the concentration increased up to a maximum value of 9.72 g/L at 20.25 hours of fermentation, right as the switch to continuous operation was initiated. After the switch a rapid decrease occurred indicating a wash-out of succinic acid. After overnight continuous fermentation the final concentration is close to zero. Looking at the curve for acetic acid it displayed similar pattern with a maximum concentration reached at the start of continuous operation followed by a large decrease. Formic acid has a similar profile but does not decrease as much after continuous fermentation overnight with an increased production just at the end. The production of lactic acid was also similar during the batch phase. The decrease in concentration was more delayed than the other metabolites and just like lactic acid the concentration increased at the end. The concentration of ethanol started at very high values at the beginning just after inoculation which would most likely be of similar nature as the ethanol concentration from the polyurethane tubing batch fermentation with contamination from sterilization. The main product formed was lactic acid which reached a maximum concentration of 15.42 g/L after 26 hours of fermentation. No results regarding the glucose concentration are presented in the results as the HPLC standard for this run was not calibrated for glucose analysis.

### 5.3.2 Results reactor B

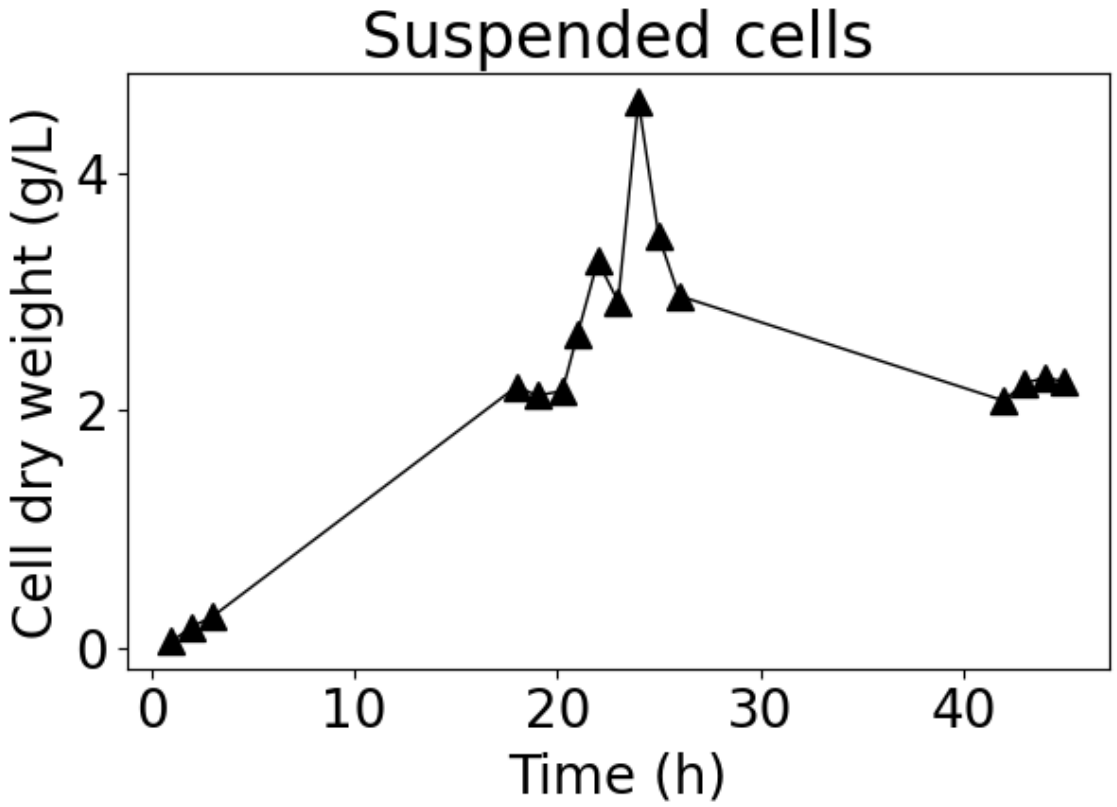


*Figure 25. Suspended cell growth in reactor B. OD was measured at 600 nm and converted to CDW/L.*

The cell concentration of samples taken from reactor B are seen in Figure 25. The cell concentration achieved after batch operation was 2.19 g/L. After switching to continuous operation at 20.25 hours of fermentation the cell started to grow exponentially. A maximum concentration of 4.61 g/L was reached after 24 hours of fermentation. This value is most likely not accurate as aggregates were present in some samples like the 24 hour one which would dissolve when measuring the OD causing a much denser solution than expected. The cells remained in stationary at around 23 hours until 26 hours. After overnight fermentation the concentration decreased for a final value of 2.24 g/L after 45 hours. The maximum specific growth rate during the exponential phase after switching to continuous operation was 0.234 $h^{-1}$.

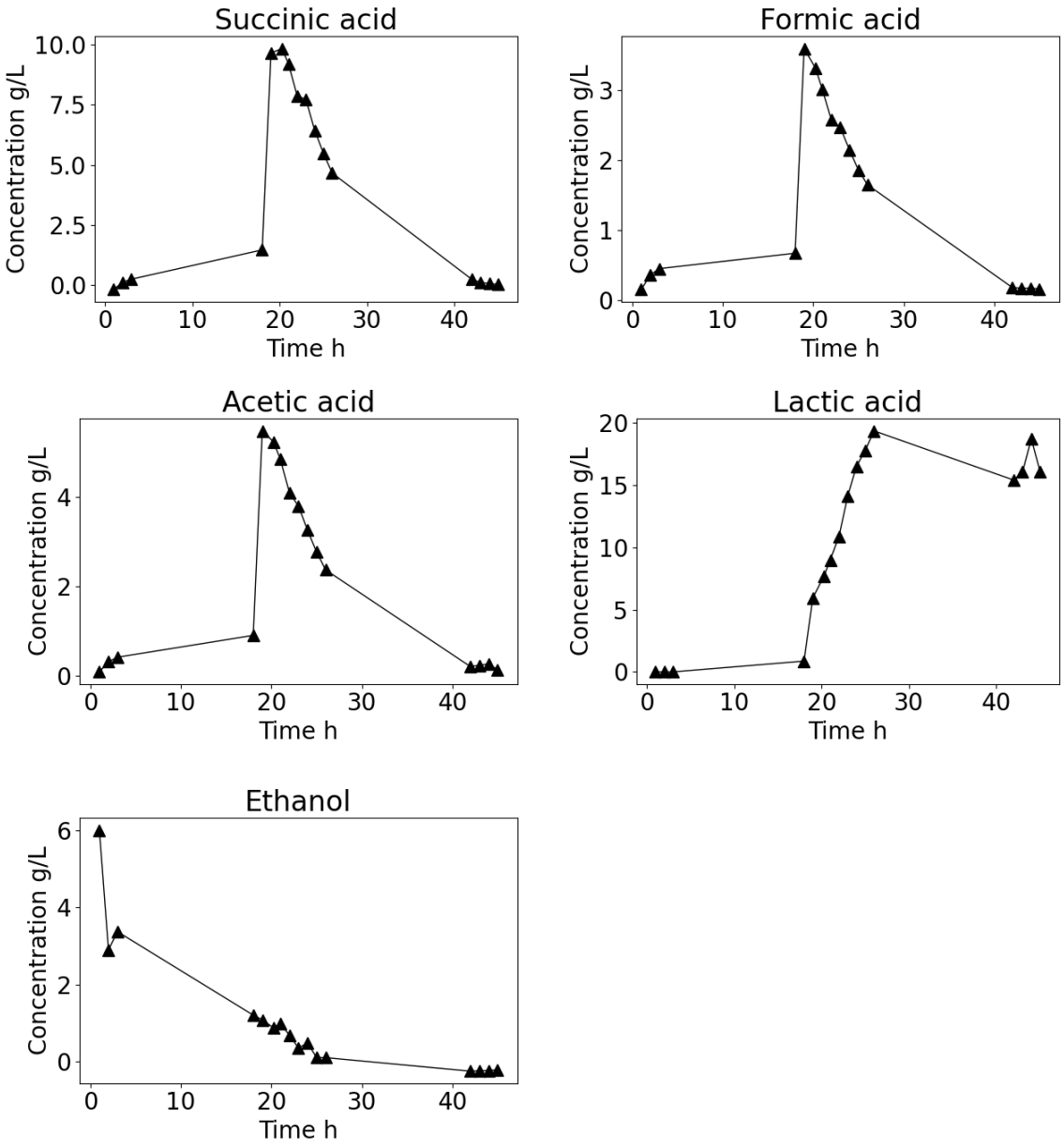


*Figure 26. Product formation in reactor B*

In Figure 26 the HPLC analysis results are shown. A sudden spike in concentration for all compounds except ethanol occurred between the sample at 18 hours and the sample at 19 hours. This was most likely due to some residual broth present in the sampling port that did not wash out properly before sampling. The production of succinic acid followed a similar trend as in reactor A. The maximum concentration achieved was 9.84 g/L after 20.25 hours of fermentation. As the operational mode was switched to continuous the same effect as described for reactor A took place. The main product of succinic acid, and main by-products of formic acid and acetic acid were washed out of the reactor. Instead, the new main product was lactic acid. The concentration of lactic acid was even higher in reactor B compared to reactor A, reaching a maximum concentration of 19.38 g/L. More wash-out of all compounds occurred during the overnight continuous fermentation.

### 5.3.3 Discussion

The suspended cell growth indicates after switching to continuous operation the cells started to grow exponentially again as new media was added to the reactor and nutrient depleted broth was removed.

The value of $\mu_{max}$ at 0.229 was slower than achieved in the 100 ml batch bottles but is comparable to values from the 20 ml batch fermentation or microplate studies.

The product formation results from reactor A indicated some form of wash-out of the product. However, this is not supported by cell growth which did not decrease in the same manner as the products. Although succinic acid was produced, the high values of by-product, especially lactic acid even before switching to continuous operation suggests a contamination present in the reactor from the start and not only from the feeding tank or effluent tubing. As a decreased concentration was seen for all main products of *A. succinogenes* while lactic acid remains constant when switched to continuous operation the contamination becomes completely dominating in the reactor.

In reactor B the suspended cell growth showed a similar trend as in reactor A. The main difference was the scale of cell concentration, which reached values of around 3 g/L after switching to continuous operation. Even after the overnight batch fermentation the cell concentration was much higher than in reactor A. Compared to reactor A the concentration did not remain stationary after overnight continuous operation as a decrease in concentration could be observed.

HPLC results from reactor B indicated some form of contamination just as in reactor A with a similar trend of wash-out of the main product of *A. succinogenes* in favour of lactic acid. The contamination seemed rather efficient in producing lactic acid at relatively high concentrations with only small formation of other products, which could be interesting for other bioproduction applications. However, there could be other by-products present in the samples other than the ones analysed in the HPLC. Since the glucose concentration was unknown a carbon balance could not be performed.

It is not certain whether the contamination was due to a contaminated preculture or from the reactor. It is possible that *A. succinogenes* was the dominating strain during the initial phase of the fermentation process only to be outrivalled over time by the contamination. The results from both reactors indicate that the switch to continuous operation is a sensitive moment during the process as the removal of fermentation broth completely seems to have favoured the contamination. Therefore, extra precaution should be taken to keep the environment as uncontaminated as possible during the switch. Since the high probability of the contamination being present in the reactor from the beginning due to the results from both this experiment and the polyurethane tubing experiment the sterilization of the reactor should also be intensified to reduce the risk for future contaminations.

## 5.4 Continuous fermentation with packing material

The second reactor run was performed with packing material present in the reactor to allow for more surface area for cell attachment. 240 grams of packing material was used in each reactor for a packing density of 160 g/L.

Some improvements were made from the previous run to mitigate the problems described. The first problem was backflow of reactor broth to the gas tubing causing decreased or fully inhibited gas flow to the reactor. This also involved gas leaks making the recirculation to the gas bags impossible, and thus running out of gas during overnight fermentations. This issue was resolved by coupling the gas inflow directly to the $CO_2$ tank instead of using gas bags. This allowed for higher gas flows, which increased the resistance for the liquid to flow into the tubing and removed the need for refilling gas bags ensuring a constant supply of $CO_2$. This solution is not optimal from an economical viewpoint as the gas recirculation is completely removed from the setup and gas flowing out from the reactor was not collected.

Due to the contamination observed in the previous run more thorough sterilization methods were applied. In addition to cleaning the reactor with ethanol and leaving it under UV light, the inside of the reactor was soaked and washed with chlorhexidine, a disinfectant with antimicrobial properties (Huang, 2022).

When inoculating the reactors for batch operation there were problems with the growth of the preculture and faulty temperature control in reactor B, causing growth to only occur in reactor A. New precultures were therefore prepared and both reactors were inoculated one more time for batch operation overnight. However, due to an accident with the tubing from the NaOH bottle, no pH control was present for reactor B overnight causing the pH to drop significantly. Despite the problems, both reactors had active cell cultures and were switched to continuous operation. The batch time for reactor A had then reached around 43 hours while reactor B had operated for around 21 hours.

To quantify biofilm formation in the reactor packing material was extracted from a port on the top of the reactor using a needle reshaped to a hook which prior to every sample was stored in 70 % ethanol and sterilized in the flame of a Bunsen burner. Triplicates of the packing material were taken from each reactor at the sampling time.

Due to results indicating contamination in the run, these results are left out and the experiment was repeated. An issue with packing material not being submerged and pushed up to the headspace was also noted during the experiment. Therefore, the amount of packing material was reduced to 150 g/reactor for a packing density of 100 g/L.

### 5.4.1 Results reactor A

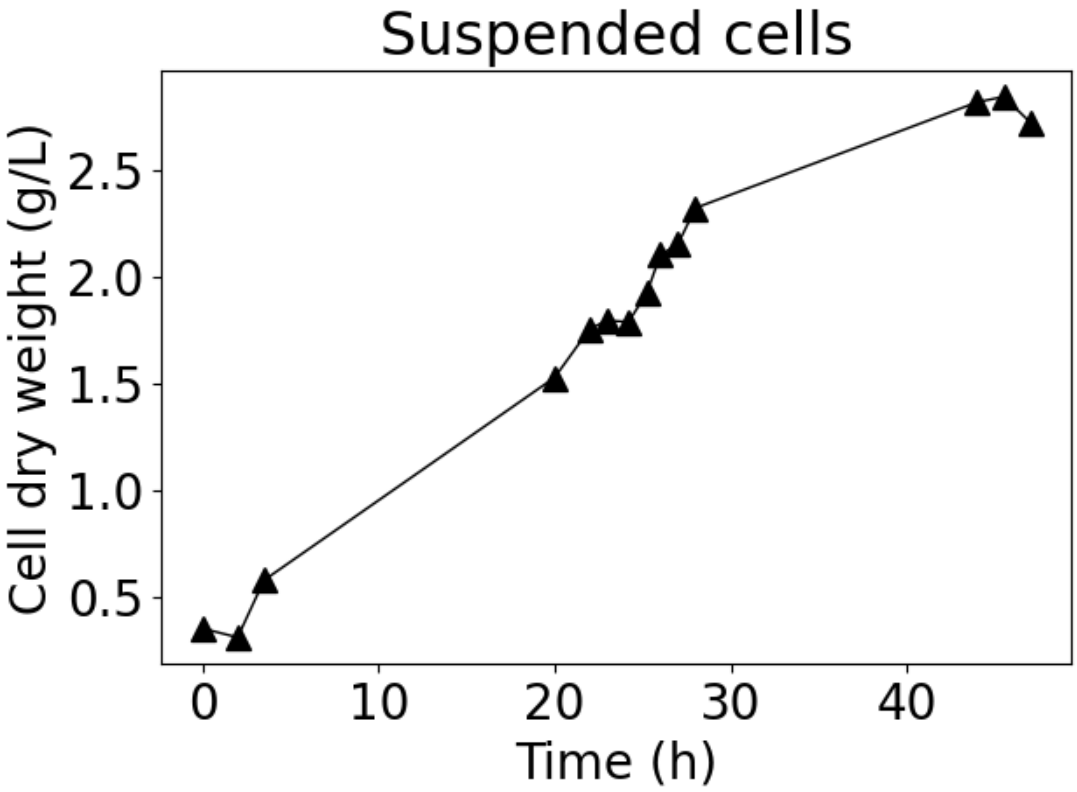


*Figure 27. Suspended cell growth in reactor A. OD was measured at 600 nm and converted to CDW/L.*

Figure 27 shows the suspended cell growth in reactor A during the fermentation. The reactor ran in batch fermentation for a total of 23 hours switching to continuous operation at sample number 6 after reaching a concentration of 1.79 g/L. After new medium was added to the reactor the concentration remained constant during the lag phase and then started increasing continuously until 28 hours after which the reactor was left to operate overnight. The concentration continued to increase during the night until maximum cell concentration was reached. The final cell concentration was 2.72 g/L after a total of 47 hours of fermentation. No wash-out of cell occurred throughout the process.

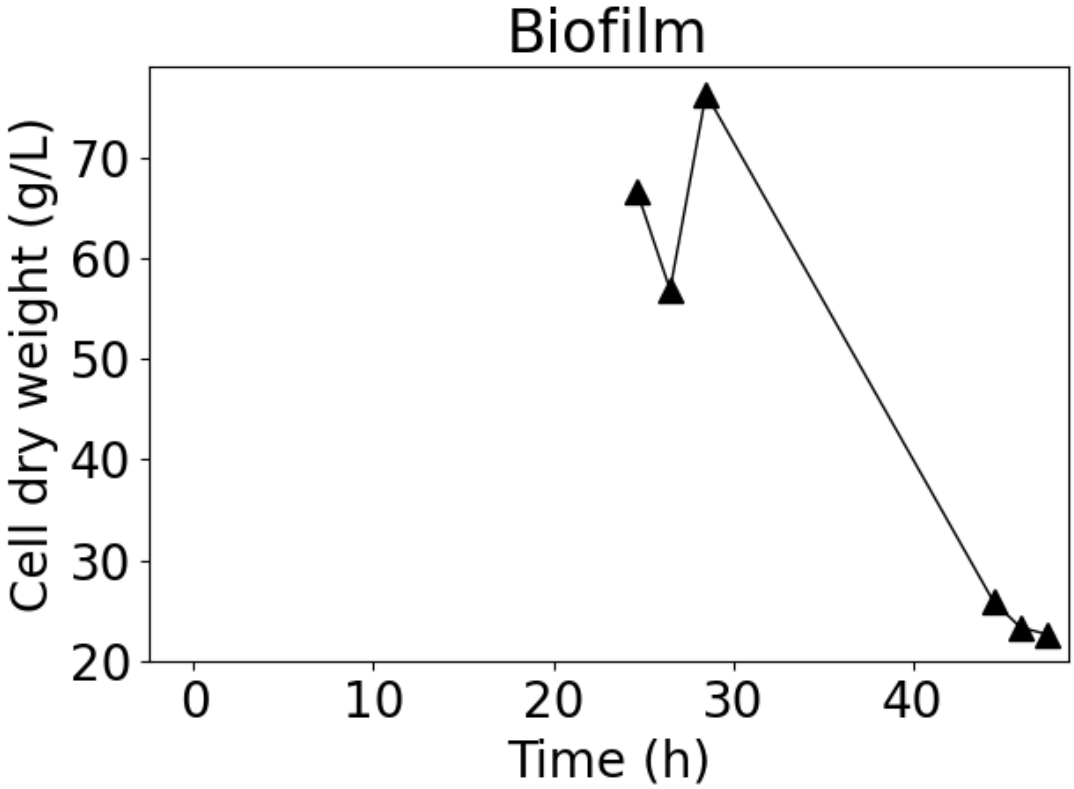


*Figure 28. Biofilm formation in reactor A. Absorbance was measured at 550 nm after crystal violet staining and converted to CDW/L.*

The biofilm growth on one packing material is presented in Figure 28 as the average value of triplicate samples and adjusted for the total amount in the reactor. The first sample was taken 1.67 hours after switching to continuous operation after a total of 24.67 hours after inoculating. The cell concentration at this point was 66.70 g/L. The concentration of biofilm decreased rapidly during the overnight continuous fermentation for a final concentration of 22.59 g/L. Normalized for the total available surface on 100 g of packing material, approximately 1833.33 $cm^2$, the final biomass concentration was 0.012 $g/L \cdot cm^2$. The magnitude of the biofilm is most likely highly exaggerated, during the biofilm formation assay concentrated dark spots were visible on the packing material, which differed from packing material examined in previous experiments.

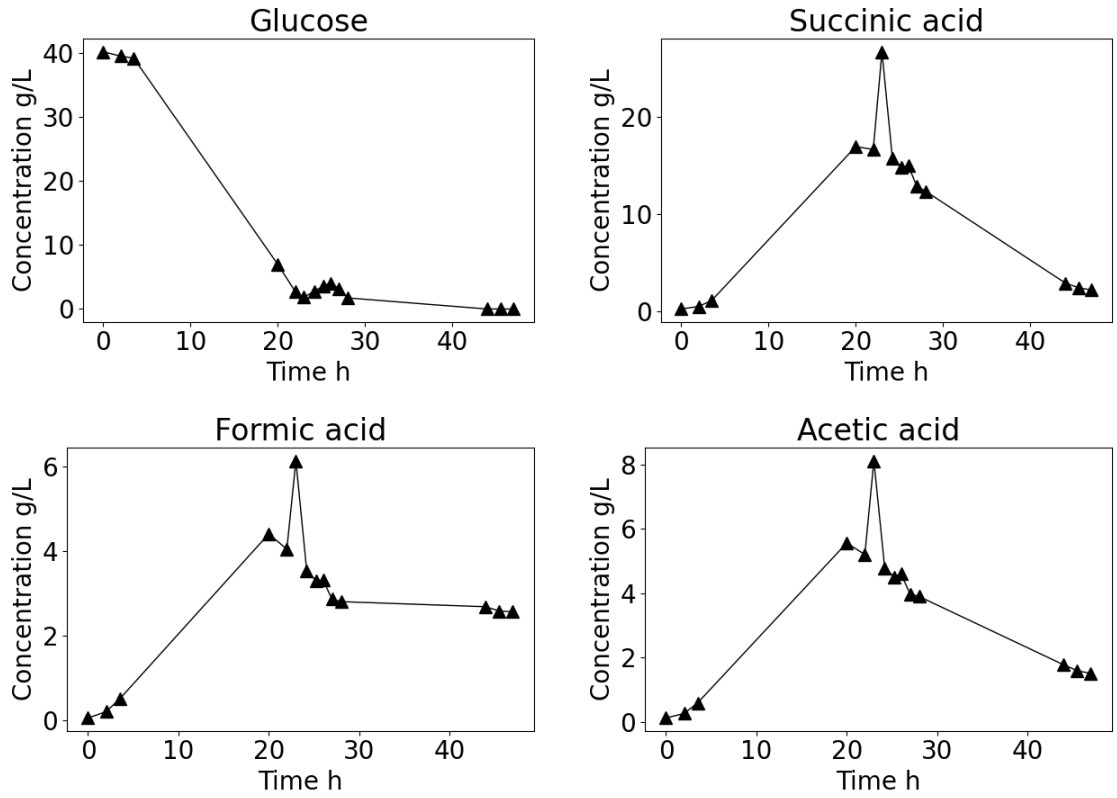


*Figure 29. Substrate consumption and product formation in reactor A.*

The consumption of glucose is seen in Figure 29. The initial concentration was 40 g/L at the start of the batch fermentation. After 23 hours the concentration had dropped to 1.82 g/L as almost all glucose had been consumed. After switching to continuous operation, the concentration increased slightly to around 3.5-4 g/L as the cells adjusted to the new mode of operation and began to grow exponentially. After overnight fermentation the glucose concentration was 0 g/L. All glucose fed to the reactor was therefore fully converted.

Throughout the fermentation the concentration of ethanol and lactic acid remained at 0 g/L. The main product was succinic acid which reached a maximum concentration of 16.97 g/L after 20 hours of fermentation. Although the sample taken at 23 hours shows a concentration of 26.72 g/L this value is not accurate as the spike in concentration for all products in this sample is much greater than expected. The cause is most likely due to errors in sampling or preparation for the HPLC samples. Formic acid and acetic acid reached a maximum of 4.40 g/L and 5.56 g/L, respectively, after 20 hours of fermentation.

After switching to continuous operation, the concentration of all products decreased. After overnight fermentation the concentration of succinic acid decreased to 2.22 g/L after 47 hours of fermentation. Formic acid did not decrease as much overnight and reached a final concentration of 2.56 g/L. The final concentration of acetic acid was 1.51 g/L.

### 5.4.2 Results reactor B

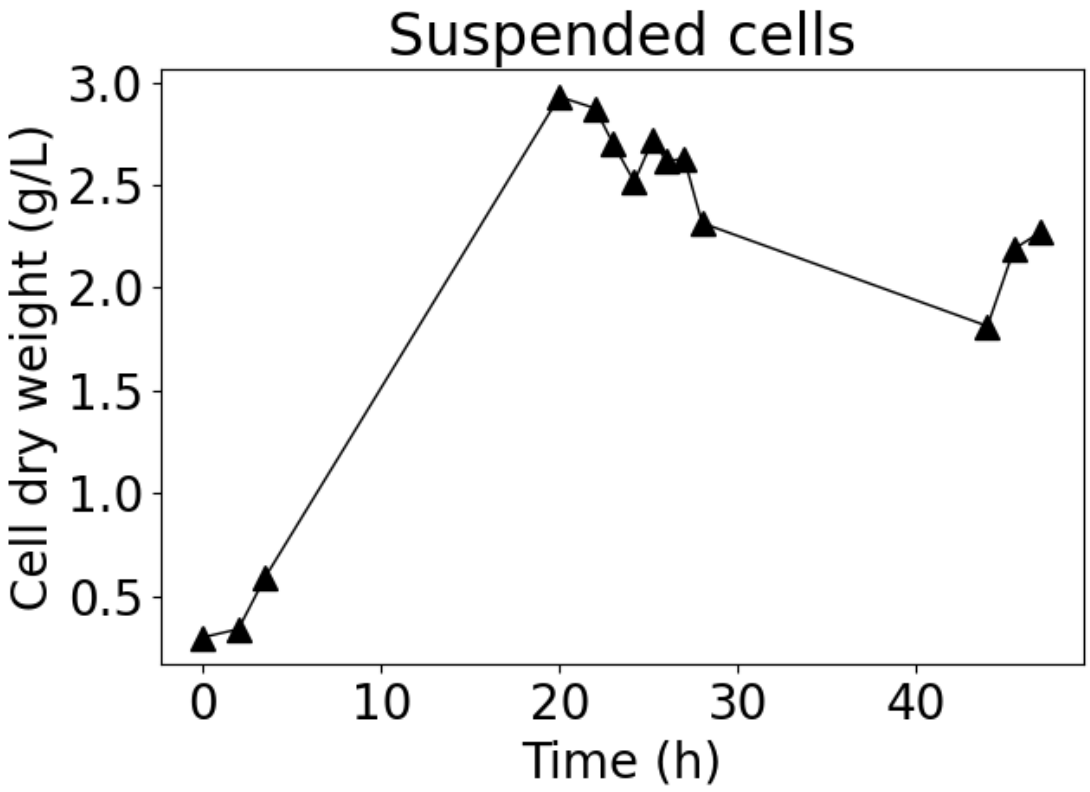


*Figure 30. Suspended cell growth in reactor B. OD was measured at 600 nm and converted to CDW/L.*

The concentration of cells in reactor B is presented in Figure 30. Under batch conditions the concentration reached a maximum of 2.93 g/L after 20 hours of fermentation. After 23 hours the concentration was 2.70 g/L when switching to continuous operation. The concentration increased slightly to 2.72 g/L but decreased again. After overnight operation the concentration had dropped further but increased again for a final concentration of 2.27 g/L after 47 hours.

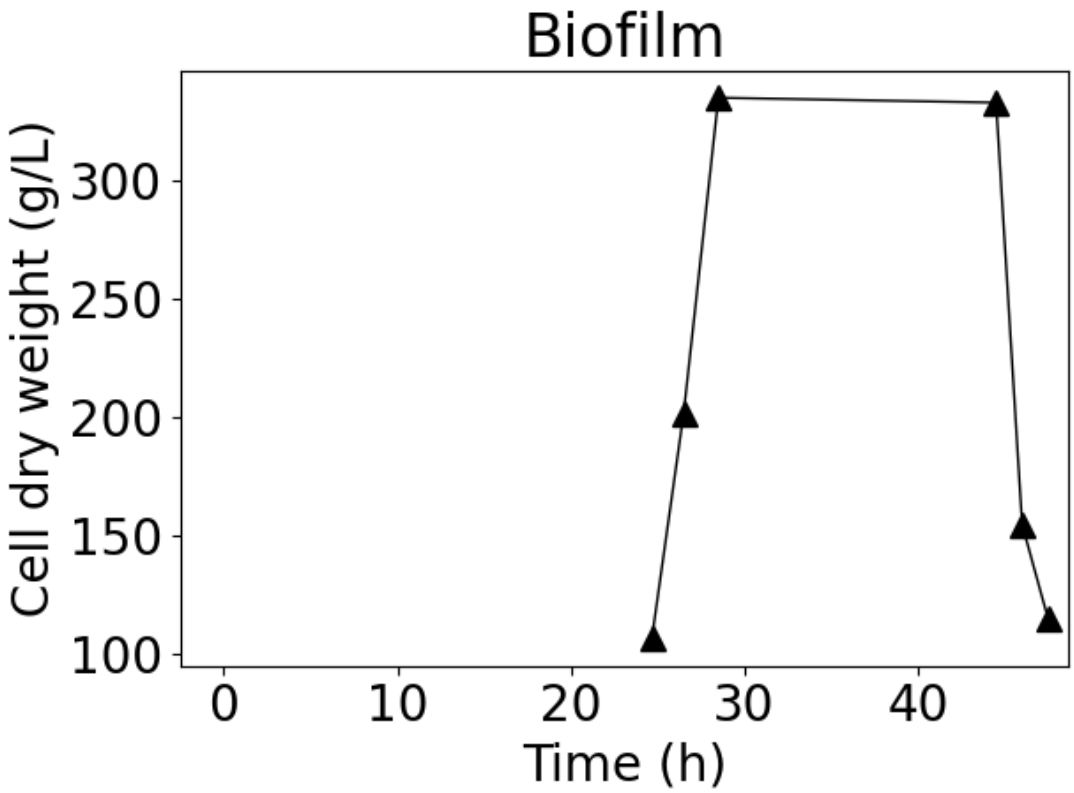


*Figure 31. Biofilm formation in reactor B. Absorbance was measured at 550 nm after crystal violet staining and converted to CDW/L.*

The biofilm formation in reactor B is shown in Figure 31 adjusted for the total amount in the reactor. The first sample measured a concentration of 106.26 g/L which further increased to 335.27 g/L in just 4 hours. The concentration remained almost constant overnight after which it decreased very rapidly for a final concentration of 114.45 g/L after 47.5 hours. Similar dark spots as in reactor A were observed on the packing material extracted from reactor B, influencing the measured absorbance. Normalized for available surface area the final concentration was 0.062 g/L·cm$^2$.

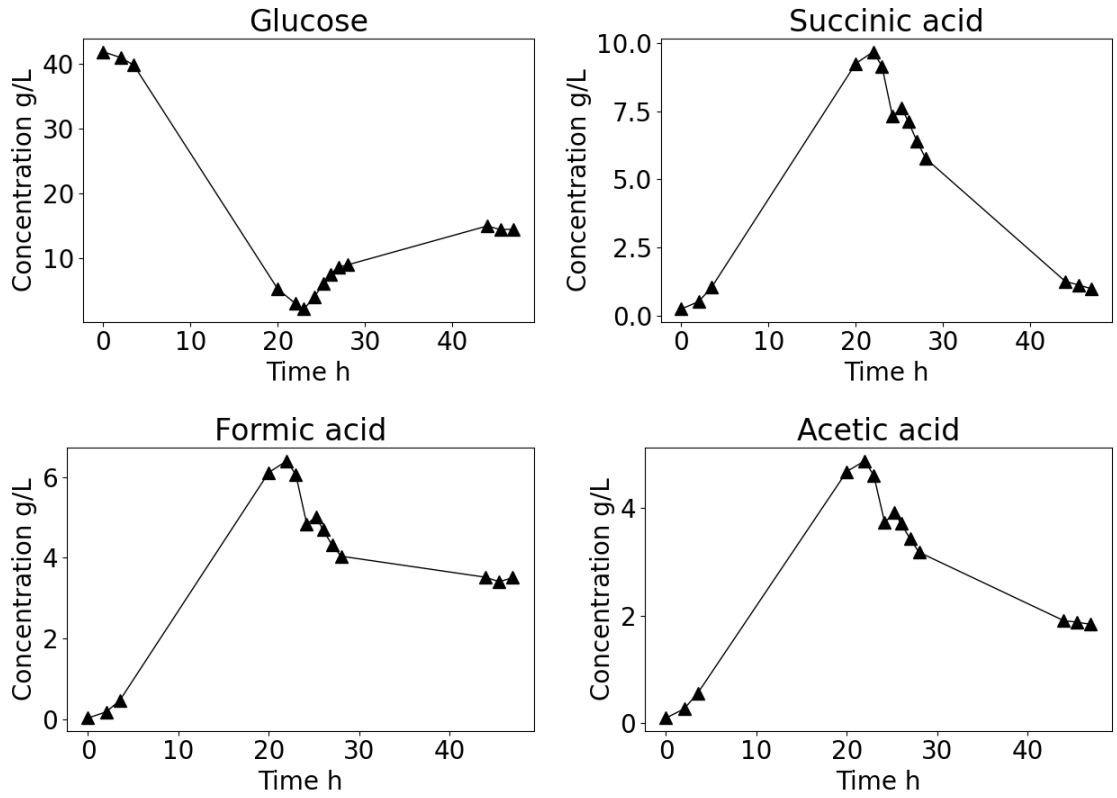


*Figure 32. Substrate consumption and product formation in reactor B.*

Figure 32 shows the results from the HPLC analysis for reactor B. The concentration of glucose started at 40 g/L at the beginning of the batch fermentation. After 23 hours it had decreased to 2.13 g/L. The concentration then increased as continuous operation was initiated. After overnight fermentation the concentration had increased further with a final concentration of 14.45 g/L. The residual glucose in the samples indicated that the system did not convert glucose efficiently.

Just as reactor A, there was no ethanol or lactic acid production in reactor B throughout the entire fermentation. The concentration of succinic acid reached a maximum value of 9.67 g/L after 20 hours of fermentation. At the same time the maximum concentration of formic acid at 6.40 g/L and acetic acid at 4.88 g/L were reached.

After switching to continuous operation, the concentration of all products decreased. During overnight fermentation the concentration of succinic acid and acetic acid continued to decrease, while the formic acid maintained at a relatively constant concentration. The final concentrations after 47 hours were 1.00 g/L for succinic acid, 3.51 g/L for formic acid, and 1.84 g/L for acetic acid.

### 5.4.3 Discussion

The results for reactor A showed similar trends as previous experiments. During the batch phase the cell concentration increased as the substrate was almost completely consumed. After switching to continuous operation, the cells were capable to grow again since new substrate was fed to the reactor.

The decrease in product concentration indicated wash-out in the reactor. However, this does not correlate with the results from the suspended cells growth. Since the glucose concentration was 0 g/L, all glucose was converted which would yield similar product concentrations as the substrate get converted by the microorganisms. These factors indicate contamination in the reactor or the feed tank. The medium in the feed tank was observed after overnight fermentation and was cloudier than expected. However, a sample of the medium was taken at the end of fermentation for HPLC analysis. The results showed that the glucose concentration was 40 g/L and 0 g/L for all products, meaning that no microorganisms were consuming glucose in the feed tank. A possible explanation would be contamination entering the reactor

when sampling the packing material. The main product of the contamination is most likely none of the product analysed in the HPLC as the concentrations are too low.

During the batch phase there seems to be no contamination present in the reactor and the microorganism growing inside showed the characteristics of *A. succinogenes 130Z* with the main product being succinic acid followed by acetic acid and then formic acid.

The results from reactor B also indicated that wash-out had occurred in the reactor when switching to continuous operation. Both product and cell concentration decreased while the substrate concentration increased. However, due to the significant drop in succinic acid compared to formic acid and acetic acid, the reactor most likely had become contaminated. Just as for reactor A the contamination did not occur in the feed tank but in the reactor itself.

The results from both reactor A and reactor B showed that some form of contamination overtook *A. succinogenes* as the dominating microorganism. The time point for contamination occurred at the same time, when the operational mode was changed from batch to continuous. The most likely source for contamination is from extracting packing material from the top of the reactor. Since the setup was not kept and operated in a sterile environment the risk for contamination when exposing the inside of the reactor to the outside is too severe. Keeping an open flame and sterilizing with 70% ethanol is not sufficient to negate microorganisms from entering.

Results from the biofilm formation assay strongly indicated some internal phenomenon affecting the packing material. As mentioned, very dark spots were present on the packing material extracted from both reactors indicating a local concentration of crystal violet. The origin of these spots is unknown, but it is highly unlikely that it would be densely packed cells. Packing material analysed during the batch fermentation in bottles displayed a more even distribution of dye on the surface of the packing material, with similar colouration. Samples from the reactor lacked colouration on most of the surface except on these highly, almost particle-like structures.

The method of extracting packing material directly from the reactor for analysis of the biofilm formation should be deemed unsuccessful for the results obtained in this study. Both for the risk of contamination and unknown aggregates adhering to the packing material. No run was successful in growing *A. succinogenes* through the entire process, and therefore, it is not known if the method is applicable as a means of quantifying biofilm inside a bioreactor.

## 5.5 Model validation using data from a continuous fermentation in a CSTR

The fermentation was not performed in the bubble column reactor as in the previous experiments but instead in a CSTR setup using no packing material. Similar control over parameters such as temperature, pH and gas supply were present throughout the run. One addition from the CSTR is constant agitation from an impeller allowing for homogeneity in the fermentation broth.

The reactor was inoculated and operated in batch for 24 hours before switching to continuous operation. The dilution rate was set to 0.05 $h^{-1}$, and the reactor was continuously fed with new media with a glucose concentration of 50 g/L. At the end of the fermentation the biofilm was quantified using a modified biofilm formation assay.

The initial conditions and dilution rate were fed into the model using a superficial approximated surface area of 400 $cm^2$. The results are presented in Figure 33.

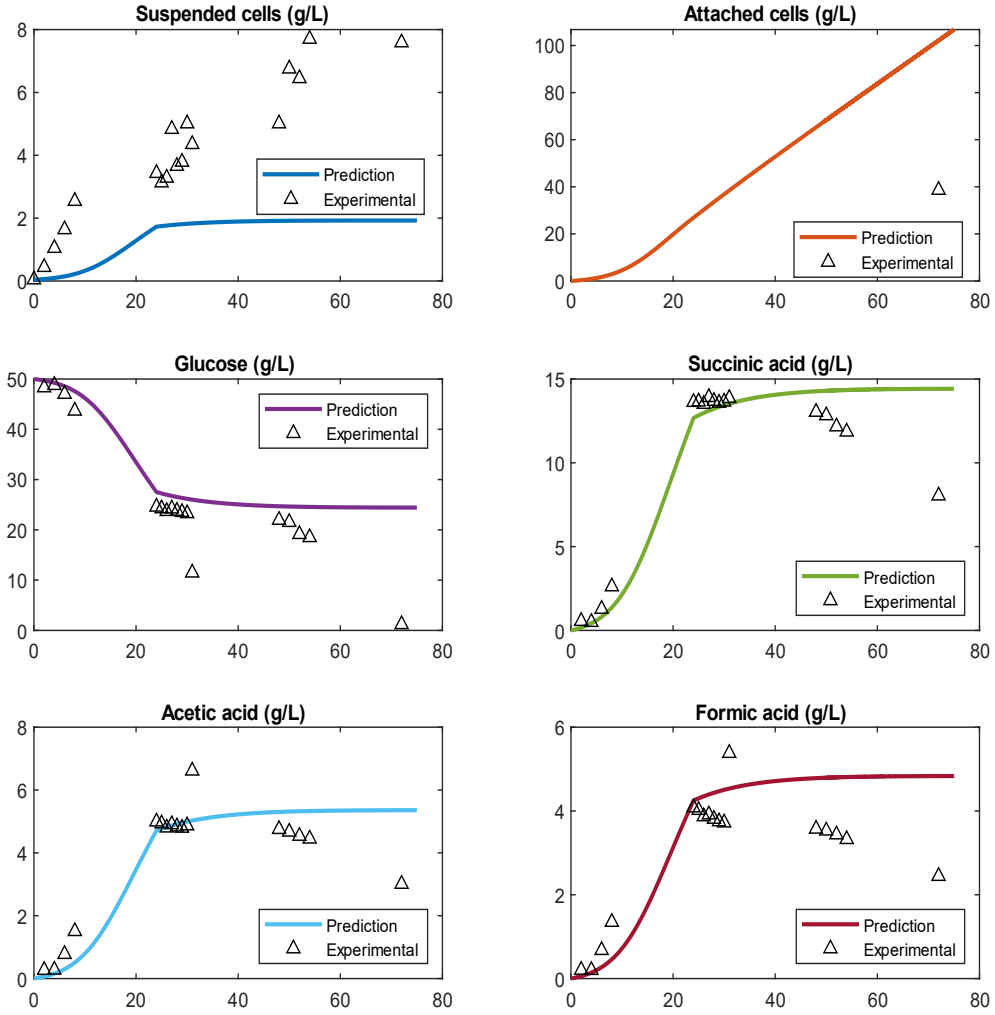


*Figure 33. Experimental results and model prediction when operating in continuous mode with a dilution rate of 0.05 $h^{-1}$ and substrate concentration of 50 g/L.*

From the results it can immediately be seen that the model has difficulties predicting the concentration of suspended and attached cells. The suspended cells are limited to a concentration of 2 g/L, with a much faster growth than the model anticipates. Since biofilm was only quantified at the end of the fermentation it is unknown how the actual kinetics behave. However, the model greatly exaggerates the final concentration of biofilm and continues to increase during the entire process. This is due to no limitations being set on either the substrate requirements for biofilm formation or any critical biofilm concentration.

Regarding substrate consumption and product formation, the model is more accurate in its prediction. The glucose is consumed during the batch phase to a concentration close to the experimental. After switching to continuous operation, the glucose concentration becomes stationary which correlates with the decreased consumption from the experimental results. The product formation also follows similar trend, predicting the final concentration during the batch phase the stationary values. The experimental data indicates a wash-out of products from the reactor under continuous phase which is not accounted for by the model.

# 6 Conclusion

The objective of this project was to investigate the bioproduction of succinic acid using *A. succinogenes* through a variety of cultivation techniques. Additionally, the focus was to characterize the formation of biofilm during fermentation along with calibration and validation of a dynamic model for the fermentation process.

During the initial phase of the project fermentation was performed in microplates to characterize kinetics of both suspended cells and attached cells while simultaneously getting familiar with the fermentation technique. A method for quantifying biofilm formation using crystal violet staining was evaluated to see if the method could be used effectively. In the experiment different initial conditions for pH and concentrations of yeast extract were tested to see how it would influence the growth.

From the experiments kinetic curves for microbial growth in both suspended form and attached form were produced in which growth phases such as lag phase, exponential phase, and stationary phase could be identified. The data were used to calculate the maximum specific growth rate $\mu_{max}$ which yielded values in the range of 0.178-0.413 $h^{-1}$ for suspended cells and 0.063-0.089 $h^{-1}$ for attached cells depending on the initial conditions. This gave an initial estimation of the range of these parameters for further experiments. Testing different initial conditions indicated that suspended cell growth was highly influenced by both the pH and concentration of yeast extract. Higher pH and more yeast extract gave a higher concentration of suspended cells, but the higher pH also yielded a slower growth. For biofilm formation the pH had a great influence on the amount formed being favoured by lower pH, however, at a slower rate.

Cultivation in microplates has been shown to be an effective method for characterizing growth kinetics of both suspended and attached cells under different conditions. The biofilm formation assay using crystal violet staining was shown to be very effective in quantifying the amount of biofilm formed on the microplates. The downside is that the system is limited to batch operation and taking samples for metabolite analysis is difficult due to the small working volume.

In the second phase of the study *A. succinogenes 130Z* was cultivated in glass bottles. Experiments were performed with added packing material for biofilm formation which would more accurately represent the conditions in a packed-bed bioreactor. During this study kinetics were measured for suspended cell growth and attached cell growth just as in the microplates. In addition to this, the substrate consumption and production formation were analysed using HPLC. The results from the experiments were used for calibration of the kinetic model.

Similar kinetics to previous results from microplates for suspended cell growth were obtained to characterize exponential growth. Biofilm formation showed that a larger quantity of biofilm was formed when packing material was present, as was expected. However, the results from the HPLC analysis did not give the expected outcome of more succinic acid being produced in correlation with the number of attached cells. The maximum specific growth rates when no packing material was present were 0.224 $h^{-1}$ and 0.413 $h^{-1}$ for 20 ml bottles and 100 ml bottles, respectively. With packing material these values were 0.209 $h^{-1}$ and 0.427 $h^{-1}$.

For the formation of biofilm, the calculated maximum specific growth rates were 0.207 $h^{-1}$ with no packing material and 0.306 $h^{-1}$ with packing material. On the packing material itself the growth rate was harder to calculate as no exponential phase was identified as initial attachment occurred rapidly. When normalizing for the available surface area it became clear that biofilm formation on the packing material was very rapid and in larger quantity compared to on the glass bottles.

Using crystal violet staining for quantification of biofilm showed great potential for application on scales not only in microplates. However, due to the time consuming and destructive analysis this method may be limited as many bottles are required to accurately determine the growth kinetics.

The third and final stage of the study was to assemble a bioreactor and perform experiments on a larger scale. The assembly of all components took longer time than expected and posed many challenges, but a working setup was produced, able to cultivate microorganism under controlled conditions. However, due to contaminations being present during all experiments this phase must be deemed unsuccessful as no data were acquired during successful cultivation of *A. succinogenes*. The main problem is the material

of the reactor being PVC which makes autoclavation impossible. The alternative sterilization techniques using ethanol, chlorhexidine, and UV-radiation were not sufficient in ensuring a sterile environment. Since biofilm was quantified by extracting packing material directly from the reactor the exposure to outside environment generated a very high risk of contamination. This method cannot be deemed as effective for biofilm quantification. However, from the results using recirculation via polyurethane tubing the formation of biofilm seemed quite promising as an ex-situ alternative to minimize risk of contamination. In the future this method could maybe be implemented to emulate the biofilm formation inside the reactor.

Using data from a successful experiment in a CSTR the model was validated. The results showed somewhat good predictions for substrate consumption and product formation but did not achieve a good prediction for suspended and attached cell growth. The model is very simple and further work should be done to describe the kinetics more precisely. Especially for biofilm formation where values of maximum specific growth should somehow be implemented which may yield a more accurate description of the process. A consequent refit of the main parameters is suggested to achieve a more accurate description of the state variables.

The main issue during the project has been time constraint. Since the fermentation process is rather long, especially if evaluating biofilm formation, the number of experiments is highly limited. In combination with the assembly of the reactors, sample preparation, and data analysis the project became excessively time consuming. However, the targets of the project are well achieved. Biofilm formation was characterized at different scales and a model was constructed which displayed a good representation of the system, even for a preliminary estimation.

For future research, the goal would be to increase the knowledge regarding the biofilm formation. Based on results from this study, the effect of pH at different scales as well as refinement of the biofilm quantification techniques would be highly relevant to better estimate and predict the fermentation process.

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