Continuous succinic acid fermentation by *Actinobacillus succinogenes*

Carel D. van Heerden

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Continuous succinic acid fermentation by *Actinobacillus succinogenes*

by

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Dissertation presented in partial fulfilment of the requirements for the degree of

Master of Engineering in Chemical Engineering

at the University of Pretoria

Faculty of Engineering, the Built Environment and Information Technology,
Department of Chemical Engineering,
University of Pretoria

Supervisor: Prof. W. Nicol

March 2012

Synopsis

Succinic acid was produced from D-glucose in a salt medium with added yeast extract and corn steep liquor, while CO_2 (g) was fed to the fermentation broth. The fermentations occurred in a novel, externally recycled reactor with the pH and temperature controlled at 6.8 (with KOH) and 38 °C, respectively. Separate fermentations were tested with and without added expanded perlite particles for cell support. The dilution rates employed varied between 0.04 and 0.76 h^{-1} at D-glucose feed concentrations of 20 or 40 $g.\ell^{-1}$.

Gradually increasing immobilised cells were observed in the calmer areas of the reactor and productivity therefore increased, but this caused significant deviation from chemostat behaviour. Unknown cell concentrations in the reactor therefore prevented any plausible kinetic analysis. At a D-glucose feed concentration of 20 $g.\ell^{-1}$ fermentations without packing in the reactor delivered productivities of up to 4.6 and 4.9 $g.\ell^{-1}.h^{-1}$ at dilution rates of 0.76 and 0.56 h^{-1}. Nevertheless, by providing more support for biofilm growth by filling 40 % of the reactor volume with packing, further increased productivities were observed. At the two dilution rates mentioned, productivities increased by 54% and 35% to 7.1 and 6.6 $g.\ell^{-1}.h^{-1}$, respectively. Yields were mostly unaffected by dilution rate and biofilm formation and remained at approximately 0.67 ± 0.05 $g.g^{-1}$. Succinic acid was produced in the same molar ratio of 1.25 : 1 to both byproducts formed: acetic and formic acid.

Several factors can increase biofilm formation of *A. succinogenes* that can further increase the productivity and efficiency of succinic acid fermentation. Type and size of packing, hydrodynamic conditions around the packing, and medium components are just some of the factors that affect cell adhesion and/or growth. Further investigations into higher substrate concentrations

and their interaction with biofilm and byproduct formation are required as well.

Keywords: succinic acid, *Actinobacillus succinogenes*, continuous, biofilm, suspended cell, carbon dioxide, D-glucose.

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Nomenclature

C_i	Concentration of component i	$g.\ell^{-1}$
D	Dilution rate	h^{-1}
MM_i	Molar mass of component i	$g.mol^{-1}$
P_i	Productivity of component i	$g.\ell^{-1}.h^{-1}$
$R_{i/j}$	Ratio of component i to component j	$mol.mol^{-1}$
t	Time	days
X	Biomass concentration	$g.\ell^{-1}$
$Y_{i/S}$	Yield: gram of component i produced per gram substrate consumed	$g.g^{-1}$

Greek

γ	Conversion	$g.g^{-1}$
ϕ	Carbon recovery fraction	$mol.mol^{-1}$

Component acronyms

AA	Acetic acid
FA	Formic acid
S	Substrate
SA	Succinic acid
SI	Initial substrate

Chapter 1

Introduction

Bioenergy and biobased chemicals have received increased attention in research, politics and industry in the last decade. Limited fossil resources, increasing greenhouse emissions and climate changes have driven change to these and other sustainable alternatives. Sources of biomass are widely available, but processes for converting it into value-added chemicals are complicated and relevant technologies are new. Integrating already proven biofuel production with high value, lower volume, biobased chemicals can justify incentive for financial investment for developing unfamiliar technology. Current successful bioprocesses (e.g. production of ethanol, lactic acid and citric acid), as well as increasing prices of petrochemical feedstocks are gradually changing the balance in favour of diversifying biochemical processes (Bechthold *et al.*, 2008; Cukalovic & Stevens, 2008; Sheldon, 2011).

Organic acids represent a large fraction of the current and future biochemical industry. Due to functional groups present in these acids, they can be used as platform chemicals. Succinic acid is naturally produced by microorganisms and is among a few other biochemicals that have been identified to have the greatest potential in future biorefineries (Bozell & Petersen, 2010). Provided that competitive bioproduction processes for succinic acid can be developed, its market can increase significantly. With a potential market size of 25 million tons per year, the most important future application is succinic acid-derived polymers. Also, some notable intermediate chemicals that can be produced from succinic acid are tetrahydrofuran, γ-butyrolactone, 1,4-butanediol, adipic

acid and various pyrrolidones. These derivatives have an estimated market size of 240 000 tons per year (Zeikus *et al.*, 1999; McKinlay *et al.*, 2007b; Sauer *et al.*, 2008; Cukalovic & Stevens, 2008). Construction or late planning phases of commercial-scale succinic acid production facilities by BioAmber, Reverdia, Myriant Technologies and a collaboration between BASF and CSM are also currently underway to start capitalising on the increasing demand (Bomgardner, 2011; DSM, 2011; Duckett, 2011).

Biochemical production, including succinic acid fermentation, has largely been carried out in suspended cell batch processes in the past. As such, most literature has focused on this mode of operation. It is the preferred operating mode where the market for the product is relatively small (Shuler & Kargi, 2002: 247). In order to further optimise process parameters such as productivity, yield and product concentration, extensive investigation into other modes of operation needs to be considered. However, research on continuous, semi-batch and multi-stage succinic acid production has been limited. Continuous systems form the basis of dedicated processing systems and are known to have increased productivities (Shuler & Kargi, 2002: 245 – 247). Studies on continuous fermentations on succinic acid-producing microorganisms include *Actinobacillus succinogenes* (Urbance *et al.*, 2004; Kim *et al.*, 2009), *Anaerobiospirillum succiniciproducens* (Samuelov *et al.*, 1999; Lee *et al.*, 2000; Meynial-Salles *et al.*, 2008; Lee *et al.*, 2009, 2010), *Basfia succiniciproducens* (Scholten *et al.*, 2009), *Enterococcus faecalis* (Wee *et al.*, 2002) and *Mannheimia succiniciproducens* (Lee *et al.*, 2003; Kim *et al.*, 2004; Oh *et al.*, 2008). All of the mentioned bacteria are natural succinic acid producers through anaerobic metabolism.

Reaction rate is related to the cell concentration inside the reactor. The use of membranes and immobilisation techniques are two popular methods to increase the cell concentration by preventing cell washout. Membranes are not often used in low cost, large volume chemical productions due to the associated costs. Cell immobilisation may be classified in two categories: active and passive immobilisation. Active immobilisation includes entrapment, adsorption and covalent bonding techniques, while passive immobilisation entails the formation of biofilms. It is defined as microbial cell layers that reversibly or irreversibly attach on surfaces and can exhibit different growth and bioactivity compared to suspended cells. Cells are embedded in a self-produced ex-

opolysaccharide (EPS) matrix. Biofilm reactors are characterised by economic and potentially long operation time compared to reactors utilising other immobilisation techniques (Shuler & Kargi, 2002; Qureshi *et al.*, 2005; Cheng *et al.*, 2010: 263 – 266).

The objective of this study was to investigate continuous, anaerobic succinic acid bioproduction with the bacterium *A. succinogenes* (130Z) as biocatalyst. The aim was to evaulate steady state conditions, cell attachment through biofilm growth, productivity and byproduct formation. It was also aimed to achieve maximum periods of stable, 'pure culture' fermentation.

Succinic acid was produced from D-glucose and CO_2 (g) in a salt medium with added yeast extract and corn steep liquor. The fermentation occurred in a novel, externally recycled reactor. The pH and temperature were controlled at 6.8 (with KOH) and 38 °C, respectively. D-glucose feed concentrations of 20 and 40 $g.\ell^{-1}$ were investigated at different dilution rates. Perlite particles were added in some fermentations to provide more area for cell attachment.

Chapter 2

Theory

2.1 Bioprocessing industry

The use of coal, gas and oil have increased by several orders of magnitude since the start of the Industrial Revolution. This is due to a substantial increase in the number of applications for these fossil fuel resources over the last two centuries. Fuel and platform chemicals produced in the petroleum industry have largely dominated the chemical industry. Although this is not expected to change for some time, the depletion of fossil fuel resources is inevitable. Some estimate its depletion in less than 50 years. Increasing greenhouse emissions worldwide and climate changes have generated much concern as well.

Locally available renewable resources as an alternative to fossil fuel resources are essential to sustainable development. The development and implementation of bioenergy and biobased chemicals have consequently received a lot of attention in research, politics and industry in the last decade. Other terms such as 'green', 'efficient', 'durable', 'white biotechnology' and 'carbon neutral' are popular in promoting the use of renewable resources. Whereas options for sustainable energy include biomass, solar, wind, geothermal, magnetic and hydropower, only biomass can be used as a source of carbon-based chemicals. Sources of biomass encompass agricultural food and feed crops, dedicated energy crops and trees, agricultural and forestry residues, aquatic plants and animals and municipal waste. Still, only a small percentage of available biomass is utilised in a few industries such as biofuels and wood and paper process-

ing. This is because conversion of renewable carbon to value-added chemicals is the least developed and most complicated of all biorefining operations. Apart from technological feasibility, the availability and cost of basic carbohydrate feedstocks and other essential nutrients, isolation and purification of endproducts and the overall costs of the process need to be considered for industrial-scale implementation. Nevertheless, proof that these problems can be overcome is evident from previous and current commercial success of large scale bioproduction processes of ethanol, lactic acid and citric acid, among others. However, many processes utilising biomass are still more expensive than equivalent petrochemical processes. This presents a major hurdle to increase biomass utilisation. However, an integrated biorefinery where essential (but low value) biofuel production is integrated with high value, lower volume biobased chemicals can overcome this hurdle. Table 2.1 summarises some advantages and disadvantages of the petrochemical and biochemical industry. Some of these advantages and disadvantages indicate a direct contrast, while others are unique to both of the industries. It is worth noting that some problems of the aging petrochemical industry are increasing, whereas most of the disadvantages of the biochemical industry are currently being addressed (Zeikus *et al.*, 1999; McKinlay *et al.*, 2007b; Bechthold *et al.*, 2008; Cukalovic & Stevens, 2008; Bozell & Petersen, 2010; Sheldon, 2011).

Table 2.1: A general comparison between petrochemical and biochemical production processes (Cukalovic & Stevens, 2008)

	Production method	
	Petrochemical	Biochemical
Origin	Non-renewable feedstocks	Renewable feedstocks – carbohydrates
Price considerations	Still cheaper than renewable resources	Downstream processing much more expensive than feedstocks
Routes	Developed routes; established technology	Routes under constant improvement; young technology
Yields and productivity	Generally high	Significant amounts of byproducts are common; diluted media; long reaction times
Major disadvantages	High energy demands (pressure and and temperature); catalyst disposal problems	Sensitive microorganisms; complex additional nutrients often needed; complicated product recovery; large amounts of wastes
Public awareness	Decreasing popularity	Increased interest

Certain functional groups are necessary in platform chemicals to produce fur-

ther intermediates and are introduced by costly oxidative processes in the petrochemical industry. Organic acids are one of the types of chemicals that contain these functional groups. Their potential as platform chemicals has already been identified and has received much attention in the literature. The previously mentioned functional groups are present in carbohydrates in biomass and are transferred to organic acids produced in bioprocesses. This involves fermentation where carbohydrates are metabolised by microorganisms and the intermediates or endproducts in the metabolic pathways are converted to organic acids in downstream processes. Apart from lactic and citric acid, some other noteworthy organic acids can be produced by microorganisms, including succinic acid, fumaric acid, malic acid, itaconic acid and 3-hydroxypropionic acid. Provided that competitive bioproduction processes for these acids can be developed, their respective markets can increase significantly (Sauer *et al.*, 2008; Cukalovic *&* Stevens, 2008).

2.2 Succinic acid

Succinic acid (butanedioic acid, 1,2-ethanedicarboxylic acid or amber acid) is a saturated dicarboxylic acid with the chemical formula $HO_2C(CH_2)_2CO_2H$ and is illustrated by a ball-and-stick model figure 2.1. The acid and its esters occur in nature in amber, animal tissues, vegetables and fruit, spring water and meteorites. It is formed in alcoholic fermentation and in the chemical and biochemical oxidation of fats. In its pure form, succinic acid occurs as colourless triclinic prisms (α-form) and monoclinic prisms (β-form) (Fumagalli, 2007: 416 – 417). Important properties of succinic acid are summarised in table 2.2.

Historically, succinic acid was obtained from the distillation of pulverised amber (Latin: *succinum*). Currently in industry, the majority of succinic acid is produced petrochemically in a process that involves the oxidation of N-butane to form maleic anhydride. The maleic anhydride is then hydrolysed to maleic acid, with the final step involving hydrogenation to produce succinic acid (Zeikus *et al.*, 1999; Vaidya *&* Mahajani, 2003). This process is illustrated by reactions 2.1 to 2.3. Other petrochemical processes involves the hydration of succinic anhydride, the hydrogenation of fumaric acid and the recovery of

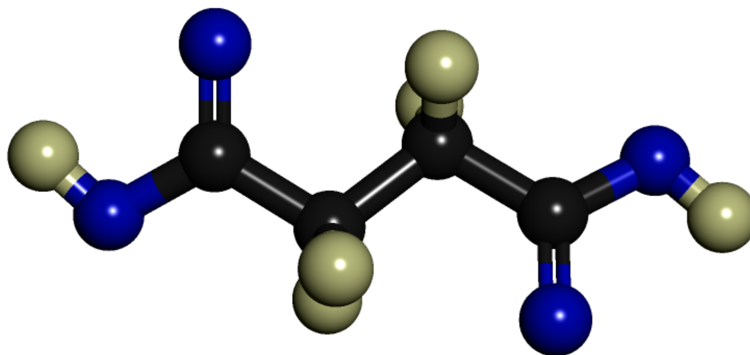


Figure 2.1: Ball-and-stick model of a succinic acid molecule

Table 2.2: Properties of succinic acid (Perry *et al.*, 1997: 2-45)

Succinic acid properties	Details	Value	Units
Acidity	pK_{a1}	4.21	-
	pK_{a2}	5.64	-
Boiling point		235	°C
Melting point		189	°C
Molar mass		118.09	$g.mol^{-1}$
Solubility	20 °C	58	$g.\ell^{-1}$
Specific gravity		1.57	$g.cm^{-3}$

succinic acid from a byproduct stream in adipic acid production (Fumagalli, 2007: 424). Succinic acid that has been produced fermentatively from carbohydrates on small scale has almost exclusively been used in the food and health industry.

$$2\,C_4H_{10} + 7\,O_2 \longrightarrow 2\,C_2H_2(CO)_2O + 8\,H_2O \qquad (R\,2.1)$$

$$C_2H_2(CO)_2O \xrightarrow{H_2O} C_4H_4O_4 \qquad (R\,2.2)$$

$$C_4H_4O_4 \xrightarrow{H_2} C_4H_6O_4 \qquad (R\,2.3)$$

2.2.1 Current and future applications and production

The main market for succinic acid in the last two decades has consisted of its application as a surfactant, detergent, extender and foaming agent. It is also used as an ion chelator and for the manufacturing of resins, coatings, inks, dyes, photographic chemicals, lacquers, plasticisers, plant growth stimulants, feed

additives, electrolyte bath additives and biodegradable plastics. In the food industry it is used as a pH modifier, a flavouring agent and an anti-microbial agent. Finally, it is used in the manufacture of health-related products, such as pharmaceuticals, antibiotics, amino acids and vitamins. 16 000 tons of succinic acid were produced in 2007 for these and other specialty applications (Zeikus *et al.*, 1999; Urbance *et al.*, 2003; Song & Lee, 2006; Sauer *et al.*, 2008; Fumagalli, 2007: 417).

Significant industrial potential for succinic acid bioproduction was already suggested in 1980. As a platform chemical, it can replace maleic acid or maleic anhydride. Chemical intermediates produced from succinic acid can replace chemicals based on benzene and other intermediate petrochemicals. This is because alternative, and in many cases environmentally safer chemicals, can be used for the same applications (Zeikus *et al.*, 1999; Sauer *et al.*, 2008). In accordance with a report in 2004 by the US Department of Energy, Bozell & Petersen (2010) agreed that succinic acid is among a few other biobased chemicals with the most potential as chemical building blocks. The main criteria used for these chemicals, which can be produced from carbohydrates, were similar in both evaluations. Important reasons for the inclusion of succinic acid in a list of ten top future chemicals include:

- The fermentative production route has received significant attention in the literature.
- Technology relevant to succinic acid production can be applied or adapted to other production processes.
- It has a strong potential as a platform chemical — its derivatives offer important flexibility and breadth to biorefineries.
- Plans for upscaling to industrial production are under way.

With a potential market size of 25 million tons per year, the most important future application is succinic acid-derived polymers. Furthermore, immediate derivatives have an estimated market size of 240 000 tons per year. Some standard succinic acid derivatives are shown in figure 2.2 (not all possible

pathways are shown). Some notable derivatives with significant current markets include tetrahydrofuran, γ-butyrolactone, 1,4-butanediol, adipic acid and various pyrrolidones. These chemicals are used in the production of solvents, resins, surfactants, paint removers, fibres, polymers and plasticisers, among other applications. Dibasic esters such as dimethyl succinate have great potential as an environmentally benign solvent that can replace other current solvents (Zeikus *et al.*, 1999; McKinlay *et al.*, 2007b; Sauer *et al.*, 2008; Cukalovic *&* Stevens, 2008).

Plans to capitalise on these opportunities are under way. Pilot scale and industrial scale plants are in various phases of operation and planning. The first two pilot plants for succinate production were those of BioAmber (DNP Green Technology and ARD) and Reverdia (Royal DSM and Roquette Frères) — both began production in France in 2009. Both of these companies used genetically engineered *Escherichia coli* for their operations (Yuzbashev *et al.*, 2011). BioAmber followed up on their pilot studies by announcing a 35 000 tons-per-year succinic acid plant in Sarnia, Canada. They claim that the process produces no significant byproducts and uses CO_2 to sustain the bacterium. It is also claimed that, compared to the petroleum process, the bioproduction of succinic acid uses 60% less energy and costs 40% less. The plant will begin operations in 2013, with an initial capacity of 17 000 tons per year. A further plan is to start utilising an unspecified yeast as a biocatalyst by 2014. The relevant technologies are being developed by Cargill (Duckett, 2011). Meanwhile, Reverdia plans to have a plant operational as early as 2012 in Cassano Spinola, Italy. The plant will have an annual capacity of 10 000 tons. Reverdia also plans on using a proprietary, anaerobic yeast-based process for succinic acid production (DSM, 2011).

Two other pilot plants in the USA include those of Myriant Technologies and MBI International. For their biocatalyst, these two companies are using *E. coli* and a rumen bacterium, *Actinobacillus succinogenes*, respectively (Yuzbashev *et al.*, 2011). Myriant Technologies is also planning to start operating an industrial-scale plant in 2012 with a capacity of 15 000 tons succinic acid per year in Louisiana, USA. The plant will use sorghum as feedstock (Bomgardner, 2011).

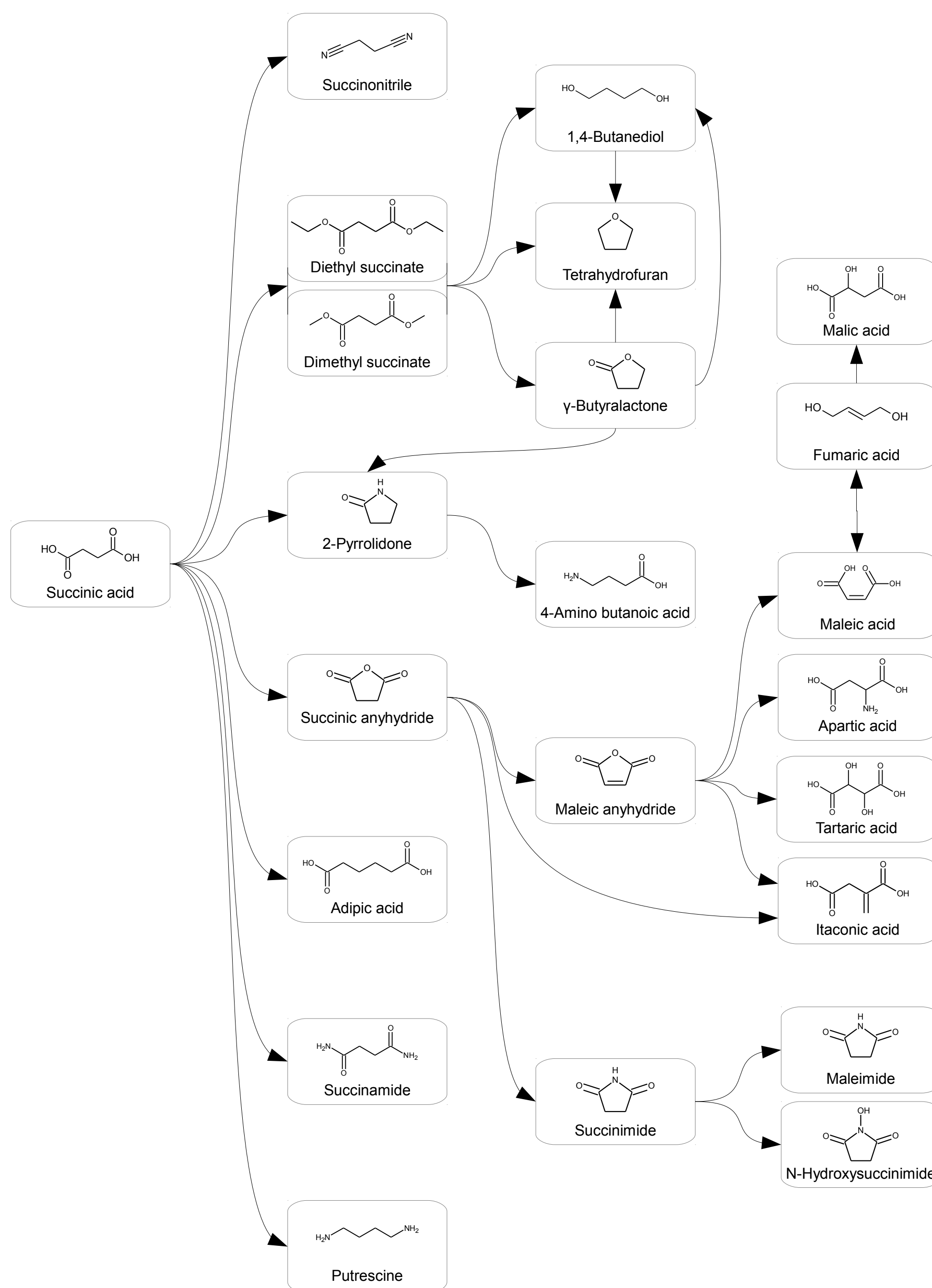


Figure 2.2: Some commodity chemicals that can be produced from succinic acid (Zeikus *et al.*, 1999; Sauer *et al.*, 2008; Bechthold *et al.*, 2008; Cukalovic & Stevens, 2008; Beauprez *et al.*, 2010)

The final two commercial implementations of succinic acid bioprocesses are collaborations between the German and Dutch companies, BASF and CSM, and two Japanese companies, Mitsubishi Chemical and Ajinomoto. The former collaboration will use the newly isolated rumen bacterium, *Basfia succinogenes*, while the latter plans on using a recombinant strain of *Corynebacterium glutamicum*. BASF and CSM started with a 4 000 ton-per-year pilot-scale plant near Barcelona, Spain, and followed up with an announcement for a 25 000 ton-per-year plant for 2013 (also in Spain). A 50 000 ton-per-year plant is already in the initial planning phase.

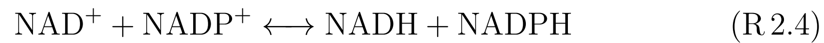
Currently all commercial biochemical succinic acid production involves anaerobic processes. Production of succinic acid by *E. coli* and *A. succinogenes* has been studied extensively (Beauprez *et al.*, 2010). In contrast, *B. succinogenes* has only been isolated recently (Scholten *et al.*, 2009; Kuhnert *et al.*, 2010) and *C. glutamicum* has received very little attention in the open literature with regard to succinic acid production.

2.2.2 Biological reactions

Metabolism of carbon sources involves catabolism and anabolism. Catabolism generates energy from processes that break down complex compounds into simpler compounds. Anabolism involves the growth of cells and consumes energy. Related processes utilise simple carbon compounds to synthesise more complex compounds (Shuler & Kargi, 2002: 134). In addition to energy requirements for biosynthesis, cells also need energy for motility, maintenance and the transport of nutrients. Energy generation from a carbohydrate such as D-glucose occurs primarily during three phases of aerobic catabolism: glycolysis (Embden-Meyerhof-Parnas or EMP pathway); tricarboxylic acid (TCA) cycle; and the electron transport chain or respiration. This generated energy is stored and transferred primarily via adenosine triphosphate (ATP). The majority of energy generation occurs through the electron transport chain. This mechanism involves transferring electrons from nicotinamide adenine dinucleotide (NADH), a coenzyme found in all living cells, to oxygen that acts as the electron acceptor (Shuler & Kargi, 2002: 134 – 142).

Seeing that glycolysis is a standard metabolic process in all living organisms,

the initial steps of metabolism is similar in anaerobic, as well as aerobic organisms. Also, in some cases anaerobic organisms may have all the enzymes necessary for a complete TCA cycle. The cycle can then be active under the right conditions. Furthermore, the electron transport chain can be active when electron acceptors other than oxygen are available and used — this is called anaerobic respiration. In contrast, when no alternative electron acceptors are utilised, the substrate must undergo a balanced series of oxidative and reductive processes, where the following reaction is in equilibrium:



$$NAD^+ + NADP^+ \longleftrightarrow NADH + NADPH \qquad \text{(R 2.4)}$$

This is called fermentation. However, the term 'fermentation' is used in a much broader context in the modern bioprocess industry. Endproducts of fermentation are formed to balance consumption and production of reducing power in cells. Ethanol, lactic acid, 2,3-butanediol and succinic acid are examples of these products. For each mol of D-glucose oxidised through the Embden-Meyerhof-Parnas (EMP) pathway to phosphoenolpuryvate, two moles of NAD^+ are reduced to NADH. The constraint of anaerobic metabolism that requires an equal amount of NADH to be oxidised to NAD^+ and this results in no net ATP formation through the electron transport chain or NAD^+ reduction. This is the main reason why anaerobic processes have such a low energy yield compared to aerobic processes. Without ATP formation from electron transport chain, the ATP produced by these organisms is produced by a process called 'substrate-level phosphorylation' (Shuler & Kargi, 2002: 148 – 152).

Succinic acid can be produced through fermentation from several carbon sources by a variety of microorganisms. Common byproducts produced alongside succinate include acetate, formate, lactate, pyruvate and ethanol. This is referred to as mixed-acid fermentation (Zeikus *et al.*, 1999). No microorganism has been discovered that is naturally capable of monosuccinate fermentation. It is important to note that industrial fermentations are usually performed at pH values close to neutral and the dissociated forms (e.g. $C_4H_4O_4^{2-}$, $C_3H_5O_3^-$, and $C_2H_3O_2^-$) are produced rather than the acids themselves.

Reaction 2.5 shows the formation of succinic, acetic and formic acid from

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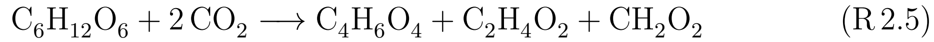
D-glucose and carbon dioxide:



$$C_6H_{12}O_6 + 2\,CO_2 \longrightarrow C_4H_6O_4 + C_2H_4O_2 + CH_2O_2 \qquad \text{(R 2.5)}$$

CO_2 fixation by the enzymes such as PEP carboxykinase is necessary to produce succinic acid from glucose through a reversed path in a partial TCA cycle. This is illustrated in figure 2.3, where an extra carbon is attached in the process to convert phosphoenolpyruvate to oxaloacetate. The desired C_4 pathway, with succinate as the endproduct, is therefore clearly distinguished from the C_3 pathway that forms a variety of undesired byproducts. Apart from CO_2 (g), other sources such as alkaline and earth alkaline carbonates can provide the necessary CO_2 for the fixation reaction (Guettler *et al.*, 1996a; Van der Werf *et al.*, 1997). Combinations of gas and carbonates have also been used (Lin *et al.*, 2008).

Reaction 2.5 depict a simplified reaction scheme where no biomass formation or maintenance is taken into account. Other products produced in minor quantities, such as ethanol, are also disregarded. Instead, a more complex situation arises in the fermentation of sugars and other substrates, as explained earlier in this section. With CO_2(g) present, an equilibrium between H_2CO_3 or CO_2 (aq) and HCO_3^- exists in the medium. The bacteria consumes glucose and HCO_3^- to produce succinate (see reaction 2.6). Theoretically, the optimal yield of approximately 1.71 mol succinate per mol D-glucose (1.12 g.g$^-$1) can be obtained without biomass formation. This is based on the available electrons — 24 electrons in D-glucose divided by 14 electrons in succinate (McKinlay *et al.*, 2007b).

$$C_6H_{12}O_6 + 0.86\,HCO_3^- \longrightarrow 1.71\,C_4H_4O_4^{2-} + 1.74\,H_2O + 2.58\,H^+ \qquad \text{(R 2.6)}$$

The theoretical yield can be increased to 2 mol succinate per mol glucose (1.31 g.g$^-$1) when CO_2 is coupled with additional reducing power (e.g. H_2):

$$C_6H_{12}O_6 + 2\,HCO_3^- + 2\,H_2 \longrightarrow 2\,C_4H_4O_4^{2-} + 2\,H_2O + 2\,H^+ \qquad \text{(R 2.7)}$$

Due to the inability of anaerobic processes to generate energy efficiently, the processes are generally characterised by poor cell growth and slow carbon

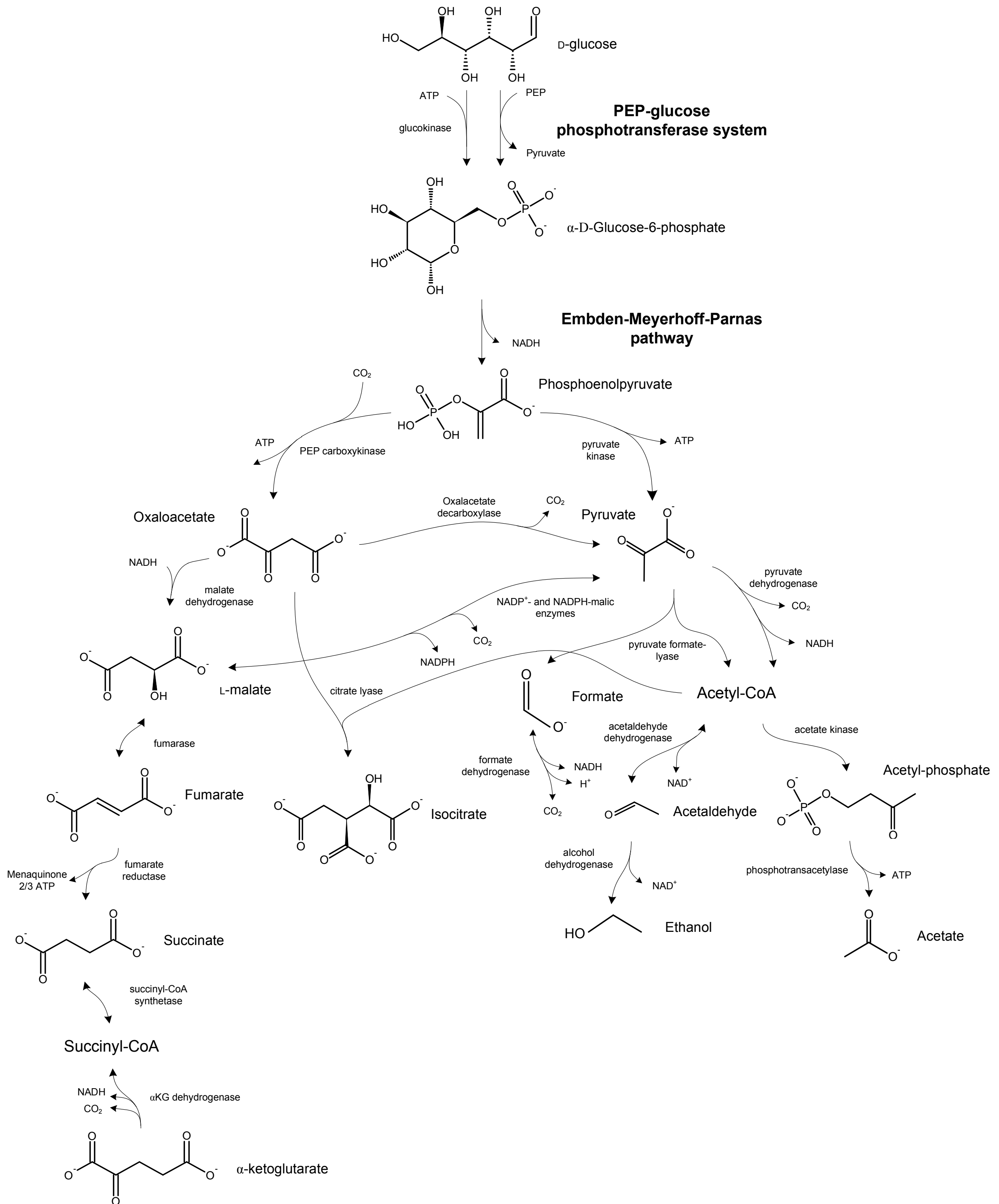


Figure 2.3: Anaerobic metabolism of *A. succinogenes* (McKinlay *et al.*, 2005, 2007a, 2010)

throughput. This leads to low production rates of the desired chemical. As an alternative to anaerobic succinate production, aerobic production has also been shown to be possible. While CO_2 and H_2O are the only products in a full, oxidative citric acid cycle, Lin *et al.* (2005a) showed that succinic acid can be produced by a genetically engineered *E. coli* (see figure 2.4). The usual path of the citric acid cycle is interrupted

before succinate can be converted to fumarate. This is accomplished by deleting the genes necessary for the conversion. In addition to the citric acid cycle, succinate is also synthesised through the glyoxylate cycle. Disadvantages introduced in the aerobic succinate production system include a lower maximum theoretical yield where carbon losses occur due to CO_2 production.

2.2.3 Biocatalysts

Many microorganisms have been investigated under different fermentation conditions to evaluate their potential for succinic acid production. These organisms are listed in table 2.3. Some additional details are also included in the table: the first publication for each specific microorganism; the approximate number of publications in the open literature; and substrates investigated for consumption in all these publications.

Song & Lee (2006) and McKinlay *et al.* (2007b) identified five bacteria as the most promising succinic acid producers: the natural succinic acid producers *Actinobacillus succinogenes*, *Anaerobiospirullum succiniciproducens* and *Mannheimia succiniciproducens*, as well as genetically engineered strains of *Corynebacterium glutamicum* and *Escherichia coli*. With the exception of *C. glutamicum*, these bacteria have been studied extensively. Also, a newly discovered species of bacteria, *Basfia succiniciproducens*, has been reported by Scholten & Dägele (2008) to have exceptional promise for the fermentation of glycerol to succinic acid.

2.2.3.1 *Actinobacillus succinogenes*

A. succinogenes, a bacterium from the family *Pasteurellaceae*, was isolated from the rumen of a cow at Michigan State University (Guettler *et al.*, 1996a, 1999). The bacterium is a gram-negative, capnophilic, facultative anaerobe.

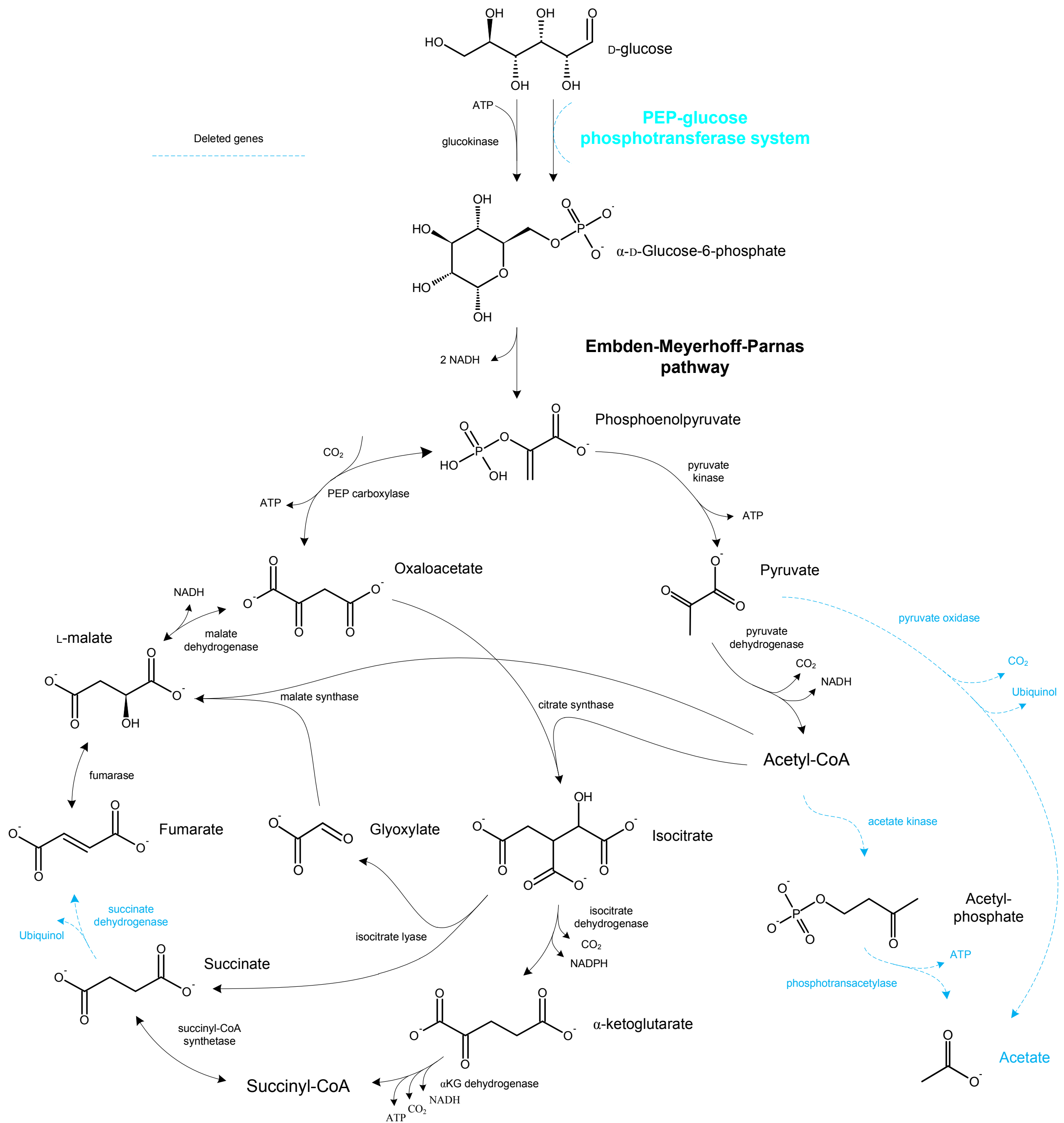


Figure 2.4: Aerobic metabolism of an *E. coli* strain engineered to produce succinic acid with a partial TCA-cycle (Lin *et al.*, 2005b)

Table 2.3: Microorganisms investigated for succinic acid production.

Microorganism	Type	Pub.	First publication	Substrates
Actinobacillus succinogenes	Bacterium	50+	Guettler *et al.* (1996a)	Various sugars (mostly D-glucose) and glycerol
Anaerobiospirillum succiniciproducens	Bacterium	20+	Samuelov *et al.* (1991)	Various sugars (mostly D-glucose) and glycerol
Aspergillus niger	Mold	1	Meijer *et al.* (2007)	Xylose
Bacteroides fragilis	Bacterium	2	Isar *et al.* (2006)	D-glucose
Basfia succiniciproducens	Bacterium	4	Scholten *et al.* (2009)	Various sugars and glycerol
Candida brumptii	Yeast	2	Sato *et al.* (1972)	N-alkanes
Candida catenulata	Yeast	1	Kamzolova *et al.* (2009)	Ethanol
Candida zeylanoides	Yeast	1	Kamzolova *et al.* (2009)	Ethanol
Clostridium thermosuccinogenes	Bacterium	3	Sridhar *&* Eiteman (1999)	D-glucose, fructose and inulin
Corynebacterium glutamicum	Bacterium	3	Inui *et al.* (2004)	D-glucose
Enterococcus faecalis	Bacterium	6	Ryu *et al.* (1999)	Fumarate
Enterococcus flavescens	Bacterium	1	Agarwal *et al.* (2007)	D-glucose
Escherichia coli	Bacterium	50+	Millard *et al.* (1996)	Various sugars (mostly D-glucose) and glycerol
Fibrobacter succinogenes	Bacterium	2	Gokarn *et al.* (1997)	Cellulose, cellubiose and D-glucose
Klebsiella pneumoniae	Bacterium	2	Thakker *et al.* (2006)	D-glucose
Mannheimia succiniciproducens	Bacterium	20+	Lee *et al.* (2002)	Various sugars (mostly D-glucose) and glycerol
Penicillium simplicissimum	Yeast	1	Gallmetzer *et al.* (2002)	D-glucose
Ruminococcus flavefaciens	Bacterium	1	Gokarn *et al.* (1997)	Cellulose, cellubiose and D-glucose
Saccharomyces cerevisiae	Yeast	4	Lupianez *et al.* (1974)	D-glucose
Selenomonas ruminantium	Bacterium	1	Eaton *&* Gabelman (1995)	L-lactic acid
Yarrowia lipolytica	Yeast	3	Yuzbashev *et al.* (2010)	Ethanol, D-glucose and glycerol

A. succinogenes produces succinic, acetic and formic acid in significant quantities and ethanol in minor quantities (Guettler *et al.*, 1996a; McKinlay *et al.*, 2005). The production of small amounts of pyruvic, propionic, as well as lactic acid, has been reported in some cases (Li *et al.*, 2010b). Four wild strains of this bacterium have been investigated: 130Z from the American Type Culture Collection (ATCC No. 55618), as well as three strains from the China General Microbiological Culture Collection Center (CGMCC), namely No. 1593, No. 1716 (NJ113) and No. 2650 (BE-1) (Guettler *et al.*, 1996a; Liu *et al.*, 2008a; Chen *et al.*, 2010a; Li *et al.*, 2010b). No attempt to compare these strains experimentally has been reported to date.

Evident from table 2.3 is the capability of *A. succinogenes* to ferment a wide variety of substrates. Guettler *et al.* (1996a) reports the most comprehensive list of pure substrates that can be fermented by the bacteria. It includes four of the most abundant plant sugars: glucose, fructose, xylose and arabinose. Consequently, a wide variety of raw materials has also been successfully fermented: corn wastes, cane molasses, cotton stalk, sake lees, wheat, and whey (Chen *et al.*, 2010a,b; Du *et al.*, 2007, 2008; Li *et al.*, 2010b; Liu *et al.*, 2008b; Wan *et al.*, 2008).

Many species of the *Pasteurellaceae* family are pathogenic. While pathogenicity has not exclusively been ruled out for *A. succinogenes*, McKinlay *et al.* (2010) state that from their study in genome sequencing, pathogenicity is unlikely. There are also no known reports of disease caused by this bacterial species. Lack of pathogenicity can lead to large cost savings in downstream processing, because the reactor effluent does not have to be sterilised (Shuler *&* Kargi, 2002: 323).

2.2.3.2 *Anaerobiospirillum succiniciproducens*

A. succiniciproducens (ATCC53488), a member of the *Succinivibrionaceae* family, is a capnophilic, mesophilic, gram-negative, spiral rod. It is also an obligate anaerobe and therefore cannot grow in the presence of oxygen. It produces succinic and acetic acid as its main products, and also minor amounts of ethanol and lactic acid (Song *&* Lee, 2006). It has been shown that *A. succiniciproducens* is able to ferment D-glucose, fructose, lactose, maltose and

glycerol to succinate.

2.2.3.3 *Escherichia coli*

E. coli, a member of the *Enterobacteriaceae* family, is one of the most studied bacteria in the literature. It is a gram-negative, facultative anaerobe. *E. coli* grows in the lower intestines of warm-blooded animals and numerous wild strains have been identified over the course of the last few decades. Natural mixed-acid fermentation occurs under anaerobic conditions and lactate, succinate, acetate and ethanol are typically produced. Considerable effort has been put into decoding the genome sequence of *E. coli*. It can therefore be genetically engineered with relative ease, and different metabolic engineering strategies have been followed to enhance succinate production. These studies have mostly aimed at operation under anaerobic conditions for the production of succinate (Vemuri *et al.*, 2002; Lin *et al.*, 2004).

Lin *et al.* (2005b) opted for an aerobic approach. The metabolic pathways of this genetically engineered strain of *E. coli* are illustrated in figure 2.4 (see section 2.2.2). Apart from complete aerobic production, a dual-phase fermentation strategy can solve the general problem of slow growth of organisms in anaerobic fermentation. The bacteria are first grown aerobically and then afterwards succinate is produced anaerobically (Vemuri *et al.*, 2002; Jiang *et al.*, 2010). However, dual-phase fermentation introduces additional production costs. Therefore, significant advantages over anaerobic processes, such as increased concentration and productivity, are needed to justify the process.

2.2.3.4 *Mannheimia succiniciproducens*

M. succiniciproducens (MBEL55E) was isolated from the rumen of a Korean cow and is, like *A. succinogenes*, a member of the *Pasteurellaceae* family. Both are capnophilic, facultative anaerobic, gram-negative rods and their genome sequences are more similar to each other than to any other sequenced genome (McKinlay *et al.*, 2007b). These bacteria are therefore mqetabolically similar: both produce succinate, formate and acetate as main fermentative endproducts when metabolising D-glucose. McKinlay *et al.* (2007b) pointed out that whereas *A. succinogenes* produces ethanol in minor quantities under oxygen deprivation conditions, *M. succiniciproducens* produces lactate.

Also, unlike *A. succinogenes* which only possesses a partial TCA cycle (see figure 2.3 in section 2.2.2), *M. succiniciproducens* has a complete TCA cycle (Beauprez *et al.*, 2010). It has been shown that this bacterium can produce succinate from D-glucose, fructose, lactose, arabitol, mannitol, glycerol and xylose. Its ability to ferment xylose efficiently is a promising prospect, because xylose can be obtained from wood hydrolysate which is very cheap and easily obtained (Samuelov *et al.*, 1999; Lee *et al.*, 2001; Kim *et al.*, 2004).

2.2.3.5 Other microorganisms

C. glutamicum and *B. succiniciproducens* are the other two bacterial species identified that showed the most promise as succinic acid producers. *C. glutamicum* is a widely studied gram-positive, facultative anaerobe from the family *Corynebacteriaceae*. The bacterium has many genetic engineering tools available for modifying its metabolic pathways. Okino *et al.* (2008) successfully engineered a strain that could produce succinic acid with a concentration and yield of 146 $g.\ell^{-1}$ and 0.92 $g.g^{-1}$ respectively. The fermentation was done in fed-batch operating mode in a salt medium with biotin and thiamine. *B. succiniciproducens*, from the family *Pasteurellaceae*, and very similar to *A. succinogenes* and *M. succiniciproducens*, has been studied for glycerol fermentation. It has been reported to produce yields of up to 1.2 gram succinate per gram crude glycerol in batch processes (Scholten & Dägele, 2008). This is close to the theoretical yield of 1.28 $g.g^{-1}$ glycerol. Also, stable continuous fermentation for up to 80 days was obtained with yields as high as 1.02 $g.g^{-1}$ crude glycerol. The optimum productivity was 0.094 $g.\ell^{-1}.h^{-1}$ at a dilution rate of 0.018 h^{-1} (Scholten *et al.*, 2009). This productivity, however, is very low compared to the highest succinic acid productivity reported during continuous fermentation of glycerol: 2.2 $g.\ell^{-1}.h^{-1}$ at 0.14 h^{-1} by *A. succiniciproducens* (Lee *et al.*, 2010).

Fermentative production of succinic acid is usually performed at pH values close to neutral. This process mostly produces succinate, rather than succinic acid, since the pK_{a1} and pK_{a2} values of succinic acid are 4.16 and 5.61 (at 25 °C) respectively (Fumagalli, 2007: 418). Depending on the neutralising agent used in the reactor (e.g. NaOH or KOH), succinate salts are then processed to succinic acid in downstream processes. Yeasts that can grow and

produce succinic acid at pH values of 4 or lower can eliminate or significantly reduce the amounts of alkali needed for pH control. The large amounts of inorganic acid required to process succinate salt to free succinic acid can be excluded as well. Yuzbashev *et al.* (2011) proposed an aerobic process with a genetically modified strain of *Y. lipolytica* to facilitate this process. The main disadvantage of this process is the inability of the yeast to utilise glucose as a substrate. At present, only glycerol can be fermented with the current available strains. The well-known yeast, *S. cerevisiae* has also been targeted for research on production of succinic acid at low pH values (Raab *et al.*, 2010; Raab *&* Lang, 2011).

2.2.4 Reaction studies

In-depth investigations into conditions present during the fermentation of succinic acid have been undertaken. How these conditions affect cell growth and acid production have been debated and opinions differ based on the differences in approach and interpretation. The effects of CO_2 concentration (Xi *et al.*, 2011), redox potential (Park *&* Zeikus, 1999; Li *et al.*, 2010a), osmotic stress (Liu *et al.*, 2008a,b; Fang *et al.*, 2011a,b), product inhibition (Corona-González *et al.*, 2008, 2010; Lin *et al.*, 2008) and substrate inhibition (Lin *et al.*, 2008) have been investigated. These are some of the studies specifically pertaining to *A. succinogenes*.

Under CO_2 limited conditions in the fermentation broth, Guettler *et al.* (1996a) suggested that *A. succinogenes* produces higher concentrations of ethanol. CO_2 concentrations are affected by medium composition (salt concentrations), agitation rate, temperature, pH, partial pressure of CO_2 (g) and the presence of additional carbonates (Xi *et al.*, 2011). Decoupling the intrinsic effect of each of these parameters on cell growth and acid production from the effect of CO_2 concentration, however, is impossible in some cases or would require more in-depth investigations for conclusive results.

Optimal redox potential in the fermentation broth has been shown to provide increased reducing power to shift metabolic flux toward the reverse TCA cycle. This causes a higher ratio of succinate to byproducts. Moreover, it has also been shown to increase substrate consumption rate and cell growth (Park *&*

Zeikus, 1999; Li *et al.*, 2010a).

In a study by Liu *et al.* (2008a), high cation concentrations of Na^+ and Ca^{2+} have been shown to have a negative impact on succinate and cell production, whereas Mg^{2+} ions had almost no effect. Seeing that $MgCO_3$ has successfully been used as a buffer in many succinic acid fermentations, this study explained its effectiveness. NH_4^+ had the most toxic effect of the cations tested and no growth was observed in its presence. In contrast to the cations, the anions that was tested (chloride, phosphate and sulphate) had very little effect on succinate and cell production.

Further inhibition are presented by the substrates used, as well as the products produced by the bacteria. The tolerance of organisms to high concentrations of succinic acid or its salts are crucial to product recovery (Zeikus *et al.*, 1999). Critical concentrations that prevented any cell growth of *A. succinogenes* by D-glucose and products formed were determined by Lin *et al.* (2008). The respective experimentally determined values for D-glucose, acetate, ethanol, formate, pyruvate and succinate were 158, 42, 14, 74, and 104 $g.\ell^{-1}$. Guettler *et al.* (1996a) reported cell growth in medium saturated with magnesium succinate (130 $g.\ell^{-1}$). Utilising a much more basic growth medium, Corona-González *et al.* (2008) found that a combined acid mixture of succinic, acetic and formic acids adding up to 22 $g.\ell^{-1}$ stopped all cell growth. Succinic acid production stopped at 45 $g.\ell^{-1}$. Many models have been proposed to quantify the effects of substrate and product inhibition (Lin *et al.*, 2008; Corona-González *et al.*, 2008, 2010). The problem is that any possible model is simplified to take into account only the specific conditions used in the experiments pertaining to all of the variables discussed in this section. To alleviate the adverse effect of high concentrations of salts, Fang *et al.* (2011a) tested different osmoprotectants which have previously successfully been used to improve fermentation: trehalose, glycine betaine and proline. Proline proved to be the most effective and increased both succinic acid concentration and production rate by approximately 22%.

Although all studies mentioned have been limited to batch operation mode, Corona-González *et al.* (2008) reports that their future work will focus on fed-batch operation. Other natural succinic acid bacteria have also been studied

regarding the discussed parameters, but not to the extent of *A. succinogenes*. In most cases, qualitative influence of the mentioned parameters should be applicable to other bacteria, especially the two species most similar to *A. succinogenes*: *B. succiniciproducens* and *M. succiniciproducens*.

2.2.4.1 Batch and semi-batch production

Batch mode operation has dominated the research in succinic acid fermentative production. This is partly due to the relative ease of batch fermentation (anaerobic bottle fermentation) on laboratory scale. Much useful data and deductions from these data can be obtained before upscaling and investigating

Table 2.4: Summary of significant results obtained in batch and fed-batch (FB) experiments

Authors	Strain	Substrate	C_{SA} (g.ℓ^{-1})	$Y_{SA/S}$ (g.g^{-1})	Time (h)	Mode
			A. succinogenes			
Guettler *et al.* (1996b)	FZ6	D-glucose	63.7	0.94	63	Batch
Guettler *et al.* (1996b)	FZ53	D-glucose	106	0.82	78	Batch
Guettler *et al.* (1998)	130Z	D-glucose	39	0.79	79	Batch
Urbance *et al.* (2003)	130Z	D-glucose	17.4	0.87	96	Batch
Liu *et al.* (2008b)	CGMCC1593	Cane molasses: fructose; D-glucose;	55.2	0.94	48	FB
Du *et al.* (2008)	130Z	Wheat: D-glucose	64.2	0.81	65	Batch
			A. succiniciproducens			
Glassner & Datta (1992)	ATCC53488	D-glucose	43.5	0.91	23	Batch
Nghiem *et al.* (1997)	ATCC53488	D-glucose	32.2	0.99	27	Batch
Lee *et al.* (2001)	ATCC53488	D-glucose; glycerol	29.6	0.97	22	Batch
			C. glutamicum			
Okino *et al.* (2005)	R	D-glucose	23	0.19	6	Batch
Okino *et al.* (2008)	R △ldhA-pCRA717	D-glucose	146	0.92	46	FB
			E. coli			
Vemuri *et al.* (2002)	AFP111-pyc	D-glucose	99.2	1.10	76	FB
Lin *et al.* (2005c)	HL27659k-pepc	D-glucose	58.3	0.62	59	FB
Sánchez (2005)	SBS550MG	D-glucose	40	1.06	95	FB
Isar *et al.* (2006)	W3110	Sucrose	24	1.20	30	Batch
Jantama *et al.* (2008a)	KJ073	D-glucose	79	0.80	96	Batch
Jantama *et al.* (2008a)	KJ060	D-glucose	73	1.10	120	Batch
Jantama *et al.* (2008b)	KJ122	D-glucose	83	0.90	93	Batch
			M. succiniciproducens			
Lee *et al.* (2002)	MBEL55E	D-glucose	14	0.70	8	Batch
Lee *et al.* (2003)	MBEL55E	Whey: lactose	13.5	0.72	11	Batch
Lee *et al.* (2006)	LPK7	D-glucose	52.4	0.76	30	FB

more complicated modes of operation.

The most successful results obtained from various investigations in batch and fed-batch operation modes are listed in table 2.4. A more comprehensive listis given by Beauprez *et al.* (2010). Also, Chimirri *et al.* (2010) summarised results for batch and fed-batch processes where complex mediums from agricultural byproducts were used as feed. No studies on continuous fermentations using complex mediums have been reported to date.

2.2.4.2 Continuous production

Continuous fermentation experiments were performed with various bacteria, including *A. succiniciproducens*, *A. succinogenes*, *B. succiniciproducens*, *E. faecalis*, and *M. succiniciproducens* (see table 2.5). However, some data obtained by Urbance *et al.* (2004) (suspended cell; 150 rpm) and data obtained by Lee *et al.* (2000) and Oh *et al.* (2008) in their fermentation experiments are suspect. This is because cross-referencing the reported graphs or data of yield, productivity and concentrations with calculated substrate conversions yielded inconsistent results. Continuous fermentation studies regarding genetically modified or engineered *E. coli* have been limited to obtaining mutants from long-term adaption in chemostats (Kwon *et al.*, 2011). No continuous studies on fungi have been reported.

Table 2.5: Summary of results in previous continuous fermentation studies

Authors; Substrate(s)	D (h^{-1})	C_{SA} ($g.\ell^{-1}$)	P_{SA} ($g.\ell^{-1}.h^{-1}$)	$Y_{SA/S}$ ($g.g^{-1}$)	C_{SI} ($g.\ell^{-1}$)	γ ($g.g^{-1}$)	X ($g.\ell^{-1}$)
		A. succinogenes					
Urbance *et al.* (2004)	0.2	5.6	1.1	0.29	20	0.97	N/A
S = D-glucose	0.4	5.2	2.1	0.29	20	0.91	N/A
125 rpm	0.6	7.2	4.4	0.64	20	0.56	N/A
	0.8	6.2	5.0	0.70	20	0.44	N/A
	1.0	6.0	6.0	0.73	20	0.41	N/A
	1.2	0	0	-	20	0	N/A
150 rpm*	0.2	3.3	0.7	0.13	20	1.25	N/A
	0.4	2.4	1.0	0.08	20	1.45	N/A
	0.6	2.2	1.3	0.4	20	0.28	N/A
	0.8	1.4	1.1	0.03	20	2.80	N/A
	1.0	7.0	7.0	0.76	20	0.46	N/A
	1.2	0	0	-	20	0	N/A

*Inconsistent data

Table 2.5 (continued)

Authors; Substrate(s)	D (h^{-1})	C_{SA} ($g.\ell^{-1}$)	P_{SA} ($g.\ell^{-1}.h^{-1}$)	$Y_{SA/S}$ ($g.g^{-1}$)	C_{SI} ($g.\ell^{-1}$)	γ ($g.g^{-1}$)	X ($g.\ell^{-1}$)
PCS 125 rpm (biofilm)	0.2	10.1	2.0	0.63	20	0.81	N/A
	0.4	9.8	3.0	0.61	20	0.81	N/A
	0.6	5.9	3.5	0.51	20	0.58	N/A
	0.8	5.5	4.4	0.53	20	0.52	N/A
	1.0	4.5	4.5	0.40	20	0.56	N/A
	1.2	7.3	8.8	0.46	20	0.80	N/A
PCS 150 rpm (biofilm)	0.2	10.4	2.1	0.72	20	0.73	N/A
	0.4	6.2	2.5	0.67	20	0.46	N/A
	0.6	4.8	2.9	0.61	20	0.39	N/A
	0.8	4.6	3.7	0.60	20	0.38	N/A
	1.0	3.5	3.5	0.48	20	0.37	N/A
	1.2	4.6	5.5	0.61	20	0.38	N/A
PP 125 rpm (biofilm)	0.2	8.7	1.7	0.46	20	0.95	N/A
	0.4	8.1	3.3	0.49	20	0.83	N/A
	0.6	5.2	3.1	0.4	20	0.65	N/A
	0.8	4.4	3.5	0.39	20	0.57	N/A
	1.0	6.3	6.3	0.57	20	0.55	N/A
	1.2	4.2	5.0	0.43	20	0.49	N/A
PP 150 rpm (biofilm)	0.2	7.5	1.5	0.40	20	0.94	N/A
	0.4	5.8	2.3	0.35	20	0.84	N/A
	0.6	4.4	2.7	0.27	20	0.81	N/A
	0.8	4.0	3.2	0.31	20	0.65	N/A
	1.0	4.2	4.2	0.58	20	0.36	N/A
	1.2	3.0	3.6	0.21	20	0.70	N/A
Kim *et al.* (2009)	0.2	18.6	3.71	0.56	60	0.55	16.4
S = D-glucose	0.3	15.0	4.50	0.55	60	0.46	13.5
	0.4	15.6	6.25	0.59	60	0.44	13.0
	0.5	13.3	6.63	0.50	60	0.44	13.1
			A. succiniciproducens				
Samuelov *et al.* (1999)	0.085	24.0	2.1	0.72	45	0.80	N/A
S = Whey (lactose)	0.150	19.8	3.0	0.62	45	0.76	N/A
Lee *et al.* (2000)*	0.030	14.0	0.4	0.94	20	1.00	0.9
S = Whey (lactose)	0.044	13.6	0.6	0.92	20	1.00	0.9
	0.060	13.3	0.8	0.94	20	1.00	0.9
	0.071	12.2	0.9	0.93	20	1.00	0.9
	0.086	12.2	1.1	0.92	20	1.00	0.9
	0.100	12.2	1.2	0.93	20	1.00	0.9
	0.113	11.7	1.4	0.92	20	1.00	0.9
	0.127	10.1	1.3	0.83	20	0.95	0.6

*Inconsistent data

Table 2.5 (continued)

Authors; Substrate(s)	D (h^{-1})	C_{SA} ($g.\ell^{-1}$)	P_{SA} ($g.\ell^{-1}.h^{-1}$)	$Y_{SA/S}$ ($g.g^{-1}$)	C_{SI} ($g.\ell^{-1}$)	γ ($g.g^{-1}$)	X ($g.\ell^{-1}$)
	0.140	8.4	1.2	0.81	20	0.85	0.5
Meynial-Salles *et al.*	0.19	16.2	3.4	0.81	20	1.00	15.8
(2008)	0.23	14.8	3.7	0.74	20	1.00	18.8
S = D-glucose	0.32	16.2	5.5	0.81	20	1.00	18.6
	0.49	16.2	8.3	0.83	20	0.98	21.3
	0.56	16.5	9.6	0.83	20	0.99	24.5
	0.81	15.9	13.2	0.82	20	0.97	35.6
	0.93	15.5	14.8	0.81	20	0.96	42.4
Includes monopolar-	0.93	6	4.8	N/R	60	N/R	7.8
electrodialysis	0.93	38	N/R	0.63	60	1.00	N/R
	0.93	20	10.4	N/R	120	N/R	5
	0.93	83	N/R	0.69	120	1.00	N/R
Lee *et al.* (2009)	0.056	15	0.8	0.79	19	1.00	0.96
S = D-glucose	0.1.0	14.7	1.5	0.77	19	1.00	1.06
	0.18	14.7	2.6	0.77	19	1.00	1.30
	0.22	14.1	3.1	0.74	19	1.00	1.35
	0.27	13.7	3.7	0.81	19	0.89	1.45
	0.29	13.1	3.8	0.80	19	0.86	1.42
	0.31	12.2	3.8	0.77	19	0.84	1.49
	0.36	11.7	4.2	0.75	19	0.82	1.47
	0.43	11.3	4.9	0.74	19	0.80	1.47
	0.52	10.2	5.3	0.81	19	0.66	1.49
	0.58	9.7	5.6	0.79	19	0.65	1.45
	0.63	8.3	5.2	0.82	19	0.53	1.27
	0.032	29.6	0.9	0.78	38	0.99	1.38
	0.064	26.5	1.7	0.80	38	0.88	1.6
	0.11	19.5	2.1	0.75	38	0.68	1.32
	0.15	18.5	2.8	0.76	38	0.64	1.29
	0.22	18.1	4.0	0.75	38	0.63	1.16
	0.41	15.9	6.5	0.73	38	0.57	1.10
	0.54	9.2	5.0	0.75	38	0.32	0.79
Lee *et al.* (2010)	0.022	16.1	0.4	1.45	11	1.00	0.48
S = crude glycerol	0.027	14.5	0.4	1.44	11	0.91	0.46
	0.042	14	0.6	1.41	11	0.90	0.42
	0.022	16	0.4	1.42	11.3	1.00	0.49
	0.032	16.1	0.5	1.42	11.3	1.00	0.50
	0.042	16	0.7	1.42	11.3	1.00	0.52
	0.053	15.2	0.8	1.35	11.3	1.00	0.51
	0.064	15.7	1.0	1.39	11.3	1.00	0.55
	0.10	15.3	1.5	1.35	11.3	1.00	0.61
	0.14	15.5	2.2	1.37	11.3	1.00	0.62
	0.19	11.1	2.1	1.34	11.3	0.73	0.46
	0.25	8.0	2.0	1.23	11.3	0.58	0.35

Table 2.5 (continued)

Authors; Substrate(s)	D (h^{-1})	C_{SA} ($g.\ell^{-1}$)	P_{SA} ($g.\ell^{-1}.h^{-1}$)	$Y_{SA/S}$ ($g.g^{-1}$)	C_{SI} ($g.\ell^{-1}$)	γ ($g.g^{-1}$)	X ($g.\ell^{-1}$)
	0.022	16	0.4	1.5	10.7	1.00	0.44
	0.027	14	0.4	1.5	10.7	0.87	0.42
	0.032	12.7	0.4	1.48	10.7	0.75	0.39
	0.042	9.4	0.4	1.46	10.7	0.60	0.27
			M. succiniciproducens				
Lee *et al.* (2003)	0.1	9.3	1.0	0.64	21	1.00	2.3
Whey	0.15	10.3	1.6	0.69	21	1.00	2.4
S = lactose	0.2	10.1	2.6	0.68	21	1.00	2.6
	0.3	9.1	2.8	0.68	21	0.95	2.9
	0.4	8.3	3.4	0.67	21	0.90	2.8
	0.5	7.3	3.7	0.64	21	0.85	2.7
	0.6	6.7	3.9	0.65	21	0.79	2.5
	0.7	5.6	3.8	0.63	21	0.72	2.2
Kim *et al.* (2004)	0.1	14.1	1.4	0.54	18; 7	1.00; 1.00	1.24
S = D-glucose +	0.2	11.0	2.2	0.49	18; 7	0.83; 1.00	1.55
xylose	0.3	9.9	2.9	0.50	18; 7	0.75; 0.80	1.45
	0.4	8.3	3.2	0.50	18; 7	0.62; 0.68	1.45
	0.5	6.3	3.1	0.43	18; 7	0.54; 0.53	1.55
	0.6	3.9	2.4	0.34	18; 7	0.46; 0.49	1.38
	0.7	3.0	2.0	0.35	18; 7	0.37; 0.29	1.34
Wood hydrolysate	0.2	9.7	1.9	0.61	18; 7	0.66; 0.51	0.7
S = D-glucose +	0.3	8.4	2.5	0.56	18; 7	0.65; 0.50	0.5
xylose	0.4	8.0	3.2	0.55	18; 7	0.61; 0.44	0.5
Oh *et al.* (2008)*	0.1	12.89	1.29	0.71	9	1.00	N/A
S = D-glucose	0.15	9.94	1.49	0.55	9	0.89	N/A
	0.2	7.83	1.57	0.43	9	0.69	N/A
	0.3	5.21	1.56	0.29	9	0.85	N/A
	0.1	10.72	1.07	0.29	18	0.91	N/A
	0.2	8.87	1.77	0.25	18	0.68	N/A
	0.3	3.51	1.05	0.10	18	0.46	N/A
Kim *et al.* (2009)	0.1	12.8	1.28	0.64	20	1.00	6.0
S = D-glucose	0.2	11.2	2.24	0.56	20	1.00	12.0
	0.3	9.5	2.85	0.48	20	0.99	19.0
			B. succiniciproducens				
Scholten *et al.* (2009)	0.004	4.01	0.016	0.79	5.10	1.00	0.21
S = crude glycerol	0.008	3.62	0.029	0.71	5.10	1.00	0.22
	0.012	4.30	0.052	0.84	5.10	1.00	0.31

*Inconsistent data

Table 2.5 (continued)

Authors; Substrate(s)	D (h^{-1})	C_{SA} ($g.\ell^{-1}$)	P_{SA} ($g.\ell^{-1}.h^{-1}$)	$Y_{SA/S}$ ($g.g^{-1}$)	C_{SI} ($g.\ell^{-1}$)	γ ($g.g^{-1}$)	X ($g.\ell^{-1}$)
	0.014	4.97	0.070	0.97	5.10	1.00	0.30
	0.018	5.21	0.094	1.02	5.10	1.00	0.25
			E. faecalis				
Wee *et al.* (2002)	0.1	30.0	3.0	1	30	0.97	N/R
S = fumarate	0.2	28.5	5.7	1	30	0.95	N/R
	0.4	27.3	10.9	1	30	0.95	N/R
				1			
	0.1	49.0	4.9	1	50	0.97	N/R
	0.2	46.0	9.2	1	50	0.87	N/R
	0.4	37.3	14.9	1	50	0.70	N/R
				1			
	0.1	72.0	7.2	1	80	0.90	N/R
	0.2	55.0	11.0	1	80	0.69	N/R
	0.4	42.8	17.1	1	80	0.50	N/R

Continuous fermentations by *A. succinogenes* have only been reported by Kim *et al.* (2009) and Urbance *et al.* (2004). Urbance *et al.* (2004) investigated suspended cell and biofilm options, while Kim *et al.* (2009) opted for an approach that involved an external membrane that facilitated cell recycle to increase cell concentration in the reactor. The setup contained a hollow fibre membrane filtration unit and was tested at various dilution and bleeding rates. Continuous fermentation was not possible for periods longer than 50 h due to membrane fouling. Also, contamination presented a major problem. *A. succinogenes* could not compete against lactic acid producing bacteria. Prior to the continuous bioreactor experiments by Urbance *et al.* (2004), an investigation to evaluate plastic composite support (PCS) blends for biofilm fermentation was undertaken. The blends consisted of polypropylene support containing various amounts of agricultural products and other microbial nutrients (Urbance *et al.*, 2003). The main variables of the continuous experiments included dilution rates from 0.2 h^{-1} to 1.2 h^{-1} at two agitation speeds of 125 and 150 rpm. Biofilms were grown on either eight PCS tubes or six PP tubes attached perpendicularly to the agitator shaft. The relevance of comparing agitation rates in suspended cell fermentation, with those in the biofilm experiments is not clear. General trends included increased final succinic acid

concentrations and productivity with increased agitation.

The only other investigation in cell immobilisation for succinic acid production was done by Wee *et al.* (2002). *E. faecalis* was studied in a hollow-fibre bioreactor (HFBR) with fumarate as the substrate. The reactor was operated in tranverse mode where the medium was pumped into the shell side. Medium and products then diffused through the membranes in and out of the fibres, after which product was collected at the end of the reactor on the lumen side. Uncirculated, one-directional flow through the reactor resulted in pH and cell, substrate and product concentration gradients. The longest stable operation (15 days) was observed at a dilution rate of 0.2 h^{-1} and a feed concentration of 50 $g.\ell^{-1}$ fumarate. Succinate concentration, productivity and yield were 46.2 $g.\ell^{-1}$, 9.2 $g.\ell^{-1}.h^{-1}$ and 0.90 $g.g^-1$ respectively.

2.3 Cell immobilisation

Cell immobilisation can provide several benefits in a bioreactor. High cell concentrations in a confined space is one advantage of immobilisation and it also prevents cell washout at high dilution rates. These two factors frequently improve reactor productivity. Moreover, cell reuse eliminates the need for cell recovery and recycle, while genetic stability is also improved in some cases. Some cells require protection from shear forces, which is provided in some forms of immobilisation. Lastly, favourable microenvironmental conditions for cells are created in some cases that result in increased performance. Two categories are distinguished: active and passive immobilisation (Shuler *&* Kargi, 2002; Qureshi *et al.*, 2005: 263).

2.3.1 Active immobilisation

Physical or chemical forces are utilised for active immobilisation. Physical entrapment in porous matrices is the most widely used method for cell immobilisation. Entrapment in microcapsules (encapsulation) and membrane-based reactors are also used. Matrix disintegration with time is a common problem with entrapment. In addition, mass transfer problems may be introduced and cells tend to leach out of the matrix that causes the requirement of centrifugation of reactor effluent in downstream processes.

Adsorption and covalent binding to the surfaces of support materials are the other two forms of active immobilisation. A major advantage of adsorption is direct contact of cells between nutrients and support materials. It is also very simple and inexpensive. However, adsorption are characterised by weak binding forces and require careful consideration in reactor design to keep hydrodynamic shear forces from removing cells from support surfaces. Covalent binding forces are stronger, but is primarily used for enzyme immobilisation. Application to cell immobilisation is not widespread. This is because functional groups on cells and support surfaces are not usually suitable for covalent binding. Also, chemicals to treat surfaces of support materials may adversely affect cells. Similarly to entrapment the previously two mentioned methods frequently exhibit mass transfer limitations (Shuler & Kargi, 2002; Qureshi *et al.*, 2005: 263 – 266).

2.3.2 Passive immobilisation: biofilms

Biofilms are defined as microbial cell layers that reversibly or irreversibly attach on surfaces. The process can also involve the formation of flocs or aggregates (granular biofilms). Cells are embedded in a self-produced exopolysaccharide (EPS) matrix and frequently exhibit different growth and bioactivity compared to suspended cells. Reactor configurations for biofilm applications include packed bed reactors, fluidised bed reactors, airlift reactors and upflow anaerobic sludge blanket reactors, among others. Compared to active immobilisation, reactors with biofilms can be operated for longer periods of time and are very economic.

Support for cells should provide favourable adhesion conditions, have high mechanical resistance and be inexpensive and widely available. Surface charge, hydrophobicity, porosity, roughness, size and density of support materials can affect cell adhesion (Qureshi *et al.*, 2005; Cheng *et al.*, 2010).

Possible disadvantages in biofilm reactors include mass transfer limitations of nutrients entering the cell layers and products exiting these layers. Also, when excess EPS formation occurs reactor space is wasted and may also result in reactor blockages. Possible fluctuations in productivity or product quality is of great concern as well. This is because process reproducibility is of great

importance in industry. Therefore, the complex and dynamic nature of biofilms needs to be studied extensively (Rosche *et al.*, 2009).

2.4 Sterility

Possible contamination is a large problem in bioreactor setups. Furthermore, compared to batch fermentations, it presents an even larger problem in continuous fermentations. Succinic acid fermentation conditions are usually favourable for lactic acid producers such as *Lactobacillus* sp. and *Leuconostoc* sp. that are present in humans and are overall widespread in the environment. These bacteria are common contaminant bacteria in many fermentation processes (Kim *et al.*, 2009). It is therefore important to be aware of different methods to prevent contamination.

Sterility is an absolute concept — a system cannot be partially or almost sterile. Practical application of the term, however, can only extend to the detection limits of available equipment. The absence of any detectable microorganism indicates a possible sterile environment. This kind of sterile environment is aimed for before the introducing a specific microorganism (inoculum) into the fermentation medium. This results in a 'pure culture' and means that only the desired microorganism can be detected (Shuler & Kargi, 2002: 314).

Heat sterilisation is preferred in large-scale fermentations, but heat-sensitive equipment and/or media frequently prevent this method. Heat-up and cool-down of media can, for instance, be damaging to necessary vitamins and proteins or lead to caramelisation of sugars. Filter sterilisation is often the only viable or practical alternative to heat sterilisation in industry. A common problem, however, is that singular defects in the filter membrane present significant risk in its use. Also, viruses and mycoplasma can pass the filter. Radiation is another method sometimes considered. However, ultraviolet radiation can in most cases not penetrate media. X-rays and gamma rays can penetrate media, but cost and safety concerns prevent their use in large-scale fermentations. Chemical sterilisation can only be used when no residues are left after the process that affect the microorganisms used in the fermentation. Also, the chemicals used should not affect the medium. For gas sterilisation,

heating or filtration are employed almost exclusively (Shuler & Kargi, 2002: 315-320).

Chapter 3

Experimental

3.1 Culture strain and growth

A. succinogenes 130Z (DSM No. 22257 or ATCC No. 55618) from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) was used in this study (Guettler *et al.*, 1996a, 1999). Stock cultures of the bacteria were cultivated approximately once a month in tryptone soy broth or TSB (Merck KgaA) at 38 °C for 16 – 20 h in an incubator (with the rotary shaker at 100 rpm). Approximately 15 mℓ of TSB in 25 mℓ screw-capped glass vials was used for this purpose. Culture purity was frequently tested by streaking on tryptone soy agar (Merck KgaA). When lactic acid and/or ethanol were formed in the broth and detected through high-performance liquid chromatography (HPLC) analysis, it also indicated contamination.

3.2 Media

A medium based on a formulation by Urbance *et al.* (2003) was used in all experiments (see table 3.1). The hemin, xylose, vitamins and fatty acids were excluded. It was aimed to have all other nutrients in excess compared to amounts of D-glucose to be added to the medium. In addition to nutritional components, 0.02% $Na_2S \cdot H_2O$ was added to ensure strict anoxic conditions (Van der Werf *et al.*, 1997). The D-glucose solution was separately sterilised in an autoclave and aseptically added to the medium. This was necessary to prevent caramelisation of the sugar during autoclaving.

Table 3.1: Succinic acid medium components

Compound	Final C_i (g.ℓ^{-1})	Source
Corn steep liquor	10.00	Sigma-Aldrich
Yeast extract	6.00	Merck KgaA
Antifoam A	0.05	Sigma-Aldrich
$CaCl_2 \cdot H_2O$	0.20	Merck KgaA
K_2HPO_4	3.00	Merck KgaA
$MgCl_2 \cdot 6\,H_2O$	0.20	Merck KgaA
NaCl	1.00	Merck KgaA
Na_2HPO_4	0.31	Merck KgaA
NaH_2PO_4	1.60	Merck KgaA
NaOAc	1.36	Merck KgaA
$Na_2S \cdot 9\,H_{20}$	0.16	Acros Organics
D-glucose	20 or 40	Merck KgaA

CO_2 (g) was supplied to the fermentation broth for the necessary CO_2 fixation required in succinic acid bioproduction. In one of the experiments 10 g.ℓ^{-1} magnesite (provided by Chamotte Holdings) was added to the medium in addition to the CO_2 (g). The magnesite contained approximately 93% $MgCO_3$.

3.3 Apparatus

The fermentation setup is illustrated in figure 3.1 and the equipment is detailed in table 3.2.

Figure 3.2 is a basic rendering of the reactor represented in figure 3.1, while the dimensions of the reactor are shown in figure 3.3. It consisted of the following components: an aluminium top and bottom section; a glass tube; two aluminium distributor plates; four 1/4" stainless steel (SS) tubes; an aluminium thermowell; a magnetic stirrer; four O-rings; and two threaded shafts. Fermentation broth entered the reactor from the recycle line through a SS tube in the bottom section. The bottom section, situated on the hotplate and stirrer, also contained a magnetic stirrer to promote heat transfer in the fermentation broth. The two distributor plates (see figure 3.4) were inserted for even broth and gas distribution. 1.0 mm holes with a 4 mm triangular pitch arrange-

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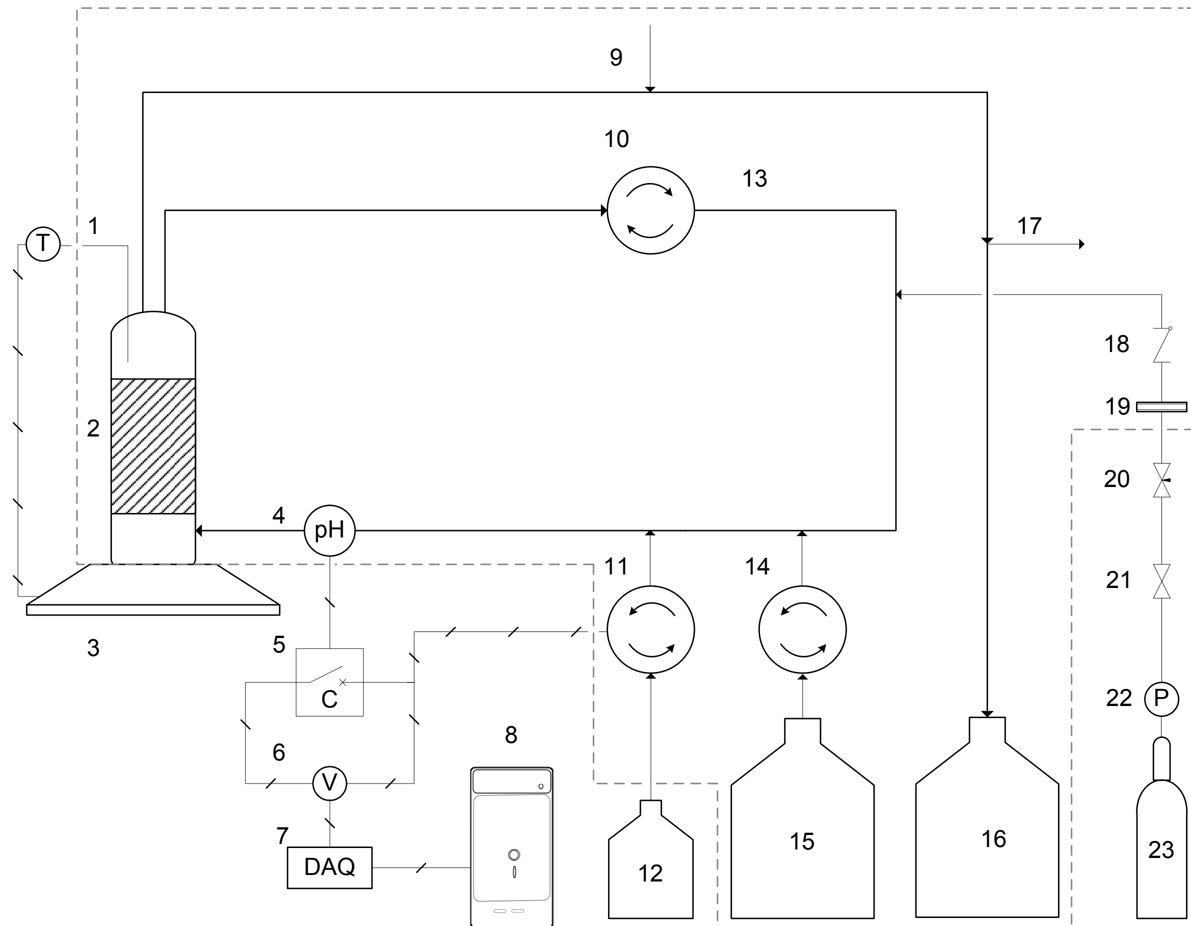

Figure 3.1: The continuous fermentation setup. The dotted lines indicate the part of the setup to be sterilised before each fermentation.

Table 3.2: Equipment used in the experimental setup (see figure 3.1).

No.	Equipment description	Details
1	Thermocouple	Integrated in hotplate (see no. 3)
2	Bioreactor	Custom made
3	Hotplate and stirrer	Heidolph Instruments - MR Hei-Standard
4	pH probe	Autoclavable Sentek Sterprobe
5	pH controller	Eutech Instruments - αlpha pH560
6	Voltage meter	Integrated in DAQ (see No. 7)
7	DAQ	National Instruments - NI USB-6008
8	Personal computer	-
9	Inoculation line	-
10	Peristaltic pump	Watson-Marlow 323
11	Peristaltic pump	Watson-Marlow 520S
12	Base reservoir	
13	Recycle line	-
14	Peristaltic pump	Watson-Marlow 120U
15	Medium reservoir	-
16	Product reservoir	-
17	Sample line	-
18	Check valve	Ozogen
19	0.22 μm filter	Sartorius - Midisart 2000
20	Needle valve	Swagelok - S Series with Vernier handle
21	Plug valve	Swagelok - P4T Series
22	Pressure regulator	African Oxygen - Afrox Scientific W01940
23	CO_2 canister	African Oxygen - Technical grade 99.95%

ment were drilled into the plates. The top section contained SS tubes for the connection of the recycle and product lines and for a thin aluminium sheath that acted as the thermowell. Finally, two O-rings sealed the space between the glass tube and the top and bottom sections of the reactor, while two more O-rings kept the two distributor plates in place.

The total working volume of the fermentation was 156 mℓ and consisted of the reactor and the recycle line. The volume of the reactor (128 mℓ) made up approximately 82% of the fermentation volume, while the tubing of the recycle line made up the rest.

Cell attachment (biofilm formation) of *A. succinogenes* was tested by adding

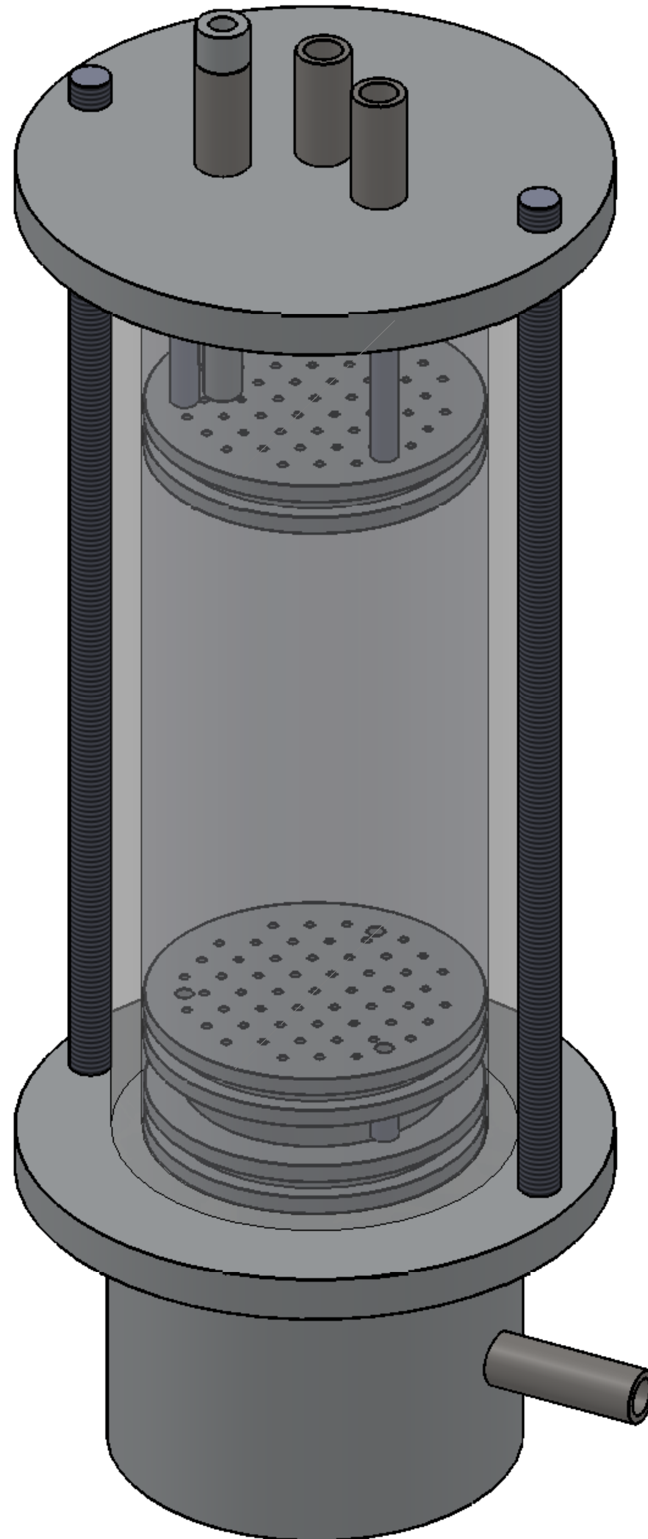

Figure 3.2: A three-dimensional rendering of the reactor used in the experimental setup

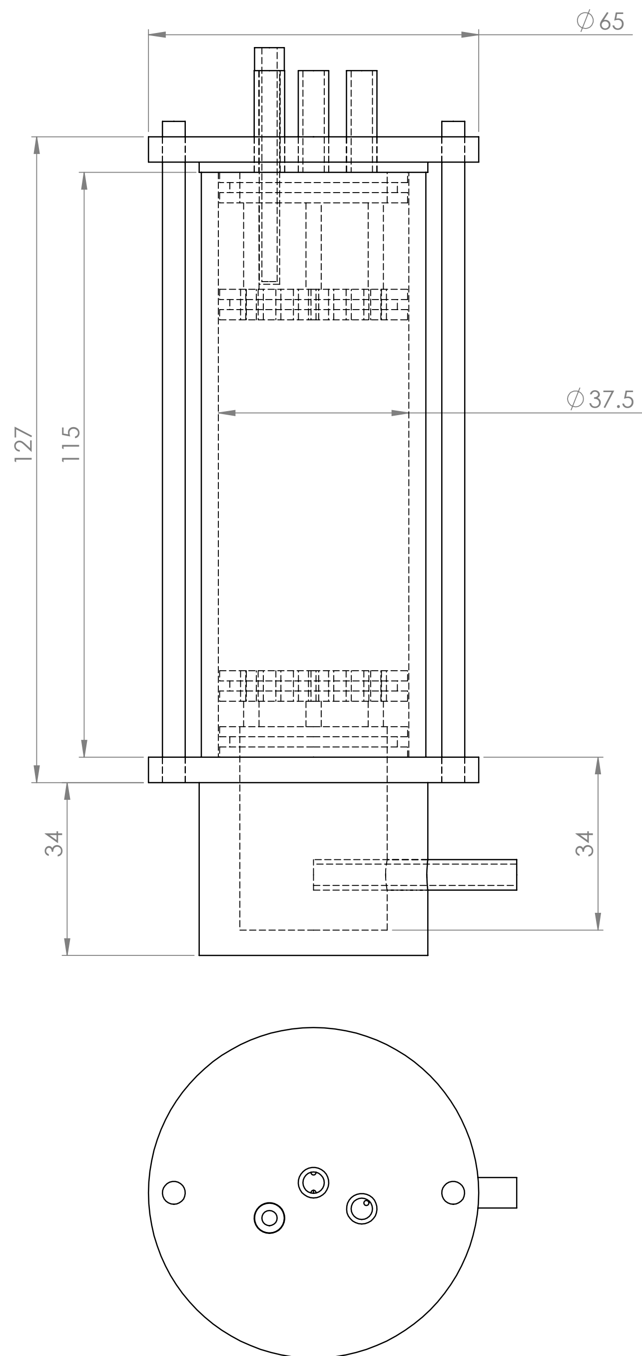

Figure 3.3: Side and top view of the reactor

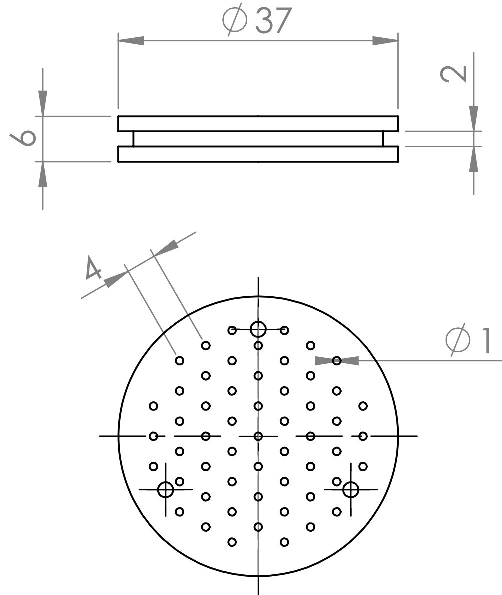

Figure 3.4: Side and top view of the two distributor plates in the reactor

expanded perlite (Genulite^{TM} Groperl from Infigro) between the two distributor plates at a height of 56 ± 1 mm (packing volume ≃ 62 mℓ or 40% of the working volume). Perlite is an amorphous, volcanic glass. When it is heated above approximately 900 °C, it greatly expands and becomes a porous solid that can hold and release liquid easily. The particles used for support had a diameter of 2 – 4 mm and a bulk density of 90 – 145 kg.m^{-3} (Infigro, 2011).

Temperature and pH in the fermentation broth were controlled at 38 ± 1 °C and 6.80 ± 0.05 respectively. The temperature was controlled by the hot plate and a thermocouple that was inserted into the thermowell at the top section of the reactor. pH was measured by inserting and sealing the bottom of the electrode in a cylinder casing in the recycle line. The controller, to which the electrode was connected, controlled the pH by intermittently activating the pump that fed 10 M KOH solution to the recycle line (10 M NaOH solution was used in the first fermentation).

3.4 Fermentation

In order to ensure a sterile environment after autoclaving for the introduction of pure cultures of *A. succinogenes*, a 'closed system' for the whole fermentation

setup was necessary. The 'working volume' of the reactor setup was completely sealed, except for filters fitted to the gas feedline and the product and medium reservoirs. This prevented the formation of vacuums or pressure build-up — the filters allowed air, steam and excess CO_2 (g) to enter and exit the containers during autoclaving and fermentations, while also preventing the introduction of contaminants.

The 'closed system' had to be opened to connect the base feedline to the reservoir, to introduce inoculum and to take samples. It was assumed that the KOH solution was sufficient to kill any possible contaminants on and inside the base feedline. The inoculum (prepared similarly to stock cultures: see section 3.2) was first transferred to a sterile syringe with an attached needle. The needle was heated with a flame and inserted into a silicone stopper that was connected to a T-piece in the product line. The gasflow was then briefly stopped and the inoculum was allowed to flow into the reactor. Sampling was done by opening the product line for brief periods with gas and liquid flowing out.

Prior to each experiment, the components in the system indicated by dotted lines in figure 3.1 were connected and sterilised together in an autoclave for 40 min at 121 °C. After setting up the sterilised equipment, the CO_2 (g) feedline was connected to the 0.22 μm filter and the flowrate of the gas was set to 0.05 ± 0.02 vvm (± 5 – 10 $m\ell.min^{-1}$). 0.05 vvm CO_2 (g) provided 0.016 $mol.h^{-1}$ CO_2 to the fermentation broth. Marprene tubing sections in the recycle line, as well as the medium and base feedlines, were mounted into the peristaltic pump heads. The reactor was then filled with the medium and recycling of the contents was started at 100 $m\ell.min^{-1}$. This resulted in an upward liquid velocity of 90 $mm.min^{-1}$ in the reactor. After connecting the base reservoir to its feedline, the pH probe to the controller, and inserting the thermocouple into the thermowell, the pH and temperature were controlled for the duration of each fermentation. Approximately 8 $m\ell$ (5% of the reactor volume) of inoculum was introduced into the reactor system after the pH and temperature remained stable at their controlled values. During the fermentations, dilution rates were varied between 0.04 and 0.76 h^{-1}. Deviations at very low liquid flowrates ($D < 0.10$ h^{-1}) resulted in deviations of up to 0.03 h^{-1} in the dilution rate. At higher flowrates lower maximum deviations

of 0.01 h^{-1} were observed. Fermentation broth from the reactor accumulated in the product reservoir through overflow.

3.5 Analysis

Samples for HPLC analysis from stock cultures, inoculation cultures and fermentation product from the reactor were first centrifuged at 12 100 g for 5 min. Afterwards the supernatant was filtered through a 0.2 µm filter attached to a syringe. HPLC analysis was then used to determine organic acid, D-glucose and ethanol concentrations. An Agilent 1260 Infinity HPLC (Agilent Technologies, USA), equipped with a 300 x 7.8 mm Aminex HPX-87H column (Bio-Rad Laboratories, USA) and a refractive index detector (RID) was used for this purpose. The mobile phase used was 0.3 $m\ell.\ell^{-1}$ (5.6 mM) sulphuric acid at a flowrate of 0.6 $m\ell.min^{-1}$ with a column temperature of 60 °C.

Dry cell weight (DCW) was determined from three 1.5 mℓ samples. The samples were centrifuged three times and cell pellets were washed with distilled water after each cycle. The sample containers were dried in an oven for more than 24 h at 90 °C. DCW measurements were only used as a rough indication of cell mass concentration. This is because the accuracy of DCW determined in broth containing noncellular solids (e.g. insoluble solids from corn steep liquor) are frequently inaccurate and inconsistent (Shuler & Kargi, 2002: 158).

Chapter 4

Results and discussion

The most important results during the course of successful fermentations are given in section 4.1. General observations, the reasons for changes between fermentations and the difficulties experienced are discussed.

The productivity and byproduct formation are discussed in more detail in sections 4.2 and 4.3. These results are then compared to previous investigations regarding continuous succinic acid fermentation in section 4.4. Finally, sterility is briefly discussed in section 4.5.

4.1 Main results

Fermentations will be referred to by their respective numbers of one to seven, as indicated in table 4.1. Differences between these fermentations are also

Table 4.1: Some details of the respective fermentations

Run no.	$C_{\text{D-glucose}}$	SC or Biofilm	Extra carbon source
1	40 $g.\ell^{-1}$	No packing	CO_2 (g)
2	40 $g.\ell^{-1}$	No packing	CO_2 (g)
3	20 $g.\ell^{-1}$	No packing	CO_2 (g)
4	20 $g.\ell^{-1}$	No packing	CO_2 (g)
5	20 $g.\ell^{-1}$	No packing	CO_2 (g); $MgCO_3$ (s)
6	20 $g.\ell^{-1}$	Packed bed	CO_2 (g)
7	20 $g.\ell^{-1}$	Packed bed	CO_2 (g)

given in the table.

Inlet concentrations of compounds that were obtained by analysing the medium in the HPLC are indicated in table 4.2. These concentrations were subtracted from the product concentrations obtained in the samples.

Table 4.2: Acid and ethanol concentrations in the medium

Compound	C_i (g.ℓ^{-1})
Acetic acid	1.0 ± 0.1
Ethanol	0.02 ± 0.01
Formic acid	0
Lactic acid	0.6 ± 0.2
Propionic acid	0.19 ± 0.04
Succinic acid	0

Continuous succinic acid fermentations by *A. succinogenes* were successful for periods between 5 and 17 days (see figure 4.1). When initial succinic acid production was observed through HPLC analysis, the fermentation continued

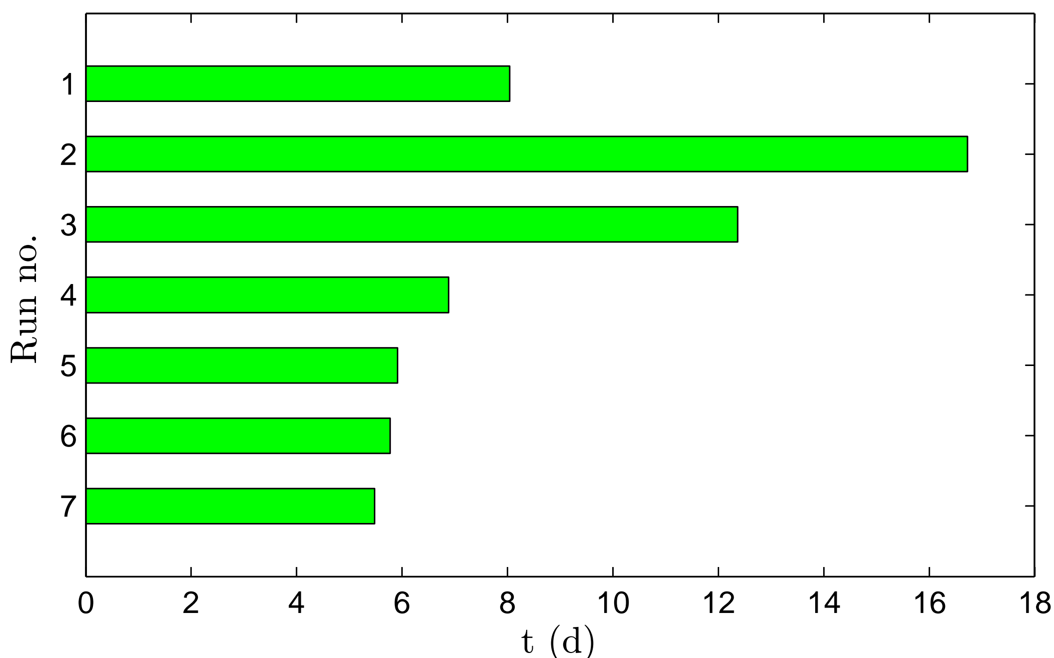

Figure 4.1: Relative length of fermentations carried out that successfully produced succinic acid

without infection, provided that medium sterilised along with the reactor was available (see section 3.4). With the addition of more medium to the original container, contaminants were introduced into the reactor. The time span of each of the fermentations was therefore limited by the size of the initial medium container and the dilution rates employed. The main results that were obtained during each of the seven fermentations are indicated in figures 4.2 to 4.4. This includes the D-glucose and succinic acid concentrations, the productivity, as well as the yield (see equation 4.1) at the specific dilution rates at which the reactor was operated. Figures A.1 to A.3 in appendix A show the concentrations of acetic and formic acid that were produced during the fermentations.

$$Y_{SA/S} = \frac{C_{SA}}{C_{SI} - C_S} \tag{4.1}$$

Successive fermentations were adapted based on results obtained from the completed fermentations. In the first two fermentations, maximum product concentrations at low dilution rates were pursued. At a dilution rate of only 0.14 h^{-1} the highest D-glucose conversions obtained in the two fermentations were 48% and 57%. Therefore, the D-glucose feed concentration was lowered to 20 $g.\ell^{-1}$, which was the concentration used in the continuous fermentations by Urbance *et al.* (2004). 100% conversion was obtained in the third fermentation at a dilution rate of 0.21 h^{-1} and therefore higher dilution rates could be tested in subsequent fermentations. Intentional biofilm formation was tested in the last two fermentations, with only two changes in dilution rate in the last fermentation.

It is widely assumed that steady state in an ideal suspended cell bioreactor or chemostat can be achieved within approximately five retention times. Kinetic interpretation and modelling require data obtained at steady state points at different dilution rates. Cell concentration measurements that are both accurate and representative of the contents of the bioreactor are required, in addition to concentrations of substrates and products. However, biofilm growth was evident from visual observation in all fermentations carried out in this investigation. Initial biomass accumulation occurred in the calmer areas of the reactor where shear forces were low, as can be seen in figure 4.5. Therefore, in addition to the suspended cells, an unknown amount of immobilised cells were

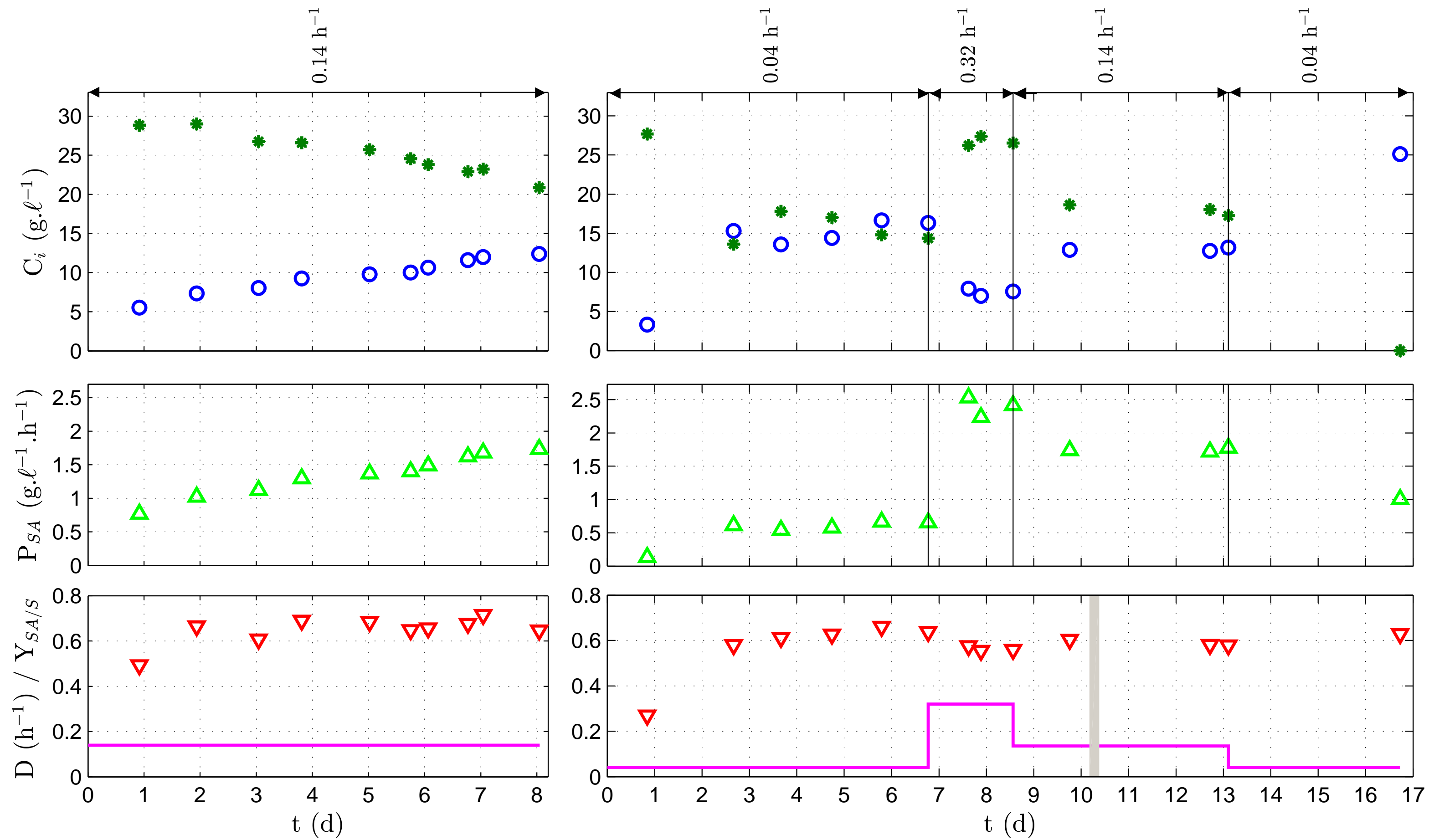

Figure 4.2: Main results in fermentations nos 1 and 2; $C_{SI} = 40$ g.ℓ^{-1}. Specific dilution rates are given above the top graphs. D-glucose ($\ast$), succinic acid ($\bigcirc$), productivity ($\triangle$), yield ($\triangledown$), dilution rate (-), blockage(s) indicated by grey area(s).

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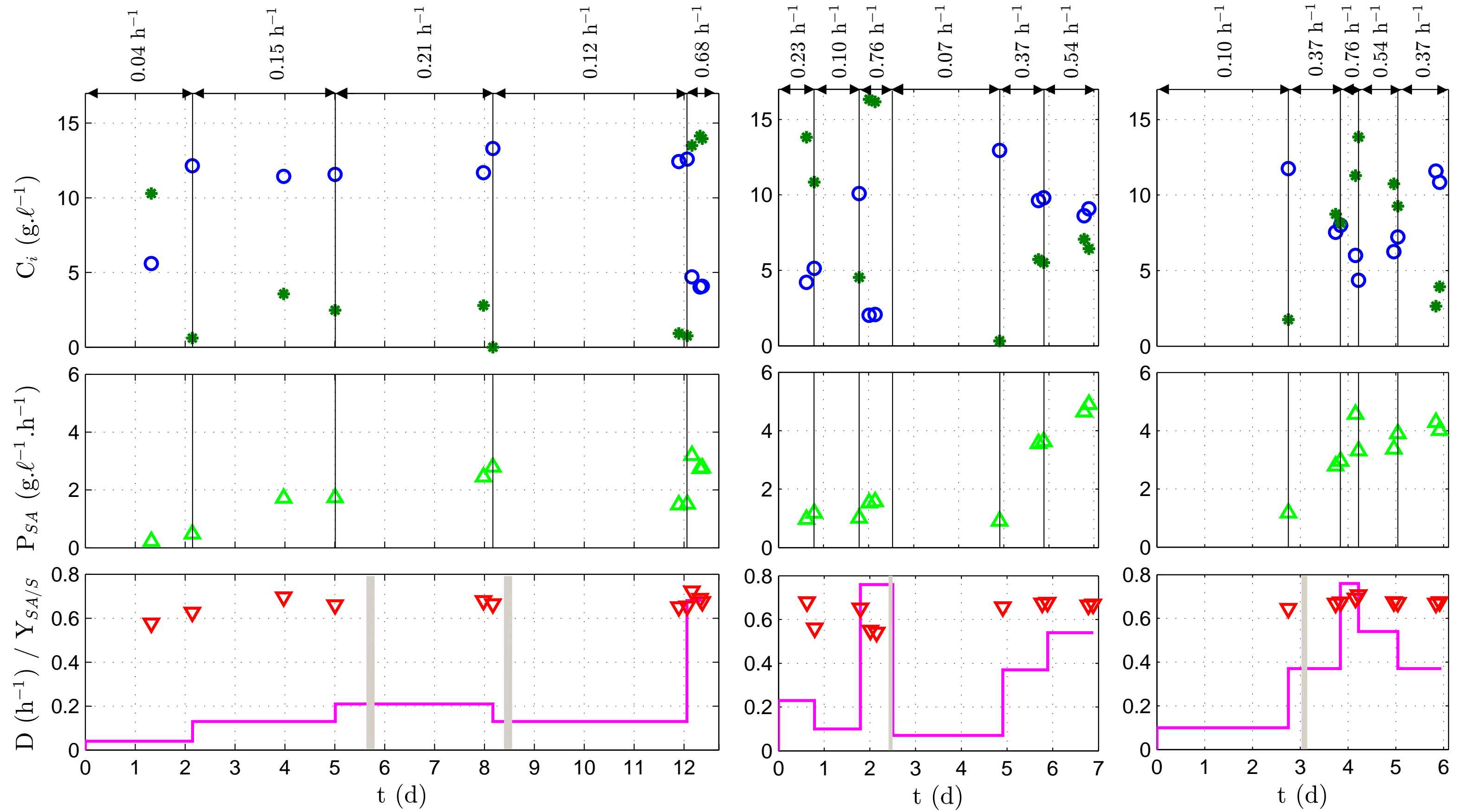

Figure 4.3: Main results in fermentations nos 3, 4 and 5; $C_{SI} = 20$ g.ℓ^{-1}. Specific dilution rates are given above the top graphs. D-glucose (∗), succinic acid (○), productivity (△), yield (▽), dilution rate (-), blockage(s) indicated by grey area(s).

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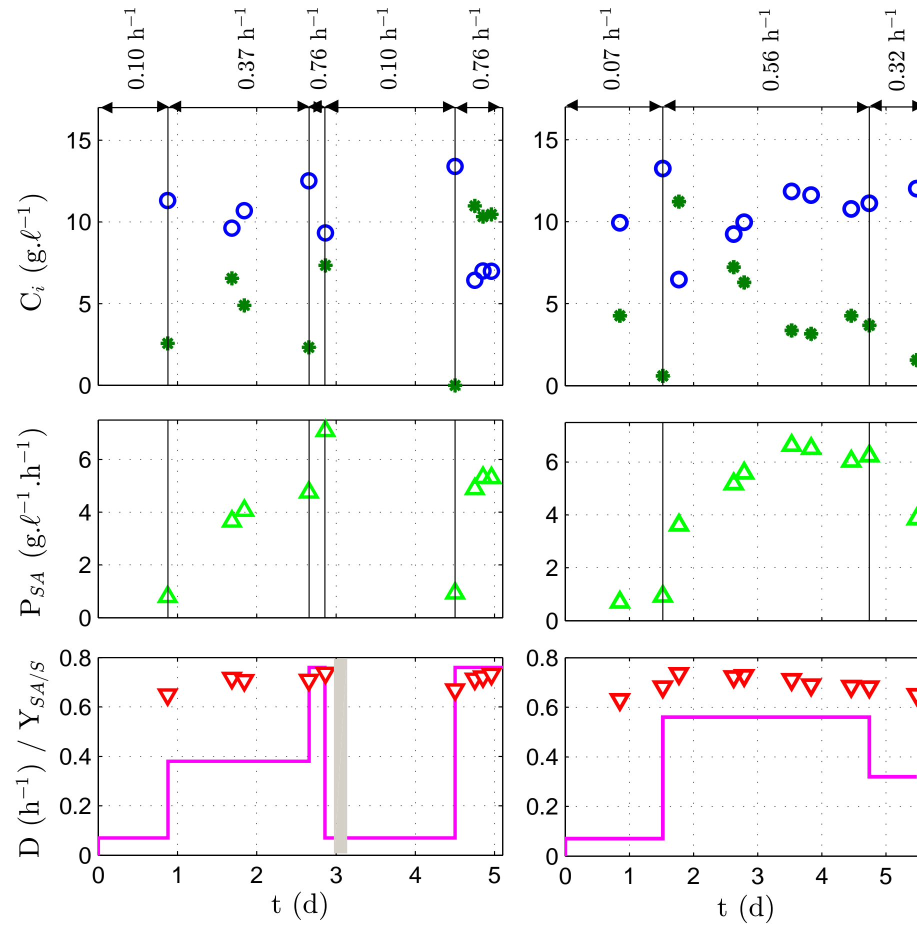

Figure 4.4: Main results in fermentations nos 6 and 7; $C_{SI} = 20$ g.ℓ^{-1}. Specific dilution rates are given above the top graphs. D-glucose ($\ast$), succinic acid ($\bigcirc$), productivity ($\triangle$), yield ($\triangledown$, dilution rate (-), blockage(s) indicated by grey area(s)).

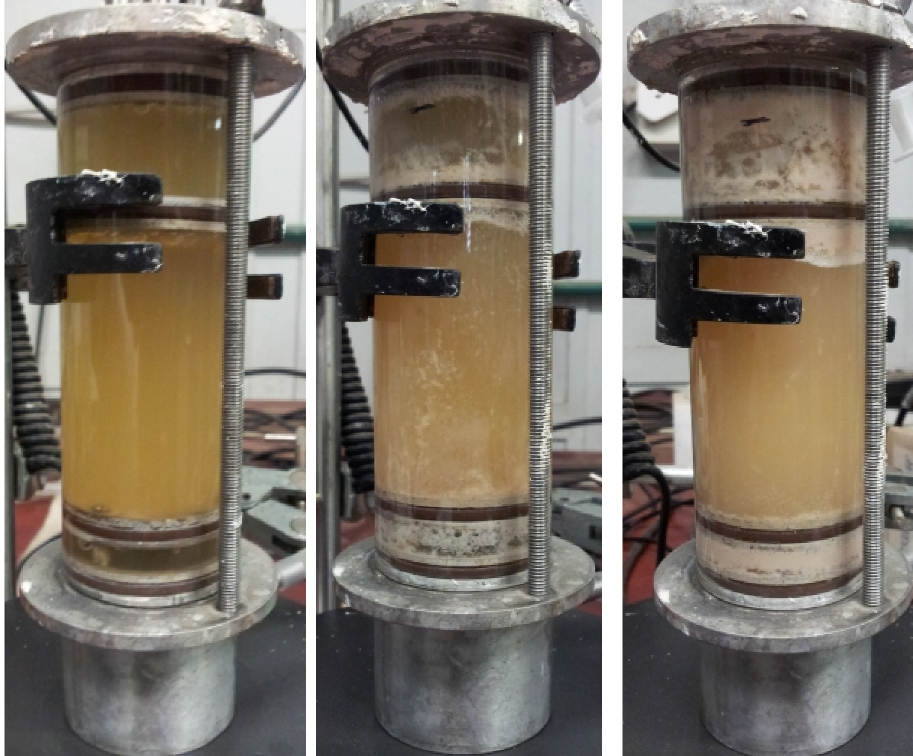

Figure 4.5: The reactor in the second fermentation at the start, after six days and after twelve days

active in the reactor. Furthermore, at later stages in the fermentations biofilms covered all areas inside the reactor, except for the recycle line. Apart from the difficulty of measuring DCW in the broth containing insoluble solids (from corn steep liquor and impurities in the magnesite), the total concentration of cells in the reactor could not be measured. Therefore no plausible kinetic analysis could be performed. Cell concentrations determined from DCW for fermentations nos 2 – 5 are shown in appendix B.

Gradual biofilm growth during the course of fermentations was accompanied by increasing succinic acid production and D-glucose conversion. For the full duration of the first fermentation a constant dilution rate of 0.14 h^{-1} was maintained (see figure 4.2). After 27 retention times steady state was still not achieved. The productivity increased more than 70% over the last 20 retention times. In the subsequent fermentation, a very low dilution rate of 0.04 h^{-1} was initially employed (see figure 4.2). The reactor seemed to have reached steady state after 6.5 retention times. Afterwards, the dilution rates were changed

twice: to 0.32 and then to 0.14 h^{-1}. The reactor was kept at these two dilution rates for 14 and 15 retention times respectively. The results indicated that steady state could have been reached. However, when the dilution rate was decreased again to 0.04 h^{-1}, the D-glucose was depleted in the reactor, which was not observed initially at this low dilution rate. This also pointed towards increased cell concentration in the later stages of the fermentation.

For several time periods in the third and fourth fermentations, possible steady state occurrences were observed (see figures 4.3). It was attempted to confirm a possible steady state period at a dilution rate of 0.37 h^{-1} in the fifth fermentation, by returning to the same dilution rate at the end of the fermentation. However, it was evident from the large difference in productivity at the end of the two time periods that the reactor was not even close to steady state. The productivity increased from 3.0 $g.\ell^{-1}.h^{-1}$ at the end of the first period to 4.0 $g.\ell^{-1}.h^{-1}$ at the end of the second period. Upsets in the reactor in the form of dilution rate changes caused changes in biofilm formation and prevented the reactor from returning to the same conditions when the dilution rate was changed back to a previous value. Steady state periods in the previous fermentations are therefore called into question.

More area for attachment was provided in the sixth and seventh fermentation. The cells attached easily to the packing and therefore more cells were retained in biofilm layers on the perlite support. This is illustrated in figure 4.6. In the sixth fermentation (see figure 4.4), the reactor reached the highest productivity of all fermentations: 7.1 $g.\ell^{-1}.h^{-1}$. In the final fermentation, the reactor was operated at a dilution rate of 0.56 h^{-1} for the 43 retention times (see figure 4.4), after which a productivity of 6.6 $g.\ell^{-1}.h^{-1}$ was achieved. It is estimated that steady state was reached at approximately 27 retention times. The high productivities attained in the reactor in the last two fermentations undoubtedly confirm a great increase in cell concentration in the reactor, as can be observed in figure 4.6.

Accumulation of solids in the feed line caused instances where blockages occurred that prevented flow into the reactor. Unless medium was continuously fed to the reactor, it emptied due to gas entrainment of fermentation broth through the product line. As the reactor emptied, the recycle line also emp-

Figure 4.6: The reactor with expanded perlite packing on the sixth day of the seventh fermentation

tied, which caused incomplete mixing in the reactor. Consequently, pH and temperature gradients severely impacted cell growth and succinic acid production. Different methods to prevent further blockages in the feed line were tested, but subsequent fermentations continued to show this problem. Filtering the medium, as was done before the second fermentation, removed most of the solids. However, after sterilising the medium, additional solids formed which also caused blockages (see figure 4.2). Further blockages in the medium feed line also occurred twice in the third fermentation and once in each of the fourth, fifth and sixth fermentations.

The effect of a blockage is best illustrated in the sixth fermentation where significantly more biofilm was present compared to the fermentations in the unpacked reactor (see figure 4.4). The reactor reached a productivity of 6.6 $g.\ell^{-1}.h^{-1}$ at a dilution rate of 0.76 h^{-1}. After the blockage and returning the dilution rate to 0.76 h^{-1} at a later stage, the reactor performance could not recover to its original state. This state indicated possible death

of cell layers in the reactor that could not be sufficiently replaced within the fermentation time span.

4.2 Productivity

Data regarding the most important points in the fermentations are shown in figure 4.7. These include points where the highest productivities were achieved, possible steady state periods, and points at full D-glucose conversions. In addition to productivity, D-glucose consumption rates are indicated (+) in the figure. Other details regarding the data points can also be deduced from the graph: conversion is the D-glucose consumption rate divided by the maximum consumption rate above each point on the diagonal line; succinic acid concentration is the productivity divided by the corresponding dilution rate; and

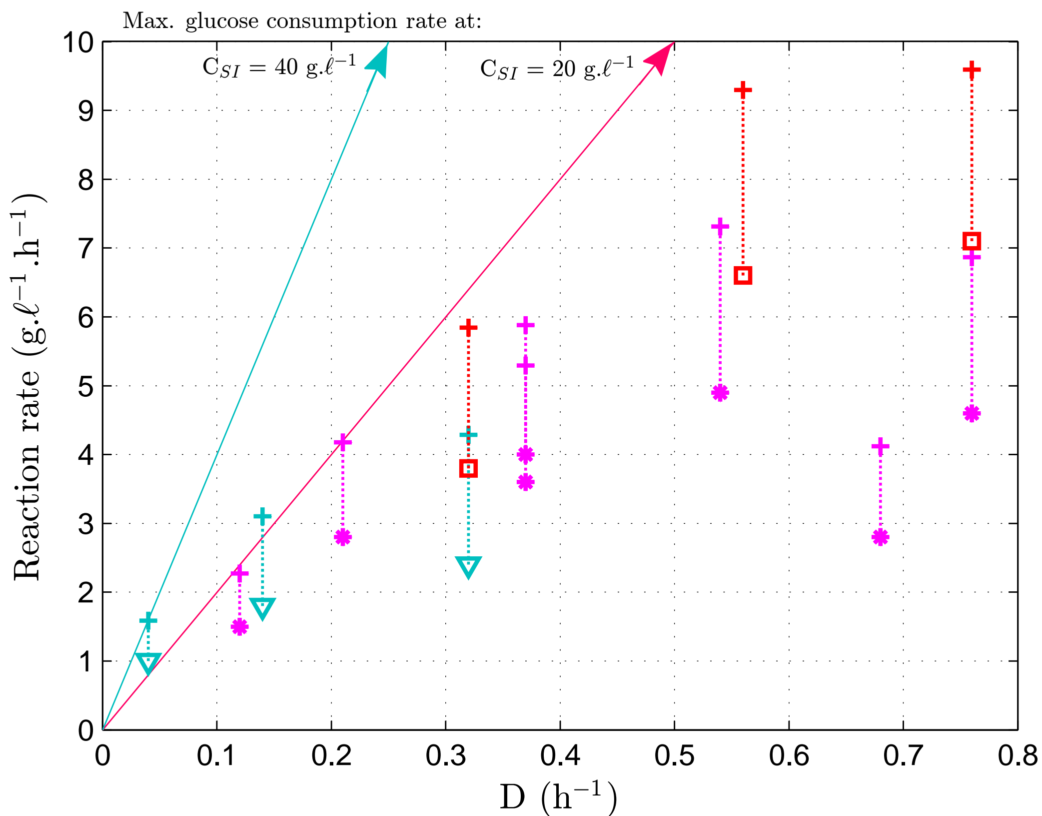

Figure 4.7: Productivities and D-glucose consumption rates (+). C_{SI} = 40 g.ℓ^{-1} (▽); C_{SI} = 20 g.ℓ^{-1} and reactor with packing (□) and without packing (∗).

yield is the productivity divided by the D-glucose consumption rate. High yields are therefore represented in the figures by short distances between the productivities and D-glucose consumption rates. The ideal situation is to have a higher productivity than the D-glucose consumption rate (yield > 1). As yet, this has only been achieved by genetically engineered *E. coli* in batch fermentations (Hong & Lee, 2002; Jantama *et al.*, 2008a).

At a feed concentration of 20 g.ℓ^{-1}, fermentations without packing yielded productivities as high as 4.6 and 4.9 g.ℓ^{-1}.h^{-1} at dilution rates of 0.76 and 0.56 h^{-1}. Productivities at these dilution rates were improved in the fermentations with packing by 54% and 35% (see fermentations nos 6 and 7 in table 4.3) . It is important to note that these increases in productivity were attained where the packing only filled 40% of the reactor volume and further increases should be possible. Specific values regarding the details of each data point in figure 4.7 are summarised in table 4.3.

Table 4.3: Details at points in the fermentations where the highest productivities were achieved, as well as productivities at possible steady state periods

No.	D (h^{-1})	C_{SA} (g.ℓ^{-1})	P_{SA} (g.ℓ^{-1}.h^{-1})	$Y_{SA/S}$ (g.g^{-1})	C_{SI} (g.ℓ^{-1})	γ
1	0.14	12.4	1.7	0.65	40	0.48
2	0.32	7.5	2.4	0.56	40	0.34
	0.14	13.2	1.8	0.58	40	0.57
	0.04	25.1	1.0	0.63	40	1.00
3	0.21	13.3	2.8	0.67	20	1.00
	0.12	12.6	1.5	0.66	20	0.96
	0.68	4.1	2.8	0.68	20	0.30
4	0.37	9.8	3.6	0.68	20	0.73
	0.54	8.1	4.9	0.67	20	0.68
5	0.76	6.0	4.6	0.67	20	0.44
	0.37	10.9	4.0	0.68	20	0.81
6	0.76	9.3	7.1	0.74	20	0.63
7	0.56	11.8	6.6	0.71	20	0.83

4.3 Yield and byproduct formation

Succinic acid yield mostly remained within the range of approximately 0.67 ± 0.05 $g.g^{-1}$ in all the fermentations. This is indicated in figure 4.8. Exceptions include the second 40 $g.\ell^{-1}$ fermentation, as well as many samples taken in the initial growth phase of some of the fermentations. The large deviations of the yields of the second fermentation can be explained by the calculated carbon recovery illustrated in figure 4.9. The cell concentration, and therefore the carbon in the cells in the product stream, was not taken into account. Also, it is assumed that the activity of both the formate dehydrogenase and pyruvate dehydrogenase enzymes is negligible. Therefore, no CO_2 is produced in the fermentation. Carbon recovery was calculated as follows:

$$\phi = \frac{6\,C_S MM_S + 3\,C_{SA} MM_{SA} + 2\,C_{AA} MM_{AA} + C_{FA} MM_{FA} + 2\,C_{EtOH} MM_{EtOH}}{6\,C_{SI} MM_S} \tag{4.2}$$

Consistently lower fractions of carbon recovery calculated from the second fermentation indicate an error in the amount of D-glucose initially added to the medium container.

Xi *et al.* (2011) found that adding $MgCO_3$ (s) to the medium, in addition to sparging CO_2 through the fermentation broth, increased the succinic acid yield. These fermentations, which were done in batch mode, had 'productivities' of approximately 1.2 $g.\ell^{-1}.h^{-1}$ and therefore low CO_2 fixation rates of 0.45 $g.\ell^{-1}.h^{-1}$. In the fifth fermentation, 10 $g.\ell^{-1}$ magnesite were added to the medium ($\pm$ 0.11 $mol.\ell^{-1}$ $MgCO_3$). A slurry was thus created, since the solubility of $MgCO_3$ is very low ($\pm$ 0.1 $g.\ell^{-1}$). From figure 4.8 ($\triangle$) it can be seen that although the yield varied in the first two days of the fermentation, it stabilised above 0.65 afterwards. This is approximately the same yield that was attained in the other fermentations, including the last two fermentations that had the highest CO_2 fixation rates. Furthermore, Guettler *et al.* (1996a) concluded that increased ethanol production was seen under CO_2 limited conditions. Therefore, ethanol production can potentially be used as an indicator of sufficient CO_2 availability, as well as incomplete mixing in the reactor. The maximum ethanol concentration in the fermentations was only 0.13 $g.\ell^{-1}$. 0.05 vvm CO_2 (g), which was used in fermentations in this study, provides a

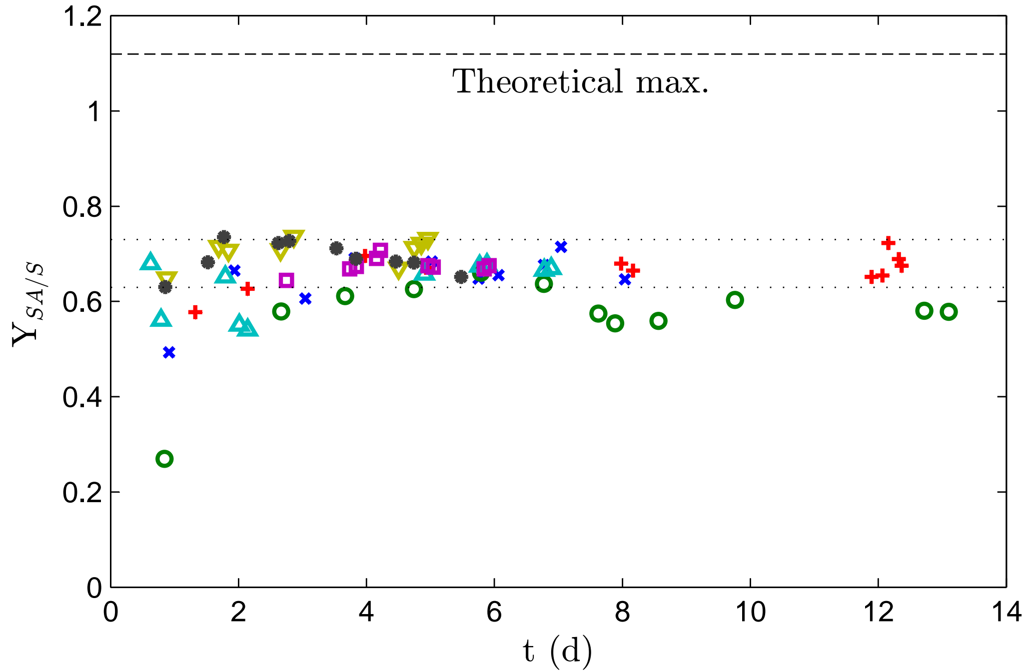

Figure 4.8: The yields of all seven fermentations in this study (see table 4.1 for details): 1 (×); 2 (○); 3 (+) ; 4 (△); 5 (□); 6 (▽); and 7 (✱)

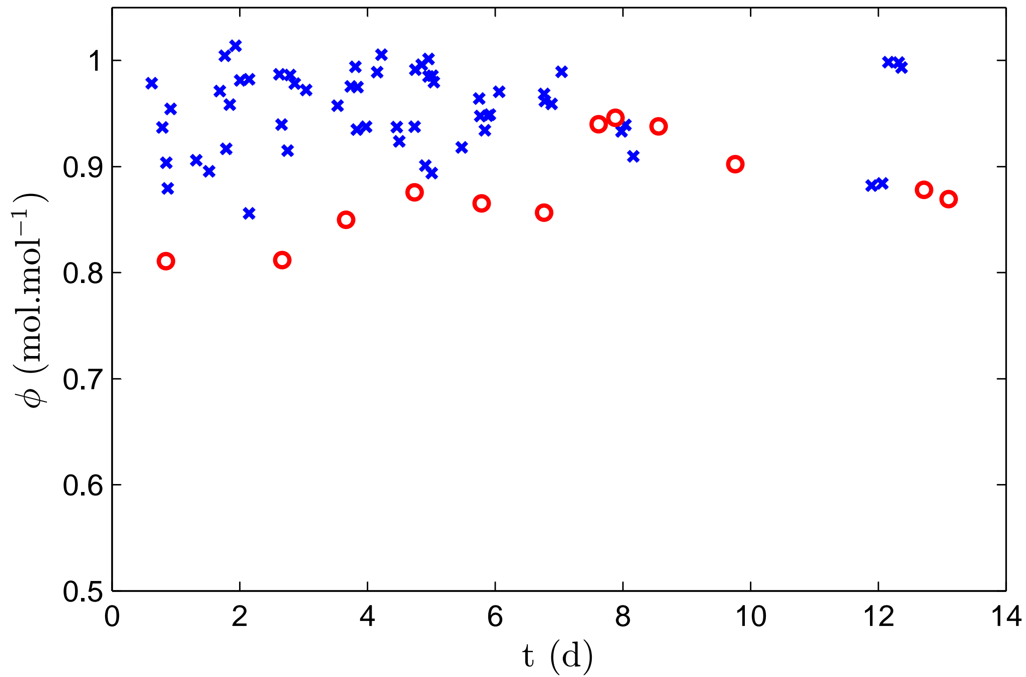

Figure 4.9: Fraction carbon recovery (ϕ) from the product stream in the fermentations: nos 1 and 3 to 7 (×); no. 2 (○)

maximum of 0.016 mol.h^{-1} CO_2 to the fermentation broth. At a productivity of 7.1 g.ℓ^{-1}.h^{-1} the required CO_2 is 0.009 mol.h^{-1}. It can therefore be concluded that approximately 0.05 vvm CO_2 (g) supplied to the reactor provided excess dissolved CO_2 in the fermentation broth for the necessary fixation reaction to produce succinic acid. Whether an increased yield can be attained by adding 0.2+ mol.ℓ^{-1} $MgCO_3$ (s) to the medium is doubtful.

Figure 4.11 shows that the molar ratio of succinic to acetic acid was in the range of 1.1:1 to 1.3:1 when the D-glucose feed concentration was 20 g.ℓ^{-1} (fermentations no. 3 – 7). Also, the acetic to formic acid molar ratio was close to 1:1. Different growth conditions for the high concentrations of immobilised cells in the sixth and seventh fermentation therefore had no effect on the product distribution. From figure 4.10 it can be deduced that the pyruvate dehydrogenase and formate dehydrogenase enzymes were mostly inactive during the fermentations. The initial assumption that no CO_2 was

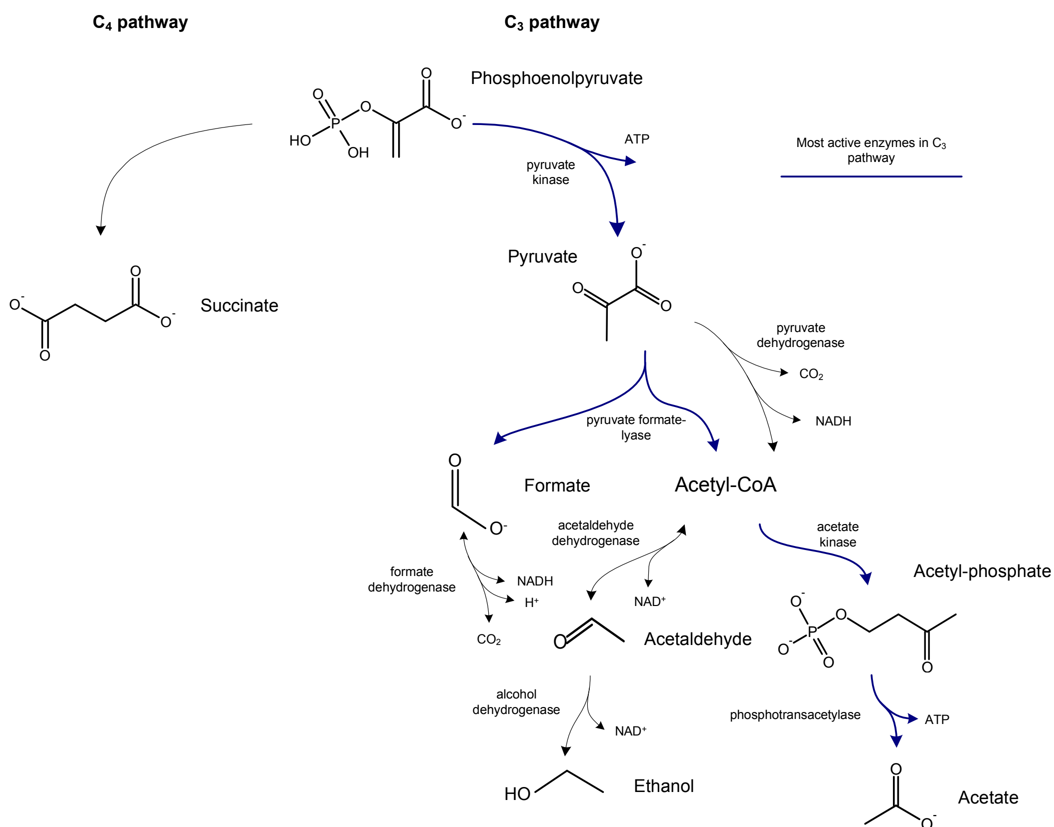

Figure 4.10: Partial metabolic map of *A. succinogenes*

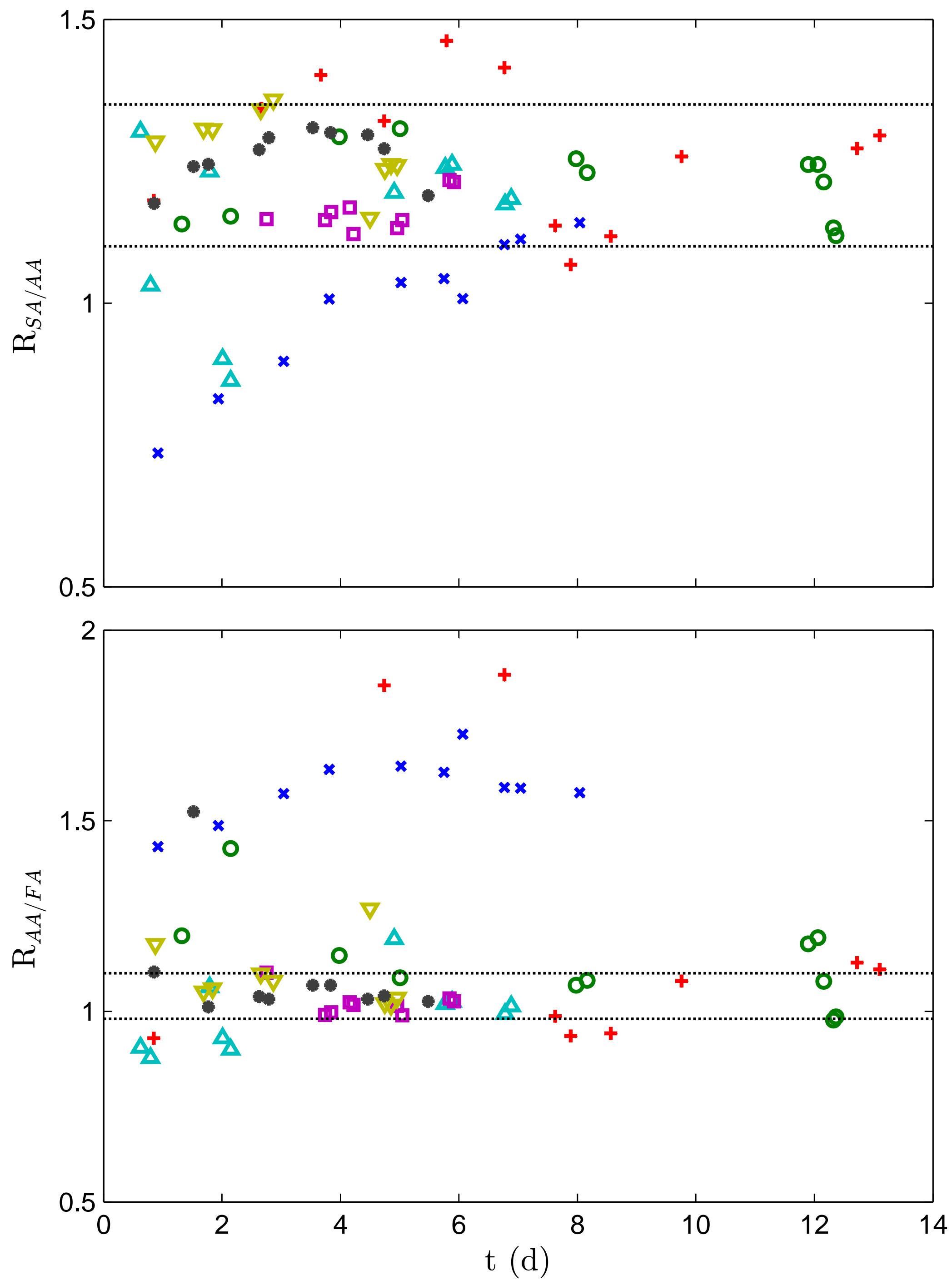

Figure 4.11: Molar product ratios in all fermentations : 1 (×); 2 (○); 3 (+) ; 4 (△); 5 (□); 6 (▽); and 7 (∗)

produced in the system was therefore correct. The most important branch in the metabolic pathway of *A. succinogenes* is undoubtedly at the point where phosphoenolpyruvate either enters the reverse TCA cycle (C_4 pathway) or the byproduct branch (C_3 pathway). The flux distribution ratio between the C_4 and C_3 pathway is therefore on average 1.25:1.

The fermentations with the D-glucose feed concentration at 40 g.ℓ^{-1} showed varied results (see figure 4.11: fermentation 1, ×; 2, +). Either the pyruvate dehydrogenase or formate dehydrogenase enzymes or both seemed to be active at higher D-glucose concentrations. However, this was not supported by results from the second fermentation — after the second day, the flux distribution ratio became similar to that obtained in fermentations nos 3 to 7.

4.4 Comparison with results from the literature

Figures 4.12 and 4.13 compare the productivities obtained in this study with that of other continuous succinic acid fermentation studies in the literature (see section 2.2.4.2) where similar feed concentrations of D-glucose were used.

In figure 4.12 the results in this study of the reactor without packing and a D-glucose feed concentration of 20 g.ℓ^{-1} are compared with the results obtained by Urbance *et al.* (2004) and Lee *et al.* (2009) (suspended cell fermentations). The productivities in this study were similar to what Lee *et al.* (2009) obtained with *A. succiniciproducens*. Considering that *A. succiniciproducens* and *A. succinogenes* are very different bacteria from different genera, the similarities in results might be coincidental. The yields in their fermentations were slightly higher: 0.74 – 0.82. The conditions in the fermentations obtained by Urbance *et al.* (2004) bear the closest resemblance to the fermentations in this study. Very low yields were attained in their suspended cell fermentations at low dilution rates. This resulted in low productivities. Nevertheless, at higher dilution rates, more similar productivities and yields were attained.

The results of biofilm fermentations obtained by Urbance *et al.* (2004) and recycled cell fermentations by Kim *et al.* (2009) (*M. succiniciproducens*) and

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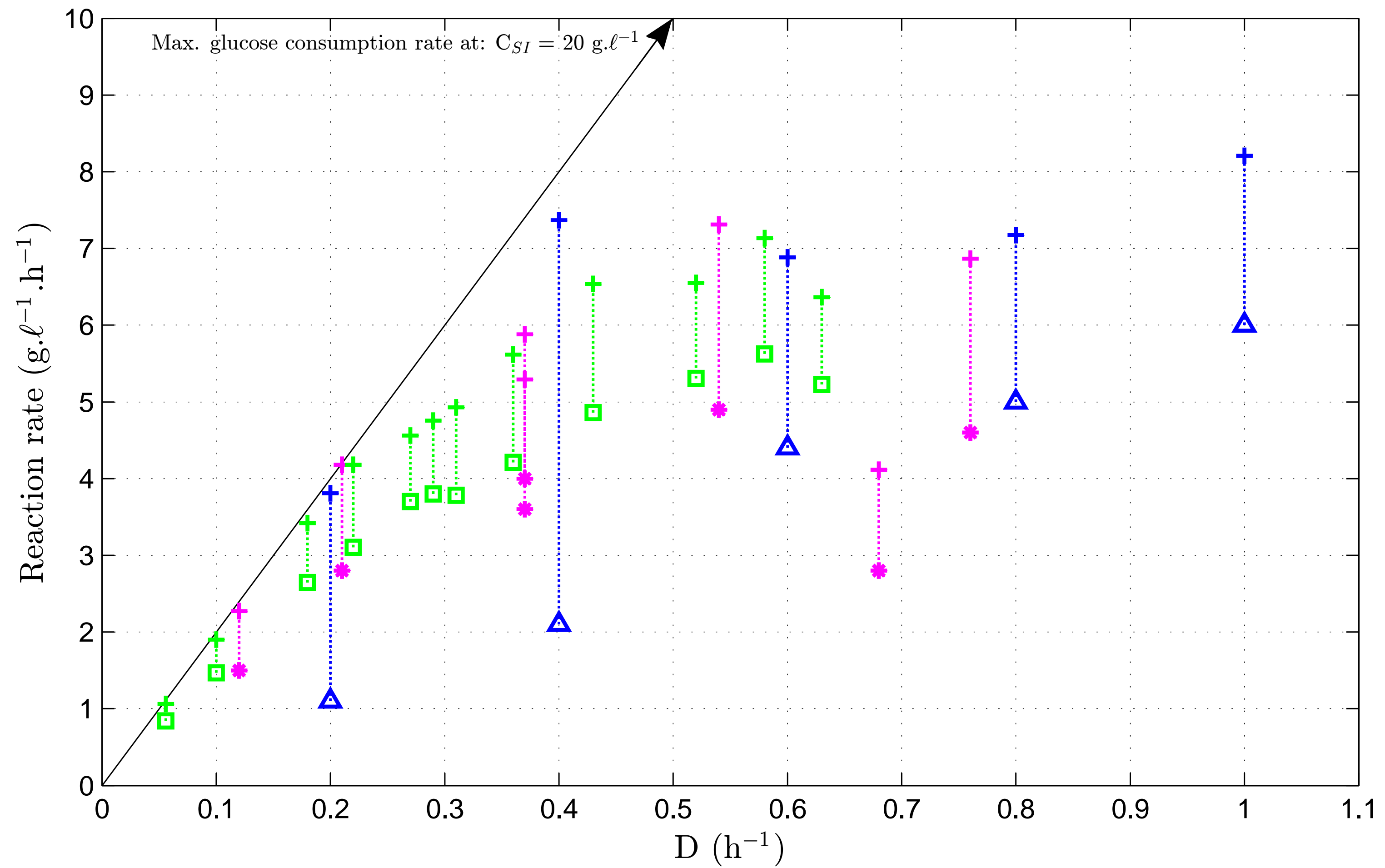

Figure 4.12: Comparison of productivities and D-glucose consumption rates (+) with those in the literature. This study ($\ast$); Urbance *et al.* (2004) ($\triangle$): $C_{SI} = 20$ g.ℓ^{-1}; and Lee *et al.* (2009): $C_{SI} = 19$ g.ℓ^{-1} ($\square$).

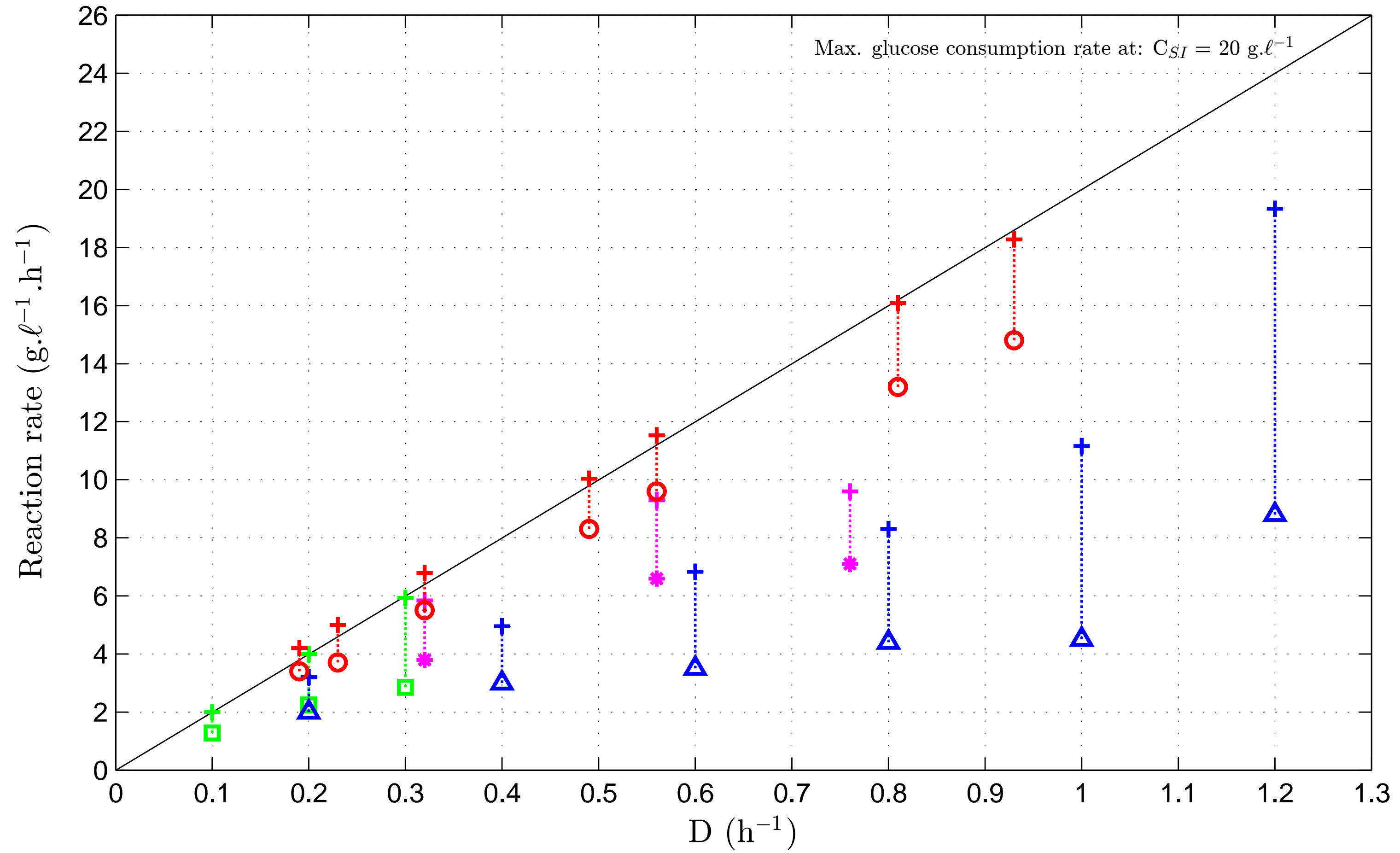

Figure 4.13: Comparison of productivities and D-glucose consumption rates (+) with literature (recycled cells and biofilms; C_{SI} = 20 g.ℓ$^{-1}$). This study (∗); Urbance *et al.* (2004) (△); Meynial-Salles *et al.* (2008) (○); and Kim *et al.* (2009) (□).

Meynial-Salles *et al.* (2008) (*A. succiniciproducens*) are shown along with the best results obtained in this study in figure 4.13. While the productivities obtained in this study compare very favourably with those obtained by Urbance *et al.* (2004), Meynial-Salles *et al.* (2008) still obtained the best continuous succinic acid fermentation results by far. The yields obtained in their fermentations were also consistent at approximately 0.80. Contrary to their suspended cell fermentations, the yields obtained by Urbance *et al.* (2004) decreased with increasing dilution rate. Nevertheless, the productivities were much higher than those obtained in their suspended cell fermentations.

It is difficult to make comparisons with the little data obtained at a feed concentration of 40 g.ℓ^{-1}. Moreover, only Lee *et al.* (2009) have published results with a similar feed concentration. They used 38 g.ℓ^{-1} and, as mentioned previously, *A. succiniciproducens* was used in their fermentations.

4.5 Sterility

As in previous investigations using *A. succinogenes*, it was found that the bacterium cannot compete for survival with microorganisms from the environment (see section 2.4). A prerequisite for a successful fermentation was therefore that *A. succinogenes* could be introduced into a sterile reactor setup. This would result in a 'pure culture' that could grow and produce succinic acid.

The addition of a second sterile batch to the initial medium container has always resulted in an infection. By adding more medium to the container, the 'closed system' was opened. Contaminants were thus allowed to enter the medium from the air. Several preventive measures, such as flames in close proximity to the bottle openings, did not prevent contaminants from entering the medium. Figure 4.14 shows that the infection can be seen by a significant, visible increase in cell mass. Lactic acid production was also observed. Seeing that extensive genome studies by McKinlay *et al.* (2010) provided no evidence of lactate-producing enzymes in *A. succinogenes* under oxygen deprivation conditions, lactic acid production alone indicates active contaminants in the fermentation.

Among others, De Oliva-Neto & Yokoya (1994) and Kim *et al.* (2009) found

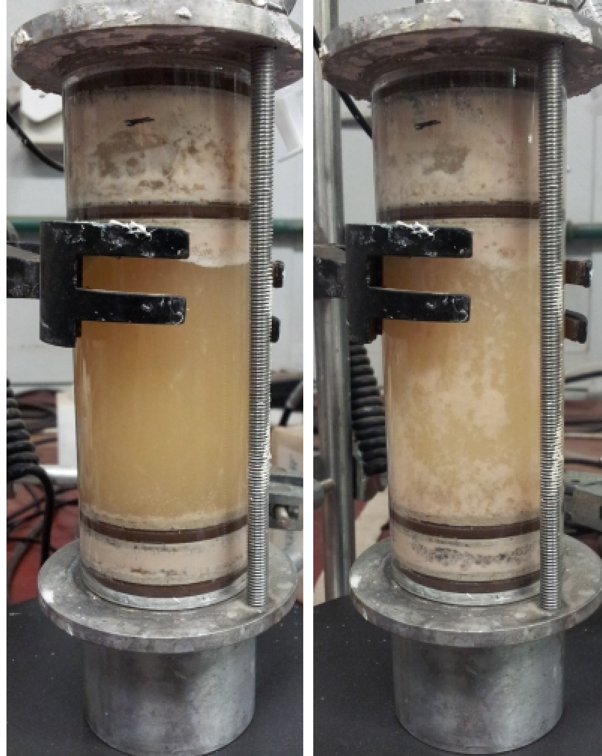

Figure 4.14: The reactor in the second fermentation after 12 and 13 days

that both short-term (batch) and long-term (continuous) successful fermentation requires an environment where contaminants will not be introduced into the reactor system. Media optimised for ethanol and succinic acid fermentation provide a suitable environment for lactic acid-producing bacteria, such as those from the generay *Lactobacillus* and *Leuconostoc*. Species from these genera are present in most open, non-sterile environments.

Chapter 5

Conclusions

Continuous succinic acid fermentations by *A. succinogenes* were successful for periods between 5 and 17 days. The length of each of the fermentations was limited by the size of the initial medium container and the dilution rates employed. The addition of more medium to the initial medium container have always resulted in an infection, despite measures taken to disinfect the area where medium transfer was attempted. This was indicated by lactic acid production from contaminants in the fermentation broth. Sterility is a challenge in both laboratory and industrial fermentation and warrants significant attention in reactor design. Blockages in the feed line caused upsets in reactor operation and contributed to unpredictable behaviour. This problem, along with sterility, highlights the challenge of stable operation in continuous bioreactors.

It is widely assumed that steady state in a chemostat (the ideal suspended cell bioreactor) can be achieved within approximately five retention times. However, this behaviour was not observed in the bioreactor. Increasing cell immobilisation could be observed over the course of each fermentation, and succinic acid productivity increased accordingly. However, it also resulted in unknown cell concentrations in the reactor that prevented any plausible kinetic analysis. A good In order to take advantage of the biofilm formation, packing was supplied in later fermentations that provided more area for attachment. It was done to further increase cell concentration, and therefore also productivity, in the reactor.

The highest productivity achieved at a D-glucose concentration of 40 $g.\ell^{-1}$

was 2.4 g.ℓ^{-1}.h^{-1} at a dilution rate of 0.32 h^{-1} (34% conversion). At a feed concentration of 20 g.ℓ^{-1}, the fermentations without packing in the reactor yielded productivities as high as 4.6 and 4.9 g.ℓ^{-1}.h^{-1} at dilution rates of 0.76 and 0.56 h^{-1}. Productivities at these dilution rates were improved in the packed bed fermentations by 54% and 35% to 7.1 and 6.6 g.ℓ^{-1}.h^{-1}. These increases in productivity were attained where the packing only filled 40% of the reactor volume and further increases should therefore be possible.

Succinic acid yield mostly remained within the range of approximately 0.67 ± 0.05 g.g^{-1} in all the fermentations. When the initial D-glucose concentration was 20 g.ℓ^{-1}, molar ratios of succinic acid to the two byproducts, acetic and formic acid, were both approximately 1.25:1. Biofilm formation did not affect the byproduct production ratios. Furthermore, fermentations at which the D-glucose feed concentration was 40 g.ℓ^{-1}, molar ratios varied and were less predictable.

Chapter 6

Recommendations

Long, uninterrupted, continuous fermentations are essential for obtaining more experimental data. Biofilms are sensitive to any changes in the conditions in the reactor. Process stability should therefore always be given priority to prevent fluctuations in productivity or product quality. Although two days (27 retention times 0.56 h^{-1}) seemed to be enough for one biofilm fermentation to reach steady state, Rosche *et al.* (2009) suggest that biofilm formation in fluidised bed reactors may take several weeks to months to establish. This suggests that a much higher concentration of biofilm can be obtained in the reactor under optimal conditions. For optimal biofilm formation, several factors such as support size, hydrodynamic conditions, and alternative medium components can be further investigated. Additionally, to obtain a material that provides optimal adhesion characteristics for *A. succinogenes*, more packing types need to investigated. Optimal cell immobilisation can increase succinic acid productivity even further.

The reactor can be modified to be able to obtain chemostat data. Previously, only Urbance *et al.* (2004) have reported non-cell recycled suspended cell reactor operation for *A. succinogenes*, but also provided no cell concentration data. More chemostat data and analysis through kinetic modelling may provide invaluable insight for further succinic acid fermentation studies.

Succinic acid yields can be improved significantly. Many strategies such as alternative medium formulations and redox control have been proven to be successful in improving batch fermentations. These methods can be tested in

continuous fermentations as well, since it is always essential to minimise the byproduct concentration to prevent wasting substrate and to simplify downstream product separation processes.

High cell concentrations in biofilms warrant the use of higher substrate concentrations. It is worth investigating whether immobilised *A. succinogenes* can better withstand higher concentrations of substrate and products than suspended cells.

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Appendices

Appendix A

Byproducts

The concentrations of acetic and formic acid produced during the course of the fermentations are shown in figures A.1 to A.3.

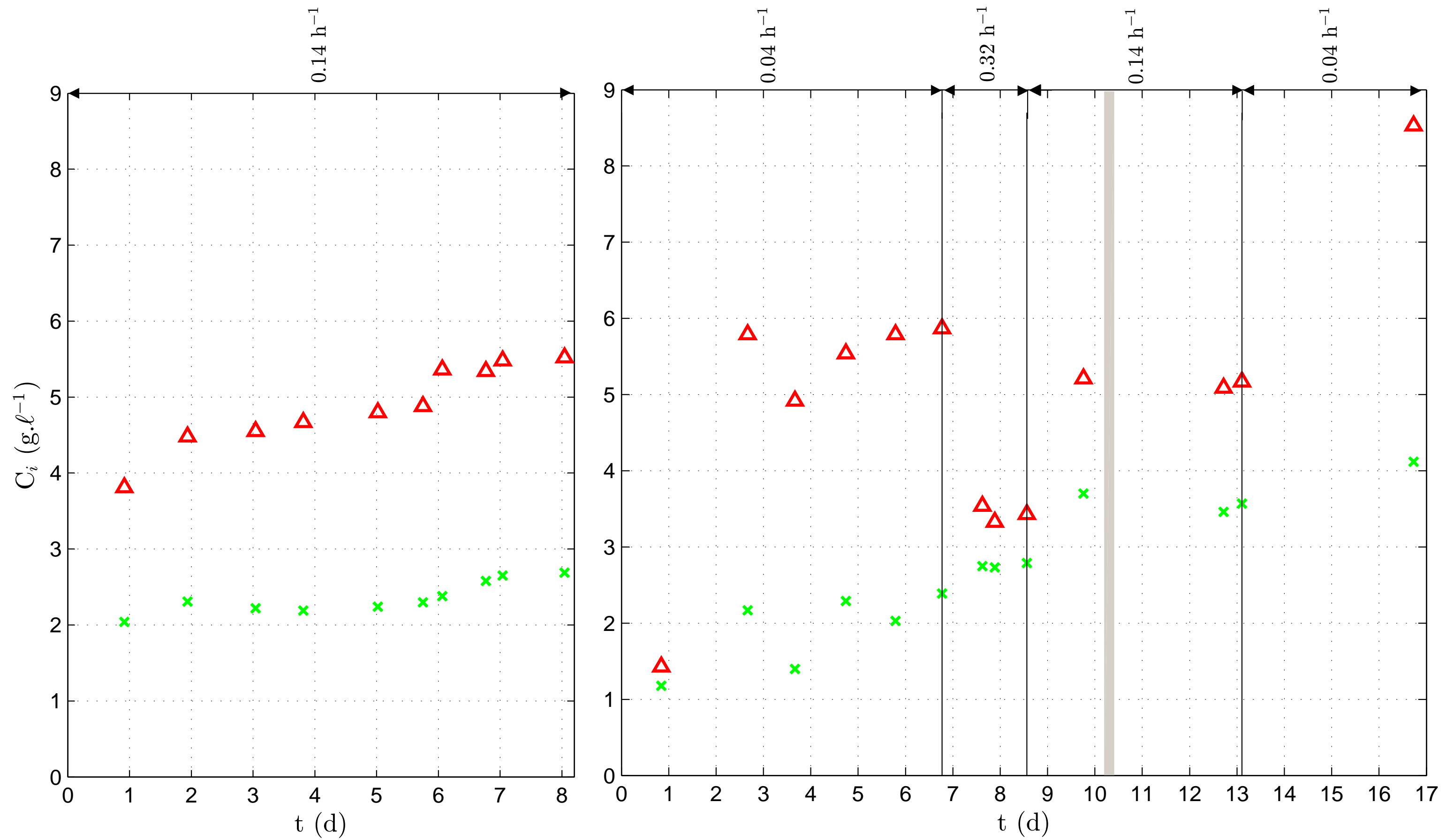

Figure A.1: Byproducts in fermentations nos 1 and 2; $C_{SI} = 40$ g.ℓ^{-1}. Dilution rates are given at the top. Acetic acid ($\triangle$), formic acid (x), blockage(s) indicated by grey area(s).

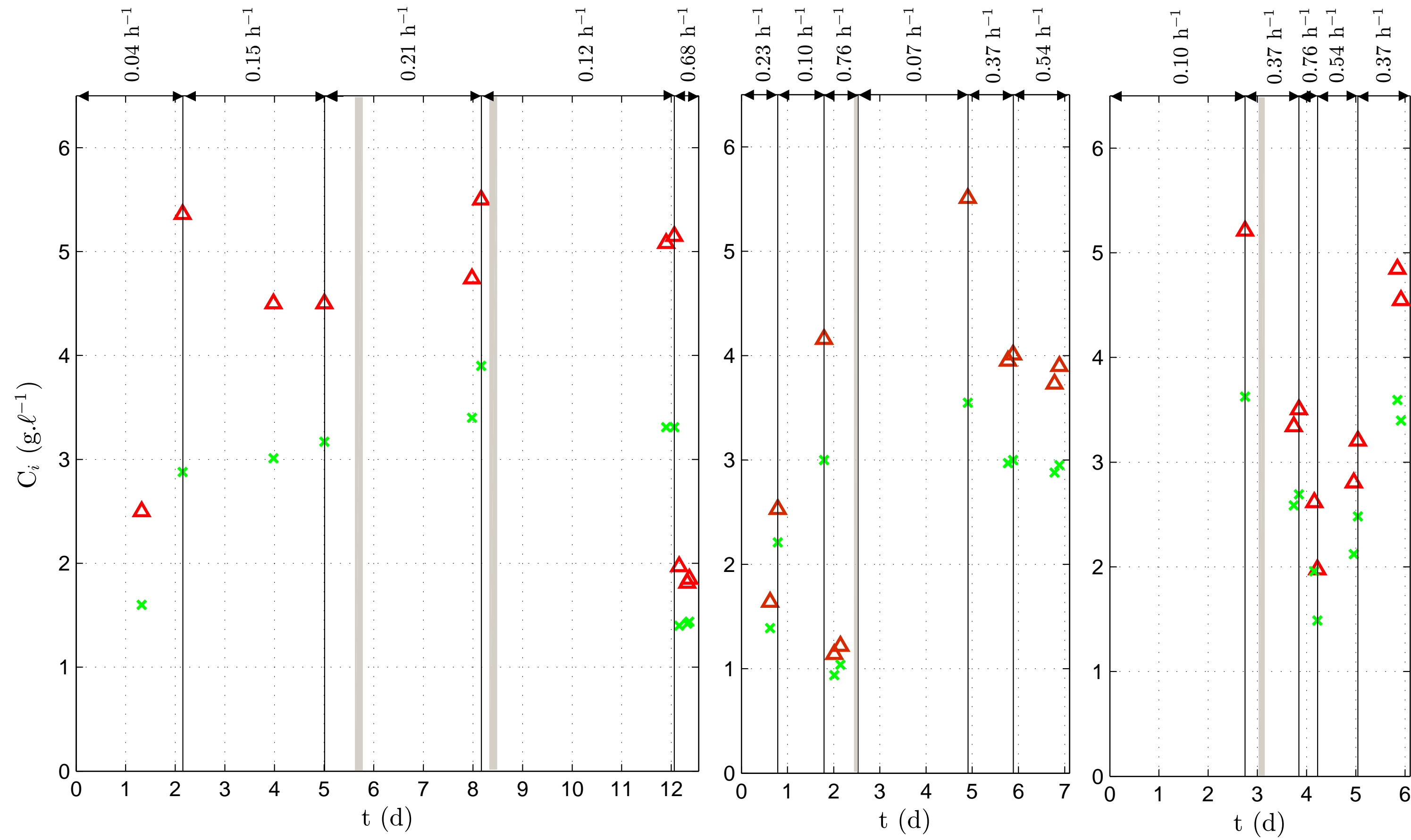

Figure A.2: Byproducts in fermentations nos 3, 4 and 5; $C_{SI} = 20$ g.ℓ^{-1}. Dilution rates are given at the top. Acetic acid ($\triangle$), formic acid (x), blockage(s) indicated by grey area(s).

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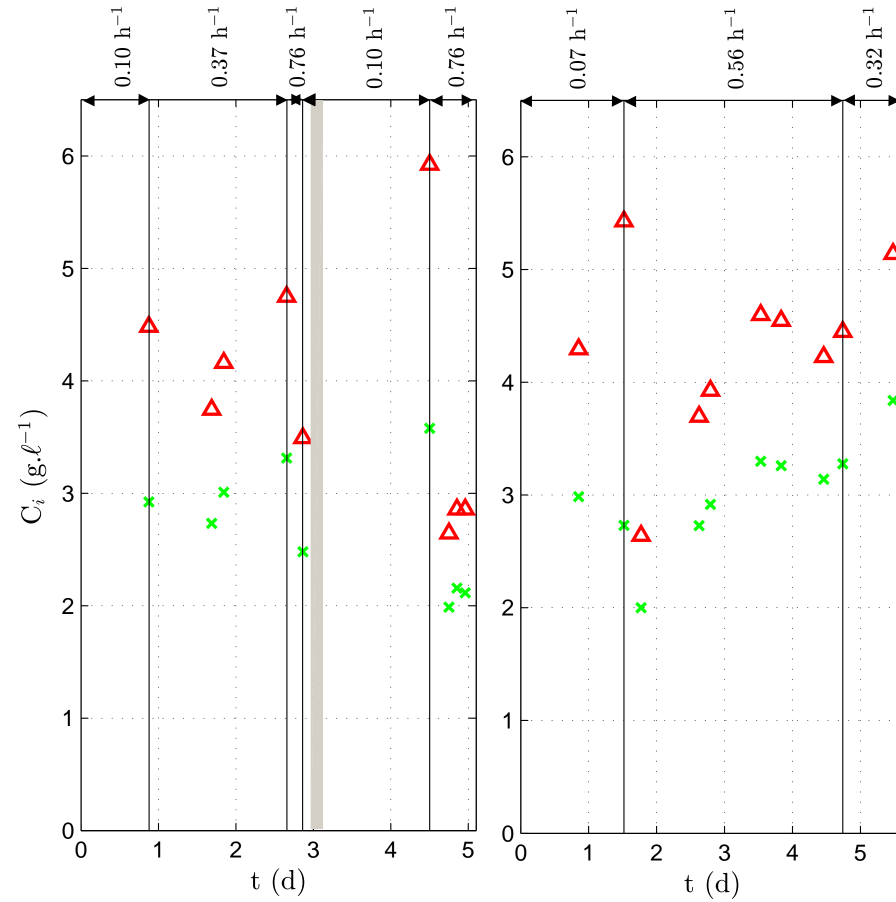

Figure A.3: Byproducts in fermentation nos 6 and 7; $C_{SI} = 40$ g.ℓ^{-1}. Dilution rates are given at the top. Acetic acid (△), formic acid (x), blockage(s) indicated by grey area(s).

Appendix B

Cells

The cell concentration calculated according to the DCW measurements during fermentations nos 2 – 5 are shown in figures B.1 and B.2. Large deviations are present in measurements at the same dilution rates. In addition to undissolved solids from the medium, lumps of cells that broke loose from the biofilm flowed out of the reactor and influenced the measurements. In the fifth fermentation the general increase in DCW measurements can be attributed to impurities in the magnesite.

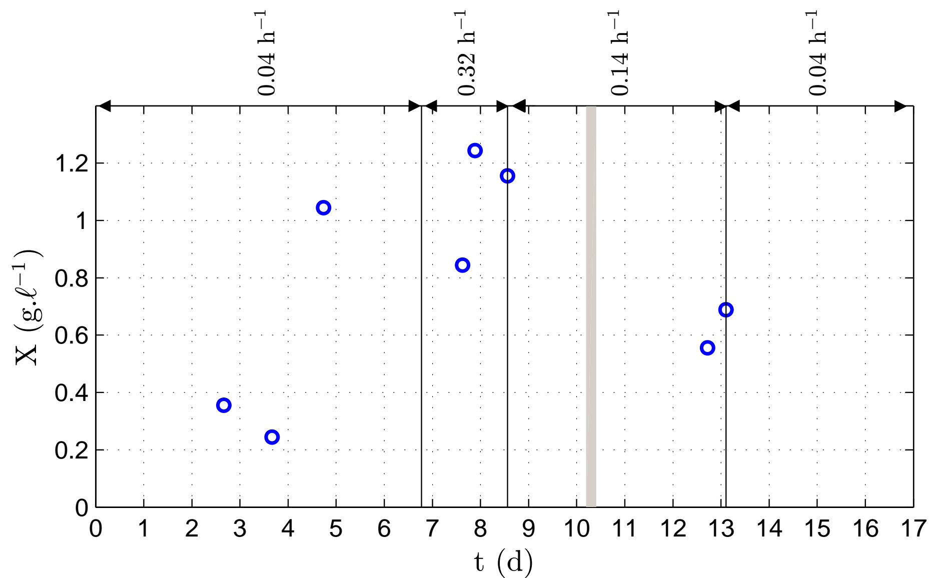

Figure B.1: Cell concentration according to DCW measurements in fermentation no. 2; C_{SI} = 40 g.ℓ^{-1}. Dilution rates are given at the top, while the blockage is indicated by the grey area.

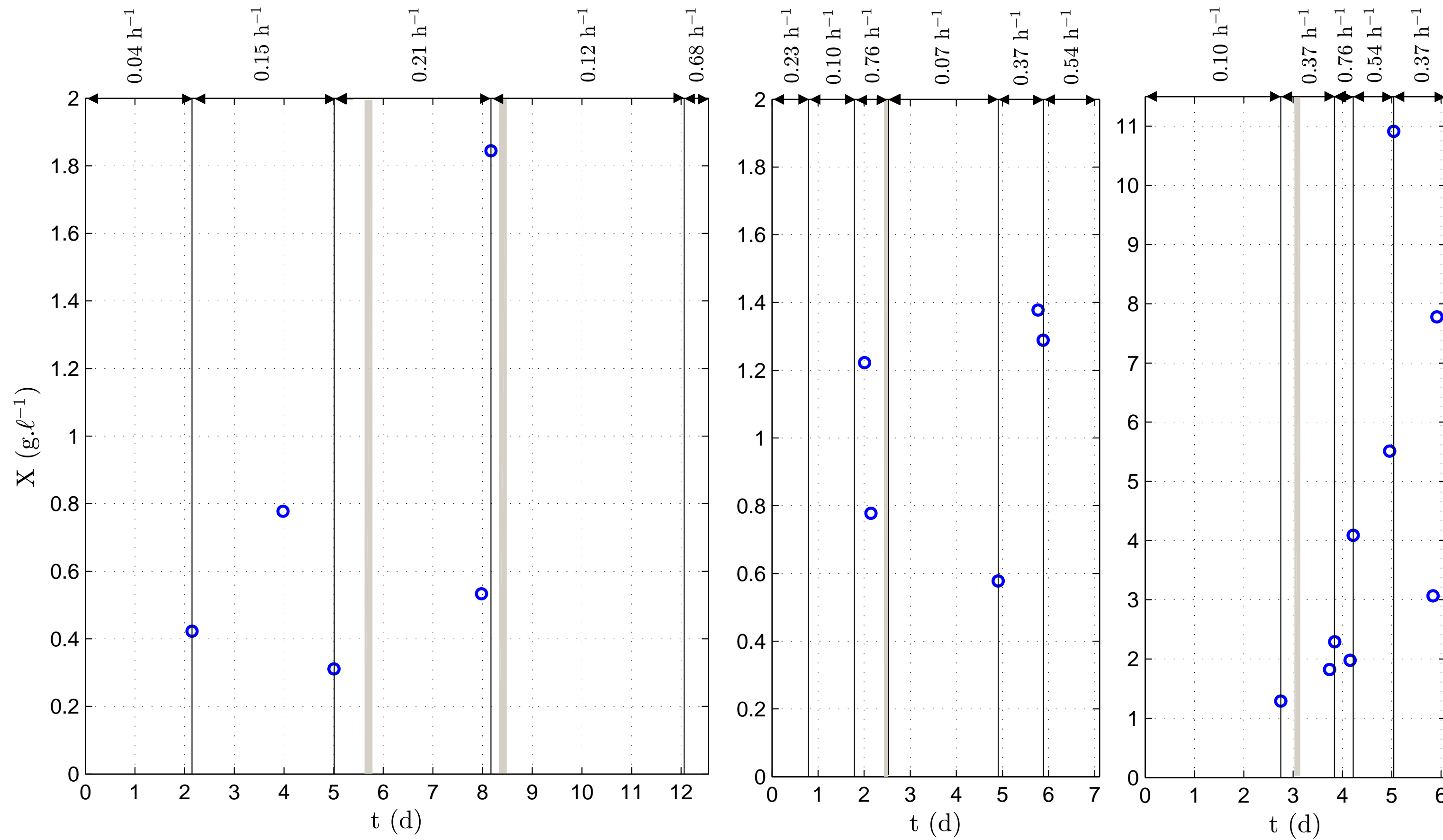

Figure B.2: Cell concentration according to DCW measurements in fermentations nos 3, 4 and 5; $C_{SI} = 20$ g.ℓ^{-1}. Dilution rates are given at the top, while blockages are indicated by the grey areas (note the scale difference in the fifth fermentation).