Continuous production of succinic acid with

Actinobacillus

succinogenes biofilms: Effect of complex nitrogen

source on yield and productivity

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Continuous production of succinic acid with *Actinobacillus succinogenes* biofilms: Effect of complex nitrogen source on yield and productivity

by

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Synopsis

Continuous fermentations were performed in an external-recycle, biofilm reactor using glucose and CO_2 as carbon substrates. The nitrogen source for the auxotrophic *Actinobacillus succinogenes* was a combination of yeast extract (YE) and corn steep liquor (CSL), and sometimes only YE or CSL was used. The total concentration of the complex nitrogen source in the growth medium remained constant at 16 g·L^{-1}, although the respective concentrations of YE and CSL were varied for all runs. In this study, the concentrations of the organic acids, especially succinic acid (SA) and its productivity, were profiled.

The succinic acid productivity of *A. succinogenes* decreased by 67% as the amount of YE in the complex nitrogen source mixture decreased from 16 g·L^{-1} to 0 g·L^{-1}. Succinic acid production increased as the CSL concentration in the nitrogen source increased, and the mass ratio of succinic acid to acetic acid exceeded the theoretical maximum limit of 3,93 g·g^{-1} when only CSL was used as the nitrogen source. The mass ratio of formic acid to acetic acid was consistently within the theoretical yield limitations (0,77 g·g^{-1}) and decreased as the CSL concentration in the nitrogen source increased.

Three fermentation runs were performed. The highest SA concentration in this study was 22,57 g·L^{-1} when only YE was used as the nitrogen source in the growth medium, and the highest SA productivity obtained in this study was 1,58 g·L^{-1}·h^{-1} when a combination of YE and CSL was used as a nitrogen source. The highest mass ratio of SA to AA achieved was 8,3 g·g^{-1} when CSL

was the sole nitrogen source. The mass ratio of FA to AA was consistently less than 0,77 $g \cdot g^{-1}$, approaching 0 $g \cdot g^{-1}$, as the CSL concentration in the nitrogen source increased.

It is assumed that surplus nicotinamide adenine dinucleotide (NADH) is required to achieve the results obtained in this study and it is likely to be provided by the activation or enhancement of an alternative metabolic pathway, i.e. the pentose phosphate pathway, in the presence of CSL or the absence of YE.

Keywords: *A. succinogenes*, Continuous fermentation, Biofilm, Corn steep liquor, Yeast extract.

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Nomenclature

C_{AA} concentration of acetic acid (g·L^{-1})

C_{FA} concentration of formic acid (g·L^{-1})

C_{S0} initial glucose concentration in feed medium (g·L^{-1})

C_{SA} concentration of succinic acid (g·L^{-1})

D_f dilution rate based on feed (h^{-1})

D_T total dilution rate(h^{-1})

MM_{AA} molar mass of acetic acid (g·gmol^{-1})

MM_{FA} molar mass of formic acid (g·gmol^{-1})

MM_{SA} molar mass of succinic acid (g·gmol^{-1})

PSA   succinic acid productivity

R1   fermentation run 1

R2   fermentation run 2

R3   fermentation run 3

vvm	volumetric flowrate of gas per reactor volume
Y_{AASA}	yield/ratio of succinic acid on acetic acid (g·g^{-1})
Y_{AAFA}	yield/ratio of formic acid on acetic acid (g·g^{-1})
Y_{GLSA}	yield/ratio of succinic acid on glucose (g·g^{-1})

Abbreviations

AA, AcA	acetic acid
Ac-CoA	acetyl coenzyme A
ATP	adenosine triphosphate
CSL	corn steep liquor
DCW	dry cell weight
FA	formic acid
FDH	formate dehydrogenase
FDH-H	formate dehydrogenase activity

FHL	formate-hydrogen lyase
Fum	fumarate
GLC, Glu	glucose
HPLC	high-performance liquid chromatography
Mal	malate
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
OXA	oxaloacetate
PDH	pyruvate dehydrogenase
PEP	phosphoenolpyruvate
PFL	pyruvate-formatelyase
PPP	pentose phosphate pathway
PYR, Pyr	pyruvate
RI	refractive index

SA	succinic acid
TCA	tricarboxylic acid
TSB	tryptone soy broth
YE	yeast extract

Greek letters

ΔGLC	glucose consumed (g·L^{-1})

1.   Introduction

Modern civilisation is highly dependent on chemical products and fuels derived from non-renewable raw materials such as crude oil and natural gas. However, the depletion of non-renewable raw materials and the potentially negative long-term environmental impacts caused by the processing of the raw materials is forcing industries to develop alternative routes to produce the fuel and chemicals that are essential for current society (Cok *et al*, 2014).

An alternative that is fast growing in popularity is the replacement of non-renewable raw materials such as fossil fuels with renewable biomass. In 2004 the U.S. Department of Energy (Werpy & Peterson, 2004) identified a wide range of chemicals that could be produced from renewable biomass and only 12 of them were listed as top platform chemicals. In 2010, the list was revised and the top 10 chemicals that had the biggest market potential were declared (Bozell & Peterson, 2010). Succinic acid (SA) was shortlisted as one of those top 10 chemicals (Bozell & Peterson, 2010) and its recognition as a potential platform chemical by the U.S. Department of Energy resulted in opportunities for SA to replace petroleum-based platform chemicals such as maleic anhydride and adipic acid (Zeikus, Jain & Elankovan, 1999).

At present commercial production of bio-SA is done mainly by four companies: BioAmber, Reverdia, Myriant and Succinity, which Succinity is the largest in terms of production capacity (Grand-View- Research, 2014). In order for fermentation processes to be economically competitive with petrochemicals-derived products, research on the biological production of

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SA has become extensive over the years because of the need to understand and refine the process of producing bio-SA.

In the literature, the choices of microbial organisms available for bio-based SA production are very diverse, although most natural production hosts described are capnophilic microorganisms (Beauprez, De Mey & Soetaert, 2010). *A. succinogenes* stood out as one of the most favourable strains for commercial production due to its ability to produce SA naturally at high titres (Zeikus *et al.*, 1999) and its tolerance to high acid concentrations (Lin *et al.*, 2008). However, one major drawback of *Actinobacillus succinogenes* is the auxotrophic requirement for amino acids and vitamins (McKinlay, Zeikus & Vieille, 2005). In fermentation studies, usually the essential amino acids and vitamins are provided by adding yeast extract (YE) or corn steep liquor (CSL) to the feed medium, but in an industrial setup, addition of YE would be highly unfeasible because of the estimated cost, which ranges between US$3.50/kg and US$67/kg (Kwon *et al.*, 2000; Sridee, *et al.*, 2011). For the successful commercial exploitation of *A. succinogenes,* the cost of the fermentation feedstock and the availability of essential and beneficial ingredients that will influence the conversion process should be taken into account. Since using only YE is not economically feasible, cheaper sources of these critical components are hence required. CSL is the first ideal candidate as its market value ranges between US$0.07/kg and US$0.075/kg (Davis *et al.*, 2013).

Most fermentation studies done on *A. succinogenes* employ a batch mode of operation (Beauprez *et al.*, 2010) in which final concentrations, yield and productivity are used as performance indicators. To date all publications in which the complex nitrogen source was varied have used batch

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fermentation (Xi *et al.*, 2013; Jiang *et al.,* 2010; Shen *et al.*, 2015; Yan *et al.*, 2013). Nevertheless, none of these studies distinguished between the nitrogen requirements for growth-related and non-growth-related SA production. It is well known that the specific growth rate of *A. succinogenes* is severely inhibited by the accumulation of organic acids in the fermentation broth (Corona-González *et al.* 2008), and that the specific growth rate approaches zero above an SA titre in the vicinity of 10-15 $g \cdot L^{-1}$ (Brink & Nicol, 2014). Most batch fermentations reach a final SA concentration well in excess of this critical SA concentration, thus indicating that a significant fraction of the SA in these studies is produced under non-growth or maintenance conditions, and that the nutrient requirements for the non-growth production phase might be different to those of the growth phase, in all likelihood less severe.

In order to study the non-growth nutrient requirements of *A. succinogenes*, steady state would be ideal; this can be achieved through a continuous mode of operation. Maharaj, Bradfield and Nicol (2014) have clearly demonstrated that prolonged steady state operation of *A. succinogenes* under non-growth conditions is feasible and stable. The concentrations of organic acids in the fermentation broth can be manipulated by altering the throughput (or dilution rate) in order to induce non-growth production of SA. Another beneficial factor with continuous operations is biofilms which are unavoidably formed in long-term continuous fermentations and can significantly enhance productivity, as observed by Van Heerden and Nicol, (2013b).

This study aims to evaluate the complex nitrogen requirements of non-growing *A. succinogenes* biofilms. Nitrogen sources used in this study will

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be restricted to YE and CSL since they are the most common sources of nitrogen sources used in the literature and because of the potential economic advantage of using CSL. Initial biomass accumulation will be achieved by using only YE for rapid growth of the micro-organism. Once non-growth production of SA commences, the YE content will be reduced and the CSL content will be increased. The total complex nitrogen source concentration in the feed will be maintained at a constant value of 16 g·L^{-1} throughout the experiments; however, the percentage of YE in the total complex nitrogen source will decrease over time. Throughout the study, the change in nitrogen source will be referred to as YE % in the nitrogen source. At a fixed dilution rate (at a chosen initial SA concentration) for the whole run, productivity and product distribution as a function of YE % in the nitrogen source will be assessed.

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2. Literature survey

2.1 Bio-based chemicals

Bio-based chemicals are platform and intermediate chemicals derived from biomass feedstocks. The U.S. Department of Energy (DOE) identified 300 potential bio-based platform chemicals, but most of the proposed chemicals did not have sufficient market potential nor was it economical to produce them. Only 30 chemicals were considered to be relevant since they had the potential to replace non-bio-based chemicals and out of those 30 chemicals, only 12 building block chemicals were identified as being the most important because of their potential market, chemical derivatives and synthesis pathways (Werpy & Peterson, 2004).

The importance for bio-based production of fuels and chemicals arises from the need to move away from petroleum-based production. The depletion of non-renewable feedstocks and the strong demand by consumers for environmentally friendly energy sources has made the production of fuels and chemicals from biomass feedstocks to be a sustainable alternative to the petrochemical production route. Government and industry are now interested in bio-based resources and production and they understand that to form a sustainable global economy, a bio-based industry should substitute the current petroleum-based routes to produces fuels and chemicals (Cok *et al.,* 2014). However, to have a bio-based economy, for the development of highly efficient and cost effective bio-refineries is essential. Petrochemical production has been optimised and refined over long period of time, making it a cheaper option compared with the relatively new bio-based production. Therefore, to enhance the development of a bio-based economy, it is essential that the biological

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processes are understood, refined and optimised through continuous research and development (Cok *et al.*, 2014).

2.2   Succinic acid

2.2.1   Application of succinic acid

One of the top 12 bio-based platform chemicals recognized by the US DOE was succinic acid (SA) (Werpy & Peterson, 2004; Bozell & Peterson, 2010). See Figure 2.1 for the molecular structure of SA. Most of the SA produced to meet global demand is manufactured through the petroleum route, i.e. it is produced by the partial oxidation of butane, followed by hydrogenation of the intermediate product which is maleic anhydride. However, the expensive conversion costs and non-renewability of this petroleum-based route for producing SA has limited the SA market to low-cost bulk application (Xu & Guo, 2010; Beauprez *et al.*, 2010).
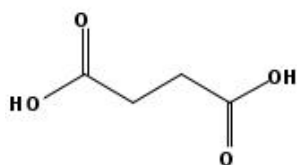
Figure 2.1:   *Succinic acid molecule*

Traditionally, the application of SA has been limited mainly to four functional regions: its largest area of application is as a surfactant, an additive to a detergent and a foaming agent; the second is as an ion chelator

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for preventing corrosion and spot corrosion of metals in the electroplating industry; the third is as an acidulant, which is a pH regulator and flavouring agent in the food industry; and the fourth area of application region is in the pharmaceutical industry, specifically in the production of antibiotics, amino acids and vitamins (Xu & Guo, 2010). As a platform chemical, it is also used as a precursor of many commodity or specialty chemicals, as seen in Figure 2.2 (Zeikus *et al.,* 1999).
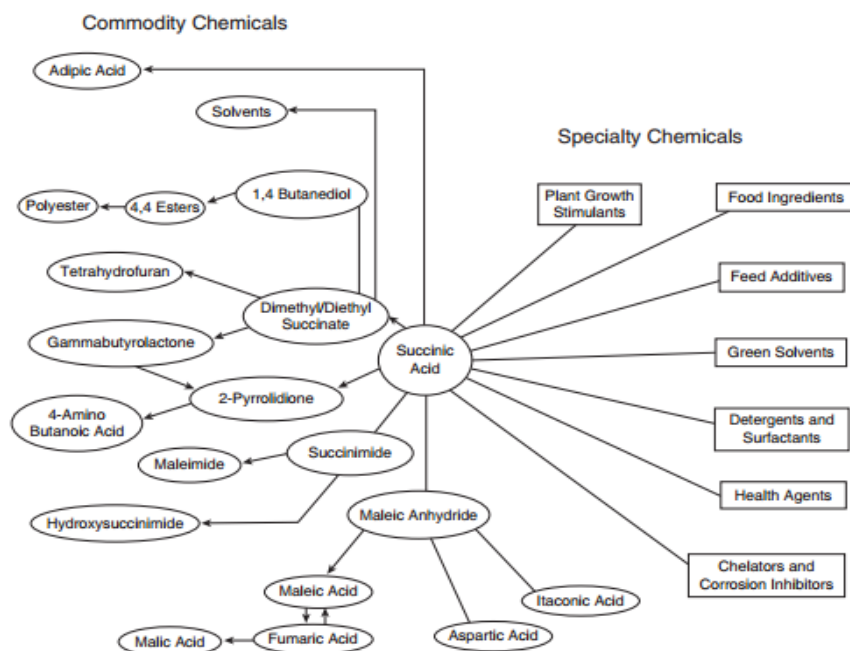
Figure 2.2: *Various chemicals and products derived from succinic acid (Zeikus et al., 1999)*

Nevertheless, there is potential for the bulk application of SA. The key to the growth of the SA market lies in its derivatives. The diamines and diols that are derived from SA can be used as monomer units of a variety of

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plastics, such as polyesters, polyamides and polyester amides (Bechthold *et al.,*2008). Among them is the SA derivative 1,4-butanediol (BDO). It is a platform chemical for tetrahydrofuran (THF), gamma-butyrolactone (GBL) and polymers such as polybutylene terephthalate (PBT) and polyurethane (PU). These chemicals are widely used in the production of engineering thermoplastics and elastic fibres and because of these applications, the BDO market is expected to increase as the demand for THF and spandex for sports apparel increase (Grand- View- Research, 2014). Another derivative of SA that is predicted to increase the SA global market is the biodegradable polybutylene succinate (PBS) and its copolymers. PBS polymers have a range of applications as supermarket bags, packaging film, mulch film and other disposable articles. Owing to the steady growth of the market for biodegradable plastic, the demand for PBS is expected to increase rapidly, which in turn could grow the SA market (ICIS, 2012).

2.2.2   Succinic acid market

The global SA market in 2011 was estimated to be worth US$240 million and it is projected to reach US$836 million by 2018 (Transparency-Market-Research, 2014), due to the development of the bio-SA. Consumption of petroleum-based SA in manufacturing various other chemicals is restricted due to unpredictability of price and carbon footprints. These concerns, however, are advancing the progression of biological manufacturing of SA. The major drivers for this growth will be the high cost of crude oil, the rise in carbon footprints, and an interest in producing "green" chemicals. Newer applications of SA as PBS, BDO, plasticizers and polyesters polyols will fast-track the future growth of the bio-SA market. However, the higher price of bio-SA and the lengthy extraction processes are the primary factors that

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will restrain the market growth for the next few years, although the market is expected to grow at a significant rate over the next seven years (Allied Market Research, 2014).

At present, there are numerous institutions researching the development of SA through bio-based raw materials and there are companies that are already manufacturing bio-SA for commercial purposes. The list of major companies involved in the production of bio-SA includes BioAmber, Myraint, DSM, Mitsui & Co, Mitsubishi, BASF, Roquette Frérese S.A, Purac and Reverdia. In 2013, the global bio-SA market volume was approximately 51 100 tons and in seven years it is expected to reach a market volume of 710 000 tons. In that same year, BDO is predicted to emerge as the largest application segment for bio-SA as it will replace maleic anhydride in the production of BDO; every 1MT of maleic anhydride will be replaced by 1,2MT of bio-SA (Allied Market Research, 2014).

2.2.3 Bio-based SA production

Bio-SA, which has the same structure as petroleum-based SA, is produced by the fermentation of a carbohydrate using a natural producer or an engineered organism. The feedstock used for the production of bio-based succinic acid can be wheat, maize, glucose, lignocellulosic-derived sugar, or sorghum grain processed to starch (BioConSepT, 2013).

Compared with the petroleum-based process, the fermentation process has the advantages of mild operating conditions, independence of the fossil feedstock and fixation of CO_2 and with development of genetic engineering, metabolic modification of microbial strains and improvement of

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purification technology, the fermentative production of SA from renewable resources can be more cost-effective than the fossil-based processes (Xu & Guo, 2010).

To date, various microorganisms have been reported to produce SA, such as typical gastrointestinal bacteria and rumen bacteria and some lactobacillus strains (Kaneuchi, Seki & Komagata, 1988; Beauprez *et al.*, 2010). Among them, *Actinobacillus succinogenes* (Guettler, Rumler & Jain, 1999), *Anaerobiospirillum succiniciproducens* (Oh *et al.*, 2008), *Mannheimia succiniciproducens* (Lee *et al.*, 2000) and genetically modified *Escherichia coli* (Lin, Bennet & San, 2005) are the most promising strains for producing SA at high yields. Fungi species such as *Aspergillus niger, Aspergillus fumigatus, Byssochlamys nivea, Lentinus degener, Paecilomyces varioti, Penicillium viniferum* and yeast *Saccharomyces cerevisia* also produce succinic acid, but not in high concentrations or yields (Song & Lee, 2006).

Nevertheless, for industrial applications *A. succinogenes* stands out because it is by far the most studied wild strain (Brink & Nicol, 2014) and its ability to produce high concentrations of SA naturally from a broad range of carbon sources (Guettler *et al.,* 1999; McKinlay, Vieille & Zeikus, 2007) further makes it ideal for commercial applications.

2.3 *Actinobacillus succinogenes*

2.3.1 Description of microorganism

A. succinogenes, a Gram-negative, rod-shaped and non-motile bacterium isolated from bovine rumen (Guettler *et al.,* 1999) is considered to be one

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of the top natural SA producing microorganisms (McKinlay *et al.,* 2007, Brink & Nicol, 2014). It produces SA as part of a mixed acid fermentation in which acetic acid (AA), formic acid (FA) and ethanol are the by-products. It is capable of digesting a wide range of carbon sources such as glucose, fructose, mannitol, arabitol, sorbitol, sucrose, xylose and arabinose under anaerobic conditions (Van der Werf *et a,.,* 1997), but most laboratory experiments use glucose, fructose, xylose and sucrose as carbon substrates.

A. succinogenes grows optimally at moderate temperatures (37 °C to 39 °C) and it is facultative anaerobic, i.e. it can survive in the presence or absence of oxygen. Furthermore, it is capnophilic because its growth is enhanced at increased CO_2 concentrations (Guettler *et al.,* 1999) and it is a chemoheterotroph that grows optimally at pH ranging from 6 to 7,4.

2.3.2   Metabolic pathway

As stated earlier, *A. succinogenes* is a promising candidate for industrial SA production. However, in addition to producing SA, it also produces formic acid (FA), acetic acid (AA) and sometimes ethanol. The summarised version of the metabolic pathway for *A. succinogenes* is shown in Figure 2.3.

A. succinogenes ferments glucose to phosphoenolpyruvate (PEP) by glycolysis. PEP is thought to serve as the point of divergence between the FA, AA and ethanol producing pathway (C3 pathway), and the SA producing (C4) pathway. The path from glucose to PEP is neutral overall in terms of adenosine triphosphate (ATP) production, but one-third of nicotinamide adenine dinucleotide (NADH) is produced per cmol of glucose consumed.

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C4 metabolic pathway (reverse tricarboxylic pathway)

Overall, this pathway leads to the formation of SA, and two molecules of NADH and one molecule of CO_2 must be consumed to form one molecule of SA. Ideally, one would prefer the carbon flux to follow this route to attain homosuccinate production.

The key enzyme in this pathway is PEP carboxykinase because it pushes the carbon flux from PEP to oxaloacetate, which later on becomes SA. This enzyme is regulated by CO_2 levels, i.e. the level of CO_2 available to A. *succinogenes* will have an influence on the SA production of the organism (Van der Werf *et al.,* 1997). Hence, it will be imperative to achieve correct extracellular and intracellular CO_2 levels during SA production.

C3 metabolic pathway

However, the C3 pathway cannot be avoided due to the redox requirement of the cell. In this pathway, PEP is converted to pyruvate by pyruvate kinase, generating one molecule of ATP during the process. Pyruvate is then converted to acetyl-CoA by either pyruvate dehydrogenase (PDH) and/or pyruvate-formatelyase (PFL).

Pyruvate conversion with PDH results in one molecule of CO_2 and one molecule of NADH being formed, alongside one molecule of AA. With PFL, FA is produced instead of CO_2 and NADH, but FA can be further broken down to CO_2 and NADH by the enzyme formate dehydrogenase (FDH).

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The FDH activity (encoded by the fdhF gene in *A. succinogenes*) is known to occur only under the following conditions (Spector, 2009):

- Absence of an electron acceptor such as oxygen or nitrate
- Acidic pH conditions
- Presence of FA

PFL expression is stated to be dependent upon two glycolysis enzyme activities, namely phosphoglucoisomerase and phosphofructokinase. Interestingly, the expression of PFL and FDH enzymes is reported to increase under carbon energy source starvation (Spector, 2009).

Acetyl-CoA is the branch point at which both ethanol and AA are later formed. In this process, a molecule of ATP is produced per molecule of AA formed. For one molecule of ethanol formed, two molecules of NADH are absorbed. However, ethanol formation can be reduced, if there is intracellular availability of CO_2 to the organism (Van der Werf *et al.,* 1997).
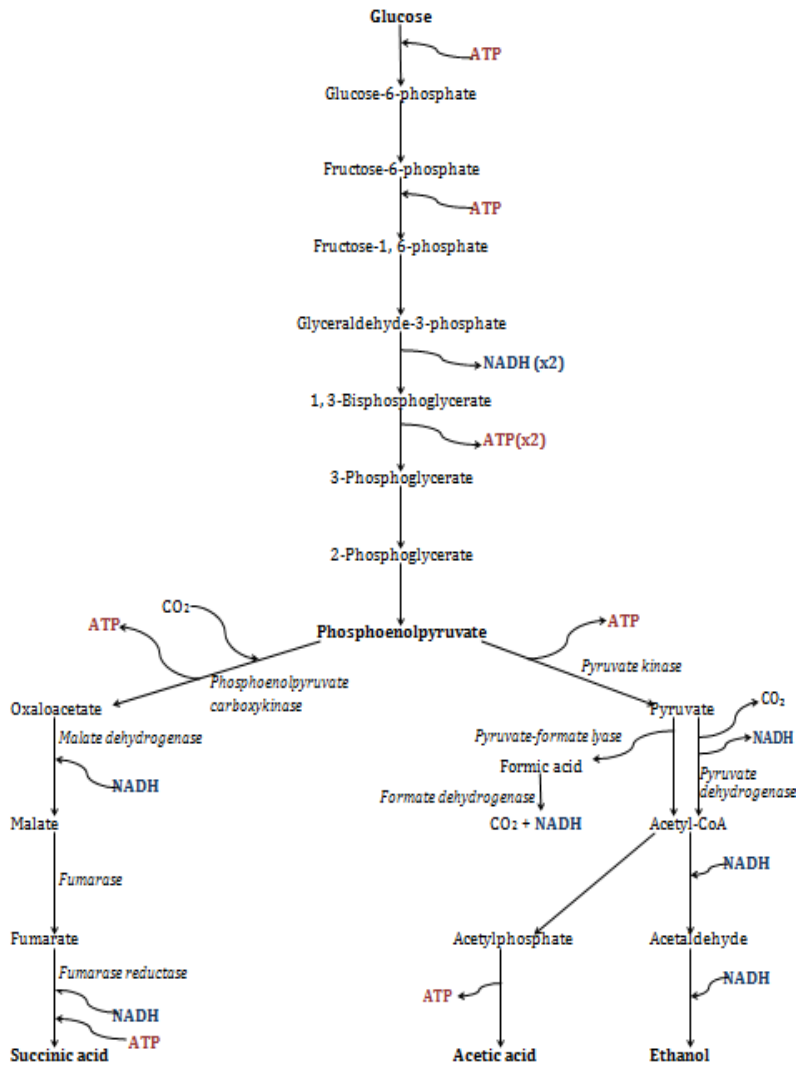
Figure 2.3: *Simplified metabolic network of A. succinogenes (based on McKinlay et al. (2007)*

Pentose phosphate pathway (PPP)

Figure 2.3 gives a simplified version of the metabolic pathway of *A. succinogenes*. However, several studies have noted that the pentose phosphate pathway is an additional pathway for the *A. succinogenes* (Brink & Nicol 2014; Bradfield & Nicol 2014; Van der Werf *et al.,* 1997; McKinlay *et al.,* 2007; McKinlay *et al.,* 2005). McKinlay (2007) stated that the PPP only contributed 20% to the NADPH required for the organism's growth. But Bradfield & Nicol (2014) and Brink & Nicol (2014) postulated that the contribution of the PPP could increase and be more than predicted when the organism enters the maintenance phase or non-growth phase.

According to Rühl *et al.* (2012), a resting, non-growing *Bacillus subtilis* cell showed constant metabolic activity without cell growth, which lead to an apparent overproduction of nicotinamide adenine dinucleotide phosphate (NADPH) (via the PPP) which is then converted by transhydrogenase into NADH. Bradfield & Nicol (2014) claim that it is possible that this can also occur with *A. succinogenes* as it does possess the transhydrogenase. The analysis by Bradfield & Nicol (2014) indicated an under-prediction of the Y_{AASA} for the growth metabolism (associated with NADH "losses"), while an over-prediction of the Y_{AASA} is achieved for the maintenance metabolism (associated with NADH "gain"). This could imply that different metabolic pathways are employed under growth and maintenance conditions. Medium contributions were previously considered as the source for the NADH disparities during the maintenance phase, but this reasoning can be ruled out given the results of the study done by Brink & Nicol (2014) in which the same medium resulted in opposite redox balance trends with regard to the growth and non-growth phases.

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2.4   SA yield considerations

The theoretical maximum yield of SA on a carbon substrate, through fermentation, can be determined by considering the net metabolic pathway involved and a redox balance, as illustrated by Villadsen, Nielsen & Lidén (2011: 159-163). The overall black box stoichiometry in converting glucose to SA with no biomass and no by-product formation is:

$$C_6H_{12}O_6 + \frac{6}{7}CO_2 \rightarrow \frac{12}{7}C_4H_6O_4 + \frac{6}{7}H_2O \qquad (2.1)$$
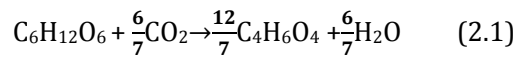
From Equation 2.1, the theoretical maximum yield of succinic acid on glucose (Y_{GLSA}) is 1,12 g·g^{-1}. However, it is not possible to achieve the maximum yield because actual SA yield will depend on the active metabolic pathway of the organism and the associated product distribution. To determine the maximum Y_{GLSA} possible with by-product formation, a redox balance is required.

Pyruvate metabolism

Assuming that there is no carbon flux to cell growth and if only the PDH enzyme is active or FDH converting all FA, the overall black box stoichiometry in converting glucose to SA and AA with no biomass formation is:

$$C_6H_{12}O_6 + \frac{2}{3}CO_2 \rightarrow \frac{4}{3}C_4H_6O_4 + \frac{2}{3}C_2H_4O_2 \qquad (2.2)$$
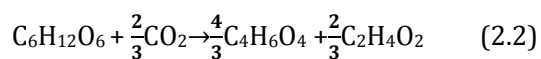
Since no FA is formed, the Y_{AAFA} will be 0 g·g^{-1}. This pathway releases one molecule of NADH for every molecule of AA produced (see Figure 2.4). The

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NADH would be consumed by the C4 pathway to produce SA, and then the theoretical Y_{GLSA} would be 0,88 g·g^{-1}.
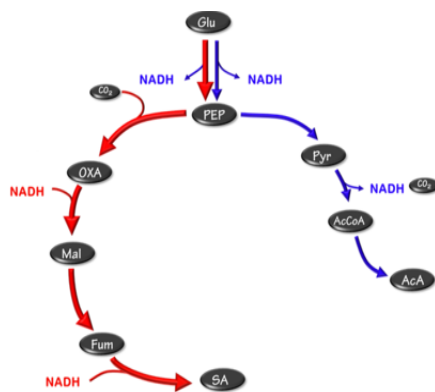
Figure 2.4:   *Simplified metabolic network of A. succinogenes (PDH active)*

On the other hand, if only the PFL enzyme is active, both FA and AA will be formed (see Figure 2.5). The overall black box stoichiometry in converting glucose to SA, AA and FA with no biomass formation is:

$$C_6H_{12}O_6 + CO_2 \rightarrow C_4H_6O_4 + C_2H_4O_2 + CH_2O_2 \qquad (2.3)$$
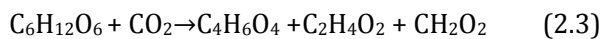
For every molecule of FA formed, a molecule of AA will be produced, so the Y_{AAFA} will be 0,76 g·g^{-1}. As a result, Y_{GLSA} will be 0,66 g·g^{-1}. If both PFL and FDH enzymes are active, then FA will be broken down to produce CO_2 and NADH, which will result in Y_{AAFA} being 0 g·g^{-1} and Y_{GLSA} being 0,88 g·g^{-1}.

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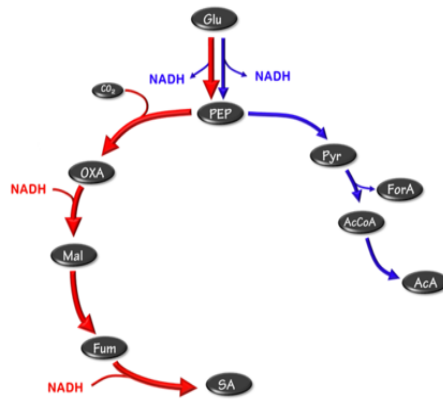
Figure 2.5:   *Simplified metabolic network of A. succinogenes (PFL active)*

Nevertheless, as mentioned by Van Heerden & Nicol (2013a),the maximum theoretical yield of SA can be obtained through the metabolic engineering of *A. succinogenes*. This can be achieved by manipulating the organism to use the oxidative part of the tricarboxylic acid (TCA) cycle under anaerobic conditions (see Figure 2.6a).
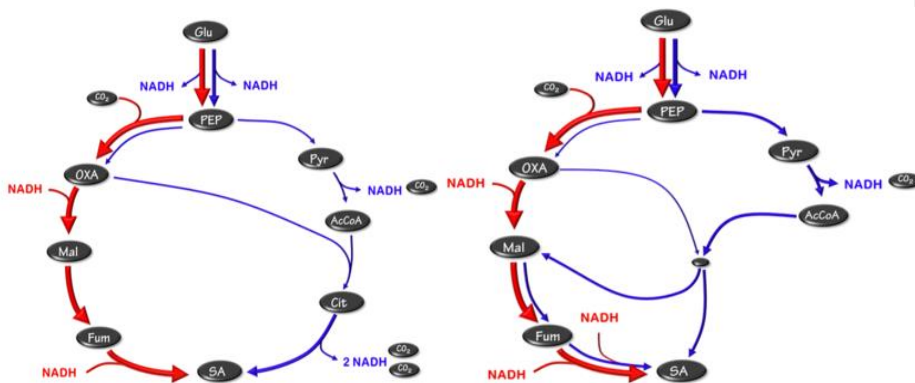
Figure 2.6: *(a) Simplified metabolic network of A. succinogenes (oxidative TCA cycle active); (b) Simplified metabolic network of A. succinogenes (glyoxylate bypass active)*

Alternatively, the glyoxylate bypass can be utilised (Figure 2.6b) to give the same result. For both these scenarios the mass-based SA-to-glucose ratio is 1,12 g·g^{-1}, which is the maximum theoretical yield of SA that can be achieved.

2.5   Nitrogen source for *A. succinogenes*

The composition of the growth medium is a key factor in microbial fermentations. An important element of the fermentation medium, which will be essential for the growth of the organism, is the nitrogen source.

In the rumen, *A. succinogenes* is surrounded by a massive source of nutrients which allow the organism to thrive. But outside the rumen, more specifically in the laboratory or in an industrial fermentation setup,

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nutrients important for growth of the organism must be provided. Since *A. succinogenes* is auxotrophic, it requires certain amino acids and vitamins to be provided for growth and SA production. McKinlay *et al.* (2010) determined the essential vitamins required for the growth to be: nicotinic acid, pantothenate, pyridoxine and thiamine, and the needed amino acids to be: cysteine, glutamate and methionine. Furthermore, they concluded that it is able to grow without biotin supplementation.

By contrast, Xi *et al. (*2012) declared the essential vitamins to be only biotin and nicotinic acid, and the essential amino acids to be only glutamate and methionine. Nevertheless, the addition of these defined components makes the growth medium expensive for industrial use (Shuler & Kargi, 2002: 52). This collection of vitamins and amino acids is thought to be better introduced to the organism in a complex form (e.g. yeast extract or corn steep liquor) where the components and concentration of the nitrogen-carrying compounds are unknown. A complex form of nitrogen source is less expensive than a defined form and it is said to produce higher cell growth compared with media with a defined nitrogen source (Shuler & Kargi, 2002: 52).

Batch fermentation studies

Table 2.1 lists batch studies that investigated alternative nitrogen sources for SA production and *A. succinogenes* growth. From the studies that used only one type of nitrogen source (either YE or YE & CSL or CSL & vitamins), it can be seen that studies that used only YE generally had a lower Y_{GLSA} compared with the studies that used a combination of YE and CSL as the nitrogen source. Addition of vitamins (Yan *et al.,* 2013) to CSL produced SA

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yields and cell growth that were better than the results produced by the YE and CSL combination.

Studies that compared different nitrogen sources within the same study, such as Jiang *et al.* (2010) and Shen *et al.* (2015), compared the performance of several nitrogen sources, inorganic and organic, and observed the following:

- Inorganic nitrogen sources such as $(NH_4)_2SO_4$ or NH_4Cl were the worst performing with regard to cell growth and SA yield (Jiang *et al.,* 2010).
- YE as the only nitrogen source in a growth medium produced the highest SA yield and the highest dry cell weight (DCW) in comparison with other nitrogen sources (Jiang *et al.,* 2010; Shen *et al.,* 2015).
- CSL, a by-product of corn starch production, as the only nitrogen source in a growth medium was the second best performer (Jiang *et al.,* 2010; Shen *et al.,* 2015).
- CSL medium did not achieve full carbon substrate conversion, compared with YE medium, at the end of the batch fermentation (Jiang *et al.,* 2010; Shen *et al.,* 2015).

These studies suggested that the most promising nitrogen source, in terms of *A. succinogenes* growth and SA yield from carbon substrate, was yeast extract (YE). YE is said to have various vitamins, amino acids, minerals and trace metals that are necessary to stimulate the growth of *A. succinogenes* and synthesize its metabolites (Kasprow, Lange & Kirwan, 1998). Although YE is considered to be the best source of nitrogen, its price ranges from US$3.50/kg to US$67/kg (Kwon *et al.*, 2000; Sridee *et al.*, 2011), while the

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price of CSL ranges from US\$0.07/kg to US\$0.075/kg (Davis *et al.*, 2013), which makes YE an expensive and unfeasible nitrogen source for long-term industrial fermentation (Jiang *et al.,* 2010; Xi *et al.,* 2013; Shen *et al.,* 2015) and CSL an economically viable alternative to YE. The feasibility of CSL feasibility as a nitrogen source was also investigated using microorganisms such as *Mannheimia succiniciproducens* (MBEL55E) and *Anaerobiospirillum succiniciproducens*. Those studies (Lee *et al.,* 2000, 2002) came to the same conclusion as Jiang *et al.* (2010) and Shen *et al.* (2015): the inexpensive CSL can replace the expensive YE as a nitrogen source to promote cell growth and SA production.

A possible reason why CSL proved to be second best to YE is because CSL contained the cofactor biotin, which is considered to be vitally important for the metabolism of protein, lipid and carbohydrate (Xi *et al.,* 2012). The biotin content in CSL is 1 mg·kg^{-1} and this is said to be sufficient to make a significant contribution to the nutritional requirement of *A. succinogenes* or other microorganisms (Nghiem *et al.*, 1996). However, Jiang *et al.* (2010) and Shen *et al.* (2015) noticed that with CSL as a nitrogen source, in contrast to YE as the nitrogen source, there was a significant amount of residual glucose that remained at the end of the batch fermentation. Shen *et al.* (2015) suggest that CSL may lack trace elements and certain nitrogen-containing compounds that prevent *A. succinogenes* from meeting its normal physiological needs. Another reason could be that the batch fermentation time was not long enough for the CSL medium to reach full carbohydrate consumption. Since no profile of the variables over time was given for these studies, it is hard to determine whether the residual glucose found in the CSL medium was due to a lack of nutrients or productivity reduction.

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Xi *et al. (*2013) stated that the heme and CSL combination in the production medium improved SA production by creating a more reductive environment (the initial redox potential was very low when heme was added to the medium).

Table 2.1   *Batch fermentation studies of A. succinogenes using different nitrogen sources, namely YE and CSL*

Study	Model	Nitrogen source (g·L⁻¹)	Carbon Source	C_{So} (g·L⁻¹)	C_{SA} (g·L⁻¹)	Y_{GLSA} (g·g⁻¹)	Y_{AASA} (g·g⁻¹)	Y_{AAFA} (g·g⁻¹)	DCW (g·g⁻¹)
Gunnarsson, Karakashev& Angelidaki (2014)	130Z	YE(20)	Synthetic hydrolysate	36	30	0.83	8.6	0.58	2.5
Zhen *et al*. (2009)	CGMCC 1593	YE(15)	Straw hydrolysate	58	45.5	0.807	7.583	0.83	5.6
Corona-Gonzalez *et al*. (2008)	130Z	YE(10)	Glucose	54.7	33.8	0.62	5.2	1.1	3.7
Liu *et al*. (2008)	CGMCC 1593	YE(10)	Sugarcane molasses	64.4	46.4	0.72	7.7	3	5
Yan *et al.* (2013)	CCTCC M2012 036	CSL(25) & vitamins mixture	Glucose	50	39	0.85	-	-	-

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Study	Model	Nitrogen source (g·L⁻¹)	Carbon Source	C_{So} (g·L⁻¹)	C_{SA} (g·L⁻¹)	Y_{GLSA} (g·g⁻¹)	Y_{AASA} (g·g⁻¹)	Y_{AAFA} (g·g⁻¹)	DCW (g·g⁻¹)
	CCTCC M2012 036	CSL(25) & vitamins mixture	Glucose	100	88.1	0.88	-	-	-
Jiang *et al.* (2014)	CGMCC 1716	YE(10) & CSL(5) mixture	Sucrose	100	55	0.69	11	0.9	3
Chen *et al.* (2010)	NJ113	YE(10) & CSL(5) mixture	Glucose	50	42	0.84	-	-	-
Urbance *et al.* (2004)	130Z	YE(6) & CSL (10) mixture	Glucose	40	33.9	0.87	-	-	-
Urbance *et al.* (2003)	130Z	YE(6) & CSL(10) mixture	Glucose	20	17.4	0.87	-	-	-

Study	Model	Nitrogen source (g·L⁻¹)	Carbon Source	C_{So} (g·L⁻¹)	C_{SA} (g·L⁻¹)	Y_{GLSA} (g·g⁻¹)	Y_{AASA} (g·g⁻¹)	Y_{AAFA} (g·g⁻¹)	DCW (g·g⁻¹)
Shen *et al*. (2015)	GXAS137	YE(12)	Sugarcane molasses	70	54.6	0.78	7.5	5	3.8
		CSL(20.6)	Sugarcane molasses	70	47.9	0.68	7.6	5.73	3
Xi *et al.* (2013)	NJ113	YE(10) & CSL(7.5) mixture	Glucose	30	17.1	0.57	3.72	-	3.7
		CSL (15)	Glucose	30	15.1	0.50	4.58	-	3.1
		CSL (15) & heme (0.001) mixture	Glucose	30	21.7	0.72	4.93	-	3.3
Jiang *et al.* (2010)	NJ113	YE(15)	Glucose	70	48.7	0.70	-	-	4.5
		CSL(15)	Glucose	70	9.6	0.14	-	-	2.1

Continuous fermentation studies

To date, all continuous fermentation studies except the study done by Yan *et al.* (2014) used a combination YE and CSL (based on the revised SA medium by Urbance *et al.*, 2003) as nitrogen sources. None of these studies investigated on the effect of varying the nitrogen sources type or the concentration on SA productivity and cell growth. In Table 2.2 it can be seen that, with a fixed combination of YE and CSL growth medium, Maharaj *et al.* (2014) achieved the highest SA yield on glucose mass ratio and highest SA concentration. However, Yan *et al.* (2014) reported even Y_{GLSA} and SA concentration and they used a combination of the inexpensive CSL and defined vitamins.

2.6   Continuous fermentation

In an economic context, the need to produce large volumes of product is the main reason for the selection of the continuous reactor mode, preferably one in which a wild strain acts as the biocatalyst to diminish mutation problems (Villadsen *et al.*, 2011: 384). Continuous systems are advantageous also on a monetary scale because they have lower capital and labour costs in comparison with batch production systems and since continuous processes are time independent, constant product quality is more attainable compared with batch or fed-batch processes (Villadsen *et al.*, 2011: 384).

A unique aspect of continuous operation is the formation of biofilm, which is inevitable during pro-longed *A. succinogenes* fermentations (Van Heerden & Nicol, 2013; Maharaj & Nicol, 2014; Urbance *et al.,* 2003;

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Bradfield & Nicol, 2014; Brink & Nicol, 2014). Biofilms are microbial cell layers that are embedded in self-produced exopolysacharide (EPS) which is prone to attaching to surfaces. Reactors with biofilms can be operated for longer periods of time and are very economical because of the self-immobilizing nature of *A. succinogenes* which results in high volumetric productivities (Rosche *et al.,* 2009).

It is well-known that the growth of *A. succinogenes* is inhibited by the total concentration of acids in the medium (Corona-González *et al.,* 2008; Lin *et al*, 2008; Urbance *et al.,* 2004) and therefore at a low throughput (or D), growth of the organism will be slow since that is when high yields and acid concentrations are found. At a high D, the biofilm is established at a quicker rate but process instability is more severe (Maharaj & Nicol, 2014). The excessive biofilm shedding at this point does not allow the system to reach steady state easily and maintain it. At a low D, the effects seen with a high D are not observed. According to Bradfield & Nicol (2014), the concentrations produced at a low D are linked to the maintenance phase and therefore only a small fraction of the biomass is replicating, which allows the system to reach and maintain steady state (Maharaj & Nicol, 2014).

Table 2.2: *Continuous fermentation studies of A. succinogenes using different nitrogen sources, namely YE and CSL*

Study	Organism model	Nitrogen source	Dilution	C_{So} (g·L⁻¹)	C_{SA} (g·L⁻¹)	Y_{GLSA} (g·g⁻¹)	Y_{AASA} (g·g⁻¹)	Y_{AAFA} (g·g⁻¹)	DCW (g·g⁻¹)
Maharaj *et al.* (2014)	130Z	YE(6) & CSL(10)	0.054 0.71		32.6 14.1	0.9 0.80	- -	- -	- -
Yan *et al* (2014)	CCTCC M2012036	CSL(25) & vitamins	0.1-0.4 0.01-0.1		18.8- 39.96 42.2- 63.6	0.84- 0.92 0.72- 0.85	- -	- -	- -
Van Heerden and Nicol (2013b)	130Z	YE(6) & CSL(10)	0.49		13	0.71	2.5	0.77	-
Urbance et al. (2004)	130Z	YE(6) & CSL(10)	0.85		10.4	0.76	-	-	-

3.   Materials and Methods

3.1   Microorganism and growth

Microorganism

In this study, *Actinobacillus succinogenes* 130Z (DSM No. 22257 or ATCC No. 55618) was used. It was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The culture was stored in a cryopreservation solution at a temperature of –75 °C.

Inoculum preparation

Inoculum was prepared by transferring 1 ml of the preserved culture to a vial containing 15 ml of sterilized tryptone soy broth (TSB). The vial was sealed and stored in an incubator at 37 °C, with a shaker speed of 100 rpm for a duration of 16–24 h. The broth was later tested for contamination and usability by performing an analysis with high-performance liquid chromatography (HPLC). If the broth contained lactic acid or ethanol, it was considered to be contaminated, and if a considerable amount of SA was found in the broth, then the culture was deemed to be viable.

3.2   Fermentation media

All chemicals used to make the fermentation medium were obtained from Merck KgaA (Darmstadt, Germany), unless otherwise indicated. The feed medium consisted of three parts: a growth medium, a phosphate buffer and

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a glucose solution. The fermentation medium is based on the composition proposed by Urbance *et al* (2003).

Six different growth media were used during the fermentation runs; each medium were composed of a different type and/or concentration of nitrogen sources. The first medium used only yeast extract (YE) as the nitrogen source and the last medium used only corn steep liquor (CSL). The first run had approximately 33 $g \cdot L^{-1}$ of D-glucose added to the medium and the last run had approximately 45 $g \cdot L^{-1}$ of D-glucose added to the medium. Table 3.1 gives details of the components and the respective concentrations used to make the feed media. $CO_2(g)$ (Afrox, Johannesburg, South Africa) was fed into the recycle line at 10% vvm to serve as the inorganic carbon source.

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Table 3.1: *Specifications of feed media[a] used during fermentations*

	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6
Glucose			$(g \cdot L^{-1})$			
Runs 1 & 2	33	33	33	33	33	33
Run 3	45	45	45	45	45	45
Growth			$(g \cdot L^{-1})$			
YE	16	10	6	3	1	0
CSL	0	6	10	13	15	16
YE % (% of YE in N_2 source)[d]	100	62,5	37,5	18,75	6,25	0
NaCl	1	1	1	1	1	1
$MgCl_2 \cdot 6H_2O$	0,2	0,2	0,2	0,2	0,2	0,2
$CaCl_2 \cdot 2H_2O$	0,2	0,2	0,2	0,2	0,2	0,2
CH_3COONa	1,36	1,36	1,36	1,36	1,36	1,36
$Na_2S \cdot 9H_2O$	0,16	0,16	0,16	0,16	0,16	0,16
Antifoam Y[b,c]	1	1	1	1	1	1
Phosphate			$(g \cdot L^{-1})$			
K_2HPO_4	1,6	1,6	1,6	1,6	1,6	1,6
KH_2PO_4	3,2	3,2	3,2	3,2	3,2	3,2

[a] Based on Urbance *et al.* (2003)

[b] Antifoam from Sigma-Aldrich, St. Louis, USA

[c] Concentration is in $ml \cdot L^{-1}$

[d] YE % in N_2 source is the % of YE in the total complex nitrogen source in the medium. The concentration of the total complex nitrogen source (a combination of CSL and powder YE) was 16 $g \cdot L^{-1}$ throughout the fermentations for all runs.

3.3   Bioreactor

The bioreactor, pictured in Figure 3.1 (a & b), was a glass cylindrical body set between an aluminium base and head, with an external recycle line to provide agitation. The working volume of the reactor (including recycle) was 356 mL. The liquid level in the reactor was maintained by using a peristaltic pump on the product line. All pumps used in the system were peristaltic pumps. A wooden stick covered in terry cloth was inserted in the glass cylindrical body for biofilm attachment. In fermentation run1 and run2, one stick was used and in fermentation run 3, three sticks were used.

CO_2 flow rates were controlled using Brooks 5850S mass flow controllers (Brooks Instrument, Hungary) and the CO_2 entered the reactor via an inlet in the recycle line, which was connected to a 0,2 μm PTFE membrane filter (Midisart 2000, Sartorius, Göttingen, Germany). The gas exited the system through a filter, with the same specifications as mentioned earlier, connected to the foam-trap.

The pH was measured using a CPS 71D-7TB21 glass combination probe (Endress+Hauser, Gerlingen, Germany) held within a stainless-steel holder connected in-line within the recycle stream. Maintaining the pH at 6,8 required the use of a Liquiline CM442 (Endress+Hauser, Gerlingen, Germany), in which an internal relay controlled the dosing of 10 M unsterilized NaOH in an on–off fashion. Temperature was controlled in a similar fashion; a hotplate was used to provide the heat required to maintain the temperature at 37 °C. For better control of temperature fluctuations, the hotplate was linked to the National Instrument (NI) module. The NI module is further discussed in Section 3.5.
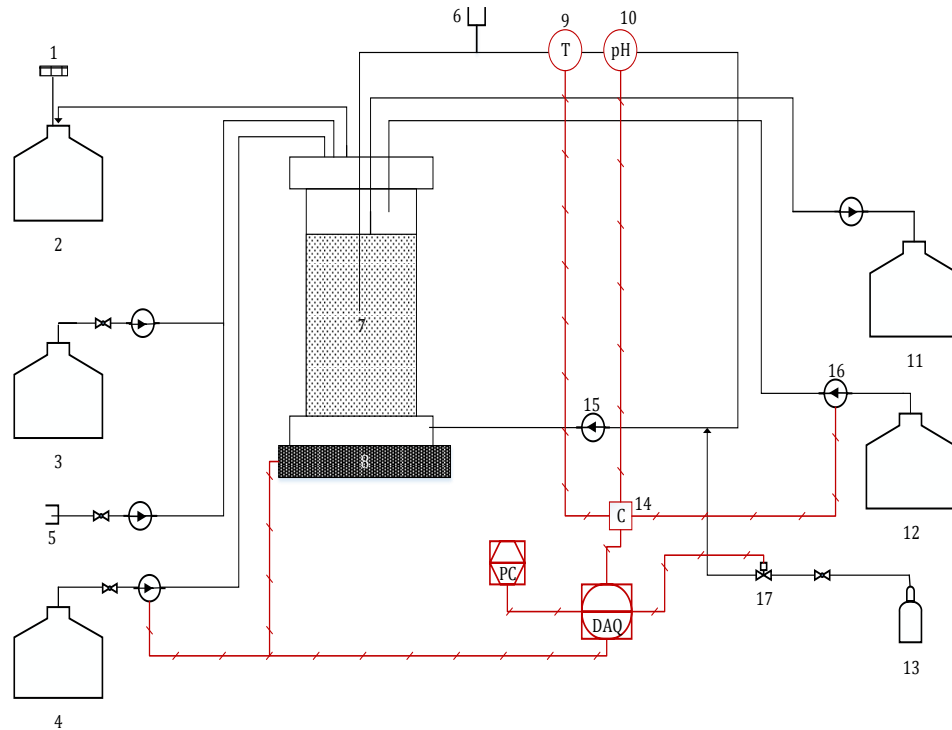
Figure 3.1a):   *Simplified schematic of bioreactor setup*

No.	Description
1	Gas filter
2	Foam-trap
3	Medium reservoir 1
4	Antifoam reservoir
5	Medium reservoir 2
6	Inoculation septum
7	Reactor body
8	Hot plate
9	Temperature control
10	pH probe
11	Product reservoir
12	NaOH reservoir
13	CO_2 cylinder
14	Controller
15	Recycle pump
16	Dosing pump
17	Gas flow controller

Figure 3.1b): *Bioreactor setup*

3.4 Fermentation procedure

The three parts of the fermentation medium (growth medium, buffer and glucose) were prepared in separate bottles and were autoclaved at 121°C for 60 minutes. To prevent unwanted reactions amongst the medium components, the three parts of the medium were only mixed once the bottles had cooled to room temperature (approx. 24 °C). The reactor system (excluding NaOH) was also autoclaved at the same temperature and duration as stated above.

The first fermentation medium (see Table 3.1.) was used to start up the fermentation. It had only YE as the nitrogen source. This was done to form a substantial amount of biofilm for full glucose consumption. The feed setup was then connected to the sterile reactor system with a sterile coupling. The coupling consisted of a U-connection, one half of which was fixed to the feed setup and the other half to the reactor system. Each half of the connection had a ball valve to isolate the system from the external environment. The half connections were then coupled and placed in an oil bath at 140 °C for 20 minutes. The reactor was then filled with medium and once the temperature and pH had stabilized to 37°C and 6,8 respectively, 10 mL of inoculum was injected into the reactor through a silicon septum attached to the reactor head.

Fermentations were started off by operating the system at batch conditions for 17 hours and then changing to continuous operation at a low dilution rate. This allowed for cell accumulation and prevented cell washout. Addition of antifoam into the reactor was only done when deemed necessary. The recycle flow rate was kept constant at 500 mL·min^{-1} in all fermentations to maintain similar shear conditions. The CO_2 flow rate was

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set at a vvm of 10% as it seemed sufficient to maintain CO_2 saturation in the reactor. After a few days of operation, biofilm appeared on internal surface of the bioreactor, as well as the wooden stick covered in terry cloth which was inserted into the glass cylinder as the intended surface for biofilm attachment.

Controlled variables

Dilution rates for the fermentation runs were determined using the first fermentation medium. Once biofilm had formed in the reactor after a few days of continuous operation, the dilution rate was adjusted until 95% of initial glucose had been consumed. For R3, 90% was the target for glucose consumption. The dilution rate was fixed for the entire experimental run if the above mentioned glucose consumption stayed more or less constant for three days or more.

Media change during fermentation

Similar to the feed connection to the reactor, the different medium setups were connected to the sterile reactor system with another sterile coupling. The half connections were then coupled and the U-connection was placed in an oil bath at 140 °C for 40 minutes.

3.5   Online monitoring

Monitoring of the process was performed in a similar fashion to that of Van Heerden and Nicol (2013b) in which the time-averaged NaOH dosing

fraction was linked to a productivity factor, allowing an estimated SA concentration to be calculated in real-time. A LabVIEW (NI module) program was used to monitor and control the reactor. The program was linked to the reactor instrumentation using a cDAQ-9184 data acquisition device (National Instruments, Hungary) with voltage and current input modules and a current output module. Temperature, dosing and antifoam flowrates were controlled with the program. Temperature, pH, gas flow rates and the time-averaged dosing of NaOH were recorded through the program.

3.6   Analytical methods

HPLC analysis

High-performance liquid chromatography (HPLC) was used to determine the concentrations of glucose, SA and other organic acids. Analyses were done using an Agilent 1260 Infinity HPLC (Agilent Technologies, USA), equipped with an RI detector and a 300 mm × 7.8 mm Aminex HPX-87 H ion-exchange column (Bio-Rad Laboratories, USA). The mobile phase (0,3 mL·L^{-1} and 1,1 mL·L^{-1}H$_2$SO$_4$) flowrate was 0,6 mL·min^{-1} and the column temperature was 60°C.

Suspended cell analysis

Once the reactor had reached a steady state, the product stream was collected in a bottle that was kept in a small bar refrigerator. The product bottle was kept cool to prevent further growth of cells and metabolic activity and depending on the dilution rate, the duration of the collection period varied from 12 to 72 hours. The collected volume was then

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thoroughly mixed, and a 12 mL sample was collected for suspended cell analysis. This was performed by splitting the 12 mL sample into 12 x 1 mL samples, and centrifuging at 4000 rpm for 2 minutes. The samples were centrifuged three times and after each centrifugation, the supernatant was poured out, and the cell precipitate washed in distilled water. Following the third centrifugation, the cell precipitates were transferred to an empty glass vial that had been measured beforehand. The cell precipitates were then dried to a constant weight in an 85 °C oven.

Mass balance analysis

Overall mass balances were performed to assess the accuracy of each sample. The mass balances were performed by comparing the stoichiometric amount of glucose required to achieve the experimental concentrations of SA, AA and FA with the experimental amount of glucose consumed.

3.7   Steady state check

When the time-averaged dosing profile, constructed by the LabVIEW program, ceased to fluctuate by a 5% standard deviation of the average dosing flow, it was assumed that the system had reached pseudo-steady state and the product stream was sampled. To further ascertain steady state, the glucose and organic acid concentrations in product samples frequently taken over a certain period were compared and if negligible differences were found between the sample data, the system was confirmed to be at steady state.

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3.8 Summary of fermentation

Results were obtained over three continuous fermentation runs. Several other runs were attempted but did not provide any results that were valuable due to upsets encountered in the system. The three runs mentioned in Table 3.2 were conducted to prove the repeatability of trends observed in the first fermentation run.

Table 3.2: *Summary of fermentation runs performed*

Run	Operating hours (h)	Glucose (g·L^{-1})	D_f (h^{-1})	Medium No.					
1	1 008	33	0,080	1	2	3	4	5	-
2	1 104	33	0,045	1	3	5	-	-	-
3	1 272	45	0,041	1	2	3	4	5	6

Five different mediums, each with a glucose concentration of 33 g·L^{-1}, were used in fermentation run 1 (R1). This gave an indication of what the general trend would be of the product distribution. The dilution rate was adjusted until steady state was achieved, with only 5% of initial glucose concentration as residual for the first medium. Therefore with the 33 g·L^{-1} initial glucose, a D_f of 0,08 h^{-1} was set. Fermentation run 2 (R2) was conducted to prove the repeatability of the trend seen in R1. Fermentation run 3 (R3) was conducted with six different media at a glucose concentration of 45 g·L^{-1} and 90% glucose conversion was only achieved when the D_f was lowered to 0,041 h^{-1}.

Biofilm attachment and appearance

Approximately 72 hours after inoculation of the bioreactor, biofilm started forming on the glass wall of the reactor. A substantial amount of biofilm inside the bioreactor only formed after ± 330 operating hours. The structure and the amount of the biofilm varied throughout the fermentations and it appeared that the biofilm was dependent on the following:

- the surface to which it is attached
- the history of the fermentation
- the composition of the fermentation medium

Figure 3.2 (a) shows the bioreactor before inoculation and without cell attachment. After operation for more than 330 hours, using only YE as the nitrogen source, a thick mass of biofilm had formed, as shown in Figure 3.2 (b). However, as the CSL concentration on the fermentation medium increased, more biofilm started to shed and eventually, when only CSL was used at the nitrogen source, there was almost no biofilm attachment on the glass surface of the bioreactor (as seen in Figure 3.2 (c)).

Although there was not much biofilm on the glass surface, there was biofilm on the internals of the reactor (the wooden sticks covered in terry cloth). Figure 3.3 (a & b) illustrates the sticks covered in biomass at the end of the fermentations.

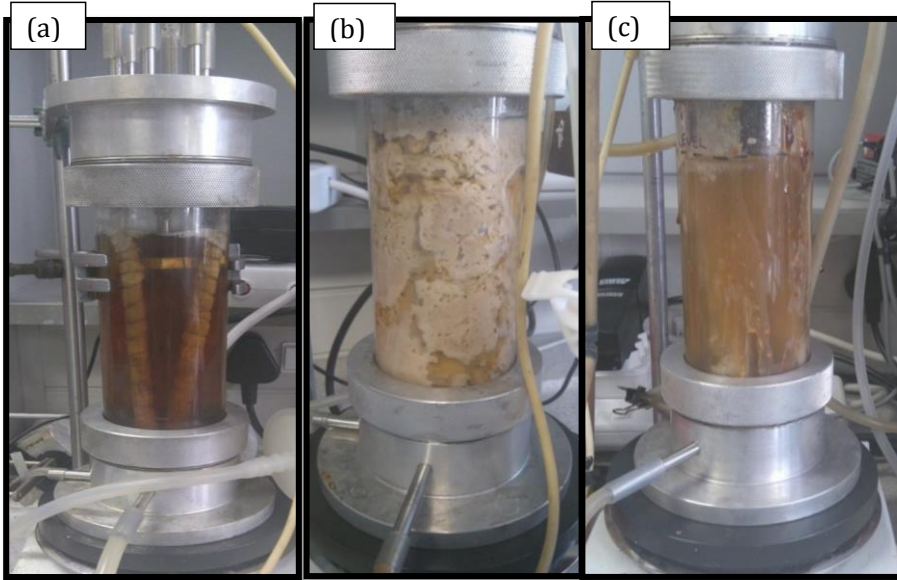41

Figure 3.2: ***a)*** *Bioreactor before inoculation;* ***b)*** *Biofilm growth using only YE as nitrogen source;* ***c)*** *Bioreactor with only CSL as nitrogen source*
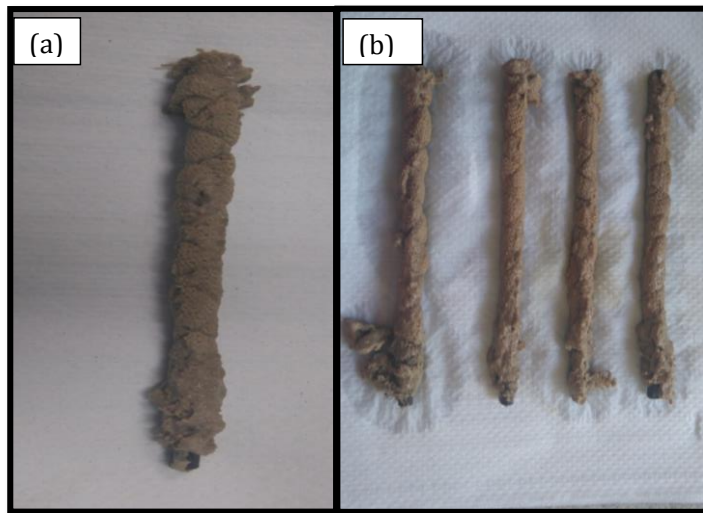
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Figure 3.3: *__a)__ Internal support for biofilm attachment (at the end of fermentation run 1); __b)__Internal supports for biofilm attachment (at the end of fermentation run 3)*

4. Results and Discussion

4.1 Experimental strategy for different runs

The objective of R1 (fermentation run 1) was to find steady state data that could be analysed to understand the influence of YE and CSL on the growth and productivity of *A. succinogenes*. As seen in Table 3.1, the media used in the experiments differed from each other regarding the concentrations of CSL and YE, but the total complex nitrogen source concentration in the media remained constant. The intended experimental plan was to report the steady state product concentrations as the concentration of YE in the growth medium varied (i.e. as the YE concentration decreased and the CSL concentration increased). R2 (fermentation run 2), which followed the same procedure as R1, was conducted to prove that the data found in R1 was repeatable. R3 (fermentation run 3) was an attempt to achieve high SA concentrations, following the same experimental plan as R1, except that R3 used a high initial glucose feed concentration and had additional internal support to increase the surface area available for biofilm attachments.

All fermentations began with a growth medium that contained only YE as the nitrogen source (100% YE) and once a healthy and stable biofilm had formed, the growth media were changed according to the procedure explained in Section 3.4 Fermentation procedure.

Initially, R1 had a high biomass content and activity (when only YE was in the fermentation medium) and as the YE content in the growth medium decreased, the biomass activity decreased. R2 was effectively a repetition of R1, but due to the low initial biomass activity, the dilution rate was adjusted to have approx. 95% glucose conversion (when the growth

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medium had only YE as the nitrogen source). Despite the increased surface area for biofilm attachment, the biomass in R3 struggled to achieve the same activity as R1. Initially, the SA concentration was slightly higher, but eventually the results of R3 coincided with those of R2 when CSL was introduced into the growth medium. The dilution rate for R3 could have been lowered further to reach higher product concentrations, but it was decided not to do this due to time constraints for achieving steady state.

Table 4.1 gives the steady state results for all three runs.

Table 4.1: *Steady state data for the three continuous fermentation runs*

Dilution (h⁻¹)	SA (g·L⁻¹)	AA (g·L⁻¹)	FA (g·L⁻¹)	PYR (g·L⁻¹)	Δ GLC (g·L⁻¹)	YE (% in nitrogen source)	Black box mass balance (%)	Average black box mass balance (%)	Average black box mass balance (%) with DCW
					Fermentation run 1				
0,08	17,31	8,14	6,08	0,48	30,41	100,00	85,29		
0,08	16,85	8,16	6,19	0,50	30,67	100,00	83,47		
0,09	16,71	8,00	6,12	0,51	30,59	100,00	82,69	83,82	
0,08	18,60	7,02	4,49	0,66	29,16	62,50	87,77		
0,08	18,72	6,67	4,35	0,79	29,17	62,50	87,16	87,47	95,81
0,08	17,64	4,92	2,63	0,45	26,19	37,50	83,43		
0,08	17,92	4,94	2,51	0,33	25,96	37,50	84,73		
0,08	17,78	4,98	2,59	0,31	25,96	37,50	84,41	84,19	
0,08	16,91	3,59	1,83	1,78	21,52	18,75	96,46		
0,08	16,96	3,49	1,68	1,75	22,23	18,75	92,78		
0,08	16,69	3,53	1,70	1,72	21,07	18,75	96,87	95,37	
0,08	15,65	2,39	0,64	2,24	18,55	6,25	99,35		
0,08	14,11	1,92	0,48	2,90	18,08	6,25	94,59		
0,08	13,28	1,76	0,56	2,93	18,22	6,25	89,21	94,38	

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Dilution (h⁻¹)	SA (g·L⁻¹)	AA (g·L⁻¹)	FA (g·L⁻¹)	PYR (g·L⁻¹)	Δ GLC (g·L⁻¹)	YE (% in nitrogen source)	Black box mass balance (%)	Average black box mass balance (%)	Average black box mass balance (%) with DCW
						Fermentation Run 2			
0,04	17,80	8,52	5,69	0,51	29,54	100,00	90,23		
0,04	18,53	8,78	5,69	0,44	29,72	100,00	92,56		
0,04	18,74	8,97	5,85	0,40	30,04	100,00	92,87	91,89	
0,04	17,96	5,64	2,48	0,42	24,89	37,50	91,54		
0,04	17,59	5,82	2,63	0,35	23,54	37,50	96,16		
0,04	17,60	5,70	2,28	0,38	25,61	37,50	87,60	91,77	
0,04	11,75	2,90	0,71	2,01	14,47	6,25	105,75		
0,04	11,06	2,89	0,74	1,99	14,55	6,25	100,81		
0,04	10,80	2,83	0,71	2,02	13,90	6,25	103,57	103,38	
						Fermentation Run 3			
0,04	22,53	10,22	4,93	0,26	36,95	100,00	86,86		
0,04	22,57	10,09	4,99	0,23	36,73	100,00	87,11		
0,04	22,05	9,76	4,77	0,25	36,56	100,00	85,22	86,40	91,38
0,04	19,27	7,87	2,98	0,00	30,43	62,50	85,38		
0,04	19,70	8,03	3,06	0,07	32,21	62,50	82,66		
0,04	19,91	8,20	3,35	0,05	32,42	62,50	83,44	83,83	92,13
0,04	17,85	6,04	1,28	0,63	29,98	37,50	85,83		

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Dilution (h⁻¹)	SA (g·L⁻¹)	AA (g·L⁻¹)	FA (g·L⁻¹)	PYR (g·L⁻¹)	Δ GLC (g·L⁻¹)	YE (% in nitrogen source)	Black box mass balance (%)	Average black box mass balance (%)	Average black box mass balance (%) with DCW
0,04	18,79	6,15	1,31	0,69	30,92	37,50	85,72	85,68	89,74
0,04	16,35	4,93	0,28	0,67	26,35	18,75	87,18		
0,04	16,28	4,86	0,22	0,77	26,05	18,75	86,44		
0,04	15,78	4,55	0,36	0,80	26,64	18,75	84,31	85,98	99,19
0,04	11,59	2,95	0,00	0,82	14,89	6,25	92,55		
0,04	11,10	2,86	0,00	0,98	19,55	6,25	85,45		
0,04	11,04	2,75	0,00	0,99	14,19	6,25	96,30		
0,04	10,44	2,59	0,00	1,14	14,33	6,25	89,08	90,85	112,40
0,04	8,17	1,37	0,00	1,77	15,82	0,00	61,86		
0,04	7,09	0,86	0,00	2,37	14,25	0,00	86,91		
0,04	7,25	0,72	0,00	2,49	14,57	0,00	61,40		
0,04	7,12	0,80	0,00	2,73	12,71	0,00	71,61	70,45	72,34

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Mass balances or redox balances were performed on all the samples and the average closures of the steady state samples are shown in Table 4.1. Mass balance closure did increase as the YE content decreased. In R3, DCWs were taken to see if the mass balance closure would reach 100%. Including the DCWs significantly improved the mass balance, but did not close it. Anything above 90% was considered a proper closure; and most figures that were below 100% may indicate unknown metabolites or non-perfect analysis of acids and sugars.

4.2 Productivity analysis

Figure 4.1 shows the average concentrations of SA, AA and FA in the product streams at steady state for all fermentation runs. For a clear comparison of the metabolite concentrations of all the fermentation runs, the results were put into a single plot, despite the differences in dilution rate and glucose feed concentration. The reasons for the difference in dilution rates were explained in Section 4.1.
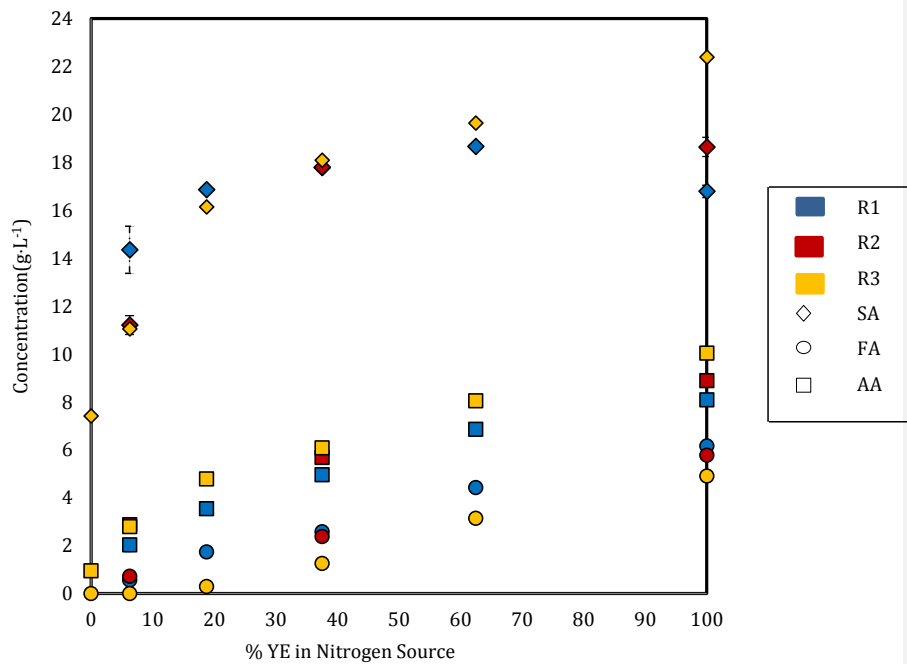
49

Figure 4.1: *Product concentration profiles for SA, AA and FA of A. succinogenes as the YE % in the growth media varied.**

* The total nitrogen source (combination of YE and CSL) concentration in the growth medium stayed constant at 16 g·L-¹only the concentrations of YE (powder form) and CSL (liquid form) varied.

Figure 4.2 depicts how SA productivity and the glucose consumption rate changed as the YE concentration decreased.

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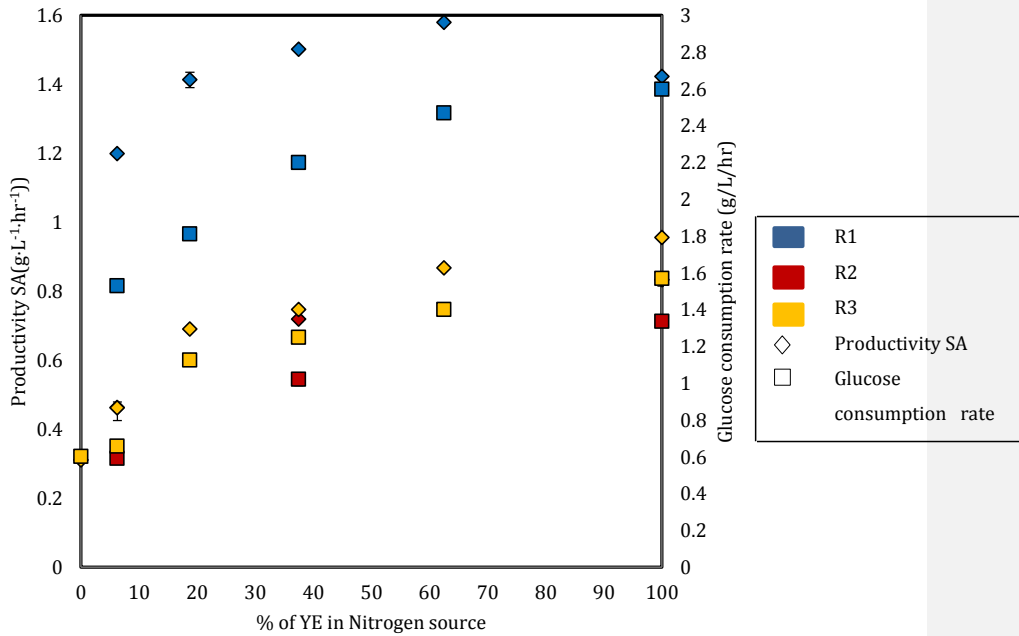
Figure 4.2: *SA productivity profile and glucose consumption rate profiles of A. succinogenes as the YE % in the growth media varied.**

*The total nitrogen source (combination of YE and CSL) concentration in the growth medium stayed constant at 16 g·L-1, only the concentrations of YE (powder form) and CSL(liquid form) varied.

SA concentration decreased as the concentration of YE was reduced in the medium. When only YE was used as the nitrogen source, which is an ideal ingredient for *A. succinogenes* growth and SA productivity, the highest SA concentration for R2 and R3 was achieved. For R1, the SA concentration was at its highest when the 62.5% of YE medium was used. Despite the difference in glucose concentration, SA concentrations for R1, R2 and R3

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were similar when CSL was added to the medium. R3 started with a high SA concentration, as expected, but then quickly dropped and displayed the trend observed in R1 and R2. AA and FA both decreased as the YE content in the medium decreased, for all runs. The concentrations for both FA and AA more or less matched in all runs, irrespective of the dilution rate and the glucose feed concentration. When only CSL was used as the nitrogen source in the growth medium in R3, no FA was produced.

The SA productivity trend, shown in Figure 4.2, is the same as the SA concentration profile. The highest SA productivity obtained in this study was 1.58 $g \cdot L^{-1} \cdot h^{-1}$, when a 62.5% YE medium was used as the nitrogen source. From Figure 4.2, it should be clear that the productivities of R1 were almost twice as high as the productivities of R2. The same can be said for the glucose consumption rates for R1 and R2. This is because the dilution rate of R1 was also twice as much as the dilution rate of R2. According to Maharaj *et al.,* (2014), a decrease in dilution should lead to an increase in product concentrations and yields. R1 and R2 do not follow that trend. Instead their product concentrations profile matched. A possible reason for the inconsistency in the trends could be that there was less biofilm activity in R2, for an unknown reason, compared with the biofilm activity in R1, resulting in a product concentrations profile match instead of higher product concentrations in R2 compared with the profile for R1.

4.3 Analysis of product distribution

Metabolite concentrations were used to determine and study the product distributions Y_{AASA}, Y_{AAFA} and Y_{GLSA} of the three fermentations.

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In Section 2.4, the theoretical metabolic flux limitations were discussed and it was noted that in the absence of biomass formation, the maximum theoretical values for Y_{AASA} and Y_{GLSA} should be $3,93 g \cdot g^{-1}$ and $0,88 g \cdot g^{-1}$ respectively. These yields would only be achieved if no FA was produced, i.e. if only PDH was active. However, if the PFL route was active and all of the FA was converted to CO_2 and NADH by FDH, then the maximum theoretical yields mentioned earlier could be still achieved. If biomass is produced, then a portion of the glucose in the growth medium would be directed to the anabolic pathway, which would influence the carbon distribution, resulting in maximum yields less than those given above.

It is illustrated in Figure 4.3 that Y_{AASA} should vary between $1,97 g \cdot g^{-1}$ (for $Y_{AAFA} = 0,77 g \cdot g^{-1}$) and $3,93 g \cdot g^{-1}$ (for $Y_{AAFA} = 0 g \cdot g^{-1}$). Using only YE as the nitrogen source, the experimental yields for all runs matched the maximum theoretical yields that were obtained when only the PFL enzyme was active. As the nitrogen source content varied (i.e. CSL concentration increased and YE concentration decreased), Y_{AASA} increased and Y_{AAFA} decreased, thus indicating a shift in the metabolic flux distribution. Y_{AASA} for R2 and R3 were similar throughout the runs, so was Y_{AAFA}. However, when the YE concentration in the nitrogen source was 18,75% for R1 and R3, Y_{AASA} exceeded the maximum theoretical yield, while Y_{AAFA} was still in the range of $0 g \cdot g^{-1}$ to $0,77 g \cdot g^{-1}$. The highest Y_{AASA} achieved in this study was $8,3 g \cdot g^{-1}$ and the lowest Y_{AAFA} was $0 g \cdot g^{-1}$, as seen in Figure 4.3; these yields were obtained in R3, when only CSL was used as the nitrogen source.

Certain batch studies in *A. succinogenes* also achieved a Y_{AASA} that exceeded the maximum theoretical yield (see Section 2.6, Table 2.1), but those studies used YE as the nitrogen source, whereas in this study it was

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achieved using only CSL. Xi *et al.* (2013) was the only study (batch fermentation), mentioned in Table 2.1, that used only CSL as the nitrogen source, with glucose as the carbon substrate, and exceeded the maximum theoretical Y_{AASA}. They attained a Y_{AASA} of 4,58 $g \cdot g^{-1}$.
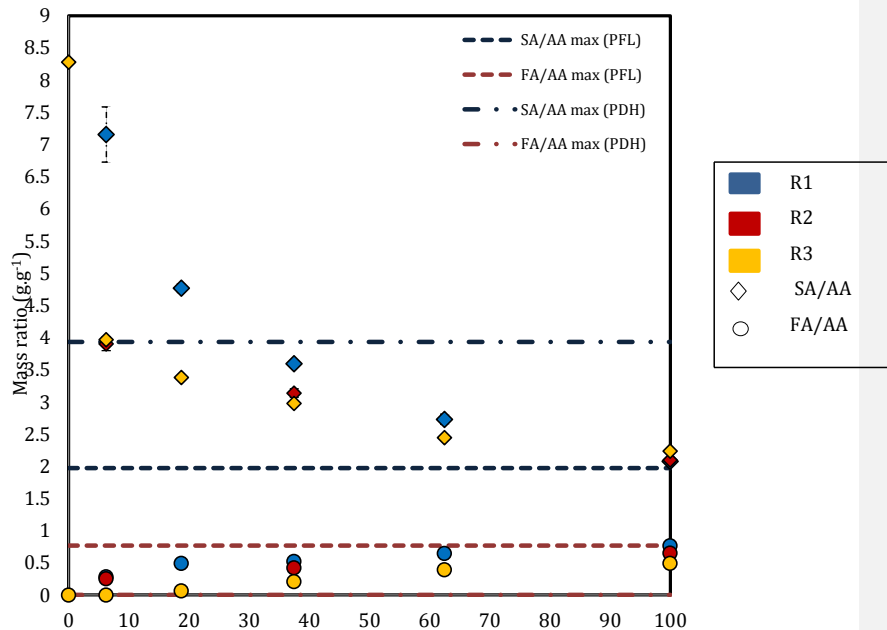
54

Figure 4.3: *Experimental yields plotted with the maximum theoretical yields profile of A. succinogenes as YE % in medium changes.**

*The total nitrogen source (combination of YE and CSL) concentration in the growth medium stayed constant at 16 g·L-¹, only the concentrations of YE (powder form) and CSL(liquid form) varied.

Considering the flux at the pyruvate node, Y_{AAFA} decreased and was approaching zero as the CSL concentration in the nitrogen source increased, as seen in Figure 4.3. This could be an outcome of an increase in either PDH or FDH activity or a decrease in activity for both PDH and PFL, resulting in an increased concentration of pyruvate in the product stream. Figure 4.4 illustrates how the pyruvate concentration increased as the CSL concentration in the growth medium increased.
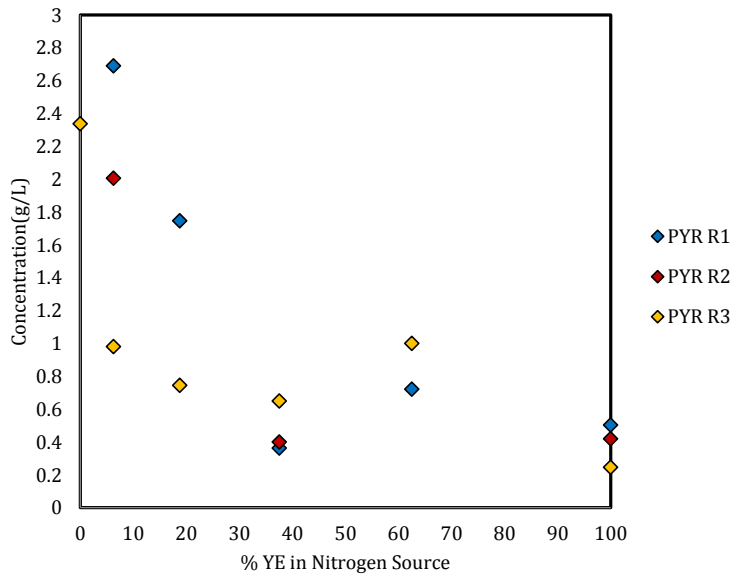
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Figure 4.4: *Pyruvate concentration profile of A. succinogenes as YE % in medium changes.**

*The total nitrogen source (combination of YE and CSL) concentration in the growth medium stayed constant at 16 g·L-1, only the concentrations of YE (powder form) and CSL(liquid form) varied.

According to the redox balance, it is expected that Y_{AASA} would increase as Y_{AAFA} decreased, and in this study the same development was observed. This could be linked to the increasing CSL concentration in the nitrogen source in this study. Bradfield & Nicol (2014) suggested that there could be an additional reducing power that produced the yields that were achieved. Using carbon and redox balances, they noticed in their study that the experimental Y_{AASA} values exceeded the predicted values, suggesting that an increase in PDH or FDH activity was not the only contribution to the increase in Y_{AASA} and that additional reducing power was present. The same

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explanation can be applied to this study, as seen in Figure 4.3. The increase in the Y_{AASA} is influenced by the decrease in the Y_{AAFA} ratio, although it is not sufficient to reach the expected ratios (according to the redox balance).
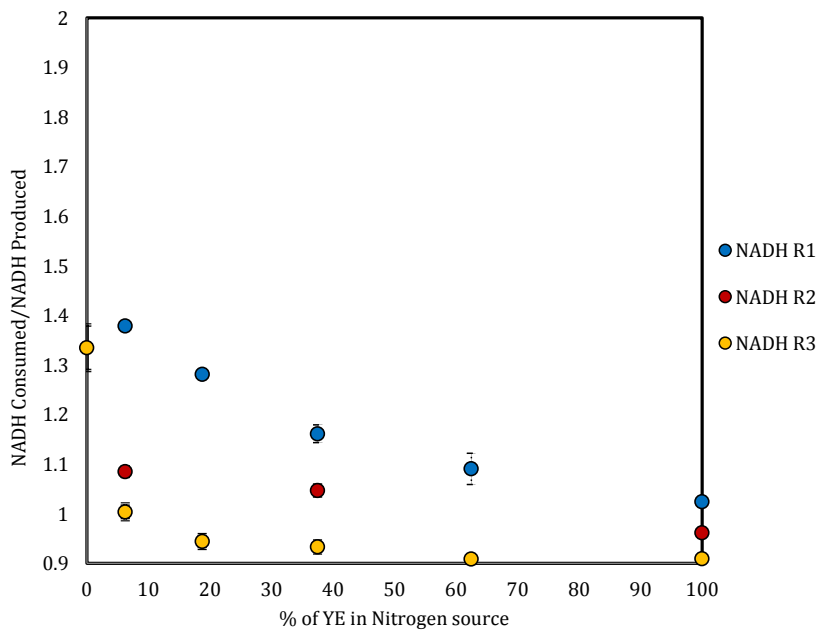

Figure 4.5:   *NADH ratio consumed during production of A. succinogenes as YE % in medium changes.**

*The total nitrogen source (combination of YE and CSL) concentration in the growth medium stayed constant at 16 g·L-1, only the concentrations of YE (powder form) and CSL(liquid form) varied.

Figure 4.5 illustrates the NADH consumed per NADH produced for all fermentation runs. The NADH consumed (mol NADH·cmol glucose-1) was calculated as follow:

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$$\frac{\frac{2C_{SA}}{MM_{SA}}}{\frac{6\Delta GLC}{MM_{GLC}}} \qquad (4.1)$$

Using Figure 2., Equation 4.1 was generated. The only pathway that consumed NADH was the path that converted PEP to SA, where 2 molecules of NADH were consumed to produce 1 molecule of SA.

The NADH (mol NADH·cmol glucose^{-1}) produced was calculated as follow:

$$\frac{\left(\frac{C_{SA}}{MM_{SA}} + \frac{2C_{AA}}{MM_{AA}} - \frac{C_{FA}}{MM_{FA}}\right)}{\frac{6\Delta GLC}{MM_{GLC}}} \qquad (4.2)$$

The path from glucose to PEP produced 2 molecules of NADH per one glucose molecule consumed and from PYR to AA, 1 molecule of NADH per one AA molecule was produced. All the inputs for these equations were the product concentrations obtained from the experimental runs.

In Figure 4.5, it is noted that for all the fermentation runs the ratio of NADH consumed to NADH produced increased as the concentration of YE in the growth medium increased. Nevertheless, there is a clear dissimilarity between the three runs. In R3, the NADH production was higher than the consumption for all growth media, except when only CSL was used as the nitrogen source. R2 and R3, in comparison with R1, did not display a massive imbalance in the NADH ratio and R1 was the only run where NADH consumed over produced ratio consistently increased significantly as the YE content in the growth medium increased. It appears that the additional NADH generation capability of R1 was not fully present in R2 and R3, and possibly the strains used in R2 and R3 had altered production

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characteristics compared with the original strain. Nevertheless, the R1 results coincide with the observation of both Bradfield & Nicol (2014) and Brink & Nicol (2014), which was that there was an "excess" of NADH that was consumed during the maintenance phase/non-growth phase, resulting in a high Y_{AASA}.
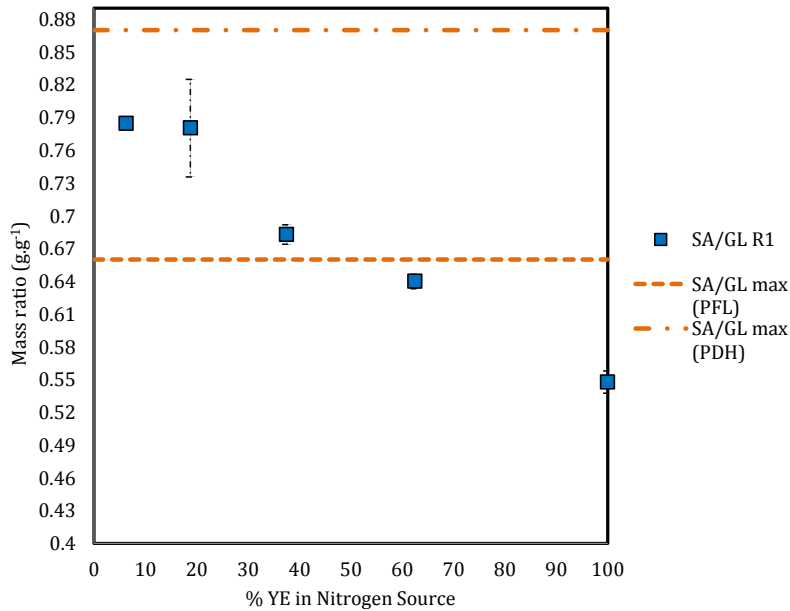
Figure 4.6: *Mass ratio of succinic acid over glucose profile of*
A. succinogenes as YE % in medium changes.*

*The total nitrogen source (combination of YE and CSL) concentration in the growth medium stayed constant at 16 g·L⁻¹, only the concentrations of YE (powder form) and CSL(liquid form) varied.

Figure 4.6 illustrates the yield of SA on glucose for R1. This graph shows how the flux from glucose to SA increases as the CSL concentration in the growth medium increases. The trend observed in Figure 4.6 can be linked to the "excess" NADH that favours the production of SA. Xi *et al.* (2013) observed that using only CSL as the nitrogen source provided high selectivity towards SA (4,58 g·g⁻¹) in comparison with other by-products. On the other hand, CSL with heme provided a high glucose-to-SA flux (0,72 g·g⁻1) and a much higher Y_{AASA} (4,93 g·g⁻¹). A similar observation was

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documented by Yan *et al.* (2013). Xi *et al.* (2012) tried to explain this observation in their study by stating that CSL contained cofactor biotin which promoted the high SA selectivity.

It is reasonable to postulate that there is an ingredient in CSL that may trigger or enhance the activity of an additional metabolic pathway, known as the pentose phosphate pathway (PPP), to produce high carbon flux to SA and high SA selectivity. The idea of PPP as the source of the additional reducing power stems from the findings of Bradfield and Nicol (2014). Their analysis has shown that if the NADPH generated in the PPP cycle is converted to NADH in order to supply redox to the SA pathway, then high SA selectivity can be achieved. *A. succinogenes* has the transhydrogenase that allows PPP to be active and, as mentioned earlier, the presence or absence of one or more ingredients in CSL may allow this enzyme to be active, consequently activating the PPP as well.

Xi *et al.* (2012) did mention biotin as the culprit for the high SA selectivity. They investigated the influence of biotin on *A. succinogenes* growth and SA productivity by varying the biotin concentration in a chemically defined medium. There was no SA production and cell growth was severely inhibited when biotin was absent from the growth medium. They reported a high SA yield in their study, but their data for AA concentration, Y_{GLAA} and Y_{AASA} were inconsistent with their observed product concentrations. The following example is extracted from their results. In the experiment using 8 mg L^{-1} biotin, the glucose consumed was 30.0 g L^{-1}, C_{SA} = 19.4 g L^{-1}, C_{AA} = 3.2 g L^{-1}, Y_{GLAA} = 0.203 g g^{-1} and Y_{AASA} = 6.1 g g^{-1}. However, the Y_{GLAA} should have been 0.107 g·g^{-1} if C_{AA} was reported correctly, but if the value for Y_{AASA} was used as the value of C_{AA}, then the reported Y_{GLAA} would be correct.

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Nevertheless, because of the inconsistency of the data, it cannot be concluded that biotin did play a role in gaining a high SA yield.

Either the presence or absence of a component in a growth medium can influence the enzymatic activity of a pathway and this concept should be used to postulate explanations for the results obtained in this study. A breakdown of the constituents of CSL and YE could help in identifying the ingredients in CSL that may have triggered or enhanced the activity of the PPP. Since both CSL and YE are complex media and there is no definite information on the ingredients, it is hard to fully confirm the following theory.

Xiao *et al.* (2013) did a study on the constituents of CSL and stated that any grade of CSL has the following amino acids: isoleucine, asparagine, methionine, lysine, proline and aspartic acid. YE was reported, by an industrial data sheet (Organotechnie® S.A.S, France), to have a high content of the following: alanine, isoleucine, leucine, methionine, phenylalanine, tyrosine and valine. Although there are common amino acids shared between CSL and YE, there is one amino acid YE has that is absent from CSL, namely leucine. This could be a reason why CSL could be related to the excess NADH.

Leucine, in *E.coli*, is said to repress the transhydrogenase activity, which in turn represses the PPP (Gerolimatos & Hanson, 1978). This could be true for *A. succinogenes*, since it also has the transhydrogenase for PPP, as mentioned in Section 2.3. *A. succinogenes* with only YE as the nitrogen source produced yields that were expected according to the carbon and redox balance. It can be postulated that as the YE content reduce, the

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leucine content decreased and the activity of the transhydrogenase increased, causing additional NADH to be generated from the enhanced PPP, resulting in improved yields.

5.   Conclusions

The complex nitrogen requirements of non-growing *A. succinogenes* biofilms were evaluated. The study restricted itself to the use of YE and CSL as nitrogen sources. The SA productivity, yields and titre were the variables assessed.

The YE concentration was decreased while simultaneously increasing the CSL concentration in the medium. Although this led to a decrease in the steady state productivity of the organism, overall SA selectivity increased. The production of certain metabolites such as SA and pyruvate also increased while FA and AA concentrations decreased, in the increasing absence of YE. This led to an increase in the SA to AA mass ratio and to a decrease in the FA to AA mass ratio. When zero amounts of YE were in the growth medium, no FA was produced.

Three fermentation runs were performed. The highest SA concentration in this study was 22,57$g\cdot L^{-1}$, when only YE was used as the nitrogen source in the growth medium, and the highest SA productivity obtained in this study was 1.58 $g\cdot L^{-1}\cdot h^{-1}$, when a combination of YE(62.5%) and CSL(37.5%) was used as the nitrogen source.The highest SA to AA mass ratio achieved was 8,3 $g\cdot g^{-1}$, when only CSL was used as the nitrogen source. The FA to AA mass ratio was consistently less than 0,77 $g\cdot g^{-1}$, approaching 0 $g\cdot g^{-1}$ as the CSL concentration in the nitrogen source increased.

Lower FA to AA mass ratio values were not fully responsible for the increase in the SA to AA mass ratio. The surplus NADH required is provided

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by the activation or enhancement of an alternative metabolic pathway, i.e. the pentose phosphate pathway.

The observations made in this study bolster the idea of using a combination of YE and CSL, where YE is the minor component, as the nitrogen source for commercialisation of bio-based succinic acid production using *A.succinogenes* as the catalyst.

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6.   References

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