ESTABLISHING A BIOCONVERSION PROCESS FOR THE PRODUCTION OF SUCCINIC ACID USING INDUSTRIAL FEEDSTOCKS

Frances Faith O'Brien

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University of KwaZulu-Natal

Durban, South Africa

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Supervisor: Prof. Bruce Sithole

Co-supervisor: Dr Santosh Ramchuran and Dr Viren Chunilall

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Supervisor

Prof. Bruce Sithole

Co-supervisor

Dr S. Ramchuran

Co-supervisor

Dr V Chunilall

Declaration 2 – Publication

This work is in preparation to be published in the following journal publications:

Publication 1

O'Brien, F.F, Ramchuran, S. and Chunilall, V. (2022) 'Assessing the succinic acid production capabilities of wild type *Corynebacterium gluctamicum* using industrial feedstocks'

Signed:

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This dissertation is dedicated to my baby girl, Hannah. Hannah, you allowed me to full-fill my biggest heart's desire of being a mother. I love you very much. Thank you for displaying how great God's love is for us.

Abstract

One of the leading challenges of the current global situation is the decline of non-renewable, fossil fuels. Due to this rapid depletion, there is a shift towards replacing petrochemical products with equivalent, ideally superior bio-based substitutes. The bio-chemical of interest that was studied in this work is bio-succinic acid which is considered a platform chemical. Bio-based procedures have the attractive advantage of potentially obtaining a high-value product from an underutilised product/waste stream. In this dissertation, the industry that was focused on was the sugar sector, this vital industry is under pressure it is therefore crucial that alternative revenue avenues are identified.

A literature study highlighted the importance of succinic acid, detailed both the upstream and downstream literature methods and addressed the impact that biochemical processes could have within South Africa. Small scale flask studies were conducted using succinate-producing microorganisms, on synthetic C5 and C6 sugar medias, namely xylose and glucose. The results from these studies showed that *L. paracasei* and *C. glutamicum* were the top performing strains on the C6 sugar (glucose) media and as a result these strains were then grown on C6 industrial material, namely sugarcane juice and molasses. These flask studies concluded that *C. glutamicum* grown on molasses was the superior combination, with a succinic acid concentration of 18.81 ± 0.75 $g.L^{-1}$ and a productivity of 0.67 ± 0.07 $g.L^{-1}.hr^{-1}$ being achieved. The process was then successfully scaled up to 30L reactors where a succinic acid concentration of 28.89 ± 3.57 $g.L^{-1}$ was reached, which was higher than the 'ideal' glucose reactor run. Downstream processing of the harvested broth was conducted using the precipitation method. Process development was performed, and the final method resulted in a final succinic acid recovery of 54.47 ± 14.02 % and 58.20 ± 2.24 % for the glucose and molasses-based medias respectively. In conclusion, molasses has the potential as an alternative carbon source in the production of succinic acid. The biochemicals sector is still a novel concept within South Africa, and as this platform gains more traction such studies show the 'value' of industry's waste/by-product streams, especially for the sugar industry.

Contents

List of Figures

List of Tables

List of Acronyms

SA	succinic acid
TCS	tricarboxylic acid cycle
BDO	butanediol
USA	United States of America
ROW	rest of world
facult.	Facultative
obli.	Obligate
GRAS	generally recognised as sage
GMO	genetically modified organism
SASA	South African Sugar Association
SACGA	South African Cane Growers Association
USP	upstream processing
DSP	downstream processing
ktpa	kilo tonne per annum
SCJ	sugarcane juice
PFD	process flow diagram
bioSA	bio-succinic acid
PUO	process unit operation
Da	Dalton
IPAP	Industrial Policy Action Plan
SARi	South African Renewable Initative
NDP	National Development Plan
CSIR	Council of Science and Industrial Research
MRS	Modified De Man, Rogosa and Sharpe

YPG	yeast extract peptone glycerol
OD	optical density
BIDF	Biorefinery Industry Development Facility
UV	ultraviolet
HPLC	high pressure liquid chromatography
UV/Vis	ultraviolet visible
Glu.	Glucose
Xyl.	Xylose

CHAPTER 1: LITERATURE REVIEW

1.1. Problem statement

Can bio-succinic acid be successfully produced and purified to replace traditional petrochemical routes of succinic acid production. Additionally, can industrial by-product streams from the South African sugar industry be used in this process as part of their current product diversification initiative.

1.2. Aims and Objectives

The main aim of this work is to ensure bio-succinic acid can be produced from one of the sugar industry's by-product streams. The objectives of this dissertation were to:

- Perform a literature review on succinic acid to gain an understanding of the chemical, its market, the traditional methods, the upstream and downstream processing options and the social and economic impact.
- Perform small-scale flask studies to determine running parameters, such as the type of microorganism and carbon source (glucose and alternative industrial by-product streams) that results in succinic acid production.
- To scale-up the process to a Biostat reactor and do comparative studies to determine the succinic acid production capabilities of the industrial stream.
- To perform the downstream processing section, to determine the optimum method based on literature and experimental work.
- To draw up an overall process flow of the determined process and make recommendations for further work required/suggested to improve the succinic acid process.

1.3. Introduction

Succinic acid (SA) is a dicarboxylic acid also known as butanedioic acid, 1,2-ethanedicarboxylic acid or amber acid, the chemical structure is shown in Figure 1-1. SA is an intermediate in the tricarboxylic acid cycle (TCA) or Krebs cycle (Beauprez, De Mey and Soetaert, 2010), hence it can be produced biologically.

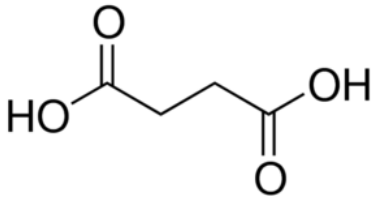

Figure 1-1: The chemical structure of succinic acid

Succinic acid has a colourless/white crystalline appearance. Other important properties that need to be known, especially for the downstream processing steps, are displayed in Table 1-1.

Table 1-1: Properties of succinic acid

Property	Unit	Value
Molecular formula	-	$C_4H_6O_4$
Molecular weight	$g.mol^{-1}$	118.09
Melting point	ºC	185 - 190
Boiling point	ºC	235
Size	Da	118.09
pKa_1	-	4.2
pKa_2	-	5.6

The carboxylic acid groups found at the ends of the molecule (see Figure 1-1) allow for numerous processing possibilities (Brink and Nicol 2014) which is why SA is regarded as a platform chemical. A platform chemical is an intermediate product in the production of various other chemicals. Due to the abundant processing options of SA, it is utilised in multiple industries including polymer, food, pharmaceutical, cosmetic, petrochemical, etc. (Song and Lee, 2006) Figure 1-2 illustrates the abundant chemical production avenues for SA.

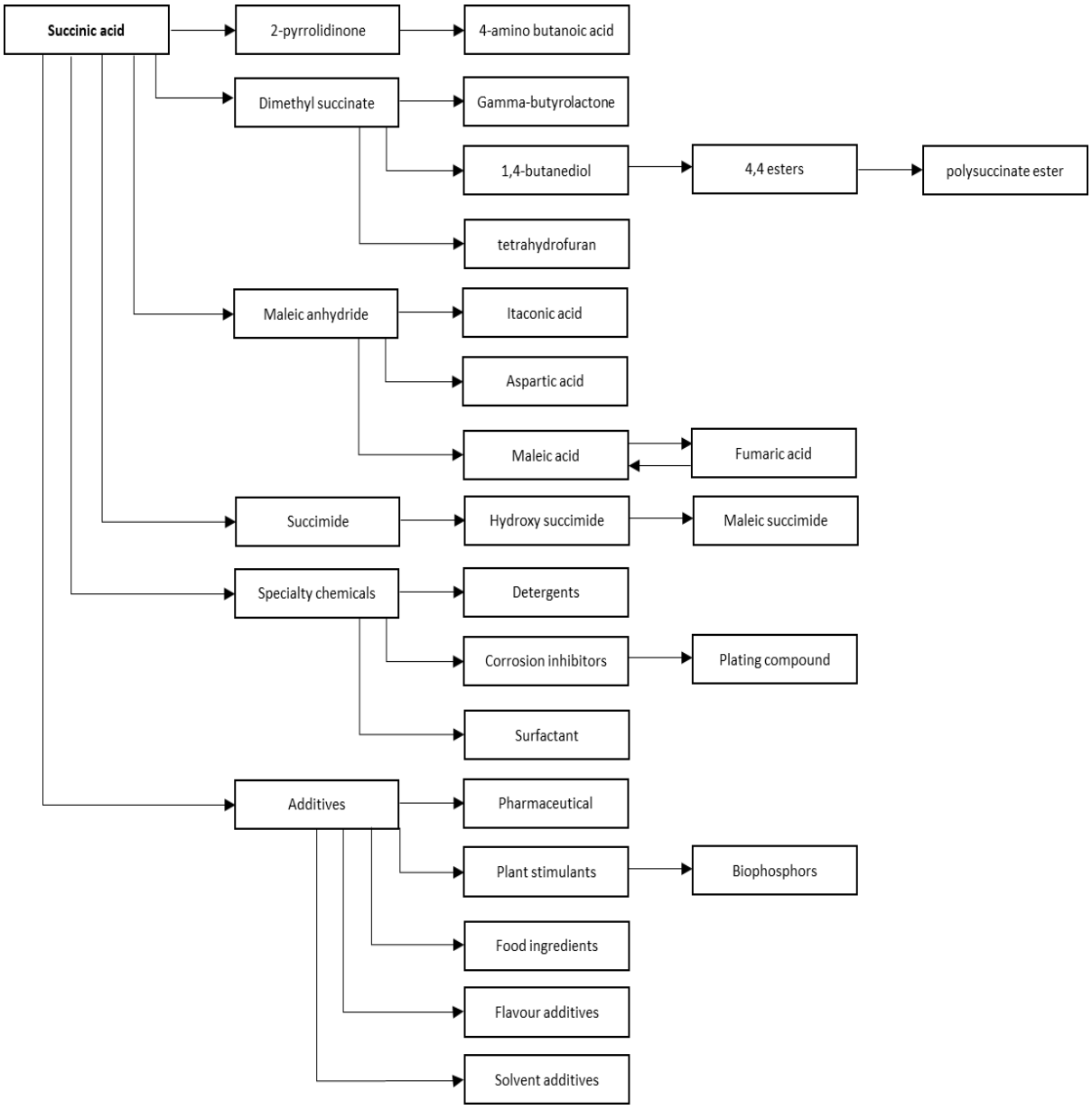

Figure 1-2: Various pathways of succinic acid use

The main end products of interest include 1,4-butanediol (BDO) and maleic anhydride. BDO is an intermediate chemical that is largely used in the production of polymers, examples include; polyesters, polyurethanes and polyethers (Silva, Ferreira and Borges, 2020). BDO is synthesised through hydrogenation reactions, that occur via succinic acid transforming to gamma-butyrolactone via hydrogenation and then successively hydrogenation of the gamma-butyrolactone with a metal catalyst to form BDO (Baidya *et al.*, 2019). BDO is utilised in a vast range of industries and is traditionally manufactured through petrochemical routes. It is

estimated that more than half of the produced SA is employed in BDO production (George, 2017) and it is the fastest growing division of the SA sector (Carvalho, Roca and Reis, 2016). Maleic anhydride is another chemical of interest, which is conventionally derived from oxidation or hydrogenation of *n*-butane and is the traditional precursor for SA. Succinic acid can be converted to maleic anhydride through heat treatment. Maleic anhydride is used in the synthesis of copolymers and resins additionally is the source for multiple acids (as depicted in Figure 2-2). Maleic anhydride and SA are very similar with regard to their chemical behaviour and structure, therefore SA can be used as an attractive substitute to maleic anhydride (Cukalovic and Stevens, 2008).

1.2. Market size

1.2.1. South African chemical market

The South African chemical industry has a significant impact on the economy. It employs roughly 105 690 people (Department of Trade and Industry, 2017) and accounts for approximately 25% of the manufacturing sales of which 55% is from petrochemical production (Majozi and Veldhuizen, 2015). The South African chemical and petrochemicals industry is expected to increase by 2 - 4% each year (Majozi and Veldhuizen, 2015). A majority of the sales is from the petrochemical sector. However, this reliance on non-renewable fossil fuels is on the decline and the use of natural resources as alternative for these chemicals in demand. South Africa's chemical industry has been slow in its transition to green chemistry and currently the prices of bio-based chemicals are higher than those of petrochemical products. However, these bio-based chemicals are becoming more and more competitive and it is predicted that these prices will outcompete the petrochemical/traditional processes in the near future (Meijer, Nielsen and Olsson, 2008; de Jong *et al.*, 2020).

Various challenges are encountered by this industry, such as large chemical import volumes compared to the export statistics, poor employment figures, a reduced demand due to global economics, increasing utility costs, poorly developed downstream processing sectors, etc. (Department of Trade and Industry, 2018). One of the main challenges of the country's chemical industry is that it largely relies on imported raw materials, which are subject to international levies therefore reducing the profit margin for the manufacturers (Majozi and Veldhuizen, 2015).

1.2.2. Bio-based chemicals market

In 2017 the global bio-based chemical market was valued at US $8.81 billion and due to the shift towards a more sustainable economy this market is expected to have a compound annual growth rate of 12.6% from 2018 to 2025 (Grand View Research, 2019). The focused drive towards a bio-based economy is as a result of various factors, which include (Department of Science and Technology, 2013):

- Depleting and non-renewable sources of fossil fuels
- Efforts to reduce the environmental impacts, such as climate change
- Stimulating development in rural areas through direct and indirect employment and the development of skills
- Increasing population figures
- Ensuring security in supply through local manufacturing

The 2011 global production of chemicals and polymers through bio-based pathways versus petrochemical routes is graphically represented in Figure 1-3 (Higson, 2011).

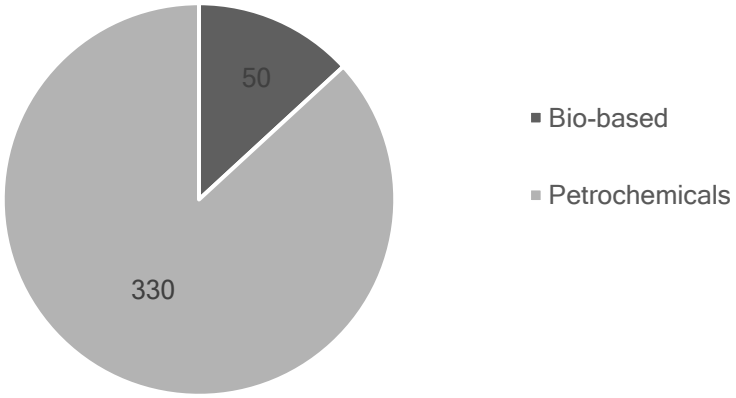

Figure 1-3: Comparison between bio-based and petrochemical production of chemicals and polymers, presented in million tonnes.

Bio-based chemicals can be produced through chemical, thermochemical or fermentation processes. Fermentation processes can utilise ‘green chemistry’ principles, which are favourable over conventional petrochemical processes due to the use of sustainably sourced materials. The global market for fermentation derived fine chemicals was estimated at $22 billion (equivalent to R330 billion) in 2013, of which the organic acid sector accounts for 22%

of this value (de Jong *et al.*, 2012). The substitution of petrochemicals with bio-based ones will need to prove superior to already optimised processes, through being cost effective and environmentally friendlier for the shift to occur (Haigh *et al.*, 2020) (Cheng *et al.*, 2012).

1.2.3. Succinic acid market

The global SA market size was 76 000 tonnes/year (Taylor *et al.*, 2015) of which 5% was bio-succinic acid (bioSA). It was estimated that the SA demand will be 600 000 tonnes/year by 2020 (E4tech Ltd, 2015) which translated to a value of $1 billion (George, 2017). Information of SA market values within South Africa is hard to find, therefore extrapolating data from predicted global information is required: Such data is represented in Figure 1-4 (Inkwood research, 2016). South Africa would fall into the Rest of World, "ROW", global category.

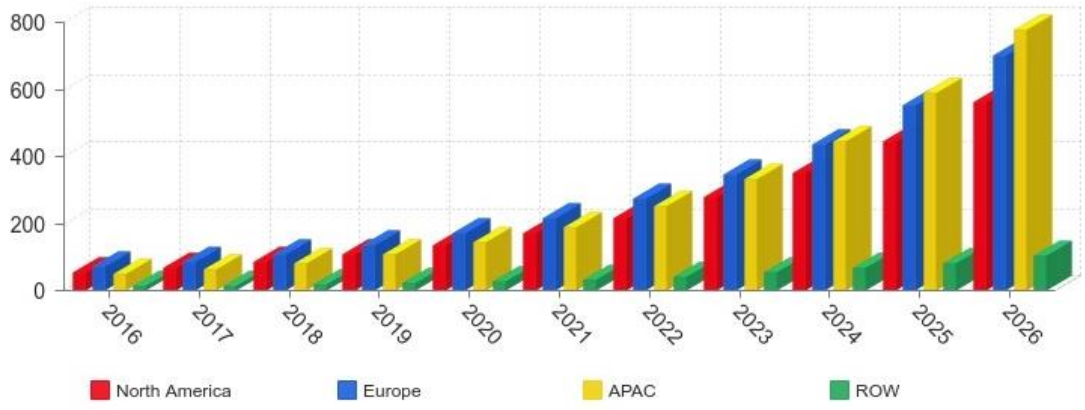

Figure 1-4: Global succinic acid predictions in $ millions, until year 2026.

Figure 1-4's data shows that "ROW" will be roughly $100 million and that SA market is expected to grow ~28.03% by the year 2026 (Inkwood research, 2016).

1.3. Succinic acid production processes

1.3.1. Traditional process

Traditionally, SA is produced through the partial oxidation of butane to maleic acid, after which hydrogenation reaction results in succinic acid, as presented in Figure 1-5 (George, 2017).

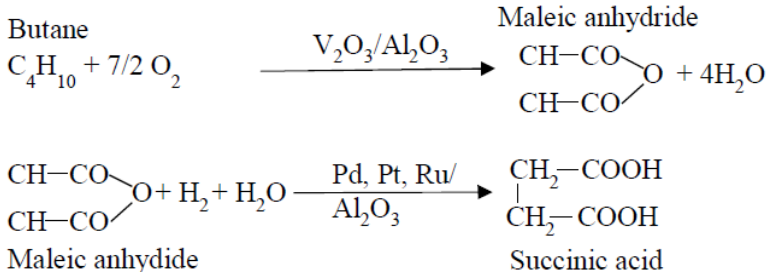

Figure 1-5: Chemical reactions for production of succinic acid from butane (George, 2017)

The traditional process has multiple disadvantages which include expensive catalysts, time consuming downstream processing due to low purities obtained, heavy pollution (Saxena *et al.*, 2017), high operating temperatures and pressures, and a yield that is less than 40% (George, 2017).

1.3.2. Biological process

SA is an intermediate product in a microorganism's TCA cycle. Production of SA through the use of microorganisms is gaining more momentum due to it being an environmentally friendlier alternative compared to the traditional method.

BioSA can be produced from the abundant sugars found in a plant's biomass, these include glucose, fructose, arabinose and xylose. Glucose is the most popular sugar used for this reaction, with an aerobic theoretical yield of 1 mole succinate per mole of glucose, which increases to 1.71 mole succinate per mole of glucose in anaerobic conditions, shown in Equation 1.1 (McKinlay, Vielle and Zeikus, 2007).

$$C_6H_{12}O_6 + 0.86HCO_3^- \rightarrow 1.71Succinate^{2-} + 1.74H_2O + 2.58H^+ \quad (1.1)$$

If carbon dioxide is present, as well as hydrogen, the theoretical yield increases to 2 moles as shown in Equation 1.2 (McKinlay, Vielle and Zeikus, 2007).

$$C_6H_{12}O_6 + 2HCO_3^- + 2H_2 \rightarrow 2Succinate^{2-} + 2H_2O + 2H^+ \quad (1.2)$$

1.3.2.1. Succinic acid producing microorganisms

SA is produced through metabolic pathways of cellular respiratory cycles, that vary according to the type of microorganism used (Song and Lee, 2006). There are various groups of microorganisms that can produce SA which include fungi, yeasts and bacteria. Generally, fungi are not focused on due to their low yields and difficulties in fermentation, separation and purification (Song and Lee, 2006; Yang *et al.*, 2016). Various yeast species have been studied to show high concentrations of SA (Song and Lee, 2006). Numerous bacteria species have been researched, it is well established that the rumen bacteria; *Mannheimia succiniciproducens*, *Actinobacillus succinogenes* and *Fibrobacter succinogenes* can sufficiently produce SA (Kaboré *et al.*, 2017). Industrially *A. succinogenes* is the most popular microorganism used due to its wide range of carbon source utilisation (Zhang *et al.*, 2016) (Ferreira and Carvalho, 2015).

SA can be produced both aerobically and anaerobically, as illustrated in Figure 1-6 (Nghiem, Kleff and Schwegmann, 2017), with the thick blue arrows indicating aerobic steps (oxidative) whereas the thick grey arrows are the anaerobic steps (reductive). Aerobic production of SA has the advantages of higher and quicker cell-biomass production, shorter doubling time, faster carbon throughput and product formation and does not, necessarily, require as complex/nutrient rich media (Leszczewicz and Walczak, 2014). The main disadvantages are that there is a decline in theoretical yield of SA, and that in many aerobes the production of SA in the TCA cycle is only as a transient molecule (Alexiou, 2017).

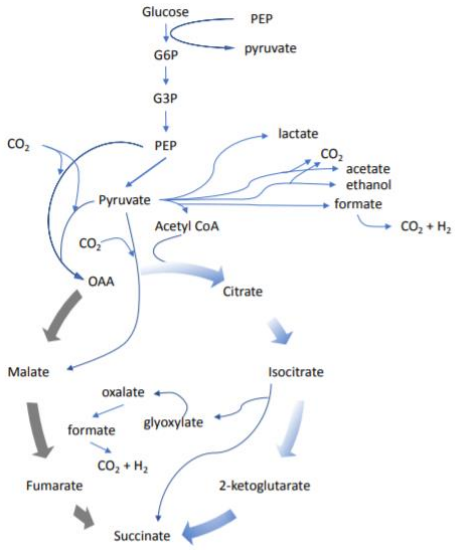

Figure 1-6: Aerobic (thick blue arrows) and anaerobic (thick grey arrows) metabolic pathways to succinic acid

A majority of the succinate producing organisms fall into the anaerobic cluster. Table 1-2 shows the classification of popular succinate microorganisms. The ‘aerobic’ and ‘anaerobic’ microorganisms can be further classified into facultative (facult.) or obligate (obli.), such classification is an important aspect to consider.

The advantages and disadvantages of each of the listed microorganisms (Table 1-2) will be examined in Table 1-3, to assist with the selection for the experimental work.

Table 1-2: Succinate producing microorganisms

Type	Species	Aerobic		Anaerobic		Reference
		Obli.	Facult.	Obli.	Facult.	
Fungi	*Aspergillus niger*				X	(Alcantara *et al.*, 2017)
Yeast	*Saccharomyces cerevisiae*				X	(Otero *et al.*, 2013)
	Yarrowia lipolytica	X				(Cui *et al.*, 2017)
Bacteria	*Escherichia coli strains*				X	(Bechthold *et al.*, 2008) (van Heerden and Nicol, 2013)
	Actinobacillus succinogenes				X	(Bechthold *et al.*, 2008) (Song and Lee, 2006)
	Mannheimia succiniciproducens				X	(Song and Lee, 2006)
	Anaerobiospirillum succinicproducens			X		(Song and Lee, 2006)
	Corynebacterium glutamicum				X	(Leszczewicz and Walczak, 2014)
	Basfia succiniciproducens				X	(Pateraki *et al.*, 2016)

Table 1-3: Advantages and disadvantages of succinate producing microorganisms

Microorganisms	Advantages	Disadvantages	References
A. niger	• Classified as a GRAS organism • Natural ability to secrete organic acids • Fast growing • Acid and thermos-tolerant • Broad pH and temperature range • Utilises different carbon sources	• Not a naturally high producer of SA • SA produced within the mitochondria (harder to excrete) • Fungi shows difficulties in fermentation, separation and purification	(Upton, *et al.,* 2017) (Roca, 2015) (Song and Lee, 2006)

Microorganisms	Advantages	Disadvantages	References
S. cerevisiae	• Classified as a GRAS organism • Robustness and endurance towards stressful environments • Rapid growth • Cell activity in acidic conditions • Utilise different carbon sources • Produces desired quality of SA	• Fewer cloning vectors available • Low yields • Less research available using this microbe for SA production • SA is produced within the mitochondria	(Jegede, 2019). (Reis *et al.*, 2013)
Y. lipolytica	• Robust • Non-pathogenic yeast • Classified as GRAS • High toleration to low pH (optimum for SA production)	• Inefficient use of glucose-based media • Sensitive to low pH • Low productivity	(Ong *et al.*, 2019) (Li, *et al.*, 2019)
E. coli	• Well researched bacteria • Fast growing • Many cloning vectors available and easy gene expression • High SA yields possible • Can be grown in inexpensive media	• High endotoxin content • Subject to bacteriophage • Wild types produce low yields of SA • Various mixed acid by-products formed, resulting in higher purification costs	(Huang *et al.*, 2019) (Jegede, 2019) (Roca 2015) (Song and Lee 2006)
A. succinogenes	• High tolerance to succinate salts • Utilise different carbon sources • Wild types can produce SA • High yields of SA reported	• Requires specific nitrogen and vitamin sources • Neutral pH required • Limited in genetic modifications	(Jegede, 2019) (Pateraki *et al.*, 2016)

Microorganisms	Advantages	Disadvantages	References
	• Natural succinate overproducer		
M. succiniciproducens	• Little/no formation of lactic, acetic, or formic acid by-products • Wild types can produce SA • Utilises different carbon sources • Natural succinate overproducer	• Exhibits many auxotrophies	(Jegede, 2019) (Beauprez, *et al.*, 2010) (Song and Lee 2006) (Pateraki *et al.*, 2016)
A. succiniproducens	• High tolerance to succinate salts • Utilise different carbon sources • Natural succinate overproducer	• Various mixed acid by-products formed, resulting in higher purification costs • Unknown genomic information	(Jegede, 2019) (Song and Lee 2006)
C. glutamicum	• Promising productivity and yields • Utilise different carbon sources	• Unable to utilise numerous renewable resources • Produces a range of metabolic waste products	(Jegede, 2019) (Leszczwicz, *et al.*, 2014)
B. succiniciproducens	• Wild types can produce SA • High natural SA yield • Ability to detoxify a few inhibitory molecules often found in hydrolysates • Utilise different carbon source	• Less research available using this microbe for SA production • Undesirable metabolic fluxes	(Carvalho, Roca and Reis, 2016) (Cimini *et al.*, 2019)

Since many SA producing microorganisms reported in literature are genetically modified organisms (GMOs), it is therefore important to compare natural (wild) versus metabolically engineered organisms. Most of the natural production hosts detailed in literature have been isolated from the rumen of ruminants (Beauprez, *et al*., 2010). Many natural bioSA producers are classified as capnophilic i.e., require carbon dioxide (Beauprez, *et al*., 2010). Wild types exhibit high tolerance to osmotic pressure which is caused by high levels of succinate (Cao, *et al*., 2013). It has been reported that wild strains do not produce SA in high enough quantities and require expensive nutrient media (Cao, *et al*., 2013). There is a strong possibility that the wild strains will not yield sufficient SA results, this is further emphasised in literature where a majority of metabolically engineered organisms are utilised (Beauprez, De Mey and Soetaert, 2010). Metabolic engineered microorganisms allow for improved production yields of SA by eliminating competitive pathways; the required genetic modifications will be different for aerobic and anaerobic processes.

1.4. Overview of the South African sugar industry

Sugarcane was first grown in South Africa in the late 1840's and was ascertained to be highly successful crop, especially in the KwaZulu-Natal province and soon after sugarcane mills were built (Lewis, 1990). The sugar industry is currently dominated by the following companies; Illovo Sugar Ltd, Tongaat-Hulett Sugar Ltd and TSB Transvaal Sugar Ltd (National Development Agency, 2018).

The South African sugar sector is one of the world's leading producers of high-quality sugar and is important from social and economic aspects. It contributes an estimated R2 billion annually to the country's foreign exchange earnings (National Development Agency, 2018). Like many industries it is facing sustainability issues, however this leads to opportunities to diversify. The industry has been experiencing troubling times mainly due to the implementation of the sugar tax in 2018, which has resulted in loss of profit.

There are multiple potential pathways to biofuels and biochemicals through the sugar platform, and so this study will focus on bio-succinic acid production using sugarcane as a feedstock material. Success could lead to additional profit lines for the sugar industry from sugar cane product/waste streams that are underutilised.

1.4.1. Challenges

The sugar industry is a significant sector within South Africa with one million people being dependent, directly and indirectly, on this industry (National Development Agency, 2018). South Africa is ranked in the top 15 countries out of the total 120 sugar-producing countries, it is therefore important to ensure that this sector thrives irrespective of the challenges faced. The South African sugar tax also known as the 'health promotion levy', came into effect from the 1 April 2018, with an inflationary increase of 5.2% occurring on 1 April 2019 (Schneider, 2019). The South African Sugar Association (SASA) estimated that the local demand for sugar will decline by 200 000 tons a year, an equivalent of R1 billion annually, due to the implementation of this sugar tax (Schneider, 2019). The South African Cane Growers' Association (SACGA) have reported a decrease in sales and prices, and has reported a 64% loss, which is the equivalent of R592 million or potentially 6500 jobs (Schneider, 2019). Along with the sugar tax, additional concerns for the sugar industry are the fluctuating and plummeting sugar price, increasing energy costs, long-standing (old) infrastructure and the increase in cheaper import options available (majority coming from ESwatini) is placing a great deal of strain on the industry. There has been a heightened apprehension amongst the small-scale growers, who account for ~25 000 out of the 26 000 sugarcane growers, i.e., 96%, (breakdown given in Figure 1-7) who rely on the crops for their livelihood (Department of Agriculture, 2014). Figure 1-7 (Department of Agriculture, 2014) illustrates how the largest majority of sugarcane crops are grown by large-scale growers, however the highest percentage of personnel are located in the 'small-scale' category. The strains on the sugar industry could results in a significant amount of job losses and consequently, lead to the closure of sugar mills.

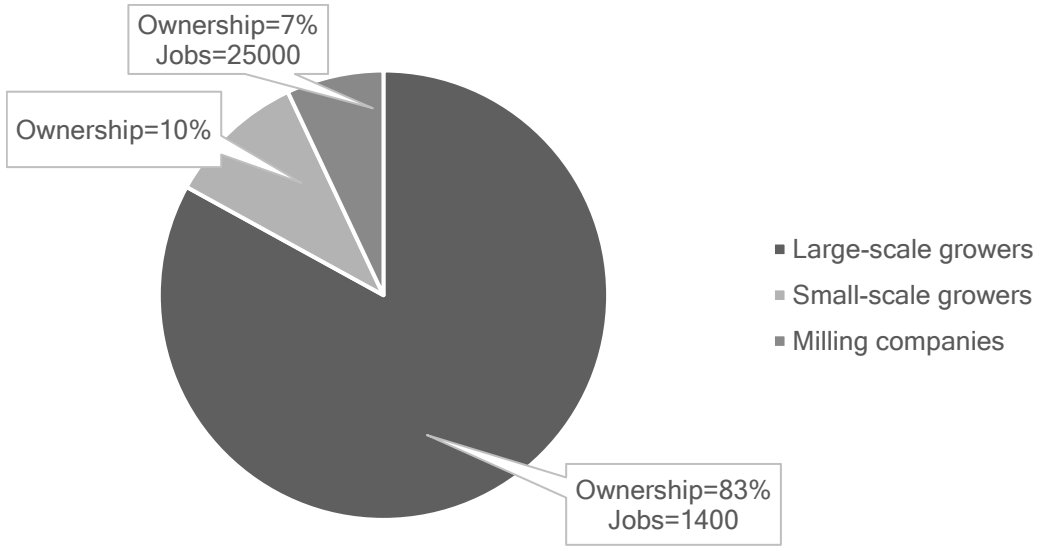

Figure 1-7: Sugarcane crop ownership within the different sugar sectors

Due to such challenges faced by the sugar industry, it is necessary for alternative downstream production opportunities to be researched and implemented to provide stability and sustainability for one of South Africa's essential industries. South Africa's government has recognised that the sugar industry is in crisis and has recently implemented a 'Sugar Industry Master Plan' to pull the industry out of its decline. The plan ensures that industrial users and retailers to a minimum off-take of 80% sugar from local sugar industry which will increase to 95% by 2023 (Department of Trade Industry and Competition, 2020). Diversification of sugar cane products beyond sugar is recognised as a pivotal route to survival of the industry under the recently promulgated Sugar Master Plan (Department of Trade, Industry and Competition, 2020).

1.4.2. Sugarcane derived products and biomass as a potential feedstock

Biosynthesis reactions of microbes require a carbon source which is utilised for reproduction, formation of products, and cell maintenance (University of KwaZulul Natal, 2014). The carbon sources used in industrial fermentations are generally purified sugars or corn syrups. Substituting this source with agricultural residues i.e., sugarcane biomass, will allow for a bio-based process to produce SA.

The sections of sugarcane, *Saccharum officinarum,* that could be utilised in bioconversions include the leaves, bagasse, sugarcane juice and molasses. Figure 1-8 (Cotlear, 2004) shows the typical processing steps that are followed from harvesting the cane to obtaining raw sugar, and the different points at which the various sugarcane unit operations, could potentially be used in this study.

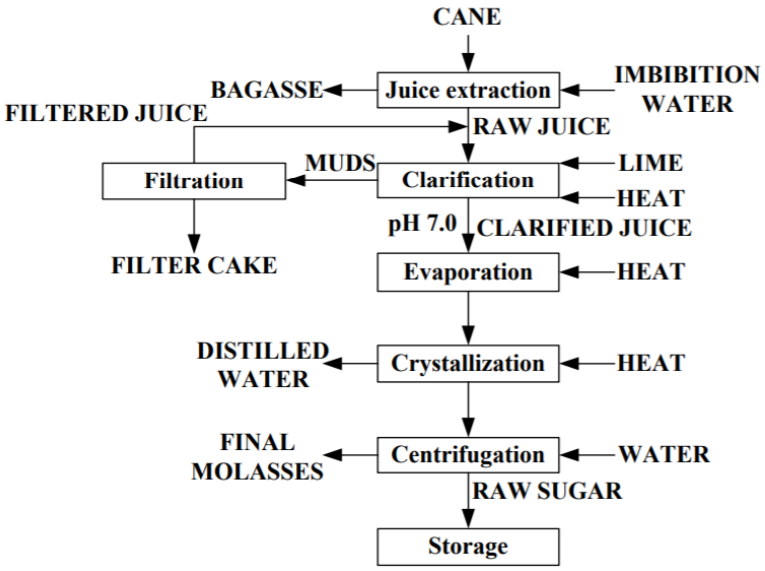

Figure 1-8: Processing steps to obtain sugar

Bagasse, sugarcane juice (SCJ) and molasses have been researched in the production of SA using various microbes, as detailed in Table 1-4.

Table 1-4: Sugarcane biomass material used as carbon sources for succinic acid production

Biomass	Microorganism	Aerobic/ anaerobic	SA titre ($g.L^{-1}$)	References
Molasses	*E. coli W3110*	Dual	26.2	(Agarwal *et al.*, 2007)
	E. coli AFP111	Dual	37.3	(Ma *et al.*, 2014)
Bagasse	*E. coli BA305*	Dual	39.9	(Liu *et al.*, 2012)
	Y. lipolytica	Aerobic	33.2	(Ong *et al.*, 2019)
SCJ	*A. succinogenes GXAS137*	Anaerobic	62.1	(Shen *et al.*, 2016)

1.5. Processing steps for utilising sugar in the production of chemicals

The production of SA is divided into upstream (USP) and downstream processing steps (DSP). The upstream steps are inclusive of all the sections that involve the growth of the microbes whereas the downstream processing steps are conducted on the material containing the product of interest, produced in the USP section, to obtain the desired quality of SA. The basic process to produce SA is shown in Figure 1-9. There will be multiple process unit operations that will be followed in the DSP section, however for simplicity and due to there being many options available, these have been summarised as 'Purification' in Figure 1-9.

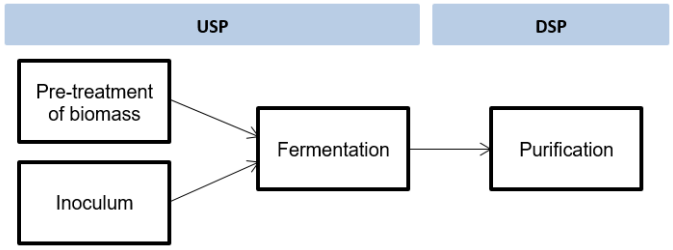

Figure 1-9: Overall upstream and downstream processing steps

Further details of each of these processing unit operations are detailed in the sections that follow.

1.5.1. Pre-treatment

Pre-treatment of biomass material will vary and will be dependent on the type of material that is being processed as well as the form in which the biomass material is received. The different pre-treatment processing steps that could theoretically be followed for each of the potential sugarcane materials are described below.

1.5.1.2. Sugarcane juice

SCJ does not require numerous pre-treatment steps, such steps could include extraction of juice and pH and sugar concentration adjustments. Extracting the juice from sugarcane is a simple process however some of the disadvantageous of using SCJ include low storability and microbial decomposition (Zabed *et al.*, 2014). A process flow diagram (PFD) of the juice extraction process is given in Figure 1-10.

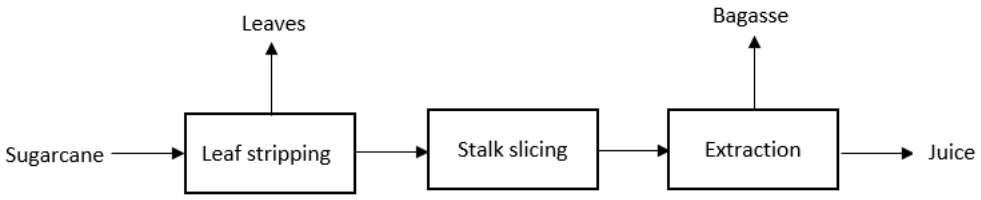

Figure 1-10: Basic sugarcane juice extraction process

The extraction method can either be done through crushing or diffusion. The crushing method is the traditional process and usually consist of roller mills whilst diffusion is a chemical process that uses osmosis to extract the sugar from the cane into water.

1.5.1.3. Molasses

Sugarcane molasses' pre-treatment will be dependent on the type of molasses obtained for the experimental work. Molasses that contains simple sugars do not require any pre-treatment steps, these fermentable sugars contain mainly sucrose, glucose and fructose. However, molasses that require pre-treatment will need either hydrolysis or enzyme treatment, which are complicated processes, to obtain such sugars from the cellulosic material (Ma, *et al.*, 2014).

1.5.2. Fermentation processes for production of succinic acid

1.5.2.1. Industry

There are several companies who have started with the production of bioSA, Table 1-5 (Pateraki *et al.*, 2016; George, 2017; Jegede, 2019) lists these companies.

Table 1-5: Major bio-succinic acid producing companies

Company	Country	Capacity (ktpa)	Strain	Biomass/ raw material
BioAmber Inc.	France	2.0	*E. coli*	Wheat glucose
	Canada	7.0	*E. coli* *S. cerevisiae*	Corn glucose
Myriant Technologies	USA	13.6	*E. coli*	Corn glucose
Reverdia	Italy	10.0	*S. cerevisiae*	Starch/ sugars
Succinity	Spain	10.0	*B. succiniproducer*	Glycerol/ sugars

The table above shows the commercial companies producing succinic acid, detailing the microorganisms and carbon sources utilised, again it should be highlighted that these are all international companies, due to no national enterprises existing.

1.5.2.2. Fermentation

The inoculum stage is known as the 'starter culture' and its primary object is for cell growth, after which it enters the biomass is transferred to the fermentation stage. The fermentation step is of great interest and a crucial stage, as it influences the quantity of SA produced. Generally the concentration of succinate in the fermentation broth is low, usually about 5 - 15% in a glucose-based media (Cheng *et al.*, 2012). The fermentation media and associated parameters will be detailed below.

1.5.2.2. Media

The media makeup is an important aspect in the experimental design as the nutrient sources are crucial in achieving the following (University of KwaZulu-Natal, 2014):

- Maximum yield and concentration of the desired product
- Maximum rate of formation of the product
- Minimum yield of undesired products

The media components may consist of the following sources: carbon, nitrogen, salts, vitamins (generally biotin and thiamine are used for SA) and minerals. A rich media may result in high yields of SA however the economical aspect needs to be considered and whether such expense could be industrially implemented.

The two media components that are of great importance are the carbon and nitrogen sources. The carbon source is the energy source required by microbes for growth. The carbon source could be sucrose, glucose, fructose, etc. however utilising an alternative inexpensive carbon source is a vital step in the process of producing SA that is competitive with the traditional, petrochemical route. A nitrogen source is vital to promote the growth of biomass. Various nitrogen sources have been researched in SA literature, these include yeast extract, corn steep liquor powder, peanut meal, soybean meal, ammonium sulphate and urea. Yeast extract is the

traditional source used and has proven successful in the increased quantity of SA however the disadvantage of yeast extract is its high cost. Corn steep liquor powder is a good alternative however it is not as successful as the yeast extract (Shen *et al.*, 2016).

1.5.2.3. Fermentation parameters

Multiple processing parameters will need to be determined prior to experimental runs being conducted, some of the important parameters are detailed below.

- pH control

SA production by fermentation is very sensitive to pH: if the pH is too high (above its pKa value, given previously in Table 2-1) then dissociation of the succinic salts occurs which results in a decline in the quality of the SA produced. The dissociated SA will require further processing to convert it back to un-dissociated form (Jegede, 2019). Ideally SA production should occur at low pH levels, ranging between 3 and 4 (Jegede, 2019), but such low values could harm the microbes, therefore it may be necessary to control the pH. Producing SA at a low pH, i.e., without buffering agents, has a cost advantage, as Yuzbashev (2011) has estimated that to produce 1 ton of succinate, 0.5 ton of pH buffering agent is required to maintain a neutral pH as well as 1 ton of sulphuric acid is required in the purification process. These inorganic salts that are produced, over 1 ton, cannot be recycled back into the system (Yuzbashev *et al.*, 2011). The pH conditions will be dependent on the microbe utilised. If the pH is controlled, the choice of base it very important as this will determine the resultant succinate salt that is produced, Table 1-6 lists common base options and subsequent form of SA produced.

Table 1-6: Alkali pH control options and their resulting form of succinic acid

Base	Succinic acid form
Ammonium hydroxide	di-ammonium succinate
Ammonia	di-ammonium succinate
Calcium	Calcium succinate
Sodium hydroxide	di-ammonium succinate
Potassium hydroxide	Potassium succinate

- Temperature

The operating temperature in the reactors is dependent on the microbe being grown. In most cases, for bacterial SA production, the experiments have been conducted between 37 and 40

ºC (Chen *et al.*, 2014) whereas for yeasts, temperatures ranging from 28 to 30 ºC are common (Raab, *et al.*, 2010). Fungal microbes, though not popular microorganism in SA production, operate over a wide temperature range between 25 and 30 ºC (Yang *et al.*, 2016) (Sazanova, Shchiparev and Vlasov, 2014).

1.5.3. Downstream processing

1.5.3.1. Industry

Commercial companies that are currently producing bio-succinic acid (as detailed in Table 2-2) utilise different downstream processing methods:

- BioAmber Inc.

BioAmber Inc., commenced operation in 2009 in France and opened a second facility in Canada in 2013. The company holds a patent (CA 02657666) for production of esters of carboxylic acids with minimum separation and purification steps. A schematic of the process is illustrated in Figure 1-11 (Dunuwila, 2009). *E. coli* produces di-ammonium succinate through fermentation with ammonium and carbon dioxide. The *E. coli* utilised by BioAmber Inc.is a GMO that prevents the formation of any by-products, therefore reducing the need for further separation steps. The produced di-ammonium succinate undergoes partial concentration via vacuum evaporation, after which an esterification reaction occurs with alcohols and carbon dioxide to form dialkyl succinate. The final step is hydrogenation where different derivatives are formed (Dunuwila, 2009).

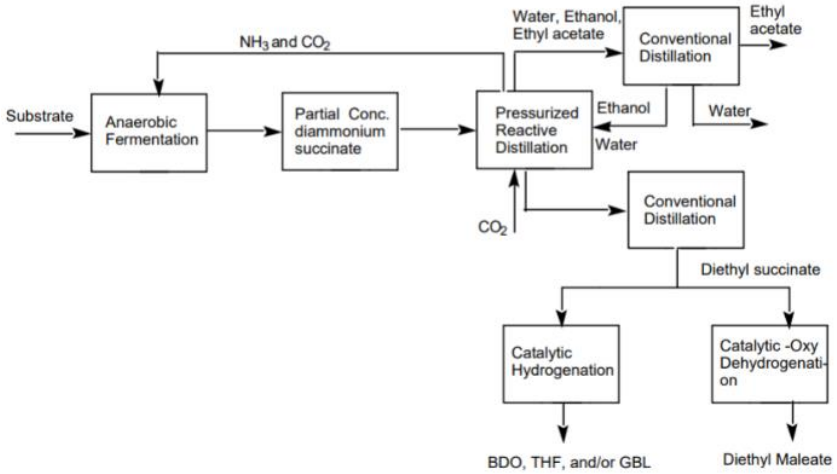

Figure 1-11: The BioAmber Inc. process for succinic acid production

- Myriant Technologies

Myriant Technologies began operations in 2013 and uses genetically engineered *E. coli* to produce succinate from sorghum grits. Their method utilises a step that converts phosphoenolpyruvate to oxaloacetate, which is the primary fermentation pathway. The process uses minimal salt media, reduces the amount of by-products formed, and increases the overall yield of the succinate to1.6 mole of succinic acid per mole of glucose (Jantama, 2009).

- Reverdia

Reverdia began operations in 2012 using a low-pH yeast instead of bacteria. The patented process is a simple and direct procedure with one of its main advantages being that feedstock is converted directly into acids unlike bacteria-based processes that require extra processing (Smidt, 2011). Other advantages include less risk of contamination, improved quality, and reduced amount of by-products. Figure 1-12 (Smidt, 2011) is a schematic of the two different processes (Smidt, 2011).

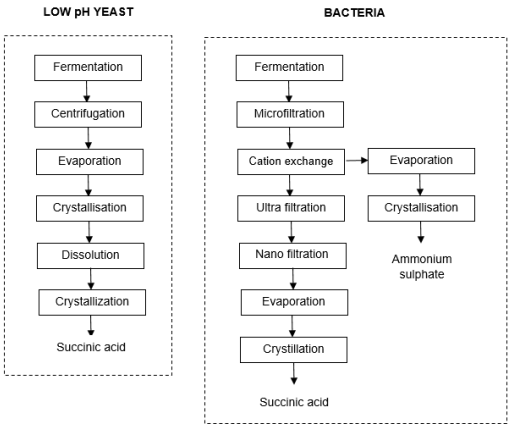

Figure 1-12: Comparison of the processing steps for yeast versus bacteria to produce succinic acid

- Succinity

Succinity was established in 2014 as a joint venture between two companies. BASF and Corbion Purac merged for the production and commercialisation of biobased succinic acid. Succinity uses various renewable substrates (allowing for flexibility) with carbon dioxide to

ferment *B. succiniciproducens*. The processing steps include downstream purification phases which result in a high quality SA with no major waste streams as it is a closed-loop process (Jegede, 2019).

1.5.3.2. Downstream processing methods

The DSP steps are the largest cost contributor in SA production, and typically account for 60-70% of the production cost (Zeikus, Jain and Elankovan, 1999). A viable industrial downstream process needs to be scalable, robust, and result in high separation yield at low cost. The isolation and purification of SA is known to be complex and difficult, due to its hydrophilicity properties and the complexity of the fermentation broth which commonly contains by-product acids, proteins, sugars, salts, cells and debris (Alexiou, 2017). There are various downstream processing steps that have been researched and experimented on for the downstream processing of SA. Usually the DSP consists of three process unit operations (PUO) (Cheng *et al.*, 2012):

1. Removal of the cells
   This step can be done through centrifugation or membrane filtration.
2. Removal of impurities and primary separation of SA from the fermentation broth.
   Filtration, precipitation, electro-dialysis, reactive extraction, solvent extraction and/or adsorption can be used.
3. Purification
   This step can be performed via vacuum evaporation or crystallisation.

Further details of each PUO are given below.

1.5.3.1. Removal of cells

Ensuring the cells are removed, if they do not contain the product, is usually the first step in the DSP activities. For the SA process, membrane filtration or centrifugation can be done to achieve this step.

1.5.3.2. Removal of impurities

This PUO entails both the removal of impurities and the primary separation of SA from the fermentation broth. Numerous methods have been tested in literature: these include filtration, precipitation, electro-dialysis, reactive extraction, solvent extraction and/or adsorption.

a. Filtration

Filtration, especially through activated carbon, is an important DSP step that is used to remove impurities such as proteins, pigments and lignosulphonates (Alexandri *et al.*, 2019). The filtration step can additionally/alternatively be done as a final clarification step.

b. Direct crystallisation

Direct crystallisation can be used as a primary recovery method for SA or as a final purification step. It is based on the solubility states of dissociated and undissociated forms of carboxylic acids that vary with pH. Studies have shown that at 4 ºC and at a pH of 2.0, the solubility of SA was only 3% whereas other by-product acids were still in their miscible form, thereby allowing for separation resulting in a 70% yield and a 90% purity of SA (Li *et al.*, 2010). High purities of SA can be achieved in commercial production when crystallisation is used in conjunction with membrane filtration and ion exchange. The advantages of direct crystallisation are the low yields of waste by-products and the few operating units that are necessary. Disadvantages are the high energy input costs, low purity yields, and the requirement for efficient removal of impurities prior to implementation of the method (Cheng *et al.*, 2012).

c. Precipitation

Precipitation is a traditional method, where a base, examples listed in Table 2-6, is used to maintain the pH of the fermentation to form succinate salts. The salts which precipitate out of the broth are removed through filtration or centrifugation, after which they are acidified with sulphuric acid to form SA. Commonly, precipitation occurs through the addition of calcium hydroxide or calcium oxide, for example, Datta et al., (1999) patented a precipitation process using calcium oxide. The subsequent acidification reaction also produces calcium sulphate (gypsum), as a low value by-product (Cheng *et al.*, 2012). The produced SA usually undergoes more downstream processing, often filtration and ion exchange, in order to obtain a further purified fraction (Alexiou, 2017).

The main advantage of precipitation is that such methods have been previously implemented in citric and lactic acid production industries, hence there is ready availability of equipment, technology and infrastructure that can be used directly or after minimal adaption for the SA sector (Cheng *et al.*, 2012). The main drawbacks are the high operational costs the due to large quantities of non-recyclable chemicals required for this procedure (Cheng *et al.*, 2012).

Patented precipitation processes (US Patent 5:034,105 and 6:265,190) avoids the formation of calcium sulphate through the addition of ammonium hydroxide with ammonia gas to form di-ammonium succinate. Ammonium bisulphate is then added to form SA and ammonium sulphate, and the SA is then recovered through methanol evaporation to form SA crystals (Yedur, Berglung and Dunuwila, 2001). These processes have higher yields than the traditional method.

d. Membrane separation

Membrane separations including microfiltration, ultrafiltration, nanofiltration or electrodialysis. Membrane filtration allows for the fermentation broth to be filtered. However, additional processing via crystallisation and ion exchange steps will be required to remove particles with lower molecular weight than SA (Alexiou, 2017). Microfiltration allows for contaminants larger than 0.1 - 0.2 µm to be retained, ultrafiltration removes impurities and proteins larger than 5000 - 15 000 Da whereas nanofiltration retains unutilised carbon sources and other compounds larger than 130 - 150 Da (Wu *et al.*, 2011). The key drawback to membrane filtration is the fouling that results in frequent replacement of membranes (Cheng *et al.*, 2012).

Electrodialysis has been investigated as a primary SA recovery method (no clarification of the fermentation broth required). Electrodialysis works with the use of charged membranes which separate a stream containing only ionic species, to produce succinate salt. Further processing such as water-splitting electrodialysis is used to convert the succinate salt to SA. Water-splitting electrodialysis results in separate streams of SA and sodium hydroxide, this has the advantage of being able to recycle the sodium hydroxide back into the process; however the SA still requires additional polishing to remove ionic contaminants (Alexiou, 2017). The advantage of electrodialysis is the high purity and the minimal waste that is formed, however there are significant membrane loss, high energy inputs, and low product yields (Cheng *et al.*, 2012).

e. Solvent extraction

Solvent extraction is based on relative solubilities of different compounds to assist with separation. Two different solvent extraction techniques are used for SA:

- Liquid-liquid extraction

Liquid-liquid extraction is conducted through three stages, namely, extraction/absorption, back extraction/desorption, and regeneration. The fermentation broth is mixed with an extractant (extractant choice is crucial), in which the SA is removed from the aqueous phase to the organic phase. A liquid-liquid separator is then used to remove the organic phase from the aqueous phase. The organic phase is mixed with a strong base or acid to separate succinate or succinic acid, respectively (Alexiou, 2017). Liquid-liquid extraction requires large volumes of the extraction agents; however, no sufficient extractants have been identified (Cheng *et al.*, 2012).

- Reactive extraction

Reactive extraction is a form of liquid-liquid extraction, which was developed to avoid the excessive use of extraction agents. This is done through the conversion of succinic acid to a chemical with no carboxyl group, after which, liquid-liquid extraction is performed (Cheng *et al.*, 2012). Reactive extraction is achieved with the addition of a reactive compound, this assists with an increase in the distribution coefficient, which allows for an improved separation. Reactive extraction requires pre-treatment (cell removal) and post treatment (concentration, crystallisation and drying) processes. Reactive extraction is a fairly complicated process and requires expensive extraction and diluent agents (Cheng *et al.*, 2012).

f. Chromatography

Various chromatography methods and resins have been researched for the purification of SA; prior to this step the fermentation broth requires some form of clarification. Different sorbents (resins) are applied in chromatography, these include chemisorbent, non-reactive adsorbents or absorptive functions. Numerous resins have been researched, the popular ones and their adsorption capacities are given in Table 1-7.

Table 1-7: Resins investigated in the purification of succinic acid through chromatography

Resin	Adsorption ($g.g^{-1}$)	References
XUS 40285 & XFS 40422	0.060-0.070	(Davison, Nghiem and Richardson, 2004)
PVP Reilex 425	0.050 - 0.080	(Davison, Nghiem and Richardson, 2004)
SBA-15 silica	0.007 - 0.058	(Jun *et al.*, 2007)
NERCB 09	0.110	(Davison, Nghiem and Richardson, 2004)
NERCB 09	0.380 - 0.560	(Li *et al.*, 2009)

Absorbent resins are more selective to other by-product acids other than SA (Table 2-7's silica resin is an example). Absorbent resins have not been exceedingly researched for SA and act through partitioning properties (Alexiou, 2017). Due to SA being a dicarboxylic acid (Figure 2-1, shows the structure) it can affect the adsorption ability within chemisorbent resins. It can be effected in either a positive way, through increased chances of hydrogen bonding due to two carboxyl groups being present on the SA molecule; or in negative way, as one molecule can bind to two sorbent sites on the column therefore reducing the column's capacity (Alexiou, 2017).

The desired properties of resins include high capacity, re-generatability and specific selectivity towards SA (Cheng *et al.*, 2012). Purification through chromatography can be applied as an additional/final purification step. The advantages to ion exchange are that it is easy to scale up and it is considered a clean process. The disadvantage of this step is the low selectivity and yields which results in a more diluted product form, expensive adsorbents (resins) and frequent resin regeneration (Cheng *et al.*, 2012) (Davison, Nghiem and Richardson, 2004).

1.5.3.3. Purification

Depending on the process followed, extra purification/concentration steps are required to obtain a higher purity of SA. Vacuum distillation is one such method to concentrate the SA, due to the high boiling temperature, see Table 1-1, it will remain in the bottom fraction allowing for the removal of the excess water/solvent, etc. Other final purification steps include direct crystallisation or filtration.

1.6. Economic impact

The economic impact of a potential SA production within South Africa, is an important aspect to consider, especially in a third world country: both the potential positive and negative sides are detailed.

1.6.1. Potential positive impacts

Globally, steps are being implemented to transition from fossil fuels towards a more sustainable economy where renewable resources are used. South Africa should place added focus on sustainable development and make a significant contribution towards this initiative. There are several driving forces for this shift, these include high oil prices, government

regulations, consumer preferences (‘greener’ products being of importance), environmental concerns, etc.

There are several policies and strategies that exist in South Africa that will support the production of biochemicals/ biofuels, these include (University of Stellenbosch Business School, 2017):

- *New Growth Path*
  Contains a goal of creating 5 million jobs by 2020, where the ‘green’ sector is a highlighted area for job creation.
- *Industrial Policy Action Plan (IPAP) and South African Renewables Initiative (SARi)*
  The focus of these plans is to develop renewable energy technologies and move towards a greener economy.
- *National Development Plan (NDP)*
  The aim of this plan is to move away from using natural resources and transition into a robust, low carbon economy.

The use of sugarcane biomass in the production of biochemicals will diversify sugar products which in turn will secure and create jobs, which is especially important in South Africa, where the unemployment figures are very high. Additionally, it could support rural development, become a global competitive nation for biochemicals, which could sequentially attract investments into the country.

1.6.2. Potential negative impacts

The following issues, could potentially emanate from the shift towards a bioeconomy in South Africa and ought to be assessed at a higher level should large scale production of biochemicals commence:

- Food security
- Water utilisation in a water-deprived country
- Agricultural stresses on the land (soil degradation, increased fertiliser, etc.)
- Impact on the biodiversity of the country
- Higher production costs

1.7. Conclusions

Succinic acid is termed a bio-based chemical, as it can be produced metabolically from a range of microorganisms; however, it is traditionally manufactured through petrochemical routes. Succinic acid is regarded as a platform chemical due to its abundant processing possibilities, which are largely attributed to carboxylic acid groups located at each end of the succinic acid molecule. The main end-product of interest is BDO (it is estimated that over half of the produced SA is used to manufacture this BDO) which is then utilised in the production of polymers. Similar to many South African industries, the chemical sector has encountered many challenges. This industry plays such an important role in the country's economy, it is therefore important that this sector thrives. With the global shift towards green chemistry, it is imperative for the focus to be placed on developing the bio-based chemical industry.

The South African sugar industry is another sector that has been struggling as of late, especially with the implementation of the sugar tax in 2018 with has seen a drop in profit. The country's sugar industry is important from a social and economic aspect, with one million people being dependent, directly, or indirectly on this industry. It is therefore necessary to find alternative downstream production opportunities to provide stability for this sector. The government have recognised the strain faced by the sugar sector and so have implemented the 'Sugar Industry Master Plan' to try to relieve some of the pressure.

Due to succinic acid being able to be produced through fermentation processes and with the need of a carbon source for this to be possible it provides an opportunity for both the chemical and sugar industry to combine and produce a product of interest. Alternative carbon sources can be obtained from sugarcane material, hence this study aims to ascertain whether bio-succinic acid can be successfully produced from sugarcane biomass. The process consists of upstream and downstream steps to obtain the succinic acid. The upstream stages will involve the growth of a variety of microorganisms to observe the optimum microbe. This microbe will then be grown on sugarcane material to observe whether it is successful in the production of succinic acid. Such upstream processes will be scaled up, and the material that is produced will then be used to conduct downstream section, which will include the removal of cells, removal of impurities and primary separation of succinic acid and then finally purification.

The production of succinic acid from sugarcane material will provide beneficial economic impacts for the country especially with the current high oil prices, government regulations, environmental concerns, and the consumer's preference to buying 'greener' products. There are potential negative impacts with a shift towards a bioeconomy, however, should the large-

scale production of bio-chemicals commence in South Africa, a higher-level assessment should be conducted to obtain an understanding of such an impact.

1.8. References

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CHAPTER 2 BENCH SCALE PRODUCTION STUDIES

2.1. Introduction

Small-scale flask studies were conducted using readily available identified succinate producing microbial strains. These were grown in synthetic media to compare their succinate producing abilities on C5 and C6 sugars. The optimum sugar group from the synthetic studies, either C5 or C6 sugars, was then compared with industrial media consisting predominantly of either C5 or C6 sugars. The experimental flow of the small-scale upstream process was based on a screening procedure where microbes were eliminated at each stage to arrive at the optimum succinate producing microbe, Figure 2-1 diagrammatically shows this process.

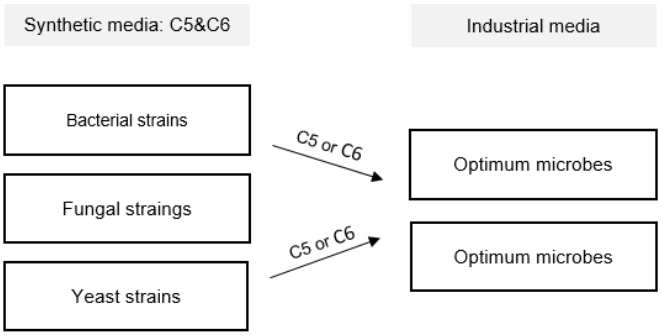

Figure 2-1: Experimental elimination process for the small-scale upstream process

Once the completion of the above-mentioned process, Figure 2-1, the 'optimum' microbe was then run in scaled up fermentations, detailed in Chapter 3.

The various methods and experimental results used/obtained for the small-scale shake flask work will be described in this chapter. Such work will include sample analysis, analytical methods, synthetic media screening, pre-treatment of feedstock and industrial media screening testing.

2.2. Methods

2.2.1. Synthetic media studies

The identified aerobic succinate producing wild type microorganism, which were readily available (at the CSIR), were used for the synthetic media studies. These include:

- *Lactobacillus paracasei*
- *Corynebacterium glutamicum*
- *Yarrowia lipolytica*
- *Aspergillus niger*

Preliminary tests were conducted using two defined synthetic media consisting of glucose and xylose, respectively, as the carbon source. Triplicate flasks were prepared for both the inoculum and fermentation steps, using the same media in each, as specified below.

Depending on the type of microorganism (bacteria, yeast or fungi), media was prepared as detailed below. Each type of microorganism had a certain quantity of the carbon source, which was constant for each microbe group. The media used for the *L. paracasei* was Modified De Man, Rogosa and Sharpe (MRS) media, Table 2-1 (De Man, Rogosa and Sharpe, 1960) details the composition. Whilst the media for *C. glutamicum* was a slight variation from the media used by Shi (2014) and is showed in Table 2-2. The vitamin solution for this media, was sterile and filtered into the media once cooled (Table 2-3) (Shi *et al.*, 2014).

Table 2-1: Synthetic media for *L. paracasei*

Component	Concentration ($g.L^{-1}$)
Glucose or Xylose	40.00
Peptone	10.00
Meat extract	8.00
Sodium acetate trihydrate	5.00
Yeast extract	4.00
K_2HOPO_4	2.00
Tri-ammonium citrate	2.00
$MgSO_4.7H_2O$	0.20
$MnSO_4.4H_2O$	0.05

Table 2-2: Media for *C. glutamicum*

Component	Concentration ($g.L^{-1}$)
Glucose or Xylose	40.0
Yeast extract	25.0
K_2HOPO_4	6.0
KH_2OPO_4	2.0
$MgSO_4.7H_2O$	2.0
$FeSO_47H_2O$	0.2
$MnSO_4.7H_2O$	0.2
Vitamin solution	5.0 mL

Table 2-3: Vitamin stock solution for the *C. glutamicum* media

Component	Value
Water	25.0 mL
Thiamin	7.0 mg
Biotin	3.5 mg

The media used for the yeast strain, *Y. Lipolytica*, was Yeast extract Peptone Glucose (YPG) media, details of which are in Table 2-4.

Table 2-4: Media for the yeasts

Component	Concentration ($g.L^{-1}$)
Glucose or Xylose	20.0
Peptone	20.0
Yeast extract	10.0

The media used for fungi, *A. niger*, was a variation on Vogel's media (Vogel, 1956), Vogel's media is commonly used for the growth of fungi, Table 2-5 (Vogel, 1956) lists the components. Media was prepared and the fungal media was pH adjusted to 6.5 using 10% $v.v^{-1}$ HCl and/or 20% $m.m^{-1}$ NaOH solutions.

Table 2-5: Defined media for fungi

Component	Concentration ($g.L^{-1}$)
Glucose or Xylose	30.00
KH_2PO_4	6.49
Trace element solution	6.00 mL
$(NH_4)H_2PO_4$	3.74
Na_3citrate.$2H_2O$	3.38
KNO_3	3.27
Biotin stock solution	3.00 mL
$MgSO_4.7H_2O$	0.26
$CaCl_2.2H_2O$	0.13
Chloroform to preserve	A few mL

The stock solutions listed in Table 2-5, are detailed in Table 2-6 and 2-7, respectively.

Table 2-6: Trace element stock solution for the fungal media

Component	Concentration ($g.L^{-1}$)
Water	1.0 L
Citric acid.H_2O	53.0
$ZnSO_4.7H_2O$	11.0
$Fe(NH_4)_2(SO_4)_2.6H_2O$	3.0
$CuSO_4.H_2O$	1.0
$MnSO_4.H_2O$	1.0
H_3BO_3 (anhydrous)	1.0
$Na_2MoO_4.2H_2O$	53.0

Table 2-7: Biotin stock solution for the fungal media

Component	Concentration ($g.L^{-1}$)
Water	1.0 L
Biotin	0.1

Triplicate flask studies were conducted for each strain. The strain specific media (700 mL) was prepared and then dispensed into a 2L Fernbach flask. The flasks were autoclaved using the SD 396 autoclave (Eins-Sci, South Africa) at 121ºC for 15 minutes. Once cooled, the biotin stock solution was aseptically added to the fungal media using a sterile syringe filter.

2.2.1.1. Inoculum preparation

Cryovials (2 mL) of each strain were removed from the – 80°C ultra-freezer (Thermo Scientific, USA) and used to inoculate triplicate flasks containing the synthetic media. The flasks were incubated on a rotary platform shaker (New Brunswick Scientific, USA) at 180rpm, 30°C for between 12 – 70 hours. Once the mid-exponential point, i.e., transfer time, was reached (as determined through the growth curve studies, experimental work not covered in this dissertation) the inoculum was used to inoculate the synthetic fermentation media. The volume of inoculum used to inoculate the fermentation flasks was calculated using Equation 2-1.

$$C_1V_1 = C_2V_2 \quad (2\text{-}1)$$

Where:

C_1 : measured cell count (concentration) of the inoculum, cells, mL^{-1}

V_1 : calculated volume required to inoculate the fermentation flasks, mL

C_2 : desired cell count i.e., approximately 10% of the maximum growth value of the inoculum, $2x10^7$ cells mL^{-1}

V_2 : volume of the inoculum, i.e., 700mL

2.1.1.2. Assessment of growth and succinic acid production

Once the fermentation flasks were inoculated, they were placed on a rotary platform shaker at 180rpm and 30ºC. Samples (4mL) were taken aseptically at different times for various durations, as listed below:

- Bacteria: *L. paracasei*, every 6 hours for 52 hours
- Bacteria: *C. glutamicum*, every 2 hours for 32 hours
- Yeast: *Y. lipolytica*, every 6 hours for 90 hours
- Fungi: *A. niger*, every 4 hours for 111 hours

Sample analysis as per section 2.2.3 was performed on each sample.

2.2.2. Industrial media studies

Following the outcome of the primary screening study, the top performing microbes and sugar substrate were selected and tested for the ability to produce succinic acid using industrial sugarcane media; sugarcane juice (SCJ) or molasses, Tables 2-8 to 2-10 were used to prepare the media for the industrial flask studies.

Table 2-8: Synthetic media for *L. paracasei*

Component	Concentration ($g.L^{-1}$)
Sugarcane juice/ molasses	40.00*
Peptone	10.00
Meat extract	8.00
Sodium acetate trihydrate	5.00
Yeast extract	4.00
K_2HOPO_4	2.00
Tri-ammonium citrate	2.00
$MgSO_4.7H_2O$	0.20
$MnSO_4.4H_2O$	0.05

* SCJ and molasses was added to obtain a total sugar concentration of $40g.L^{-1}$, as per sugar analysis

Table 2-9: Media for *C. glutamicum*

Component	Concentration ($g.L^{-1}$)
Yeast extract	25.0
Sugarcane juice/ molasses	40.0*
K_2HOPO_4	6.0
KH_2OPO_4	2.0
$MgSO_4.7H_2O$	2.0
$FeSO_47H_2O$	0.2
$MnSO_4.7H_2O$	0.2
Vitamin solution	5.0 mL

* SCJ and molasses was added to obtain a total sugar concentration of $40g.L^{-1}$, as per sugar analysis

Table 2-10: Vitamin stock solution for the *C. glutamicum* media

Component	Value
Water	25.0 mL
Thiamin	7.0 mg
Biotin	3.5 mg

2.2.2.1. Biomass material

Sugarcane biomass materialwas used as the carbon source in the secondary screening, however prior to utilisation, pre-treatment was required where necessary.

Pre-treatment of Sugarcane juice

Sugar cane was obtained from the South Africans Farmers Development Association (KwaZulu-Natal, South Africa). The leaves of the stalks were removed and sliced to a length of about 50 cm, at an angle of 45º. The sliced stripped cane was collected and the mass recorded. The stripped cane was then fed through a decorticator, which assisted in crushing the cane, and increasing the surface area for extraction efficiency during further processing. The crushed cane was collected and the mass recorded. The crushed cane was loaded into a tincture press (Hubert Schwanke, Germany), set at a pressure of 400 bar that was used to extract the sugar cane juice. The sugar cane juice and the bagasse (the pressed stalks) were collected, and the masses recorded. The efficiency of the extraction process was calculated using Equation 2-2.

$$\text{Extraction efficiency (\%)} = \frac{\text{Mass of juice (kg)}}{\text{Mass of stripped stalks (kg)}} \times 100 \qquad (2\text{-}2)$$

Pre-treatment of molasses

The high-test molasses was obtained from Tongaat Hulett (KwaZulu-Natal, South Africa) and did not require any pre-treatment steps. It was stored at -20 ºC until used.

Biomass analysis

The sugarcane juice (SCJ) and molasses were sent for sugar analysis to the BIDF (KwaZulu-Natal, South Africa). The sugar analysis was done using Dionex ICS5000+ Ion Chromatograph (ThermoFisher Scientific, USA) equipped with a CarboPac PA1 column and a Borate trap (ThermoFisher Scientific, United States of America). Analysis was done using 200mM sodium hydroxide as the mobile phase at a flow rate of 1mL.min^{-1} and a column temperature of 25ºC. The calibration curve was set up using glucose, fructose and sucrose standards and Millipore water for dilutions.

2.2.2.2. Industrial media preparation

Similar to synthetic screening, the composition of the media was the same and was detailed in Tables 2-8 to 2-10. The SCJ and molasses was used as the carbon source instead of glucose/ xylose, it was supplemented with these materials to obtain a total sugar concentration of 40 $g.L^{-1}$ as per the synthetic screening. The volume of SCJ or molasses was calculated based on the sugar results that was done on the treated biomass as described in Section 2.3.1.3.

Industrial fermentation media (700 mL) supplemented with SCJ or molasses was dispensed into each 2L Fernbach flask and prepared according to Section 2.2.2.

2.2.2.3. Inoculum preparation

Inoculum flasks were prepared with the same composition and method as per the synthetic media studies as detailed in Section 2.2.1. The industrial media flasks were then inoculated using Equation 2-1, to ensure it resulted in a final cell concentration of 2×10^7 $cell.mL^{-1}$ in each flask.

2.2.2.4. Assessment of growth and succinic acid production

Once the industrial media fermentation flasks were inoculated, they were placed on rotary platform shaker at 180 rpm and at 30ºC. Samples (4mL) were aseptically taken at different times for various durations, as listed below:

- *L. paracasei*, every 2 hours for 38 hours
- *C. glutamicum*, every 2 hours for 38 hours

Sample analysis as per section 2.2.3. was performed on each sample.

2.2.3. Sample analysis

Numerous analyses were conducted on samples extracted from the fermentation studies for both the synthetic and industrial media studies.

2.2.3.1. Dry cell weights

All samples were prepared in duplicate from each of the triplicate flasks sampled, the following protocol was followed:

Cellulose acetate (25 mm diameter) micro filters with 0.22 µm pore size were weighed accurately to 4 decimal places. The pre-weighed filter paper was then placed in a multi-filter filtration (Millipore Corporation, USA). The system was attached to a 50 Hz (230 V) in-house vacuum pump, fitted with appropriate safety traps. 0.5 mL of the sample was aliquoted onto the filter paper. This process was repeated for each sample and was done in duplicate. Once all the liquid was filtered through, the filter paper was carefully removed and dried in a HS153 halogen moisture analyser (Mettler Toledo, Switzerland). The biomass content was calculated by measuring the difference in the mass of the material before and after drying (as displayed on the moisture analyser), Equations 2-3 was used. The dried biomass values were calculated using Equation 2-4.

$$M_{\text{Dry biomass i,j}} = M_{i,j} - M \quad (2\text{-}3)$$

Where,

i = microorganism; L. paracasei, C. glutamicum, Y. lipolytica or A. niger

j = substrate: glucose, xylose, SCJ or molasses

$M_{\text{Dry biomass i, j}}$ = mass of dry biomass obtained for strain i using substrate j, g

$M_{i,j}$ = mass of filter paper after filtering broth obtained from fermentation using strain I and substrate j, g

M = tare mass of filter paper, g

$$\text{Biomass}_{i,j} = \frac{M_{Dry\ biomass\ i,j}}{\text{Vol}} \quad (2\text{-}4)$$

Where,

i = microorganism; *L. paracasei*, *C. glutamicum*, *Y. lipolytica* or *A. niger*

j = substrate: glucose, xylose, SCJ or molasses

Biomass *i*,*j* = the concentration of biomass obtained for strain i and substrate j, $g.L^{-1}$

Vol = sample volume of 0.5mL

$M_{Dry\ biomass\ i,j}$ = mass of dry biomass obtained for strain i using substrate j, g

2.2.3.2. Glucose

Glucose measurements were done only for the flasks that contained glucose, sugarcane juice (SCJ) and molasses as the carbon source in the media. A glucose meter and test strip, Accu-chek Active, was used to test an aliquoted sample. To calculate the correct glucose measurement, Equation 2-5 was utilised, and the glucose measurements recorded.

$$\text{Glucose}_j = c \times MM_j \quad (2\text{-}5)$$

Where,

j = substrate: glucose, SCJ or molasses

Glucose_j = the glucose concentration whilst using substrate j, $g.L^{-1}$

c= glucose concentration obtained from meter, $mmol.L^{-1}$

MM_j = molar mass of the substrate j, $g.mmol^{-1}$

The molar mass (MM) for glucose and the industrial media was 0.198 $g.mmol^{-1}$ (molar mass of glucose monohydrate, which is the substrate used) and 0.1802 $g.mmol^{-1}$ (molar mass of glucose), respectively.

2.2.3.3. pH

The pH measurements were done using a FiveEasy pH meter (Mettler Toledo, Switzerland). Prior to use and in between samples, the probe was rinsed with distilled water and dried with paper towel. The probe was inserted into the extracted samples and the pH was recorded.

2.2.3.4. Optical density

Optical density (OD) measurements were done on a Spectroquant ® Pharo 300 (Merck, Germany) with dilutions been made up as required using distilled water. The wavelength at which the analysis was done for each microbial group is listed below:

- Bacteria, 660nm
- Yeast, 600nm
- Fungi, 660 nm

2.2.3.5. Cell count concentrations

Cell counts were completed using an Olympus BX40 Microscope (Olympus-Life Science, Japan) at 40x magnification to determine cell concentration. Cells were counted using a Haemocytometer (Neubauer-Improved, Lasec, South Africa).

2.2.3.6 Succinic acid analysis

Sample preparation

A sample (1.5 mL) from each test flask was dispensed into an Eppendorf tube. A micro-centrifuge (Eppendorf, Germany) was used to centrifuge the Eppendorf tube for 10 minutes at 12000 x g to enable pellet formation of the solid material. 1 mL of the liquid phase was pipetted and passed through a 0.22 µm syringe filter into an amber vial. The sample was analysed using an UltiMate High Pressure Liquid Chromatography (HPLC).

High pressure liquid chromatography

The operating conditions for the UltiMate 3000 HPLC (Thermo Scientific, USA) with an Ultra AQ C18 5µm column (Restek, USA), 150 x 4.6mm are detailed in Table 2-11. The column was equilibrated with the mobile phase which consisted of 50mM potassium phosphate (pH 2.5) and 99% acetonitrile at a ratio of 99:1 respectively. Equilibration occurred at a flow rate of 1.0 $mL.min^{-1}$ until a stable ultraviolet (UV) was observed.

Prior to any samples being run, a standard curve was set up using succinic acid standard. Samples containing distilled water with 2.5, 5.0, 10.0, 25.0 and 50.0 $g.L^{-1}$ total succinic acid were analysed according to the operating conditions of the HPLC (Table 2-11).

Table 2-11: Operating conditions of the HPLC

Parameter	Value	Unit
Injection volume	10.0	µL
Injection draw speed	7.0	$\mu L.s^{-1}$
Injection draw delay	3.0	s
Dispense speed	7.0	$\mu L.s^{-1}$
Dispense delay	0.0	s
Dispense to waste speed	32.0	$\mu L.s^{-1}$
Sample height	2.0	mm
Injection wash volume	100.0	µL
Wash speed	20.0	$\mu L.s^{-1}$
Column temperature	25.0	ºC
Column equilibration time	0.5	min
Ready temperature delta	1.0	ºC
Flow rate	1.0	$mL.min^{-1}$
UV/Vis	210.0	nm
Sampler temperature	4.0	ºC
Run time	30.0	min
Retention time	5.0	min

Succinic acid yields

The yield of succinic acid achieved per g of sugar substrate was calculated as follows, in Equation 2-6.

$$\text{Yield}_{i,j} = \frac{\text{SA}_{\text{max}i,j}}{\text{mass of substrate}_j} \quad (2\text{-}6)$$

Where,

i = microorganism; L. paracasei, C. glutamicum, Y. lipolytica or A. niger

j = substrate: glucose, xylose, SCJ or molasses

Yieldi, j = yield obtained for strain i using substrate j based on total substrate used, g.g-1

SAmaxi,j = maximum succinic acid produced for strain i using substrate j the fermentation time, g

mass of substrate$_j$ = quantity of substrate used, g

Succinic acid productivity

The succinic acid productivity was calculated as shown in Equation 2-7:

$$\text{Productivity}_{i,j} = \frac{\text{SAmax}_{i,j}}{\text{Vol} \times \text{Time}} \quad (2\text{-}7)$$

Where,

i = microorganism; L. paracasei, C. glutamicum, Y. lipolytica or A. niger

j = substrate: glucose, xylose, SCJ or molasses

Productivity $_{i,j}$ = productivity obtained for strain i and substrate j, based on succinic acid produced per volume and time

$SAmax_{i,j}$ = maximum succinic acid produced for strain i using substrate j, across the fermentation time, g

Vol = reactor volume, L

Time = fermentation time, hr

Succinic acid titre

The succinic acid titre was calculated as follows in Equation 2-8:

$$\text{Titre}_{i,j} = \frac{\text{SAfinal}_{i,j}}{\text{Vol}} \quad (2\text{-}8)$$

Where,

i = microorganism; L. paracasei, C. glutamicum, Y. lipolytica or A. niger

j = substrate: glucose, xylose, SCJ or molasses

$Titre_{i,j}$ = productivity obtained for strain i using substrate j, based on succinic acid produced per reactor volume

$SAfinal_{i,j}$ = final succinic acid produced for strain i and substrate j, g

Vol = reactor volume, L

2.3. Results and discussion

2.3.1. Sample analysis

The standard calibration curve generated on the HPLC for the quantification of succinic acid is detailed in Figure 2-2.

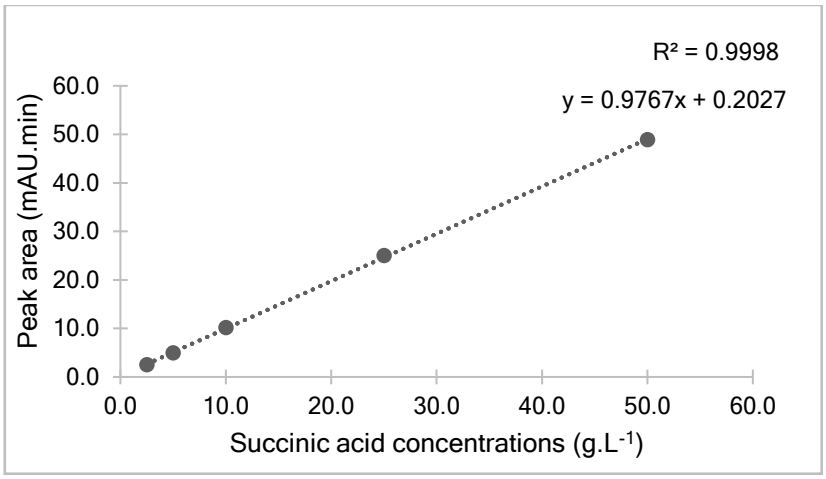

Figure 2-2: Standard calibration curve of succinic acid's peak area (mAU.min) versus the concentration values

The average retention time for the succinic acid was 5.333 minutes; an example of a succinic acid chromatogram at a succinic acid concentration of 2.5 $g.L^{-1}$ is shown in Figure 2-3, a citric acid standard was prepared at the same time therefore the citric acid chromatogram peak is present in Figure 2-3. The coefficient of determination (R^2) obtained from the regression of the data was 0.9998 which indicates that the calibration curve has a very high degree of linearity. The standard calibration curve could therefore, be used to determine the concentration of succinic acid produced.

From the regressed data, the equation of the calibration curve is shown in Equation 2-9:

$$Y = 1.8723\,X - 0.1352 \tag{2-9}$$

Where:

Y=Peak area of the succinic acid at the retention time in mAU.min

X=Concentration of succinic acid measured in $g.L^{-1}$

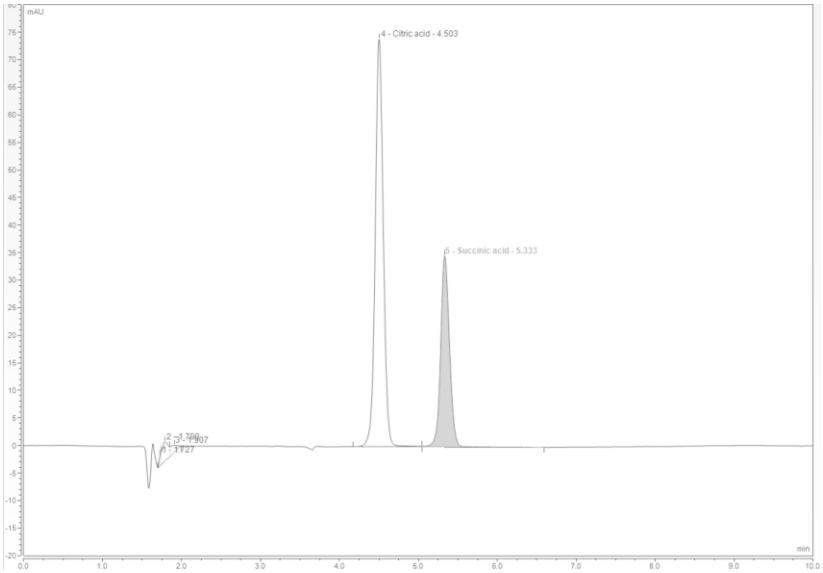

Figure 2-3: Chromatogram of the succinic acid standard

2.3.2. Synthetic media studies

All strains were able to produce succinic acid in the synthetic media for both the glucose and xylose-based media. *C. glutamicum* showed the highest production in both types of media, with the highest concentration of 18.81 ± 1.27 $g.L^{-1}$ and 17.17 ± 0.60 $g.L^{-1}$ for glucose and xylose respectively.

Assessment of the maximum biomass formation results displayed in Figure 2-4, showed that *A. niger* produced the highest with 20.40 ± 2.25 $g.L^{-1}$ and 19.93 ± 1.71 $g.L^{-1}$ for the glucose and xylose media respectively. This was expected due to the filamentous growth of fungi, however it produced the lowest concentrations of succinic acid for both groups of sugars. *C. glutamicum* produced the second highest biomass of 16.36 ± 1.74 $g.L^{-1}$ and 17.20 ± 2.49 $g.L^{-1}$ for glucose and xylose respectively, at 16 hours of growth for both sets of studies. The cell growth rate is comparable for both groups of sugar mediums, with the maximum biomass being achieved at identical fermentation ages. In a study conducted by Briki (2020) with *C. glutamicum*, the dry cell weight reached a maximum of 16.00 $g.L^{-1}$ in the aerobic phase, which is on par with the results from this study.

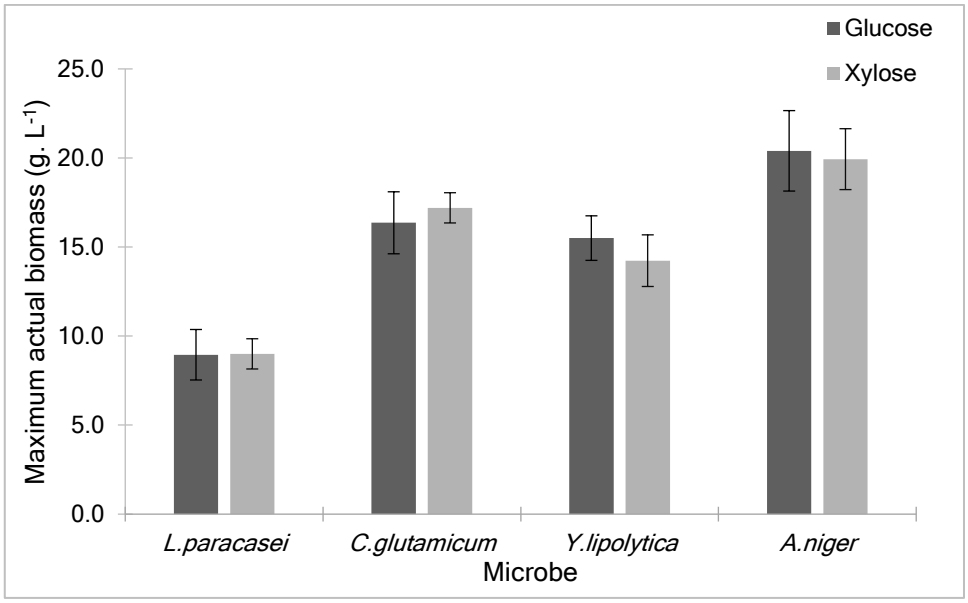

Figure 2-4: Maximum dry biomass concentration ($g.L^{-1}$) obtained using glucose and xylose for *L. paracasei, C. glutamicum, Y. lipolytica* and *A. niger*

Comparison of the maximum succinic acid yields for the two sugar substrates is shown in Figure 2-5. *C. glutamicum* produced the highest yields across both sugars, of 0.47 ± 0.01 $g.g^{-1}$ and 0.43 ± 0.01 $g.g^{-1}$. The yeast, *Y. lipolytica*, proved satisfactory when comparing the succinic acid yields and obtained the second highest results for the synthetic screening study.

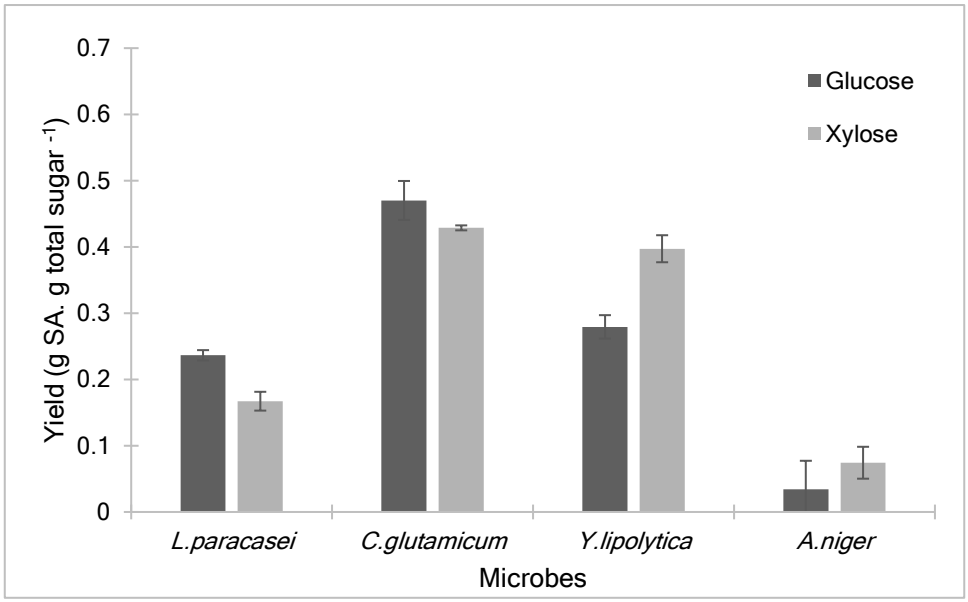

Figure 2-5: Maximum succinic acid yields obtained for glucose and xylose as the carbon feed for *L. paracasie, C. glutamicum, Y. lipolytica* and *A. niger* over the fermentation period for the small-scale synthetic fermentation studies

The top performing microbe, *C. glutamicum*'s achieved yields of 0.51 $g.g^{-1}$ and 0.47 $g.g^{-1}$ for glucose and xylose respectively, are comparable to literature, as summarised by Table 2-12. The maximum SA concentrations produced throughout the fermentation run for the glucose media was 18.81 ± 1.27 $g.L^{-1}$, at a fermentation age of 28 hours. The maximum SA concentration at specified fermentation time can be translated into productivity, as depicted in Figure 2-6.

Table 2-12: Succinic acid yields for *C. glutamicum* studies within a glucose media

Microorganism	Wild or GMO	Yield ($g.g^{-1}$)	Reference
C. glutamicum R	GMO	0.19	(Okino, M and Yukawa, 2005)
C. glutamicum R	GMO	0.92	(Okino *et al.*, 2008)
C. glutamicum 2262	GMO	0.94	(Kaboré *et al.*, 2017)
C. glutamicum 2262	Wild	0.22	(Briki *et al.*, 2020)
C. glutamicum R	Wild	0.29	(Okino, M and Yukawa, 2005)
C. glutamicum 534	GMO	0.56	(Shi *et al.*, 2014)

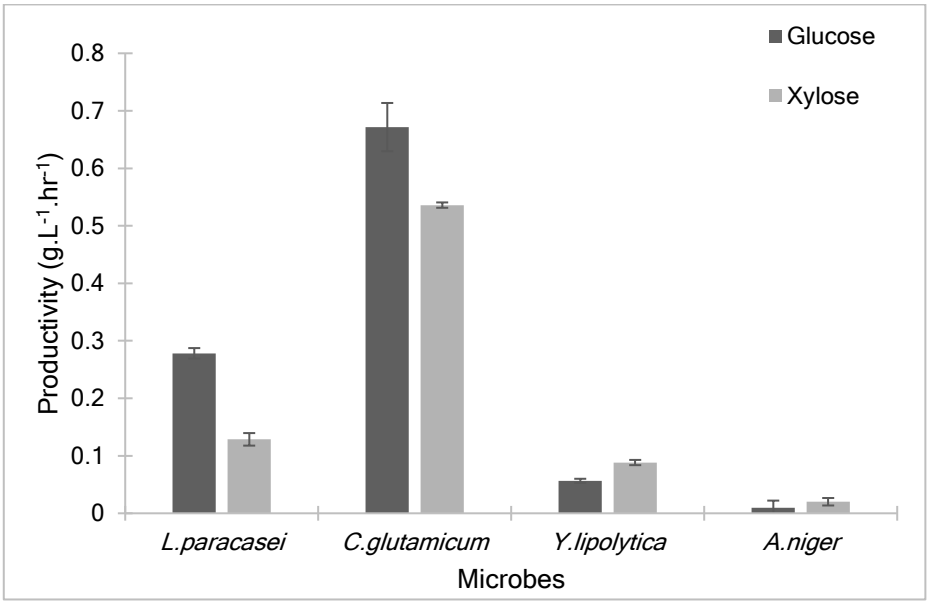

Figure 2-6: Productivity for succinic acid production obtained for glucose and xylose as the carbon feed for *L. paracasie, C. glutamicum, Y. lipolytica* and *A. niger* for the small-scale synthetic fermentation studies

The productivity values are of high importance, as it is inversely proportional to the fermentation time, and significant for economic reasons. The longer the fermentation time, the higher the running expenses that are involved, including capital and operational costs. Productivity calculation shows how efficient the production capacity is, therefore, high productivity values are desired as this reduces such costs.

A comparison between the synthetic media flask studies is detailed in a favourability table in Table 2-13, where '+' represents the least favourable outcome to '+ + +' the most favourable outcome, for a variety of growth parameters for glucose (glu.) and xylose (xyl.) synthetic flask studies for each microorganism.

Table 2-13: A favourability table for the synthetic glucose (glu.) and xylose (xyl.) flask studies

	L. paracasei		*C. glutamicum*		*Y. lipolytica*		*A. niger*	
	Glu.	Xyl.	Glu.	Xyl.	Glu.	Xyl.	Glu.	Xyl.
Biomass growth	+	+	+ +	+ +	++	++	+++	+++
Yield	++	++	+++	+++	++	++	+	+
Productivity	++	+	+++	+++	+	+	+	+

Y. lipolytica was favourable in the yield category, however the productivity values are very low, at 0.06 ± 0.01 $g.L^{-1}.h^{-1}$ and 0.09 ± 0.01 $g.L^{-1}.hr^{-1}$, for glucose and xylose respectively, therefore proving to be an unfavourable option. *C. glutamicum* showed favourable results across the majority of the growth parameters, detailed in Table 2-13, especially in the yield and productivity values across the two different media.2.3.3. Industrial media studies

2.3.3.1. Pre-treatment of biomass

A basic process flow diagram of the pre-treatment process of the sugar cane is shown in Figure 2-7.

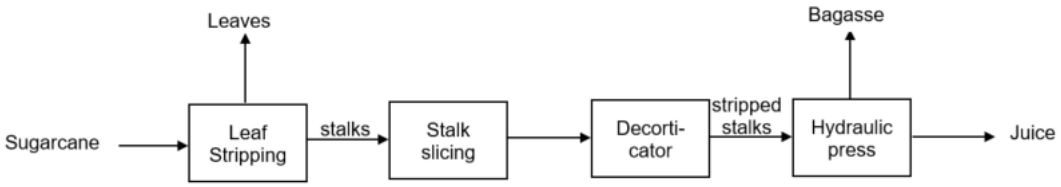

Figure 2-7: Pre-treatment process for the extraction of sugar cane juice from sugar cane stalks

The mass balance for the pre-treatment of the sugar cane is detailed in Table 2-14. The estimated extraction efficiency was calculated using Equation 2-7, and aligns to reported juice extraction values of approximately 50% (Khare, 2012). The losses amounted to 9.11% which was a reasonable value due to it being less than 10%.

Table 2-14: Recovery (%) of sugar cane juice

Description	Unit	Value
Mass stripped stalks	kg	19.20
Mass bagasse	kg	7.32
Mass juice extracted	kg	10.13
Losses	kg	1.75
Juice extracted	%	52.79

2.3.3.2. Biomass sugar analysis

The breakdown of the sucrose, fructose and glucose percentages of the SCJ and molasses material is shown in Figure 2-8, results of which are in line with literature (Palmonari, *et al.*, 2020). The sum of these sugars, determined for both the extracted sugarcane juice and the molasses, were used to determine the total volume to obtain a total sugar concentration of 40.0 $g.L^{-1}$, to match that which was used in the synthetic media studies.

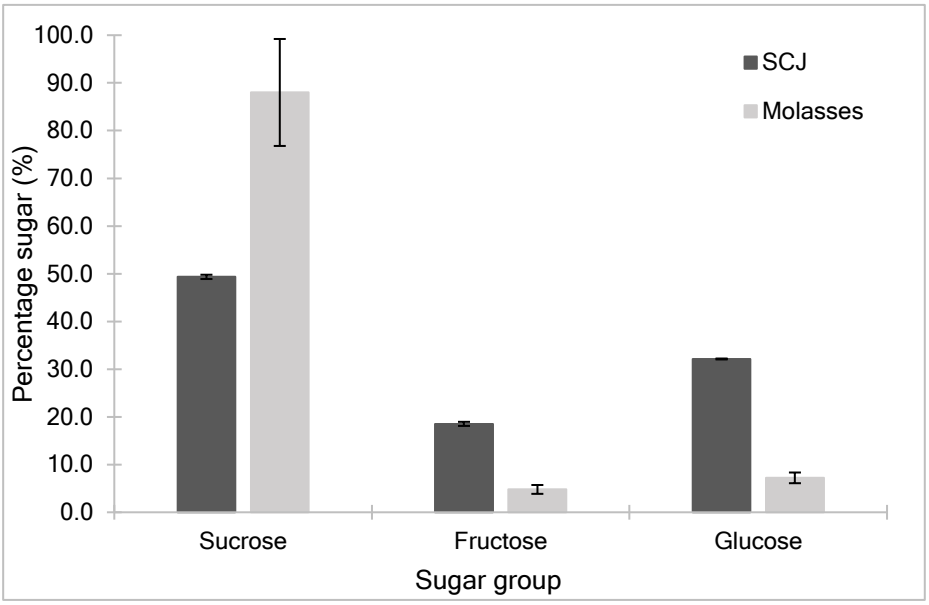

Figure 2-8: Percentage of sugar groups for sugarcane juice and molasses

The sugar content of the molasses is ten folds higher than that of the SCJ, this is an important aspect to consider, as an increased volume of SCJ will be required to obtain the same quantity as molasses. Due to the cost of raw materials being on a quantity basis, the higher volumes will be directly proportional to the raw material costs.

2.3.3.3. Shake flask studies

The two bacterial strains, *C. glutamicum* and *L. paracasei*, were both able to produce succinic acid in the industrial medias, SCJ and molasses. *C. glutamicum* showed the highest production, with a concentration of 20.31 ± 0.75 $g.L^{-1}$ in the molasses media.

Assessment of the biomass concentrations, results shown in Figure 2-9, showed that the two strains produced very similar quantities of biomass, except for the *L. paracasei* whilst in the SCJ media. *C. glutamicum* produced 31.15 ± 1.60 $g.L^{-1}$ and 31.44 ± 4.41$g.L^{-1}$ for SCJ and molasses, respectively and *L. paracasei* produced 32.65 ± 2.60 $g.L^{-1}$ in the molasses media. The molasses maximum biomass point was delayed by four hours compared to the SCJ for both bacterial strains, indicating that it took slightly longer for the material to be broken down.

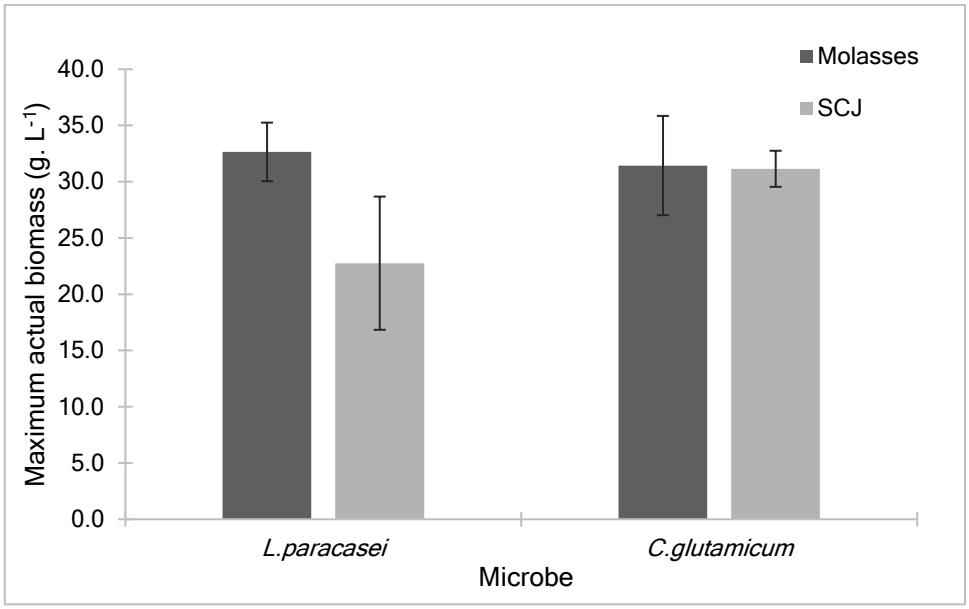

Figure 2-9: Dry cell weights ($g.L^{-1}$) obtained using sugarcane juice and molasses for *L. paracasei* and *C. glutamicum*

Comparison of the maximum succinic acid yields for the two sugar substrates is shown in Figure 2-10. C. *glutamicum* produced the highest yield when using molasses media, a 0.51 ± 0.06 $g.g^{-1}$. An evaluation between the synthetic and industrial media screening studies, shows that molasses and glucose (shown in Figure 2-5) yields were the same. Such a result shows that molasses could be used as a substitute for the 'ideal' glucose carbon source, the result could be due to *C. glutamicum* being reported to be able to grow on a variety of different sugars as a single or combined carbon source (Briki *et al.*, 2020). The yield calculation is an important aspect due to the raw materials accounting for a large portion of the total cost of production.

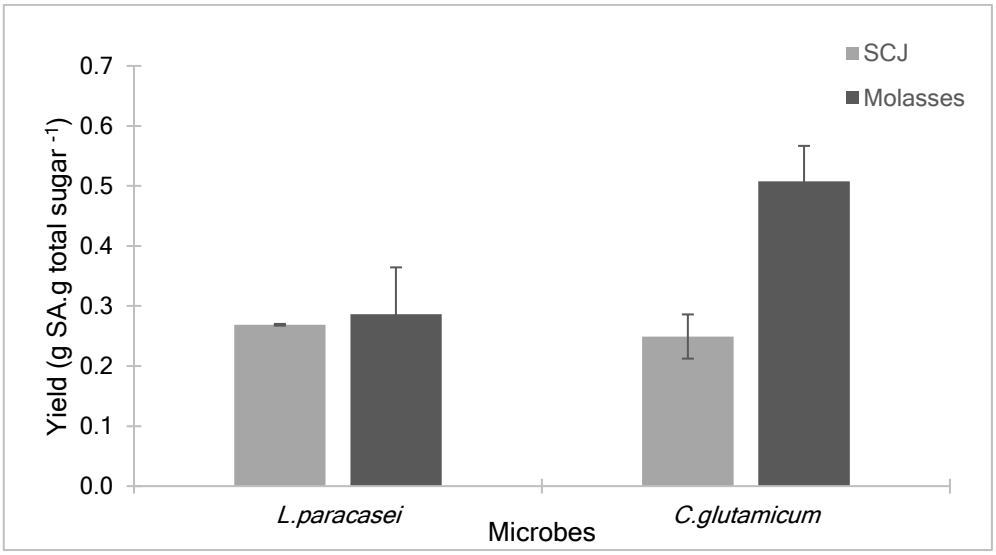

Figure 2-10: Maximum succinic acid yields obtained for sugarcane juice and molasses as the carbon feed for *L. paracasie* and *C. glutamicum* over the fermentation period for the small-scale industrial media studies

The bacterial strains are on par with literature results when using an industrial media as the carbon source, Table 2-15 (repeated from the Literature Chapter), shows microorganisms that contain sugarcane biomass, molasses, bagasse and SCJ, as the energy source in the fermentations. The SA titres for *C. glutamicum* in the molasses media are comparable to literature, Table 2-15, with a titre of 20.29 ± 1.73 g.L^{-1}, although these results are slightly lower it is to be expected at a flask level, and it is envisaged as scale-up occurs, that the succinic acid concentrations would increase.

Table 2-15: Literature comparison for the ability of microorganisms to produce succinic acid with industrial medias as the carbon source

Biomass	Microorganism	Aerobic/ anaerobic	SA titre (g.L^{-1})	References
Molasses	*E. coli W3110*	Dual	26.20	(Agarwal *et al.*, 2007)
	E. coli AFP111	Dual	37.30	(Ma *et al.*, 2014)
Bagasse	*E. coli BA305*	Dual	39.90	(Liu *et al.*, 2012)
	Y. lipolytica	Aerobic	33.20	(Ong *et al.*, 2019)
SCJ	*A. succinogenes GXAS137*	Anaerobic	62.06	(Shen *et al.*, 2016)

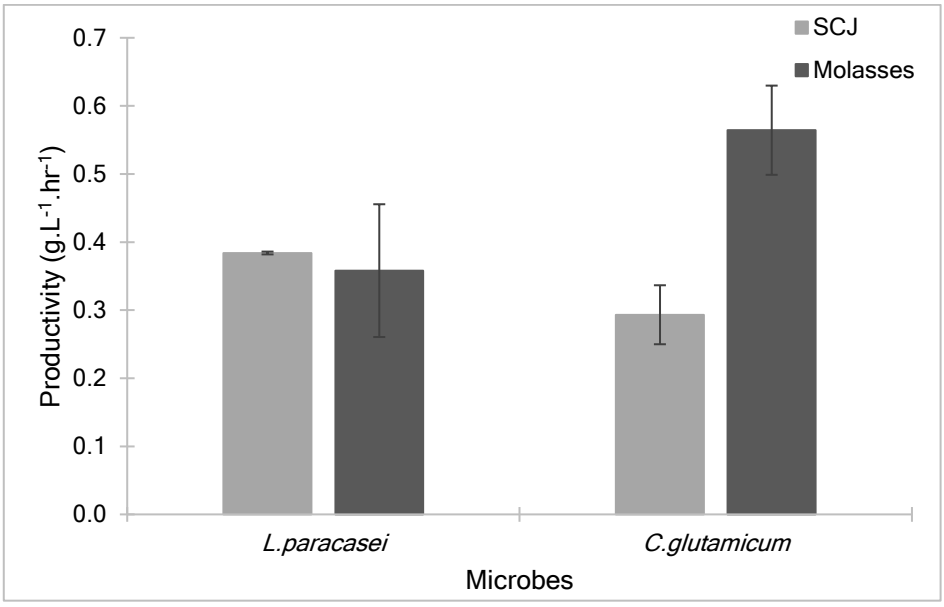

Figure 2-11: Productivity for succinic acid production obtained for sugarcane juice and molasses as the carbon feed for *L. paracasie* and *C. glutamicum* for the small-scale industrial media fermentation studies

Due to productivity being an indicator of how efficient the production capacity is, it is important to increase it, the values obtained from the study are shown in Figure 2-11, all of which are decent, and the highest is from *C. glutamicum* in the molasses study, obtaining 0.56 ± 0.07 $g.L^{-1}.hr^{-1}$.

A comparison between the industrial media flask studies is detailed in Table 2-16 where '+' represents the least favourable outcome to '+ + +' the most favourable outcome, for a variety of growth parameters for glucose and xylose synthetic flask studies for each microorganism.

Table 2-16: A favourability table for the industrial flask studies

	L. paracasei		*C. glutamicum*	
	SCJ	**Molasses**	**SCJ**	**Molasses**
Biomass growth	+	+ + +	+ + +	+ + +
Yield	+	+	+	+ + +
Productivity	+ +	+ +	+ +	+ + +

Across the growth parameters *C. glutamicum* with the molasses industrial media was the favoured option, with yield and productivity being of the utmost importance.

2.4. Conclusions

In production of biochemicals, in this case succinic acid, the growth parameters that are of high importance are yield and productivity. *C. glutamicum* resulted in the highest yield across both sugars (glucose and xylose), of 0.47 ± 0.01 $g.g^{-1}$ and 0.43 ± 0.01 $g.g^{-1}$ respectively, and although *Y. lipolytica* had the next highest yield results its productivity values were very low. A comparison of the different growth parameters for each microbe and sugar group is depicted in Table 2-13 and it was concluded that the two bacterial strains, namely *C. glutamicum* and *L. paracasei* were the most favourable option, with the C6 sugar group, glucose, proving superior.

These two identified microorganisms were then used in the industrial shake flask studies where C6 industrial sugar materials from the sugar industry, molasses and sugarcane juice, were used as the substitute carbon source. The results of which clearly showed that *C. glutamicum* was the top performing microbe. The molasses media was the preferred industrial material, yielding higher succinic acid concentrations as well as having a greater overall total sugar concentration, therefore requiring less material compared to the sugarcane juice to obtain the same sugar concentration.

The small-scale flask studies concluded that glucose was the preferred synthetic carbon source and would be used as the ‘ideal’ case. *C. glutamicum* proved to be the superior microbe from this study and would be grown in molasses, the top performing industrial material, , in a scaled-up study covered in Chapter 3.

2.5. References

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CHAPTER 3 SCALED-UP FERMENTATION STUDIES

3.1. Introduction

Based on the small-scale shake flasks studies, covered in Chapter two, the optimum succinate producing microbe and the suitable feedstock for the bioconversion was demonstrated. Additionally, the results were indicative that industrial media from the sugar industry, namely sugarcane juice (SCJ) and molasses could both successfully produce succinic acid. A summary of the experimental flow of these flask studies and how it leads into the next stage of work, specifically demonstration in controlled bioreactors as part of scale-up fermentation, covered in this chapter, is shown in Figure 3-1.

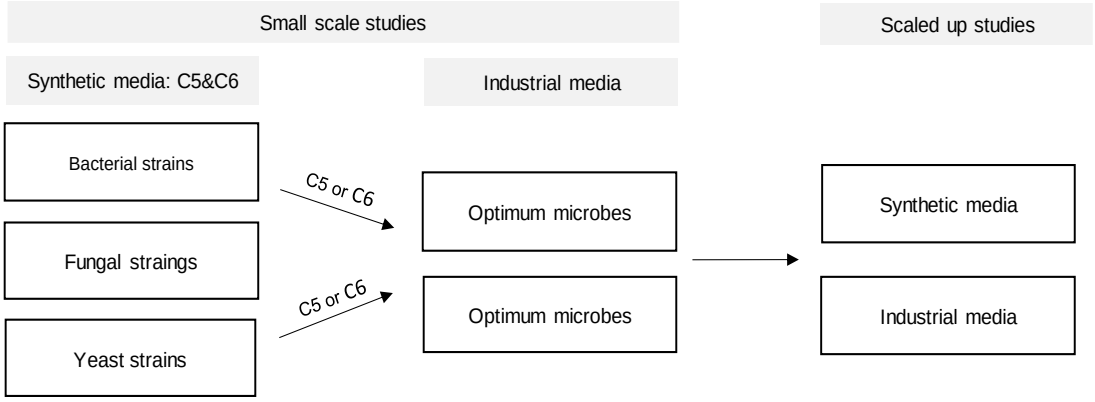

Figure 3-1: Experimental procedure from the small-scaled fermentation studies to the scaled-up studies

Upon completion of the above-mentioned process, the broth produced from the 'ideal' carbon source, i.e., from the scaled-up synthetic media study, will be used to develop a suitable downstream process (DSP) method.

The various methods and experimental results obtained for the scaled-up reactor studies will be detailed in this chapter, with references to methods done in Chapter 2. This work will include sample analysis, application of analytical methods, synthetic and industrial media scaled-up reactor fermentations. Scale-up is a critical part of bioprocessing as it is important to determine if a process can be a feasible option and to observe the performance of the strain at a larger scale. The scale-up processes for the work covered in this chapter were performed on 30L biostat reactors.

3.2. Methods

3.2.1. Growth assessment

Numerous analyses were conducted on samples extracted from the fermentation studies for both the synthetic and industrial fermentations. Every two hours, four sets of twenty millilitre samples were removed from the reactors and analysed for dry cell weights, glucose, pH, optical density, cell counts and succinic acid, the method for each analysis is detailed in Chapter 2, section 2.2.3.

3.2.2. Fermentation

3.2.2.1. Fermentation setup

The Biostat C+ reactors, Figure 3-2, underwent water sterilisation to ensure the sterility of the reactors. Thereafter, the pH and dissolved oxygen probes were calibrated, the jacket primed, the inlet and outlet filters added, and the pH controls were attached to the system. The two 30 L Biostat C+ fermenters had the initial media loaded to obtain a volume of 24.3 L (as detailed below in Table 3-1). The reactors were pressure tested and thereafter underwent media sterilisation at 121 ºC for 45 minutes. Once the reactors were cooled, they were ready for fermentation.

Figure 3-2: Photograph of the 30LBiostat reactor used for the scaled-up fermentations.

3.2.2.2. Fermentation media

The synthetic media used in the reactor was of the same composition as that used in the flask studies, albeit a slight variation from the media used by Shi (2014). The initial charge to the reactor included antifoam, the details of the initial charge are shown in Table 3-1. The carbon source was either glucose monohydrate or the top-performing industrial media, molasses.

Table 3-1: Initial media make-up for the scaled-up reactor runs

Source	Component	Unit	Value
Protein	Yeast extract	$g.L^{-1}$	25.0
Sugars	Carbon source	$g.L^{-1}$	40.0
Salts and others	K_2HOPO_4	$g.L^{-1}$	6.0
	KH_2OPO_4	$g.L^{-1}$	2.0
	$MgSO_4.7H_2O$	$g.L^{-1}$	2.0
	$FeSO_4.7H_2O$	$g.L^{-1}$	0.2
	$MnSO_4.7H_2O$	$g.L^{-1}$	0.2
	Antifoam	$mL.L^{-1}$	1.0
Vitamins	Thiamine	$mg.L^{-1}$	1.4
	Biotin	$mg.L^{-1}$	0.7

3.2.2.3. Inoculum media

Four flasks of inoculum media were prepared for the *C. glutamicum* strain as per Chapter 2, and incubated at 30 ºC, at 180 rpm for 12 hours. Once the flasks were ready, as per the OD measurements, the monoseptic status was checked using a microscope. The inoculum (700 mL) was aseptically added to the 2 L transfer vessels and attached to the reactor and steamed on for 20 minutes to ensure sterility.

3.2.2.4. Fermentation parameters

The bioreactors were inoculated with the inoculum, as prepared above, to achieve a working volume of 25 L. The cultivation temperature was set at 30 ºC, pressure at 0.5 bar and the stirrer at 100 rpm. The dissolved oxygen was also monitored. The pH was maintained at 7.5 with 10 % $v.v^{-1}$ H_2SO_4 and 5 M NaOH. The airflow was set at 12.5 slpm to maintain approximately

0.5 $v.v^{-1}.min^{-1}$ in the bioreactor. The vessels were harvested once the consumption of glucose was observed.

3.3. Results and discussion

3.3.1. Growth assessment

Assessment of the biomass concentrations for the batch reactor fermentations is shown in Figure 3-3. *C. glutamicum* produced 28.13 $g.L^{-1}$ and 62.1 $g.L^{-1}$ using glucose and molasses, respectively. The high biomass values reached by the molasses media are similar to those obtained in literature, Okino (2009) recorded 60 $g.L^{-1}$. The biomass concentrations showed similar trends to the optical density measurements. This was an expected result as absorbance (660 nm) is a rough indication of microbial growth and is directly related to biomass. The slight decline in biomass that occurs between 3 - 11 hr, in Figure 3-3, occurs over the lag phase of growth and can be accounted to change in media and/or dilution error prior to analysis.

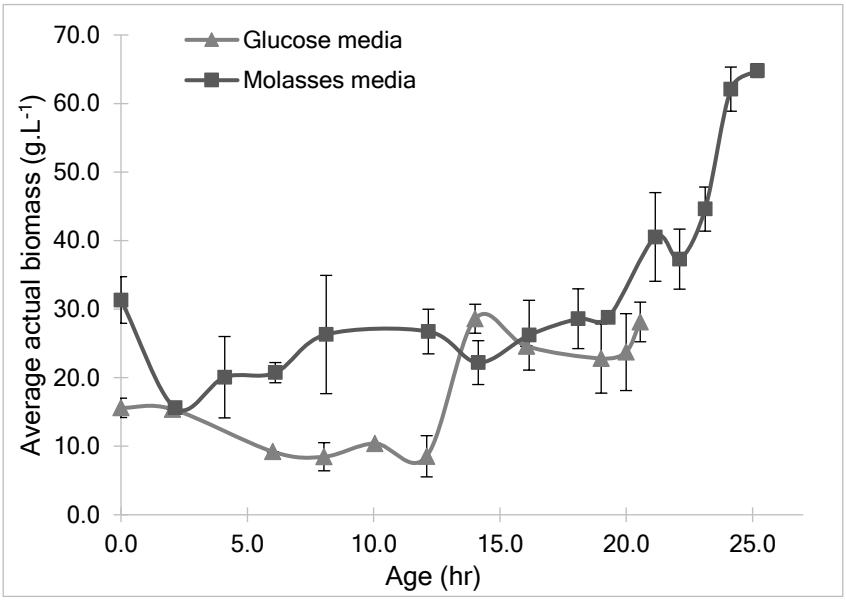

Figure 3-3: The actual biomass for the glucose and molasses-based media across the fermentation age

3.3.2. Fermentation performance

The dissolved oxygen recorded by the reactors during the fermentation runs is another indication of cell growth. The dissolved oxygen was monitored and once the level reached below 30 %, the stirrer was cascaded to increase and maintain the level above the set point. The decrease in the dissolved oxygen correlates to a slight increase in absorbance indicating

the growth of cells has begun, this occurs between 6.00 - 10.00 hours for both types of media which additionally correlates to the biomass increases (given in Figures 3-4 and 3-5).

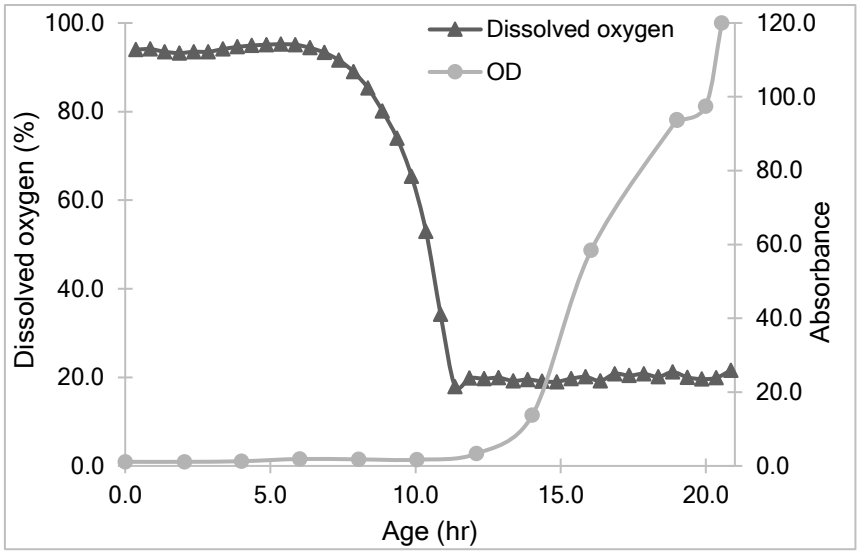

Figure 3-4: The dissolved oxygen percentage and the absorbance for the glucose-based media across the fermentation age

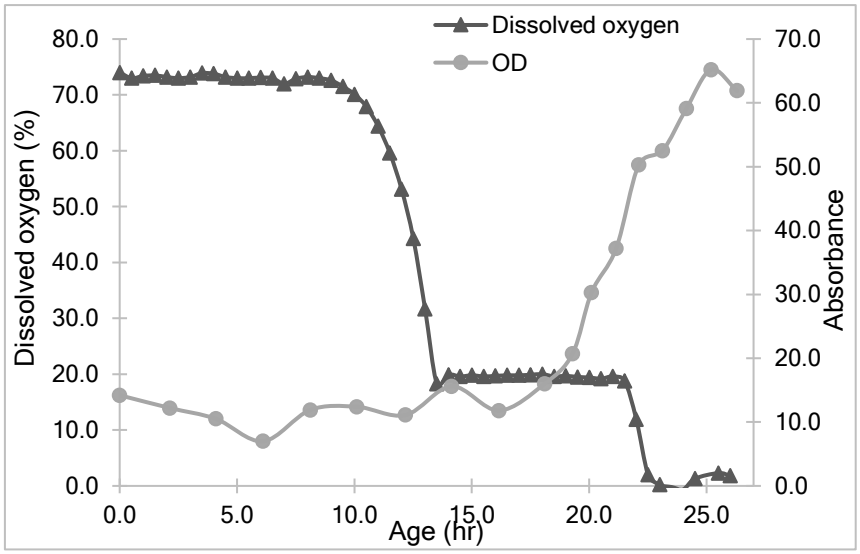

Figure 3-5: The dissolved oxygen percentage and the absorbance for the molasses-based media across the fermentation age

The produced succinic acid concentrations against the fermentation ages for the glucose and molasses media are shown in Figures 3-6 and 3-7, respectively. The maximum produced succinic acid concentration for the glucose-based media was 24.32 ± 0.20 $g.L^{-1}$ whilst the

molasses media showed higher results of 28.89 ± 3.57 $g.L^{-1}$. This is similar to the small-scale flask studies where the molasses proved superior on a concentration basis. The starting glucose concentration (time 0 hr), within the molasses media is lower compared to the glucose media, due to the molasses being made up of multiple types of sugars (Chapter 2). The glucose was depleted at 19 hr and 24 hr for the glucose and molasses media and so the biomass within the reactors was harvested so after. The depletion of glucose was an indication of when to harvest the reactors, to avoid the succinic acid from being converted to other downstream products in its TCA cycle, and although the measured OD did not display a constant peak prior to harvesting, as shown in Figure 3-3, it was important to harvest at that time-point to ensure a high succinic acid titre was achieved.

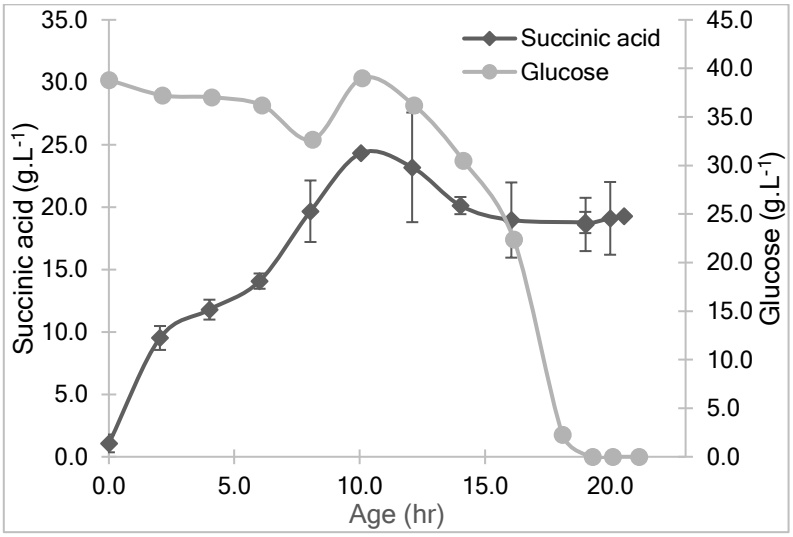

Figure 3-6: Succinic acid produced and the glucose levels across the fermentation age for the reactor using glucose as the carbon source

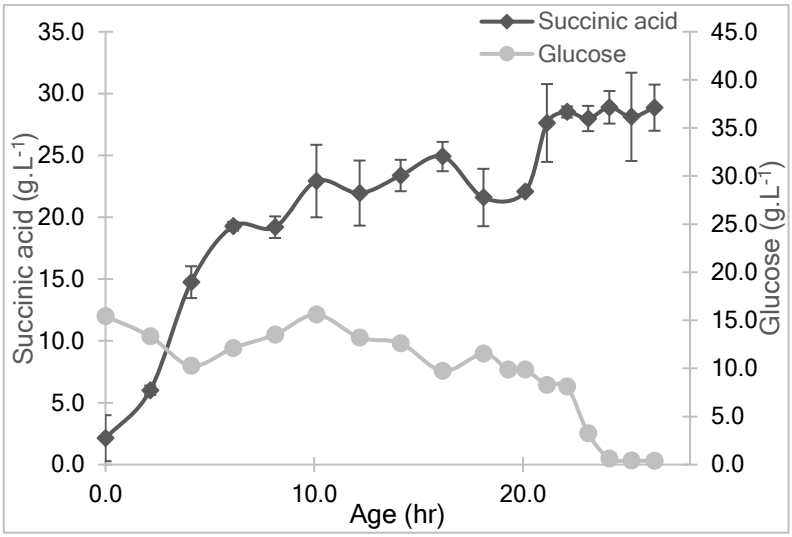

Figure 3-7: Succinic acid produced and the glucose levels across the fermentation age for the reactor using molasses as the carbon source

The fermentation age at which the maximum concentrations of succinic acid occurred at 10.05 hr and 24.14 hr for the glucose and molasses reactors. This equated to productivity values of 2.42 $g.L^{-1}.hr^{-1}$ and 1.20 $g.L^{-1}.hr^{-1}$. Molasses proved superior in yields and titres, however it was inferior with regards to the productivity aspect.

A proportional relationship between the cell density, shown by the absorbance and the dry biomass values (Figures 3-3 and 3-4 show the cell density), is observed between the succinic acid, especially for the molasses-based media. A similar trend was also observed by Okino (2005).

Multiple base options that included ammonium hydroxide, ammonia, calcium, sodium hydroxide and potassium hydroxide (as detailed in Chapter 1, Table 1-6) were considered prior to NaOH being selected. The calcium options for pH control have been reported to be toxic to *C. glutamicum* during succinic acid production (Song *et al.*, 2007). The magnesium alkaline selections showed similar concentrations of succinic acid to the sodium choices, and the magnesium cation is an activator for enzymes required in the pathway for succinic acid (Bazaes *et al.*, 2007). The ammonium bases resulted in inhibited cell growth and low concentrations of carboxylic acids (Shi *et al.*, 2014). When the sodium alkaline options were compared; NaOH, Na_2CO_3 and $NaHCO_3$, the sodium hydroxide resulted in the highest succinic acid concentration (Liu *et al.*, 2008). The sodium cation plays a crucial role in the cell's metabolism as it maintains the pH gradient across the membranes as well as its osmotic

pressure (Liu *et al.*, 2008). Thus, NaOH and $MgCO_3$ were the two preferred options with the magnesium ion already being present in the fermentation media and so sodium hydroxide was chosen as the base control. The pH control of the 5 M NaOH increased in dosing as the production of succinic acid increased, this trend for the molasses reactor is shown in Figure 3-8. During the period where a slight decrease in succinic acid was observed, around 18 hours, the quantity of base added to the reactor slightly decreased in comparison to other stages during the fermentation where the succinic acid concentration increased, *i.e.*, between 18.10 - 20.01 hours the base addition was 20mL and 80mL respectively, in comparison to the 100 - 220 mL base consumption over 21.15 - 24.14 hrs. Additionally, in controlling the pH to maintain a constant environment in the fermenter, other by-products are likely to be produced as a result of overflow metabolism which could result in an increase in base demand. The quantity of base consumed is an important factor, as this will influence the operating costs. The glucose media consumed a total of 1300 mL whilst molasses used 1900 mL. These totals included the initial pH adjustment of the initial media charge, prior to inoculating, to 7.5 (400 mL and 600 mL for glucose and molasses media, respectively). Lower quantities of base were consumed within the glucose-based media as a result of the shorter fermentation time as well as the initial media having a higher pH value, requiring less base for the preliminary pH adjustment. Through the use of pH control, which is required when bacteria is being grown, due to the sensitivity aspect of this microorganism to its environment, this will increase the raw material cost aspect of the process. The economic impact of pH controls will have to be considered in future work where it is recommended that techno economics is done (recommendations made in Chapter 5).

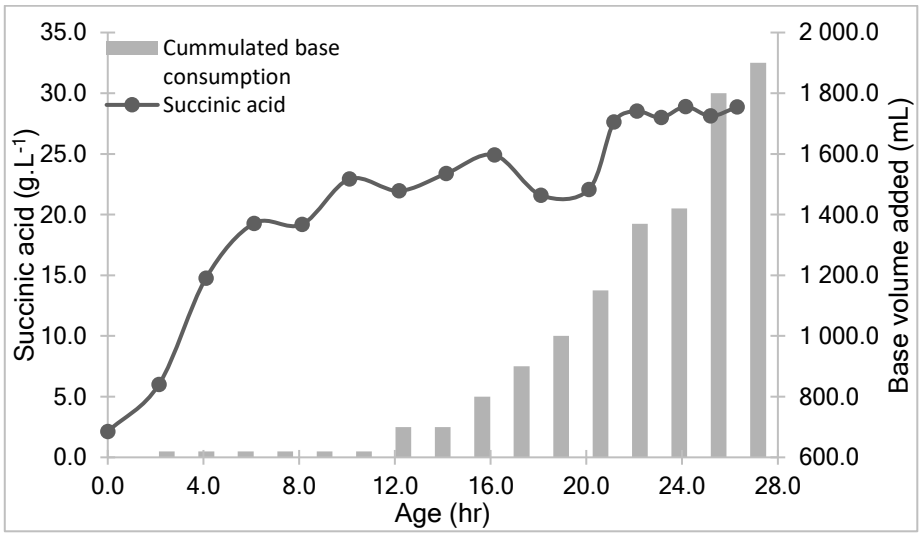

Figure 3-8: The produced succinic acid concentrations and the cumulative base consumed by the molasses reactor against the fermentation age

Table 3-2 shows a comparison between concentrations and productivity values across literature for *C. glutamicum* using a range of substates. Variations in succinic acid concentrations observed within the table can be accounted to a range of differing process components which including the microorganism itself (wild type versus varied GMOs), substrate and different fermentation modes (batch versus fed-batch). The highest succinic acid concentration obtained in literature was by the strain BOL-3/pAN6-gap at 146 $g.L^{-1}$. This was produced in oxygen deprived conditions. The highest succinic acid concentration, produced by a wild strain of *C. glutamicum*, i.e., *C. glutamicum 2262*, was 93.6 $g.L^{-1}$. The results from this study, especially the molasses batch proved successful with succinic acid concentrations in the ranges observed in literature (Table 3-2), showing that sugarcane material can effectively produce succinic acid.

Table 3-2: The succinic acid concentrations and productivity of *C. glutamicum* in literature

Microbe	Process	Substrate	Maximum concentration ($g.L^{-1}$)	Productivity ($g.L^{-1}.hr^{-1}$)	Reference
C. glutamicum 2262	Fed-batch	Glucose	93.6	1.42	(Briki *et al.*, 2020)
C. glutamicum R	Batch	Glucose	23	11.70	(Okino, Inui and Yukawa, 2005)
C. glutamicum BOL-3/pAN6-gap	Fed-batch	Glucose	146	2.48	(Litsanov, Brocker and Bott, 2012)
C. glutamicum BL-1/pVWEx1-glpFKD	Fed-batch	Glycerol	9.3	0.43	(Litsanov, Brocker and Bott, 2013)
C. glutamicum	Batch	Cassava bagasse hydrolysate	22.5	0.42	(Shi *et al.*, 2014)
C. glutamicum NRRL 11472	Batch	Glucose	24.32	2.42	This study
C. glutamicum NRRL 11472	Batch	Molasses	28.89	1.20	This study

3.4. Conclusions

The fermentation was successfully scaled up from the small-scale flask study (Chapter 2) for both the glucose and molasses-based medias. A maximum succinic acid concentration of 24.32 ± 0.20 $g.L^{-1}$ was produced in the glucose (ideal) substrate and the industrial media molasses produced superior concentration results of 28.89 ± 3.57 $g.L^{-1}$. Molasses achieved higher succinic acid yields which could be due to the molasses being of a complex nature, in terms of sugar (Van Wouwe, *et al.*, 2016) and other components, such as mineral and vitamins (Xu, *et al.*, 2015) in comparison to the glucose. These values equated to very satisfactory yield results, which can be seen in Figure 3-9. The improved yield from the small-scale flask studies to the biostat reactors displays a successful scaled-up process.

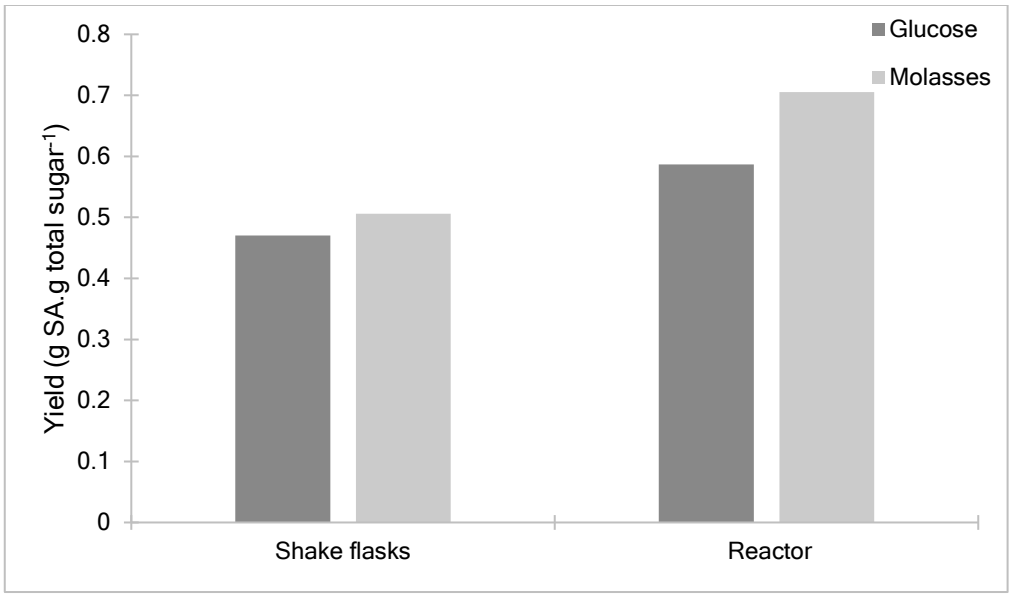

Figure 3-9: Succinic acid yield comparison of the *C. glutamicum* for the shake flask studies (synthetic and industrial medias) and the scaled-up reactor

The maximum succinic acid values were reached at a shorter fermentation age in the glucose media, therefore resulting in higher productivity values compared to the molasses media. The fermentation broth produced in the bioreactors will be used to determine the optimum downstream processing steps required to obtain succinic acid in a purified form (Chapter 4).

The 30 L reactor fermentation runs proved that succinic acid can be successfully produced at high concentrations whilst using an alternative carbon source, i.e., industrial material. The study shows that the sugar industry's biomass material has the potential to be used in alternative processing resulting in increased revenue possibilities. This study can therefore

inform a range of policies and strategies to be implemented in the country. This is especially significant to the recent Sugar Master Plan which was implemented by the government to ensure this vital South African sector survives.

3.5. References

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CHAPTER 4 DOWNSTREAM PROCESSING

4.1. Introduction

The harvested material from the scaled-up fermentations (Chapter 3), will require purification through the downstream processing (DSP) activities. There are multiple options available for these purification steps therefore a selection will be made based on literature, followed by experimental work.

4.2. Methods

4.2.1. Selection of method

The selection of the DSP method to be implemented is vital due to the recovery and purification steps being a major cost contributor to the overall process. The DSP steps commonly account for 50-80 % of the production costs (Cheng *et al.*, 2012; Lopez-Garzon and Straathof, 2014). A comparison of the DSP options for succinic acid is tabulated below in Table 4-1, the literature aspect of each was detailed in Chapter 1. A few issues that are associated with a majority of the purification methods include:

- Complexity, which results in issues for scaling up the process for industrial purposes
- Cost and time issues
- Use/ formation of large quantities of chemicals

There are numerous purification options, as detailed in Chapter 1 and no method has proven superior due to individual challenges associated with each (Londono, 2010; Cheng *et al.*, 2012).

Table 4-1: Comparison of the downstream processing steps available, detailing the advantages, disadvantages, succinic acid yields and purities

Method	Positives	Negatives	Recoveries (%)	Purities (%)	References
Filtration/ membranes	High purity	Low recoveries High equipment costs Very environmentally unfriendly	75.0	99.5	(Cheng *et al.*, 2012) (Wu *et al.*, 2011) (Yao *et al.*, 2008)
Crystallisation	Few unit operations Higher researched process	Low recoveries Not sufficient method on its own, requires removal of impurities Additional required methods have high energy costs	70.0 57.0-79.0	90.0 90.0-96.0	(Cheng *et al.*, 2012) (Li *et al.*, 2010) (Alexandri *et al.*, 2019)
Precipitation	Low technology barriers Depending on the type of precipitation, recycling of reagents/by-products could be possible. Easy to scale up	Large quantities of reactants High operational costs Erosion of equipment	93.3 13.0	48.7-60.0 81.0	(Cheng *et al.*, 2012) (Yedur, Berglung and Dunuwila, 2001) (Alexandri *et al.*, 2019) (Sosa-Fernandez and Velizarov, 2018)
Electro-dialysis	Membranes expensive Membranes a pollutant	High membrane and electricity costs Low recoveries Environmentally unfriendly - high membrane pollution	60.0		(Cheng *et al.*, 2012) (Zeikus, Jain and Elankovan, 1999) (Li *et al.*, 2009)

Solvent/ reactive extraction	Low energy costs	Requires complicated pre-treatment and post treatment High reactant costs Complicated method Not easy to scale up	78.0-85.0 73.0	97.2	(Cheng *et al.*, 2012) (Kurzrock and Weuster-Botz, 2011) (Alexandri *et al.*, 2019)
Adsorption	Clean process Reduced reagents High purity Easy to scale up Quick recovery	Co-adsorption of other by-products Regeneration of chromatographic medium - large quantities of chemicals Usually results in dilution due to low selectivity	99.0	89.5	(Efe *et al.*, 2011) (Davison, Nghiem and Richardson, 2004) (Cheng *et al.*, 2012) (Lin *et al.*, 2010)

4.2.2. Method evaluation

4.2.2.1. Succinic acid recovery

The recovery (%) across each step and the overall process for each downstream processing method was calculated as shown in Equation 4-1:

$$\textbf{Recovery}\ (\%) = \frac{\textbf{mass of SA purified}_{i,j}}{\textbf{mass of SA starting material}_{i,j}} \times \mathbf{100} \quad (4\text{-}1)$$

Where,

i = fermentation broth; glucose or molasses

j = downstream processing method

mass of SA purified i, j = mass of succinic acid purified using the fermentation broth, i, and the downstream processing method j, $g.g^{-1}$

mass of SA starting material i,j = mass of succinic acid in the starting material of fermentation broth, i, for the downstream processing method, j, $g.g^{-1}$

4.2.2.2. Succinic acid purity

The purity (%) was calculated for each downstream processing method as shown in Equation 4-2:

$$\textbf{Purity}\ \% = \frac{\textbf{mass of SA purified}_{i,j}}{\textbf{mass of purified product}_{j}} \times \mathbf{100} \quad (4\text{-}2)$$

Where,

i = fermentation broth; glucose or molasses

j = downstream processing method

mass of SA purified i, j = mass of succinic acid purified using the fermentation broth, i, using downstream processing method, j, g.

mass of purified product i, j = mass of the final material obtained from the fermentation broth, i, using the downstream processing method, j, g

4.2.3. Downstream processing methods

The downstream processing method was determined using the fermentation broth from the ideal carbon source, i.e., glucose. Once the method had been determined it was then repeated using the fermentation broth containing the industrial media (molasses) to compare recoveries and purities, and to determine whether the downstream processing of the succinic acid from an underutilised sugar stream is a viable option.

4.2.3.1. Cell removal methods

Two cell removal methods were performed to determine the superior method, Figure 4-1 shows a process flow diagram (PFD) of the two options, after which clarification methods were followed (Section 4.2.2.).

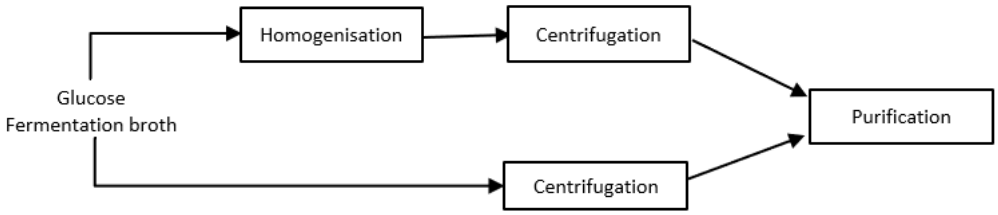

Figure 4-1: Cell removal through a combination of homogenisation and centrifugation compared to just centrifugation

Homogenisation and Centrifugation

There are numerous references to fermentation broth being homogenised prior to centrifugation (Yang *et al.*, 2016; Sosa-Fernandez and Velizarov, 2018), therefore this additional step prior to centrifugation was tested.

Triplicate studies were performed where two hundred millilitres of fermentation broth was homogenised using an Ultra Turrax IKA T18 at 6000 rpm for 15 minutes, after which centrifugation was performed at 4500 rpm, 20 ºC for 30 minutes. Samples were taken on each of the triplicate studies after each process unit operation, for succinic acid analysis on the HPLC.

Centrifugation

Triplicate studies were performed where two hundred millilitres of fermentation broth were centrifuged at the same parameters as detailed above in section 4.1.3.1. Samples were taken before and after centrifugation and analysed for succinic acid was done.

4.2.3.2. Purification methods

Traditionally, ultrafiltration is applied as a clarification step especially in water treatment operations, however, due to the pore size of the membranes and that which would be required for succinic acid, it cannot be used as such (Prochaska *et al.*, 2017). Multiple studies as well as the industrial producer, Myriant, uses ultrafiltration as a pre-treatment step. This is due to ultrafiltration being used for the removal of suspended solids, microorganisms, proteins and also to improve turbidity (Prochaska *et al.*, 2017). The ultrafiltration step was applied to all the purification methods, as shown in Figure 4-2.

Two methods for the ammonium precipitation method were tested. Due to conflicting literature methods for the sequence of processes (Sosa-Fernandez and Velizarov, 2018; Shmorhun, 2015) one method setup included pH adjustment to 11.0 and one method setup without. The methods followed for the DSP activities were based of these two sources (Sosa-Fernandez and Velizarov, 2018; Shmorhun, 2015). The placement of the decolourisation step was tested on both methods at the beginning of the purification process and at the end of the process. Figure 4-2 is a PFD of these different processes.

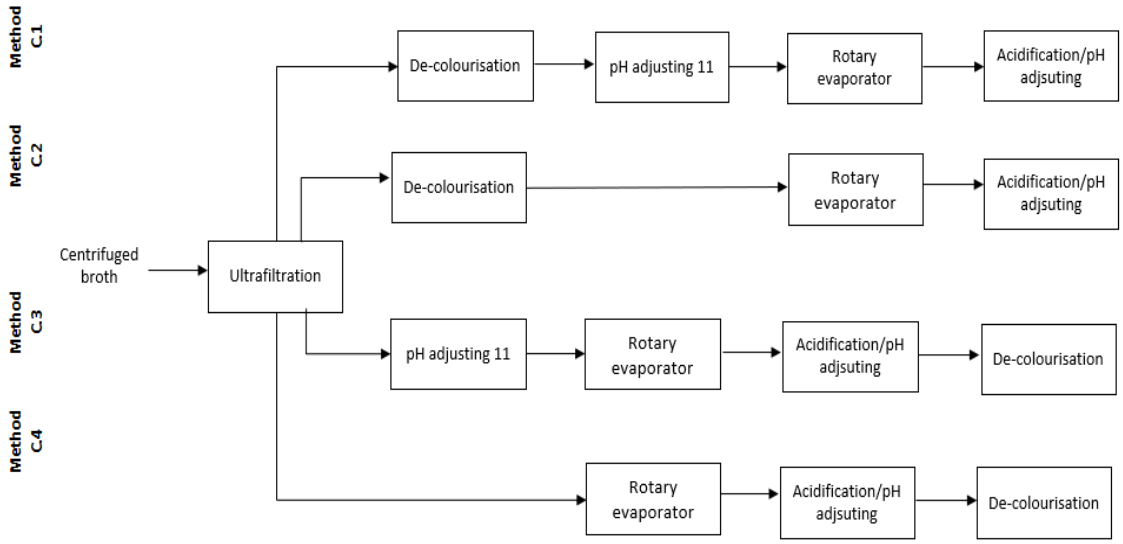

Figure 4-2: Process flow for purification methods (C1-C4)

In methods C1 and C2, de-colourisation was performed at the beginning of the purification process, whilst C3 and C4 concluded with this step. In methods C1 and C3, pH adjustment with ammonium was conducted whilst methods C2 and C4 had no adjustment in the process. All the methods were performed in triplicate.

The pH of the de-colourised/filtered broth (method C1/C3) was measured, and a 25 % ammonium solution was used to adjust the pH to 11.0 – the volume of ammonium solution was recorded. Due to the fermentation broth having a combination of succinic acid (free form) and diammonium succinate, due to the addition of the ammonium hydroxide (fermentation's pH control), the pH is adjusted to obtain a solution containing only di-ammonium succinate (Sosa-Fernandez and Velizarov, 2018).

The pH adjusted solution was concentrated to roughly a third of its volume using a rotary evaporator set at 50 ºC, vacuum of 80 kPa and stirring at 90 rpm. The vacuum evaporation assisted in the removal of volatile carboxylic acids, such as formic and acetic acid (Alexandri *et al.*, 2019). The removed volatile acids, have a potential to be a by-product stream from the succinic acid process, further work could be done in the future to determine if these could be effectively separated and purified as other product pure streams during the production of succinic acid. Should this be possible it will have a positive effect in lowering the overall techno-economics of the process due to multiple final products being recovered during the fermentation process. The concentration of the solution results in a decreased amount of acid required in the acidification step.

The pH of the concentrated fraction was then pH adjusted to 1.5 using concentrated sulphuric acid (98 %), the volume of acid used was recorded. The addition of the sulphuric acid results in the formation of succinic acid in its free form, from the di-ammonium succinate. This free form is the required state of the succinic acid.

The pH adjusted solution was then de-colourised using activated carbon. Various studies utilise activated carbon for the clarification step. Different loadings have been tested to determine the optimum loading ratio, Alexandri (2018) reported that 12.5 % ($w.v^{-1}$) resulted in almost complete colour removal. Karp (2018) determined that the activated carbon step should be used after ion exchange thereby reducing the quantity required. They determined that 3.0 % ($w.v^{-1}$) was the optimum ratio for 4 hours. The removal of colour bodies is highly effective with the use of activated carbon. However, the drawback is that absorption of succinic acid by the activated carbon will occur, thereby lowering the overall product yield

(Karp *et al.*, 2018). Table 4-2 shows the reported succinic acid loss due to absorption. Although Alexandri (2018) reported the higher ratio of activated carbon was effective in the removal of colour, it was not specified whether succinic acid was lost in this step.

Table 4-2: Activated carbon loading and the resultant succinic acid lost in literature

Activated carbon ($w.v^{-1}$ %)	Succinic acid lost (%)	References
1.0	6.0	(Karp *et al.*, 2018)
7.0	21.0	(Karp *et al.*, 2018).
12.5	Not specified	(Alexandri et al., 2018)

Samples were taken throughout the process before and after each unit operation to determine the succinic acid recovery and the final purity of the succinic acid obtained for each of the purification methods C1 to C4. When the pH is below 2, i.e., below succinic acid's pKa values, the solubility at 4 ºC is only 3 % whilst other acids, produced as by-products, will still be in their miscible form (Li, *et al*., 2010). The solution from the optimum purification method was therefore agitated at 4 ºC and 500 rpm and resulted in the formation of succinic acid crystals, i.e., the crystallisation step. The solidified succinic acid was then dried at 60 ºC for 24 hours. These steps are shown in Figure 4-3 below.

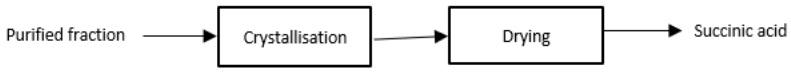

Figure 4-3: Crystallisation and drying stages from the purified fraction

Samples were taken as each step of the crystallisation and drying stages and the final recovery and purity of the crystals was determined.

4.2.4. Downstream purification of molasses-based fermentation broth

The optimum method that was determined with the glucose-based fermentation broth was then conducted using the molasses-based broth. This was performed in triplicate studies.

4.3. Results and discussion

4.3.1. Selection of methods

The companies that are currently producing succinic acid at an industrial scale, given in Table 1-4 in Chapter 1, are an important decision-making tool due them successfully producing and purifying succinic acid at a commercial large scale. Two of the companies, BioAmber (Sarnia, Canada) and Reverdia (Cassano Spinola, Italy), both use yeast fermentation processes that can survive in low pH environments, therefore removing the need for large amounts of base control and reducing the downstream processing steps. The other two commercial companies, Succinity and Myriant, use bacterial fermentations processes followed by precipitation for downstream purifying. Succinity utilises magnesium precipitation whilst Myriant conducts ammonia precipitation (Sosa-Fernandez and Velizarov, 2018). A comparison between the different precipitation methods available, is detailed in Table 4-3 below.

Sosa-Fernandez (2018) experimentally compares the three precipitation methods (Table 4-3) and concludes that ammonia precipitation results in the highest yield and purity (Sosa-Fernandez and Velizarov, 2018). Myriant and Succinity successfully produced succinic acid utilising precipitation as their downstream purification method. Literature (Table 4-1) also revealed that the ammonium process resulted in the highest recoveries. Hence, these methods will be experimentally tested.

Table 4-3: The advantages and disadvantages of the precipitation methods

Method	Advantages	Disadvantages	SA recoveries (%)	References
Calcium	High yields of SA Use existing infrastructure from lactic and citric industries	High consumption of reactants No regeneration/ recycling Poor quality of by product, calcium sulphate, therefore unlikely that it will be commercialised Slow process	33.67 36.00	(Sosa-Fernandez and Velizarov, 2018) (Luque *et al.*, 2009) (Cheng *et al.*, 2012)
Magnesium	Lowest chemical costs comparing the precipitation methods	Heating required, increases cost	75.10 86.00	(Sosa-Fernandez and Velizarov, 2018) (De Haan *et al.*, 2013)
Ammonia	Lower quantities of waste by products By product, di-ammonium sulphate, can be used in fertiliser industry or thermally cracked to ammonia and ammonium bi-sulphate Cheap cost for ammonia	Higher chemical costs Equipment erosion	83.58 78.00	(Cheng *et al.*, 2012) (Sosa-Fernandez and Velizarov, 2018) (Yedur, Berglung and Dunuwila, 2001)

4.3.2. Downstream processing methods

4.3.2.1. Cell removal methods

A comparison of the two methods for the cell removal method is shown in Figure 4-4 where a comparison of the succinic acid and mass recovery for each method is displayed. By comparison, the addition of the homogenisation step prior to centrifugation was not a necessary step to include, as it did not result in higher succinic acid concentrations.

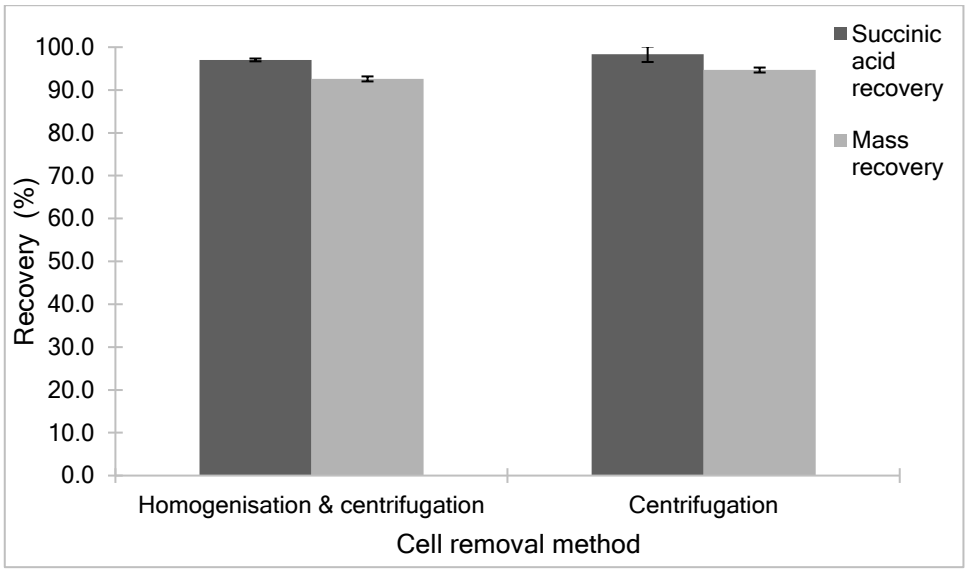

Figure 4-4: Recovery of succinic acid and mass recovery of the supernatant for the two cell removal methods

The recovery of succinic acid in both methods was very similar with the homogenisation combined with the centrifugation resulting in 97.02 % ± 0.32 % and the centrifugation only method, resulting in 98.35 % ± 1.81 %. From a mass balance perspective in relation to the supernatant mass, again, the results are similar, which is an expected outcome, due to the homogenisation step not including any removal of material. The centrifugation only method was therefore determined to be the preferred technique especially from an economical aspect.

4.3.2.2. Purification methods

Initial experimental de-colourisation steps were performed using the 12.5 % activated carbon loading as suggested by Alexandri (2019). Triplicate studies of 100 mL of ultrafiltered supernatant was stirred at 700 rpm, at 21 ºC for 60 minutes, after which the activated carbon

was removed through filtration. Such a loading resulted in a 97.87 ± 0.09 % succinic acid adsorption. This loading percentage did result in visually clear solutions, Figure 4-5. However, due to extremely high loss, this process step will not be a viable option.

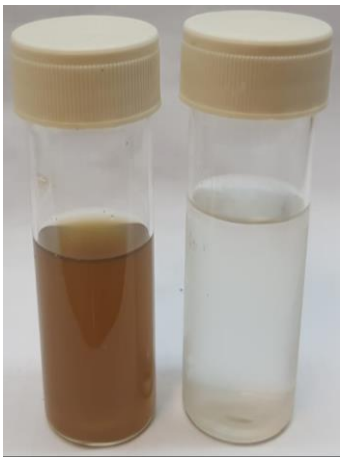

Figure 4-5: Photograph of ultrafiltered supernatant (left) and the activated carbon treated solution at a 12.5% loading (right)

The activated carbon loading that resulted in the lowest succinic acid loss, reported in Table 4-2 was therefore tested, a 1 % ($w.v^{-1}$) loading amount was used. Repeating the same procedure resulted in a 49.81 ± 0.67 % succinic acid loss. Even though this is significantly less than the 12.5 % loading amount, such a loss in succinic acid will still not be a viable option. It has been reported that to avoid the adsorption of succinic acid by the activated carbon, that hydration of the activated carbon should be applied to reduce this loss (Alexandri *et al.*, 2019). The activated carbon was therefore hydrated and the 1% loading was then repeated, the hydration step proved to be an essential step with 18.85 ± 1.26 % loss of succinic acid being recorded. The only downfall of the low carbon loading percentage was that the material was not as visually clear as that shown in Figure 4-5.

Due to various stirring times, reported in literature, for this activated carbon step, initial experiments were then conducted to determine the required stirring time. The stirring time was then increased to observe the effect at a low hydrated carbon loading (1 %). The results of which are presented in Figure 4-6.

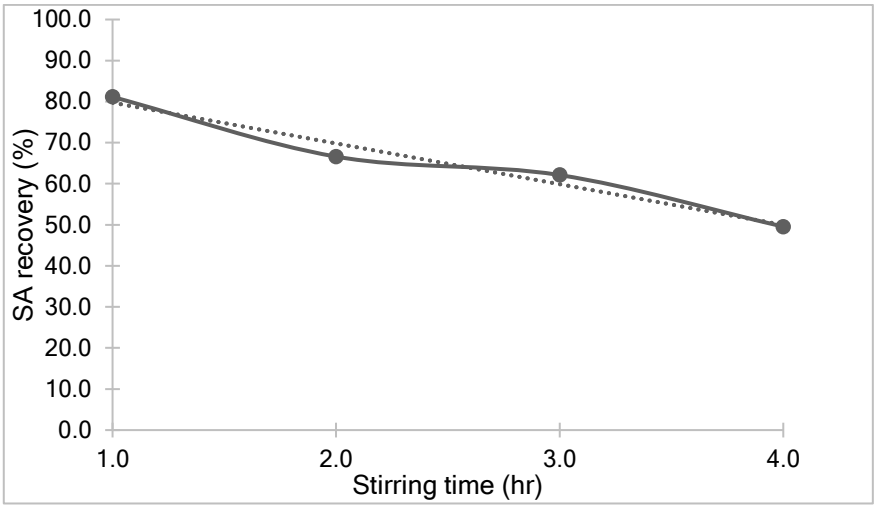

Figure 4-6: Succinic acid recovery percentage using a 1% hydrated activated carbon loading with increased stirring time

As the stirring time increased, the succinic acid recovery decreased in a linear fashion. The linear trendline fitted to the date resulted in an R^2 value of 0.9659. Visually there was no improvement over the increased mixing time, the filtrate from the activated carbon loading was read on a spectrophotometer at 280 nm. This UV wavelength tracks the relative amount of aromatic components which contributes to the colour (Karp *et al.*, 2018). The UV absorbance results confirmed that there was not a significant improvement in the colour reduction over the increased stirring time. Therefore, for the de-colourisation steps in the purification processes, 1 % loading, using hydrated activated carbon, at one hour of stirring is recommended.

The four purification processes that were evaluated (methods C1-C4) and the succinic acid recoveries obtained are displayed in Figure 4-7.

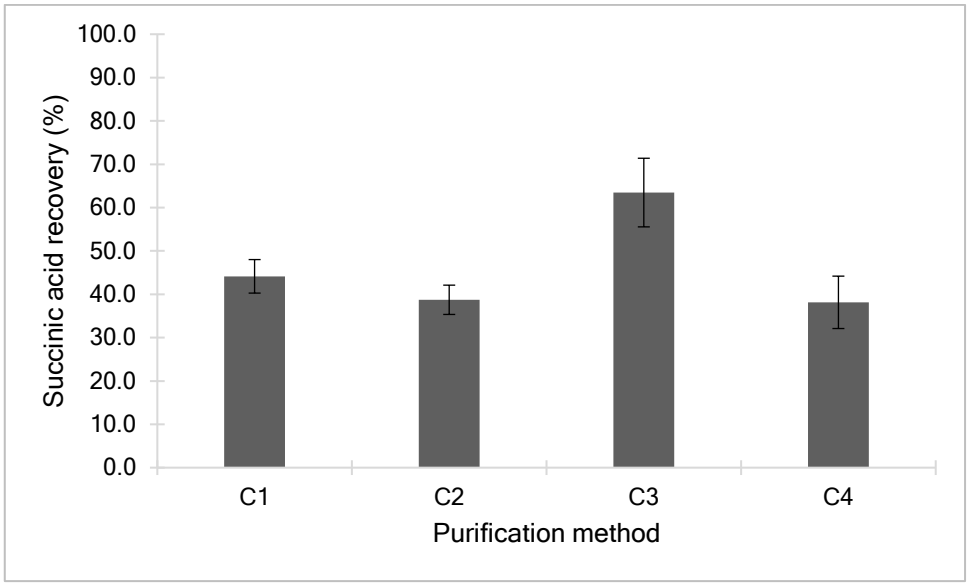

Figure 4-7: Succinic acid recoveries of the four purification methods tested (C1-C4)

Method C3, namely with a pH adjustment to 11.0 at the beginning of the process and ending in a de-colourisation step produced the highest succinic acid recovery of 63.47 ± 7.91 %. There was a significant improvement seen in the succinic acid recovery when the pH was increased to 11.0, i.e., C3 vs C4, a 25.33 % difference. These results were confirmed, less evidently, through methods C1 and C2, where only a 5.41 % improvement was seen when the pH adjustment was conducted, however all these purification methods confirm that such a step should be performed. The pH altering stages are necessary steps to ensure the correct chemical reactions take place to have the solution/solid in the desired state. For the alkaline addition (pH adjusting the solution to 11.0), through the addition of ammonium hydroxide results in di-ammonium succinate. The acidification step, using sulphuric acid, which converts the di-ammonium succinate into succinic acid, in its free acid form, and di-ammonium sulphate. The di-ammonium sulphate can be a potential by-product stream as it can be used as a fertiliser or thermally cracked into ammonia and ammonium bisulphate (Sosa-Fernandez and Velizarov, 2018).

Based on circular economy thinking, that strategy during commercial implementation of this process needs to include waste valorisation steps such as acid extraction from the final broth and recycling of the acid into the process. There has been advances in commercial scale acid recycling processes which will need to be assessed for applicability in this process (Avantium, 2019).

The de-colourisation step through hydrated activated carbon at a 1 % ($w.v^{-1}$) loading, proved more successful based on succinic acid recovery, at the end of the purification process. Additionally, from an economic perspective, performing this step at this point is a more feasible option due to the decreased amount of activated carbon required, as it is then done after the vacuum evaporation, therefore a third of the volume is used compared to Methods C1 and C2. The vacuum evaporation step also results in an increase in the viscosity and therefore the colour of material is darker due to volume reduction. Figure 4-8 compares material before and after rotary evaporation, to show the colour change that occurs. Therefore, from a practical perspective it makes more sense to include the de-colourisation after the step that results in an increase in the colour and turbidity.

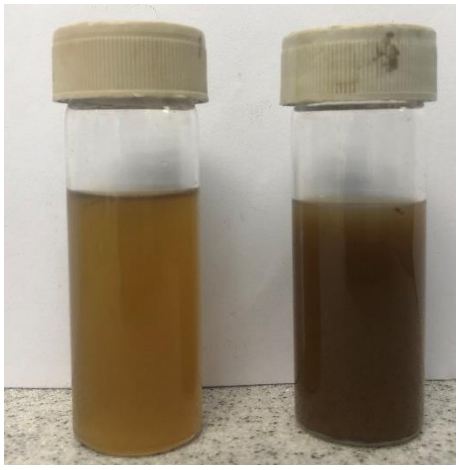

Figure 4-8: Photograph of solution before vacuum distillation (left) and after vacuum distillation (right)

The anticipated process for the activated carbon is absorption and therefore di-colourisation of the solution occurs. The used activated carbon is then a waste stream, it is due to this fact, that was a contributing factor in the C3 DSP method being chosen as a reduced amount of activated carbon is used and therefore created as waste from this step. It is recommended that future work focuses on this process unit operation, and determines if alternative de-colurisation methods could be implemented, for example anion exchange or bleaching practises (Karp *et al.*, 2018).

The results obtained by the optimum purification method, namely C3, are comparable to literature results using ammonia precipitation, with SA recoveries ranging from 58.6 – 83.6 % (Sosa-Fernandez and Velizarov, 2018; Yedur, Berglung and Dunuwila, 2001) and purities ranging from 14.1 – 48.7 % (Sosa-Fernandez and Velizarov, 2018).

Crystallisation and drying of the superior method (C3) were then performed and resulted in an overall recovery of 54.47 ± 14.02 % and a purity of 22.21 ± 1.13 %. Due to Method C3 proving superior to the other purification methods, this was then repeated using the broth produced from the industrial media source, the molasses-based media.

4.3.3. Downstream purification of molasses-based fermentation broth

The preferred downstream purification process, as determined using the glucose-based media, was then repeated using the molasses, Figure 4-9 shows the PFD for the downstream processing method.

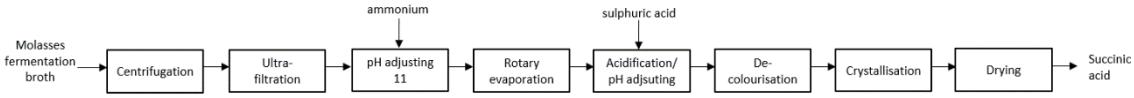

Figure 4-9: Process flow diagram of the downstream purification for the molasses-based fermentation media

The overall recovery (%) for the succinic acid for each step shown in Figure 4-9 above, is displayed in Figure 4-10, this recovered amount is based on the succinic acid concentrations present in the harvested fermentation broth.

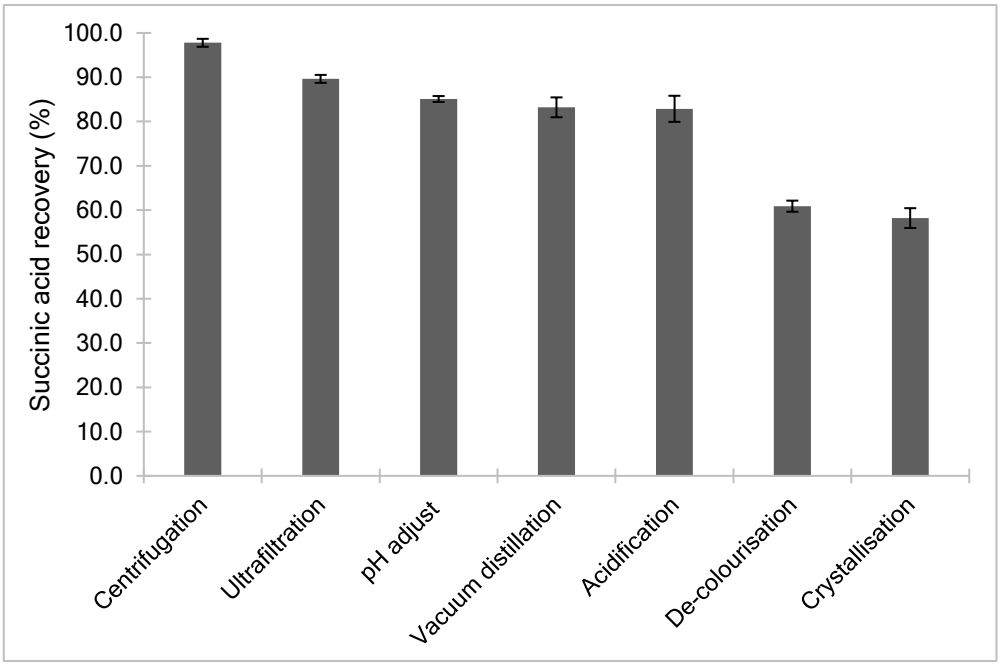

Figure 4-10: Succinic acid recoveries across each step in the purification process

The highest loss in succinic acid was observed over the de-colourisation step which accounted for a 21.98 % reduction in succinic acid. This absorption is an expected result as it was observed in the glucose-based fermentation media. The molasses-based broth's purification process resulted in succinic acid recovery of 58.20 ± 2.24 % which is a similar result obtained from the glucose process. This shows that the determined purification method was successfully replicated using the industrial media source.

4.4. Conclusions

The ammonium type precipitation was the chosen downstream purification method due to the higher succinic acid yields and purities reported in literature. The two cell removal methods for the initial stage of the downstream process, namely homogenisation followed by centrifugation versus just centrifugation, showed that the results were similar within the analysis error. Hence, the removal of the homogenisation step will result in a more cost-effective option due to decreased capital and operational costs.

The purification method C3, which included the pH adjustment to 11.0 and concluded in the de-colourisation step at the end of the process, proved to be the superior method due to the highest recovery of succinic acid of 63.47 ± 7.91 %. The de-colourisation step through hydrated activated carbon, proved more successful at the end of the purification process. From an economic perspective, performing this step at this point is a more feasible option due to the decreased amount of activated carbon required as it is then done after volume reduction step. The crystallisation and drying stages performed on method C3's material resulted in a succinic acid recovery of 54.47 ± 14.02 % and a purity of 22.21 ± 1.13 %. Once the process was determined, broth from the molasses-based media was then used for the purification of succinic acid, the selected process flow is presented in Figure 4-11.

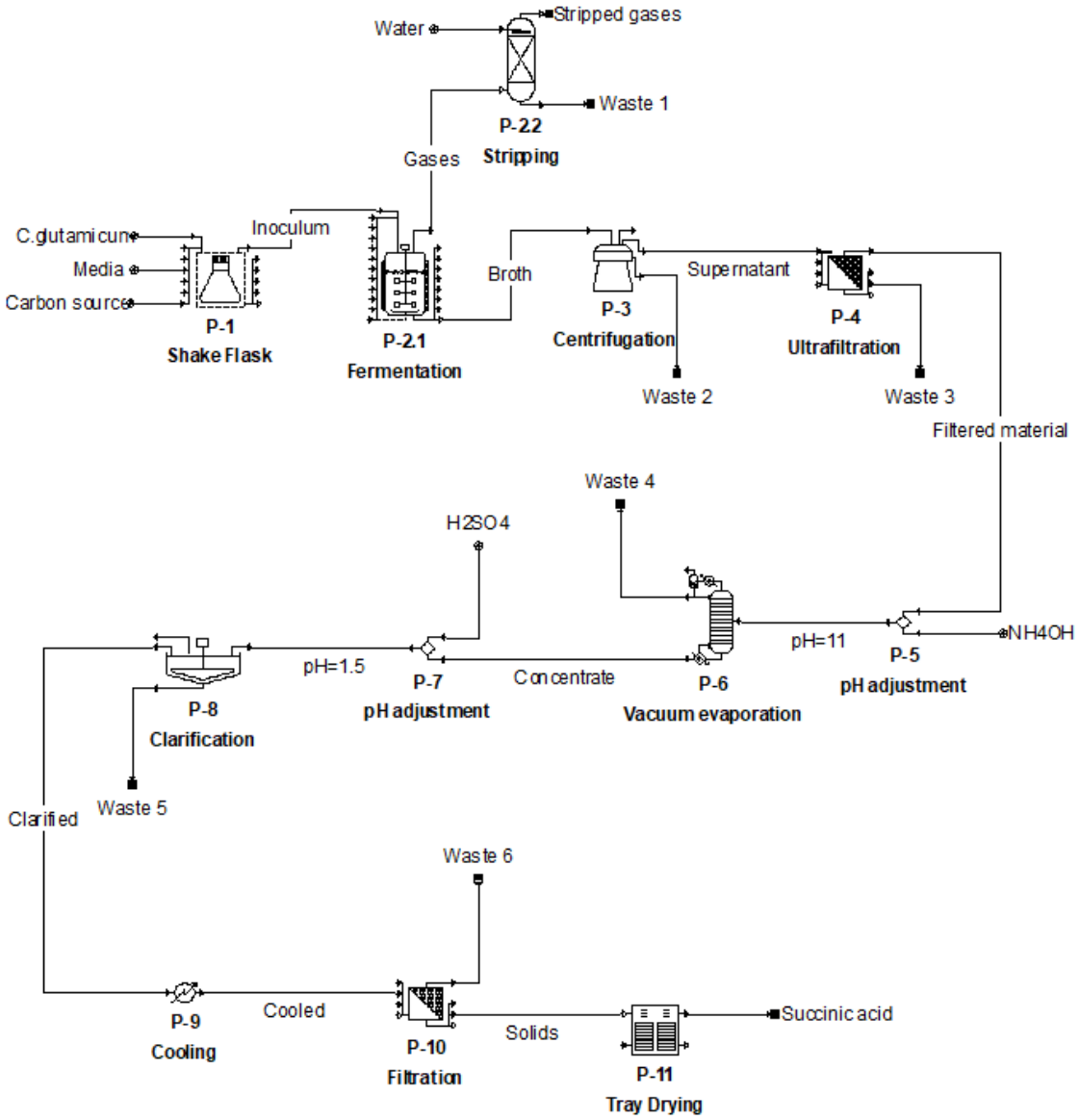

Figure 4-11: The determined process flow displaying the upstream and downstream selected methods for the bench scale production of bio-succinic acid

Method C3 (displayed in Figure 4-11) was then repeated using the fermentation broth from the molasses-based media and proved to be successful with a similar and slightly improved succinic acid recovery of 58.20 ± 2.24 %. This shows that succinic acid can be successfully recovered from a fermentation broth containing succinic acid produced using an under-utilised feedstock as the carbon source.

4.4. References

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CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The following dissertation assessed the production of succinic acid using available sugar sources from the South African sugar industry as a potential industry-diversification option. Succinic acid is traditionally manufactured through petrochemical routes however this chemical can also be produced through certain microorganisms' metabolic pathways, these cellular respiratory cycles will be dependent on the type of microorganism. The literature review section (Chapter 1) highlighted the importance of succinic acid and the different processing options for this high-value platform chemical.

The South African chemical industry is a sector of great importance for the country and with the global shift towards green chemicals, it is important that this industry adapts and thrives in this environment. The transition towards greener processes requires bio-based production with the integration and utilisation of South African feedstocks. The sugar industry has had a tough couple of years due to the recent implementation of legal regulations as well as cheaper export options being available. This has thus resulted in the sector having multiple waste streams or underutilised product streams available. Therefore, assessing alternative production options is a key output of this work. The sugar feedstock material is a potential carbon source for the upstream process, in the production of bio-succinic acid. The downstream steps are the largest cost contributor in the succinic acid process, there are multiple researched purification processes however none of these methods have proved superior due to complex nature of succinic acid.

The literature study resulted in the identification of a range of succinic acid producing microorganisms including fungi, yeasts, and bacteria, being selected from those that were readily available at the CSIR (Pretoria, South Africa). The identified microorganisms were then grown at a flask scale level (700 mL) in synthetic media, on both C5 and C6 sugars, namely xylose and glucose. The biomass production, pH, absorbance, glucose consumption and succinic acid production abilities were assessed. The results of which showed that all strains were capable of producing succinic acid on both the glucose and xylose-based medias, the C6 sugar group and *L. paracasei* and *C. glutamicum* showed superior performance. These microorganisms were then grown at a flask scale level (700mL) using industrial feedstock, molasses and extracted sugarcane juice, as the carbon source. These studies showed that *C. glutamicum* on the molasses-based media was the favoured option reaching a concentration of 20.24 ± 0.75 $g.L^{-1}$ and a productivity of 1.09 ± 0.07 $g.L^{-1}.hr^{-1}$. From a technoeconomic point of view, molasses biomass material is a cheaper industrial feedstock to use compared to the sugarcane juice for two reasons: Firstly, molasses is a lower value by-product from the

industry during primary production of sugar: Secondly, molasses is concentrated and has a higher sugar content than SCJ in its present form. For SCJ to be consider an additional concentration step will have to be introduced before the feedstock can be used for the bioconversion to succinic acid.

During the scale-up process (30L biostat reactors) *C. glutamicum* showed favourable results on the molasses-based media when compared to the 'ideal' carbon source, glucose. Reactors were run under controlled conditions where pH, agitation, temperature, pressure, and aeration were maintained. The maximum production levels of succinic acid using the glucose-based media and molasses-based media were 24.32 ± 0.20 g.L^{-1} and 28.89 ± 3.57 g.L^{-1} respectively.

The yields achieved in the shake flasks are relatively low but are considered satisfactory for an uncontrolled environment. An improvement was observed under controlled fermentation conditions in the bioreactor which is an expected/desired result as conditions are monitored and controlled. The yield achieved in the molasses biostat reactor run was slightly higher than the aerobic theoretical yield of 1 mol succinate per mol of glucose (0.66 g.g^{-1}). This slightly higher recorded value could be due to the molasses being a complex media which may have additional carbon and reductant sources within the material and/or additional sugar groups not accounted for within the biomass screening stage.

The harvested broth from the fermentation was used to assess the downstream processes for recovery of the bio-succinic acid. The literature chapter (Chapter 1) established that the downstream processing steps are a vital part of the succinic acid cycle, due to the high costs associated with the purification section. The multiple downstream purification options were explored through a literature review, each purification option had challenges associated with it, so no method has proved superior to the others. The downstream processing method was experimentally assessed was ammonium precipitation, due to its easy operations, cost, and commercial applicability. The downstream processing can be classified into three main process unit operations: the removal of cells and impurities, primary separation of succinic acid and finally purification. Various methods were tested using the harvested glucose broth, to determine the optimum process. Once the process was determined, broth from the molasses-based media was then used for the purification of succinic acid.

For solid-liquid separation two methods were compared, centrifugation versus a combination of centrifugation and homogenisation. The centrifugation method was selected due to higher recoveries, additional, from a techno-economic perspective this is saving on time, energy, manpower and capital costs that are involved with the addition of an extra processing step.

The centrifuged fermentation broth then went through ultrafiltration, for the removal of impurities after which the purification phase was entered. Ammonium precipitation was the purification method utilised. This was determined through literature studies and observing what is currently done internationally at industrial level. Four variations of the ammonium precipitation method were experimentally tested, at each stage of these methods, succinic acid analysis was performed using the HPLC to determine quantities which were used for mass balance calculations. The method 'C3', which is shown in the process flow above (Figure 5-2), proved to be the optimum ammonium precipitation process. The succinic acid recovery for the glucose-based fermentation broth was 54.47 ± 14.02 % and 58.20 ± 2.24 % for the molasses-based broth, the deviation in these recoveries could be due to difference in the broth's viscosity and nature.

5.2. Recommendations

The production of succinic acid was successfully demonstrated with the use of products/by-products from the sugar industry. This further reveals the processing possibilities for this industrial sector, which is not only limited to this platform chemical but could be used in the production of a vast range of bio-based chemicals. To aid in the process of achieving commercial reality for bio-succinic acid and other bio-based chemicals the following recommendations are made:

- Hyper-production possibilities of the microbial strains

  Based on the advances in Industrial Synthetic Biology, tools such as CRISPR-Cas9 that allows for genome editing can now be applied to metabolic engineer microorganisms, thus allowing hyper-production of succinic acid or other bio-based chemicals. This provides an exciting opportunity for the commercialisation of a number of bio-based chemicals due to overall process improvements in terms of bioconversion yields and techno-economics because of hyper-synthesis.

- Identification of additional potential feedstocks

Carbon sources account for a large percentage of the raw material cost for fermentations, so the identification of additional cheaper or currently discarded (industrial waste streams) options that are renewable resources is an important aspect to consider. Although this study focused on the sugar industry's feedstocks, there are unlimited potential biomass feedstock options from forestry, agricultural, industrial waste, domestic waste and the aquacultural industry, that could be utilised in such processes.

- Optimisation of fermentation process

Further work is recommended in the upstream processing sector, where different feeding strategies can be explored to limit the effects of overflow metabolism and direct the carbon flux more efficiently to succinic acid production.

- Scaling-up process

It is recommended that both the upstream and downstream process be scaled-up to demonstrate the economies at that scale, with the potential for commercial toll manufacturing. The information from these suggested studies could be used for the techno-economic evaluation (mentioned below).

- Improvements in DSP activities

The DSP activities, for a large majority of bio-based chemical processes, require simplification and a reduction in the number of processing steps, this sector accounts for the majority of the production costs.

- Process modelling and techno-economic evaluation

Engineering software should be used to draw up the process model and perform an in-depth techno-economic assessment of the process. The information obtained from such a task will determine whether the process is economically viable. Additionally, it can be used for the optimisation of the process through the identification of bottle-necking operations.

The recommendations stated above are suggested with the main aim of improving succinic acid concentration, yield, titre, and productivity values, additionally ensuring that the process is feasible.

Globally the drive towards ‘sustainability’ is the focus in many sectors, the traditional manufacturing methods require re-working and adapting to ensure cleaner and greener processes. For such systems to be adopted into the economy they need to be cost competitive. Having processes that are economically viable is a huge challenge especially as traditional processes have been optimised over the years and the knowledge and infrastructure is ‘cemented’ into place. These are the issues that are faced by many biomaterials, due to industry-low technology readiness as well as pushback from the markets associated with these products. This is largely due to higher costs, uncertainty linked to new technologies and a general lack of knowledge. The chemical industry plays a crucial role in the global economy, thus research that focuses on one of the important platform chemicals, succinic acid, is imperative in the demonstration of the processing possibilities of South Africa industries. The work covered in this dissertation highlighted South Africa’s sugar sector, however there are multiple industries that have underutilised products/by-product streams that could have the same potential. However, like many other biomaterial processes, it requires additional research to ensure that the process is optimised. Additional research will result in the minimal generation of by-products, minimal consumption of raw materials, processing steps are reduced, decrease in environmental impact and an overall reduction in costs. This optimisation helps the strive towards industrial symbiosis.

The work presented in this dissertation is a demonstration of succinic acid production which could potentially be integrated into the South African sugar industry as part of their diversification portfolio.