2015 Bioproduction of succinic acid from renewable feedstocks by *Actinobacillus succinogenes* 130Z Margarida Carvalho 

Margarida da Silva Ferreira Morais de Carvalho

Mestre em Biotecnologia e Biologia Celular

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Dissertação para obtenção do Grau de Doutor em
Química Sustentável

Orientador : Christophe François Aimé Roca, Investigador,
Universidade Nova de Lisboa

Co-orientadora : Maria Ascensão Miranda Reis,
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A todas as pessoas que me inspiram a dar o meu melhor,
todos os dias.
Aos meus pais, que nunca me cortaram as asas para poder voar.
Ao Rodolfo, a minha luz ao fundo do túnel.

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" I'm walkin' back down this mountain, with the strength of a turnin' tide
Oh the wind's so soft on my skin, the sun so hard upon my side.
Oh lookin' out at this happiness,
Oh feelin' blind and realize,
All I was searchin' for was me.
...all I was searchin' for was me.
Keep your head up, keep your heart strong."
Ben Howard

E passados quase 5 anos, cá estou eu. Que aventura, uma bióloga no meio de engenheiros! O desafio era grande e entrei nele ciente de que iria encontrar grandes obstáculos pelo caminho, encontramos sempre, mas um doutoramento é isso mesmo: perdermo-nos pelo caminho, questionarmo-nos, considerar caminhos alternativos... e redescobrir a paixão que nos move e a matéria de que somos feitos. Não é um caminho fácil, mas no final vale todos os segundos de cansaço, dúvida e às vezes mesmo, desespero. Se chegamos até aqui é porque a prova foi superada e objectivo principal alcançado: não desistimos!

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"This journey has always been about reaching your own other shore, no matter what it is...
Never, ever give up!
Find a way."

Diana Nyad,
World record swimmer at 64 years old.

Abstract

Succinic acid (SA) is a highly versatile building block that is used in a wide range of industrial applications. The biological production of succinic acid has emerged in the last years as an efficient alternative to the chemical production based on fossil fuels. However, in order to fully replace the competing petro-based chemical process from which it has been produced so far, some challenges remain to be surpassed. In particular, one main obstacle would be to reduce its production costs, mostly associated to the use of refined sugars. The present work is focused on the development of a sustainable and cost-effective microbial production process based on cheap and renewable resources, such as agroindustrial wastes. Hence, glycerol and carob pods were identified as promising feedstocks and used as inexpensive carbon sources for the bioproduction of succinic acid by *Actinobacillus succinogenes* 130Z, one of the best naturally producing strains. Even though glycerol is a highly available carbon source, as by-product of biodiesel production, its consumption by *A. succinogenes* is impaired due to a redox imbalance during cell growth. However, the use of an external electron acceptor such as dimethylsulfoxide (DMSO) may improve glycerol metabolism and succinic acid production by this strain. As such, DMSO was tested as a co-substrate for glycerol consumption and concentrations of DMSO between 1 and 4% (v/v) greatly promoted glycerol consumption and SA production by this biocatalyst. Aiming at obtaining higher succinic acid yield and production rate, batch and fed-batch experiments were performed under controlled cultivation conditions. Batch experiments resulted in a succinic acid yield on glycerol of 0.95 g SA/g GLY and a production rate of 2.13 g/L.h, with residual production of acetic and formic acids. In fed-batch experiment, the SA production rate reached 2.31 g/L.h, the highest value reported in the literature for *A. succinogenes* using glycerol as carbon source. DMSO dramatically improved the conversion of glycerol by *A. succinogenes* and may be used as a co-substrate, opening new perspectives for the use of glycerol by this biocatalyst. Carob pods, highly available in Portugal as a residue from the locust bean gum industry, contain a significant amount of fermentable sugars such as sucrose, glucose and fructose and were also used as substrate for succinic acid production. Sugar extraction from raw and roasted carobs was optimized varying solid/water ratio and extraction time, maximizing sugar recovery while minimizing the extraction of polyphenols. Kinetic studies of glucose, fructose and sucrose consumption by *A. succinogenes* as individual carbon sources till 30 g/L were first determined to assess possible metabolic differences. Results showed no significant differences related to sugar consumption and SA production between the different sugars. Carob pods water extracts were then used as carbon source during

controlled batch cultivations. Maximum production rates were obtained by the consumption of 15 - 30 g/L sugars, 1.61 - 1.67 g/L.h, showing no sugar preference. Moreover, a fed-batch strategy to increase SA yield, final titer and production rate was developed by taking advantage of the *A. succinogenes* metabolism, uncoupling cell growth from succinic acid production, redirecting this way all carbon to SA. This strategy resulted in a SA yield of 0.93 g SA/ g sugars, the highest value reported in the literature for fed-batch and continuous experiments, with the arrest of acetic and formic acids production. The results obtained in this thesis show that glycerol and carob pods are suitable feedstocks for the bio-production of succinic acid by *A. succinogenes*. Considerations on the improvement of the process will be presented and discussed, in particular how to maximize succinic acid production while maintaining by-products concentration at residual values.

Keywords: Succinic acid; *A. succinogenes* 130Z; Fermentation; Glycerol; Carob pods

Resumo

O ácido succínico é um composto extremamente versátil utilizado numa vasta gama de aplicações industriais. A produção biológica de ácido succínico emergiu nos últimos anos como uma alternativa eficiente à produção química baseada em combustíveis fósseis. Contudo, alguns obstáculos têm ainda de ser ultrapassados de forma a substituir o processo petroquímico na sua totalidade. Um destes obstáculos, em particular, consiste na redução dos custos relativos à sua produção, normalmente associados ao uso de açúcares refinados. O presente trabalho tem como objectivo o desenvolvimento de um processo de produção biológico sustentável e economicamente viável, baseado em matérias-primas renováveis tais como resíduos e sub-produtos agroindustriais. Desta forma seleccionaram-se o glicerol e a vagem de alfarroba como substratos de baixo custo para a produção biológica de ácido succínico pela estirpe bacteriana *Actinobacillus succinogenes* 130Z. Apesar de ser uma fonte de carbono abundante, como sub-produto da indústria do biodiesel, o consumo de glicerol pela estirpe *A. succinogenes* é limitado devido a um desequilíbrio redox durante o crescimento celular. No entanto, a utilização de um aceitador de electrões externo, como por exemplo dimetilsulfóxido (DMSO), pode melhorar o metabolismo do glicerol e a produção de ácido succínico nesta estirpe. Assim, o composto DMSO foi testado como co-substrato no consumo de glicerol, tendo-se verificado que concentrações de DMSO entre 1 e 4% (v/v) promoveram o consumo de glicerol e a producão de ácido succínico por este microorganismo. Tendo em vista o aumento do rendimento e da taxa de produção de ácido succínico, realizaram-se fermentações em condições controladas em modo batch e fed-batch. Em modo batch obteve-se um rendimento de 0.95 g /g GLY e uma taxa de produção de 2.13 g/L.h, associados a uma produção residual de ácido acético e fórmico. Em modo fed-batch, a taxa de produção de ácido succínico atingiu 2.31 g/L.h, o valor mais elevado descrito na literatura para a estirpe *A. succinogenes* utilizando glicerol como fonte de carbono. A utilização de DMSO melhorou significativamente a conversão de glicerol desta estirpe, podendo ser usado como co-substrato na produção de ácido succínico, abrindo novas perspectivas para a conversão de glicerol através deste microorganismo.

A vagem de alfarroba, um sub-produto agroindustrial muito abundante em Portugal como resultado da indústria da goma de alfarroba, contém uma quantidade significativa de açúcares, incluindo sacarose, glucose e frutose, tendo sido usada também como substrato na produção de ácido succínico. A extracção aquosa dos açúcares a partir de vagens e de farinha comercial foi testada e optimizada, variando a proporção sólido/líquido e o tempo de extracção, maximizando a recuperação de açúcares e minimizando a extracção de compostos fenólicos. Foram realizados

estudos cinéticos do consumo de glucose, frutose e sacarose como fontes de carbono individuais até 30 g/L pela estirpe *A. succinogenes*, por forma a verificar possíveis diferenças metabólicas. Os resultados obtidos mostraram não haver diferenças significativas relativamente ao consumo de açúcar e produção de ácido succínico para os diferentes açúcares testados. Os extractos aquosos das vagens de alfarroba e da farinha comercial foram então utilizados como substrato durante fermentações controladas em modo batch. As taxas de produção de ácido succínico mais elevadas foram obtidas pelo consumo de 15 - 30 g/L de açúcares, 1.61 - 1.67 g/L.h, consumindo simultaneamente sacarose, glucose e frutose. Adicionalmente, desenvolveu-se uma estratégia de fermentação em modo fed-batch de forma a aumentar o rendimento de ácido succínico, taxa de produção e concentração final. Esta estratégia envolveu o desacoplar do crescimento celular de *A. succinogenes* da produção de ácido succínico, redireccionando o carbono consumido para a produção de ácido. Assim, foi obtido um rendimento de ácido succínico de 0.93 g/ g açúcares, o valor mais elevado descrito na literatura para ensaios em modo fed-batch e contínuo, sem produção de ácido acético e fórmico. Os resultados obtidos nesta tese mostram que o glicerol e a vagem de alfarroba são matérias-primas adequadas à produção biológica de ácido succínico pela estirpe *A. succinogenes*. São também apresentadas e discutidas algumas considerações acerca do melhoramento do processo, em particular como maximizar a produção de ácido succínico, mantendo a produção de sub-produtos em valores residuais.

Palavras-chave: Ácido succínico; *A. succinogenes* 130Z; Fermentação; Glicerol; Vagem de alfarroba

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Nomenclature

Abbreviations

1,3-PD 1,3-Propanediol

3HPA 3-Hydroxypropionaldehyde

AA Acetic Acid

AcCoA acetyl-coenzyme A

ACE Acetate

Acetal Acetaldehyde

ANOVA Analysis of variance

ATP Adenosine Triphosphate

BDO 1,4-Butanediol

CAGR Compound Annual Growth Rate

DHA Dihydroxyacetone

DHAP Dihydroxyacetone Phosphate

DMSO Dimethylsulfoxide

DOE Department of Energy

DR Dimethylsulfoxide reductase

EtOH Ethanol

FA Formic Acid

FDH Formate Dehydrogenase

FOR Formate

Fru Fructose

Fru1,6PP Fructose-1,6-biphosphate

Fru1P Fructose-1-phosphate

Fru6P Fructose-6-phosphate

FUM Fumarate

G3P Glycerol-3-phosphate

GA3P Glyceraldehyde-3-phosphate

GAE Gallic Acid Equivalents

GF Glycerol Facilitator

Glu Glucose

Glu6P Glucose-6-phosphate

GLY Glycerol

HPLC High Performance Liquid Chromatography

MAL Malate

ME Malic Enzyme

MIC Minimum Inhibitory Concentration

NADH Nicotinamide Adenine Dinucleotide

NADP Nicotinamide Adenine Dinucleotide Phosphate

OAA Oxaloacetate

OPEC Organization of the Petroleum Exporting Countries

pK_a Logarithmic acid dissociation constant

PBS Polybutylene Succinate

PDH Pyruvate Dehydrogenase

PEP Phosphoenolpyruvate

PFL Pyruvate Formate Lyase

PFL Pyruvate Formate-Lyase

PPC Phosphoenolpyruvate Carboxylase

PPP Penthose Phosphate Pathway

PTS Phosphotransferase system

PYR Pyruvate

PyrDH Pyruvate dehydrogenase

RI Refractive Index

SA Succinic Acid

Suc Sucrose

Suc6P Sucrose-6-phosphate

SUCC Succinate

TCA Tricarbolyxic Acid Cycle

TH Transhydrogenase

UV Ultraviolet

Variables and parameters

μ Specific growth rate (h^{-1})

μ_{max} Maximum specific growth rate (h^{-1})

AA/SA Acetic to succinic acid production ratio (g/g)

D Dilution rate ($h-1$)

F Inlet feed rate in the fed-batch phase (ml/h)

FA/SA Formic to succinic acid production ratio (g/g)

K_S Substrate saturation constant or Monod coefficient (g/L)

P Fermentation product

q_{SA} Specific succinic acid production rate (grams of SA/grams of DCW.h or Cmol SA/Cmol biomass.h)

r_P Volumetric production rate of fermentation product (SA, AA or FA), expressed in g/L.h

r_{SA} Volumetric production rate of succinic acid (g/L.h)

r_S Volumetric consumption rate of substrate (g/L.h)

r_X Volumetric production rate of biomass (g/L.h)

S Fermentation substrate (g/L)

S_0 Initial substrate concentration (g/L)

V Volume (ml)

X Active biomass

$Y_{AA/GLY}$ Yield of acetic acid on glycerol (g/g)

$Y_{AA/S}$ Yield of acetic acid on substrate (Cmol/Cmol)

$Y_{FA/GLY}$ Yield of formic acid on glycerol (g/g)

$Y_{FA/S}$ Yield of formic acid on substrate (Cmol/Cmol)

$Y_{S/X}$ Substrate yield on biomass (Cmol/Cmol)

$Y_{SA/GLY}$ Yield of succinic acid on glycerol (g/g)

$Y_{SA/S}$ Yield of succinic acid on substrate (Cmol/Cmol)

$Y_{SA/X}$ Yield of succinic acid on biomass (Cmol/Cmol)

$Y_{X/GLY}$ Yield of produced biomass on glycerol (g DCW/g GLY)

$Y_{X/S}$ Yield of biomass on substrate (Cmol/Cmol)

DCW Dry Cell Weight (g/L)

OD Optical Density (dimensionless)

pH pH (dimensionless)

f

1 State of the art

Summary *This chapter provides an introduction to the concepts presented and discussed in this thesis. It aims to put into perspective the driving forces that gave rise to a biological succinic acid production process. Additionally, it serves the purpose of reviewing the pertinent literature, and therefore, the main advances put forward in the last years, so as to create the necessary base for a constructive and supported discussion of the results found from the current work. A summary of the motivation for this work is also presented in this section, together with an outline of the contents of each chapter that constitute the thesis.*

1.1 INTRODUCTION

Early in the 20th century, before petroleum was discovered as an "unlimited and ubiquous" source of energy, the chemical industry was dependent on coal and renewable resources (Figure 1.1). Important bulk chemicals such as ethanol and butanol as well as lactic, citric and acetic acids were produced from biomass [Kale et al., 2008]. Following the dramatic development of the petroleum industry after 1930, many bio-based processes were replaced by petrochemical methods and new oil-derived products were developed: natural fibers such as wool and silk in clothes were replaced by synthetic polymers (Nylon, Perlon) coloured with artificial dyes; wood and metal were replaced by plastics in many consumer items, furniture and buildings [Rowe, 1998, Glaser, 2005].

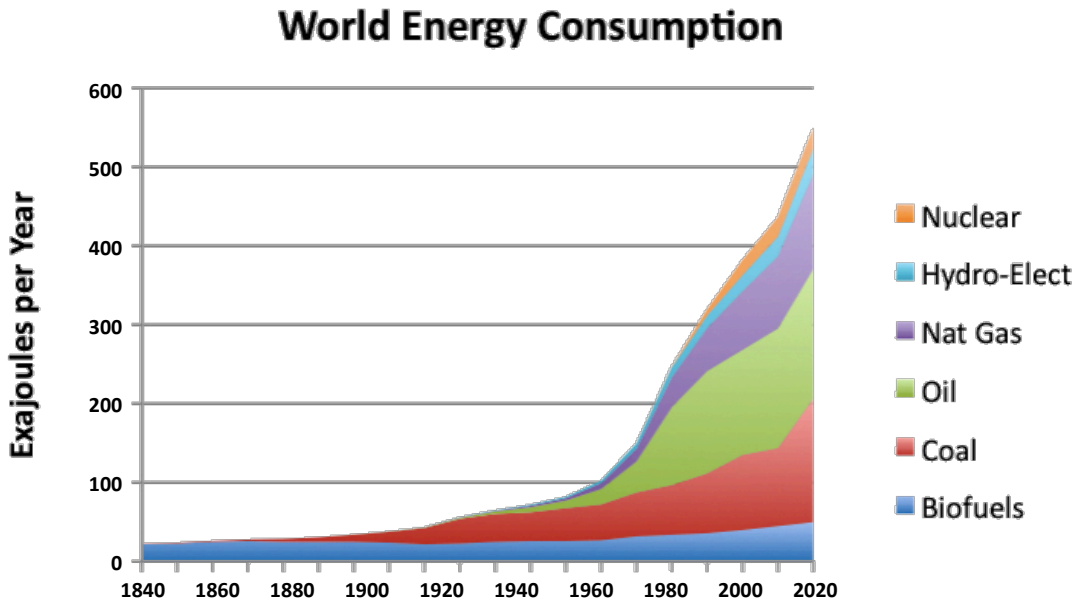


Figure 1.1: World energy consumption by source (adapted from [Smil, 2010])

However, during the first oil crisis in the 1970s, it became clear that oil resources are limited. According to the latest OPEC annual statistical bullet from 2015, the current world oil supplies account for 1,500 billion barrels, which if consumed at the actual consumption rate, will be exhausted in the next 50 years. Beside its scarcity, the use of fossil feedstocks leads to worldwide environmental problems, as the accumulation of non-degradable synthetic compounds in landfills and increasing CO_2 concentrations in the atmosphere [Webb et al., 2013]. As such, stricter environmental regulations imposed by governments together with growing concerns about the dependence on imported oil, particularly in the U.S., started an increasing demand for new CO_2

neutral technologies to explore nature's potential and replace petroleum-based processes.

White biotechnology is a branch of the biotechnology industry dedicated to the production of biodegradable products by living cells (yeast, bacteria, fungi and plants) or enzymes, that require less energy and create less waste during their formation, representing a promising alternative to petro-based processes [Glaser, 2005]. In this way, several countries evaluated the potential of biobased chemicals by governmentally funded projects according to three main aspects: national availability, white biotechnology and integrated biorefinery concepts. The U.S. Department of Energy (DOE) and the BREW project reports led to the identification of several chemicals that can be produced from sugar by biological or chemical conversion (Table 1.1) [Werpy and Petersen, 2004, Patel, 2006].

Table 1.1: Top 12 sugar-derived building blocks identified by the U.S. Department of Energy (DOE) [Werpy and Petersen, 2004]

Buiding Blocks
1,4 Diacids (succinic, fumaric and malic)
2,5 furan dicarboxylic acid
3 hydroxy propionic acid
Aspartic acid
Glucaric acid
Glutamic acid
Itaconic acid
Levulinic acid
3-Hydroxybutyrolactone
Glycerol
Sorbitol
Xylitol/Arabinitol

These chemicals are named building blocks, as they can be further converted into high-value bio-based chemicals or materials. The most promising buiding blocks that can be produced from biomass are fumaric, malic and succinic acids. In particular, succinic acid obtained much attention in the last years.

1.2 THE SUCCINIC ACID MARKET

Succinic acid (IUPAC name: butanedioic acid) is a dicarboxylic acid with the molecular formula $C_4H_6O_4$. It was firstly purified by pulverization and distillation of amber, in 1546 by Georgius Agricola and because of that it is also known as amber acid [Song and Lee, 2006]. With a linear and saturated structure, it can be converted into a wide variety of compounds with innumerous industrial applications (Figure 1.2): from acidulant or neutralizing agent in food and beverages, to growth regulator in seeds treatment or anticarcinogenic agent in pharmaceuticals [Zeikus et al., 1999, Cok et al., 2014, Jansen and van Gulik, 2014]. In the chemical industry, succinic acid can be used for the production of commodity chemicals such as 1,4-butanediol, tetrahydrofuran, adipic-acid, gamma-butyrolactone, N-methyl pyrrolidinone and 2- pyrrolidinone [Bechthold et al., 2008, Song and Lee, 2006]. Often designated as "green" building block, it is also a precursor of green

solvents, polyurethanes, biodegradable polymers such as polybutylene succinate (PBS, a polymer used for flexible film applications) and polyamides (Nylon) [Bechthold et al., 2008].

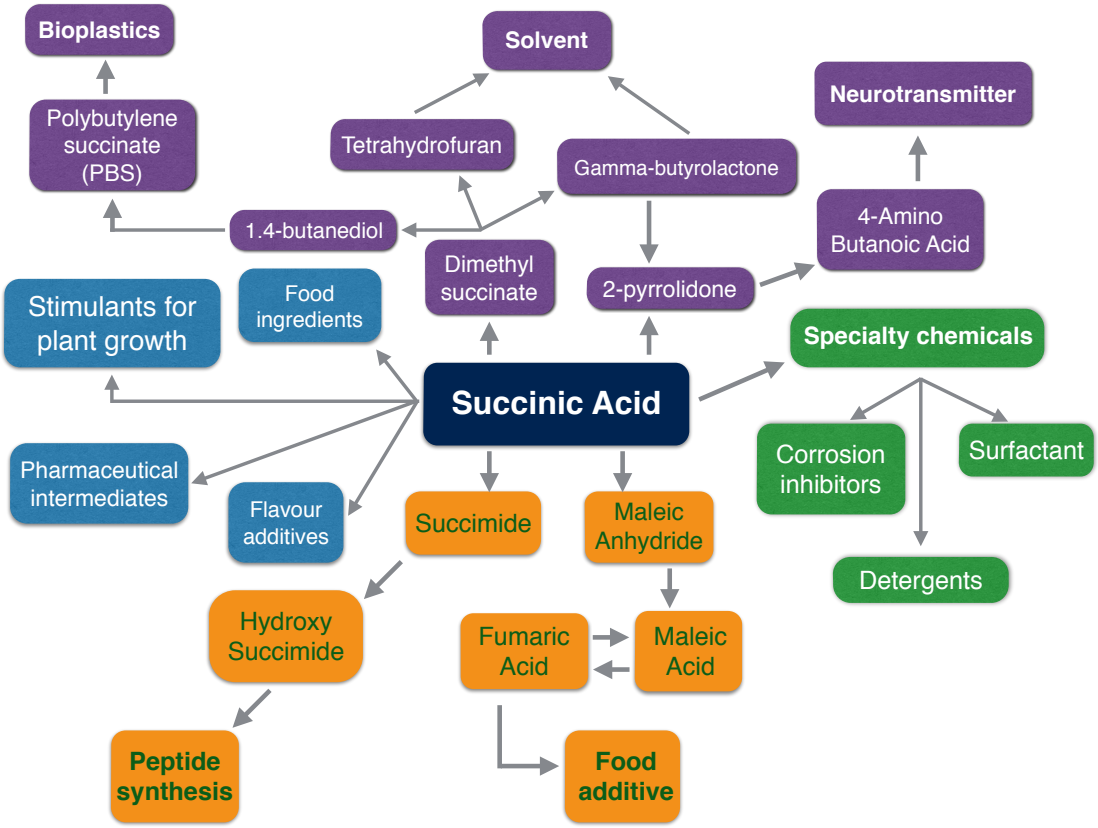


Figure 1.2: Succinic acid derived products (adapted from [Zeikus et al., 1999])

According to a 2013 survey from the Transparency Market Research agency, in 2011 the succinic acid market was valued US$ 240.3 million, with resins, coatings, dyes and inks accounting for 20.8% of total SA global consumption, followed by pharmaceuticals, the second largest application of succinic acid, with 13.0% share. The growing demand in emerging economies such as China, Brazil and India has been responsible for the market growth during the last years by increasing the demand in resins, coatings and polyurethanes, required for construction and infrastructure development activities.

Currently the global production of succinic acid is estimated between 30 to 50 kton per year with a price range of 6-9 US$/ kg, depending on its purity [Cao et al., 2013]. According to the 2015 survey report from Research and Markets agency, the global succinic acid market is expected to grow at a Compound Annual Growth Rate (CAGR) of 24% by 2020, generating US$ 496 millions in revenue.

The instability of oil prices and the ever-increasing demand for greener and more sustainable technologies are also major drivers for the growth of this market. Succinic acid could substitute many commodities based on benzene and intermediate compounds, resulting in the replacement of more than 250-benzene derived chemicals [Morris et al., 1994]. Among the industrial applications,

1,4 - butanediol (BDO) has the largest market share and is the fastest growing segment of the SA market, with an estimated CAGR of 67.9% between 2013 and 2020 (Allied Market Research). 1,4 - butanediol is an important industrial solvent and raw material for the production of resins. It can also be used for the production of biodegradable polybutylene succinate (PBS), a polymer with excellent thermal and mechanical properties [Cheng et al., 2012]. N-methyl pyrrolidinone, for instances, has been regarded as an alternative to the solvent methylene chloride because it is much less volatile, allowing its capture and recycling without further toxic emissions to the atmosphere. Another possible derivative is tetrahydrofuran, solvent and key ingredient in the formulation of inks, adhesives and magnetic tapes [Cao et al., 2013, Zeikus et al., 1999].

Strong regulations related to environmental practices and carbon footprints have made Europe the largest succinic acid consumer with 33.6% share in 2013, followed by Asia-Pacific (29.2%) and North America (29.2%). Asia Pacific is however, the fastest growing market with an expected CAGR of 49.5% from 2013 to 2020 (Allied Market Research). Overall, the increasing demand for succinic acid is encouraging the development of more sustainable and clean technologies, promoting the replacement of petroleum-based processes.

1.3 Succinic acid production: Petrochemical vs. biological production

The rising greenhouse gas concentrations in the atmosphere and the limited nature of fossil fuels urge the need to develop a more sustainable global economy based on cleaner technologies. At present, industrial scale production of succinic acid is mainly obtained by chemical synthesis based on the hydrogenation of petroleum-derived maleic anhydride or maleic acid (Figure 1.3) [Zeikus et al., 1999].

Although high yield (95% w/w), high purity levels and selectivity of succinic acid are obtained, this process requires different types of noble metal-based catalysts such as palladium (Pd) and rubidium (Rb) and occurs under high pressure and temperature conditions, which poses environmental concerns and increases the production costs [Cheng et al., 2012]. An alternative method to obtain succinic acid is by electrolytical reduction of maleic anhydride in acidic medium, but the significant amount of consumed electric power turns it into an expensive and non-environmental friendly process [Muzumdar et al., 2004].

Biomass is one of the most ubiquitous and important renewable resources, which can be transformed into energy, chemicals and materials in biorefineries, a promising alternative concept to petro-based processes. Since succinic acid was identified as one of the most important building blocks which can be produced from biomass (Table 1.1) [Werpy and Petersen, 2004, Patel, 2006], extensive research has been conducted on the fermentative production of this molecule [Zeikus et al., 1999, Bechthold et al., 2008, Beauprez et al., 2010, Cok et al., 2014]. Indeed, succinic acid is an intermediate of the tricarbolyxic acid cycle (TCA) and one of the end-products of bacterial anaerobic metabolism, being produced by virtually all microbial, plant and animal cells [Beauprez et al., 2010]. A very interesting feature of the bioproduction of this molecule is that it represents several of the 12 principles of Green Chemistry, developed by the American Chemical Society,

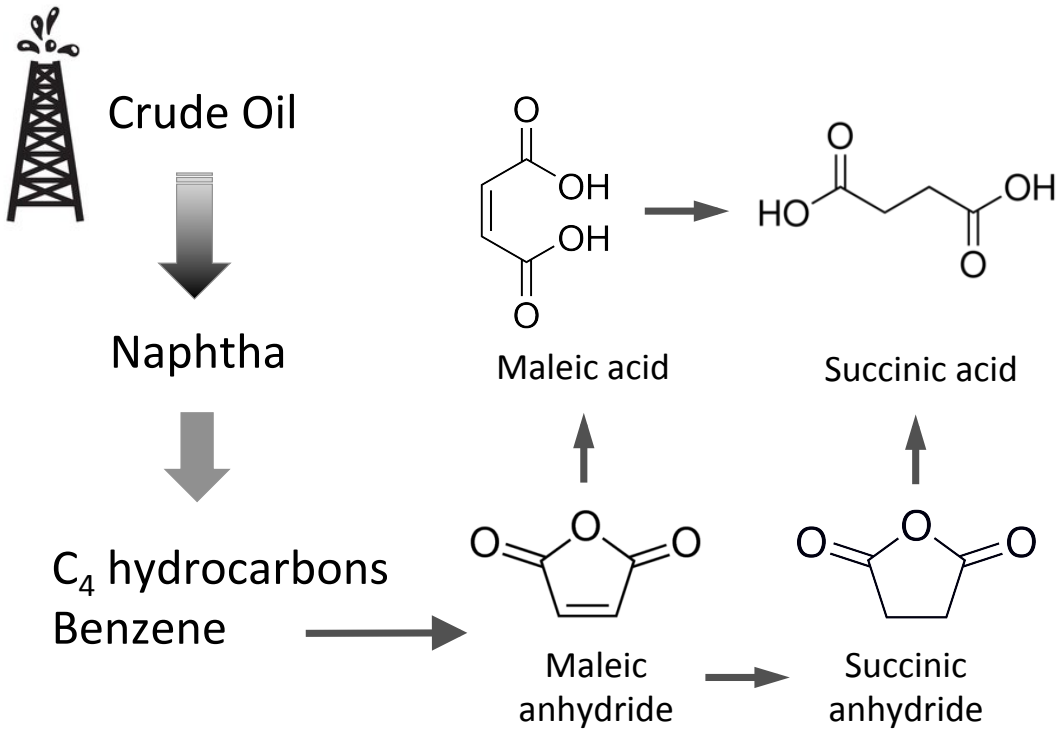


Figure 1.3: Production of succinic acid by chemical conversion of petro-derived compounds

aiming at the development and implementation of processes and/or products that reduce or eliminate hazardous substances (Table 1.2).

The use of renewable feedstocks and industry by-products as substrates for succinic acid production by microbial fermentation is one of the most important aspects of the process, being not only more sustainable by preventing waste formation and recycling by-products, but also reducing the process costs by using cheaper substrates. Moreover, the production of bio-succinic acid requires the consumption of CO_2 as cosubstrate, an additional environmental benefit [Bechthold et al., 2008, Beauprez et al., 2010].

Three important factors determine the economical viability of a bioprocess: product yield, final titer and production rate. While the product yield is related to variable cost of the raw feedstock, the titer and production rates are associated to the fixed costs and overall investment of the project. Low production rates demand greater energy consumption and costs, while low titers will result in a larger investment to ensure a significant plant capacity. Based on the petrochemical analogue, maleic anhydride, the U.S. Department of Energy have set the production price of bio-succinic acid at 0.45€/kg [Beauprez et al., 2010]. As such, fermentations targets were estimated for the process to be cost-effective: a minimum SA productivity of 2.5 g SA/L.h, a succinic acid yield of 100% (mol/mol) and high final titers (although not specified, 50 to 250 g/L is normally accepted) [Werpy and Petersen, 2004, Beauprez et al., 2010]

Table 1.2: The 12 Principles of Green Chemistry

1. Prevent waste
2. Atom Economy
3. Less hazardous chemical synthesis
4. Designing safer chemicals
5. Safer solvents and auxiliaries
6. Design for energy efficiency
7. Use of renewable feedstock
8. Reduce derivatives
9. Catalysis
10. Design for degradation
11. Real-time analysis for pollution prevention
12. Inherently safer chemistry for accident prevention

1.4 Downstream processing of bioproduction

The downstream cost for the purification of succinic acid is the most expensive part of the process, accounting for more than 60% of the total production costs. The separation process starts with the removal of cells, normally by centrifugation of microfiltration, followed by an ultrafiltration step where protein-like impurities and polysaccharides are eliminated. The isolation of succinic acid from the remaining by-products, namely acetic, formic, lactic and pyruvic acids, may follow different strategies, including precipitation, extraction and electrodialysis. On the other hand, the majority of SA applications require the free acid form instead of the salt form that is normally produced during anaerobic fermentations operated at neutral pH values, requiring further treatment for the conversion of succinate into the free acid [Zeikus et al., 1999]. Precipitation is the most common recovery method based on crystallization of calcium salt upon addition of calcium hydroxide to the culture broth to neutralize pH [Datta, 1992, Kurzrock and Weuster-Botz, 2010]. A simple filtration step recovers the calcium crystals, which can be further washed and liquidized by the addition of a concentrated sulfuric acid solution. The free succinic acid is then filtered and pure crystals formed by evaporation. The main disadvantages of this method are the manipulation of acids, production of gypsum and large amounts of chemicals and the low yield and purity of succinic acid crystals, as other organic acids present in the fermentation broth may be precipitated together with succinic acid.

Electrodialysis is based on separation of ionic species from non-ionic species (sugars) and molecules with higher masses, like proteins and polyssacharides, under the effect of an electric current. As such, after fermentation, the culture broth is clarified, removing cells and other particles by centrifugation, filtration or using micromembranes. Then, the succinate salt is concentrated and together with other ions transported across the electrodialysis membrane to be separated from sugars, proteins and amino acids. The succinate salt is then converted into the free acid through a bipolar, water-splitting membrane [Huang et al., 2007, Cok et al., 2014].

Separation and purification of succinate from the fermentation broth is still an economical obstacle for its biological production. The downstream processing methods employed for commercial production are mainly based on the classical crystallization strategy [Cao et al., 2013].

The challenge to successfully isolate succinate from the fermentation broth is how to apply these technologies to large-scale industrial processes in a cost and time-effective manner.

1.5 THE BIO-SUCCINIC ACID MARKET

Currently the biotechnological production of succinic acid is experiencing significant progress in light of business joint ventures and recent development activities (Figure 1.4). For instance, BioAmber Sarnia, a joint venture between BioAmber and Mitsui, will supply bio-succinic acid to Olean France for the production of succinate lubricants. This contract was signed in December 2014 and will run in exclusive supply until 2018. Moreover, BioAmber signed a 210,000 tonnes per year contract for bio-based succinic acid with Vinmar International. Under the terms of the 15-year agreement, Vinmar has committed to purchase and BioAmber Sarnia has committed to sell 10,000 tonnes of succinic acid per year from the plant that is currently under construction in Sarnia, Canada.

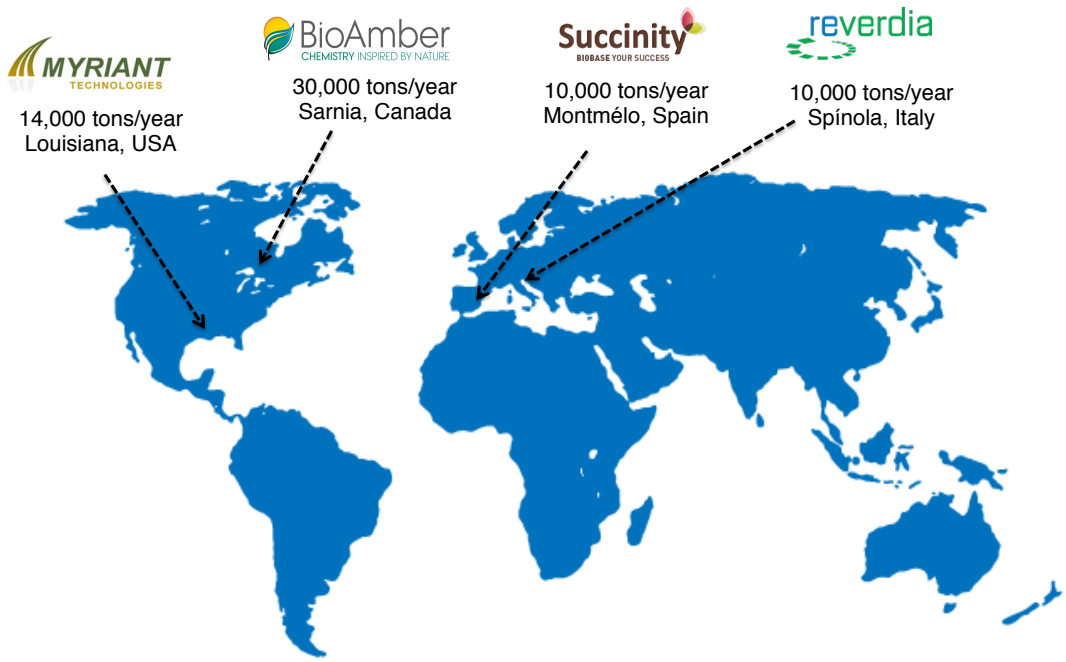


Figure 1.4: Current distribution of bio-succinic acid production worldwide

Reverdia, a joint venture between DSM and Roquette, started to operate a commercial-scale bio-succinic acid plant in 2012. The plant is located in Cassano Spinola, Italy and has a capacity to produce 10,000 tonnes of sustainable Biosuccinium^{TM} succinic acid per year. Together with BioAmber, in February 2015, they were awarded the Chemical of the Year for bio-succinic acid

by the Biofuels Digest. Also in 2012, BASF and Purac, a subsidiary of Corbion, also established a joint venture for the production and sale of biobased succinic acid. The company is called Succinity GmbH and started operations in 2014 in an existing fermentation facility, at Purac's Montmélo, Spain. This plant has an annual capacity of 10,000 tonnes of SA and a second facility is already being planned.

Myriant was awarded U.S.$ 50 million by the U.S. Department of Energy to build a succinic acid plant with an initial capacity of 14,000 tonnes per year in Louisiana, 2010. However, it started to produce and sale bio-succinic acid at ThyssenKrupp Uhde's facility, Germany, in 2013. Later that year, the Louisiana facility started to operate and is now supplying bio-SA for Oxea, where they produce phthalate-free plasticizers. Considering the current economic and political climate of oil-based industries and the recent developments in bio-succinic acid production, it seems possible to establish a sustainable and economically viable process for bio-succinic acid production in the near future, which will be able to compete with existing petrochemical markets.

1.6 Bio-succinic acid production: natural and metabolic engineered producers

Succinic acid is an intermediate of the tricarboxylic acid cycle (TCA) and a fermentation end-product of some bacterial strains during anaerobic metabolism. Several microorganisms have been screened for succinic acid production, but only a few produce enough quantities to be considered suitable for industrial production. The choice of host is diverse, with natural and genetically engineered producers available. *Actinobacillus succinogenes* 130Z [Guettler et al., 1999] and *Mannheimia succiniciproducens* [Lee et al., 2002] are Gram-negative bacteria, capnophilic (thrive in the presence of CO_2) and two of the most promising natural succinic acid producing bacteria as they produce it as a major fermentation product. Both strains were isolated from bovine rumen, a special environment found in the gut of ruminant animals, rich in carbon dioxide, methane and hydrogen, which favor the production of succinic acid. The later is then converted to propionic acid, which is absorbed through the rumen wall and used in the energy and biosynthesic pathways of the animal [Zeikus et al., 1999]. *Anaerobiospirillum succiniciproducens* has been isolated from the throat of a beagle dog and differs from the above strains by being a strict anaerobe, being harder to handle as they only live in the absence of oxygen [Davis et al., 1976]. These three strains produce a mixture or organic acids and as capnophilic microorganisms they are able to fix carbon dioxide and use it as a carbon source along with sugars, an additional environmental benefit [Guettler et al., 1999, Lee et al., 2002, Lee et al., 2001].

Fungi as *Aspergillus niger* [Bercovitz et al., 1990] and *Penicillium simplicissimum* [Gallmetzer et al., 2002] are also known to produce succinic acid. However, the productivity is much lower compared to the bacterial strains. On the other hand, succinic acid production takes place inside the cell's mitochondria, which means it has to cross two membranes in order to be excreted. Therefore, bacteria are more suitable for succinate production instead of fungi.

In addition to the natural producers, many microorganisms can be metabolically engineered to produce succinate as a fermentative end-product. However, every engineering strategy requires

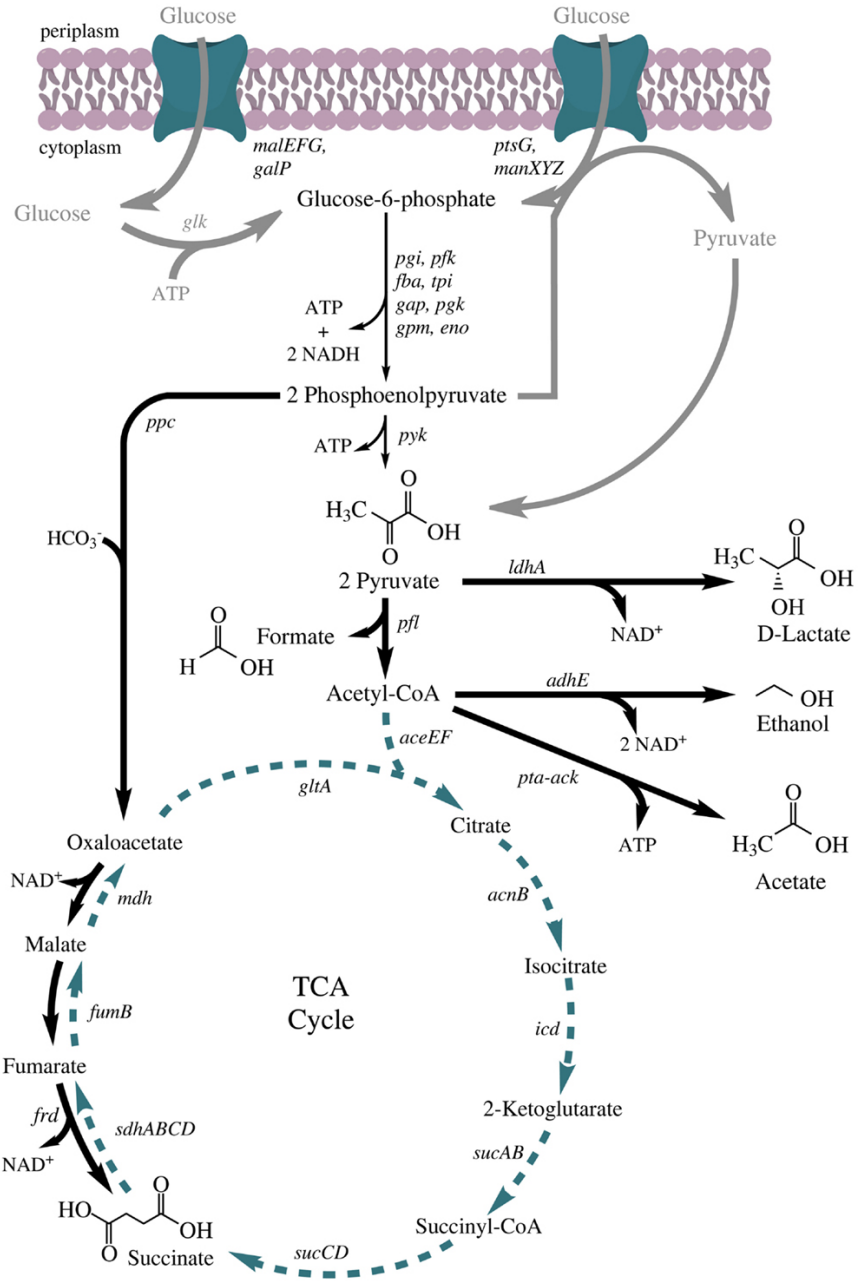


Figure 1.5: *Escherichia coli* metabolic pathways involved in mixed-acid fermentations. Black arrows represent fermentative reactions;dashed, green arrows represent reactions totally active during aerobic conditions (adapted from [Förster and Gescher, 2014]).

the essential genetic tools to modify the organism accordingly, which is the reason why model microorganisms are preferred. *Escherichia coli*, a reference bacterial strain, is selected by molecular biologists due to its fast growth rate and ease of manipulation. In the wild type strain, succinic acid is a minor fermentation product under anaerobic conditions (Figure 1.5), but metabolic engineered strains resulted in significantly improved productivities, yield and final titers, either by amplifying the activity of phosphoenolpyruvate carboxylase (PPC) activity for CO_2 fixation or inhibiting by-products formation [Chen et al., 2013, Blankschien et al., 2010]. More recently, commercial production using proprietary *E. coli* strains has been reported for BioAmber and Myriant companies. *Corynebacterium glutamicum* is one of the few Gram-positive bacteria, which have been tested for succinate production. With a long history in the microbial fermentation industry for amino acids and nucleotides, this strain is able to produce a mixture of organic acids under oxygen deprivation, including succinic acid [Jeon et al., 2013]. Genetic manipulation of the strain allowed the production of higher succinic acid amounts from glucose [Litsanov et al., 2012]. Aiming at producing succinic acid from lignocellulosic biomass, the strain has also been metabolically engineered to broaden its substrate utilization range to include L-arabinose and xylose [Kawaguchi et al., 2008, Kawaguchi et al., 2006]. In this context, *A. succinogenes* has a distinct advantage, as it is naturally able to metabolize more than 20 industrially relevant sugars including glucose, fructose, mannose, arabinose, sucrose and glycerol, probably a consequence from its symbiotic role in the bovine rumen [McKinlay et al., 2010, Guettler et al., 1999, Vlysidis et al., 2011, Carvalho et al., 2014a].

The yeast *Saccharomyces cerevisiae* is a robust and well-established industrial microorganism, in particular for the production of bioethanol. As *A. succinogenes*, it is able to use a broad range of substrates and exhibits an excellent osmotolerant capacity. The high tolerance towards acidity is an excellent advantage, since it minimizes the requirement for neutralization strategies, improving the downstream processing [Raab et al., 2010]. Production of succinic acid at low pH avoids the formation of gypsum, greatly improving the product recovery and therefore reducing the production costs. However, the formation of ethanol is an obstacle to the production of higher succinic acid titers [Oud et al., 2012]. The joint venture Reverdia, established between DSM and Roquette, developed a high succinic acid producing *Saccharomyces cerevisiae* strain by genetic engineering and is currently using this biocatalyst for the commercial production of bio-succinic acid. BASF/Purac (Succinity joint venture) isolated a naturally Gram-negative succinic acid producing strain from the bovine rumen, which was named *Basfia succiniciproducens* [Scholten et al., 2009, Scholten and Dägele, 2008]. Although this strain is naturally able to produce significant quantities of succinic acid, it has also been subjected to metabolic engineering to eliminate undesired metabolic fluxes, increasing the succinic acid yield by 45% [Becker et al., 2013]. It is worth noting that the choice of the microbial host will influence to a certain extent the process configuration and as a consequence, the overall process cost and environmental impact.

1.7 CURRENT CHALLENGES AND OPPORTUNITIES FOR BIO-SUCCINIC ACID PRODUCTION

The biotechnological potential for succinic acid production is huge considering the market demand, environmental benefits of the process and availability of succinic acid producing microorganisms. Often the costs are still the problem. In the case of bulk chemicals (<US $1/kg) the product price is mainly affected by the cost of raw material and for succinic acid, in particular, the price per kilogram must drop below 0.45€ to open up the commodity markets for succinic acid derived products [Beauprez et al., 2010].

The cost of bio-based succinic acid production relies on two main factors: the cost of the fermentation process and the cost of the downstream processing. As mentioned in section 1.4, the downstream processing accounts for 60-70% of the total production costs. Therefore, the development of cheaper and more efficient separation technologies will be responsible for a major reduction in bio-succinic acid production costs and its establishment as an industrial process.

On the other hand, improving the fermentation process will certainly have its impact on the separation process. Increasing the succinic acid titer, while maintaining other organic acids concentrations at low values, improves the driving force for separation and purification. As such, the development of fermentation strategies and microbial strains that can produce large quantities of succinic acid even in the presence of high concentration of organic acids is essential. Moreover, replacing the use of refined sugars as glucose and sucrose by industrial residues and by-products as substrates for microbial fermentation is another attractive opportunity to reduce production costs, as industrial residues are significantly cheaper than refined sugars.

The availability of microbial strains which are able to metabolize a wide range of sugars, such as *A. succinogenes* , provides great potential. Extensive research has been conducted in the last decade for the production of succinic acid from different renewable resources: glycerol from the biodiesel industry [Vlysidis et al., 2011], sugarcane molasses [Ma et al., 2014, Chan et al., 2012, Liu et al., 2008a], whey [Wan et al., 2008] and also lignocellulosic biomass including straw hydrolysate [Zheng et al., 2009], corn stover [Zheng et al., 2010] and corn fiber [Chen et al., 2011].

As most of the available strains are unable to ferment cellulosic biomass, a pretreatment step is required to release the sugars from the plant biomass. As a consequence, a variety of impurities such as furfural, 5-hydroxymethylfurfural and lignin products are released and may act as antimicrobial agents. Moreover, the hydrolysis of biomass prior to sugar release generates large amounts of waste. Even though efforts have been made to improve the microbial resistance to inhibitory substances such as the above, increasing the range of available low-cost feedstocks for succinic acid production will certainly help to overcome this obstacle. Although there are still some technical challenges to overcome, the production of bio-succinic acid has shown its potential with long-term prospects. The establishment of several joint ventures who decided to start to commercialize bio-succinic acid proves that this bioprocess is no longer an R&D program, but a reality. The continuous effort of researchers and entrepreneurs from different areas will certainly contribute for the replacement of the petrochemical process in the near future.

1.8 Thesis Motivation and Outline

1.8.1 Motivation

The main objective of this Ph.D. thesis was to develop a sustainable and cost-effective microbial process for succinic acid production from renewable feedstocks. The costs of the substrate, as well as its composition, have a great impact on the final price of succinic acid and on process performance. Agroindustrial wastes and by-products are complex substrates but with a high potential to be used as cheap carbon sources for the production of succinic acid. *A. succinogenes* 130Z was chosen as biocatalyst for succinic acid production as it holds some features which are suitable for an industrial process: it consumes a wide range of relevant sugars present in complex feedstocks; it is osmotolerant, being able to thrive in the presence of high concentration of salts; it requires the simultaneous consumption of CO_2, an additional environmental benefit; its genome sequence is available since 2010, allowing the study of the metabolic pathways involved in the succinic acid production process and its further improvement.

Having this in mind, the main goal of the work presented in this thesis was to study and optimize the succinic acid production by *A. succinogenes* using cheap renewable feedstocks such as glycerol and carob pods. These substrates were selected as substrates for succinic acid production by *A. succinogenes* since their avaibility as by-products of national industries. Glycerol consumption by this biocatalyst is, however, limited because of a redox imbalance during cell growth. The use of an external electron acceptor may improve the metabolism of succinic acid by *A. succinogenes* in glycerol. As such, DMSO was tested as co-substrate for glycerol consumption aiming at optimizing succinic acid production. Furthermore, carob pods were used as sugar based substrate that contains a mixture of sugars including sucrose, glucose and fructose. The behaviour of this strain in the presence of multiple sugars and succinic acid production was studied.

1.8.2 Outline

This Ph.D. thesis comprises the following chapters:

- **Chapter 1** introduces the key concepts addressed in this work and motivation for this Ph.D. thesis.
- **Chapter 2** covers the production of succinic acid by *A. succinogenes* from glycerol using dimethylsulfoxide (DMSO) as electron acceptor. The metabolism of *A. succinogenes* is revised, highlighting the metabolic pathways involved in this process. Glycerol and DMSO concentrations were optimized for SA production in serum flask experiments. Batch and fed-batch experiments were performed under controlled cultivation conditions, aiming at optimizing the succinic acid production. The possibility of using alternative sources of glycerol and DMSO instead of the pure compounds is also adressed in this chapter [Carvalho et al., 2014a]. *DOI: 10.1016/j.nbt.2013.06.006*
- **Chapter 3** describes the production of SA by *A. succinogenes* using roasted carob flour water extracts as carbon source. Sugar extraction from carob flour was optimized varying

solid/liquid ratio and extraction time, maximizing sugar recovery while minimizing the extraction of polyphenols. Kinetic studies of glucose, fructose and sucrose consumption by *A. succinogenes* as individual carbon sources were performed. Succinic acid production from carob water extracts was studied during controlled batch cultivations [Carvalho et al., 2014b]. *DOI: 10.1016/j.biortech.2014.07.117*

- **Chapter 4** studies the production of SA from raw carob pods by *A. succinogenes* , in similarity with chapter 3 where roasted pods were used. Sugar water extraction from carob pods was optimized and batch fermentations performed. Moreover, a strategy to increase SA final production was developed by taking advantage of the *A. succinogenes* metabolism, uncoupling cell growth from SA production.

- **Chapter 5** presents the final remarks and main conclusions of this thesis, along with some questions and suggestions for future research that have emerged during the present work.

The scientific work developed in this Ph.D. project is described in Chapters 2 to 4. These chapters are written in the format of scientific papers, Chapters 2 and 3 being already published, while Chapter 4 is the basis of a manuscript to be published. The methodology used in each individual chapter is detailed in the context of the respective subject and, when appropriate, is related to that used in previous chapters.

2 Succinic acid production from glycerol

Summary *Glycerol, a by-product of the biodiesel industry, constitutes today a cheap feedstock for biobased succinic acid (SA) production. Actinobacillus succinogenes is one of the best succinic acid producers. However, glycerol consumption by this biocatalyst is limited due to a redox imbalance during cell growth. The use of an external electron acceptor may improve the production of succinic acid synthesis by A. succinogenes in glycerol. In this study, the effect of dimethylsulfoxide (DMSO), an electron acceptor, on glycerol consumption and succinic acid production by A. succinogenes under controlled fermentation conditions was investigated. Concentrations of DMSO between 1 and 4% (v/v) greatly promoted glycerol consumption and succinic acid production. During fed-batch cultivation, succinic acid concentration reached 49.62 g/L with a maximum production rate of 2.31 g SA/L.h and a product yield on glycerol of 0.87 g/g, the highest values so far reported in the literature for A. succinogenes using glycerol as carbon source. These results show that using DMSO as external electron acceptor significantly promotes glycerol consumption and succinic acid production by A. succinogenes and may be used as a co-substrate, opening new perspectives for the use of glycerol by this biocatalyst.*

The contents of this chapter were adapted from the publication: Carvalho, M., Matos, M., Roca, C. and Reis, M.A.M. (2014). Succinic acid production from glycerol by *A. succinogenes* using dimethylsulfoxide as electron acceptor. New Biotechnology, 31(1), 133-139

2.1 INTRODUCTION

Production of biobased succinic acid (SA) has become today a reality and industrial scale is being developed by chemical companies such as BASF, Bioamber or DSM. Chemical properties of this diacid make the molecule very attractive and versatile for a wide range of applications from deicing agent, precursor for polymers and fibers to additives in paints [Zeikus et al., 1999, McKinlay et al., 2007b]. Present production at industrial scale is based on the oxidation of maleic anhydride and remains limited due to its production costs. However, succinic acid can also be produced by microbial fermentation using microorganisms like *Mannheimia succiniciproducens* [Lee et al., 2002], *Anaerobiospirillum succiniciproducens* [Nghiem et al., 1997, Lee et al., 2010b] *Actinobacillus succinogenes* [Guettler et al., 1999, Lin et al., 2008] or *Basfia succiniciproducens* DD1 [Scholten and Dägele, 2008, Scholten et al., 2009]. Metabolic engineered strains have also been developed in order to produce succinic acid, like *Escherichia coli* [Blankschien et al., 2010], *Yarrowia lipolytica* [Yuzbashev et al., 2010] or *Saccharomyces cerevisiae* [Raab et al., 2010].

The utilization of renewable raw materials as carbon source for fermentation has appeared as a solution to reduce production costs and obtain a sustainable production of succinic acid. The majority of feedstocks used today for the bioproduction of succinic acid are rich in sugars. Corn stover, straw and corn fiber hydrolysates are just a few examples of renewable materials used for bio-based succinic acid production [Zheng et al., 2009, Zheng et al., 2010, Chen et al., 2011].

Glycerol has appeared as a cheap feedstock for microbial fermentation after the biodiesel boom over the last decade (Figure 2.1). Since 2003, new directives on the promotion of the use of biofuels for the transport sector were implemented in the European Union and followed by the U.S. countries. For each 10 L of biodiesel produced, almost 1 L glycerol is generated, causing a rapidly increasing glycerol oversupply that could not be absorbed by the refined glycerol markets: pharmaceutical and personal care industries. The glycerol coming from the biodiesel industry contains significant amounts of methanol (23 to 38%) and salt which makes it unsuitable for traditional markets and its purification by distillation is highly expensive [Ciriminna et al., 2014]. As such, one way of valorizing this abundant by-product is to convert it into high added-value bioproducts as succinic acid.

Few microorganisms, such as *Basfia succiniproducens* DD1 or *Anaerobiospirillum succiniciproducens* are able to produce succinic acid from glycerol [Scholten et al., 2009, Lee et al., 2010b]. Even though high yields were reported (1.02 g SA/g GLY), several obstacles have to be surpassed to turn the process economically viable, in particular low production rates (0.094 g SA /L.h) [Scholten et al., 2009]. *A. succinogenes* is one of the most efficient strains to produce succinic acid. However, succinic acid production from glycerol by this microorganism remains limited to 29.3 g/L with a low production rate (0.27 g SA/ L.h) [Vlysidis et al., 2011], even if its genome reveals the existence of glycerol utilization pathway [McKinlay et al., 2010].

This low efficiency in using glycerol compared to using glucose, where succinic acid production can reach concentration as high as 106 g/ L with production rate of 1.36 g/ L.h [Guettler et al., 1996], lays in a redox imbalance during biomass formation, impairing glycerol conversion. In all known glycerol fermenting microorganisms, one reducing equivalent is generated for one glycerol

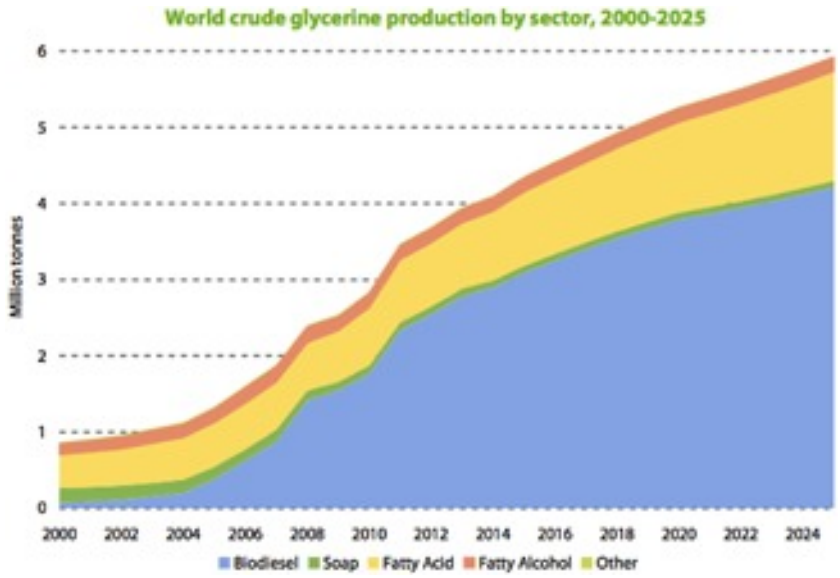


Figure 2.1: World crude glycerol production values from 2000 to 2025 (projected) (adapted from [Ciriminna et al., 2014])

entering glycolysis (Figure 2.2, dashed lines). Recycling of the generated molecule to maintain redox balance can be accomplished by reducing glycerol to 1,3-propanediol (1,3 – PD), which is then excreted and in parallel oxidizing glycerol to dihydroxyacetone phosphate (DHAP), that is further metabolized through glycolysis.

A. succinogenes lacks the 1,3-propanediol metabolic pathway, so glycerol consumption is only possible via DHAP production in the presence of an external electron acceptor (Figure 2.2, dark line). The use of an external electron acceptor may therefore improve the metabolism of succinic acid synthesis by *A. succinogenes* in glycerol as observed by Schindler [Schindler, 2011].

In that study dimethylsulfoxide (DMSO) was used as electron acceptor as *A. succinogenes* genome reveals the existence of a DMSO reductase [McKinlay et al., 2010]. However experiments were performed under uncontrolled culture conditions and rather low glycerol (11 g/L) and DMSO concentrations (0.14 % v/v) were used, resulting in low succinic acid concentration (3.3 g/L) with limited production rate (0.09 g/L.h), without further optimization. The objective of this work was to understand the influence of dimethylsulfoxide (DMSO), an electron acceptor, on glycerol consumption and succinic acid production by *A. succinogenes* under controlled fermentation conditions. DMSO and glycerol were tested in different initial concentrations and batch and fed-batch experiments were performed in order to maximize succinic acid production, aiming at obtaining the best yield and production rate of succinic acid on glycerol, taking full advantage of *A. succinogenes* as succinic acid producer.

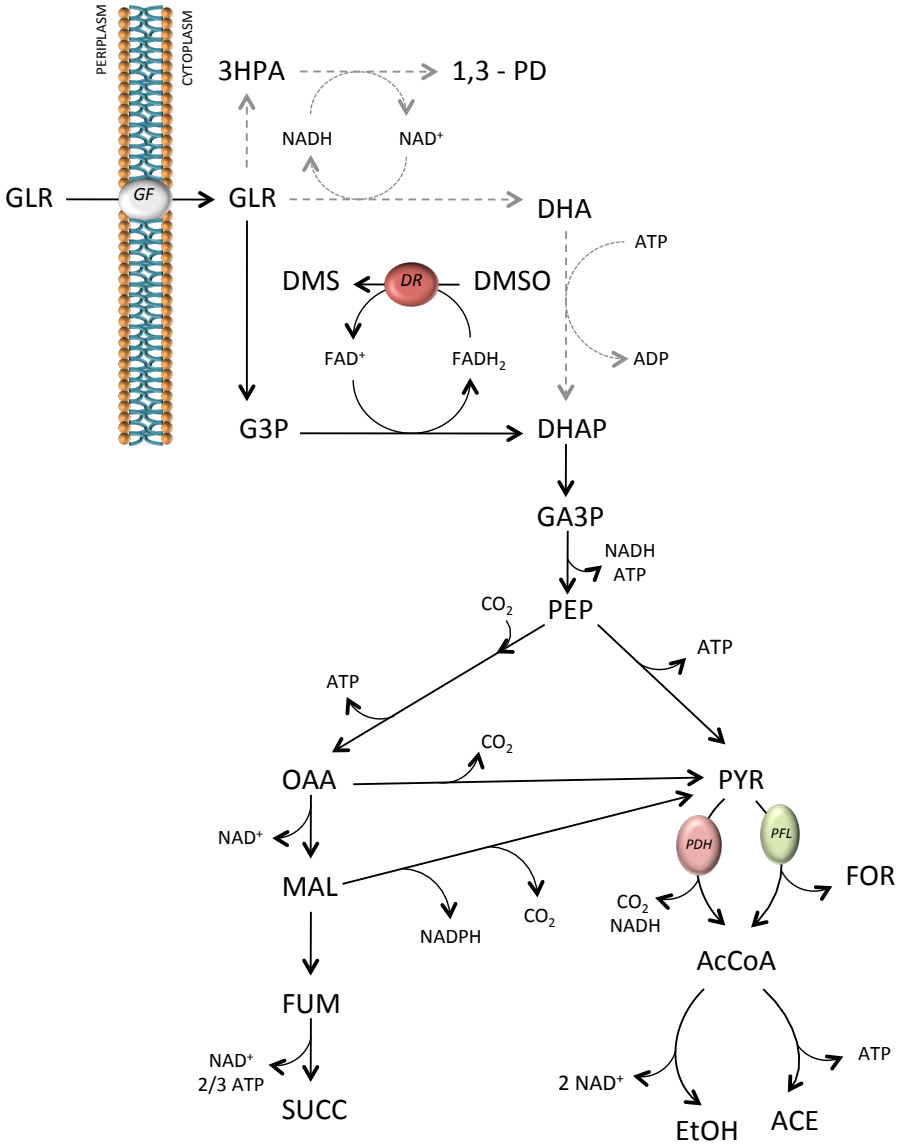


Figure 2.2: Metabolic pathways involved in glycerol utilization. Dashed lines: 1,3 – PD pathway (absent in *A. succinogenes*). Black lines: DHAP pathway in the presence of an electron acceptor (present in *A. succinogenes*). Abbreviations: GLY, glycerol; 3HPA, 3-hydroxypropionaldehyde; 1,3–PD, 1,3-propanediol; G3P, glycerol-3-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; FOR, formate; AcCoA, acetyl-coenzyme A; ACE, acetate; EtOH, ethanol; OAA, oxaloacetate; MAL, malate; FUM, fumarate; SUCC, succinate. Enzymes: GF, glycerol facilitator; DR, DMSO reductase; PFL, pyruvate formate-lyase; PDH, pyruvate dehydrogenase. (adapted from [McKinlay et al., 2010])

2.2 Materials and Methods

2.2.1 Strain and inoculum preparation

Actinobacillus succinogenes 130Z (ATCC 55618) was used in all experiments. Inoculum was prepared by incubating cells from a -80°C glycerol stock culture in 50 mL rubber sealed serum flasks, containing 50 mL of culture medium. The medium contained per liter: 6.0 g glucose, 10.0 g yeast extract, 8.4 g $NaHCO_3$, 8.5 g $NaH_2PO_4 \cdot H_2O$, 15.5 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2$ [Guettler et al., 1999]. Medium was heat sterilized at 121°C for 20 min. Glucose and $CaCl_2/MgCl_2$ solution were sterilized separately and added aseptically. After inoculation, the rubber sealed flasks were incubated at 37°C in a rotary shaker at 220 rpm, for 12 to 16h, until late exponential phase was reached and confirmed by optical density measurements at 660 nm.

2.2.2 Cultivation conditions

2.2.2.1 Serum flasks

Dimethylsulfoxide (DMSO) was initially tested to evaluate its effect on bacterial growth, glycerol consumption and succinic acid production. In this way, 50 mL serum flasks containing 50 mL of medium similar to the one for inoculum preparation were used, except that glycerol was used instead of glucose as carbon source. Flasks containing 4 g/ L glycerol were supplemented with DMSO at final concentrations between 0 and 7.44% (v/v) as summarized in Table 2.1. To evaluate the effect of glycerol concentration on succinic acid production, DMSO was used at 1.6% (v/v) and different initial concentrations of glycerol were tested: 17.78, 32.14, 50.67, 63.42 and 83.08 g/ L (Table 2.2). Each flask was inoculated with 2.5 ml of inoculum and was placed at 220 rpm for 24h, at 37°C .

2.2.2.2 Bioreactor cultivations

In order to assess with more precision the effect of DMSO on glycerol consumption and succinic acid production and in an attempt to obtain higher succinic acid concentration than previously attained in serum flasks, anaerobic fermentations were performed under controlled cultivation conditions. Three independent batch experiments were carried out in a 1 L bioreactor sparged with CO_2 at 0.05 vvm, at 37°C and with stirring at 220 rpm. The pH was automatically controlled at 6.8 by the addition of 5M NaOH solution. The culture medium contained per liter: 18 mL DMSO (1.8% v/v), 10.0 g yeast extract, 8.4 g $NaHCO_3$, 8.5 g $NaH_2PO_4 \cdot H_2O$, 15.5 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2$. Glycerol was used as carbon source at 24 g/L (Table 2.3). Additionally, pulses of glycerol and DMSO were performed during a fed-batch cultivation. A 2 L bioreactor was used with an initial volume of 1.5 L, sparged with CO_2 at 0.05 vvm, at 37°C and with stirring at 220 rpm. The pH was set to 6.8 and automatically controlled by the addition of 5M NaOH solution. The culture medium was the same as for the batch experiments, except that initial DMSO and glycerol concentrations were 1% (v/v) and 33.70 g/L, respectively.

The bioreactor was supplemented twice with additional 25 g/L of glycerol and 1% (v/v) of DMSO, 30h and 60h after the start of fermentation. All bioreactor experiments were inoculated with 5% (v/v) inoculum.

2.2.3 Analytical methods

Glycerol, succinic acid, formic and acetic acids were analyzed by HPLC using an ion exchange column (Aminex HPX-87H; 300mm x 7.8mm, 9 μm; Biorad) and a refractive index detector (RI-71, Merck). The mobile phase was 0.01 N H_2SO_4 solution at a flow rate of 0.5 mL/min and the column was operated at 30°C. DMSO was also analyzed by HPLC in the same conditions but using UV detection (L-4250, Merck) at 210 nm. Cell concentration was estimated by dry cell weight. Broth samples were collected and centrifuged for 10 min at 10.000 rpm, after which the supernatant was collected for HPLC analysis. The pellet was washed with distilled water and centrifuged for 10 min at 10.000 rpm, twice and dried for 24h at 70°C. Cell growth was also estimated by optical density (OD) measurements at 660 nm (Spectronic Helios Alpha UV/Vis spectrophotometer, Fisher Scientific). From these data, an experimentally determined correlation factor of 0.811 g/ L for an OD660 of 1 was estimated.

2.2.4 Calculation of kinetic parameters

The specific growth rate μ (h^{-1}) was determined by linear regression of the curve ln (X/X_0) versus time (X representing the biomass calculation and X_0 the biomass at time zero). Succinic acid production rate (r_{SA}), expressed in g/L.h, was calculated as the maximum increase in SA concentration for a determined time interval. Maximum specific production rate (q_{SA}) was obtained by dividing the maximum production rate by the average biomass amount produced at that time. Yields of produced acids on glycerol ($Y_{SA/GLY}$, $Y_{AA/GLY}$, $Y_{FA/GLY}$) were determined as the amount of organic acid produced from one gram of consumed glycerol. Ratios of produced acids on succinic acid (FA/SA and AA/SA) were determined as the amount of organic acid produced for one gram of produced succinic acid. Biomass yield on glycerol ($Y_{X/GLY}$) was determined as the amount of biomass produced from one gram of consumed glycerol.

2.3 Results and discussion

As mentioned previously, *A. succinogenes* lacks the 1,3-propanediol metabolic pathway, so glycerol consumption is only possible via DHAP production in the presence of an external electron acceptor (Figure 2.2, dark line). In this study, DMSO was used as electron acceptor. In order to assess the influence of DMSO on the conversion of glycerol into succinic acid by *A. succinogenes* , different concentrations of DMSO were tested using the same initial glycerol concentration (4 g/L). DMSO concentration clearly affects glycerol consumption and succinic acid production by *A. succinogenes* (Table 2.1).

Except for the extreme DMSO concentrations (0 and 7.44%), after 24h of incubation, all glycerol was consumed. The yield of succinic acid on glycerol ($Y_{SA/GLY}$) increases with DMSO

Table 2.1: Effect of DMSO concentration on glycerol consumption and succinic acid production by *A. succinogenes* during fermentation with 4 g/L glycerol

DMSO % (v/v)	Final [SA] (g/L)	Final [GLY] (g/L)	$Y_{SA/GLY}$	r_{SA}	$Y_{AA/GLY}$	$Y_{FA/GLY}$
0.00 ± 0.00	0.36 ± 0.08	2.36 ± 0.20	0.09 ± 0.03	0.01 ± 0.01	-	-
0.96 ± 0.02	3.52 ± 0.02	0.00 ± 0.00	0.88 ± 0.01	0.15 ± 0.00	0.09 ± 0.01	0.12 ± 0.00
1.36 ± 0.14	4.76 ± 0.04	0.00 ± 0.00	1.19 ± 0.01	0.20 ± 0.00	0.06 ± 0.01	0.16 ± 0.01
1.83 ± 0.11	4.08 ± 0.14	0.00 ± 0.00	1.02 ± 0.05	0.14 ± 0.01	0.08 ± 0.01	0.20 ± 0.02
2.27 ± 0.22	3.76 ± 0.27	0.00 ± 0.00	0.94 ± 0.10	0.19 ± 0.02	0.11 ± 0.02	0.10 ± 0.02
4.45 ± 0.27	3.21 ± 0.15	0.00 ± 0.00	0.80 ± 0.05	0.17 ± 0.01	0.10 ± 0.01	0.10 ± 0.02
7.44 ± 0.28	0.00 ± 0.01	4.01 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	-	-

Data are mean values in duplicates represented as mean ± standard deviation.
DMSO % (v/v) - Volumetric percentage of DMSO in the culture media.
$Y_{SA/GLY}$ - Succinic acid yield on glycerol expressed in grams of produces acid per gram of glycerol
r_{SA} - Maximum succinic acid production rate expressed in g/L.h
$Y_{AA/GLY}$ - Yield of produced acetic acid (AA) on consumed glycerol (GLY) (g/g)
$Y_{FA/GLY}$ - Yield of produced formic acid (FA) on consumed glycerol (GLY) (g/g)

concentration from 0 to 1.36%, reaching a maximum of 1.19 g SA/ g GLY. The maximum concentration of succinic acid (4.76 g/L) was also achieved for 1.36 % of DMSO, with an associated production rate of 0.198 g SA/L.h. In the control experiment, without DMSO, SA concentration was below 0.36 g/ L with a corresponding production rate of 0.015 g/L.h. These results show that glycerol consumption by *A. succinogenes* , and consequently succinic acid production are limited to the addition of an external electron acceptor, as also verified by Schindler [Schindler, 2011]. In that study, DMSO concentration between 0.14% (20 mM) and 0.64% (80 mM) was tested on *A. succinogenes* glycerol consumption and succinic acid production. Results showed that SA production increased with DMSO concentration, resulting in a product yield of 0.76 g SA/g GLY with 0.64 % DMSO (80 mM) and an initial glycerol concentration of 9 g/L. However, as DMSO concentration increased from 0.14 to 0.64 %, more acetate was produced, with succinate/acetate ratio decreasing from 3.5 (with 0.14% DMSO) to 1.5 (with 0.64 %). In our study, acetate formation was the highest in the presence of 2.27 % DMSO (0.11 g acetate/g glycerol), whereas formate production was highest for 1.83 % DMSO (0.20 g formate/g glycerol) (Table 2.1). These results suggest that an excess of electron acceptor might actually disturb cells' internal redox state, promoting by-products formation, probably in order to provide enough reducing equivalents to reduce oxaloacetate to succinate. For high concentrations of DMSO (e.g. 7.44% v/v), no glycerol consumption was observed, suggesting an inhibitory effect of this electron acceptor. Bacteriostatic effect of DMSO in Gram negative bacteria was reported in *Escherichia coli* and *Pseudomonas aeruginosa* strains, where virtually all growth was absent at DMSO concentration of 13% [Ansel et al., 1969]. The authors observed severe cytological modifications in *E. coli* grown for 24h in the presence of 5 and 10% of DMSO.

The values obtained in our study, using DMSO as electron acceptor, were above of those recently reported by Vlysidis et al. using an adapted strain of *A. succinogenes* to glycerol, without using an electron acceptor [Vlysidis et al., 2011]. For 4 g/ L glycerol, the reported strain was able to produce 3.9 g/L of succinic acid, with a corresponding yield and production rate of 1.04 g SA/ g GLY and 0.13 g SA/ L.h, respectively. This would actually suggest that the use of DMSO to balance intracellular redox state confers to the cell a more favorable situation to convert glycerol than the unknown "mutation" acquired during adaptation. The stability of the adapted clone was not reported by authors. Furthermore, the adapted clone may not be suitable for industrial applications, as long fermentation times and other carbon sources which might be present may result in a reversion of the mutated clone, whereas the addition of DMSO is relatively straightforward.

In this study, it was confirmed that the use of an electron acceptor such as DMSO has a positive effect on glycerol consumption and succinic acid production in *A. succinogenes* in concentrations between 1 and 4% (v/v).

2.3.1 Effect of glycerol concentration on succinic acid production

The effect of glycerol on the production of succinic acid by *A. succinogenes* in the presence of DMSO was evaluated in serum flasks at different initial glycerol concentrations: 17.78, 32.14, 50.67, 63.42 and 83.08 g/L with 1.6% DMSO (Table 2.2). Specific succinic acid production rate (q_{SA} increased when initial glycerol concentration rose from 17.78 to 32.14 g/ L and kept almost constant up to 50.67 g/ L glycerol (aproximatelly 0.5 g SA/ g DCW.h), decreasing for higher concentrations of substrate. The yield of succinic acid (0.87 g SA/g GLY) was highest at 32.14 g/ L of glycerol, slowly decreasing for concentrations over this value of substrate concentration (Table 2.2). Acetate and formate production increased as glycerol rose from 17.78 to 32.14 g/ L, reaching the highest values in the presence of 32.14 g/ L glycerol (0.09 g acetate/ g glycerol and 0.11 g formate/ g glycerol). For glycerol concentrations over 32.14 g/ L, by-products and biomass formation decreased, probably because of an inhibition effect of glycerol (Table 2.2). These results show that glycerol above 50.78 g/ L has a negative impact on the succinic acid metabolism of *A. succinogenes* , which agree with Vlysidis et al. results, where almost no glycerol consumption occurred and succinic acid was produced at a rate of 0.08 g succinic acid/ L.h, in the presence of 66 g/L glycerol [Vlysidis et al., 2011].

Table 2.2: Effect of initial glycerol concentration on succinic acid production by *A. succinogenes* during fermentation with 1.6% DMSO.

S_0 (g/L)	Final [SA] (g/L)	$Y_{SA/GLY}$	$Y_{X/GLY}$	r_{SA}	q_{SA}	$Y_{AA/GLY}$	$Y_{FA/GLY}$
17.78 ± 0.4	4.63 ± 0.33	0.67 ± 0.05	0.09 ± 0.01	0.22 ± 0.02	0.425 ± 0.06	0.06 ± 0.02	0.07 ± 0.01
32.14 ± 0.9	5.02 ± 0.42	0.87 ± 0.05	0.12 ± 0.00	0.23 ± 0.03	0.505 ± 0.05	0.09 ± 0.01	0.11 ± 0.01
50.67 ± 2.5	5.44 ± 0.05	0.49 ± 0.00	0.06 ± 0.00	0.26 ± 0.00	0.496 ± 0.05	0.06 ± 0.00	0.06 ± 0.00
63.42 ± 0.2	3.97 ± 0.30	0.31 ± 0.02	0.04 ± 0.01	0.18 ± 0.02	0.396 ± 0.06	0.03 ± 0.00	0.03 ± 0.00
83.08 ± 5.6	4.61 ± 0.21	0.21 ± 0.01	0.03 ± 0.00	0.22 ± 0.01	0.348 ± 0.02	0.01 ± 0.00	0.03 ± 0.00

Data are mean values in duplicates represented as mean ± standard deviation.
S_0 - Initial glycerol concentration
$Y_{SA/GLY}$ - Succinic acid yield on glycerol expressed in grams of SA per gram of glycerol
$Y_{X/GLY}$ - Biomass acid yield on glycerol expressed in grams of dry cell weight (DCW) per gram of glycerol
r_{SA} - Maximum succinic acid volumetric production rate expressed in g SA/L.h
q_{SA} - Specific succinic acid production rate expressed in g SA/g DCW.h (grams of succinic acid per gram of dry cell weight per hour)
$Y_{AA/GLY}$ - Yield of produced acetic acid (AA) on consumed glycerol (GLY) (g/g)
$Y_{FA/GLY}$ - Yield of produced formic acid (FA) on consumed glycerol (GLY) (g/g)

2.3.2 Effect of DMSO on glycerol consumption and succinic acid production during controlled batch cultivations

In order to investigate more in details the effect of DMSO on glycerol consumption and succinic acid production, and to understand its influence on cell growth, three independent batch cultivations were performed with *A. succinogenes* under anaerobic conditions in 1L controlled benchtop reactor. Fermentations started with 1.8% DMSO and around 24 g/ L glycerol (Figure 2.3 and Table 2.3). Initial DMSO concentration was slightly higher than the optimal value obtained in serum flasks (1.4% DMSO with 4 g/L glycerol) to compensate for higher glycerol concentration in the bioreactor. However, only 0.8% (v/v) DMSO was actually consumed by the end of the fermentations (Figure 2.3). Succinic acid was the major product formed, reaching a mean value of 24 g/L and approximately 23 g/L glycerol were consumed (Figure 2.3), resulting in a product yield of 0.95 g SA/g GLY (Table 2.3).

Vlysidis and colleagues reported a succinic acid yield of 0.85 g SA/g GLY in the presence of 22 g/L glycerol, with a corresponding productivity of 0.24 g SA/ L.h using their glycerol adapted *A. succinogenes* strain [Vlysidis et al., 2011]. In our study, we obtained a 9 fold higher succinic acid production rate of 2.13 g/ L.h for similar cell concentration, with a correspondent specific production rate of 1.13 g SA/ g DCW.h (Table 2.3). It seems that using DMSO as electron acceptor allows faster glycerol consumption by *A. succinogenes* than the reported adapted strain, probably generating a significant amount of reducing equivalents, resulting in a dramatically higher succinic acid production rate.

In comparison, *A. succiniciproducens* was able to reach, using glycerol as substrate, yields of 1.3 and 1.6 g SA/g GLY in a batch and fed-batch experiments, respectively, although displaying reduced SA production rate of around 0.14 g/L.h [Lee et al., 2001]. Higher production rates using glycerol as substrate were only attained with *A. succiniciproducens* in a continuous operating mode, where yeast extract was also supplemented, reaching 2.1 g SA/L.h [Lee et al., 2010b]. *B. succiniciproducens* DD1 was also capable of producing high succinic acid yields in a batch experiment using 10.2 g/ L pure glycerol as carbon source (1.2 g SA/g GLY) with a production rate of 0.7 g SA/ L.h [Scholten and Dägele, 2008]. Here, we report a succinic acid production rate of 2.13 g/ L.h, reinforcing the positive effect of DMSO on glycerol consumption and succinic acid production by *A. succinogenes* .

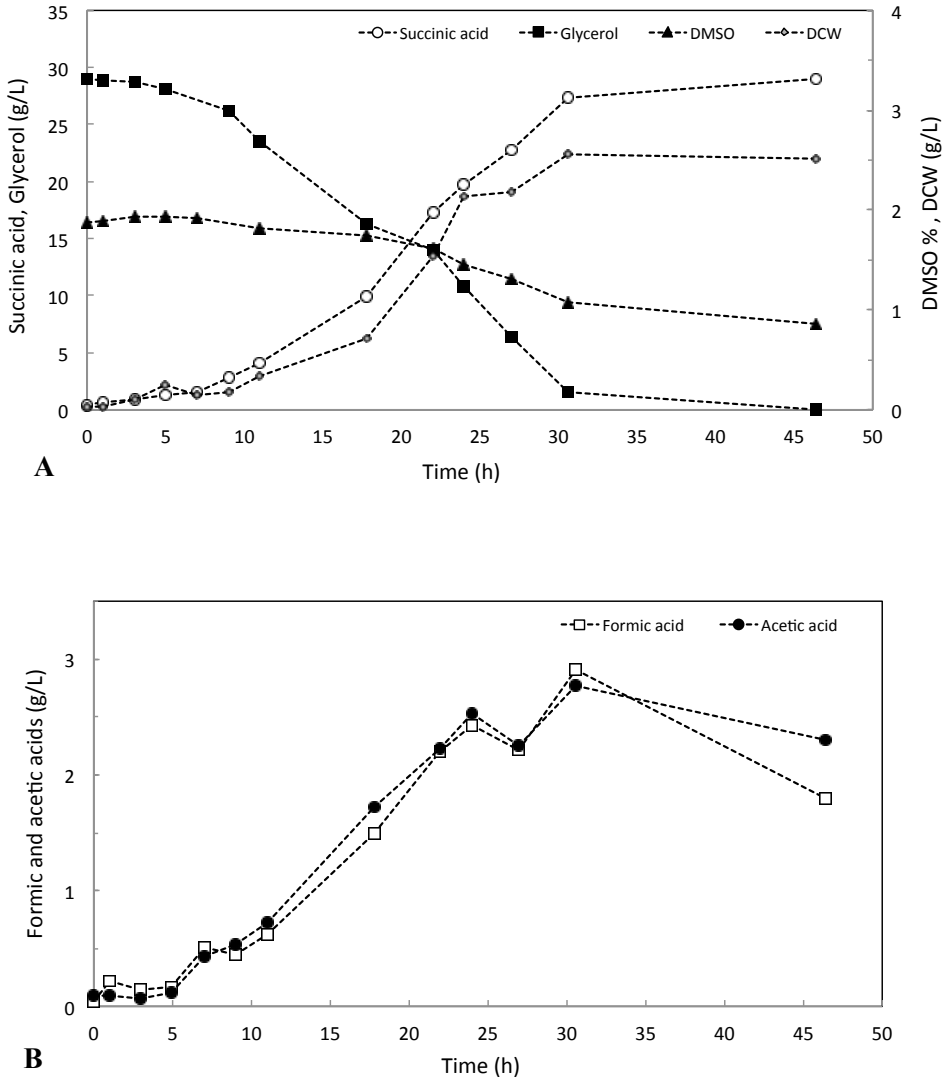


Figure 2.3: Batch fermentation profile of *A. succinogenes* using glycerol as carbon source and DMSO as electron acceptor. Mean value of triplicate experiments is represented. (A) Glycerol, DMSO, dry cell weight (DCW) and succinic acid; (B) by-products:formic and acetic acids.

Acetic (AA) and formic (FA) acids were also produced during fermentation, but concentration was below 2.5 g/L (Figure 2.3 B), with yields of 0.10 g AA/g GLY and 0.09 g FA/g GLY, respectively, and ratios of 0.11 g AA/g SA and 0.09 g FA/g SA (Table 2.3). Vlysidis et al. reported similar values in a batch experiment using 22 g/L glycerol, producing 0.06 g AA/g SA and 0.03 g FA/g SA [Vlysidis et al., 2011]. Acetic and formic acids production in *A. succinogenes* is mediated by two key enzymes: pyruvate formate lyase (PFL), which generates formate and acetyl-coA from pyruvate and coA, and pyruvate dehydrogenase (PyrDH), which generates acetyl-coA and CO_2 from pyruvate and coA, along with one NADH (see pyruvate branch point in Figure 2.2). In *E.coli*, it has been shown that a higher internal cell redox potential inactivates PyrDH but PFL becomes activated, resulting in an equivalent production of formate and acetate [Sawers and Clark, 2004]. The production of nearly equimolar concentrations of acetate and formate in our study suggests that a high proportion of pyruvate consuming flux is still going through PFL, probably because of a higher redox state of the cell induced by DMSO concentration. Guettler et al. developed a metholodogy for the isolation of resistant variants of *A. succinogenes* strain 130Z to sodium monofluoroacetate. The variant produced succinic acid from glucose with a 20% higher product yield than the wild type strain, at the expense of formate and acetate production, reinforcing the importance of reducing the carbon flux through the C_3 pathway to increase succinic acid production [Guettler et al., 1996]. Here, using 1.8% DMSO seems to favor the recycling of excess reducing equivalents through DMSO reductase, decreasing the flux through the reductive pathway of TCA, therefore reducing the production of succinic acid.

2.3.3 Fed-batch cultivation

In order to improve succinic acid production from glycerol by *A. succinogenes* , a fed-batch experiment was performed with controlled addition of DMSO and glycerol. Cell growth, succinic acid and by-products formation are depicted in Figures 2.4 A and B. Results are summarized in Table 2.3.

The reactor was firstly operated as batch with initial glycerol and DMSO concentrations of 33.70 g/L and 1%, respectively. 1% DMSO (v/v) was used as only 0.8% was consumed in batch experiments with 24 g/L glycerol and in order to minimize acetic and formic acids formation throughout fermentation, according to serum flask assays (Table 2.1). This batch phase resulted in the production of 24.33 g/ L succinic acid after 30h cultivation (Figure 2.4 A). After DMSO has been totally exhausted, glycerol consumption stopped, confirming that DMSO plays an essential role in glycerol consumption. A high succinic acid yield was obtained by the end of the batch phase, 0.87 g SA/g GLY, in agreement with the obtained values in the previous batch experiments and serum flasks assays (Tables 2.3 and 2.1). Volumetric and specific succinic acid maximum production rates were the highest obtained so far, 2.31 g SA/ L.h and 1.29 g SA/g DCW.h, surpassing by a 10 fold factor the ones reported by Vlysidis using an adapted *A. succinogenes* strain, 0.24 g SA/L.h. In that study, 22 g/L glycerol produced 18 g SA/ L, with a corresponding product yield of 0.85 g SA/g GLY [Vlysidis et al., 2011]. Improved performance of this fed-batch experiment in comparison with the previous batch experiment may be related to the lower initial DMSO

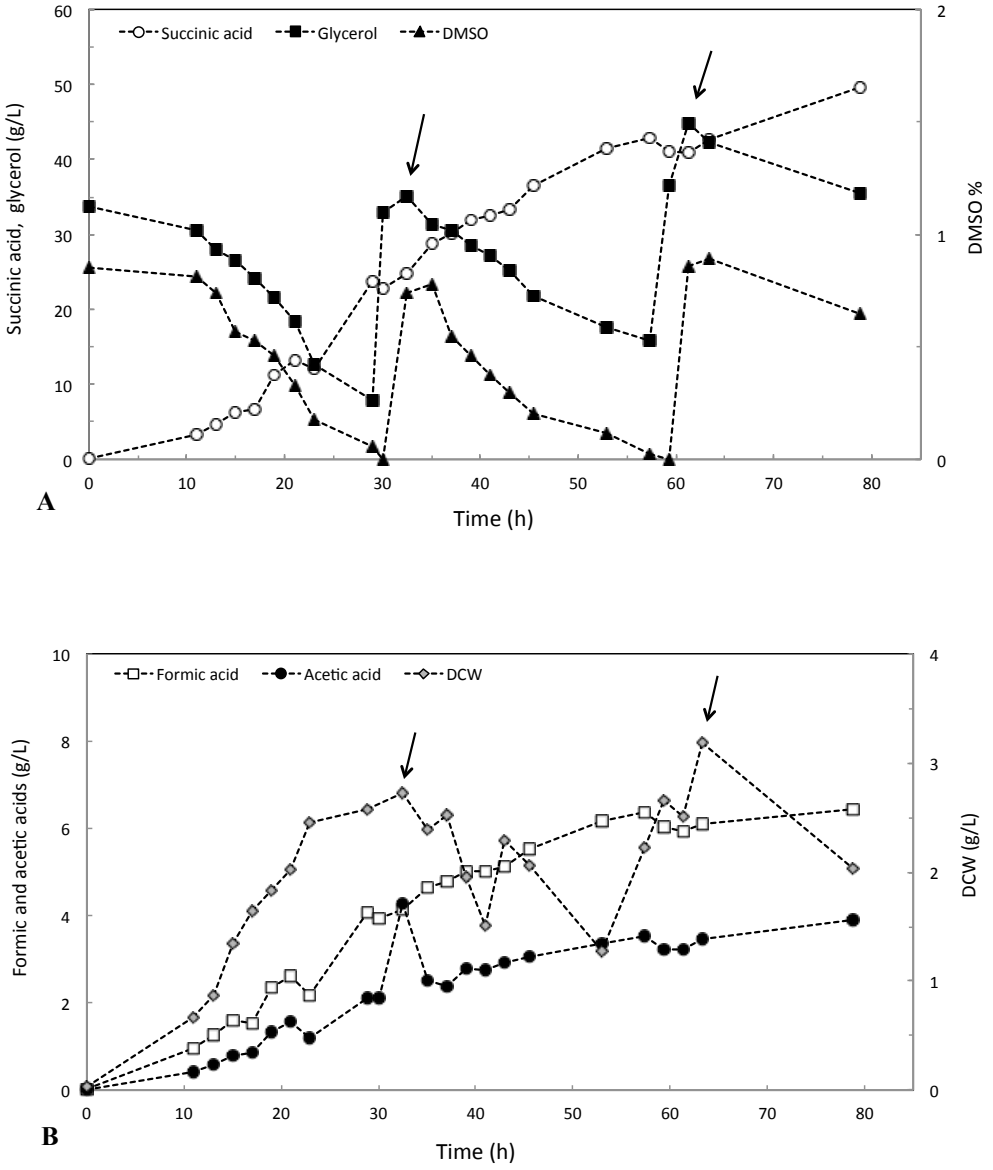


Figure 2.4: Fed-batch fermentation profile of *A. succinogenes* using glycerol as carbon source and DMSO as electron acceptor. At 30 and 60 h of fermentation (represented by arrows) additional 25 g/L of glycerol and 1% of DMSO were supplemented to the culture broth. (A) Glycerol, DMSO and succinic acid; (B) by-products: formic and acetic acids, dry cell weight (DCW).

concentration used (1%), which generated enough reducing power for glycerol to enter glycolysis and direct carbon flux through the reductive branch of TCA cycle, increasing succinic acid formation. On the other hand, biomass yield (0.09 g X/g GLY) was the lowest obtained, suggesting that succinic acid production was higher probably also at the expense of biomass formation.

Table 2.3: Final products and kinetic parameters of succinic acid production from glycerol in controlled fermentations using DMSO as electron acceptor.

System	S_0	μ (h^{-1})	Final [SA] (g/L)	Consumed GLY (g/L)	$Y_{SA/GLY}$
Batch	24.03 ± 4.6	0.15 ± 0.01	24.39 ± 4.5	23.31 ± 5.7	0.95 ± 0.2
Fed-batch: Batch phase	33.70	0.15	24.33	25.70	0.87
Fed-batch: 1st pulse	35.05	n.d.	44.78	18.56	0.83
Fed-batch: 2nd pulse	45.84	n.d.	49.62	8.32	0.64

$Y_{X/GLY}$	r_{SA}	q_{SA}	$Y_{AA/GLY}$	$Y_{FA/GLY}$	AA/SA	FA/SA
0.15 ± 0.06	2.13 ± 0.56	1.13 ± 0.05	0.10 ± 0.03	0.09 ± 0.03	0.11 ± 0.01	0.09 ±0.03
0.09	2.31	1.29	0.08	0.14	0.09	0.20
n.d.	1.63	0.60	0.07	0.11	0.07	0.12
n.d.	0.96	0.32	0.07	0.05	0.12	0.08

Data are mean values in duplicates represented as mean ± standard deviation.

S_0 - Initial glycerol concentration (g/L)

$Y_{SA/GLY}$ - Succinic acid yield on glycerol expressed in grams of SA per gram of glycerol

$Y_{X/GLY}$ - Biomass acid yield on glycerol expressed in grams of dry cell weight (DCW) per gram of glycerol

r_{SA} - Maximum succinic acid volumetric production rate expressed in g SA/L.h

q_{SA} - Specific succinic acid production rate expressed in g SA/g DCW.h $Y_{AA/GLY}$ - Yield of produced acetic acid on consumed glycerol (g/g)

$Y_{FA/GLY}$ - Yield of produced formic acid on consumed glycerol (g/g)

AA/SA - Stoichiometry of produced acetic acid on succinic acid (g/g)

FA/SA - Stoichiometry of produced formic acid on succinic acid (g/g)

After this batch phase where all DMSO was consumed, 26 g/L glycerol and 1% DMSO were pulsed inside the fermentation broth (Figure 2.4 A, 30h). As a consequence, succinic acid production continuously increased, reaching a concentration of almost 45 g/L, consuming all the supplemented DMSO but only 18.56 g/ L of glycerol, in 27 hours (Figure 2.4 A). Product yield and maximum specific production rate decreased from 0.87 to 0.83 g SA/g GLY and from 1.29 to 0.60 g SA/g DCW.h, respectively, in comparison with the initial batch phase, resulting in a lower volumetric production rate (1.63 g SA/ L.h). by-products formation was also lower than in the batch phase (Table 2.3). A second pulse with additional 25 g/L glycerol and 1% DMSO was supplemented to the broth at 60h of fermentation (Figure 2.4 A). Succinic acid concentration increased to 49.62 g/ L, the highest concentration reached so far with *A. succinogenes* on glycerol or any other microorganism. However, product yield and SA production were lower than the ones obtained during the first pulse and consequently cultivation was finished (Table 2.3).

Biomass concentration reached its highest value by the end of the batch phase, 2.52 g/L, and then slowly decreased until the end of the fermentation (Figure 2.4 B). This decrease might be

associated with the accumulation of acids during the fermentation, which may have a negative impact on cell growth. In this study, pH was maintained at 6.8 and the produced organic acids were therefore almost completely dissociated (pK_a of formic, acetic acids being 3.84, 4.75 and succinic acid 4.21 (pK_a1) and 5.64 (pK_a2)). Eventhough the undissociated forms of acids are known to be the most inhibitory, Lin and colleagues recently demonstrated that even the salt form of acids can be inhibitory for *A. succinogenes* , possibly interfering with phosphate transport and therefore ATP production. Sodium formate was shown to cause the strongest inhibition effect among all the produced acids, with concentration above 16 g/L inhibiting totally cell growth. Disodium succinate had the least inhibitory effect on cell growth by *A. succinogenes* , only inhibited at concentration as high as 42 g/L [Lin et al., 2008]. Here, formic, acetic and succinic acids reached 6.45, 4.12 and 49.62 g/L, respectively, by the end of the fermentation, clearly suggesting an inhibition by acids accumulation (Figure 2.4 B). Similar effect was also reported in other strains such as *M. succiniciproducens*, where cell growth was totally inhibited when total acids concentration exceeded 17.2 g/ L [Song et al., 2008]. In our study, total acids concentration was over 30 g/L by the end of the batch phase, reinforcing this hypothesis.

The lower yields and productivities obtained during the first and second pulses of glycerol and DMSO, in comparison with the batch phase might then be the result of a combination of high glycerol concentration (the second pulse resulted in a glycerol concentration of almost 46 g/L) and organic acids accumulation in the broth. One strategy to overcome this problem would be to continuously remove the organic acids from the broth, preventing inhibition of cell growth and succinic acid production. Moreover, the addition of glycerol at a constant and controlled feeding rate would prevent the accumulation of substrate over inhibitory concentrations.

Nevertheless, the succinic acid final concentration and production rates in this study were the highest described in literature so far using *A. succinogenes* for glycerol fermentation, highlighting this strain's natural ability to produce succinic acid and reinforcing its potential as an industrial biocatalyst.

2.4 CONCLUSIONS

This is the first report where DMSO was used for the bioproduction of succinic acid during controlled fermentations. Results show that DMSO dramatically improved glycerol consumption and succinic acid production by *A. succinogenes* , resulting in the highest final concentration of succinic acid and production rates reported so far using glycerol as carbon source. More than 80% of glycerol was converted to succinic acid which opens new perspectives on the use of this feedstock for the production of high value products. Further optimization of the process can play a significant role in the sustainability of the biodiesel industry. Moreover, the incorporation of a cheaper source of DMSO as a co-substrate for biobased succinic acid production as, for instance kraft paper process by-products where DMSO is extracted from, might turn succinic acid production into a cost-effective and environmental-friendly process.

3 *Succinic acid production from roasted carob pods flour*

Summary *Carob pods are a by-product of locust bean gum industry containing more than 50% (w/w) sucrose, glucose and fructose. In this work, carob pod water extracts were used, for the first time, for succinic acid production by A. succinogenes 130Z. Kinetic studies of glucose, fructose and sucrose consumption as individual carbon sources till 30 g/L showed no inhibition on cell growth, sugar consumption and succinic acid production rates. Sugar extraction from carob pods was optimized varying solid/liquid ratio and extraction time, maximizing sugar recovery while minimizing the extraction of polyphenols. Batch fermentations containing 10-15 g/L total sugars resulted in a maximum specific SA production rate of 0.61 Cmol/Cmol X.h, with a yield of 0.55 Cmol SA/ Cmol sugars and a volumetric productivity of 1.61 g SA/L.h. Results demonstrate that carob pods can be a promising low cost feedstock for bio-based SA production.*

The contents of this chapter were adapted from the publication: Carvalho, M., Roca, C. and Reis, M.A.M. (2014). Carob pod water extracts as feedstock for succinic acid production by *A. succinogenes* 130Z. Bioresource Technology, 170, 491-498

3.1 INTRODUCTION

Succinic acid (SA), a four-carbon dicarboxylic acid, is currently considered a key platform chemical as it is used in the production of a wide range of products, from pharmaceuticals to green solvents, fibers and bioplastics [McKinlay et al., 2007a]. In 2004, the U.S. Department of Energy (DOE) listed succinic acid as one of the most important building blocks that can be produced from biomass [Werpy and Petersen, 2004]. Industrial scale production is presently based on the hydrogenation of maleic anhydride, a petrochemical, which poses environmental and economical problems. Hence, there is a strong demand for the development of a bio-based process for succinic acid production. Microorganisms like *A. succinogenes* [Guettler et al., 1999] and *Mannheimia succiniciproducens* [Lee et al., 2002] are naturally succinic acid producers, while metabolic engineered strains of *Escherichia coli* [Blankschien et al., 2010] and *Saccharomyces cerevisiae* [Raab et al., 2010] have been developed for the production of this diacid. The use of renewable feedstocks as carbon source for the production of SA via fermentation constitutes a solution to reduce production costs and develop a sustainable SA production process. Feedstocks used in the bioproduction of this diacid include glycerol [Carvalho et al., 2014a], corn stover [Zheng et al., 2010], sugarcane and molasses [Chan et al., 2012], rice straw [Zheng et al., 2009], among others. Lignocellulosic biomass as corn stover and rice straw require, however, a pretreatment step where considerable lignin degradation products are released and could inhibit SA production from microorganisms [Zheng et al., 2010, Zheng et al., 2009].

The carob tree (*Ceratonia siliqua* L.) is a Mediterranean perennial tree which produces pods containing seeds (10% w/w) and a highly rich sugar pulp (90% w/w), mainly containing sucrose, glucose and fructose, which may constitute as much as 50% of the carob pod mass [Avallone et al., 1997, Manso et al., 2010]. According to recent data, carob pod production worldwide accounts for almost 400,000 tons yearly of which 50,000 tons/year are produced in the Algarve, Portugal, turning the region into the third largest producer in the world [Santos et al., 2005]. The carob pods are a by-product of carob locust bean gum industry, a highly viscous polysaccharide extracted from carob seeds and used as thickener, stabilizer and gelling agent in the food, cosmetics and pharmaceutical industries [Correia and Martins-Loução, 2005, Avallone et al., 1997, Santos et al., 2005]. Currently used as raw material for syrups production, cocoa substitute in the food industry [Bernardo-Gil et al., 2011] or animal feed [Roseiro et al., 2013a], most carob pods are, however, discarded and therefore constitute an inexpensive and available feedstock for the production of added-value biological products. Dextran [Santos et al., 2005] and ethanol [Turhan et al., 2010] are successful examples of biological conversion of carob pulp aqueous extracts into added-value products.

This work reports, for the first time, the production of succinic acid by *A. succinogenes* 130Z using carob pulp water extracts as feedstock. Sugar extraction from roasted carob pulp was optimized varying solid/liquid ratio and extraction time, in order to maximize sugar recovery while minimizing the extraction of polyphenols. The best extracts were selected for further bioreactor experiments. Kinetics of fructose and sucrose consumption were initially determined in serum

flasks as sole carbon sources with concentration up to 30 g/L and compared with glucose, in order to assess possible substrate inhibition on the production of SA and formulate the amount of carob pulp added to culture medium. Afterwards, controlled batch cultures were performed using the obtained carob pulp extracts, with concentrations up to 30 g/L total sugar, maximum sugar concentration possible after extraction.

3.2 Materials and Methods

3.2.1 Carob pulp water extraction procedures

Carob pulp was purchased from a local store as roasted carob flour. In order to maximize sugar extraction from carob pulp while minimizing the quantity of removed phenolic compounds, carob powder was suspended in distilled water at solid/liquid ratio 1:10 (w/v) and incubated at 25°C in a rotary shaker at 200 rpm [Manso et al., 2010, Roseiro et al., 2013b]. Moreover, different times of extraction were tested: 0.5, 1, 3, 5, 7 and 9 h. The carob pulp extracts were then centrifuged at 7500 rpm for 15 min at 4°C (Beckman J2-MC Centrifuge with a JA14 rotor) and the clarified supernatant was stored at -20°C. Aiming at obtaining richer sugar aqueous extracts, carob pulp powder was suspended in distilled water at different solid/liquid ratios (w/v). These mixtures were incubated at 25°C in a rotary shaker at 200 rpm for 1 h and then processed as described above. All experiments were performed in duplicate. The yield of sugar extraction was calculated by considering that half of the carob pulp mass (50%) is composed of sugars [Avallone et al., 1997, Yousif and Alghzawi, 2000].

3.2.2 Strain and inoculum preparation

A. succinogenes 130Z (ATCC 55618) was used in all experiments. Inoculum was prepared by incubating cells from a -80°C glycerol stock culture in rubber sealed serum flasks, containing 50 mL of a semi-modified culture medium adapted from [McKinlay and Vieille, 2008] , with CO_2 as the gas phase. Briefly, one liter of culture medium contained: 6.0 g glucose, 15.0 g yeast extract, 8.5 g $NaH_2PO_4.H_2O$, 15.5 g K_2HPO_4 and 2.1 g $NaHCO_3$. Medium was heat sterilized at 121°C for 20 min. Glucose solution was filtered through a 0.22 μm membrane filter and added aseptically. After inoculation, the rubber sealed flasks were incubated at 37°C in a rotary shaker at 200 rpm for 12 h, until late exponential phase was reached and confirmed by optical density measurements at 660 nm.

3.2.3 Cultivation conditions

3.2.3.1 Batch fermentations of glucose, fructose and sucrose in serum flasks experiments

Glucose, fructose and sucrose were individually used as fermentation substrates by *A. succinogenes* 130Z and their effect on cell growth, sugar consumption and organic acids production rates was estimated. Fermentations were carried out in serum flasks with working volume of 100 mL each. Culture medium contained per liter: 10.0 g yeast extract, 8.5 g $NaH_2PO_4.H_2O$ and 15.5 g

K_2HPO_4. Glucose, fructose and sucrose were added as sole carbon sources, individually, in initial concentrations between 0.5 and 30 g/L (0.02 and 1 Cmol/L). To keep pH constant and acids neutralization, $MgCO_3$ was used as buffering agent at 40 g/L [Zou et al., 2011]. Medium was heat sterilized at 121°C for 20 min. Glucose, fructose and sucrose solutions were filtered through a 0.22 μm membrane filter and added aseptically. After inoculation, the rubber sealed flasks were incubated at 37°C in a rotary shaker at 250 rpm. 10% (v/v) inoculum was used in all experiments. Samples were taken at regular intervals to determine cell growth, consumption of sugars and formation of products, as described in section 3.2.4. All experiments were performed in duplicate.

3.2.3.2 Bioreactor cultivations

The obtained aqueous carob pulp extracts were used as substrate for succinic acid production by *A. succinogenes* 130Z. All batch experiments were performed twice, in a 1L benchtop-bioreactor with working volume of 900 mL, sparged with CO_2 at 0.05 vvm, at 37°C and with stirring at 250 rpm.

The pH was automatically controlled at 6.8 by the addition of 5 M NaOH solution. The culture medium composition was adapted from [McKinlay and Vieille, 2008] and contained per liter: 10.0 g yeast extract, 8.5 g $NaH_2PO_4.H_2O$, 15.5 g K_2HPO_4 and 12.6 g $NaHCO_3$. Aqueous carob pulp extract was supplemented to the bioreactor to obtain final sugar concentrations of 5, 10, 15 and 30 g/L (0.15, 0.27, 0.5 and 1.01 Cmol/L). Samples were taken at regular intervals to determine cell growth, sugar consumption and formation of products, as described in section 3.2.4. All bioreactor experiments were inoculated with 5% (v/v) inoculum.

3.2.4 Analytical methods

Fermentation products (succinic, formic and acetic acids) were analyzed by high-performance liquid chromatography (HPLC) using a Metacarb 87H column (Varian) and a refractive index detector (RI-71, Merck). The column was eluted at 30°C with 0.01 N H_2SO_4 solution at a flow rate of 0.5 mL/min. Glucose, fructose and sucrose were also analyzed by HPLC, in the same operating conditions but using a Hi-Plex H column (Agilent).

Regarding serum flasks fermentations, cell growth was estimated by optical density (OD) measurements at 660nm (Spectronic Helios Alpha UV/Vis spectrophotometer, Fisher Scientific). The samples were diluted with 7% HCl to dissolve $MgCO_3$ and the biomass concentration was calculated from OD values using an experimentally determined correlation factor of 0.381 g DCW/L for an OD 660 of 1. For bioreactor experiments, cell concentration was estimated by dry cell weight. Broth samples were collected and centrifuged for 5 min at 12,000 rpm, after which the supernatant was collected for HPLC analysis. The pellet was washed with distilled water, centrifuged for 5min at 12,000 rpm and dried for 24 h at 70°C .

Total phenolic compounds were determined in all carob pulp extracts, as well as bioreactor experiments, using a modified Folin–Ciocalteu colorimetric method [Roseiro et al., 2013a]. Briefly, 100 μL of sample (or water for blank) was mixed with 400 μL of water, 250 μL 1/1 (v/v) diluted Folin-Ciocalteu reagent and 1.25 mL 20% Na_2CO_3. Absorbance was measured at 725 nm after 40

min of incubation in the dark. Total phenolic compounds were expressed as mg GAE (gallic acid equivalents)/mL by comparison to a gallic acid standard curve and converted to mg GAE/g carob pod. Experiments were performed in duplicate.

3.2.5 Calculation of kinetic parameters

The fermentation kinetics can normally be described by a cell growth model (r_X), a substrate consumption model (r_S) and a product formation model (r_{SA}). A Monod growth model was assumed to describe cell growth:

$$\mu = \mu_{max} * \frac{S}{(S + K_s)} \tag{3.1}$$

where μ is the specific growth rate (h^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), S is the substrate concentration (g L^{-1}) and K_S is the substrate saturation constant (g L^{-1}). Specific growth rate μ (h^{-1}) was determined for each experiment by linear regression of the curve ln ($X/X0$) versus time (X representing the biomass calculation and X_0 the biomass at time zero, expressed in C) for the exponential growth phase. The typical microbial composition of $CH_{1.8}.O_{0.5}N_{0.2}$ was assumed for biomass [McKinlay et al., 2007a]. Succinic acid production rate (r_{SA}), expressed in g/L.h, was calculated as the maximum increase in SA concentration for a determined time interval. Specific succinic acid production rate (q_{SA}) was calculated as follows:

$$q_{SA} = \mu * Y_{SA/X} \tag{3.2}$$

where ($Y_{SA/X}$) is the yield of succinic acid on biomass. Specific substrate consumption rate (q_S) was also determined using Eq. 3.2, using instead the substrate yield on biomass ($Y_{S/X}$). Yields of fermentation products (succinic ($Y_{SA/S}$), acetic ($Y_{AA/S}$) and formic ($Y_{FA/S}$) acids) and biomass ($Y_{X/S}$) were calculated from the slope of the straight line obtained by plotting the increasing amount of product (P/P_0), namely succinic, acetic and formic acids and biomass (X/X_0) as a function of sugar consumption ($S/S0$), respectively. Using the obtained data from batch fermentations of different sugars (glucose, fructose and sucrose), μ_{max}, q_{SAmax}, q_{Smax} and K_S were estimated for each substrate using an in-house developed program for MATLAB (Matworks, Inc), minimizing an objective function of Z:

$$Z_q = \sum_{j=1}^{n} (qj, \exp - qj, \mathrm{sim})^2 \tag{3.3}$$

where Z_q represents the least-square differences between experimental data and simulated results of biomass, product or substrate; j denotes the number of experimental data points.

3.2.6 Statistical analysis

The obtained data for carob pulp extraction procedures as well as kinetic parameters for glucose, fructose, sucrose and carob pulp extract fermentations were subjected to statistical analysis of variance according to one-way ANOVA ($P < 0.05$).

3.3 RESULTS AND DISCUSSION

3.3.1 Composition of carob pulp water extracts in sugars and total phenolic compounds

The main purpose of the carob pulp extraction procedures was to obtain highly rich sugar extracts for succinic acid production, while minimizing the quantity of recovered soluble polyphenols,as they may act as antimicrobial agents [Henis et al., 1964]. Different methods for polyphenols extraction are reported in literature, including conventional methods using water or acetone [Henis et al., 1964, Roseiro et al., 2013a, Avallone et al., 1997] , ultrasound extraction [Roseiro et al., 2013a] and supercritical extraction [Roseiro et al., 2013a, Bernardo-Gil et al., 2011]. Polyphenols extraction from carob pods is the lowest when performed with water in shake flasks at room temperature. This method was therefore selected for this study.

Table 3.1: Sugars and total phenolics content of water extracts from roasted carob pulp (solid/liquid ratio 1:10) at 25°C for 1–9 h

Time (h)	Sucrose (g/ L)	Glucose (g/ L)	Fructose (g/ L)	Total sugars (g/ L)	Yield sugar extraction (%)	GAE (mg/mL)	mg GAE/ g carob pulp mass
0.5	12.47 ± 0.3a	7.25 ± 0.1a	1.99 ± 0.1a	21.71± 1.2a	43.42 ± 3.2a	1.39 ± 0.0a	13.76 ± 0.2a
1	13.59 ± 0.9a	7.33 ± 0.1a	2.64 ± 0.1a	23.55 ± 1.1a	46.31 ± 1.9a	1.46 ± 0.0a	13.11 ± 0.4a
3	12.44 ± 0.7a	6.81 ± 0.0b	2.45 ± 0.0a	21.70 ± 0.1a	43.60 ± 1.8a	1.36 ± 0.0a	12.25 ± 0.3a
5	12.60 ± 0.9a	6.89 ± 0.1b	2.48 ± 0.1a	21.97 ± 1.1a	43.69 ± 1.9a	1.42 ± 0.1a	12.79 ± 0.7a
7	12.70 ± 0.9a	6.95 ± 0.1b	2.51 ± 0.1a	22.16 ± 1.1a	44.15 ± 2.1a	1.37 ± 0.0a	12.32 ± 0.1a
9	12.54 ± 0.9a	6.84 ± 0.1b	2.48 ± 0.0a	21.86 ± 1.0a	43.69 ± 2.1a	1.40 ± 0.0a	12.62 ± 0.3a

Data are duplicates from two independent experiments represented as mean ± standard deviation.
Within a column, values followed by the same letter are not statistically
different according to one-way ANOVA ($P < 0.05$).
Yield of sugar extraction (%) = total sugar concentration (g/L)/ theoretical sugar concentration,
i.e. half of the carob pulp mass (g/L), multiplied by 100.
GAE: gallic acid equivalents

Sugar composition and total phenolics content of aqueous carob pulp extracts obtained for different extraction times and solid/ liquid ratios are presented in Tables 3.1 and 3.2, respectively. The obtained water extracts are characterized by a high content of soluble sugars, including sucrose (more than 50% of total sugars), glucose and fructose (Table 3.1), similarly to what has been reported for deseeded carob pods [Avallone et al., 1997, Manso et al., 2010]. Theoretically, sugar content of carob pulp can reach up to 50% of its mass content. Here, we report a 45% yield of sugar, lower than the one reported by Manso et al., where nearly 95% sugars was obtained. This decrease is probably related to the Maillard reaction and caramelisation occurring during the carob roasting process [Yousif and Alghzawi, 2000]. Using instead deseeded carob kibbles directly from the industry would be the best option in order to obtain richer sugar extracts. Different extraction times had no effect on the sugar content of extracts (Table 3.1), suggesting that the extraction efficiency is not affected by time under these operating conditions, also reported for deseeded carob

pods [Manso et al., 2010]. Increasing solid/liquid ratio significantly decreases the yield of sugar extraction (Table 3.2). However, it results in water extracts more concentrated in sugars, often necessary for biological fermentations.

Table 3.2: Sugar and total phenolic compounds content of roasted carob pod water extracts with different solid/liquid ratios, at 25°C for 1 h

Ratio (w/v)	Sucrose (g/ L)	Glucose (g/ L)	Fructose (g/ L)	Total sugars (g/ L)	Yield sugar extraction (%)	GAE (mg/mL)	mg GAE/ g dry mass
1:10	13.02 ± 0.3a	6.57 ± 0.2a	1.93 ± 0.0a	21.53 ± 0.5a	43.05 ± 1.0a	1.43 ± 0.0a	13.12 ± 0.3a
2:10	19.70 ± 0.2b	9.89 ± 0.1b	2.87 ± 0.0b	32.47 ± 0.4b	32.66 ± 0.1b	2.04 ± 0.2b	9.19 ± 0.7b
3:10	17.31 ± 0.3c	8.72 ± 0.2c	2.53 ± 0.0c	28.57 ± 0.5c	18.96 ± 0.5c	2.38 ± 0.0b	7.14 ± 0.0b

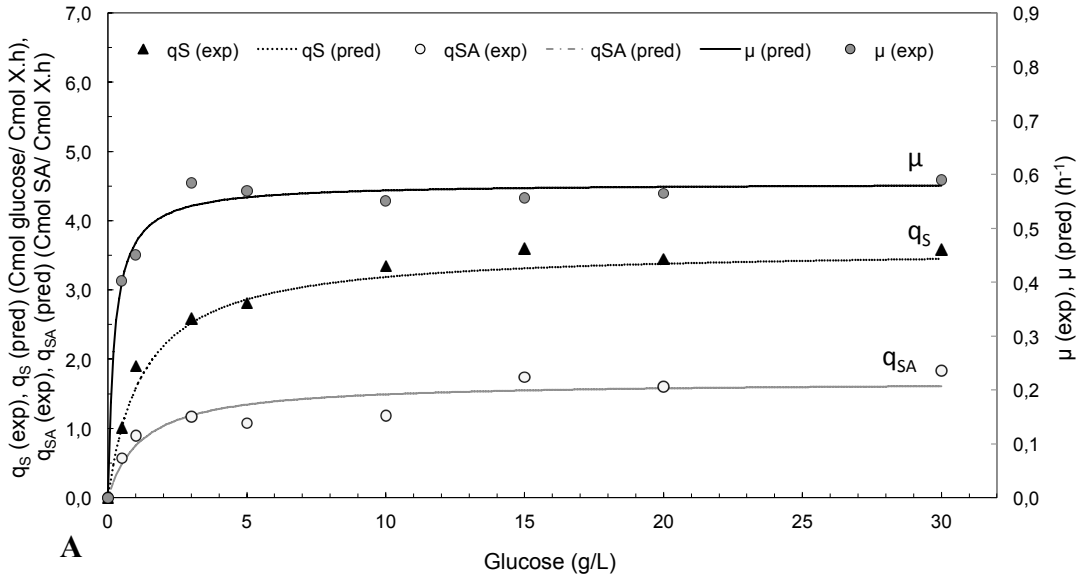
Data are duplicates from two independent experiments represented as mean ± standard deviation.
Within a column, values followed by the same letter are not statistically different
according to one-way ANOVA ($P < 0.05$).
Yield of sugar extraction (%) = total sugar concentration (g/L)/ theoretical sugar concentration,
i.e. half of the carob pulp mass (g/L), multiplied by 100.
GAE: gallic acid equivalents

Regarding soluble phenolic compounds extraction from carob pulp, the extraction time had no effect on the quantity of extracted phenolic compounds at a ratio of 1:10 (v/v), obtaining an average of 12.6 mg GAE/g carob pulp mass (Table 3.1). Increasing the solid/ liquid ratio of carob pulp/water mixtures produced extracts richer in soluble polyphenolic compounds, although there were no significant differences between ratios 2 and 3:10, suggesting that polyphenolic compounds reached saturation (Table 3.2). The reduced extraction of polyphenols at higher solid/liquid ratios can then be used as an advantage in order to reduce this amount, while recovering high sugar content.

Altogether, these results show that aqueous extracts from carob pulp at room temperature is a simple and environmental friendly methodology to recover highly sugar rich extracts, while minimizing the extraction of polyphenols. Furthermore, the solid/liquid ratio of the carob pod/water mixture may be adjusted to obtain more concentrated extracts, depending on fermentation requirements and non-roasted carob pods might also be used to obtain extracts more concentrated in sugars. In fact, the use of carob pods at a specific physiological maturity, when sugar content is maximum, will result in more concentrated extracts.

3.3.2 Fermentation kinetics of glucose, fructose and sucrose as sole carbon source

Glucose, fructose and sucrose are the main sugar compounds extracted from carob pulp. *A. succinogenes* 130Z is naturally able to produce succinic acid from a broad range of carbon sources including glucose, fructose and sucrose [Guettler et al., 1999], likely a consequence of its symbiotic role in the bovine rumen. Several sugar transporters have already been identified in its genome [McKinlay et al., 2010] but, while glucose or glycerol fermentation studies have been


qS (exp)
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qSA (exp)
qSA (pred)
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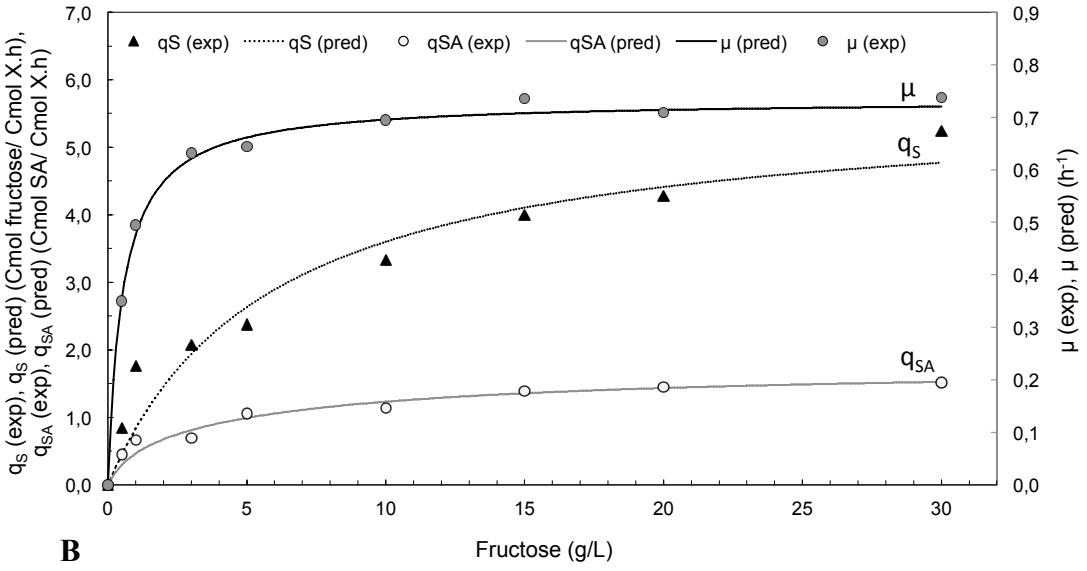
μ (exp)
μ
q_S
q_{SA}
q_S (exp), q_S (pred) (Cmol glucose/ Cmol X.h),
q_{SA} (exp), q_{SA} (pred) (Cmol SA/ Cmol X.h)
μ (exp), μ (pred) (h^{-1})
Glucose (g/L)
A


qS (exp)
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q_S
q_{SA}
q_S (exp), q_S (pred) (Cmol fructose/ Cmol X.h),
q_{SA} (exp), q_{SA} (pred) (Cmol SA/ Cmol X.h)
μ (exp), μ (pred) (h^{-1})
Fructose (g/L)
B

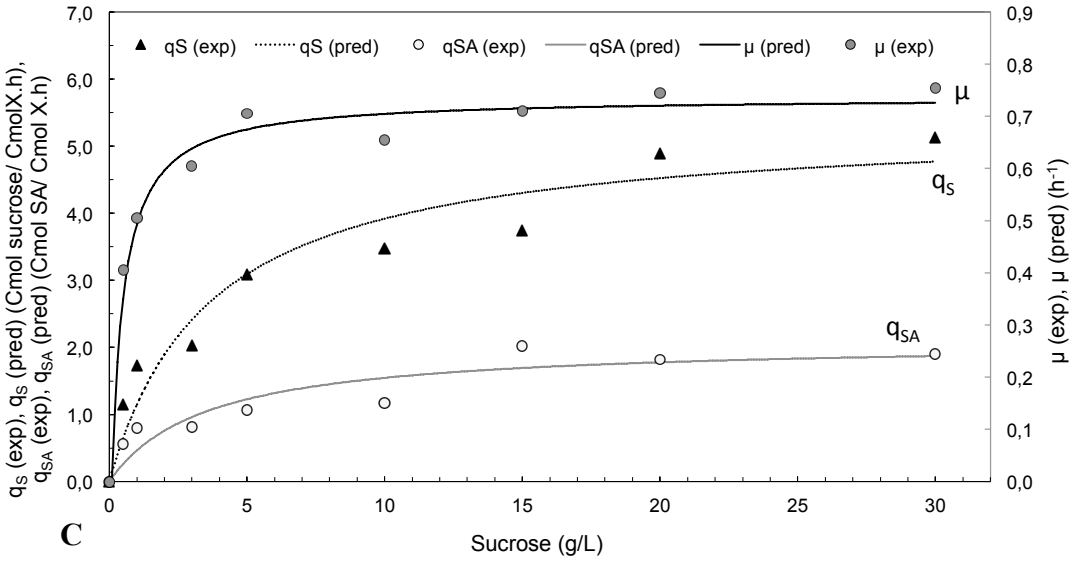


Figure 3.1: Experimental (exp) and predicted (pred) profiles for specific growth rate (μ), substrate consumption rate (q_S) and succinic acid production rate (q_{SA}) of *A. succinogenes* 130Z fermentation of glucose (A), fructose (B) and sucrose (C) as sole carbon sources at different initial concentrations.

reported in literature [Lin et al., 2008, Mckinlay et al., 2005, McKinlay et al., 2007a, McKinlay and Vieille, 2008, Carvalho et al., 2014a], little is known about fructose or sucrose consumption kinetics by this biocatalyst, apart from a report where cane molasses fermentation was assessed [Liu et al., 2008a]. Recently, sucrose utilization for SA production was reported in the different bacterial strain *A. succinogenes* NJ113 [Jiang et al., 2014]. Understanding glucose, fructose and sucrose metabolism is crucial to establish a productive fermentation from fructose and/or sucrose containing substrates, in order to assess possible sugar inhibition and to maximize the conversion of a cheap substrate into a commercially valuable product.

Kinetics of fructose and sucrose consumption by *A. succinogenes* 130Z as individual carbon source was compared with glucose utilization. Initial sugar concentration varied from 0.5 to 30 g/L and results are summarized in Tables 3.3 and 3.4. Experimental and estimated values for specific growth rates (μ),sugar consumption (q_S) and SA production rates (q_{SA}) for glucose, fructose and sucrose at different initial sugar concentrations are illustrated in Figure 3.1.

Maximum specific growth rates rapidly increased between 0 and 5 g/L sugar, regardless of the tested sugar, obtaining 0.58 (h^{-1}) for glucose (Figure 3.1 A), 0.72 and 0.73 (h^{-1}) for fructose and sucrose, respectively, at 5 g/L sugar (Fig. 3.1 B and 3.1 C). Specific growth rates remained maximum up to 30 g/L sugar, suggesting no substrate inhibition, as reported in [Lin et al., 2008]. However, a specific growth rate of 0.50 (h^{-1}) on glucose was reported, slightly lower than what we obtained here. Interestingly, specific growth rates were higher on fructose and sucrose than on glucose, which might be explained by the different sugar uptake systems for fructose, sucrose and glucose. In fact, although kinetic profiles for specific sugar consumption rates (q_S) were similar for all three sugars, different maximum estimated values were obtained for glucose (3.5 Cmol glu/Cmol X.h), fructose (4.7 Cmol fru/Cmol X.h) and sucrose (4.8 Cmol suc/Cmol X.h). Glucose specific consumption rate was maximum already after 10 g/L (Figure 3.1 A), while fructose or sucrose consumption rate was maximum after 20 g/L sugar (Figures 3.1 B and 3.1 C, respectively). The associated substrate saturation constant values calculated for each of the sugar species reflect therefore the differences in sugar uptake rates, with corresponding K_S values of 1.28 g/L for glucose, slightly lower than what has been reported in literature (2.03 g/L in [Lin et al., 2008]), 5.82 g/L for fructose and 3.65 g/L for sucrose.

The uptake of glucose, fructose and sucrose in *A. succinogenes* 130Z is mediated by a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), which has recently been identified [McKinlay et al., 2010]. This system is responsible for sugar transport into the cell and simultaneous phosphorylation, preventing sugar efflux. It consists of cytoplasmic energy-coupling enzymes EI and HPr, which lack sugar specificity and membranous enzyme II complexes (EII), composed of three different domains named IIA, IIB, and IIC, each specific for one or a few sugars (Figure 3.2).

Sugar uptake occurs in a sequential process where the phosphoryl group from phosphoenolpyruvate is transferred to EI, HPr, IIA, IIB, and finally, the incoming sugar, which is transported across the membrane via the transmembrane domain IIC. Distinct PTS families have been identified and characterized but the most representative among bacteria are the glucose family, followed by fructose, mannose and lactose families [Barabote and Saier, 2005]. While *A. succinogenes* 130Z

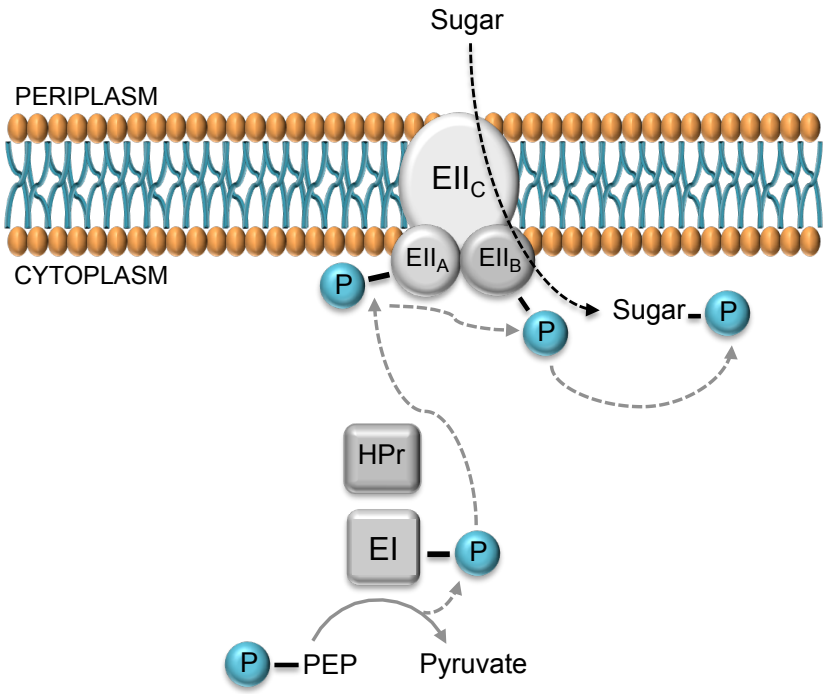


Figure 3.2: Generic representation of the phosphotransferase system (PTS) for sugar uptake in Gram-negative bacteria. The phosphoryl group (P) from phosphoenolpyruvate (PEP) is transferred to the imported sugar in a sequential way via several proteins.

genome encodes for all components required for specific fructose and sucrose PTS uptake, it lacks a homolog domain to EIIBC in *E. coli* for glucose. PTS-dependent glucose uptake is believed to take place using the mannose-specific proteins ManXYZ [McKinlay et al., 2010], which might be the cause of a lower glucose consumption rate compared to fructose or sucrose. Jiang and colleagues recently studied sucrose consumption by *A. succinogenes* NJ113 [Jiang et al., 2014]. The authors reported a PTS system for sucrose, along with sucrose-6-phosphate hydrolase and a fructose PTS to complete transport and metabolize sucrose. They also reported specific PTS activities for glucose, fructose and sucrose by enzymatic assays. Also in this case, sucrose and fructose PTS activities were always significantly higher (6 to 5-fold, respectively) than glucose PTS activity, even for cells grown on glucose. It is likely that these different PTS activities are also present in *A. succinogenes* 130Z, explaining the different sugar uptake rates obtained for glucose, fructose and sucrose. Different values for glucose consumption were obtained in a study by McKinlay and colleagues, where a (q_S) of 1.41 Cmol glu/Cmol X.h was reported, along with a (q_{SA}) of 0.71 Cmol SA/Cmol X.h. However, 100 mM $NaHCO_3$ were used for pH control instead of $MgCO_3$ [McKinlay and Vieille, 2008]. $MgCO_3$ is known to alleviate organic acids detrimental effect on cell morphology but it is also an excellent CO_2 donor, which might lead to an increase in

PEP carboxylase flux and a higher sugar consumption rate [Zou et al., 2011], as obtained here.

Metabolic studies on *A. succinogenes* 130Z using mass balances, enzyme assays and ^{13}C-metabolic flux analyses show that glucose uptake also occurs via a PTS-independent permease, through which glucose enters the cell, which is then phosphorylated by an hexokinase [Mckinlay et al., 2005, McKinlay et al., 2007a, McKinlay and Vieille, 2008]. In *M. succiniciproducens*, the EIIA domain for glucose PTS might be also used for sucrose uptake into the cell [Lee et al., 2010a]. Our results reflect not only different transport affinities for each substrate, but also the activity of different sugar transport systems acting simultaneously in sugar uptake, as illustrated by different K_S values for each sugar. Further genetic and biochemical studies are however necessary to unravel all the pathways by which these sugars might enter the cell in order to precisely characterize the kinetic parameters of sugar transporters of glucose, fructose and sucrose in *A. succinogenes* 130Z.

Regarding succinic acid production, similar maximum q_{SA} values of 1.7, 1.8 and 2.0 Cmol SA/Cmol X.h were determined for glucose, fructose and sucrose, respectively, above 10 g/L sugar. This demonstrates that SA production rate is not influenced by the sugar species consumed and that higher sugar consumption rates are actually not coupled to higher SA production, as (q_{SA}) rates plateaued at 10 g/L sugar (Figure 3.1). On the other hand, as sugar concentration increases from 10 to 30g/L, the biomass yield is significantly reduced, for fructose and sucrose, as well as acetic and formic acids, but no significant changes on the succinic acid yield were observed (Table 3.3). Over 10 g/L sugar, sugar uptake and SA production rates remain unchanged (Figure 3.1) suggesting, therefore, that biomass formation is not limited by sugar uptake or higher SA production. The accumulation of other organic acids such as formate (FA) or acetate in the broth might however affect biomass formation, as already described in literature [Lin et al., 2008]. *A. succinogenes* 130Z growth was completely inhibited when total acids concentration reached 17.2 g/L, but FA had the strongest effect, inhibiting cell growth by 30% at 5 g/L. It is known that non-dissociated organic acids can penetrate the lipidic membrane of bacteria cells and be dissociated at intracellular pH (7.5), decreasing intracellular pH. As a consequence, energy (ATP) will be required to adjust the intracellular pH and anaerobic microorganisms such as *A. succinogenes* 130Z will use more energy to expel protons instead of using energy for biosynthesis and growth [Sivakumar et al., 1994]. Our results are in agreement with these data, as organic acids only started to accumulate in the broth over 17 g/L at higher sugar concentrations (15–30 g/L) (Table 3.3). As cell growth stops, flux towards C_3 pathway also decreases as there is no further need to maintain such NADH demand via acetate and formate production [McKinlay and Vieille, 2008].

Consequently, the available carbon is diverted towards SA production and SA yield increased. Such change in final products ratios is better illustrated in Table 3.4, where biomass, SA, and acetic acid yields were normalized to formic acid production (in Cmol product:Cmol formic acid). Hardly any changes are noticed on acetic acid and biomass formation as sugar concentration increases, obtaining 2 Cmol of acetic acid and 1.5 Cmol biomass per Cmol of formic acid formed, whereas SA production nearly doubles for glucose and fructose, changing from 2.5 and 2.4 Cmol at 0.5 g/L sugar, respectively, to 4.6 and 3.9 Cmol at 30 g/L sugar. This difference is even more

Table 3.3: Fermentation yields of glucose, fructose and sucrose consumption by *A. succinogenes* 130Z as sole carbon sources, in serum flasks experiments

Sugar concentration		$Y_{SA/S}$ (Cmol SA/ Cmol sugar)			$Y_{X/S}$ (Cmol X/ Cmol sugar)			$Y_{AA/S}$ (Cmol AA/ Cmol sugar)			$Y_{FA/S}$ (Cmol FA/ Cmol sugar)		
g/L	Cmol/ L	Glu	Fru	Suc	Glu	Fru	Suc	Glu	Fru	Suc	Glu	Fru	Suc
0.5	0.02	0.49a	0.36a	0.48a	0.35a	0.21a	0.39a	0.43a	0.23a	0.48a	0.19a	0.15a	0.28a
1	0.03	0.47a	0.32a	0.47a	0.24b	0.16b	0.27b	0.33b	0.24b	0.45a	0.18a	0.15a	0.25b
3	0.10	0.47a	0.36a	0.41a	0.22b	0.22b	0.31b	0.30b	0.28b	0.37b	0.15b	0.20a	0.24b
5	0.17	0.50a	0.42a	0.38a	0.22b	0.27b	0.24c	0.30c	0.31b	0.28c	0.14b	0.15a	0.17c
10	0.33	0.53a	0.43a	0.39a	0.17c	0.27b	0.24c	0.24c	0.27c	0.26d	0.12b	0.16a	0.15c
15	0.50	0.52a	0.46a	0.47a	0.16c	0.24c	0.12d	0.26c	0.26c	0.19d	0.13b	0.14a	0.12c
20	0.67	0.53a	0.43a	0.44a	0.18c	0.16c	0.13d	0.25c	0.25c	0.17d	0.12b	0.13a	0.09c
30	1.00	0.53a	0.42a	0.35a	0.16c	0.16c	0.13d	0.23c	0.20c	0.14d	0.11b	0.11a	0.07c

Data are average values from two independent experiments.

Standard deviation was below 10% for all values.

Within a column, values followed by the same letter are not statistically different according to one-way ANOVA ($P < 0.05$).

Table 3.4: Normalization of succinic acid (SA), acetic acid (AA) and biomass (X) yields to formic acid for glucose, fructose and sucrose fermentation experiments in serum flasks

Sugar concentration		SA/FA (Cmol /Cmol)			X/FA (Cmol /Cmol)			AA/FA (Cmol /Cmol)		
g/L	Cmol/ L	Glu	Fru	Suc	Glu	Fru	Suc	Glu	Fru	Suc
0.5	0.02	2.5a	2.4a	1.7a	1.8a	1.4a	1.4a	2.2a	1.5a	1.7a
1	0.03	2.7a	2.1a	1.9a	1.3a	1.1a	1.1a	1.9a	1.6a	1.7a
3	0.10	3.1a	1.8a	1.7a	1.5a	1.1a	1.3a	1.9a	1.4a	1.5a
5	0.17	3.7a	2.8a	2.2a	1.6a	1.8a	1.4a	2.1a	2.0a	1.6a
10	0.33	4.5b	2.7a	2.5a	1.4a	1.7a	1.5a	2.0a	1.7a	1.7a
15	0.50	4.1b	3.2a	4.1b	1.2a	1.6a	1.0a	2.0a	1.8a	1.6a
20	0.67	4.3b	3.3a	4.8b	1.5a	1.3a	1.5a	2.0a	1.8a	1.8a
30	1.00	4.6b	3.9b	5.0b	1.4a	1.5a	1.6a	2.0a	1.8a	2.0a

Data are average values from two independent experiments.
Standard deviation was below 10% for all values.
Within a column, values followed by the same letter are not statistically different according to one-way ANOVA ($P < 0.05$).

significant for sucrose, changing from 1.7 to 5 Cmol SA/ Cmol FA when sugar concentration increases from 0.5 to 30 g/L (Table 3.4). These data clearly illustrate a change in *A. succinogenes* metabolism as sugar concentration increases, decoupling SA production from cell growth, favoring the C_4 reductive pathway and as a result, succinic acid production. It is worth noting that yeast extract and $MgCO_3$ are additional carbon sources and may be responsible for a carbon recovery over 1 (Table 3.3). A one-way ANOVA was conducted to compare the effects of higher sugar concentration on succinic acid yield by *A. succinogenes* 130Z. Normalization of SA to FA production clearly demonstrates a higher C_4 flux in *A. succinogenes* 130Z at higher sugar concentration (Table 3.4).

As a conclusion, our results show that glucose, fructose and sucrose consumption by *A. succinogenes* 130Z is not inhibited even up to 30 g/L, neither is SA production. Carob pulp water extracts may be of major interest for SA production as they contain amounts of glucose, fructose and sucrose (up to 30 g/L sugar) high enough to promote a significant SA production decoupled from cell growth without triggering any inhibition of SA production.

3.3.3 Succinic acid production from carob pulp extracts in bioreactor

To investigate in details the feasibility of carob pulp water extracts as substrate for succinic acid production by *A. succinogenes* 130Z and to understand its influence on cell growth, various independent batch cultivations were performed under anaerobic conditions in 1 L controlled benchtop reactor using carob pulp extracts as carbon sources. Extracts were added to bioreactor in order to have an initial concentration in total sugars between 5 and 30 g/L (0.15 - 1.00 Cmol/L) including

glucose, fructose and sucrose, corresponding to the concentrations tested in serum flasks. Table 3.5 summarizes the main fermentation results and kinetic parameters. *A. succinogenes* 130Z growth between 5 and 10 g/L sugar was identical to the one on pure sugars in serum flasks, increasing with sugar concentration and exhibiting a maximum specific growth rate of 0.74 (h^{-1}) at 10 g/L sugar. Glucose, fructose and sucrose were simultaneously consumed, showing no sign of diauxic growth (data not shown). However, specific sugar consumption and SA production rates were threefold lower the ones obtained on individual sugars (Table 3.5). Biomass yield on sugar was three times higher, 0.61 Cmol X/Cmol sugar and no significant changes were observed on SA yield, the later reaching 0.57 Cmol per Cmol of consumed sugar.

As sugar concentration increased from 10 to 30 g/L, specific growth rate decreased from 0.74 (h^{-1}) to 0.55 (h^{-1}). Biomass yield was also affected, decreasing from 0.61 to 0.38 Cmol X/Cmol sugar. Sugar consumption and SA production were however unaffected, with specific rates (q_S) and (q_{SA}) remaining constant at 1.22 Cmol sugar/Cmol X.h and 0.61 SA/Cmol X.h, respectively, till 30 g/L sugar. Previous experiments in serum flasks demonstrated that this decrease on biomass cannot be associated to higher sugar concentrations, as μ_{max} remained unchanged even at 30 g/L, regardless of the sugar nature (Figure 3.1). Instead, it might be related to the accumulation of organic acids in the broth, as mentioned for pure sugars. In serum flasks, 40 g/L $MgCO_3$ was used to buffer culture medium, but also to neutralize the organic acids production during fermentation [Zou et al., 2011]. In bioreactor, $NaHCO_3$ was used as buffering agent and carbon source for SA production, along with gaseous CO_2. Although the use of $NaHCO_3$ instead of $MgCO_3$ is economically beneficial, it could promote a significant accumulation of Na^+ ions in the broth in addition to NaOH solution used to neutralize pH and organic acids synthesis. Na^+ ions are known to be involved in intracellular pH regulation, their accumulation resulting in a hyperosmotic environment and subsequently damage of the cell morphology. The effect might be so severe that could actually affect cells even at an early stage of fermentation, reducing specific growth rates. In fact, the accumulation of sodium in the broth at 30 g/L sugar surpasses 0.6 M by the end of the fermentation (data not shown).

Beside, as extract concentration increased to get higher sugar concentration, the content in total phenolic compounds also increased. Water extractable polyphenols of carob pulp include several compounds, with gallic acid as the main detectable component [Avallone et al., 1997]. Several authors reported the antimicrobial effect of different phenolic compounds on bacterial strains, including Gram-positive and negative microorganisms [Campos et al., 2009, Borges et al., 2013].

Phenolic acids are thought to act both at the membrane and cytoplasmic levels. Their minimum inhibitory concentration (MIC), at which no growth is detected, varies according to the bacterial strain and phenolic compound, but the authors found that in general, Gram-positive bacteria are more resistant than Gram negative, probably associated to differences in cell membrane structure. In fact, MIC values of 1.5 mg/mL gallic acid were reported for *E. coli*, which led to irreversible changes in membrane properties (charge, intra and extracellular permeability, and physicochemical properties) through hydrophobicity changes, decrease of negative surface charge and events

Table 3.5: Fermentations results of succinic acid production by *A. succinogenes* 130Z in a roasted carob pods based medium with different sugar concentrations

Sugar concentration		Final SA	μ_{max}	q_S	q_{SA}	r_{SA}
g/L	Cmol/ L	(g/L)	(h^{-1})			(g SA/L.h)
5 ± 0.2	0.15 ± 0.01	2.28 ± 0.14	0.56 ± 0.06a	0.95 ± 0.18a	0.44 ± 0.07a	0.54 ± 0.02a
10 ± 0.5	0.27 ± 0.03	4.42 ± 0.86	0.74 ± 0.02b	1.23 ± 0.05b	0.63 ± 0.03b	0.99 ± 0.13b
15 ± 1.2	0.50 ± 0.06	7.93 ± 0.47	0.57 ± 0.00a	1.22 ± 0.02b	0.61 ± 0.04b	1.61 ± 0.01c
30 ± 1.2	1.01 ± 0.04	13.50 ± 0.33	0.55 ± 0.04a	1.19 ± 0.00b	0.61 ± 0.07b	1.56 ± 0.05c

$Y_{SA/S}$	$Y_{X/S}$	$Y_{AA/S}$	$Y_{FA/S}$	GAE (mg /mL)	Carbon balance
0.47 ± 0.05a	0.60 ± 0.09a	0.20 ± 0.02a	0.08 ± 0.02a	0.28 ± 0.13a	1.34 ± 0.1
0.57 ± 0.02b	0.61 ± 0.07a	0.25 ± 0.00b	0.12 ± 0.00b	0.63 ± 0.10b	1.55 ± 0.1
0.53 ± 0.03b	0.48 ± 0.00b	0.24 ± 0.00b	0.11 ± 0.00b	1.39 ± 0.33c	1.36 ± 0.0
0.42 ± 0.03a	0.38 ± 0.09b	0.16 ± 0.03a	0.08 ± 0.02a	2.01 ± 0.32c	1.04 ± 0.2

Data are duplicates from two independent experiments represented as mean ± standard deviation.

Within a column, values followed by the same letter are not statistically different according to one-way ANOVA ($P < 0.05$).

q_S - Specific sugar consumption rate in Cmol sugar per Cmol X.h.

q_{SA} - Specific succinic acid procution rate in Cmol SA per Cmol X.h.

$Y_{SA/S}$ - Succinic acid yield on sugar expressed as Cmol SA per Cmol of consumed sugar.

$Y_{X/S}$ - Biomass yield on sugar in Cmol of biomass per Cmol of consumed sugar.

$Y_{AA/S}$ - Acetic acid yield on sugar in Cmol of acetic acid per Cmol of consumed sugar.

$Y_{FA/S}$ - Formic acid yield on sugar in Cmol of formic acid per Cmol of consumed sugar.

Carbon balance was calculated from the ratio of (carbon out)/ (carbon in) where the formula $CH_{1.8}O_{0.5}N_{0.2}$ was used for *A. succinogenes* 130Z [McKinlay and Vieille, 2008].

GAE: gallic acid equivalents.

of local rupture in the cell membrane with consequent leakage of essential intracellular components [Borges et al., 2013]. The same effects were detected on lactic acid bacteria exposed to phenolic acids from wine [Campos et al., 2009]. Using the present substrate, concentration of total phenolic compounds reached 2 mg/mL in bioreactors containing 30 g/L sugar. Gallic acid was tested for antimicrobial action on *A. succinogenes* 130Z between 0.25 and 2 mg/mL, using cells grown without gallic acid as control group. Results showed absolute no difference on the specific growth rate of cells grown in the presence of gallic acid, for all the concentrations tested (data

not shown). These results suggest that *A. succinogenes* 130Z cells may not be sensitive for gallic acid, the main component of water carob pulp extracts, below 2 mg/mL. Moreover, the absence of changes in specific sugar consumption rate as total phenolic compounds content increase (Table 3.5) also indicates no physiological change. Succinic acid yield was highest between 10 and 15 g/L sugar and succinic acid production rate (r_{SA}) was at its maximum between 15 and 30 g/L, 1.61 g SA/L.h, suggesting 15 g/L sugar as an optimal concentration for SA production from carob pulp water extracts (Table 3.5). Jiang et al. recently reported the same production rate in *A. succinogenes* NJ113, 1.60 g SA/L.h, using 100 g/L pure sucrose as sole carbon source [Jiang et al., 2014]. Sugarcane molasses have also been used for SA production by *A. succinogenes* CGMCC1593, reaching a production rate of 1.15 g SA/L.h [Liu et al., 2008a]. However, this is the first time that SA is produced from carob pulp. Presence of phenolic compounds in the extracts did not affect SA production, demonstrating also the robustness of *A. succinogenes* as biocatalyst.

3.4 CONCLUSIONS

Carob pod extracts were successfully used for SA production by *A. succinogenes* 130Z. Glucose, fructose and sucrose consumption as sole carbon sources showed no inhibition on cell growth, sugar consumption and SA production till 30 g/L sugar. Carob extracts with 10 – 15 g/L sugar allowed a SA production of 0.61 Cmol/Cmol X.h, with a yield of 0.55 Cmol SA/Cmol sugar. Sugars were simultaneously consumed, producing 1.61 g SA/L.h, without any effect of phenolic compounds on SA production. Further improvements are needed to increase productivity and reduce costs, as increasing initial sugar concentration and removing acids from broth to overcome toxicity effects.

4 Improving succinic acid production from raw carob pods

Summary

Carob pods are an inexpensive by-product of locust bean gum industry that can be used as renewable feedstock for the bioproduction of succinic acid. In this study, unprocessed raw carob pods were, for the first time, used as substrate to produce succinic acid, after extraction of a highly enriched sugar solution. In batch experiments, sugar extracts containing 30 g/L sugars resulted in a maximum production rate of 1.67 g SA /L.h and a succinic acid yield of 0.37 Cmol SA /Cmol sugars. A strategy for increasing succinic acid yield and production rate was further developed, taking advantage of A. succinogenes' metabolism by uncoupling cell growth from succinic acid production. A fed-batch reactor operation mode was set up, resulting in a maximum succinic acid yield of 0.94 Cmol SA/Cmol sugars. The later was the highest succinic acid yield reported so far in the literature for fed-batch and continuous experiments, while maintaining production of acetic and formic acids at minimum values. These results showed that raw carob pods are a highly efficient bioresource to produce succinic acid.

The contents of this chapter were adapted from the publication: Carvalho, M., Roca, C. and Reis, M.A.M. (2015). Improving succinic acid production from raw carob pods by *A. succinogenes* 130Z (in preparation)

4.1 INTRODUCTION

Succinic acid has received a great deal of attention during the past decade as a green feedstock for the manufacture of biodegradable polymers, bioplastics and chemical intermediates [McKinlay et al., 2007b]. Although it has been listed in 2004 as one of the top 12 chemicals that can be produced from biomass by the US Department of Energy (DOE) [Werpy and Petersen, 2004], current industrial scale production is still based on the hydrogenation of maleic anhydride, a petrochemical process, due to high production costs for biobased succinic acid. The use of renewable feedstocks as carbon source for the production of succinic acid via microbial fermentation constitutes a solution to reduce production costs and develop a sustainable succinic acid production process [Zeikus et al., 1999, Beauprez et al., 2010, Cok et al., 2014]. Among the most reported industrial wastes for succinic acid production in literature are glycerol [Carvalho et al., 2014a, Vlysidis et al., 2011], from the biodiesel industry, sugar cane molasses [Liu et al., 2008a, Chan et al., 2012, Ma et al., 2014] and lignocellulosic derivatives, including straw [Zheng et al., 2009] and corn stover [Zheng et al., 2010]. Carob pods, a by-product of the locust bean gum industry, have been recently reported as a promising feedstock for succinic acid production as they enclose a highly rich sugar pulp containing water extractable sugars, namely sucrose, fructose and glucose [Carvalho et al., 2014b]. Current worlwide production of carob pods accounts for almost 400,000 tons/year, of which 50,000 tons are produced in the Algarve, Portugal, the third largest producer in the world [Santos et al., 2005]. Carob pods are therefore an inexpensive local carbon source for succinic acid production.

A. succinogenes , one of the naturally occurring succinic acid producing strains, is able to ferment a wide variety of relevant sugars including glucose, sucrose and fructose [McKinlay et al., 2010], an excellent trait for an industrial succinic acid production process based on renewable feedstocks. It has also been shown that *A. succinogenes* is capable of simultaneous consumption of sucrose, glucose and fructose, a crucial step to reduce fermentation time and increase productivity [Carvalho et al., 2014b]. In that study, a volumetric production rate of 1.61 g SA/L.h was reached by the consumption of 10-15 g/L total sugars, along with a product yield of 0.55 Cmol SA/Cmol sugars, using water extracts from commercial roasted carob flour. These results were obtained in the presence of up to 2 mg/ml of extracted phenolic compounds, resulting from the roast imposed to commercial flours [Sahin et al., 2009]. No apparent detrimental effects were observed on *A. succinogenes* , even though phenolic compounds are known for having anti-microbial activity [Henis et al., 1964]. However, using raw carob pods directly from the industry instead of the roasted flour would be the best option to develop a SA industrial process, not only reducing the energy costs associated to the pretreatment of the carbon source, but also preventing the extraction of undesirable substances such as phenolic compounds.

On the other hand, optimising carbon flux to succinic acid is an essential step to develop a commercially competitive succinic acid bioproduction process. Even though promising results were obtained from roasted carobs, almost 50% of carbon was used for biomass and by-products formation, namely in formic and acetic acids [Carvalho et al., 2014b]. One way to overcome this obstacle would be to develop a cultivation method where high concentration of succinic acid

is achieved by reducing by-products formation, improving not only the upstream but also the downstream purification steps. The use of high cell density reactor, such as membrane bioreactors, was proposed to improve the SA productivity to values as high as 10 g/L.h [Maharaj et al., 2014]. However, on an industrial scale, the use of membranes is also challenging, as they are prone to clogging as biomass accumulates inside the membrane system. This is particularly relevant with *A. succinogenes* prone to form biofilms during chemostat cultivations [Urbance et al., 2004, Maharaj et al., 2014, Brink and Nicol, 2014].

Here we report, for the first time, an efficient strategy for the bioproduction of succinic acid from raw carobs, obtained from a local industry. Sugar extraction was optimized while minimizing the amount of extracted phenolic compounds. Kinetics of sugars consumption was determined in batch experiments and compared with the results obtained with roasted carob flour, in order to assess possible effects of phenolic compounds on cell growth and succinic acid production. In order to further increase the succinic acid yield from raw carobs, fed-batch cultivation was performed. The effect of this strategy on *A. succinogenes* ' metabolism and by-products formation is further discussed.

4.2 Materials and Methods

4.2.1 Carob pods water extracts

Raw carob pods were obtained from a local factory and stored at room temperature in burlap sacks in a dry place prior to use. The pods were then chopped, deseeded and dried overnight at 70°C. The dried kibbles were milled to a fine powder, which was further used in the carob extracts. Sugars extraction was performed by suspending carob powder in distilled water at solid/liquid ratios 1:10 or 2:10 (w/v), depending on the desired sugar concentration of the extracts, and incubated at 25°C in a rotary shaker at 200 rpm for 1h [Carvalho et al., 2014b, Manso et al., 2010, Roseiro et al., 2013a]. The extracts were centrifuged at 8,000 rpm for 15 min at 4°C (Beckman J2-MC Centrifuge with a JA14 rotor) and the clarified supernatant was stored at -20°C. The yield of sugar extraction was calculated by considering that half of the carob pulp mass (50%) is composed of sugars [Avallone et al., 1997, Yousif and Alghzawi, 2000].

4.2.2 Strain and inoculum preparation

Actinobacillus succinogenes 130Z (ATCC 55618) was used in all experiments. Inoculum was prepared by incubating cells from a -80°C glycerol stock culture in rubber sealed serum flasks, containing 50 mL of culture medium with CO_2 as the gas phase. In short, one liter of culture medium contained: 5.0 g sucrose, 15.0 g yeast extract, 8.5 g $NaH_2PO_4.H_2O$, 15.5 g K_2HPO_4 and 2.1 g $NaHCO_3$. Medium was heat sterilized at 121°C for 20 min. Sucrose solution was autoclaved separately and added aseptically. After inoculation, the rubber sealed flasks were incubated at 37°C in a rotary shaker at 200 rpm for 12 - 16h, until late exponential phase was reached and confirmed by optical density measurements at 660nm.

4.2.3 Bioreactor cultivations

4.2.3.1 Batch fermentations of raw carob pod water extracts

The obtained sugar extracts were used as carbon source for succinic acid production by *A. succinogenes* 130Z. All batch experiments were performed three times, in a 1 L benchtop-bioreactor with working volume of 900 mL, sparged with CO_2 at 0.05 vvm, at 37°C and with stirring speed at 250 rpm. The pH was controlled at 6.8 by the automatic addition of 5M NaOH solution. The culture medium contained per liter: 10.0 g yeast extract, 8.5 g $NaH_2PO_4.H_2O$, 15.5 g K_2HPO_4 and 12.6 g $NaHCO_3$ [Carvalho et al., 2014b]. The carob extracts were added to the bioreactor to obtain final sugar concentrations of 5, 10, 15 and 30 g/L (0.15, 0.27, 0.5 and 1.01 Cmol/L). Samples were taken at regular intervals to quantify cell growth, sugars and acids concentration, as described in section 4.2.4. 5% (v/v) inoculum was used in all experiments.

4.2.3.2 Succinic acid production in fed-batch fermentation of raw carob pod extracts

To further increase succinic acid final titer, production rate and yield, a fed-batch experiment was performed in a 1.3 L benchtop-bioreactor with working volume of 700 mL, sparged with CO_2 at 0.05 vvm, at 37°C and with stirring speed at 250 rpm. Fermentation started with 35 g/L sugars (1.17 Cmol/L) and when biomass reached early stationary growth, the feeding-phase was initiated. An optimized feeding flow rate of 20 ml/h (1.75 g sugars/h) was used, based on sugar consumption kinetic parameters obtained for previous fed-batch experiments, maintaining a constant sugar concentration around 15 g/L inside the bioreactor. Moreover, 15 g/L yeast extract was used to ensure enough nitrogen availability for biomass growth during batch phase and maintenance processes during the feed phase.

4.2.4 Analytical methods

Fermentation products (succinic, formic and acetic acids) were analyzed by high-performance liquid chromatography (HPLC) using a Metacarb 87H column (Varian) and a refractive index detector (RI-71, Merck). The column was eluted at 30°C with 0.01 N H_2SO_4 solution at a flow rate of 0.5 mL/min. Glucose, fructose and sucrose were also analyzed by HPLC, in the same operating conditions but using a Hi-Plex H column (Agilent). Cell growth was estimated by optical density (OD) measurements at 660 nm (Spectronic Helios Alpha UV/Vis spectrophotometer, Fisher Scientific) and the biomass concentration was calculated from OD values using an experimentally determined correlation factor of 0.381 g DCW/L for an OD660 of 1.

Total nitrogen concentration was determined using commercial photochemical Hach-Lange test kits (Hach Lange DR 2800, UK).

A previously described Folin-Ciocalteau method [Carvalho et al., 2014b] was used to quantify total phenolic compounds in carob pulp extracts and bioreactor experiments. Briefly, 100 of sample (or water for blank) was mixed with 400 μL of water, 250 μL 1/1 (v/v) diluted Folin-Ciocalteau

reagent and 1.25 mL 20% Na_2CO_3. Absorbance was measured at 725 nm after 40 min of incubation in the dark. Total phenolic compounds were expressed as mg GAE (gallic acid equivalents)/mL by comparison to a gallic acid standard curve and converted to mg GAE/g carob pod. Experiments were performed in duplicate.

4.2.5 Calculation of kinetic parameters

Growth kinetics was assumed to follow a Monod growth model:

$$\mu = \mu_{max} * \frac{S}{(S + K_s)} \tag{4.1}$$

where μ is the specific growth rate (h^{-1}), μ_{max} (h^{-1}) is the maximum specific growth rate, S is the substrate concentration (g L^{-1}) and K_S is the substrate saturation constant (g L^{-1}). Specific growth rate μ (h^{-1}) was determined for each experiment by linear regression of the curve of ln ($X/X0$) versus time for the exponential growth phase (where X is the biomass at each cultivation time and X_0 at time zero, expressed in Cmol). The typical microbial composition of $CH_{1.8}.O_{0.5}N_{0.2}$ was assumed for biomass [McKinlay and Vieille, 2008]. The specific substrate consumption rate was described by the Herbert-Pirt equation, assuming that substrate is used for growth, acids production and maintenance:

$$q_S = \frac{\mu}{Y_{X/S}} + \frac{q_{SA}}{Y_{SA/S}} + \frac{q_{AA}}{Y_{AA/S}} + \frac{q_{FA}}{Y_{FA/S}} + m_S \tag{4.2}$$

q_{SA}, q_{AA} and q_{FA} are the specific production rates of succinic, acetic and formic acids, respectively, expressed in Cmol acid /Cmol X.h. Yield of biomass ($Y_{X/S}$) is expressed in Cmol X/Cmol sugar; yields of fermentation products (succinic ($Y_{SA/S}$), acetic ($Y_{AA/S}$) and formic ($Y_{FA/S}$) acids) are expressed in Cmol acid/Cmol sugar. m_S is the maintenance coefficient (Cmol sugar/Cmol X). A Leudeking-Piret model was used to calculate the specific production rate of succinic acid (q_{SA}) taking into account growth associated (α) and non-growth associated (β) terms:

$$q_{SA} = \alpha\mu + \beta \tag{4.3}$$

where α represents the yield of SA on biomass ($Y_{SA/X}$) expressed in Cmol SA/Cmol X. Succinic acid production rate (r_{SA}), expressed in g/L.h, is the maximum succinic acid production rate calculated from SA concentration vs time. Yields of fermentation products (succinic ($Y_{SA/S}$), acetic ($Y_{AA/S}$) and formic ($Y_{FA/S}$) acids) and biomass ($Y_{X/S}$) were calculated from the slope of the straight line obtained by plotting the increasing amount of product ($P - P_0$), namely succinic, acetic and formic acids and biomass ($X - X_0$) as a function of sugar consumption ($S - S_0$), respectively.

4.2.6 Mass balance equations

The dynamics of biomass (X), substrate (S) and fermentation products (P) are described by the following mass balance equations assuming a perfectly mixed stirred reactor operated in batch/fed-batch mode:

$$\frac{dX}{dt} = (\mu - D)\, X \tag{4.4}$$

$$\frac{dS}{dt} = -q_S X + D\,(S_f - S) \tag{4.5}$$

$$\frac{dP}{dt} = q_P X - D\,(P) \tag{4.6}$$

The dilution rate (D) is represented by the quotient of the volumetric feed with the culture medium volume ($D = F/V$). For batch experiments, $F = 0\ \mathrm{h}^{-1}$.

4.3 Results and discussion

4.3.1 Composition of carob pods water extracts in sugars and total phenolic compounds

In a previous study, commercial carob flour was successfully used as feedstock for succinic acid production by *A. succinogenes* [Carvalho et al., 2014b]. Sucrose, glucose and fructose from carob extracts were simultaneously consumed and the presence of phenolic compounds in the extracts till 2 mg/mL showed no effect on *A. succinogenes* growth or SA production, even though they are known for their anti-microbial properties [Henis et al., 1964]. However, the sugar extraction yield from carob flour was 50% lower than the one reported in literature for raw carobs [Manso et al., 2010]. Prior to its commercialization the carob flour is subjected to a roasting process, normally above 135ºC, which triggers chemical reactions such as Maillard reaction and sugar caramelization, decreasing the sugar content of the carobs [Yousif and Alghzawi, 2000]. Moreover, Sahin and colleagues demonstrated that exposing carob pods for at least 1 h to roasting temperatures increased the phenolic content by more than 2 fold [Sahin et al., 2009]. The hydrophylic phenolic compounds may then be simultaneously extracted with the sugars during the water extraction process, an undesirable consequence as they may have inhibitory effects on biomass [Kang et al., 2008, Campos et al., 2009, Borges et al., 2013].

From an economic point of view, costs associated to the pretreatment of the feedstock should be reduced and the yield of carbon recovery be maximized. Having this in mind, raw carob pods, obtained from a local factory, were used in the production of highly rich sugar extracts for further microbial fermentations, instead of the roasted commercial flour. Soluble sugars were extracted from the carob powder by suspending it in distilled water at two solid/liquid ratios (1:10 and 2:10 (w/v)) and incubating the shake flasks in a rotary shaker at 25ºC for 1h, as described in Carvalho et al. [Carvalho et al., 2014b].The resulting extracts presented a much higher amount of soluble sugars compared to the extracts obtained with roasted commercial flour (58%), with sugars

Table 4.1: Sugar and total phenolic compounds content of raw carob pod extracts with different solid/liquid ratios, at 25°C for 1h

Ratio (w/v)	Sucrose (g/ L)	Glucose (g/ L)	Fructose (g/ L)	Total sugars (g/ L)	Yield sugar extraction (%)	GAE (mg/mL)	mg GAE/ g dry mass
1:10	33.82 ± 0.5	10.36 ± 0.1	5.48 ± 0.2	49.65 ± 0.2	99.31 ± 0.3	0.212 ± 0.0	1.57 ± 0.2
2:10	62.54 ± 0.7	19.38 ± 0.7	10.18 ± 0.6	92.11 ± 0.5	92.11 ± 0.5	0.348 ± 0.0	1.05 ± 0.1

Data are duplicates from two independent experiments represented as mean ± standard deviation.

Yield of sugar extraction (%) = total sugar concentration (g/L)/ theoretical sugar concentration, i.e. half of the carob pulp mass (g/L), multiplied by 100.

GAE: gallic acid equivalents

concentration ranging from 49.7 to 92.11 g/L, depending on the solid/liquid ratio (Table 4.1).

The extraction yield of sugars was above 92% for both solid/liquid ratios and reaching almost 100% sugar recovery at 1:10, demonstrating the feasibility and sustainability of this extraction process. Similar results are also reported in the literature, reinforcing the robustness of this process [Avallone et al., 1997, Manso et al., 2010]. The concentration of extracted phenolic compounds was below 0.4 mg/mL for both extracts, ranging between 1 and 1.5 mg of GAE/ g carob powder (Table 4.1). These values were significantly lower than the ones previously reported with carob flour from commercial use, where 13.12 mg of GAE/ g carob flour were extracted in the same conditions [Carvalho et al., 2014b]. The results here obtained show that using raw carobs resulted in highly rich sugar extracts while minimizing the recovery of phenolic compounds, being the best option for a succinic acid bioproduction process.

4.3.2 Batch fermentations of raw carob pods extracts

The sugar extracts obtained from raw carob pods were used as carbon source for succinic acid production by *A. succinogenes* . Batch cultivations were performed under anaerobic conditions in 1L controlled benchtop reactor supplemented with the extracts in order to have an initial concentration in total sugars between 5 and 30 g/L (0.15 to 1 Cmol/L) including glucose, fructose and sucrose. Figure 4.1 depicts the fermentation profiles of sugars (A), biomass (B) and organic acids (C, D and E), for the different sugar concentrations, over cultivation time. Table 4.2 summarizes the main fermentation results and kinetic parameters.

The extracted sugars from raw carob pods were totally consumed by *A. succinogenes* in less than 12 hours (Figure 4.1 A), producing succinic, acetic and formic acids (Figures 4.1 C, D and E). Cell growth slightly increased with sugar concentration, reaching a specific growth rate (μ) of 0.67 h^{-1} at 30 g/L sugars (1 Cmol/L). Sucrose, glucose and fructose were simultaneously consumed (data not shown), showing no sign of diauxic growth. The specific sugar consumption rate (q_S) and succinic acid production rate (q_{SA}) were identical independently of the sugar concentration tested,

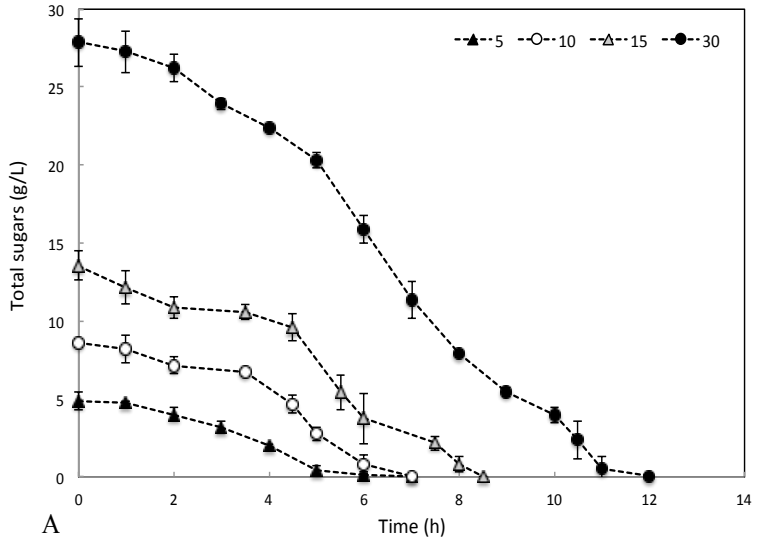
with average values of 1.17 Cmol sugars/ Cmol X.h and 0.56 Cmol SA/Cmol X.h, respectively (Table 4.2). These results show there is no significant dependency (inhibition or limitation) on substrate from 5 to 30 g/L sugars.

Interestingly, very similar values of specific cell growth, sugar consumption and SA production rates were obtained in our previous study while using extracts from roasted carobs, in the presence of a 2 to 10 fold higher concentration of phenolic compounds [Carvalho et al., 2014b]. It is known that phenolic compounds may act as anti-microbial agents by disrupting membrane permeability and inhibiting cell growth [Papadopoulou et al., 2005, Campos et al., 2009]. However, by the results here obtained we can conclude that water extracted phenolic compounds from carob pods had absolute no effect on *A. succinogenes* metabolism, in concentrations up to 2 mg/mL, reinforcing the robustness of this strain as an industrial biocatalyst for SA production using feedstock rich in phenolic compounds.

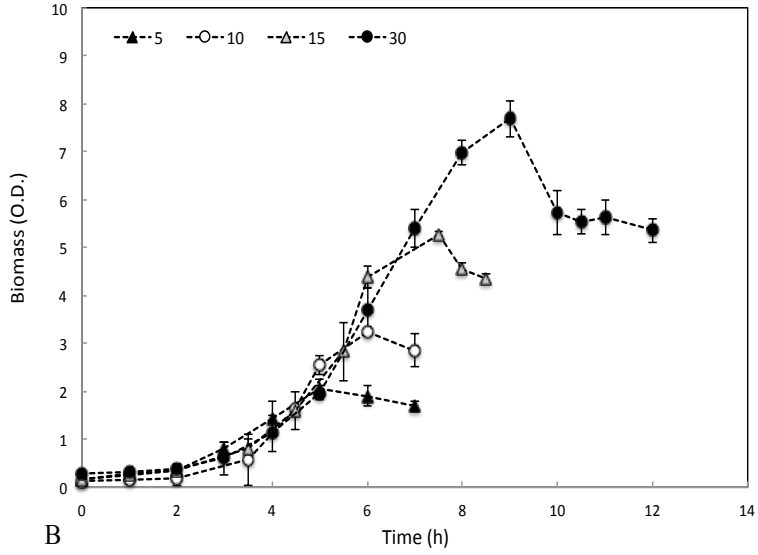
As depicted in Figures 4.1 B-E, biomass concentration increased with sugar concentration from 5 to 30 g/L, so as succinic, formic and acetic acids. The yields of biomass and acids formed were however found to decrease with higher sugar concentrations (Table 4.2). However, it is worth noting that 10 g/L of yeast extract was used in these experiments and that additional carbon sources other than the supplied sugars may be present in the culture medium, resulting in carbon balances for each condition significantly higher than 1. For sugar concentration of 30 g/L, the carbon balance value gets however very close to 1 (1.07 ± 0.03). Indeed, the fraction of additional carbon supplied by yeast extract is reduced as the concentration of the sugar carbon source becomes higher. Besides, even biomass yield and products yield calculations are affected by the presence of yeast extract, this does not have any effect on the stoichiometry of SA/AA and FA/AA: for each mole of succinic acid that is produced, one mole of each acetic acid and formic acid are formed, independently of the sugar concentration tested (Table 4.2). These data suggest a constant metabolic flux distribution within the cell in the presence of up to 30 g/L sugars.

By the end of fermentations with 30 g/L sugars (1Cmol/L) it is however worth noting a sudden change in by-products and biomass formation, in particular after 9 hours of experiment: while biomass concentration decreases by 25%, (Figure 4.1 B), succinic acid production carries on, only stopping when carbon is totally exhausted (Figure 4.1 C). Acetic and formic acids production also diminished (Figure 4.1 D and E, respectively). One hypothesis to explain this metabolic change would be to assume there is a nitrogen limitation, causing the biomass production to cease, redirecting the NADH coming from the substrate catabolism for the reduction of malate to succinate along the C_4 pathway of the TCA cycle, explaining the succinic acid production (Figure 4.2). However, there is still 275 mg/L total nitrogen available by the end of the experiments (data not shown). As such, another factor influencing cell growth other than nitrogen limitation may have occurred.

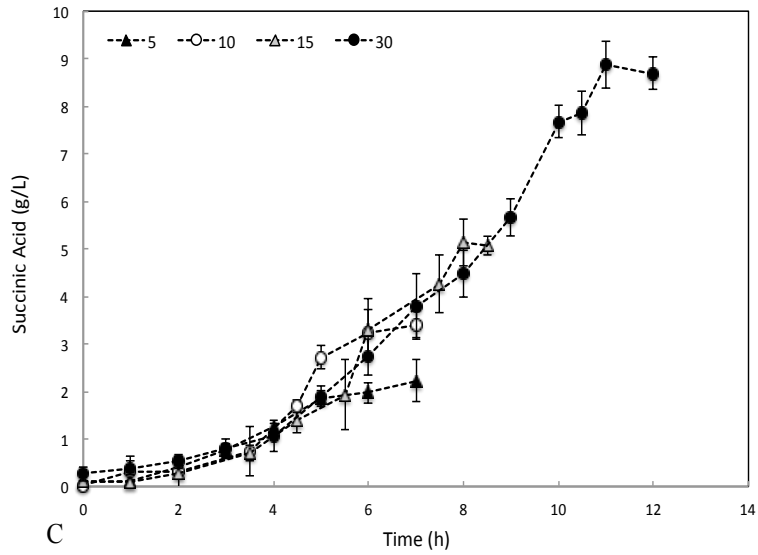
The effect of organic acids accumulation on the growth of *A. succinogenes* , in particular succinic, formic and acetic acids has been studied in details [Lin et al., 2008]. Formic and acetic acids exhibit the stronger inhibition effect. In fact, 5 g/L of formic acid decreased cell growth by 30% [Lin et al., 2008]. A maximum biomass formation was also reported in batch experiments in the presence of 22 g/L of produced acids, after which the biomass concentration started to


5
10
15
30
Total sugars (g/L)
Time (h)

A


5
10
15
30
Biomass (O.D.)
Time (h)

B


5
10
15
30
Succinic Acid (g/L)
Time (h)

C

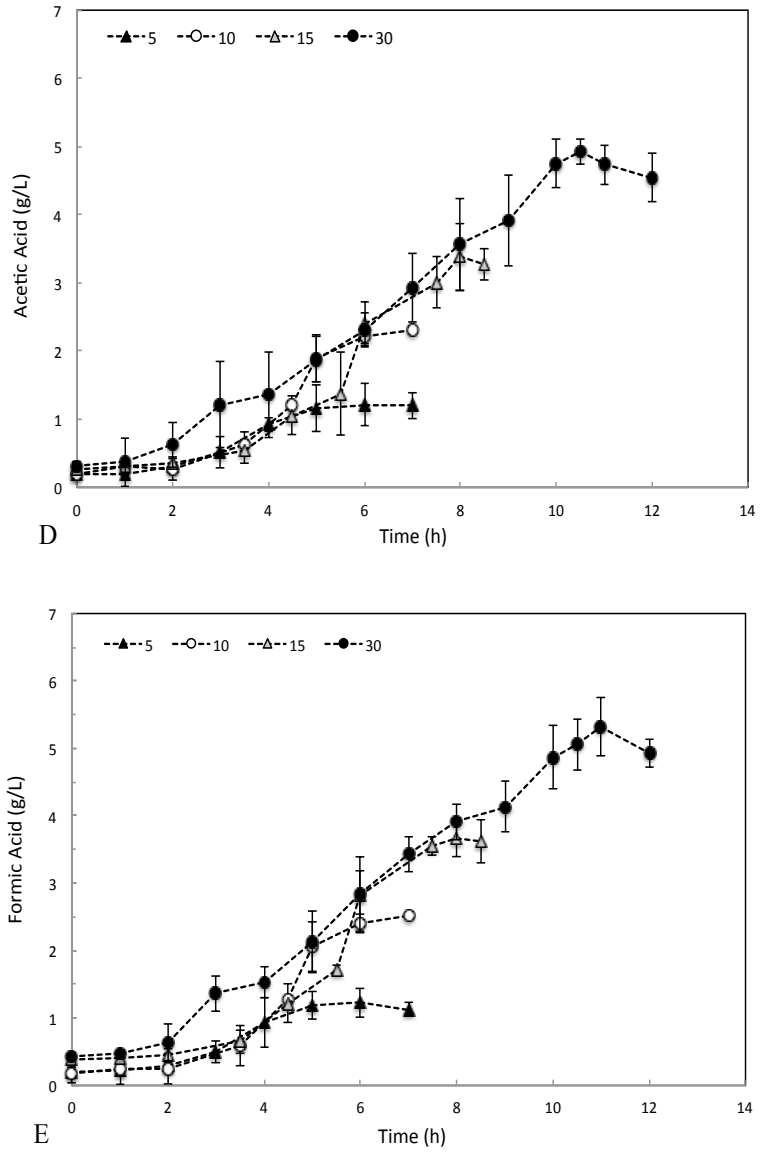


Figure 4.1: Fermentation profiles of succinic acid production by *A. succinogenes* in a raw carob pod extract based medium. Fermentations started with different initial sugar concentration, ranging from 5 to 30 g/L total sugars. Data are average values for two independent experiments for each sugar concentration. A) Total sugars, B) Biomass, C) Succinic acid, D) Acetic acid, E) Formic acid.

Table 4.2: Fermentation results of succinic acid production by *A. succinogenes* in a raw carob based medium with different initial sugar concentrations

Sugar concentration		Final SA	μ	q_{SA}	q_S	r_{SA}
g/L	Cmol/ L	(g/L)	(h^{-1})			(g SA/L.h)
5 ± 0.2	0.15 ± 0.01	2.11 ± 0.3	0.55 ± 0.01	0.52 ± 0.05	1.06 ± 0.05	0.51 ± 0.04
10 ± 0.5	0.27 ± 0.03	3.38 ± 0.03	0.56 ± 0.05	0.54 ± 0.01	1.21 ± 0.06	1.08 ± 0.07
15 ± 1.2	0.50 ± 0.06	5.37 ± 0.4	0.61 ± 0.05	0.55 ± 0.05	1.06 ± 0.13	1.01 ± 0.01
30 ± 1.2	1.01 ± 0.04	9.04 ± 0.5	0.67 ± 0.02	0.64 ± 0.1	1.35 ± 0.02	1.67 ± 0.02

$Y_{SA/S}$	$Y_{X/S}$	$Y_{AA/S}$	$Y_{FA/S}$	Carbon balance	GAE (mg/ml)	SA/AA	FA/AA
0.44 ± 0.05	0.50 ± 0.03	0.25 ± 0.03	0.20 ± 0.03	1.39 ± 0.04	0.18 ± 0.05	1.02 ±0.05	0.93 ± 0.08
0.45 ± 0.04	0.48 ± 0.07	0.27 ± 0.03	0.20 ± 0.03	1.40 ± 0.04	0.23 ± 0.05	0.84 ±0.11	1.07 ± 0.09
0.48 ± 0.09	0.50 ± 0.06	0.26 ± 0.02	0.19 ± 0.01	1.42 ± 0.05	0.24 ± 0.05	0.92 ± 0.08	1.06 ± 0.03
0.37 ± 0.05	0.41 ± 0.05	0.17 ± 0.02	0.12 ± 0.02	1.07 ± 0.03	0.19 ± 0.05	1.10 ± 0.06	1.23 ± 0.02

Data are duplicates from three independent experiments represented as mean ± standard deviation.

q_{SA} - Specific succinic acid procution rate (Cmol SA per Cmol X.h)

q_S - Specific sugar consumption rate (Cmol sugar per Cmol X.h)

r_{SA} - Succinic acid production rate (g /L.h)

$Y_{SA/S}$ - Succinic acid yield on sugar (Cmol SA per Cmol of consumed sugar)

$Y_{X/S}$ - Biomass yield on sugar (Cmol of biomass per Cmol of consumed sugar)

$Y_{AA/S}$ - Acetic acid yield on sugar (Cmol of acetic acid per Cmol of consumed sugar)

$Y_{FA/S}$ - Formic acid yield on sugar (Cmol of formic acid per Cmol of consumed sugar)

Carbon balance was calculated from the ratio of (carbon out)/ (carbon in) where the formula $CH_{1.8}O_{0.5}N_{0.2}$ was used for *A. succinogenes* 130Z [McKinlay and Vieille, 2008].

SA/AA - Stoichiometry of produced succinic acid to acetic acid (mol per mol)

FA/AA - Stoichiometry of produced formic acid to acetic acid (mol per mol)

GAE: gallic acid equivalents.

decrease [Corona-González et al., 2008]. Our results are in agreement with these data, as organic acids concentration was over 15 g/L showed inhibitory effect on cell growth.

Despite the effect of acids inhibition on biomass growth, the production rate of SA increased with the sugar concentration, as a result of a higher amount of biomass inside the bioreactor, reaching a volumetric productivity of 1.67 g SA/L.h at 30 g/L of sugars (Table 4.2). These are similar values to what was reported using pure sucrose, 1.60 g SA/L.h [Jiang et al., 2014] and roasted carobs 1.61 g SA/L.h [Carvalho et al., 2014b], but higher than what was obtained with sugarcane molasses, 0.97 g SA/L.h [Liu et al., 2008b], reinforcing the potential of carob pods as feedstock for SA production. Still, there is a large portion of carbon that is being directed to by-products formation. Optimising carbon flux to succinic acid is an essential step to develop a commercially competitive bioproduction process.

4.3.3 Improving succinic acid production from carob extracts in a fed-batch experiment

In order to increase final succinic acid concentration, yield and production rate from carob extracts, a fed-batch culture was performed, having in mind the uncoupling of cell growth of *A. succinogenes* from succinic acid production. Fermentation started with 35 g/L (1.17 Cmol/L) sugars and the feeding-phase was initiated when biomass reached early stationary growth (Figure 4.3). An optimized feeding flow rate of 20 ml/h was used (1.75 g sugars/h), based on sugar consumption rates obtained for previous fed-batch experiments (data not shown), maintaining a constant sugar concentration around 15 g/L inside the bioreactor. Moreover, 15 g/L yeast extract was used to ensure enough nitrogen availability for biomass growth during batch phase and maintenance processes during the feed phase.

During the batch phase, as depicted in Figure 4.3, *A. succinogenes* consumed approximately 20 g/L sugars in 10 hours, producing succinic, formic and acetics acids, along with biomass. As expected, specific growth rate was identical to the previous batch experiments (0.64 h−1), as well as sugar consumption and SA production rates, reaching 1.23 Cmol sugars/Cmol X.h and 0.66 Cmol SA/Cmol X.h, respectively (Table 4.3). A succinic acid yield of 0.53 Cmol SA/Cmol sugar was obtained, slightly higher than for batch experiments, probably owing to a lower biomass formation (0.30 Cmol X/Cmol sugar), as acetic and formic acid yields remained in the same range of values previously obtained. In fact, cell growth stopped around 13h of fermentation, while there was still 500 mg/L of total nitrogen left, suggesting there was no nitrogen limitation at that point. The organic acids concentration was already 20 g/L, which might have promoted sooner the inhibition of cell growth by acids accumulation, resulting in a decrease of biomass yield. The SA production rate was also identical to the previous experiments, at 1.32 g/L.h (Table 4.3).

During the feed phase, succinic acid yield was dramatically increased, reaching 0.94 Cmol SA/Cmol sugars (0.93 g SA /g sugars), while formic and acetic acids formation was practically arrested (Table 4.3). The specific sugar consumption (q_S) and succinic acid production (q_{SA}) rates were identical, 0.66 Cmol sugars/Cmol X.h and 0.67 Cmol SA/Cmol X.h (Table 4.3), suggesting that all consumed sugar was being directed to succinic acid production. In fact, succinic

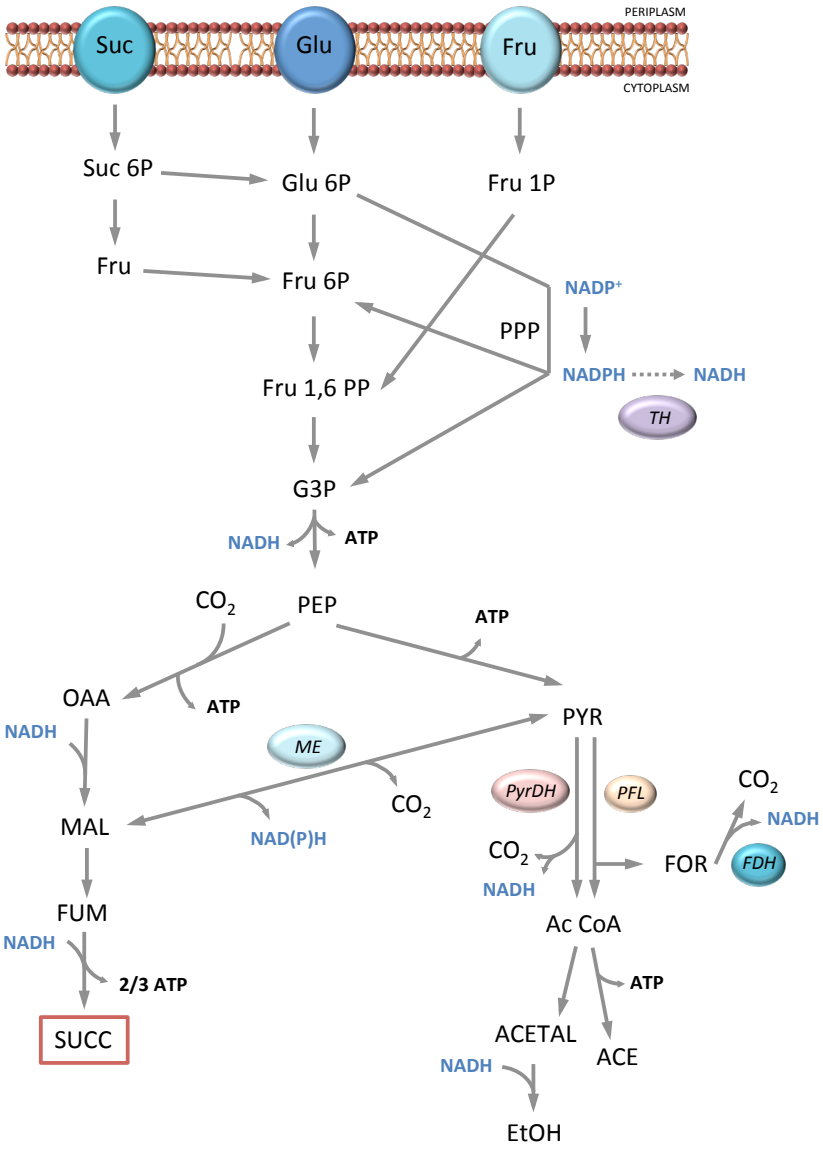


Figure 4.2: *A. succinogenes* metabolic pathways adressed in this chapter. Suc, Glu and Fru: sucrose, glucose and fructose transporters. ME: malic enzyme, PyrDH: pyruvate dehydrogenase, PFL: pyruvate formate lyase, FDH: formate dehydrogenase, TH: transhydrogenase. PPP: Penthose Phosphate Pathway. Metabolites: Suc6P: sucrose-6-phosphate, Fru: fructose, Glu6P: glucose-6-phosphate, Fru6P: fructose-6-phosphate, Fru1P: fructose-1-phosphate, Fru1,6PP: fructose-1,6-biphosphate, G3P: glyceraldehyde-3-phosphate, PEP: phosphoenolpyruvate, OAA: oxaloacetate, Mal: malic acid, Fum: fumaric acid, Succ: succinic acid, PYR: pyruvate, For: formic acid, Ac-CoA: acetyl coenzyme-A, ACETAL: acetaldehyde, ACE: acetic acid, EtOH: ethanol.

acid is formed in the C_4 pathway of *A. succinogenes* ' central metabolism, via carboxylation of phosphoenolpyruvate and consumption of NADH along the reductive branch of the TCA cycle (Figure 4.2). Acetic and formic acids are formed in the C_3 pathway where pyruvate conversion is mediated by two key enzymes: the pyruvate dehydrogenase (PyrDH) pathway or the pyruvate formate-lyase (PFL) pathway. The C_3 and C_4 pathways are connected by two additional reactions: the interconversion of malate and pyruvate by NADP-dependent malic enzyme and oxaloacetate decarboxylation to pyruvate by the OAA decarboxylase (Figure 4.2).

Table 4.3: Kinetic parameters of succinic acid production by *A. succinogenes* in a fed-batch experiment using raw carob pods as carbon source, with 15 g/L of yeast extract

	μ (h^{-1})	q_{SA}	q_S	r_{SA}	$Y_{SA/S}$	$Y_{X/S}$	$Y_{AA/S}$	$Y_{FA/S}$	SA/AA	FA/AA
Batch	0.64	0.66	1.23	1.32	0.53	0.30	0.19	0.11	1.18	1.19
Fed-batch	-	0.67	0.68	1.43	0.94	-	0.05	0.02	2.05	1.02

q_{SA} - Specific succinic acid procution rate (Cmol SA per Cmol X.h)

q_S - Specific sugar consumption rate (Cmol sugar per Cmol X.h)

r_{SA} - Succinic acid production rate (g /L.h)

$Y_{SA/S}$ - Succinic acid yield on sugar (Cmol SA per Cmol of consumed sugar)

$Y_{X/S}$ - Biomass yield on sugar (Cmol of biomass per Cmol of consumed sugar)

$Y_{AA/S}$ - Acetic acid yield on sugar (Cmol of acetic acid per Cmol of consumed sugar)

$Y_{FA/S}$ - Formic acid yield on sugar (Cmol of formic acid per Cmol of consumed sugar)

SA/AA - Stoichiometry of succinic acid to acetic acid (mol per mol)

FA/AA - Stoichiometry of formic acid to acetic acid (mol per mol)

In a recent report, Bradfield and Nicol estimated maximum yield values for succinic acid production and by-products formation according to the different metabolic pathways inside the cell [Bradfield and Nicol, 2014]. In that study, a maximum succinic acid yield of 1.12 g SA/g glucose was estimated in the event of no by-products and biomass formation, where the required reducing power for succinic acid production would be generated in an alternative pathway, eliminating the need for the oxidative C_3 flux to satisfy the redox balance (Figure 4.2). The production of acetic and formic acids during the fed-batch phase only accounts for 4% of the total carbon.

The reducing power necessary for a succinic acid yield of 96% would come from an alternative pathway. It can be hypothesized that malate is converted into pyruvate by the NADP-depent malic enzyme (Figure 4.2), producing NADPH and CO_2. However, given the reduced formation of acetic and formic acids, this hypothesis is unlikely to be taking place. Besides, McKinlay and Vieille demonstrated an increase of the C_4 decarboxilating reverse flux from pyruvate to malate in the presence of high $NaHCO_3$ concentrations (100mM). In the present study, 150mM $NaHCO_3$ were used, providing enough CO_2 availability to increase the C_4 reductive flux [McKinlay et al.,

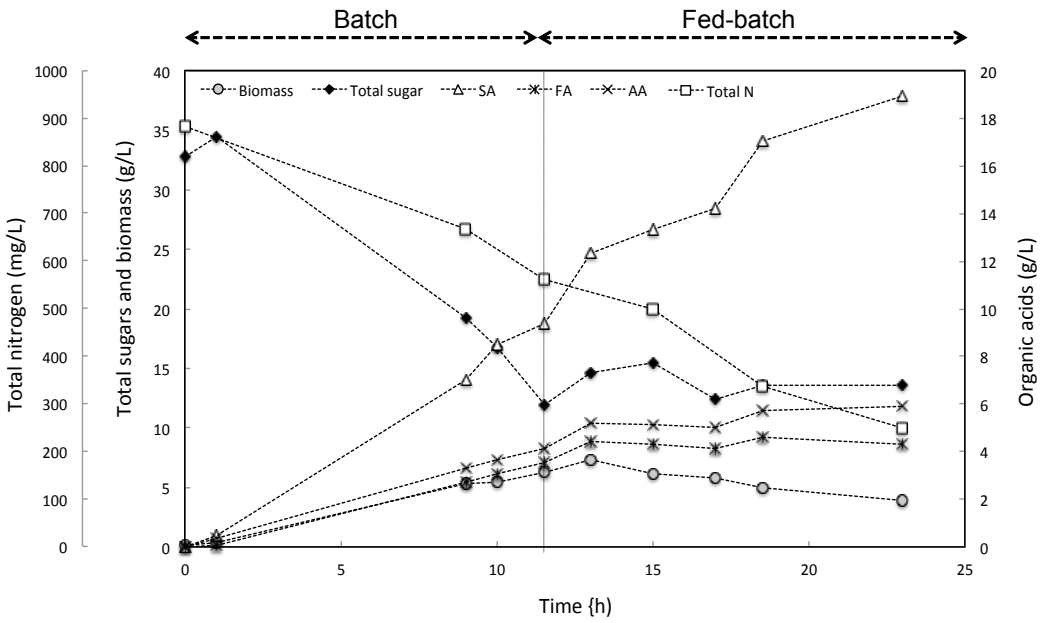


Figure 4.3: Fermentation profile of succinic acid production of in a raw carob based medium by *A. succinogenes* with 15 g/L yeast extract

2007a]. The most likely hypothesis is that the reducing power that is generated by sugar catabolism and would be used for biomass formation was now channeled into succinic acid production. In fact, the pentose phosphate pathway (PPP) generates NADPH by a NADH dehydrogenase, which would then be consumed in anabolic processes such as biomass formation [Nielsen et al., 2003]. However, in the absence of cell growth, the excess NADPH could be reoxidized to NADH by a membrane bound-transhydrogenase (Figure 4.2). This hypothesis was shown to occur in resting cells of *Bacillus subtilis*, who overproduce NADPH as a result of sugar catabolism [Rühl et al., 2012]. Moreover, McKinlay et al. identified the transhydrogenase gene in the *A. succinogenes* ' genome, along with enzyme activity in cell extracts [McKinlay et al., 2007a, McKinlay et al., 2010].

A specific SA production rate of 0.67 Cmol SA/Cmol X.h (0.791 g SA/g X.h) was obtained during the feed phase, identical to what was reported for the batch phase (Table 4.1). This value, however, corresponds only to the non-growth associated term (β) of the Luedeking-Piret model (Equation 4.3), as cell growth has already been arrested (Figure 4.3). Succinic acid is produced both during the growth and the non-growth phase of *A. succinogenes* , being classified as a metabolic by-product. For products coupled to the generation of ATP, which is the case, the rate of production is related to cellular energy demand. Growth is usually the major energy-requiring function of cells. As such, the production of SA is coupled to energy metabolism and it will be produced during cell growth. However, being a metabolic by-product, it will also be produced after cell growth arrest as a result of maintenance processes. Lin et al estimated a value for non-growth associated productivity, 0.299 g SA/g X.h, for the consumption of pure glucose [Lin et al., 2008]. However, this value was obtained for batch experiments, when organic acids other than SA are being produced, besides biomass. Here, the value was estimated for the feed phase, when all carbon that is being consumed is diverted to SA production.

Currently, only two similar studies to this have been reported in literature. A succinic acid yield of 83% (g/g) was obtained on pure sucrose, in a fed-batch experiment performed using yeast extract and corn steep liquor as nitrogen sources [Jiang et al., 2014]. A production rate of 2.16 g SA/L.h was obtained, associated to the high amount of biomass generated in the batch phase, together with 60 g/L of succinic acid after 28h of fermentation. Sugar cane molasses were also used in a fed-batch experiment for succinic acid production with *A. succinogenes* CGMCC1593 [Liu et al., 2008b]. A similar feeding strategy to the one here described was used, starting with a batch phase with 35 g/L sugars, after which the sugar concentration was maintained at 10-15 g/L by adding feeding solution at a constant flow rate. A volumetric productivity of 1.15 g SA/L.h was obtained, identical to what was here obtained (1.43 g SA/L.h), but no succinic acid yield was reported. It must be pointed out, however, that $MgCO_3$ was used as CO_2 donor, which is known to alleviate the detrimental effect of organic acids on biomass [Zou et al., 2011]. This might explain the constant biomass concentration maintained throughout the fermentation and the absence of cell death, even when the concentration of acids was over 60 g/L. The use of $MgCO_3$ can be regarded as a solution to maintain the viability of biomass in the presence of organic acids during long periods of time, allowing a constant productivity and high SA final titer and yield.

4.4 CONCLUSIONS

This study shows that raw carob pods are suitable carbon source for succinic acid production by *A. succinogenes* : they require no pretreatment and the sugar content of the carobs allow a yield of sugar recovery of almost 100% of the theoretical value, 50% (w/w). *A. succinogenes* has a versatile metabolism, which adjusts to the environmental conditions and allows the production of high succinic acid yield. Besides, when cell growth stops, the generated reducing power is redirected to succinic acid formation. A succinic acid yield of 0.94 Cmol SA/Cmol sugars (0.93 g SA/g sugars) was obtained in a fed-batch experiment, the highest succinic acid yield reported in the literature for fed-batch and continuous experiments. This is a very promising result from an industrial perspective, as it significantly reduces the production costs associated to the purification steps, helping to develop a more sustainable and cost-effective bio-succinic acid production process.

5 Conclusions and Future Work

Summary *This chapter presents some of the main overall conclusions that resulted from this thesis. In addition, some aspects that could be addressed in the future are discussed.*

The work performed during this Ph.D. thesis allowed to get a better understanding of *A. succinogenes* metabolism, evaluating its capacity of using a wide range of substrates and producing significant amounts of succinic acid. Several scientific contributions were provided constituting a step forward towards the development of a more sustainable and cost-effective bio-succinic acid production process.

The production of succinic acid by the strain *A. succinogenes* 130Z from glycerol was investigated and significantly improved by the addition of an external electron acceptor, DMSO. Without electron acceptor, *A. succinogenes* is unable to use glycerol anaerobically. However, concentrations of DMSO between 1 and 4% (v/v) promoted glycerol consumption and succinic acid production. Production of acetic and formic acids remained residual as glycerol metabolism results in the maintenance of a tight redox balance, turning the production of by-products unnecessary. Batch experiments resulted in the highest value reported so far in the literature for *A. succinogenes* using glycerol as carbon source.

By the addition of electron acceptor, more than 80% of glycerol was converted to succinic acid, opening new perspectives on the use of this feedstock for the production of high value products. Complementary to this study, the incorporation of crude glycerol from the biodiesel industry for succinic acid production from *A. succinogenes* should be considered. In fact, crude glycerol from biodiesel production contains more than 20% (wt) methanol, making it unsuitable for most traditional glycerol markets, together with a high content of salts and free fatty acids [Ciriminna et al., 2014]. Even though the biodiesel industry is experiencing a 10% reduction in its production worldwide, as a consequence of lower output in the US, Argentina and Indonesia, more than 2 million tonnes are still being generated annually. Recent market analysis estimates that demand for glycerine by-product of oleochemicals and biodiesel production will expand at an annual average rate of 7% between 2007–2021, with a 6 million tonnes overall production in 2025 [Ciriminna et al., 2014]. As such, glycerol is still expected to be used as raw material for the production of value added chemicals during the forthcoming years. Additionally, the use of an alternative source of DMSO as a co-substrate for biobased succinic acid production from glycerol should also be assessed in future works. Indeed, pure DMSO was merely used as a proof of concept but the cost efficiency at industrial scale needs to be evaluated . By-products of kraft paper process for instance where DMSO is extracted from, could be an interesting alternative. In conclusion, the use of both glycerol from biodiesel industry and industrial by-products containing DMSO will definitively decrease the cost of bio-succinic acid production.

In addition to glycerol, carob pods extracts were also successfully used for succinic acid production by *A. succinogenes* 130Z. Sucrose, glucose and fructose were easily extracted from raw carob pods through a simple water extraction step, demonstrating the feasibility and sustainability of this extraction process. Roasted carob flour was also used to obtain sugar extracts. However, the roasting imposed to carob pods prior to its commercialization significantly reduces its sugar content and increases the amount of undesirable compounds such as polyphenolic compounds.

Raw carob pods are therefore the best option as substrate for biological fermentations.

Glucose, fructose and sucrose were simultaneously consumed by *A. succinogenes* , producing succinic acid, with acetic and formic acids as by-products. The results obtained for fermentations using extracts from raw and roasted carobs were very similar, showing that *A. succinogenes* is an extremely suitable strain for succinic acid production, even in the presence of polyphenolic compounds, known for their anti-microbial properties. By operating the reactor in a fed-batch mode resulted in the highest succinic acid yield reported so far in the literature with *A. succinogenes* .

This Ph.D. work clearly demonstrated that *A. succinogenes* has a versatile and dynamic metabolism, which adjusts itself to the environmental conditions. This phenomenon allows the production of high succinic acid yields by decoupling succinic acid production from cell growth in the presence of low amounts of acetic and formic acids. This is a very promising result from an industrial perspective, as it significantly reduces the production costs associated to the purification steps.

Further optimization of the overall production process with both substrates, glycerol and carob extracts, should involve the replacement of yeast extract as nitrogen source. Corn steep liquor and spent yeast cells, industrial residues, have already been tested as nitrogen sources for succinic acid production by *A. succinogenes* with positive outcomes. This step would significantly help to decrease the cost of succinic acid fermentation, associated to substrate high costs. Moreover, given the ability of *A. succinogenes* to produce succinic acid even in the presence of high concentration of organic acids, $MgCO_3$ should be regarded as inorganic carbon source, as it is known for its buffering capacities, alleviating the effect of acids inhibition on *A. succinogenes* biomass and thereby increasing the succinic acid productivity.

The development of more efficient and cost-effective separation processes for the downstream of succinic acid production remain an issue to be solved. Even though high yield and productivity are obtained, the presence of by-products and other fermentation contaminants influences the quality of the final product and its future utilisation. Work still has to be performed to obtain an efficient purification process: membrane based technology might be a solution as it may significantly reduce the amount of generated wastes and can be coupled to the fermentation bioreactor to alleviate product inhibition and achieve high cell density.

In conclusion, the work here presented certainly helped in developing a more sustainable and cost-effective bio-succinic acid process and opened the way for further studies. This was probably the first study made in Portugal, demonstrating that succinic acid could be easily integrated in the product portfolio of emerging national biorefineries.

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