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Almqvist, Henrik

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Dicarboxylic acids from xylose, using natural and engineered hosts

DOCTORAL DISSERTATION
2018

Henrik Almqvist

Department of Chemical Engineering
Lund University, Sweden

Doctoral dissertation which, by due permision of the Faculty of Engineering of Lund University, will be publicly defended on Friday the 12th of January 2018 at 09:00 in lecture hall K:B at Kemicentrum, Naturvetarvägen 14, Lund, for the degree of Doctor of Philosophy in Engineering

Faculty opponent is Prof. Willie Nicol, Department of Chemical Engineering, University of Pretoria, South Africa.

Title and subtitle
Dicarboxylic acids from xylose, using natural and engineered hosts:

Abstract

Chemical building blocks for plastics can be produced from renewable biomass feedstocks using microbial production organisms, such as yeast or bacteria, in a biorefinery. One class of chemical building blocks that are suitable for production of biobased and biodegradable plastics are dicarboxylic acids, e.g. succinic acid. In order to avoid competition with food and feed production it is desirable to use hydrolysates of lignocellulosic feedstocks which often not only contain hexose sugars but also pentoses, out of which xylose is the most common. One example of such a feedstock is spent sulphite liquor (SSL), a side stream from sulphite pulping of Eucalyptus, which is rich in xylose. In this thesis, microbial production of dicarboxylic acids from xylose-rich feedstocks has been studied using different host organisms.

The natural succinic acid producing bacterium *Actinobacillus succinogenes* was found able to produce succinate from a xylose rich synthetic model medium mimicking sugar composition in SSL, at a titer of 31 g L^{-1} and yield of 0.71 g g^{-1}. In addition, *A. succinogenes* was tested for tolerance towards inhibiting by-products along with a related succinate producer, *Basfia succiniciproducens*. Of the by-products, both organisms were found to be most sensitive to formate (18-22 g L^{-1}), while high concentration of acetate (38 g/L^{-1}) and succinate (55 g L^{-1}) were tolerated. Succinate production with *A. succinogenes* was also tested in SSL, and titers above 22 g L^{-1} of succinate were obtained in fed-batch cultivations.

A strain of *Saccharomyces cerevisiae* engineered for xylose utilization and formation of dicarboxylic acids was assessed and found rather tolerant to SSL even at acidic conditions. The relative distribution between malate and succinate was affected by cultivation conditions, with succinate strongly favoured at carboxylating conditions at high pH.

Genes encoding enzymes of the Weimberg pathway, an orthogonal xylose degradation pathway, were introduced in *S. cerevisiae*. The complete pathway was not functional and growth on xylose was not obtained. However, the intermediate compound xylonate was formed at close to stoichiometric yields. In addition, *Caulobacter crescentus*, the natural host of the Weimberg pathway, was characterised. Activity of the Weimberg pathway was found during growth on both xylose and arabinose, but not on glucose. Interestingly, high yields of α-ketoglutarate (up to 0.43 g g^{-1}) were formed during growth on xylose.

Key words
Xylose, dicarboxylic acids, biorefineries, *Actinobacillus succinogenes*, *Basfia succiniciproducens*, *Saccharomyces cerevisiae*, *Caulobacter crescentus*

Classification system and/or index terms (if any)

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Cover illustration front: "The Forest Biorefinery" by Henrik Almqvist

"Science is magic that works"

– Kurt Vonnegut, Cat's Cradle

Abstract

Chemical building blocks for plastics can be produced from renewable biomass feedstocks using microbial production organisms, such as yeast or bacteria, in a biorefinery. One class of chemical building blocks that are suitable for production of biobased and biodegradable plastics are dicarboxylic acids, e.g. succinic acid. In order to avoid competition with food and feed production it is desirable to use hydrolysates of lignocellulosic feedstocks which often not only contain hexose sugars but also pentoses, out of which xylose is the most common. One example of such a feedstock is spent sulphite liquor (SSL), a side stream from sulphite pulping of Eucalyptus, which is rich in xylose. In this thesis, microbial production of dicarboxylic acids from xylose-rich feedstocks has been studied using different host organisms.

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Populärvetenskaplig sammanfattning på svenska

Forskningen som presenteras i denna avhandling handlar om mikroorganismer som kan användas i bioraffinaderier för att producera kemikalier, som i sin tur kan användas för att tillverka plast. Vad är då mikroorganismer och bioraffinaderier, och framförallt vad ska de vara bra för? Vårt moderna samhälle innebär stora risker för miljön. Att utsläpp av växhusgaser påverkar klimatet har det rapporterats om under en längre tid. Under de senaste åren har det dessutom rapporterats att våra sjöar och hav fylls med plast som inte bryts ner. En gemensam nämnare för dessa problem är olja. Mycket av vårt moderna samhälle är beroende av bränsle och material som tillverkas av olja. I ett oljeraffinaderi tillverkas olika sorters bränslen som t.ex. bensin och diesel men även så kallade plattformskemikalier. Dessa plattformskemikalier används som startmaterial för en stor mängd produkter där tillverkning av olika sorters plaster är ett av de största användningsområdena. Mycket av den plast som görs av olja bryts ner mycket långsamt eller inte alls i naturen och om den inte återvinns kan det leda till allvarlig miljöförstöring. Det har sagts att om vi fortsätter att skräpa ner haven som vi gör idag kommer det att finnas mer plast än fisk i haven år 2050.
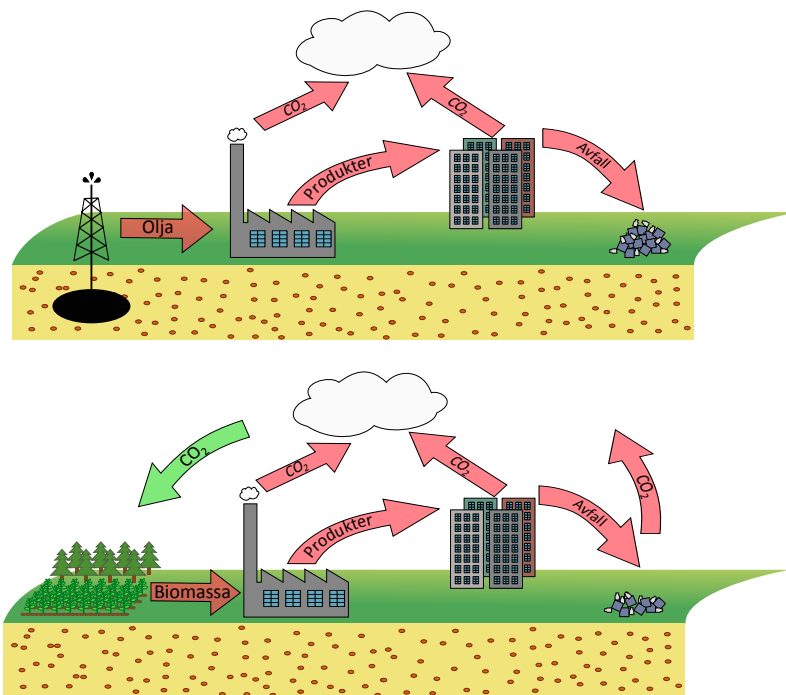
Figur 1: När olja används för att tillverka produkter som energi, plaster och kemikalier tillförs koldioxid till atmosfären (Övre bilden). Genom att basera produktionen på biomassa kan koldioxiden återgå till kretsloppet då ny biomassa odlas (Nedre bilden). Biologiskt nedbrytbar plast kan också delta i kretsloppet eftersom den kan brytas ned och inte förblir i miljön.

Det är här som bioraffinaderiet kommer in. Ett bioraffinaderi har samma syfte som ett oljeraffinaderi, att producera bränsle och kemikalier. Den stora skillnaden är att det här inte är olja som används utan material från växtriket, biomassa, som t.ex. socker från sockerrör, stärkelse från spannmål eller växtfiber från trä eller halm.

Det finns många sorters bioraffinaderier, och många av dem använder mikroorganismer för att omvandla biomassan till produkter. Mikroorganismer är små encelliga organismer som till exempel jäst eller bakterier. Människan har använt mikroorganismer i tusentals år för att tillverka olika produkter, ofta livsmedel. Typiska produkter är alkohol som produceras med jäst och olika organiska syror som mjölksyra och ättiksyra som främst produceras av bakterier. Just organiska syror är intressanta eftersom vissa lämpar sig väl för tillverkning av en grupp av plaster som kallas polyestrar. Polyestrar har länge tillverkats av oljebaserade startmaterial men eftersom startmaterialet till bio-plasten är tillverkat med hjälp av mikroorganismer så är det ofta lättare för mikroorganismer i naturen att kunna bryta ner plasten igen.

För att mikroorganismerna ska kunna använda biomassan måste den brytas ner till socker och det finns många olika slags sockerarter. I sockerrör är det lätt, de innehåller redan sackaros, vanligt socker. Stärkelse i spannmål är ganska lätt att bryta ner och består av långa kedjor av glukos, druvsocker. Problemet är att dessa råvaror också är mat, och om för mycket av dem används i bioraffinaderi kommer maten att bli dyr och det finns risk för svält. Växtfibrer är till stor del uppbyggda av långa kedjor av blandade sockerarter, men är svårare att bryta ner. När man väl lyckats bryta ner dem får man inte bara en blandning av sockerarter utan även en mängd biprodukter som kan vara skadliga för mikroorganismer. En vanlig sockerart i växtfiber är xylos, träsocker, som är näst vanligast efter glukos. Till skillnad från glukos är det betydligt färre mikroorganismer som kan använda xylos och det är just xylos som den här avhandlingen handlar om.

När det gäller valet av mikroorganism kan man antingen välja en som från början både kan använda xylos och tillverka organiska syror. Vi har testat två bakterier som kan göra detta, *Actinobacillus succinogenes* och *Basfia succiniciproducens* som naturligt lever i magen på kor. De kan vara svåra att odla och behöver ofta tillsats av näring som kan göra processen dyr. De kan också vara känsliga för de biprodukter som bildats i nedbrytningen av växtfiber. Man kan då istället välja en organism som är mer tålig och som inte behöver tillsats av så mycket näring. En populär organism i bioraffinaderier som har just de egenskaperna är vanlig bagerijäst, *Saccharomyces cerevisiae*. Den kan dock varken använda xylos eller producera större mängder organiska syror, därför behöver man genmodifiera den. I den här avhandlingen har vi utvärderat två olika sätt att genmodifiera jäst för att kunna tillverka organiska syror från xylos, ett mer beprövat sätt och ett mer nyskapande.

List of publications

This thesis is based on the following publications, referred to by their Roman numerals:

Paper I **Succinic acid production by** *Actinobacillus succinogenes* **from batch fermentation of mixed sugars**
Almqvist H, Pateraki C, Alexandri M, Koutinas A, Lidén G
J Ind Microbiol Biotechnol. 43:1117–1130, 2016 doi:10.1007/s10295-016-1787-x

Paper II **Modelling succinic acid fermentation using a xylose based substrate**
Pateraki C, **Almqvist H**, Ladakis D, Lidén G, Koutinas AA, Vlysidis A
Biochem Eng J 114:26-41, 2016. doi:10.1016/j.bej.2016.06.011

Paper III **The effect of fermentation conditions on production of di-carboxylic acids in the carboxylating pathway from xylose in engineered** *Saccharomyces cerevisiae*
Almqvist H, Stovicek V, Borodina I,Lidén G
Manuscript

Paper IV **Exploring xylose oxidation in** *Saccharomyces cerevisiae* **through the Weimberg pathway**
Wasserstrom L, Portugal-Nunes D, **Almqvist H**, Sandström AG, Lidén G, Gorwa-Grauslund MF
Submitted

Paper V **Characterization of the Weimberg pathway in** *Caulobacter crescentus*.
Almqvist H, Jonsdottir-Glaser S, Tufvegren C, Wasserstrom L, Liden G
Submitted

Paper VI **A rapid method for analysis of fermentatively produced D-xylonate using ultra-high performance liquid chromatography and evaporative light scattering detection**
Almqvist H, Sandahl M, Lidén G
Biosci Biotechnol Biochem 81:1078-1080, 2017.
doi:10.1080/09168451.2017.1292839

Related publications not covered in this thesis

Review **Saccharomyces cerevisiae: a potential host for carboxylic acid production from lignocellulosic feedstock**
Sandström AG, **Almqvist H**, Portugal-Nunes D, Neves D, Lidén G, Gorwa-Grauslund MF
Appl Microbiol Biotechnol 98:7299-7318, 2014 doi:10.1007/s00253-014-5866-5

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Author contributions

Paper I **Succinic acid production by** *Actinobacillus succinogenes* **from batch fermentation of mixed sugars**

I participated in the design of the study and the experimental work. I wrote the manuscript.

Paper II **Modelling succinic acid fermentation using a xylose based substrate**

I participated in the experimental work and critically reviewed the manuscript.

Paper III **The effect of fermentation conditions on production of di-carboxylic acids in the carboxylating pathway from xylose in engineered** *Saccharomyces cerevisiae*

I designed the study and performed the experimental work. I participated in the preparation of the manuscript.

Paper IV **Exploring xylose oxidation in** *Saccharomyces cerevisiae* **through the Weimberg pathway**

I designed and performed the bioreactor experiments and performed the UHPLC analysis. I drafted the section of the manuscript regarding the bioreactor experiments. I critically reviewed the manuscript.

Paper V **Characterization of the Weimberg pathway in** *Caulobacter crescentus*.

I designed the study, coordinated the experimental work and performed parts of it. I wrote the manuscript.

Paper VI **A rapid method for analysis of fermentatively produced D-xylonate using ultra-high performance liquid chromatography and evaporative light scattering detection**

I designed the study, performed the experimental work and wrote the manuscript.

Acknowledgements

This thesis carries my name as sole author. However, it's completion would not have been possible without the support, in both little and big matters, from a long list of people.

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Contents

Chapter 1

Introduction

1.1 Using renewable carbon sources for chemical production - one step toward sustainability

Today's society is heavily dependent on fossil carbon sources, not only for energy but also for materials, especially plastics. About 6% of the global oil consumption is used for manufacturing of plastics. When oil is used to make materials, where plastics are the most abundant, there is not so much CO_2 released - if the materials are completely recycled. Recycling of plastic material is, however, not commonly practised (World Economic Forum, 2016). Only 14% of the plastic packaging material on a global level is recycled, the rest is discarded, normally after a single use. The fraction that is recycled is even lower for plastics used for other purposes then packaging. This should be compared with other important materials such as paper, of which 58% is recycled, or iron and steel, of which 70-90% is recycled globally. Each year, 8 million tons of plastic material is released into the oceans and it is estimated that 150 million tons of plastic has accumulated in the oceans today. If the current trend is not changed, the estimates project that there will be more plastic than fish, by weight in the oceans by 2050 (!) (World Economic Forum, 2016). Reports of marine plastics are not new, it was reported as early as in the 1970s, but has recently received more widespread attention in media (Jambeck et al., 2015). Plastic debris both on land and in the oceans, can be physically harmful to animals and fish if the plastic is eaten, but there are also other less obvious effects (Rochman et al., 2013). One type of released into water is by so called microplastics, which are plastic particles less than 5 mm (Kooi et al., 2016). Microplastics can both be intentionally produced or result from physical fragmentation of larger pieces in the environment. Microplastics can also contain toxic additives, such as softeners and can furthermore absorb persistent organic pollutants from environment causing poisoning

of organisms that ingest the microplastics (Jambeck et al., 2015; UNEP, 2015; Rochman et al., 2013).

The problem with plastics is therefore two-fold; a) the use of non-renewable material for production, and b) environmental problems caused by non-biodegradable plastics. Fossil material for production of plastics can be replaced by renewable feedstocks, and the problem with release of non-biodegradable plastics to the environment can be solved – or at least diminished – by introduction of bio-degradable plastics in applications where recovery is problematic. Both these solutions are part of the transition to a bio-based economy. Transition from a fossil based economy to a bio-based economy is a challenge but also an opportunity. The transition to a bio-economy is predicted to not only be beneficial for the environment but also to create new jobs, open new markets for bio-based product and increase the competitiveness of the industry (European Commission, 2012).

Another factor discussed is the security of supply chains. In today's volatile global political situation, a complete dependence on oil producing countries for supply of such an essential group of products as plastics may be unwise. Locally sourced raw materials reduce a country or regions dependency on foreign politics (Chu & Majumdar, 2012). Some reports forecast the demise of the oil industry in a near future as road transportation is electrified, stating that technological revolutions often come about quicker than key players realise (Arbib & Seba, 2017). The platform chemicals used in today's chemical industry almost entirely comes from oil refineries. These platform chemicals are however a by-product to the main products of the oil refinery, namely the liquid fuels such as petrol, jet fuel and diesel oil, which make up as much as 80-90% of the oil use (Alfke et al., 2007). A large decrease in fossil fuel production would therefore proportionally reduce the amount of available platform chemicals. Thus, an early investment in other sources for platform chemicals may be very profitable.

The European Commission launched its bio-economy strategy in 2012. As part of that, research programmes to strengthen the European position were launched. One such programme was the knowledge-based bio-economy (KBBE) which was part of the 7th framework programme.

1.2 Scope and outline of this work

The work behind this thesis has been conducted within two of the projects in the KBBE programme; the project BRIGIT (New tailor-made biopolymers produced from lignocellulosic sugars waste for highly demanding fire-resistant applications), and the project BioREFINE-2G (Development of 2nd Generation Biorefineries - Production of Di-
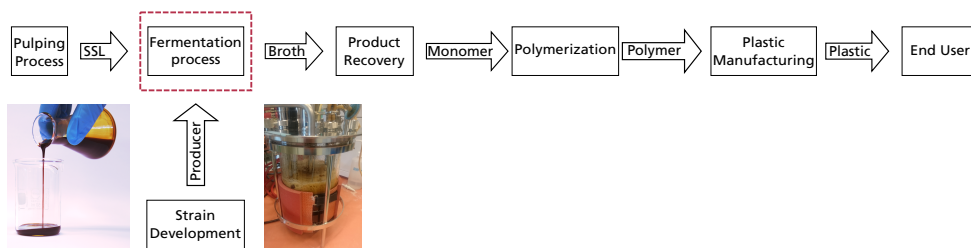
Figure 1.1: Project development chain in the BRIGIT and BioRefine2G projects. The work in this thesis regards the fermentation processed, marked with a red box.

carboxylic Acids and Bio-based Polymers Derived Thereof) projects. The aim of both these projects was valorisation of lignocellulosic waste streams by the production of bio-polymers, and the consortia contained partners from each step in the chain from producer of the raw material to the final user (Figure 1.1).

The research described in this thesis concerns the conversion of lignocellulosic sugars (provided by project partners) into carboxylic acids using different microorganisms. Both wild-type organisms and organism engineering by project partners were used.

The specific research goals of the present work were to:

- Develop process engineering strategies for improved titer, yield and productivity.

- Support the strain development by characterising the performance of production organisms in synthetic and industrially relevant media.

- Improve methods for assessment of cultivation experiments.

Conducted work was mainly experimental. Papers I–V are based on cultivation of a range of organisms, both wild-type and engineered ones. Simple cultivation methods in shake flasks were used, as well as state-of-the-art bioreactor setups which allows application of process engineering techniques to characterise and improve the process performance. In Paper II an *in silico* model of the kinetics of two wild-type organisms was developed and model parameters were fitted to experimental data using statistical tools. Experiments have been evaluated using a range of laboratory analytical methods such as spectrophotometry, enzyme activity assays and liquid chromatography. Paper VI describes the development of a novel analysis method based on liquid chromatography that was developed as the existing methods were found insufficient for the purpose.

The thesis is divided into six chapters. Chapter 2 gives a brief introduction to the bio-economy concept and gives an overview of biorefineries in terms of feedstocks, products and production organisms. The novel research performed is summarised in Chapters 3

to 5. Work on natural succinate producers is summarised in Chapter 3, whereas Chapters 4 and 5 describe the work on two different strategies for carboxylic acid formation from xylose in yeast. Conclusions and a brief outlook is finally given in Chapter 6.

Chapter 2

The Bioeconomy and Biorefineries

2.1 Bioeconomy - a brief introduction

The requirements of human society on earth today is provided by input of five categories; water, food, energy, fuels and materials. Throughout history, humans have relied on renewable resources for the supply of all their requirements - minerals and metals being the only exceptions. Up until the industrial revolution in the mid-18th century the impact of the human population on the environment was limited (Chu & Majumdar, 2012). Initially, combustion of charcoal made from wood provided energy for the industry, but the energy demand soon exceeded the possible supply of charcoal in countries like England. By the 1820s English iron industries were yearly burning coke from coal equivalent to the yearly charcoal production of a forest the size of England (Quivik, 2003). Coal also enabled powered transportation in the form of steam engines.

Industrial oil refining dates back to the 1850s, about 100 years after the start of the industrial revolution (Alfke et al., 2007). The main use of refined oil during the second half of the 1800s was production of kerosene for use as lamp oil. In the beginning of the 1900s the product demand from oil shifted quickly towards fuels for transportation using the internal combustion engine. Industrialisation, enabled by innovation and powered by fossil fuels has led to the development of the modern society and a 10-fold increase of the human population, from 700 million at the start of the industrial revolution to 7 billion today (Chu & Majumdar, 2012). This development is - as is becoming increasingly evident - unfortunately not sustainable, but returning to a pre-industrialised society is not an option either. Therefore, another revolution which transforms today's economy into a sustainable economy is necessary (Chu & Majumdar, 2012).

The concept of "bioeconomy" is based on the idea that for an economy to be truly sus-

tainable it must use renewable resources (Bosman & Rotmans, 2016). There are two main drivers behind the bioeconomy; the protection of the climate by reduction of emission of greenhouse gases (GHG) and the replacement of fossil-based feedstock with renewable ones (Kircher, 2014). The word bioeconomy is increasingly discussed in the literature, but the definition is not always clear and tends to change over time. To better understand what the bioeconomy is Bugge and co-workers analysed the literature and found three bioeconomy visions (Bugge et al., 2016).

- A bio-*technology* vision that emphasises the importance of bio-technology research and application and commercialisation of bio-technology in different sectors.

- A bio-*resource* vision that focuses on the role of research, development, and demonstration (RD&D) related to biological raw materials in sectors such as agriculture, marine, forestry, and bioenergy, as well as on the establishment of new value chains. Whereas the bio-technology vision takes a point of departure in the potential applicability of science, the bio-resource vision emphasises the potentials in upgrading and conversion of the biological raw materials.

- A bio-*ecology* vision that highlights the importance of ecological processes that optimise the use of energy and nutrients, promote biodiversity, and avoid monocultures and soil degradation. While the previous two visions are technology-focused and give a central role to RD&D in globalised systems, this vision emphasises the potential for regionally concentrated circular and integrated processes and systems.

The visions are partly overlapping, but different stakeholders tend to view bioeconomy predominantly from one of these viewpoints. For example, the European Commission regards bioeconomy mainly from a bio-resource vision(Bugge et al., 2016). This can be seen in the strategy adopted by the European Commission, "Innovating for Sustainable Growth: A Bioeconomy for Europe" which focuses on the development of an innovative, resource efficient and competitive society in terms of production of food and industrial products as well as protection of the environment (EU, 2017). The European Union is far from the only stakeholder to develop a policy for bioeconomy, several countries have developed and are developing bioeconomy strategies that are either dedicated bioeconomy strategies or bioeconomy-related ones (Figure 2.1).

The current bioeconomy is mainly focused on the food and forestry sectors as can be seen in the compilation from the European Union below (Table 2.1). The contribution from bio-products is small in comparison to the other sectors. Many of the commercialised chemicals are fine chemicals and speciality chemicals, such as enzymes and monoclonal antibodies, but some examples of bulk chemicals exist, for example bio-ethanol and citric acid (Kircher, 2014).

Bioeconomy Policies around the World
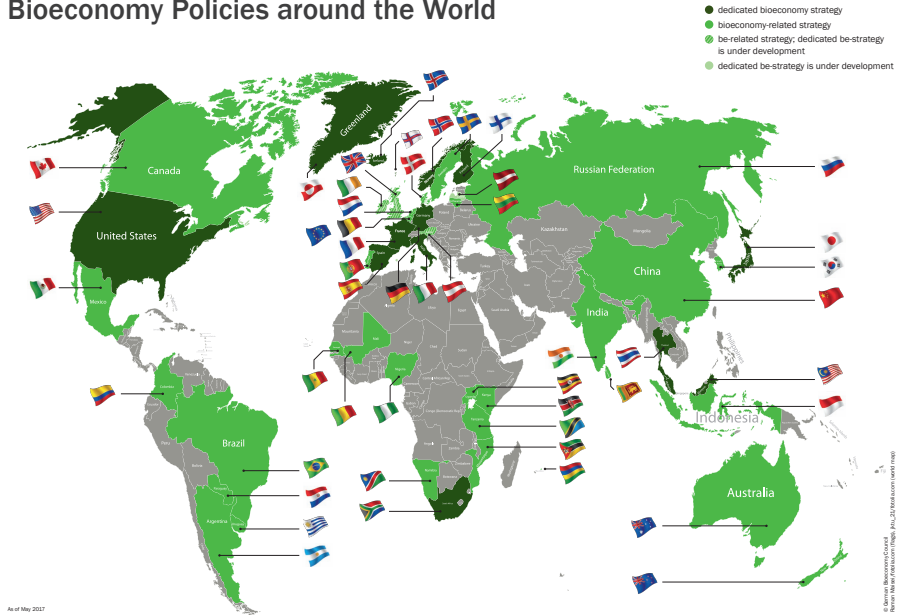
Figure 2.1: Bioeconomy policies around the world (©German Bioeconomy Council, 2017, reprinted with permission)

There are clearly huge challenges for the bioeconomy, but also large opportunities (Scarlat et al., 2015). Among the opportunities are the possibility to reduce the environmental impact working towards the goal of sustainable society by efficient use of feedstocks. Also, the development of new bio-based industries and markets for bio-based products can bring prosperity. Possibly, the greatest challenge that the bioeconomy faces is to make products that can compete with established fossil based products in terms of cost, while maintaining a small ecological footprint. Another very significant challenge is the supply of feedstock. Transition to a bioeconomy will put more strain on the supply of biomass, as well as the supply chain, and competition for arable land for production of food and biomass will increase. Intensified production of biomass can also have a negative impact on the soil quality, water accessibility, and lead to an increase in use of pesticides and non-renewable fertilisers (Scarlat et al., 2015).

Materials can be produced from mineral, fossil or vegetal sources. Some materials can only be made from one of these sources (e.g. metal from mineral sources) while other can be made from several raw materials. Another option is replacement with another material with comparable properties (Rouilly & Vaca-Garcia, 2015). By wisely selecting the source for each material produced the environmental impact can be minimised. Organic chemicals and materials such as plastics, can only be made from carbon contain-

Table 2.1: The value of the European bioeconomy (Scarlat et al., 2015)

Sector	Annual turnover (€ billion)	Value added (€ billion)	Employment (1000 s)
Agriculture	404	157	10200
Food and beverage	1040	207	468
Agro-industrial products	231	62	2092
Fisheries and aquaculture	36,6	9,7	199
Forestry logging	42	22	636
Wood-based industry	473	136	3452
Bio-chemicals	50	-	120
Bioplastics	0,4	1,4	-
Biolubricants	0,4	0,6	-
Biosolvents	0,4	0,4	-
Biosurfactants	0,7	0,9	-
Enzymes	1,2	-	-
Biopharmaceuticals	30	50	142
Biofuels	16	-	132
Bioenergy	34	-	350
Total	2357	-	21790

ing feedstocks, and for such products, a transition from fossil to renewable feedstocks is technically feasible.

Another concept often appearing together with bioeconomy is "circular economy". This is an even broader term which includes bioeconomy but also use of non-renewable materials such as minerals and metals (World Economic Forum, 2016). All materials used today are not possible to produce from renewable resources. In order to achieve a sustainable society, the circular economy must be implemented for such materials. It can also be profitable to apply circular economy to renewable materials too, thereby reducing cost and energy needed in the sourcing and refining stages if the material can be reused, refurbished and recycled (Figure 2.2).

Figure 2.2: The circular economy is based on that the materials are to be retained within the circular economy with as little loss as possible. This can be done by reuse, refurbishment and recycling of materials at different levels in the supply chain. The goal is to minimise the leakage to landfill and combustion of non-renewable materials.

2.2 Biorefineries

An oil refinery is a chemical plant where crude oil is separated into fractions with different uses e.g. liquid fuels, natural gas, platform chemicals etc. (Alfke et al., 2007). Not only separations occur in the oil refinery but also chemical conversion of certain compounds into others. Example of such processes are cracking, which is used to convert heavier hydrocarbons into lighter, and reforming which is used to convert low-octane naphtha into high-octane reformate. The main outputs of oil refineries are fuel products which amount to 80-90% of the production. The remainders are products such as solvents, paraffin waxes, lubricants and greases, bitumen and asphalt, and petroleum coke (Alfke et al., 2007). The lighter fraction called naphtha is a feedstock to produce platform chemicals for the petrochemical industry (Figure 2.3). All major bulk chemicals used in the industry are derived from these rather few platform chemicals (Cherubini, 2010).

Figure 2.3: Product spectrum from oil refineries. The six most common platform chemicals for the petrochemical industry derived from naphtha are shown at the bottom of the figure.

A term connected to bioeconomy is "biorefinery". The purpose of a biorefinery is the same as an oil refinery, i.e. to convert and separate a feedstock into a variety of products that can be used as fuels or platform chemicals for the chemical industry, but the feedstock is not oil but instead biomass (Kamm et al., 2016). Biorefining may, however, have other meanings as well. Fermentation of beer and wine, and production of vegetable oils has been done for thousands of years, and can be considered biorefining (Jong & Jungmeier, 2015). Paper mills established in the nineteenth century, where wood is used to produce paper, can be seen as the first industrial biorefineries (Jong & Jungmeier, 2015). In paper mills, the importance of recovery of by-products apart from paper was early recognised. Example of such co-products from paper mills are lignin, which is burnt for generation of power, tall oil which can be used as liquid fuel or a feedstock for chemical production, and residual sugars in the cooking liquid, which can be fermented to ethanol (Ragnar et al., 2014). There have been several attempts to more clearly define the term biorefinery. The International Energy Agency, IEA Bioenergy Task 42 has defined it as below (Jong & Jungmeier, 2015):

> "Biorefinery is the sustainable processing of biomass into a spectrum of marketable products and energy".

Table 2.2: Biorefining process categories (Jong & Jungmeier, 2015; Cherubini, 2010).

Category	Examples of processes	Examples of intermediate and end-products
Thermochemical processes	Gasification	Syngas (H_2, CO, CO_2 CH_4), Power, Fischer Tropsch-fuels, DME, ethanol
	Pyrolysis	Pyrolysis oil, charcoal, pyrolysis gas
Biochemical processes	Fermentation	Alcohols (Ethanol), organic acids, biogas (CH_4, CO_2)
	Enzymatic catalysis	Sugars
Mechanical processes	Size reduction	Particulate biomass
Chemical processes	Acid hydrolysis	Sugars
	Transesterification	Biodiesel

The spectrum of marketable products consists of food, feed, materials, chemical, fuels, heat and power (Jong & Jungmeier, 2015). According to this definition, the product spectrum covers all products produced in oil refineries, and also adds food and feed. The definition also adds the criterion of sustainable processing. Fulfilment of the sustainability criterion, depends heavily on the design of the processes, selection of feedstock and product (Moncada et al., 2016). Biorefinery processes, feedstocks and products will be discussed in more detail in this chapter.

2.2.1 Biorefinery categories

Just as in oil refineries, biorefining consists of a range of processes. The conversion processes in biorefineries can be grouped into four categories (Table 2.2). Most commonly, biorefineries described in the literature are stand-alone biorefineries, relying mainly on one process (Moncada et al., 2016). Two platform concepts are commonly mentioned in biorefinery literature, the sugar platform and the syngas platform (Jong & Jungmeier, 2015). The syngas platform relies mainly on thermochemical processes where gasification of the biomass to generate syngas is the core process.

This thesis work concerns the sugar platform, where sugars derived from biomass form a common starting point for fermentation processes where a wide range of products can be obtained, thanks to the diversity of microbial metabolism (Jang 2012). The sugar platform is not limited to biological processes, sugars can also be chemically converted to a range of products (Jong & Jungmeier, 2015).

2.3 Feedstocks

2.3.1 Starch, glucose and sucrose

Oil consists of biomass that has been degraded over millions of years to form a fairly homogeneous liquid. Biomass is, in comparison to oil, more heterogeneous. Its composition varies with both type of biomass (e.g. lignocellulosics, starch crops and oil seeds) as well as with the fraction of each plant (e.g. wood, bark, leaves and seeds). The main macro-molecular building blocks of biomass are carbohydrates, lignin, proteins and fats, but biomass also contains small amounts of vitamins, dyes, flavours and aromatic essences as well as inorganics - found as ash after combustion (Kamm et al., 2016). Therefore, biomass processing requires diverse processing methods (Moncada et al., 2016). It has been estimated that the global biosynthesis produces $170 \cdot 10^9$ tons of biomass per year. Out of this 75% is carbohydrates and 20% is lignin, leaving only 5% for all the remaining categories of compounds (Kamm et al., 2016).

Any biomass that contains sugars or carbohydrate polymers can theoretically be used in the sugar platform, making it a potential feedstock for (large volume) production of fuels and chemicals. Depending on the source of biomass used, a biorefinery can be classified in different generations (ElMekawy et al., 2013). First generation biorefineries use classical agricultural crops, such as sugar cane, wheat and corn, as feedstocks. In contrast to sucrose from sugar cane that can be directly used in fermentation processes, starch from cereals must be hydrolysed to glucose. Efficient starch hydrolysis processes that use enzymatic catalysis are well established, making starch an easily available feedstock for biorefining (Kamm et al., 2016). The selection of feedstock usually depends on the dominant local agricultural crop. In Brazil sugar cane is a popular biorefinery feedstock, whereas in the USA corn dominates and in Canada and Europe wheat is the main feedstock (ElMekawy et al., 2013; Kamm et al., 2016). Feedstocks for biorefineries do not need to be dedicated feedstocks, where a crop is grown with the sole intention to become feedstock for biorefining, by-products and side-streams from other activities can be used as well.

2.3.2 Lignocellulosic biomass

Lignocellulosic biomass is the most abundant renewable carbon source available on earth (Isikgor & Becer, 2015). The plant cell wall provides structural stability to the plant, protection against biological and physical harm and also participate in the transport of water and nutrients in the plant (Guerriero et al., 2016). The cell wall consists mainly of three polymers, cellulose, hemicellulose and lignin, hence the name lignocellulose (Figure 2.4). Cellulose is built up of glucose units linked together by β-1,4 glycosidic
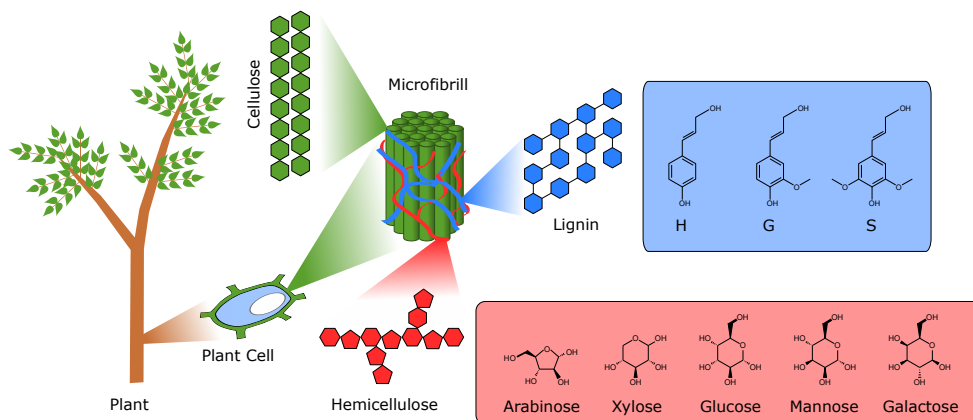
Figure 2.4: Structure of lignocellulosic biomass. Adapted from (Rubin, 2008; Isikgor & Becer, 2015).

bonds without side chains. The repeating unit is - due to the orientation of the sugar moieties - in fact not glucose, but the disaccharide cellobiose (Sorek et al., 2014). The linear cellulose molecules are tightly packed in bundles called microfibrils with high crystallinity (Guerriero et al., 2016). The cellulose microfibrils are covered in hemicellulose and lignin. Hemicellulose is like cellulose a carbohydrate polymer, but consists of a variety of monomers instead of just glucose and there are different types of hemicellulose, e.g. xylan, galactoglucomannan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan (Isikgor & Becer, 2015; Ragnar et al., 2014). The monomers forming hemicellulose are pentoses (C5) e.g. arabinose and xylose, hexoses (C6) e.g. mannose, glucose and galactose, and also sugar acids like glucuronic acid. Hemicellulose residues are, in contrast to cellulose, frequently acetylated and the polymer is often a branched polymer which furthermore is often amorphous. Lignin is not a polymer of carbohydrates, but is composed of phenylpropanoid (C9) units. Lignin is synthesised from three main lignin precursors; p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which in the polymerised lignin are found in the form of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, differing in the number of methoxy groups on the benzene ring (Pu et al., 2013). During polymerisation of lignin precursors, various types of ether bonds and C-C bonds are formed, resulting in a three-dimensional network without defined primary structure (Sorek et al., 2014). Lignin functions as a glue that strengthens the microfibril and there is also crosslinking between lignin and carbohydrate polymers (Isikgor & Becer, 2015; Li et al., 2016).

Sources of lignocellulosic materials, e.g. wood, grasses or residues from agriculture have varying composition (Table 2.3). Cellulose is usually the most abundant compound followed by hemicellulose and lignin. Together they make up most of the dry weight of biomass, the remainder being composed to a large extent of proteins, oils and ash. Not only the amount of hemicellulose and lignin varies among different types of biomass;

the composition of both hemicellulose and lignin also varies.

Table 2.3: Composition of different types of lignocellulosic biomass. Adapted from (Isikgor & Becer, 2015).

Category	Type	Cellulose (% of DW)	Hemicellulose (% of DW)	Lignin (% of DW)
Hardwood	Poplar	50.8 - 53.3	26.2 - 28.7	15.5 - 16.3
Xylan rich hemicellulose	Oak	40.4	35.9	24.1
S&G type lignin	Eucalyptus	54.1	18.4	21.5
Softwood	Pine	42.0 - 50.0	24.0 - 27.0	20.0
Galactoglucomannan rich hemicellulose	Douglas fir	44.0	11.0	27.0
G type lignin	Spruce	45.5	22.9	27.9
Agricultural crops & grasses	Wheat Straw	35.0 - 39.0	23.0 - 30.0	12.0 - 16.0
Xylan rich hemicellulose	Barley Hull	34.0	36.0	13.8 - 19.0
G,S & H type lignin	Barley Straw	36.0 - 43.0	24.0 - 33.0	6.3 - 9.8
	Rice Straw	29.2 - 34.7	23.0 - 25.9	17.0 - 19.0
	Rice Husks	28.7 - 35.6	12.0 - 29.3	15.4 - 20.0
	Oat Straw	31.0 - 35.0	20.0 - 26.0	10.0 - 15.0
	Ray Straw	36.2 - 47.0	19.0 - 24.5	9.9 - 24.0
	Corn Cobs	33.7 - 41.2	31.9 - 36.0	6.1 - 15.9
	Corn Stalks	35.0 - 39.6	16.8 - 35.0	7.0 - 18.4
	Sugarcane Bagasse	25.0 - 45.0	28.0 - 32.0	15.0 - 25.0
	Sorghum Straw	32.0 - 35.0	24.0 - 27.0	15.0 - 21.0
	Grasses	25.0 - 40.0	25.0 - 50.0	10.0 - 30.0
	Switchgrass	35.0 - 40.0	25.0 - 30.0	15.0 - 20.0

2.3.3 Pretreatment

In order to obtain sugars from lignocellulose, the carbohydrate polymers must be hydrolysed. This can be done either chemically or enzymatically. The enzymatic process is often considered the most attractive due to the milder operating conditions needed (Galbe & Zacchi, 2012). However, due to the recalcitrance of lignocellulose, it is not sufficient with only enzymatic hydrolysis. A "pre-treatment" of the biomass intended to reduce the recalcitrance is needed (Mutturi et al., 2014). There are a range of different pre-treatment methods that can be categorised into physical (e.g. milling, mechanical extrusion), chemical (e.g. dilute acide, organosolv, ionic liquids, mild alkali), physico-chemical (e.g. liquid hot water, steam explosion - STEX, ammonia fiber explosion - AFEX, sulphite pretreatment to overcome recalcitrance of lignocellulose - SPORL) and biological (e.g. fungal, bacterial) pretreatment processes (Kumar & Sharma, 2017).

The pre-treatment reduces the recalcitrance of the biomass and increases the digestibility of the material in enzymatic hydrolysis. However, pre-treatment also potentially generates degradation products that can act as inhibitors in the following fermentation processes. Such inhibitors are furan derivatives (furfural and 5-hydroxymetylfurfural), weak acids (acetic, formic and levulinic acid) and phenolics from degraded lignin (Almeida et al., 2007). Lignocellulosic biorefineries do not have to use dedicated feedstocks for biorefining, but can also operate using lignocellulosic waste-streams. One such waste-

stream is spent liquor form sulphite pulping, which has been the feedstock used in this thesis work.

2.3.4 Spent sulfite liquor (SSL)

An important part of the work in the thesis involves the valorisation of the hemicellulose monosaccharides in SSL from *Eucalyptus spp*. Sulfite pulping is done by cooking biomass in a liquor consisting of dissolved sulfur dioxide and a counter ion such as calcium, magnesium, sodium or ammonium (Ragnar et al., 2014). Cooking with magnesium bisulfite (most common) is done at acidic conditions (pH 1.5–4.0). During cooking the wood is delignified by sulfonation of the lignin molecules to form lignosulfonates which are soluble and can be separated from the pulp. In, addition lignin-carbohydrate complexes are broken and some lignin depolymerisation reactions take place. As the process takes place under acidic conditions, hemicellulose is hydrolysed and even some reduction of cellulose fiber length can occur. The pulp therefore mainly consists of cellulose and the spent cooking liquor will contain soluble lignosulfonates as well as - importantly for this thesis work - monosaccharides from hemicellulose (Rueda et al., 2014). Not only the pulp has commercial value, the lignosulfonates can be used in a variety of applications, e.g. as a dispersant in concrete (Gargulak et al., 2001). Recovery of the lignosulfonates is commonly done using the Howard process where lignosulfonates are precipitated with $Ca(OH)_2$ or NaOH (Pateraki et al., 2016a). This process, however, degrades the monosaccharides from the hemicellulose, eliminating the possibility to fully utilise this resource if the monosaccharides are not used before recovery of the lignosulphonates. Sulfite pulping today makes up a relatively small portion of the amount of pulp produced. Approximately 95% of the total amount of chemical pulp ($130 \cdot 10^6$ ton) that was produced in 2012 was produced with the Kraft process while the remaining 5% is made up by different version of sulphite pulping (Ragnar et al., 2014). Even though the proportion is small, the total amounts are fairly large.

2.3.5 Xylose - an important pentose

Spent sulphite liquor from hardwoods such as Eucalyptus contains large amounts of xylose (Rueda et al., 2014). Among the five most common monosaccharides in biomass, only xylose and arabinose are pentoses, and of these two, xylose is most abundant and it is in fact the second most abundant carbohydrate in lignocellulosic biomass (Zhang & Geng, 2012). However, as mentioned above, the amount is strongly dependent on feedstock. In hemicellulose hydrolysate from softwoods, xylose only makes up 6-16% of the obtained sugars after dilute acid hydrolysis, whereas from hardwoods, 36-95% of the obtained sugars from hemicellulose are xylose. Also in hemicellulose hydrolysates of

agricultural crops xylose is common, making up 66-86% of the sugars (Carvalheiro et al., 2008). Thus, xylose utilization is very important in biorefineries where such feedstocks are used, especially if the cellulose is used for other purposes than as a source of glucose. Still, research on microbial biorefinery processes focus heavily on glucose as substrate. During the opening lecture at the 2nd Lund Symposium on Lignin and Hemicellulose Valorisation in 2015, Michael O'Donohue of the INRA Toulouse, described biorefinery research as "glucocentric". A notable exception where the xylose utilization has been extensively investigated is in the field of ethanol production from lignocellulosic biomass. The most commonly used microorganism in ethanol fermentation, *Saccharomyces cerevisiae*, does not utilise xylose and significant research efforts have been spent on creating engineered strains which can efficiently utilise xylose - a work which is still on-going (Kwak & Jin, 2017). We will return to both the use of xylose, and the engineering of *S. cerevisiae* for xylose utilization later on in this thesis.

2.4   Products

Products from biorefineries can be placed in a value pyramid of bioproducts depending on their value and production volume (Bosman, 2016). At the bottom are fuels that, have a low value but are needed in large volumes. At the top of the pyramid are high-value products, such as pharmaceuticals and fine chemicals which are needed only in small amounts. Products with intermediate volume and value, such as chemicals, performance materials and food, are placed in between (Figure 2.5).
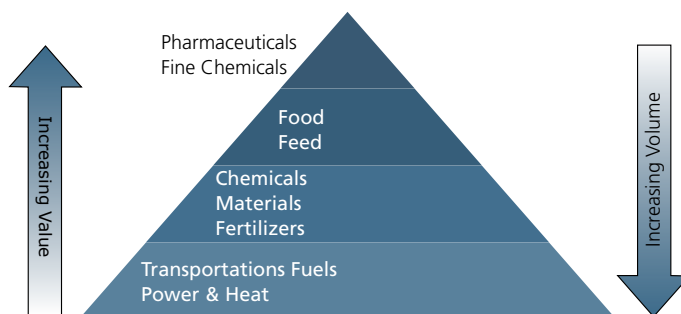
Figure 2.5: Bio-products value pyramid. Adapted from (Bosman & Rotmans, 2016)

Due to the small profit margin between feedstock and the lowest valued products (fuels, power and heat), the economics of a biorefinery is dependent on production of co-products higher up in the pyramid for generating revenue. Production of co-products can enable efficient utilisation of the entire feedstock (Kamm et al., 2016). One such product category is platform chemicals for the chemical industry.

Table 2.4: Top platform chemicals from sugar selected in the DoE report 2004 and the updated list from 2010 (Werpy & Petersen, 2004; Bozell & Petersen, 2010)

2004	2010
1,4-diacids (Succinic, fumaric, malic)	Succinic acid
2,5-furan dicarboxylic acids	2,5-furan dicarboxylic acids
3-hydroxypropionic acid	3-hydroxypropionic acid
Aspartic acid	-
Glucaric acid	-
Glutamic acid	-
Itaconic acid	-
Levulinic acid	Levulinic acid
3-hydroxybutyolactone	-
Glycerol	Glycerol/derivatives
Sorbitol	Sorbitol
Xylitol/arabinitol	Xylitol
-	Ethanol
-	Lactic acid
-	5-hydroxymetylfurfural (HMF)
-	Furfural
-	Isoprene
-	Biohydrocarbons

2.4.1 Platform chemicals

A highly influential report from the US Department of Energy (DoE) was published in 2004. In the report over 300 potential platform chemicals from biomass were analysed and a list of the 30 most promising candidates from a chemical market perspective was assembled, which was then narrowed down to 10 (Table 2.4). The chemicals were evaluated in terms of both technical and economic aspects as well as current market and future potential (Werpy & Petersen, 2004). A revised list taking into account technological progress since the first report was published using a similar methodology (Bozell & Petersen, 2010). Both lists, especially the first, include several carboxylic acids, which are of particular interest in this work.

2.4.2 Carboxylic acids

Carboxylic acids have traditionally been used in the preservation of food, and the production of acetic acid from ethanol in the form of vinegar, is more than 5000 years old (Sandström et al., 2014). Use of lactic acid bacteria for fermentation of food products (such as milk and vegetables) also has a long tradition - although the role of the bacterium in the fermentation was not necessarily known (Nair & Prajapati, 2003). The first carboxylic acid to be produced in industrial scale volumes through microbial fermentation was citric acid (Sauer et al., 2008). Citric acid had previously been extracted from lemons, but fermentative citric acid production by the fungus *Aspergillus niger* was developed in the 1930s (Sandström et al., 2014). Citric acid is still produced by fermentation and the annual production of citric acid is approximately 1.6 million tons

(Sauer et al., 2008). Carboxylic acids are common in the cell metabolism. All intermediates in the tricarboxylic acid cycle (TCA-cycle) are for example carboxylic acids, and many secreted fermentation end-products are carboxylic acids including e.g. lactic, acetic, formic, and succinic acid. In addition to the original food usage, carboxylic acids have found new industrial applications as e.g. precursors for synthesis of pharmaceuticals, and also as monomers in the production of various polymers (Sandström et al., 2014). The reactive carboxylic group can form ester and amide bonds. Dicarboxylic acids, or carboxylic acids containing an additional functional group, e.g. an alcohol or amine group, can thus form polyesters or polyamides, with suitable co-monomers (Lee et al., 2011). Polylactic acid (PLA) produced from bio-based lactic acid has lately reached production in industrial scale and is used in a range of applications especially as packaging material (Sauer et al., 2010; Jang et al., 2012). PLA is today the most important bioplastic in terms of production volume and in 2014 the annual production volume was around 120 000 tons (E4tech et al., 2015).

2.4.3 Succinic acid

A highly interesting platform chemical is succinic acid. Succinic acid (butanedioic acid or amber acid) is a four-carbon dicarboxylic acid (Figure 2.6). As a platform chemical, succinate can be used to produce industrially relevant compounds such as tetrahydrofuran (THF), γ-butyrolactone (GBL), N-methyl-2-pyrrolydone (NMP), 2-pyrrolydone, maleic anhydride, succinimide and 1,4-butanediol (Mazière et al., 2017; E4tech et al., 2015; Nghiem et al., 2017). The dual carboxylic groups located at each end of the carbon chain makes it ideal for polymerisation, especially with 1,4-butanediol (BDO) to form polybutylene succinate (PBS). The biotechnical production of succinate from glucose has moved into commercial (or near-commercial scale production) and there are currently four large-scale plants for succinate production with a total annual production capacity of 80 000 tonnes (Table 2.5). The production cost of bio-based succinic acid has been reported to be equal or even lower than the fossil based production (E4tech et al., 2015; Nghiem et al., 2017). Succinic acid was the main target product in this thesis work and is further described in chapters 3 and 4 (Papers I, II and III). In contrast to the commercial processes based on glucose, the work in this thesis targets conversion of *xylose* into succinate. Also the related acids, malic and fumaric acid, were of interest in Chapter 4.

2.4.4 Xylonic acid and α-ketoglutaric acid

A new oxidative pathway for xylose degradation was also explored in this work - further described in Chapter 5. Intermediates in this pathway are carboxylic acids, and in
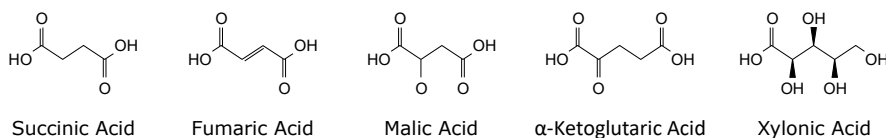
Figure 2.6: Structural formula of succinic acid, fumaric acid, malic acid, alpha-ketoglutaric acid ans xylonic acid.

particular xylonic acid and α-ketoglutaric acid are products of industrial interest (Figure 2.6). Xylonic acid was taken up on the DoE top 30 list, and is currently produced by oxidation of xylose (Werpy & Petersen, 2004). Xylonate has possible applications as a complexing agent or as dispersing agent in concrete, and it can also be an alternative to gluconic acid which has a market of 80 000 ton/year with applications in areas such as pharmaceuticals, food product, solvents, adhesives, dyes, paints and polishes (Toivari et al., 2012). The bioconversion of xylose to xylonate can reach very high yields, over 0.95 g g^{-1} (Toivari et al., 2010).

The other carboxylic acid, α-ketoglutarate, is an important intermediate in the TCA-cycle as well as an important node for amino acid synthesis and protein metabolism (Otto et al., 2011; Kamzolova et al., 2012). Chemical synthesis of α-ketoglutarate is possible through various routes, but problematic since it is a multi-step reaction involving partly toxic chemicals (Otto et al., 2011). Microbial production of α-ketoglutarate can be done with a variety of bacteria and yeasts, where engineered *Yarrowia lipolytica* has been pointed out as the promising host, being able to produce α-ketoglutarate at high concentrations (134 g L^{-1}) and yields (1.3 g g^{-1}) from rapeseed oil (Otto et al., 2011). Potential applications for α-ketoglutarate are uses as dietary supplement, in medical products, and as platform chemical for synthesis of heterocyclic compounds (Otto et al., 2011).

2.4.5 Bioplastics

Many dicarboxylic acids, such as succinic acid, are intended for the production of polymers. The main area of use for such polymers is production of plastics. Plastics have in many applications, replaced other materials such as wood, metals and glass, and new functionalities have been introduced (Elias & Mülhaupt, 2015). Plastics are very versatile materials whose properties can be altered by changing composition and processing methods and the production of plastics has increased rapidly since the introduction of the first industrial plastics (Figure 2.7).

However, as discussed in Chapter 2, the large - and increasing - production of plastics is not problem-free due to both consumption of fossil resources and accumulation of plastics in the environment. For several applications, one is now aiming to replace fossil-based with bio-based plastics. The term "*bioplastics*" is somewhat ambiguous and

Table 2.5: Large-scale succinate production (Mazière et al., 2017; E4tech et al., 2015; Ahn et al., 2016). Capacities are given in metric tonnes per year.

Company	Bioamber	Myriant	Succinity	Reverdia
Joint venture	DNP Green Technology ARD		BASF Corbion Purac	DSM Roquette
Location	Sarnia, Canada	Lake Providence, USA	Montmeló, Spain	Cassano, Italy
Established (y)	-	2013	2013	2008
Capacity (t/y)	30 000	30 000	10 000	10 000
Projected Expansion (t/y)	70 000 by 2018 200 000 by 2020	64 000	50 000	
Organism	*Pichia kudriavzevii* (*Candida krusei*)	*Escherichia coli*	*Basfia succiniciproducens*	*Saccharomyces cerevisiae*

requires explanation. IUPAC, the International Union of Pure and Applied Chemistry, defines bioplastics as being derived from "biomass or …monomers derived from the biomass and which, at some stage in its processing into finished products, can be shaped by flow" (Lackner, 2015). Bioplastics Europe, an industry association representing the bioplastics industry stakeholders in Europe, define bioplastics as "polymers that are bio-based, biodegradable, or both" (Lackner, 2015). Bio-based means that part of the material is derived from biomass. The source can be fully bio-based, like in the case of bio-polyethylene (PE) which is made from ethylene obtained by dehydration of ethanol. Plastics can also be partially bio-based, like in poly-ethylene terephthalate (PET) used in beverage containers under the name PlantBottle®, where one of the monomers (mono-ethylene glycol) is bio-based and the other (terephthalic acid) is fossil based. It should be noted that the type of plastic is not determined by whether the feedstock is bio-based or fossil-based. Some plastics are made from both bio and fossil sources, e.g. PE.

Biodegradability is another property of the plastic. Both bio-based and fossil-based plastics can be biodegradable (Figure 2.8). Biodegradation refers to the degradation of the material by biological activity, typically microorganisms (Lackner, 2015). The rate of biodegradation not only depends on the composition of the plastic but also on the environment and temperature. Biodegradability is tested against standards such as EN13432 (European Bioplastics e.V., 2015). For a bioplastic to be considered compostable it must, in addition to being biodegradable, also degrade at a rate comparable with compostable organic material and not leave fragments or toxic residues. Apart from bio-degradation, degradation of plastics in the environment can also occur from physical and chemical processes such as thermal degradation, oxidation, light induced degradation etc. Although, biodegradability may sound like an attractive property, this is not always the case. For some applications with products with a long intended life span, resistance to degradation is a key property, e.g. for material in contact with soil. The main area of
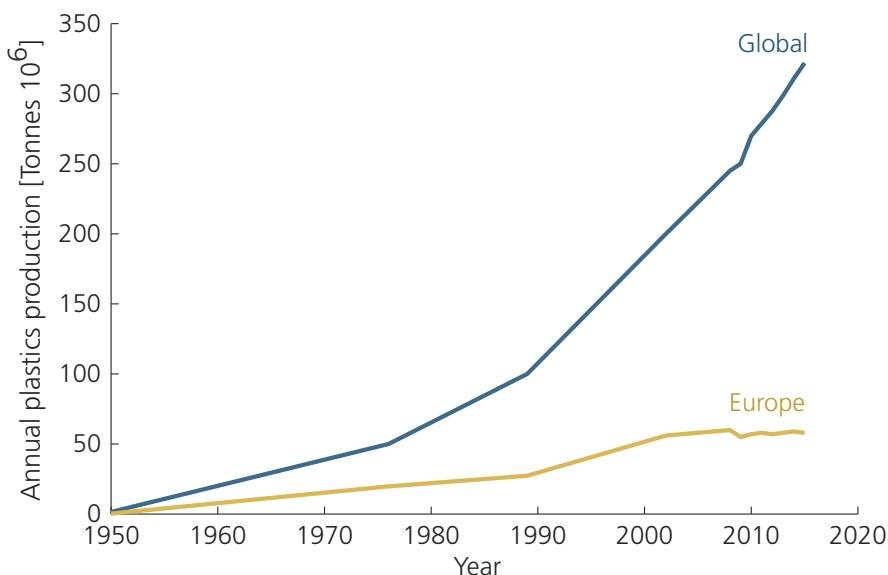
Figure 2.7: Annual plastic production from 1950 to 2015 (PEMRG, 2016).

interest for biodegradable plastics is the packaging market, where the intended life time of the plastic is short and the recycling process unfeasible (Lackner, 2015). The global production of bioplastics was 4 156 000 tons in 2016 is expected to grow with almost 50% to 6 111 000 tons already in 2021 (EuropeanBioplastics & Nova-Institute, 2016).

A third sustainability dimension to bioplastics is the production method. There are two main steps in the production of plastics, the production of monomers and the polymerisation. Both can be biological or chemical or a mix. In the case of bio-PE, the first step of the monomer production is fermentation of sugars to ethanol, a biological step, whereas the second is chemically catalysed dehydration of ethanol to ethylene (Storz & Vorlop, 2013). Polymerisation to PE is also a chemically catalysed reaction. For other polymers, like poly-hydroxyalkanoates (PHA), both the production of monomers and polymerisation are biological and take place inside the production organism (Storz & Vorlop, 2013).

2.5   Microbial production organisms

Microbial conversion (or fermentation) of sugars into target products is often assumed in sugar platform biorefineries, and either a "natural" (or wild-type) microorganism or a genetically engineered host microorganism can be used. An example of natural producers are used are lactic acid bacteria (LAB), which are efficient producers of lactic acid able to
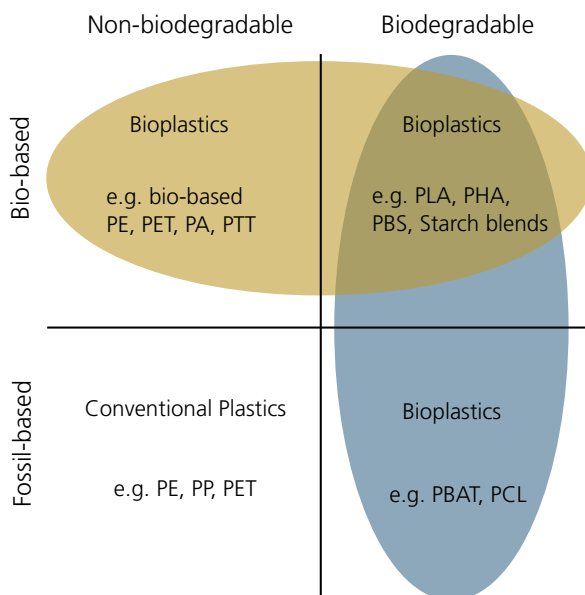
Figure 2.8: The relationship of bio-based vs. biodegradable plastics and some examples. Adapted from (EuropeanBioplastics, 2017).

reach 100 g L^{-1} at a productivity of 23 g L^{-1} h^{-1} (Sauer et al., 2010). The LAB, however, have some drawbacks for example their complex medium requirement, and problems with tolerance to high concentrations of the product at low pH-values. Therefore, engineered yeast strains have been developed and are now used in large scale production by e.g. the companies Cargill (Chen & Nielsen, 2016) and NatureWorks (Becker et al., 2015).

Filamentous fungi from the *Aspergillus* species are known for their ability to naturally produce large amounts of carboxylic acids under certain conditions (Yang et al., 2017). For example, *Aspergillus niger* can produce citric acid at a rate of 2.7 g L^{-1} h^{-1} and gluconic acid at a rate of 4.5 g L^{-1} h^{-1}. The production of acids in *A. niger* is related to stress conditions and regulation of the metabolic flux is not fully understood, which is a drawback (Yang et al., 2017).

Although there has been much work on improving natural producers using metabolic engineering, the top candidates for metabolic engineering to produce small molecules so far have been the industrial workhorses *Saccharomyces cerevisiae*, *Escherichia coli* and *Corynebacterium glutamicum* (Buschke et al., 2013). The cultivation procedures for these organisms are all well-known and genetic tools are well developed. There is also large experience and acceptance for the use of these organisms in numerous application for production of alcohols, organic acids, amino acids and pharmaceuticals.

In this thesis, work has been done with both natural producers and genetically engineered strains. For the succinate production described in Chapter 3, two natural succinate producing bacteria were used, *Actinobacillus succinogenes*, and *Basfia succiniciproducens*, whereas for the work discussed in Chapters 4 and 5, genetically engineered *Saccharomyces cerevisiae* was used.

2.5.1   Natural succinate producers

As discussed previously, succinic acid is a chemical with many potential uses. Succinic acid is also a natural metabolite found as an intermediate in the TCA-cycle in the central carbon metabolism. Many microorganisms in fact secrete low amounts of succinate, e.g. during mixed acid fermentation (Ward, 2014) and a number of succinate-producing, wild-type organisms have been described in the literature (Nghiem et al., 2017). The first isolated organism able to produce higher titers of succinic acid was *Anaerobiospirillum succiniciproducens* (Glassner & Datta, 1992; Guettler & Jain, 1996). This strictly anaerobic organism was however not considered robust enough for industrial purposes. (Nghiem et al., 2017). Later isolated organisms capable of naturally producing high titers of succinic acid include *Actinobacillus succinogenes*, *Mannheimia succiniciproducens* and *Basfia succiniciproducens*, all three belonging to the family *Pasteurellaceae*. (Nghiem et al., 2017; Mazière et al., 2017). *B. succiniciproducens* was found to have high genetic similarity to *Mannheimia succiniciproducens*, but is still considered to be a distinct species (Nghiem et al., 2017). The bacteria above were all isolated from the rumen of cattle and the natural environment of these organisms is anaerobic and contains high concentrations of partially degraded plant material (Duffield et al., 2004).

This environment, with its complex composition, selects for various features, some of which are positive and some are negative for industrial applications of the organisms. Due to the multitude of carbon sources present, the organisms have evolved towards flexibility in terms of carbon sources utilisation, which is an excellent trait for an industrial succinate producer. On the downside, however, such a rich environment also provides minerals, vitamins and easily accessible nitrogen sources such as amino acids. As a result, these organisms are often auxotrophic for a number of nutrients and therefore need a complex medium (McKinlay et al., 2005). Requirements of complex medium may result in increased production costs.

Another characteristic is that these organisms are all capnophilic, i.e. they thrive in a CO_2-rich environment and they are able to fixate CO_2 by carboxylation. In the carboxylation, phosphoenolpyruvate (PEP) is converted to oxaloacetate using PEPCK (PEP-carboxykinase) enzymes which are essential for succinate formation in these organisms (Ahn et al., 2016; Pateraki et al., 2016b).

The pH of the healthy cattle rumen is slightly below neutral - around pH 6.4 (Duffield et al., 2004). Hence, it is no surprise that the optimal pH for the succinate producing bacteria lies in this area. For industrial purposes however, acid production at neutral pH is not optimal as significant amounts of base will be needed for neutralisation of the fermentation broth as acids are produced. Typical neutralising agents are hydroxides or carbonate salts that result in high concentrations of metal ions such as sodium, potassium, calcium or magnesium that may increase the costs of purification of the succinate from the broth.

2.5.2   Metabolically engineered production organisms

Out of the four industrial scale plants for bio-succinate production described in section 2.4.3, three use engineered organisms (*S. cerevisiae*, *Pichia kudriavzevii* and *E. coli*) that are not natural succinate producers, the fourth being *Basfia succiniciproducens* (Table 2.6). Although *B. succiniciproducens* is a natural succinate producer, it has been engineered in the case described in Table 2.6 by deletion of *pfl* and *ldh* to reduce by-product formation of formate and lactate.

Saccharomyces cerevisiae is an often-used production host for several reasons. From a cultivation perspective, *S. cerevisiae* has a range of beneficial traits (Sauer et al., 2010; Sandström et al., 2014). Its cultivation conditions are well known; it has high fermentation capacity and is very robust. As a eukaryote, *S. cerevisiae* is not at risk of infection of bacteriophages, a common problem with prokaryotes in industrial processes (Moysés et al., 2016). A trait especially important to carboxylic acids production is the ability of *S. cerevisiae* to grow at low pH (Sauer et al., 2010). The advantage to operate at low pH is two-fold. Tolerance to low pH reduces the need for addition of a neutralising agent to the broth, thereby simplifying the purification process as the medium will contain less salts. The second advantage is the reduced risk of bacterial contamination, as the most contaminating bacterial species are effectively inhibited at low pH, around 4 (Narayanan et al., 2016). When using lignocellulosic feedstocks, one is often faced with the issue of inhibitory compounds produced in the hydrolysis such as furaldehyde, weak organic acids and phenolics. Tolerance to such compounds is an advantageous feature of *S. cerevisiae*, which can even detoxify e.g. furaldehydes in the feedstock (Sandström et al., 2014). It has been shown that adaptation of *S. cerevisiae* can improve the tolerance to inhibitors even at low pH (Narayanan et al., 2016)

Wild-type *S. cerevisiae* does not consume xylose, nor does it produce dicarboxylic acids to any larger extent. Metabolic engineering is thus needed to enable xylose conversion into these compounds. From a genetic engineering perspective, *S. cerevisiae* is a suitable organism since the genetics are well studied and documented in databases such as the Saccharomyces Genome Database (SGD, http://www.yeastgenome.org). *S. cerevisiae* was in

fact the first eukaryote to be fully sequenced and a vast array of molecular techniques and tools are available (Sandström et al., 2014). Efficient cloning toolkits based on CRISPR-Cas9 have recently been developed (Stovicek et al., 2015). There are also mathematical modelling tools, such as mature genome scale models available for *S. cerevisiae*, that can be used for guided metabolic engineering (Sandström et al., 2014).

In this thesis work, two principally different strategies, based on completely different introduced pathways for xylose assimilation, were investigated. In the first strategy, further discussed in Chapter 4, xylose assimilation was enabled by introduction of xylose isomerase (XI) and overexpression of the pentose phosphate pathway (PPP). This is a well-known strategy to enable ethanol production from xylose (Brat et al., 2009). Formation of dicarboxylic acids was enabled through introduction of a cytosolic reductive TCA route (Zelle et al., 2008), involving the re-targeting of peroxisomal malate dehydrogenase to the cytosol and overexpression of a pyruvate carboxylase as well as the introduction of a dicarboxylic acid transporter (Zelle et al., 2008). This pathway has previously been studied in yeast using glucose as substrate, but not for xylose consuming strains. The second strategy investigated was the introduction of the so-called Weimberg pathway in *S. cerevisiae*. This is an oxidative xylose assimilation pathway, which is completely different from the XI/PPP pathway. The Weimberg pathway is found in bacteria and archaea.

Table 2.6: Performance of engineered succinate producers. Abbreviations: Ms – *Mannheimia succiniciproducens*, Ro – *Rhizopus oryzae*, Sp – *Schizosaccharomyces pombe*, Lm – *Leishmania mexicana*, Rd – *Rhizopus delemar*, Ym – *Yersinia molaretii*. *Pichia kudriavzevii* is synonymously known as *Issatchenkia orientalis* and is an anamorph of *Candida krusei*)

Organism	Engineering	Conditions	Substrate	Titer	Yield	Rate	Ref
Glucose							
S. cerevisiae SUC-297 (Reverdia)	Δadh1 Δadh2 Δgpd1 ↑↑pckA(Ms) ↑↑gsh1 ↑↑cys3 ↑↑glr1 ↑↑mdh3 ↑↑pyc2p ↑↑fumR(Ro) ↑↑frdm1(Tb) ↑↑mae1(Sp)	Fed-batch, 30°C, aerobic, dual phase, (1. pH5; NH3; pO_2 = 20%, 2. pH 3, KOH, 50% CO_2)	Glucose	43	-	0.45	Van De Graaf et al. (2015)
*P. kudriavzevii** 13723 (Bioamber)	Δura Δpdc ↑↑pyc1 ↑↑fum ↑↑mae(Sp) ↑↑frd(Lm) ↑↑mdh(Rd)	Batch, 30°C, pH 3, aerobic (DO<10%, OUR = 18 mmol/*L*/h, 10%CO_2), KOH	Glucose	48.2	0.45	0.97	Rush & Fosmer (2014)
E. coli TG400 (Myriant)	ΔaspC ΔcitF ΔtdcDE ΔsfcA Δ(focA-pflB) ΔackA ΔpoxB ΔgalP(G297D)	Batch, 39°C, pH 7, anaerobic, K_2CO_3 + KOH	Glucose + xylose	96	0.96	0.80	Grabar et al. (2012)
B. succiniciproducens (Succinity)	Δpfl Δldh ↑↑glyoxylate shunt operon ↑↑ malate synthase (Ym),	Batch, 37 °C, pH 6.5, anaerobic, NH_4OH	Glucose	46.3	0.87	-	Scholten et al. (2014)
C. glutamicum	Δldh ↑↑pyc	Batch, 33°C, pO_2<0.01 ppm, $NaHCO_3$	Glucose	146	0.92	3.2	Okino et al. (2008)
Y. lipolytica	Δsdh2, Mutagenesis	Batch, 30 °C, aerobic, $CaCO_3$	Glycerol	45.5	0.36	0.27	Yuzbashev et al. (2010)
S. cerevisiae	Δgre3 ↑↑Cp xylA ↑↑rki1 ↑↑rp11 ↑↑tkl1 ↑↑Ps tal1 ↑↑Ps sut1 ↑↑Ps xyl3 ↑↑Sp Mae1 ↑↑mdh3Δskl ↑↑pyc2	Batch, 30 °C, aerobic, 50% CO_2	Xylose	3.5	-	-	Paper III
Hydrolysates							
E. coli	ΔpflB, ΔldhA, Δppc, ΔptsG ↑↑pepck	Fed-batch	Sugar cane bagasse	83	0.9	2.3	Maziere 2017
E. coli	ΔpflB, ΔldhA, Δppc, ΔptsG ↑↑pepck	Fed-batch	Corn stalk	61	-	1.7	Maziere 2017
E. coli	ΔpflB, ΔldhA, Δppc, ΔptsG ↑↑pepck	Fed-batch	Sugar cane bagasse	18.88	-	0.96	Tan 2014
E. coli	ΔpflB, ΔldhA, Δppc, ΔptsG ↑↑pepck	Fed-batch	Sugar cane bagasse	39.3	-	0.66	Tan 2014
S. cerevisiae	Δgre3 ↑↑Cp xylA ↑↑rki1 ↑↑rp11 ↑↑tkl1 ↑↑Ps tal1 ↑↑Ps sut1 ↑↑Ps xyl3 ↑↑Sp Mae1 ↑↑mdh3Δskl ↑↑pyc2	Batch, 30 °C, aerobic	Eucalyptus Spent sulphite liquor	3	-	-	Paper III

Chapter 3

Succinate production by *A. succinogenes* and *B. succiniciproducens*

3.1 Factors affecting fermentation performance

In this thesis work, natural succinate producers, mainly *A. succinogenes* but also *B. succiniproducens* were characterised to evaluate their suitability as producing organisms for succinic acid from xylose rich waste streams. A number of factors for industrial application were identified as important.

- Carbon source utilization (Paper I)

- Carbonate supply mode (Paper I)

- Product distribution (Paper I)

- Substrate and product inhibition (Paper II)

These factors were then studied in synthetic medium to evaluate the organisms in absence of the inhibitory effects that would arise from use of SSL. Data from the substrate and product inhibition experiments was used together with batch experiments to create a model able to predict the performance of the strains under different conditions (Paper II). Finally, experiments in SSL were performed to evaluate the performance in an industrially relevant substrate (Paper I).

3.2 Carbon source

To efficiently utilise biomass hydrolysates, it is of great importance that the production organism can utilise as many of the available carbon sources and as completely as possible. In the case of hardwood SSL which is of special interest in this thesis, the most predominant carbon sources are the monosaccharides arabinose, galactose, glucose, mannose and xylose, where xylose is the most abundant.

As mentioned in Chapter 3, *A. succinogenes* is a metabolically flexible organism, able to ferment a wide range of substrates. Genes encoding for transporters for several of monosaccharides, including all five of the most common monosaccharides found in SSL, as well as a number of disaccharides, sugar acids and polyalcohols have been identified in *A. succinogenes* (McKinlay et al., 2010). In paper I, the growth characteristics on arabinose, galactose, glucose, mannose and xylose were studied separately and as an industrially relevant mixture (Figure 1). It was found that *A. succinogenes* could grow well and produce succinate at high yields from four out of five of the monosaccharides investigated when tested individually, and some previous work also support this (Table 3.1). Galactose as a sole carbon source did not support growth, and neither was it consumed in experiments with mixed monosaccharides or SSL as substrate (Figure 3.1).

Succinate production by *A. succinogenes* has been studied in both simulated and real corn stover hydrolysate containing, xylose, glucose, arabinose and galactose (Salvachúa et al., 2016a). The results showed utilization of all four sugars, although conversion of galactose was very low. A similar study on *B. succiniciproducens* using the same substrates revealed that it could utilise all four sugars including galactose (Salvachúa et al., 2016b).

Thus, both *A. succinogenes* and *B. succiniciproducens* fulfil the criterion of being able to utilise a majority of the monosaccharides in SSL, and are suitable production organism from that perspective.

3.3 Enhancing carboxylation

Succinate can in principle be formed in the central metabolism through either the oxidative or the reductive route in the TCA cycle. In the metabolism of *A. succinogenes* C4 compounds are formed through the reductive branch of the TCA cycle, starting with oxaloacetate which has been formed from carboxylated PEP. The reason is that the organism lacks a complete TCA cycle and C4 compounds are therefore necessarily formed by the reductive route (McKinlay et al., 2007). The TCA cycle is interrupted leading to that *A. succinogenes* cannot synthesise α-ketoglutarate from glucose (McKinlay et al., 2005). In order to maximise succinate yield, it is of importance that the supply of CO_2
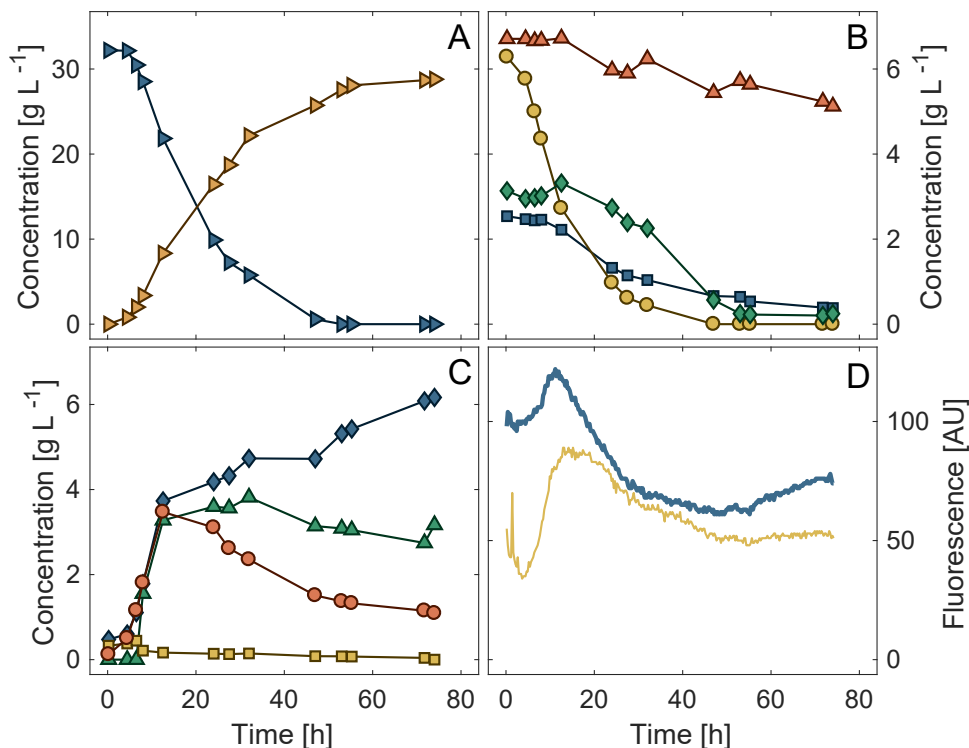
Figure 3.1: A typical fermentation with *A. succinogenes* using 50 g L⁻¹ mixed sugars as substrate and 0.5 vvm $CO-2$ sparging as sole carbonate supply. A – Xylose (blue right-pointing triangle) and succinate (yellow right-pointing triangle). B – Galactose (red upward-pointing triangle), glucose (yellow circle), mannose (green diamond) and arabinose (blue square). C – Acetic acid (blue diamond), formic acid (green upward-pointing triangle), lactic acid (yellow square) and biomass (red circle). D – Measured signal from BioView fluorimeter. Fluorescence at 450 nm from excitation at 350 nm (thick blue line) corresponding to NAD(P)H and back-scatter at 590 nm from excitation with white light (thin yellow line) corresponding to optical density. Fluorescence is reported in arbitrary units.

is sufficient. The fact has been shown not only for *A. succinogenes*, but seems general for many succinate producing organisms (Tan et al., 2014). CO_2 can be supplied to the fermentation broth in two ways, either gaseous CO_2 is sparged through the reactor or a carbonate salt is added. Both modes of CO_2 supply have their advantages and disadvantages.

An advantage of using carbonate salts is that it acts both as CO_2 source as well as a neutralising agent. Among the carbonate salts, $MgCO_3$ used in a majority of studies on *A. succinogenes*, has been found to give the highest succinate yield with glucose as carbon source (Li et al., 2011). Li et al. (2011), however, also argue that $MgCO_3$ is too expensive and found that it could be replaced with a mixture of $Mg(OH)_2$ and NaOH using glucose as carbon source and CO_2 sparging. This agrees well with the findings in Paper I, where $MgCO_3$ could be fully replaced with CO_2 sparging without loss of succinate yield when xylose rich sugar mixture was used. The main disadvantages with

Table 3.1: Comparison of fermentation performance in terms of titer, yield and productivity of succinate under batch, fed-batch and continuous conditions. YE - Yeast Extract, CSL - Corn Steep Liquor, number in brackets indicate concentration in g L^{-1}.

Carbon source	Organism	Process conditions	Nitrogen source	Sugar (g L^{-1})	Titer (g L^{-1})	Yield (g g^{-1})	Productivity (g L^{-1}h)	Reference
Glucose	*A. succinogenes* 130Z	Batch	YE(5)	10	5.6	0.56	0.70	Paper I
Xylose	*A. succinogenes* 130Z	Batch	YE(5)	10	3.94	0.42	0.15	Paper I
Mannose	*A. succinogenes* 130Z	Batch	YE(5)	10	4.07	0.38	0.16	Paper I
Arabinose	*A. succinogenes* 130Z	Batch	YE(5)	10	3.8	0.44	0.12	Paper I
Mixed sugars Xyl, Gal, Glu Man, Ara	*A. succinogenes* 130Z	Batch	YE(5)	50	31	0.71	0.53	Paper I
Mixed sugars Xyl, Gal Glu, Ara	*A. succinogenes* 130Z	Batch	YE(6) CSL(10)	80	47	0.72	1.44	(Salvachúa et al., 2016a)
Mixed sugars Xyl, Gal Glu, Ara	*B. succiniciproducens*	Batch	YE(6) CSL(10)	60	26	0.6	-	(Salvachúa et al., 2016b)
Mixed sugars Xyl, Gal, Glu Man, Ara	*A. succinogenes* 130Z	Fed-batch	YE(5)		26.9	0.55	0.58	(Pateraki et al., 2016a)
Mixed sugars Xyl, Gal, Glu Man, Ara	*B. succiniciproducens*	Fed-batch	YE(5)		34.2	0.65	0.59	(Pateraki et al., 2016a)
Glucose	*A. succinogenes* 130Z	Biofilm	YE(6)	30-	48.5	0.91		(Bradfield & Nicol, 2014)
		Continuous	CSL(10)	60				
Xylose	*A. succinogenes* 130Z	Biofilm	YE(6)	43-	10.9-	0.55-	1.5-	(Bradfield & Nicol, 2015)
		Continuous	CSL(10)	84	29.4	0.68	3.4	

$MgCO_3$ apart from its price and the fact that, in an industrial setting it is impractical to handle high concentrations of insoluble salts as solids are difficult to dose by pumping and sterilise with steam (Pateraki et al., 2016b).

Sparging with CO_2 gas has the advantage that a low-cost CO_2 source can be used. CO_2 is a by-product in several large-scale bioprocesses such as biomass combustion, bioethanol fermentation (Tan et al., 2014) and biogas production (Gunnarsson et al., 2014). In an integrated biorefinery, CO_2 utilisation can increase carbon efficiency through fixation of the CO_2 that otherwise would have been released to the atmosphere. One example of such process integration is production of algal oil by microalgae from CO_2 generated as a by-product in biomass pyrolysis where biomass to fuel conversion could be increased from 55% to 73% by CO_2 utilization (Sharifzadeh et al., 2015). Such process integration can be applied in succinate production as well. A biogas upgrading process has been described, in which succinate fermentation broth of *A. succinogenes* was used to separate CO_2 from raw biogas, resulting in high purity methane gas (95%) at the same time as the

dissolved CO_2 was used to produce succinate (Gunnarsson et al., 2014). The solubility of CO_2, C_{CO_2} in the fermentation broth can be estimated from the partial pressure of CO_2, p_{CO_2} using Henry's law and Henry's constant for CO_2 in pure water (Equation 3.1).

$$C_{CO_2} = p_{CO_2} * \frac{1}{H_0} \tag{3.1}$$

By increasing the partial pressure of CO_2, dissolved CO_2 concentration, and thereby the mass transfer of CO_2 can be increased (Gunnarsson et al., 2014). One way to increase p_{CO_2} is to use pure CO_2 as sparging gas. Unfortunately, CO_2 sparging increases the cost of the reactor since sparging equipment needs to be installed. If the CO_2 is not delivered already in a compressed form, a compressor needs to be installed and powered. It is therefore important to apply a well-controlled sparging strategy. It was reported that a dissolved CO_2 concentration of 36.8% of saturation or more was sufficient to maintain optimal succinate yields under continuous conditions (Herselman et al., 2017).

3.4 Product distribution

Wild-type strains of both *A. succinogenes* and *B. succiniciproducens* are mixed acid fermenting organisms which – as the name implies – means that they produce a mixture of acids. *A. succinogenes* produce, apart from succinate, also acetate and formate and *B. succiniciproducens* produces lactic acid in addition to these. The metabolic pathways show PEP as an important node where the carbon flux is diverted to different pathways (Figure 3.2).

The formation of succinate through the C4 branch of the metabolism is not redox balanced, but requires a net of conversion one mole of NADH per mole of succinate formed. The need for regenerating NADH, prevents the complete conversion of substrate to succinic acid (SA). The NADH requiring formation of succinate from glucose can be schematically written:

$$1 \; Glu + 2 \; CO_2 + 2 \; NADH \rightarrow 2 \; SA + 3\tfrac{1}{3} \; ATP \tag{3.2}$$
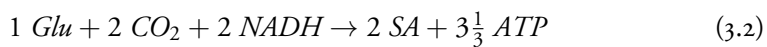
During mixed acid fermentation, this redox deficiency is balanced by the C3 branch of the metabolism, where several alternative pathways are available. The pathway from glucose to acetate (AA) via pyruvate dehydrogenase (PDH) reduces NAD^+ to NADH through the overall reaction:

$$1 \; Glu \rightarrow 2 \; AA + 2 \; CO_2 + 4 \; NADH + 4 \; ATP \tag{3.3}$$
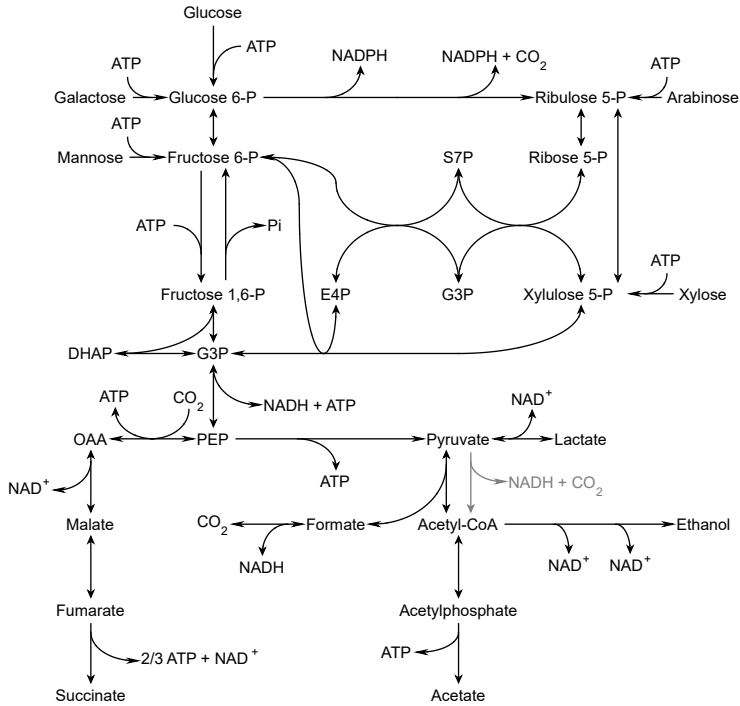
Figure 3.2: Overview of the central carbon metabolism of *A. succinogenes* and *B. succiniciproducens* (Paper I).

Another option for regeneration of NADH is the reaction catalysed by pyruvate formate lyase (PFL), which splits pyruvate into equimolar amounts of formic (FA) and acetic acid (AA). The overall reaction from glucose is in this case:

$$1 \ Glu \rightarrow 2 \ AA + 2 \ FA + 2 \ NADH + 2 \ ATP \tag{3.4}$$

Formate can be further oxidised by formate dehydrogenase (FDH). The combined activity of PFL and FDH however, results in the same overall reaction as PDH.

Lactate (LA) formation is redox neutral.

$$1 \ Glu \rightarrow 2 \ LA + 2 \ ATP \tag{3.5}$$

The maximum theoretical succinate yield under redox constraints is given by a combination of the succinate pathway (equation 3.2) and the PDH pathway (equation 3.3) resulting in an overall reaction of:

$$\tfrac{3}{4} \, Glu + \tfrac{1}{2} \, CO_2 \rightarrow 1 \, SA + \tfrac{1}{2} \, AA \tag{3.6}$$
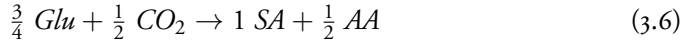
Hence, the maximum theoretical yield of succinate on glucose is 1.25 mol mol^{-1} or 0.87 g g^{-1}, while on xylose the maximum yield is 1.11 mol mol^{-1} or 0.87 g g^{-1}. These calculations are obviously valid in the absence of formation of biomass, i.e. for non-growing cells. In paper I, the effect of biomass formation on redox balance was approximated by calculation to give a small contribution of 3 mmol NADH per g biomass. In paper I, the measured product distribution seemingly violates the redox balance. A similar observation was also reported by (Bradfield & Nicol, 2014, 2015) and a few tentative explanations for this have been proposed. One such is that the fermentation broth might contain reduced compounds which provide the extra reduction potential missing in the calculations but this has not yet been experimentally confirmed. Another explanation is that there are alternative pathways that can produce reducing equivalents. The oxidative pentose pathway (OPPP) has been pointed out as a plausible source, generating NADPH that could be converted to NADH since *A. succinogenes* and *B. succiniciproducens* both have transhydrogenase activity, resulting in an overall reaction of:

$$1 \, Glu \rightarrow \tfrac{5}{3} \, PEP + CO_2 + NADH \tag{3.7}$$

This reaction results in loss of carbon and does not contribute with any ATP – and would as such not be energetically favourable for cell growth. In the current work, the activity of OPPP was measured using an enzymatic activity assay for the first enzyme in the pathway, glucose-6-phosphate dehydrogenase, which was found to be active in cultivations on mixed sugars (Paper I). Even though an active oxidative PPP may theoretically allow homosuccinate fermentation, by-products are almost always found in experiments with both *A. succinogenes* and *B. succiniciproducens*. In mathematical terms, this can be expressed by saying that the "objective function" of cells is normally not to maximise product formation but rather to maximise biomass formation. Indirectly this favours a high ATP generation needed for cell growth. The maximum theoretical yield of ATP under redox constraints is achieved by linear combination of equations 3.2 and 3.3 resulting in 3⁵/₉ mole ATP per mole of glucose.

Four main strategies to decrease by-product formation have been investigated.

- The first is changing substrate to one that is highly reduced such as glycerol which gave a higher succinate yield (1.25 g g^{-1}) in fermentations with *A. succinogenes* (Vlysidis et al., 2011). However, in the current study that was not an available option since the substrate to be used was already set to be SSL

- The second is to introduce extra reduction potential by adding an additional substrate. This has been tried for *A. succinogenes* in the form of hydrogen (Van der Werf et al., 1997), dimethylsulfoxide – DMSO (Carvalho et al., 2014), and also by changing the redox potential of the medium by electrical means using neutral red as mediator (Park & Zeikus, 1999).

- As mentioned earlier, the state of the cell also affects the metabolism. Immobilised, non-growing cell have been reported to give higher yields of succinate from both glucose (Bradfield & Nicol, 2014) and xylose than growing cells (Bradfield & Nicol, 2015) (cf Table 3.1). In paper I, it was also found that during the stationary phase at the end of batch fermentations the succinate yield increased and acetate yield decreased. Formate yield was especially affected and was close to zero.

- The fourth and final strategy to change the metabolic flux is through mutation or metabolic engineering. One such mutated strain of *A. succinogenes* is FZ53. This strain was isolated in screening experiments with fluoroacetate and it produces more succinic acid (105.8 g L^{-1}) and less formate (Guettler & Jain, 1996). Recent metabolic engineering of *A. succinogenes* where pathways for acetate and formate production were knocked out, in combination with overexpression of the reductive TCA-cycle, resulted in an enhanced flux to succinic acid and reduced the yield of the normal by-products acetate and formate. However, other by-products were formed, notably pyruvate and lactate (Guarnieri et al., 2017). Metabolic engineering of *A. succinogenes* is still fairly uncommon, but more work has been done on *B. succiniciproducens*. Deletions of the genes encoding for lactate dehydrogenase and pyruvate formate lyase increase succinate yield, but similar to *A. succinogenes*, new by-products appear, in this case pyruvate (Buschke et al., 2013).

Even though homofermentative production of succinate in *A. succinogenes* and *B. succiniciproducens* is yet to come, important progress has indeed been made. To fully realise homofermentative production, a combination of process engineering and metabolic engineering is needed.

3.5   Substrate and product inhibition

Reaching a *high titer* of the desired product is of great importance to achieve an economically sound process. Purification costs often represent a large fraction of the operating costs, and increase the more dilute the product stream is. Product inhibition sets an upper limit on product titer in the primary fermentation and is therefore important to consider. Apart from the main product, the by-products may be inhibiting and also,

Table 3.2: Critical concentrations from inhibition analysis. Critical concentration is the threshold concentration above which growth is completely inhibited.

Organism	*A. succinogenes*	*A. succinogenes*	*B. succiniciproducens*
Substrate	Glucose	Xylose	Xylose
Reference	Lin 2008	Paper II	Paper II
Critical concentrations in g L^{-1}			
Substrate	155 (Glucose)	160 (Mixed sugars)	150 (Mixed sugars)
Succinate	104.2	55	55
Formate	16	18	22
Acetate	44	38	38
Lactate	-	60	58
Pyruvate	74.1	-	-
Ethanol	42.1	-	-

substrate inhibition need to be considered. In a simple batch process, the substrate inhibition will determine maximum initial substrate concentration, and thus also the final product concentration. However, substrate inhibition can be circumvented by applying a fed-batch or continuous mode of operation, by which the substrate concentration is maintained at a suitable, non-inhibitory level.

In paper II, a sugar mixture - representing sugar levels in an industrially relevant medium - SSL - was used to assess substrate inhibition for the bacteria *B. succiniciproducens* and *A. succinogenes*. It was found that both organisms could tolerate high concentrations of sugars which is important in industrial settings (Table 3.2). Furthermore, the tolerance to the main product, succinate, as well as to all major by-products - acetate, formate and lactate - was evaluated using xylose as substrate (Table 3.2). Formate was the most inhibiting compound followed by acetate. Fortunately, succinate was well tolerated by both strains. Lactate, was also well tolerated by both strains. In table 3.2 the results are compared to a similar study on *A. succinogenes* grown on glucose (Lin 2008). The inhibition effects of acetate and formate were similar for both *A. succinogenes* and *B. succiniciproducens* grown on xylose as well as for *A. succinogenes* grown on glucose. However, a substantially higher tolerance towards succinate for fermentation of glucose was reported - almost twice as high as that found in Paper II. The reason for this difference is not obvious, but could possibly be related to acquired tolerance although the same strain background was used. An important conclusion from the work in Paper II is the fact that the by-products are the most inhibiting, especially formic acid. This adds extra importance to minimising by-products as discussed in section 3.4. By-product formation not only leads to a loss of carbon but also inhibits the production organism.

Table 3.3: Description of variables and parameters for the mathematical model. Critical concentration is the threshold concentration above which growth is completely inhibited. The index i, indicates product: SA, LA, FA, AA.

Symbol	Unit	Description
Variables		
S	g L^{-1}	Substrate concentration (Sum of all sugars)
X	g L^{-1}	Biomass concentration
P$_i$	g L^{-1}	Product concentration
Parameters		
μ	h^{-1}	Specific growth rate
μ_{max}	h^{-1}	Maximum specific growth rate
K$_s$	g L^{-1}	Substrate saturation constant
K$_i$	g L^{-1}	Substrate inhibition constant
P$_i^*$	g L^{-1}	Critical product concentration
n$_i$	-	Inhibition power designation
α	g g-1	Growth associated parameter for product formation
β	g g-1 h^{-1}	Non-growth associated parameter for product formation
γ	g g-1	Growth associated parameter for substrate consumption
δ	g g-1 h^{-1}	Non-growth associated parameter for substrate consumption

3.6   Modelling

Modelling of fermentation processes is a powerful tool which can be used to summarise experimental data, to guide the design of experiments, and to designing actual processes – e.g. feed-profiles in fed-batch cultures. It can also help to give a more quantitative understanding of the characteristics of an organism. In paper II, a simple unstructured model was developed to describe the behaviour of both *A. succinogenes* and *B. succiniciproducens* grown on a xylose-rich monosaccharide mixture, representing an industrially relevant carbon source.

As has been described in section 3.5, both organisms are subject to both substrate and product inhibition. A modified Monod kinetics expression was used, taking both substrate and product inhibition into account. The growth kinetics are described by equation 3.8 and the batch cell growth by equation 3.9. Explanation of parameters are given in Table 3.3.

$$\mu = \mu_{max} \cdot \left(\frac{s}{s + K_s + \frac{s^2}{K_i}} \right) \cdot \prod_{i=1}^{n} \left(1 - \frac{P_i}{P_i^*} \right)^{n_i} \tag{3.8}$$

$$\frac{dX}{dt} = \mu \cdot X \tag{3.9}$$

In paper I it was found that the metabolic flux was different in growing and non-growing cells. Therefore, a Luedeking-Piret model was selected to account for the non-growth coupled metabolism, and substrate consumption and product formation were described using equation 3.10 and 3.11.

$$\frac{dS}{dt} = -\gamma \cdot \frac{dX}{dt} - \delta \cdot X \tag{3.10}$$

$$\frac{dS}{dt} = \alpha_i \cdot \frac{dX}{dt} + \beta_i \cdot X \tag{3.11}$$

Estimation of the kinetic parameters was done based on experimental data from batch fermentations in shake flasks using mixed sugars with a total concentration ranging from about 1 g L^{-1} to about 70 g L^{-1}. The critical product concentrations in the model were based on experimental data presented in Table 3.3. The rest of the parameters were estimated by minimising the objective function (Eq 3.12)

$$G(pp) = \sqrt{\sum_{e=1}^{n_e} \sum_{v=1}^{n_v} \sum_{p=1}^{n_p} \left(w_{evp} \cdot \left(Q_{evp}^{pred} - Q_{evp}^{exp} \right) \right)^2} \tag{3.12}$$

Where G is the root of the weighted sum of squared error between predicted values, Q_{pred}, and measured values, Q_{exp}. The parameters are labelled pp, n_e are the number of experiments, n_v are the number of variables, and n_p are the number of measurement points. The weight of each datapoint (w_{evp}) was calculated using equation 3.13 to avoid overfitting of low concentration where relative measurement errors are larger than at high concentrations.

$$w_{evp} = \frac{1}{Q_{evp}} \tag{3.13}$$

The model was then *validated* by predicting the outcome of bioreactor experiments (Figure 3.3). It was found that the model could predict the outcome of the bioreactor experiment for both *A. succinogenes* (R^2 = 0.96) and *B. succiniciproducens* (R^2 = 0.94). Since the parameters, estimated in shake flask experiments with 250 ml working volume, were valid for describing bioreactor experiments in 1 L working volume, the model was robust enough to cope with both differences in scale and reactor design. This is promising results, indicating that the model can be used in the development of succinate production from xylose rich streams.

Other kinetic models of *A. succinogenes* do exist, but they are few. A model for *A. succinogenes* was developed for succinate production from glucose (Lin et al., 2008). The model was developed using results from experiments using glucose (0-160 g L^{-1}) in small scale bioreactors and data from inhibition experiments. It could predict not only scaled-up experiments in normal sized bench-top reactors where glucose (85 g L^{-1}) was used as
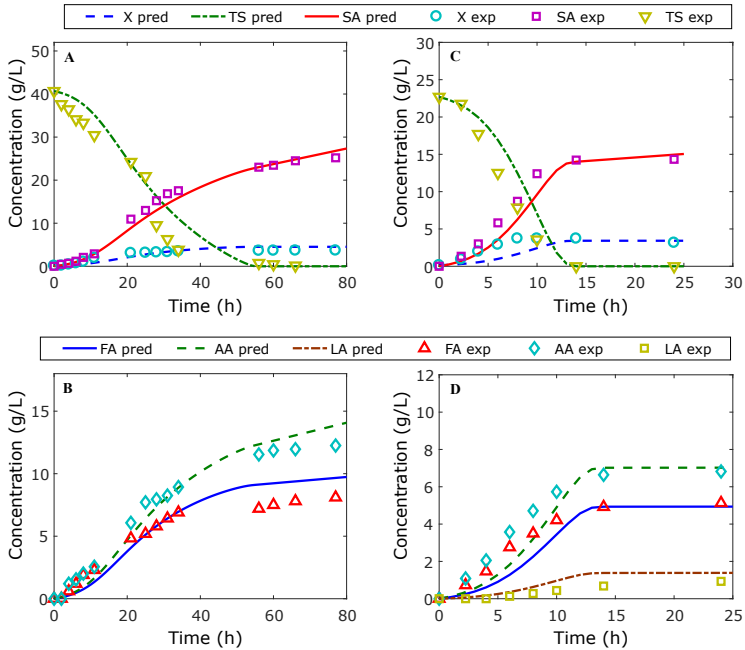
Figure 3.3: Validation experiment in scaled-up system for *A. succinogenes* (Panels A & B) and *B. succiniciproducens* (Panels C & D). Comparison of predicted outcome from the model (lines) and experimental data (points).

substrate, but also experiments where wheat flour hydrolysate (100 g L^{-1} glucose) was the substrate. A similar model was developed for succinate production from glycerol using *A. succinogenes* (Vlysidis et al., 2011). The model based on small scale bioreactor experiments with 70 ml working volume, could well predict the outcome of experiment in larger 1.8 L reactors at several initial concentrations of glycerol ranging from 9.5 to 31.0 g L^{-1}. A kinetic model of *M. succiniciproducens* for growth on glucose, has been reported (Song et al., 2008), but no kinetic models have so far been reported For *B. succiniciproducens* except the one described in Paper II.

3.7 Tests in industrially relevant medium

As discussed in Chapter 2, it is of interest to utilise lignocellulosic feedstocks for production of biochemicals. Succinate production with *A. succinogenes* and *B. succiniciproducens* from a range of agricultural hydrolysates, such as corn stover and sugar cane bagasse, has been investigated but there is to the author's knowledge not much work reported on wood hydrolysates (Table 3.4)

In Paper I, spent sulphite liquor (SSL) from sulphite pulping of *Eucalyptus* (Llano et al.,

2012) was evaluated as a substrate. A range of inhibiting compounds are present in the SSL, including furfural, HMF, methanol, acetic acid, phenolic compounds and lignosulfonates (Pateraki et al., 2016a). Addition of a combination of HMF (0.17 g L^{-1}), furfural (1.7 g L^{-1}) and acetic acid (5.8 g L^{-1}) to synthetic medium has been shown to give an extended lag-phase and lowered productivity in *A. succinogenes* (Salvachúa et al., 2016a). The same study concluded that other inhibitors e.g. phenolics and xylooligosaccharides are important since corn stover hydrolysate with the same amount of HMF, furfural and acetic acids was more inhibiting than the synthetic medium.

In the present work, to minimise the inhibitory effect, it was decided to develop a fed-batch process scheme. In a fed-batch process, the inhibitor concentration is low at the beginning of the process. Gradually, more SSL is fed to the reactor as the fermentation progresses which allows the cell culture to adapt and also to partially detoxify the medium by action of various enzyme systems. For example *A. succinogenes* is able to convert furfural into the less toxic furfuryl alcohol (Salvachúa et al., 2016a). The challenge in any fed-batch process is to determine a suitable feed rate profile, i.e. feeding as fast as the culture tolerates - but not faster. A fixed feed rate was tested, but that resulted in overfeeding in the beginning of the process and underfeeding towards the end of the process. Overfeeding will give an unnecessarily high inhibitor concentration, and as a result will also give residual unconsumed sugars in the broth at the end.
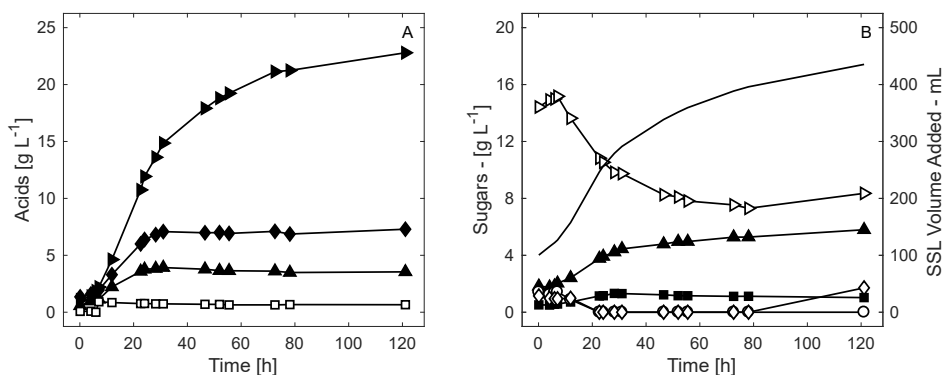
Figure 3.4: Fermentation profile of fed-batch experiment with *A. succinogenes* using SSL as substrate. SSL feed was proportional to acid production. A - Succinate (black right-pointing triangle), acetic acid (black diamond), formic acid (white upward-pointing triangle), lactic acid (white square). B - Xylose (white right-pointing triangle), galactose (black upward-pointing triangle), glucose (white circle), mannose (white diamond) and arabinose (black square). SSL feed volume is shown as a black line.

As neither substrate nor product could be effectively measured on-line in the complex medium used in Paper I, an indirect measurement of substrate consumption or product formation was needed. Formation of the products, which are all acids, will lead to acidification of the medium which is monitored by a pH electrode. In a pH-controlled fermentation, this acidification is continuously counteracted by feeding a base solution

to the reactor. By measuring the amount of base fed to the reactor and the amount of substrate consumed in earlier batch experiments, a correlation factor between the amount of base pumped and substrate consumed was calculated. This factor was then used to design a closed-loop controller for the feed pump using the signal from the base pump. The strategy proved very effective in keeping the substrate concentration within a suitable range in the bioreactor during the whole fermentation (Figure 3.4).

The results show that an initial lag-phase, which would be found in a batch fermentation, was avoided. The concentration of the most abundant substrate, xylose, was maintained between 8 and 16 g L^{-1} at all times. Glucose and mannose were quickly consumed and remained low, except for a small increase of mannose at the very end of the experiment. Arabinose concentration was kept constant at all times. Interestingly, galactose was partly utilised in this experiment. The degree of utilization was however low, leading to accumulation of galactose during the fermentation. By-products in form of acetate and formate were formed mainly during the initial phase after which the formation rate match the rate of dilution due to feeding. Succinate, however, was produced during the whole fermentation and reached a final titer of 22.7 g L^{-1}. The yield and titer of succinate in SSL experiment were similar to those found in synthetic medium. However, the productivity was markedly lower, demonstrating the inhibitory effect of the SSL.

In another study performed within the same project, SSL was pre-treated through ultrafiltration or nanofiltration to remove inhibiting compounds Pateraki et al. (2016a). The filtrate was then fermented using both *A. succinogenes* and *B. succiniciproducens* in a fed-batch setup. Yield and titer for *A. succinogenes* were similar to what was reported in paper I, but productivity was improved (Table 3.4). Compared with experiment on other hydrolysates presented in Table 3.4, the SSL fermentation was comparable in yield and titer, even if the titer was in the lower range. The volumetric productivity was however, notably lower. This may indicate that SSL is a difficult feedstock to use but there may be other causes. Initial biomass concentration has a pronounced effect on volumetric productivity. Addition of corn steep liquor is also typical in many of the experiments which can improve volumetric production though increased biomass formation, although at expense of increased costs for media preparation.

3.8   Remarks on natural producers

In this thesis, the natural succinate producing bacteria *A. succinogenes* and *B. succiniciproducens* were evaluated for production of succinate from xylose rich substrates.

- High yields of succinate were obtained from xylose rich sugar mixtures using *A. succinogenes*, in both synthetic medium (0.71 g g^{-1}) as well as in SSL (0.60 g g^{-1})

Table 3.4: Examples of succinate fermentations using hydrolysates as substrate. YE - Yeast Extract, CSL - Corn Steep Liquor, number in brackets indicate concentration in g L^{-1}

Carbon source	Organism	Process conditions	Nitrogen source	Titer (g L^{-1})	Yield (g g^{-1})	Productivity (g L^{-1}h)	Reference
SSL	A. succinogenes	Fed-batch	YE(5)	22.7	0.60	0.18	Paper I
Pretreated SSL Nanofiltration (500 Da MWCO)	A. succinogenes	Fed-batch	YE(5)	25.2	0.52	0.47	Pateraki 2016
Pretreated SSL Nanofiltration (500 Da MWCO)	B. succinicproducens	Fed-batch	YE(5)	33.8	0.58	0.48	Pateraki 2016
Corn stover hydrolysate	A. succinogens	Batch	YE(6) CSL(10)	~15	0.52	0.74	Salvachua 2016 a
Corn stover hydrolysate deacetylated	A. succinogens	Batch	YE(6) CSL(10)	43	0.74	0.27	Salvachua 2016 a

- The obtained succinate titer using *A. succinogenes* in SSL (22.7 g L^{-1}) was lower than the obtained in synthetic medium (31 g L^{-1}). Improving the tolerance to SSL is therefore an attractive strategy to obtain higher succinate titers.

- Volumetric productivity of *A. succinogenes* was the factor most negatively influenced by SSL (0.18 g L^{-1} h^{-1}) compared with synthetic medium (0.53 g L^{-1} h^{-1}). Higher cell density has a potential to improve the productivity. This could be achieved by immobilisation of the cells which has proved to enable high productivity in synthetic medium (Bradfield & Nicol, 2015).

- Formation of by-products was influenced by growth and it was found that non-growing cells produces less by-products. Limiting by-product formation is important, since these not only decrease the succinate yield but also act as inhibitors

- Cultivation of the bacteria requires complex medium which can become costly in an industrial process. Optimisation of the supply of complex nutrient would therefore be of great value for a potential industrial application.

Chapter 4

Succinate and malate from xylose in engineered *S. cerevisiae*

In the previous chapters, work on production of succinate from xylose by natural hosts was discussed. In this chapter we will turn to the use of metabolically engineered yeast, S. cerevisiae, for production of the dicarboxylic acids malate and succinate. As previously mentioned (cf Table 2.6), metabolic engineering efforts for the purpose of production of C4-dicarboxylic acids in *S. cerevisiae* has targeted production of malate or succinate from glucose primarily. Production from xylose has been looked at using other engineered organisms, e.g. *E. coli*, (Liu et al., 2012) and also the yeast *P. kudriavzevii* (Rush & Fosmer, 2014), which naturally utilises xylose (Ruyters et al., 2015). Engineered *E. coli* has also been tested in lignocellulosic hydrolysates such as sugar cane bagasse and corn stalk (Table 2.6).

4.1 Engineering of *S. cerevisiae*

In Paper III, characterisation of an engineered *S. cerevisiae* (Stovicek et al. in preparation) for conversion of xylose to dicarboxylic acids was made. Here, a short description of the needed engineering is first given, and the fermentation results are then discussed. The metabolic engineering of *S. cerevisiae* has to address two main issues: a) to enable xylose conversion in *S. cerevisiae*, b) to enable overproduction (and secretion) of di-carboxylic acids, starting from pyruvate or PEP. It is essential to use a suitable host yeast strain, which is robust towards the substrate to be used (Almeida et al., 2011). The tolerance of a number of yeast strains were tested towards a range of industrial substrates, including spent sulphite liquor from both bagasse and *Eucalyptus*, as well as spruce and pine hy-

drolysates, in the BioREFINE-2G project (Elin Johansson, unpublished). The industrial yeast strain Ethanol Red (ER), was found to be tolerant to most of the tested substrates and was selected as a suitable host organism for SSL conversion.

4.1.1   for xylose utilization

The possibility to engineer *S. cerevisiae* for xylose utilization has been investigated since the 1990s (Kötter & Ciriacy, 1993; Tantirungkij et al., 1993), primarily in the context of ethanol production from lignocellulosic material (recently reviewed by e.g. (Sànchez Nogué & Karhumaa, 2015)). The engineering required for efficient xylose conversion is in fact rather extensive, but fundamental is to achieve conversion of xylose to xylulose, which then enters the PPP. The initial conversion can be achieved through heterologous expression of genes encoding either a two-step conversion starting with reduction of xylose to xylitol, followed by an oxidation of xylitol to xylulose, or a one-step isomerisation of xylose to xylulose. The former pathway is typically fungal, whereas the latter pathway is typically bacterial – although exceptions exist. Large research efforts have been spent on both these pathways, and commercial xylose fermenting yeast strains are today a reality Sànchez Nogué & Karhumaa (2015). In addition to introducing the critical missing conversion of xylose into xylulose, several additional modifications in the PPP are necessary to remove metabolic bottle-necks and reach reasonable conversion rates. This has been achieved through overexpression of five enzymes in the PPP; xylulokinase, ribulose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transketolase and transaldolase (Kuyper et al., 2005). Also xylose transport is an issue. Xylose is taken up with low affinity glucose transporters (Farwick et al., 2014).

4.1.2   for carboxylic acid production

The second part of the engineering needed, aims at channelling the carbon flux towards dicarboxylic acid production, and also to enable its secretion. The dicarboxylic acids can be obtained through three main routes; the TCA cycle operating reductively, the TCA cycle operating oxidatively, or the glyoxylate pathway (Abbott et al., 2009). The maximal theoretical yield of malate from glucose varies depending on the route used, and is 2 mol mol^{-1} for the reductive TCA route, 1 mol mol^{-1} for the oxidative TCA route and 1–1^{1}/3 mol mol^{-1} for the glyoxylate route, depending on whether oxaloacetate pool is replenished from the glyoxylate cycle or from carboxylation of pyruvate. In the strain used in paper III, overexpression of the reductive TCA cycle in the cytosol was made according to the strategy described by (Zelle et al., 2008), i.e. the native PYC2 and malate dehydrogenase (the peroxisomal MDH3, retargeted to the cytosol) were overexpressed to increase the flux from pyruvate to malate. In addition, a documented well performing
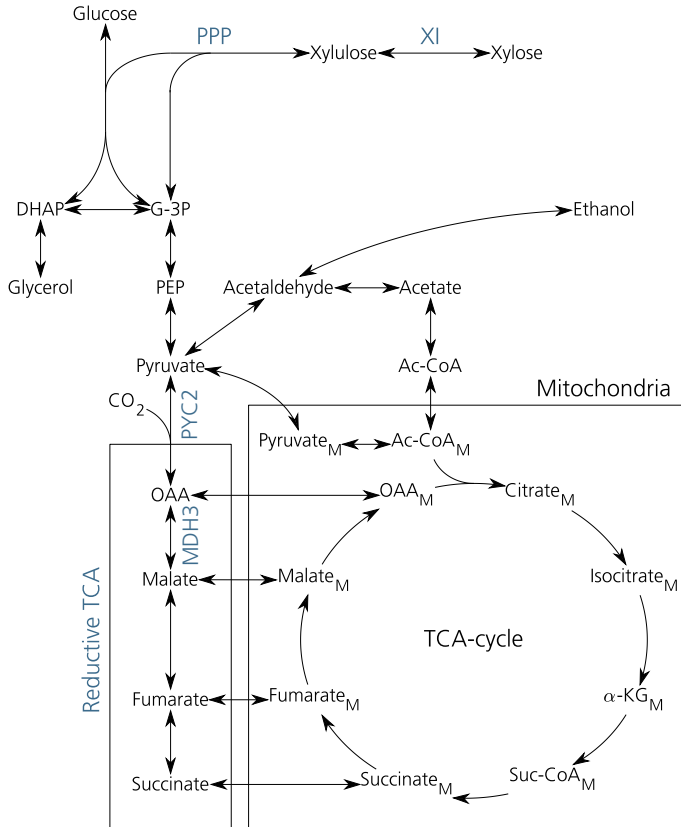
Figure 4.1: Overview of the central carbon metabolism of engineered *S. cerevisiae*.

dicarboxylic acid transporter (SpMAE1) from *Schizosaccharomyces pombe* was introduced (Zelle et al., 2008). The engineering made is summarised in Figure 4.1.

4.2 Process conditions for improved dicarboxylic acid production

Process conditions have a large impact on carboxylic acid production in natural hosts. It deserves to be pointed out that there is a major principal difference between xylose conversion to ethanol and xylose conversion to malate and succinate. Ethanol is the primary anaerobic end-product, and its formation is directly driven by cellular needs for ATP and co-factor balancing. The carboxylic acid production, on the other hand, is not an end product coupled to ATP production. In contrast, the ATP required for transport likely results in a net need of ATP for production of malate and succinate (Abbott et al.,

2009). Overproduction and secretion in natural hosts typically occur under conditions at which biomass growth is restrained or limited by availability of an essential nutrient. This could be e.g. a trace metal (as iron) or a main component, such as the nitrogen source. For example, carboxylic acid production in Aspergillus spp are often governed by parameters such as concentration of the carbon and nitrogen sources (Yang 2017). Also, fumarate formation in *Rhizopus spp* is increased by nitrogen limitation (Roa Engel et al., 2008).

An alternative use of the carbon source under aerobic conditions when growth is constrained is obviously respiration. *S. cerevisiae* is known to increase respiration of glucose when the available glucose exceeds the minimum requirements for biomass formation in nitrogen limited experiment under aerobic conditions Larsson et al. (1993).

An important factor to drive the carbon flux towards the reductive TCA cycle is a highly active carboxylation. In natural succinate producing organisms such as *A. succinogenes*, carboxylation is important in order to achieve high yields of succinic acid (Paper I). Formation of oxaloacetate from PEP by carboxylation catalysed by PEPCK is the first step in the C4 branch of metabolism enabling formation of succinate. Also in the current metabolic engineering strategy for *S. cerevisiae*, a carboxylation reaction is desired, in this case catalysed by PYC2 that carboxylates pyruvate to form oxaloacetate (Paper III). However, two main differences remain. Firstly, *S. cerevisiae*, in comparison to *A. succinogenes* harbours a complete TCA cycle, and secondly the cultivation is performed under aerobic conditions to enable use of the TCA cycle.

Both conversion rates and yields of dicarboxylic acids are thus likely affected by; limitation of biomass formation (e.g. by nutrients); controlling respiration (e.g. by oxygen limitation) and a well functioning carboxylation (affected by supply of carbonate/CO_2).

4.2.1 Enhanced carboxylation through CO_2 sparging

An increased concentration of CO_2 in the medium could enhance use of the reductive TCA route which has the highest theoretical yield (Zelle et al., 2008). In paper III, enrichment of CO_2 in the sparging gas was tested at two levels, 10% and 50%, and product distribution were compared to fully aerobic conditions. 10% CO_2 enrichment had little effect, whereas enrichment by 50% CO_2 notably improved the yield of succinate (Figure 4.2). Apparently, the CO_2 formed from respiration will not be enough to provide CO_2 for the carboxylation, and an additional supply to the medium is needed. Apart from direct effects on the carboxylation, it has also been hypothesised that the elevated CO_2 concentration may inhibit succinate dehydrogenase thereby preventing re-oxidation of succinate to fumarate and increasing the succinate yield (Zelle et al., 2010).
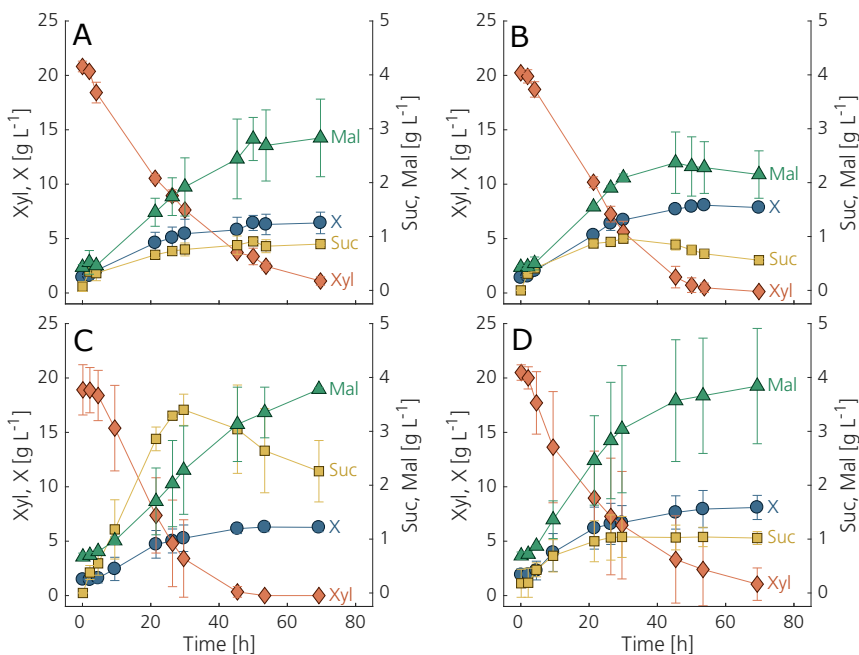
Figure 4.2: Experiments with *S. cerevisiae* engineered for dicarboxylic acid formation from xylose. Xylose (Xyl), Biomass (X), Succinte (Suc) and Malate (Mal). Aerobic conditions (Panel A), 10% CO_2 sparging (Panel B), 50% CO_2 (Panel C) and oxygen limited conditions (Panel D).

The summary of industrial succinate processes in table 2.6 shows that most processes have in common that additional CO_2 is supplied in various ways. For *E. coli*, CO_2 is supplied in the form of K_2CO_3 whereas for the two yeasts, *S. cerevisiae* and *P. kudriavzevii* gaseous CO_2 is used (Ahn et al., 2016). The mode of CO_2 supply is determined in large by the pH of the process. In carboxylic acid production, a low pH (3-5) is desired. The equilibrium of carbonate prevents the use of carbonate and bicarbonate salts below a pH value of about 6, due to the pK_a (6.3) of the hydrogen carbonate ion (Figure 4.3).

4.2.2 Oxygen limitation

The high CO_2 concentration (50%) in the sparging gas may also affect the cells by decreasing the available oxygen causing oxygen limitation. It has been reported that moderate oxygen limitation was beneficial for malate production in engineered *S. cerevisiae* (Zelle et al., 2010). Oxygen limitation in *Yarrowia lipolytica* engineered for succinate production has also been shown to increase succinate yield (Jost et al., 2014). When oxygen is limited, the carbon source cannot be fully respired and more carbon will be available for formation of dicarboxylic acids. Oxygen limitation was tested in bioreactors by controlling the dissolved oxygen tension (DOT) to 1-2% but had no effect on succin-
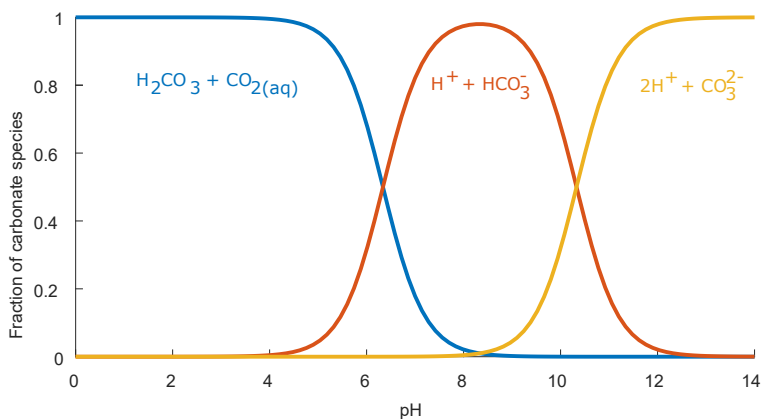
Figure 4.3: Equilibrium of carbonate species depending on pH.

ate yield and only a small increase in malate yield was found (Figure 4.2). Thus, the effect of increased CO_2 levels to 50% could not be attributed to reduced oxygen availability.

4.2.3   Limitation of biomass formation by nitrogen limitation

As the formation of dicarboxylic acids in the engineered strain is coupled to the respiratory metabolism, nitrogen limitation was studied as a means of increasing dicarboxylic acid yields. Little effect on dicarboxylic acids yield was however found, and the limitation of nitrogen simply led to a decreased uptake of xylose.

4.3   Production based on spent sulphite liquor

For use in a lignocellulosic-based industrial process, not only must the producer organism be able to utilise the substrate and produce the products, it must also have good tolerance to the substrate matrix. In Paper III, spent sulphite liquor was used to assess the feedstock tolerance (Figure 4.4). The SSL supplied by Borregaard (Sarpsborg, Norway), was produced through calcium based acid sulphite pulping of *Eucalyptus spp* and had been concentrated by evaporation to a dry matter content of over 700 g L^{-1}. The majority of the dry matter is made up of lignosulfonates but the SSL also contains 100 g L^{-1} of monosaccharides where xylose (80 g L^{-1}) is the most prominent.

In contrast to the malate-overproducing strain by (Zelle et al., 2008), the current strain has a functional pyruvate dehydrogenase (PDC), and thus ethanol is produced from the overflow metabolism of glucose if this is present in the medium. This is indeed the
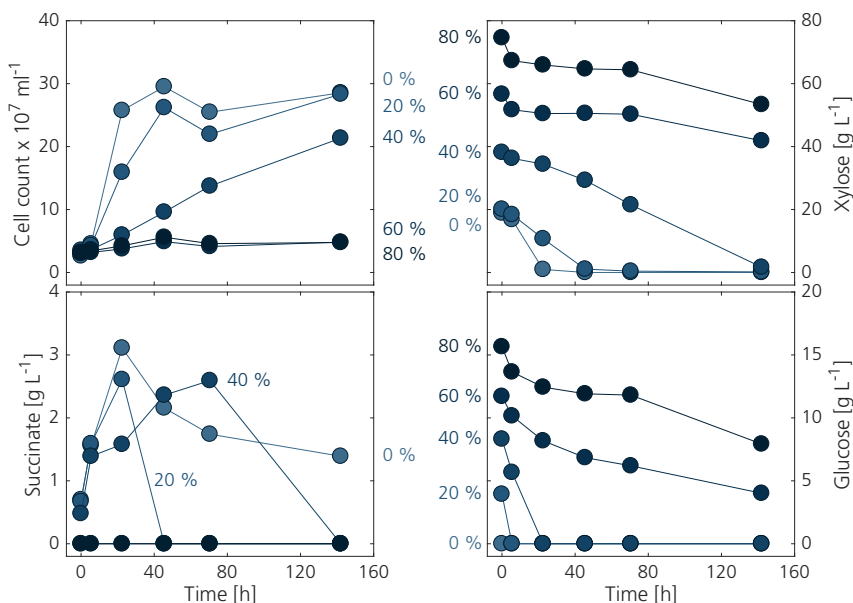
Figure 4.4: Tolerance testing of *S. cerevisiae* in a series of dilutions of SSL from 0-80%. The 0% experiment was supplemented with xylose.

case in SSL, although the xylose concentration is approximately five-fold higher. It was found that the strain could consume all glucose and xylose within 150 hours in SSL up to 40% (cf. Figure 4.4). At 60 and 80% the growth was severely inhibited but part of the glucose was consumed as well as small amounts of xylose. Succinate was the main product reaching concentrations above 2.5 g L^{-1} in all cases up to 40% SSL.

S. cerevisiae has previously been used to produce ethanol from SSL carbohydrates. *S. cerevisiae* isolated from sulphite pulping plants has shown high tolerance to SSL due to long term adaptation (Linden et al., 1992; Sànchez I Nogué et al., 2012). It has also been found that short term adaption and adaptive lab evolution of *S. cerevisiae* to common lignocellulosic inhibitors such as acetate, furaldehydes and phenolics at low pH (3.7) can effectively improve the tolerance to such compounds (Narayanan et al., 2016). One of main the inhibitors in the SSL was acetic acid ($\sim$10 g L^{-1}). The mechanism behind the inhibitory effect of acetic acid and other weak acids has been described as caused by uncoupling and intracellular anion accumulation (Almeida et al., 2007). Since acetic acid is a weak acid it is partially present in its un-dissociated form which can diffuse across the cell membrane. The effect is more pronounced at low extracellular pH since the equilibrium of weak acids is shifted towards the un-dissociated form. Intracellular accumulation of weak acids causes acidification and the cell responds by pumping protons out of the cell at an expense of ATP. Thus, less ATP is available for growth. Another group if inhibitors often found in lignocellulosic hydrolysates are phenolics that are re-

leased from depolymerisation of lignin (Almeida et al., 2007). The main mechanism of inhibition is reported to involve damage to the cell membrane and negative influence on DNA repair mechanisms (Sandström et al., 2014). Since HMF (<0.5 g L^{-1}) and furfural (<0.1 g L^{-1}) concentrations where low in the SSL, they are not believed to contribute to the inhibiting properties of the SSL.

4.4 Remarks on engineered *S. cerevisiae*

The engineered strain of *S. cerevisiae* tested in this study could produce malate and succinate from xylose. It was found that:

- Carboxylation by sparging with gaseous CO_2 has a clear positive effect on the carboxylic acids yield, mainly by increasing the yield of succinate.

- The distribution between succinate and malate changes with the carboxylation conditions. If re-consumption of excreted acids occur, succinate is preferred over malate.

- The evaluated strain could grow in SSL up to 40% and produced succinate from the sugars. However, the acids were re-consumed by the strain if given long enough time.

Chapter 5

Investigation of the Weimberg pathway from *Caulobacter crescentus* for xylose utilization in *Saccharomyces cerevisiae*

As has been discussed in Chapter 4, xylose utilization in the yeast *S. cerevisiae* has been extensively studied in relation to lignocellulosic ethanol production. In these studies, the heterologous pathway introduced has been either the XR/XDH or the XI pathway. Another, and much less studied, xylose utilization pathway is the so-called Weimberg pathway found in a number of bacteria and archaea. In this thesis work, the Weimberg pathway was investigated in both *Caulobacter crescentus*, a bacterial species that naturally carries the pathway (Paper V) and in engineered *S. cerevisiae* (Paper IV). A rapid liquid chromatography based analysis method for some of the pathway metabolites (Paper VI).

5.1 The Weimberg pathway

The Weimberg pathway, named after a paper by the researcher Ralph Weimberg at USDA, was first described in the bacterium *Pseudomonas fragi* (Weimberg, 1961). It has later been found in bacteria such as *Caulobacter crescentus* (Stephens et al., 2007), *Pseudomonas taiwanensis* (Köhler et al., 2015) and *Azospirillum brasilense* (Watanabe et al., 2006a,b) as well as in archaea such as *Haloferax vocanii* (Johnsen et al., 2009) and *Sulfolobus sulfaticus* (Brouns et al., 2006). So far, the pathway has not been found in fungi (Moysés et al., 2016).

The Weimberg pathway consists of five reactions converting xylose to α-ketoglutarate. (Figure 5.1). The first step of this oxidative pathway is the oxidation of xylose to xylono-lactone – a reaction catalysed by the enzyme xylose dehydrogenase which uses NAD$^+$ as cofactor. The second reaction is a ring opening of the lactone (via hydrolysis) to form (linear) xylonate. This is catalysed by xylonolactone lactonase. The third step is a dehydration of xylonate to 2-keto-3-deoxyxylonate – catalysed by xylonate dehydratase. This is followed by a second dehydration step, catalysed by 2-keto-3-deoxyxylonate dehydratase, to form α-ketoglutarate semialdehyde. Finally, there is a second oxidation step, in which α-ketoglutarate semialdehyde is oxidised to α-ketoglutarate by an NAD$^+$-dependent α-ketoglutarate semialdehyde dehydrogenase.

Some organisms use the Weimberg pathway also to degrade arabinose, an isomer of xylose (Watanabe et al., 2006a,b). The difference between xylose and arabinose is the steric configuration of the hydroxyl groups on carbon atom 2 and 3 counting from the aldehyde side. The chirality of these carbons atoms is lost in the third step of the Weimberg pathway when 2-keto-3-deoxyxylonate is formed. Another xylose pathway, called the Dahms pathway shares the three first steps with the Weimberg pathway. 2-keto-3-deoxyglutarate is then split by an aldolase to pyruvate and glycolaldehyde (Dahms, 1974; Watanabe et al., 2006a,b).

Both xylose and α-ketoglutarate contain five carbon atoms, implying that no carbon is lost in the Weimberg pathway. As two moles of NADH are formed from one mole of xylose, aerobic conditions are necessary to re-oxidise NADH to NAD$^+$. Furthermore no ATP is formed in the pathway, which means that ATP must be supplied from other routes, e.g. by complete oxidation of the generated α-ketoglutarate and NADH via respiration, also under aerobic conditions.

The other two known pathways for xylose degradation (Figure 5.1), i.e. the oxido-reductive pathway (XR/XDH) and the isomerase pathway (XI), have been extensively used to enable xylose utilization in *S. cerevisiae* under anaerobic conditions to allow ethanol production. The oxido-reductive pathway uses two enzymes to produce xylulose from xylose (Karhumaa et al., 2007). First xylose reductase (XR) catalyses the reduction of xylose to xylitol. Xylitol is then reduced to xylulose by xylitol dehydrogenase (XDH). This pathway is common in xylose-utilising fungi such as *Scheffersomyces (Pichia) stipitis* (Kötter & Ciriacy, 1993). Since XR prefers the co-factor NADPH whereas XDH only uses NAD$^+$, a cofactor imbalance can result in xylitol excretion. A multitude of strategies to counteract this imbalance have been developed in order to lower the xylitol formation by either adding a redox balancing route or by engineering the cofactor affinity of XR and/or XDH enzymes.

The isomerase pathway uses only one enzyme, xylose isomerase, that catalyses the isomerisation of xylose to xylulose (Karhumaa et al., 2007). This pathway is more common in
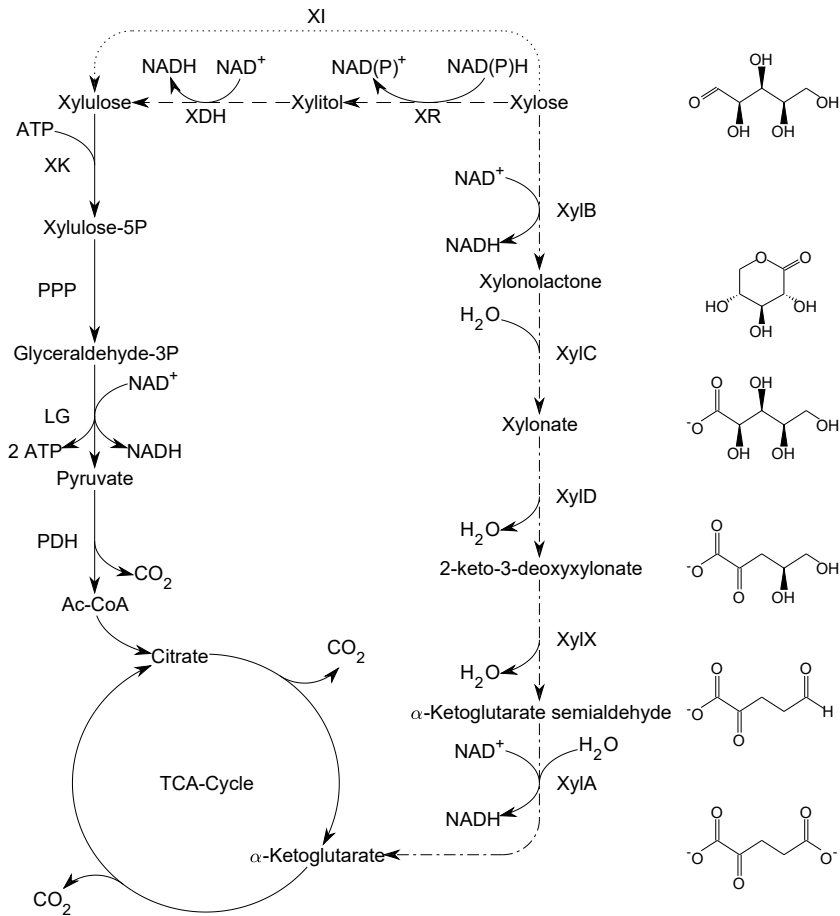
Figure 5.1: An overview of pathways for xylose metabolism and its connections to the central carbon metabolism (-): The Weimberg pathway (-··-·), the XR/XDH pathway (- - -) and the XI pathway (···). XK – xylulokinase, PPP – Pentose Phosphate Pathway, LG – Lower Glycolysis, PDH – Pyruvate Dehydrogenase.

bacteria although it has been found in some fungal species such as *Piromyces sp.* (Kuyper et al., 2005). The main challenge with XI, has been to find an enzyme that exhibits sufficiently high activity in *S. cerevisiae* at the temperatures which allows growth of this organism (Moysés et al., 2016). Both the XR/XDH and the XI pathways require xylulokinase (XK) to convert xylulose to xylulose 5-phosphate, which is then further metabolised in the non-oxidative pentose phosphate pathway (PPP) and the lower glycolysis (LG). These pathways are suitable for anaerobic conditions, since ATP is generated in the glycolysis. However, there is a carbon loss as CO_2 in the conversion of pyruvate (via PDC) or acetyl-CoA (via PDH).

In comparison to the XI or XR/XDH pathways, the Weimberg pathway by-passes the
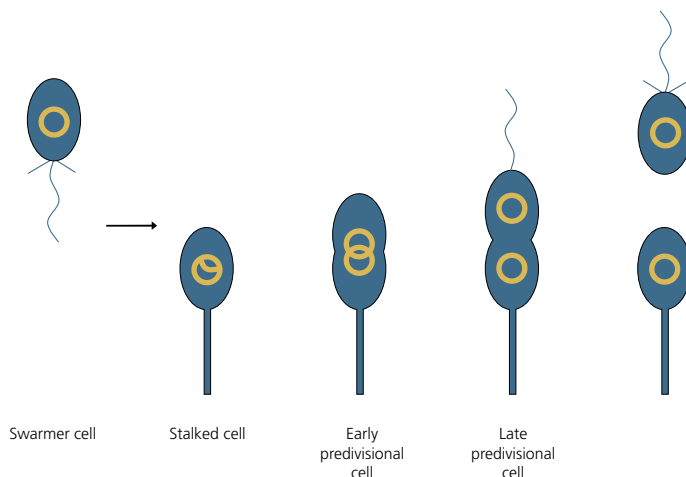
Figure 5.2: The cell cycle of *C. crescentus* starts when a motile swarmer cell sheds its flagellum attaches to a surface by formation of a hold-fast stalk. The cell division is asymmetric resulting in one stalked cell and one swarmer cell. Adapted from (Skerker & Laub, 2004)

PPP and lower glycolysis. As it does not share any metabolites with the other pathways, it can be regarded as an orthogonal pathway (Pandit et al., 2017). Introduction of an orthogonal pathway has the main advantage that the interaction with the normal pathways network is minimised thereby decreasing the risk of negative influence on the cell growth. Also, it allows decoupling product formation from growth, which may be an advantage in the design of production processes. The benefits of separating a process into an efficient biomass accumulation phase and a zero-growth production phase was discussed in a recent review by Lange and coworkers (Lange et al., 2017). Another potential advantage compared to other xylose degradation pathways is that there is no loss of carbon in the Weimberg pathway. Several interesting products, that can be formed within or via the Weimberg pathway, have been identified as discussed in Chapter 2. Xylonate, for example, has been pointed out as a potential platform chemical placed in the extended top 30 list of potential chemicals from the US DoE (Werpy & Petersen, 2004). Many of the TCA-cycle intermediates, e.g. succinate, fumarate, malate, have also been listed as potentially important platform chemicals (Werpy & Petersen, 2004). Furthermore, the Weimberg intermediate α-ketoglutarate semialdehyde can in two steps, be converted to butanediol, using 2-ketoacid decarboxylate followed by alcohol dehydrogenase (Tai et al., 2016). The wide range of product reachable via the Weimberg pathway makes it an interesting metabolic option for xylose utilization in production hosts.

5.2 Physiology of the natural host *Caulobacter crescentus*

C. crescentus is a gram-negative bacterium which habitats nutrient-poor fresh-water eco-systems (Hughes et al., 2012). The asymmetric cell cycle of *C. crescentus* (Figure 5.2) has been studied extensively and is used as a model for understanding the prokaryotic cell cycle (Skerker & Laub, 2004). As a result, its genetics has also been well studied and the genome has been completely sequenced (Nierman, 2001). However, the physiology of *C. crescentus* has not been investigated to a large extent. Understanding the physiology of a natural host carrying the Weimberg pathway and the operation of this pathway in presence of various carbon sources, is likely useful for metabolic engineering of another organism. This was the motivation for the work reported in Paper V, where the Weimberg pathway was characterised in *C. crescentus*.

To characterise *C. crescentus*, growth tests were performed using the pentose sugars xy-lose and arabinose, as well as the hexose glucose as a reference substrate. Glucose is not metabolised in the Weimberg pathway, but rather through the Entner-Doudoroff-pathway (Stephens et al., 2007). As mentioned earlier some organisms metabolise arabinose through the Weimberg pathway. It is however, unclear how arabinose is metabolised in *C. crescentus*, but there are indications that the Weimberg pathway might be involved (Stephens et al., 2007). To investigate this further, arabinose was added as a substrate in this study. Growth was tested in minimal M2 medium supplemented with 5,10 or 20 g L^{-1} of glucose, xylose or arabinose (Figure 5.3). The growth measurements gave two important results:

- Glucose was the best substrate, both in terms of growth rate and final OD. Growth on xylose was slightly slower and resulted in lower final OD. Growth on arabinose was poor with a longer lag-phase, lower growth rates and low final OD.

- All substrates caused inhibition of growth at the highest concentration tested (20 g L^{-1}). *C. crescentus* was also found to be sensitive to increased salt concentration (data not shown). These results indicate that the mechanism behind the inhibition is low osmo-tolerance which correlates well with earlier reports (Kohler et al., 2015). This agrees well with the natural habitat of *C. crescentus* which consists of a highly dilute aquatic environment.

Enzymatic assays were also made to detect the presence of an active Weimberg pathway. Enzyme activity was tested in crude cell extract (Figure 5.4). XylB activity was assayed using either xylose or arabinose as substrate and NAD^+ as cofactor. Activity of the lower part of the pathway, i.e. the combined activity of XylD, XylX and XylA hereafter de-nominated XylDXA, was tested using xylonate as substrate. During growth on glucose, there was no indication of activity in either of the assays, indicating that the Weimberg
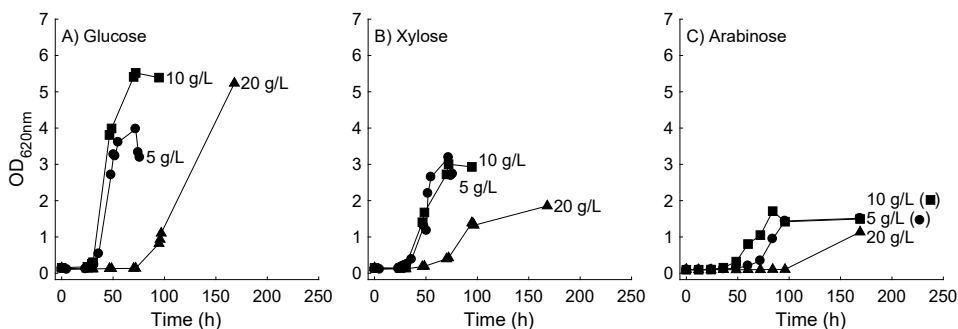
Figure 5.3: Optical density (620 nm) of cultures of *Caulobacter crescentus* in defined M2 medium supplemented with glucose (A), xylose (B) or arabinose (C) as substrate. Growth was monitored in three levels of substrate, 5 g L^{-1} (circles), 10 g L^{-1} (squares) or 20 g L^{-1} (triangles). Data shown in this figure represents experiments performed in biological duplicates.

enzymes are not synthesised on glucose. In contrast, cells grown on either xylose or arabinose showed similar enzyme activity for both substrates, and regardless of which substrate was used to grow the cells. This suggests that the same enzymes (pathway) were used to metabolise both xylose and arabinose.

C. crescentus was also evaluated in bioreactors with glucose or xylose as substrate (Figure 5.5). It was found that *C. crescentus* performed worse in terms of substrate consumption and growth under these conditions than in shake flask cultures even though the medium composition was identical. The variability between experiments was also high. As the main differences between shake flasks and bioreactors are stirring and aeration, *C. crescentus* might be sensitive to these factors. During bioreactor experiments using xylose as a substrate, substantial amounts of α-ketoglutarate were formed. This was not the case when glucose was used as substrate. Formation of α-ketoglutarate usually started during mid-exponential growth phase and continued even after growth has ceased. In one case the excreted α-ketoglutarate was re-consumed after complete consumption of xylose.

The results from the study of *C. crescentus* clearly show that although the organism harbours the Weimberg pathway and is indeed capable of producing and secreting α-ketoglutarate, it is not a suitable production organism. Its low substrate tolerance and slow growth rate is highly unfavourable. The Weimberg pathway, on the other hand, is a very interesting pathway to introduce in other – better suited – cell factories. We have notable indications that the pathway could enable utilization of both xylose and arabinose with one set of genes in e.g. *S. cerevisiae*. In contrast, other strategies for combined xylose and arabinose metabolism in *S. cerevisiae* require one set of heterologous genes for each substrate (Bettiga et al., 2009; Becker & Boles, 2003). The Weimberg pathway thus gives an advantage in cases where both xylose and arabinose are found in the substrate, e.g. in many hemicellulose-derived feedstocks.
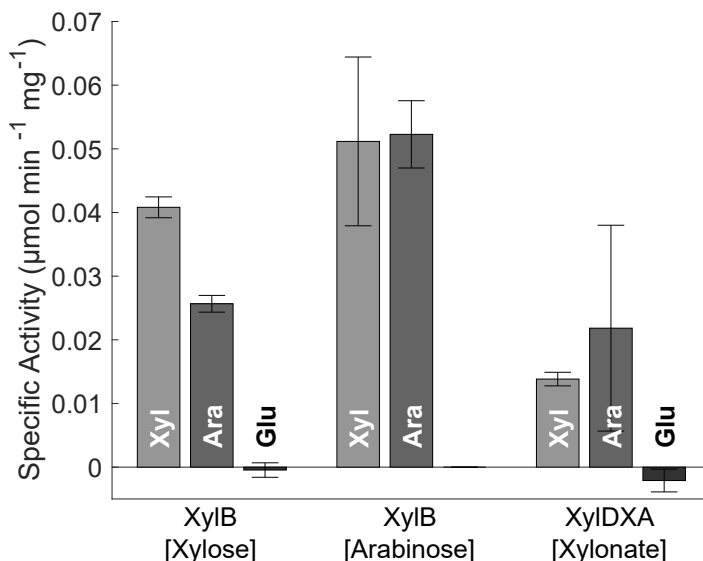
Figure 5.4: Enzyme activity in crude cell extract from *C. crescentus* grown on xylose (Xyl), arabinose (Ara) or glucose (Glu). Xylose dehydrogenase (XylB) was assayed with both xylose and arabinose as substrate and the compound activity of three enzymes (XylDXA), xylonate dehydratase (XylD), 2-keto-3-deoxyxylonate dehydratase (XylX) and α-ketoglutarate semialdehyde dehydrogenase (XylA). Substrates are indicated within square brackets. Error bars represent standard deviation.

5.3 Metabolic engineering for introduction of the Weimberg pathway in heterologous hosts

The Weimberg pathway has already been introduced, completely or partially, in several host organisms such as *Corynebacterium glutamicum* (Radek et al., 2014), *Escherichia coli* (Liu et al., 2012; Cao et al., 2013; Tai et al., 2016), *Pseudomonas putida* (Meijnen et al., 2009), *Saccharomyces cerevisiae* (Nygård et al., 2014; Toivari et al., 2010, 2012) and *Kluyveromyces lactis* (Nygård et al., 2011). A summary of such studies is presented in Table 5.1 and detailed in the text below.

5.3.1 Partial introduction of the Weimberg pathway

Introduction of the upper part of the Weimberg pathway mostly aimed at the production of xylonate. In the fungal species *S. cerevisiae* (Toivari et al., 2012) and *K. lactis* (Nygård et al., 2011), xylonate production required only addition of a heterologous xylose dehydrogenase (XylB from *C. crescentus* in *S. cerevisiae* and Xyd1 from *T. reesei* in *K. lactis*). Both studies reported high yields of xylonate. Interestingly, it was shown that the second step in the Weimberg pathway, i.e. the conversion of xylonolactone to xylonate occurred
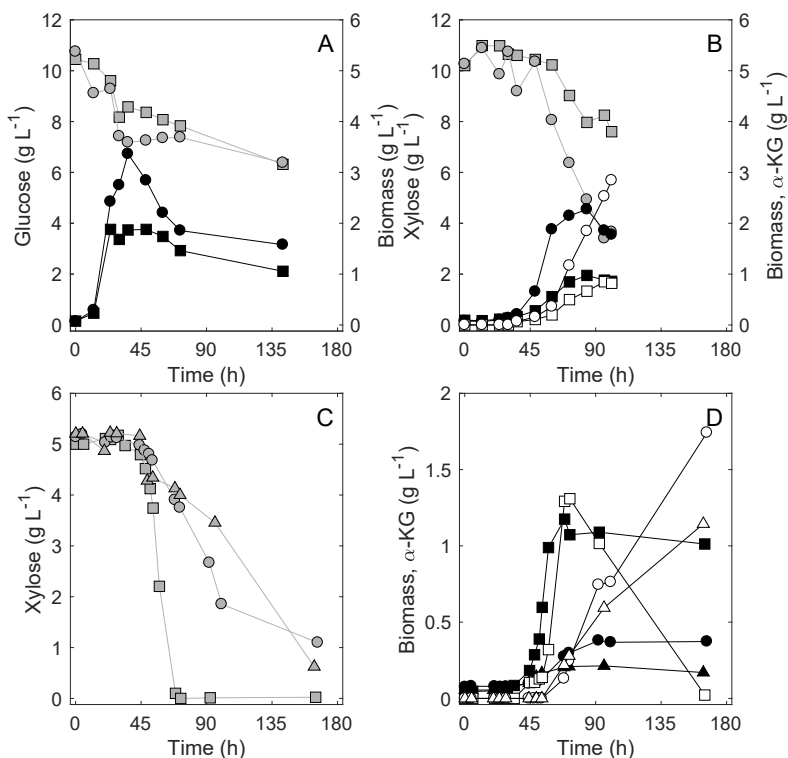
Figure 5.5: Metabolite profile from bioreactor experiments with *C. Crescentus* in three conditions. The substrate was 10 g L^{-1} of glucose (Panel A), 10 g L^{-1} of xylose (Panel B) or 5 g L^{-1} of xylose (Panels C & D). Substrate is marked with grey symbols, biomass with black symbols and α-ketoglutarate (α-KG) with white symbols. Circles, squares and triangle are used to distinguish between different replicates of the experiment.

spontaneously. Hence, introduction of xylonolactone lactonase was not required. It was found that absence of xylonolactone lactonase in *S. cerevisiae* reduced the formation rate of xylonate which simultaneously improved cell viability (Nygård et al., 2014). In *E. coli*, that can naturally metabolise xylose, deletion of the genes for the endogenous xylose pathway, i.e. *Ec xylA* encoding for xylose isomerase and *Ec xylB* encoding xylulokinase were deleted to prevent xylose being channelled to the pentose phosphate pathway (Liu et al., 2012; Cao et al., 2013). Xylonate formation was introduced using genes from *C. crescentus*. In one of the studies only *Cc* XylB was introduced and in the other both *Cc* XylB and *Cc* XylC were introduced (Liu et al., 2012; Cao et al., 2013). Both resulted in high xylonate yield and volumetric productivity. The productivities were much higher than what was achieved in the studies with the fungal producers. However, in terms of xylonate productivity, *E. coli* cannot compete with the natural xylonate producer *G. oxydans* (Buchert et al., 1988).

In one study, the objective of introducing part of the Weimberg pathway, has been to

enable butanediol formation. For that reason, the first four genes from *C. crescentus* Weimberg pathway (*Cc* XylBCDX) was introduced in *E. coli* in combination with two genes (*Kl kivD* and *Ec yqhD*), whose products open up for production of butanediol from the α-ketoglutarate semialdehyde (Tai et al., 2016). Also here the native xylose pathway was blocked by deletion of *Ec xylA*. This resulted in a highly orthogonal pathway for butanediol formation from xylose with a yield of 0.37 g g^{-1}.

5.3.2 Complete Weimberg pathway

The *C. crescentus* complete Weimberg pathway has been introduced in both *C. glutamicum* and *P. putida* (Radek et al., 2014; Meijnen et al., 2009). In both cases specific growth rates comparable with those of *C. crescentus* and *P. taiwanensis* were obtained. It should also be noted that in *P. putida*, it was found that only *Cc* xylD was needed for growth, although at lower rates. Omission of *Cc* XylB and *Cc* XylD had no negative effect on neither growth rate nor biomass yield. In both engineered organisms, as well an in *P. taiwanensis*, xylonate was produced as a by-product during growth on xylose. In all cases the xylonate was re-consumed once xylose was depleted. The bottleneck in these strains thus seemed to be dehydration of xylonate leading to xylonate excretion when xylose uptake exceeds the carbon flux downstream of xylonate, i.e. the carbon flux for growth and ATP production. In contrast, *C. crescentus* did not accumulate xylonate; instead α-ketoglutarate was excreted when xylose uptake exceeded the carbon flux for growth. Balancing the activity of the enzymes in the Weimberg pathway to allow growth without excretion of intermediates, therefore seemed to be difficult to achieve in both engineered organisms as well as native Weimberg organisms. This is of course not necessarily a drawback in case the intermediates are the desired products.

5.4 Establishing the Weimberg pathway in *S. cerevisiae*

Introduction of parts of the Weimberg pathway in *S. cerevisiae* for xylonate production has been investigated previously (Toivari, 2012, Salusjärvi 2017), but heterologous introduction of the full Weimberg pathway has not been reported yet. This was investigated in the present study and reported in Paper IV.

5.4.1 Strain construction

A number of *S. cerevisiae* constructs based on the laboratory strain CEN.PK 113-7D were generated (Table 5.2). A strain with *C. crescentus* Weimberg genes without the xylono-lactone lactonase gene (*xylC*) was a constructed (TMB4530) as well as two strains with a

Table 5.1: Summary of organisms, natural and engineered, harbouring the Weimberg pathway. Weimberg genes are labelled W1–5. *G. oxydans* naturally harbours the Dahms pathway which shares the three reactions with the Weimberg pathway Zhang et al. (2013). Abbreviations indicate origin of genes. *Cc - Caulobacter crescentus, Tr - Trichoderma reesei, Ll - Lactococcus lactis, Ec - Escherischia coli, Lh - Lactobacillus helveticus*

Organism	Metabolic Engineering	Xylose (g L^{-1})	Product (g L^{-1})	Yield	Productivity	Reference
Partial Weimberg						
E. coli	Δ*xylAB* Δ*yagF* Δ*yjhG* ↑↑*Cc xylB* (W1)	40	Xylonate	0.98 g g^{-1}	1.09 g L^{-1} h^{-1}	Liu et al. (2012)
E. coli	Δ*xylAB* ↑↑*Cc xylBC* (W1–2)	30	Xylonate	88%	1.8 g L^{-1} h^{-1}	Cao et al. (2013)
S. cerevisiae	↑↑*Cc xylB* (W1)	49	Xylonate	0.8 g g^{-1}	0.44 g L^{-1} h^{-1}	Toivari et al. (2012)
K. lactis	↑↑*Tr xyd1* (W1)	40	Xylonate (19)	0.60 g g^{-1}	0.158 g L^{-1} h^{-1}	Nygård et al. (2011)
G. oxydans	Natural Dahms*	100	Xylonate	98%	2.5 g L^{-1} h^{-1-1}	Buchert et al. (1988)
E. coli	Δ*xylA* Δ*yjhH* Δ*yagE* ↑↑*Cc xylB-CDX* (W1-4) ↑↑*Ll kivD*(V461I) ↑↑*Ec yqhD*		1,4-butanediol	0.37 g g^{-1}	-	Tai et al. (2016)
S. cerevisiae	↑↑*Cc xylBD* ↑↑*Ec yjhH* ↑↑*Ec yagE* ↑↑*Ec aldA* ↑↑*Lh dhL*	Xylose	Glycolic acid (∼1)	-	-	Salusjärvi et al. (2017)
Complete Weimberg						
C. crescentus	Natural Weimberg	Xylose	Growth		0.11 h^{-1}	Paper V
			α-ketoglutarate	0.43 g g^{-1}	0.03 g g^{-1} h^{-1}	
P. taiwanensis	Natural Weimberg	Xylose	Growth	0.3 g g^{-1}	0.18 h^{-1}	Köhler et al. (2015)
C. glutamicum	↑↑*Cc xylXABCD* (W1-5)	Xylose	Growth	-	0.07 h^{-1}	Radek et al. (2014)
P. putida	↑↑*Cc xylXABCD* (W1-5)	Xylose	Growth	0.53 g g^{-1}	0.21 h^{-1}	Meijnen et al. (2009)
P. putida	↑↑*Cc xylXAD* (W3-5)	Xylose	Growth	0.57 g g^{-1}	0.21 h^{-1}	Meijnen et al. (2009)

partial Weimberg pathway, either upper (TMB4511) or lower part (TMB4515) that were used as controls. It was anticipated that the XylD from *C. crescentus* could be difficult to functionally be expressed in *S. cerevisiae* since it relies on a iron-sulfur (Fe-S) cluster for activity. Therefore, several variants of XylD were tested: one originating from the archaeon *Haloferax volcanii*, which relies on Mg^{2+} instead of Fe-S (TMB4531) and three more XylD from *Burkholderia cenocepacia* (XylD), *E. coli* (YjhG) and an unknown bac-

terium named Ellin329 (XylD) were tested. In addition, XylC was deleted in strains with the full pathway since previous studies showed that XylC could accelerate the formation of xylonate and cause the intracellular pH to drop. (Nygård et al., 2014). Deletion of *gre3*, which encodes for an unspecific aldose reductase known to reduce xylose to xylitol, was also done in all strains with the full pathway.

Table 5.2: List of engineered strains of *S. cerevisiae* with full or partial Weimberg pathway. *Cc* – *C. crescentus*, *Hv* – *H. volcanii*,*Bc* – *B. cenocepacia*, *Ec* – *E. coli*, *El* – Ellin329.

Strain name	Introduced gene(s)	Comments
TMB4511	↑↑*Cc xylBC*	Upper part of the Weimberg pathway
TMB4512	↑↑*Cc xylB*	Upper part except *xylC* (xylonolactone lactonase)
TMB4515	↑↑*Cc xylDXA*	Lower part of the Weimberg pathway
TMB4530	↑↑*Cc xylBDXA* Δ*gre3*	Complete pathway, *xylC* (xylonolactone lactonase)
TMB4531	↑↑*Cc xylBXA* ↑↑*Hv xad* Δ*gre3*	Same as TMB4530 but with *xylD* variant from *H. volcanii*
TMB4569	↑↑*Cc xylBXA* ↑↑*Bc xylD* Δ*gre3*	Same as TMB4530 but with *xylD* variant from *B. cenocepacia*
TMB4570	↑↑*Cc xylBXA* ↑↑*Ec yjhG*) Δ*gre3*	Same as TMB4530 but with *xylD* variant from *E. coli*
TMB4571	↑↑*Cc xylBXA* ↑↑*El xylD*Δ*gre3*	Same as TMB4530 but with *xylD* variant from Ellin329

Although all constructed strains were viable on glucose, none of them were able to grow on xylose as a sole carbon source (Paper IV). Thus, it was concluded that the Weimberg pathway was either not functional or not efficient enough to support growth. Testing in a xylose and glucose mixture showed that only strains where the XylC was omitted were even viable in the presence of xylose. This agrees well with previous reports that XylC increases xylonate formation rate, leading to a level the cells cannot cope with, resulting in intracellular acidification and loss of viability (Nygård et al., 2014). Due to the lack of growth, bioconversion of xylose was tested using in a two-step process. Biomass was first accumulated by growth on glucose after which washed cells were transferred to fresh medium containing xylose as a sole carbon source. All the strains performed similarly and converted the xylose to xylonate within 100 hours.

5.4.2 Characterisation of xylose consumption in combination with feeding of glucose

To further investigate xylose consumption, both with and without glucose, experiments were set up in bioreactors in which pH was maintained at 5.5 to prevent medium acidification. During a first phase, biomass was accumulated by growth on glucose. After glucose was consumed a pulse of xylose was added to study metabolism on xylose as a sole carbon source. The third phase was fed-batch phase were glucose was fed to maintain viability. Two strains, TMB4530 and TMB4531 that only differ by the type of XylD they carry, were tested (Results from TMB4531 are shown in Figure 5.6).

Once the xylose pulse was added the biomass concentration started dropping slowly in both strains, showing that xylose metabolism was detrimental to cell viability. The biomass concentration recovered quickly as soon as the fed-batch phase started. No dif-
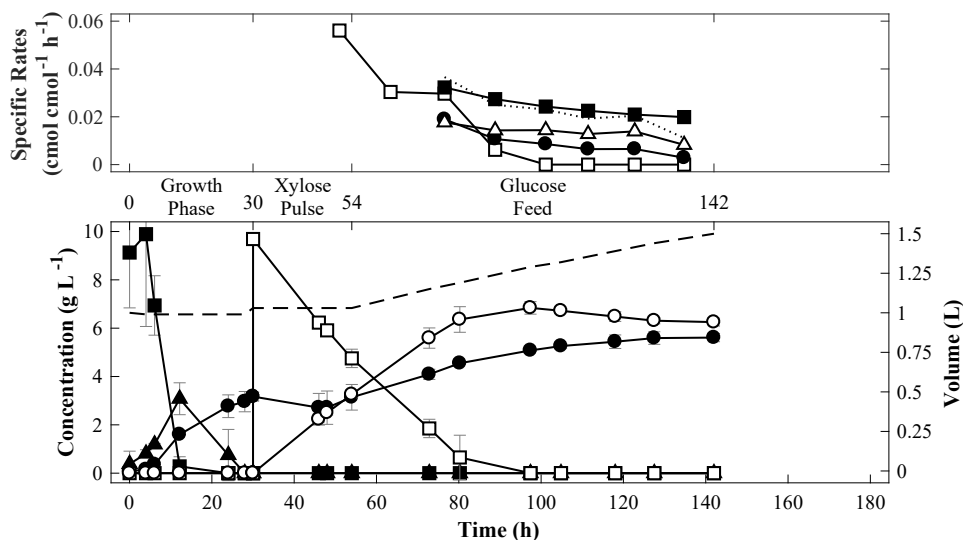
Figure 5.6: Fed-batch experiments with strain TMB4531. Glucose (filled squares), biomass (filled circles), ethanol (filled triangles), xylose (open squares), xylonate (open circles), CO_2 (open triangles) and liquid volume (- - -). The dotted line ($\cdot \cdot \cdot$) represents the sum of specific productivity of biomass and CO_2. All experiments were performed in biological duplicates and standard deviations are indicated with error bars.

ference in xylose consumption rate was found between the xylose phase and the glucose feed phase in TMB4530. In TMB4531, the xylose consumption rate was proportional to the xylose concentration. The fact that xylose consumption was not improved by the glucose feed indicated that NADH re-oxidation was not the issue. Glucose rather helped maintaining the cell viability. At the start of the fed-batch phase, the carbon flux from glucose was equally split between biomass and CO_2. As the experiments progressed and xylonate concentration increased, the carbon flux from glucose consumption shifted to less biomass and more CO_2. This indicated an increased energy requirement for transporting acid out of the cell. The overall xylonate production rate was 0.10 g L-1 h^{-1} for TMB 4530 and 0.14 g L^{-1} h^{-1} for TMB 4531, which is considerably lower than the 0.44 g L-1 h^{-1} previously reported for *S. cerevisiae* (Toivari et al., 2012). However, both the biomass concentration and, importantly, the initial xylose concentration was roughly twice as high in that study. Xylose uptake in *S. cerevisiae* has often been found to be limiting the xylose metabolism in ethanol fermentation of xylose (Moysés et al., 2016). The reason is that *S. cerevisiae* does not have any native transporters for xylose, but must rely on native hexose transporters. The xylonate yield on xylose was above 0.9 g g^{-1} in both strains which is higher than what has been reported earlier (Toivari et al., 2012).

5.4.3   Conclusions

The introduced Weimberg pathway in *S. cerevisiae* was not fully functional in either of the two evaluated *S. cerevisiae* strains carrying different XylD. The carbon flux seemed to stop at xylonate in both tested strain, indicating that XylD was a major bottleneck. As RT-PCR confirmed that all genes were transcribed to RNA (Paper IV), the issue was rather related to either the translation or the activity of one or more of the proteins in the lower pathway. The XylD from *C. crescentus*, used in TMB4530, contains a Fe-S cluster, and functional expression of enzymes containing bacterial Fe-S clusters in yeast has been proven difficult (Benisch & Boles, 2014). The XylD from *H. volcanii*, used in TMB4531 did not seem to be active either, at least not at measureable level. It is suspected that it may be due to the native environment of *H. volcanii*, which grows at 42 °C and at high salinity (Zaigler et al., 2003), conditions that are not compatible with *S. cerevisiae* physiology. As a functional Weimberg pathway in *S. cerevisiae* would indeed be of significant importance from both a fundamental and an applied perspective. Further work based on a more fundamental understanding of the transfer of this bacterial route into the eukaryotic host clearly will be needed.

5.5   New method for analysis of xylonic acid

In fermentation research, the dominating method for analysis of extracellular metabolites is liquid chromatography using ion exclusion columns such as the Aminex HPX-87H (Sluiter et al., 2008). For example, in 37 of the research papers cited in this thesis, ion exclusion chromatography was used to analyse metabolites. During the work with the yeast strains harbouring the Weimberg pathway xylonate appeared as an important metabolite that needed accurate quantification. As ion exclusion chromatography gave poor separation between xylonate and xylose a new analysis method, described in Paper VI, was developed. The goal was to develop a method that could be used to quantify all metabolites found in experiments with both *C. crescentus* and *S. cerevisiae* in a single run. The important metabolites to analyse were, xylose, xylonate, xylitol, α-ketoglutarate and glucose.

5.5.1   Selection of separation and detection method

Metabolites from microbial cultivations such as sugars, alcohols and organic acids are typically highly polar compounds. As such they are difficult to separate using RP-LC, reversed phase liquid chromatography (Buszewski & Noga, 2012). For many applications, the use of ion exclusion chromatography satisfies the needs for separation of metabolites, but in this case, that option was not available due to poor separation (Paper VI).

Ion exclusion chromatography has relatively few parameters that can be varied to modify the retention time of the analytes (Glód, 1997). A more versatile separation method for polar analytes is HILIC - hydrophobic interaction liquid chromatography (Buszewski & Noga, 2012). Separation can here be modified by changing composition of the mobile phase in terms of both organic solvent content and pH as well as column temperature. Further separation options are available through the use of gradient elution. For the work in Paper VI, an amide column was used.

The choices of detector are limited when analysing metabolites. Sugars and alcohols are usually detected by refractometry using RI-detectors as they lack UV-chromophores. In this study however, RI-detectors could not be used since they are incompatible with gradient methods. Organic acids are possible to detect using UV-spectroscopy. Xylonate however, has a low UV-absorbance limiting the sensitivity of the method.. The choice of detector therefore fell upon ELS - evaporative light scattering (Megoulas & Koupparis, 2005). ELS detection operates by mixing of the mobile phase with a gas flow in a nebuliser, creating a fine mist of solvent containing the analyte. The solvent is then evaporated leaving solvent-free particles of analyte that are subsequently detected by light scattering. The detection principle of the ELS detector depends on the light scattering properties of small particles. Because of the non-linear nature of light scattering the peak area (A) relates to the analyte mass (m) in a correlation best described by a power function with two factors, α and β (Equation 5.1).

$$A = \alpha \cdot m^{\beta} \tag{5.1}$$

The detector is regarded as universal in the sense that any compound can be detected as long as it is less volatile than the mobile phase. Since all the compounds of interest have low volatility, the ELS-detector was found to be a good choice.

5.5.2   Method

The method was tested using a mix of all five compounds of interest and it was found that all components could be efficiently (Rs > 1.5) separated (Figure 5.7B). Xylonate could be quantified in a range from 0.2 g L^{-1} to 7.0 g L^{-1} (Figure 5.7C). The mixture of analytes was analysed on an ion exclusion column (Aminex HPX-87H), resulting in less separated peaks. Especially xylose and xylonate who co-eluted preventing accurate quantification (Figure 5.7A). The developed method is also faster, requiring only 12 minutes per sample compared to the ion exclusion method which requires 30-50 minutes depending on sample composition. The method was also tested with samples from real cultivations with xylonate producing *S. cerevisiae* (Paper IV). Medium components did not interfere with the analysis of the selected metabolites (Figure 5.7D).
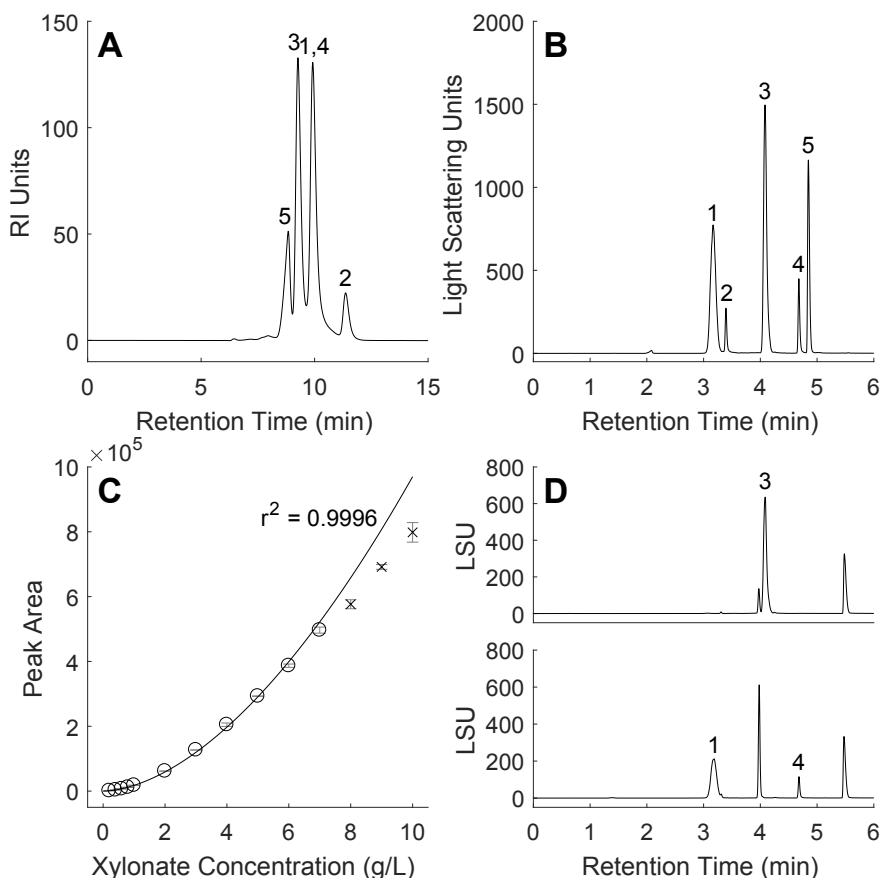
Figure 5.7: Separation of xylose (1), xylitol (2), glucose (3), D-xylonate (4) and α-ketoglutaric acid (5) on (A) HPLC ion exclusion column and (B) UHPLC amide column using the method developed in this study. (C) Good correlation (r^2 = 0.9996) to power curve fitting was found from 0.2 - 7 g L^{-1} (O) excluding concentrations outside the dynamic range (X). (D) Chromatograms from samples of fermentation broth from a xylonate producing yeast strain showing glucose or xylose and xylonate. The first sample (upper) was taken during the growth phase on glucose and the second sample (lower) during the xylose conversion phase. Unlabelled peaks are non-metabolite media components.

5.6 Remarks on the Weimberg pathway

Introduction of the Weimberg pathway, an orthogonal xylose degradation pathway, was characterised in a natural host *C. crescentus* and it was attempted to introduce the pathway in *S. cerevisiae*.

- The Weimberg pathway is active in *C. crescentus* grown on xylose and arabinose but not on glucose.

- *C. crescentus* excreted substantial amounts of α-ketoglutarate from xylose. It is however, not suited as an industrial production organism as it is highly osmo-sensitive and growth is slow. Excretion of α-ketoglutarate indicates that the channelling of carbon from Weimberg pathway to the TCA cycle is adapted to an environment with low concentrations of substrate.

- Implementation of the Weimberg pathway in *S. cerevisiae* was not completely successful. Although all genes were transcribed all were not translated to functional proteins. The main cause is likely a non- or low-functional xylonate dehydratase. The Weimberg pathway did however, enable efficient bioconversion of xylose to xylonate in *S. cerevisiae*.

Chapter 6

Concluding remarks and outlook

The aim of this thesis was to develop process engineering strategies to improve yield and productivity of dicarboxylic acids from xylose in three different production systems using different host organisms (Table 6.1).

A fundamental difference between the three strategies is that different metabolic pathways were used to convert xylose into the carboxylic acid. In the natural succinate producers, the central carbon metabolism of the organisms is harnessed to produce succinic acid. The inherent ability to fixate CO_2 and incorporate that into the product is a significant advantage as it not only increases the product yield, but also enables the use of CO_2 from for example ethanol fermentation thereby increasing the utilisation of available carbon sources in an integrated biorefinery. As the process is anaerobic, other acids are formed as by-products to maintain redox balance. With *S. cerevisiae* engineered to

Table 6.1: Summary of the three strategies for carboxylic acids formation from xylose investigated in this thesis work.

Production Organism	*A. succinogenes* *B. succiniciproducens*	*S. cerevisiae*	*S. cerevisiae*
Chapter	Chapter 3	Chapter 4	Chapter 5
Production pathway	XI, PPP, glycolysis, mixed acid fermentation	XI, PPP, glycolysis, reductive TCA-cycle	Weimberg Pathway
Metabolic engineering	None, natural producer	Δgre3 ↑↑Cp xylA ↑↑rki1 ↑↑rp11 ↑↑tkl1 ↑↑Ps tal1 ↑↑Ps sut1 ↑↑Ps xyl3 ↑↑Sp Mae1 ↑↑mdh3Δskl ↑↑pyc2	↑↑Cc xylBDXA Δgre3
Aerobic/Anaerobic	Anaerobic	Aerobic	Aerobic
pH	Neutral	Acidic	Acidic
Medium	Complex	Semi-Defined	Defined
Tested in industrial medium	Yes	Yes	No

produce dicarboxylic acids via the TCA cycle, a production pathway similar to the natural producing bacteria was used. In this case, however, the process was aerobic. This removes the redox constraint, which (in theory), could enable higher succinate yields and simplify purification due to less by-products formed. The strain can potentially achieve a net CO_2 fixation, although this cannot be verified until higher product yields are reached. A drawback is the risk of loss of substrate in respiration. The Weimberg pathway is orthogonal in the sense that it by-passes the PPP and glycolysis and connects directly to the TCA cycle without loss of carbon. As the complete pathway was not functional in *S. cerevisiae*, the intermediate compound xylonate was accumulated as the sole product.

The industrial suitability of a host organism depends on many characteristics. *C. crescentus*, the natural host of the Weimberg pathway, was clearly unsuitable as a production organism, although it produced α-ketoglutarate at rather high yield from xylose. However, it is adapted to an aquatic environment with low concentrations of solutes, which is intrinsically incompatible with industrial processing conditions. This can be compared to *A. succinogenes* and *B. succiniciproducens*, which are also natural producers, but adapted to a substrate-rich environment in the cattle rumen. Unfortunately, their natural environment is also rich in nutrients and the pH is close to neutral - not optimal for industrial diacid production. Of the organisms in this thesis, *S. cerevisiae* appears as the best suited organism for industrial carboxylic acid production, due to both its minimal nutrient requirements and its ability to grow at low pH.

Thus, the key to a successful industrial production organism is the combination of an efficient production pathway with a host organism well-suited for industrial conditions. The following points summarise some suggestions for how this could potentially be achieved.

- Succinate production from xylose rich substrates by *A. succinogenes* and *B. succiniciproducens* can be further improved. For example, slow- or non-growing cells were shown to produce higher succinate yields and evaluating immobilised cells in combination with SSL as substrate would be highly interesting. Also, high initial pitch of cells combined with nitrogen limitation could possibly increase both yield and volumetric productivity. Adaptation to SSL has showed to increase tolerance in yeast and might be a viable strategy for these organisms as well.

- In *S. cerevisiae* engineered for production via the TCA-cycle, a mix of succinate and malate was formed. Better understanding of the metabolic fluxes - including compartmentalisation - and dicarboxylic acid transport will be needed for metabolic engineering towards higher yielding strains and fermentation strategies limiting carbon losses as biomass or through respiration.

- Further research on bacterial and archaeal xylonate dehydratases in eukaryotes that

lead to the introduction of a fully functional Weimberg pathway in *S. cerevisiae* would be of great interest. α-ketoglutarate is a not much studied and very interesting gateway molecule for pentose metabolism, just a few steps away from compounds such as succinate, malate, fumarate as well as the α-ketoglutarate family of amino acids.

The ongoing research on biotechnological production of platform chemicals, where the work in this thesis is a part, has the potential to lead to future sustainable production of chemicals and materials. The expanding interest in both academia and industry such as the recent rapid development in bio-succinate production capacity can be seen as an indicator of the state of biorefining for production of platform chemicals. Potentially, we are currently at the beginning of an emerging bioeconomy based around the biorefinery.

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