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Yeast-based conversion of galacturonic acid in sugar beet pulp hydrolysate to galactaric acid: a theoretical investigation

Dissertation for Master's Degree in Bioengineering
Specialization in Biological Engineering

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Mais vale uma má decisão do que uma não decisão.

Pai

Preface

This preface was written with the purpose of explaining how the COVID-19 pandemic affected the initial objectives defined for the thesis project, while contextualizing how this situation was solved leading to the thesis hereby presented.

In the beginning, the main goal of the present thesis was to deliver the proof of concept for the yeast-based conversion of galacturonic acid in sugar beet pulp hydrolysate to galactaric acid. To achieve that, an extensive experimental work divided in seven parts was planned for the 6-months internship at the DSM Biotechnology Centre in Delft (The Netherlands):

1. Genetic engineering for yeast strain construction.
2. Enzymatic hydrolysis of sugar beet pulp.
3. Fermentation of original strains in synthetic media.
4. Fermentation of original strains on sugar beet pulp hydrolysate.
5. Fermentation of built strains in synthetic media.
6. Fermentation of built strains on sugar beet pulp.
7. Additional improvements to the final strains.

However, returning to Portugal upon the imminent strike of the pandemic in March made it impossible to carry out any of the work originally planned. Although the hope of returning to Delft was kept alive for the following months, strict measures for trainees imposed by DSM did not allow the return as soon as desired. In fact, only in mid-July the authorization for trainees to work again at the site was issued. By then, it seemed unfeasible to return and develop the project initially planned with quality, even with a special deadline prolongation.

Therefore, considering this timeline and the uncertainty regarding the development of the pandemic in the next months, it was finally decided in mid-July to adapt to the current situation and rescope the thesis objectives, developing a theoretical exploration of the initial topic that would feature the literature study work carried out until then. In addition, it was decided to make a techno-economic analysis which started in the beginning of August, since this not only revealed interesting for DSM but also functioned as a good complement to the literature study.

Acknowledgements

First and foremost, I would like to thank everyone with whom I had the pleasure of working at DSM. To Hans, thank you for choosing me to take on this project, I am grateful for having had you as a supervisor. Since day one you provided me with support whenever I needed, and your scientific insights and critical thinking are invaluable learnings that I will surely retain from this experience. Thank you for keeping in touch with me regularly during the whole quarantine period and for your comprehension whenever my weeks were harder. It is funny that now I look back and recall that we were together personally only on my first day at DSM but still we managed to go through it all!

To Tiemen, a huge thank you for your help on the techno-economic analysis part, I would not have been able to do it without your input and feedback. To Adriana, Jan, Dick, Tjeerd and Ingrid, I appreciate the time you took to meet me for the first time through a Teams meeting. Thank you for hearing my questions and helping me out whenever I needed – you made me feel like I was working in a team!

Ao Prof. Nuno, tenho a agradecer o seu papel preponderante em fazer com que o meu estágio se tornasse possível. Obrigado pela sua paciência e cooperação e, sobretudo, obrigado por defender os meus interesses. Agradeço ainda a forma próxima como sempre me permitiu comunicar consigo, que foi fundamental para resolver todos os imprevistos que foram surgindo com o desenrolar da tese.

E porque esta tese não é só o resultado dos últimos meses, mas também o culminar dos últimos seis anos, resta-me agradecer aos que me acompanharam neste percurso - vocês sabem quem são. Ao pessoal do secundário, obrigado termos mantido uma amizade próxima ao longo destes anos, que continuemos a criar histórias para mais tarde recordar. À malta do Princess, obrigado pela amizade desde crianças e que os míticos jantares de *pizza* nunca acabem. Aos amigos do curso, obrigado por todo o convívio dentro e fora da FEUP, todos os convívios e jantares de turma – obrigado por termos sido muito mais do que colegas. Ao Paulo, obrigado por seres um verdadeiro amigo.

Por último, os meus maiores e mais sentidos agradecimentos vão para as quatro pessoas mais importantes, sem as quais nada disto teria sido possível. À minha mãe, pelo carinho e apoio incondicional que só ela sabe dar. Ao meu pai, pelas valiosas lições de vida que até hoje me ensinou e que certamente continuará a ensinar. À minha irmã Kika, obrigado por todos estes anos que cresci ao teu lado, não há sentimento igual ao de ter uma irmã de quem tanto gostamos. E por fim, ao avô Alfredo, por ser uma enorme inspiração e uma prova de que mesmo partindo do zero, à custa de muito trabalho, qualquer um pode chegar muito longe...

A todos vocês,

OBRIGADO!

Abstract

Since the middle of the 20th century, a growingly intensified fossil fuels consumption has led to a current dependency on such resources for supplying global energy systems. The concerning environmental impacts of this unbridled use of fossil fuels has urged a call for transition towards a bio-based economy in which biotechnological processes are used to produce (bio-based) products and energy using biomass as feedstock.

Sugar beet is a plant grown commercially for sugar production due to the high sucrose concentrations it accumulates in its root. Sugar beet pulp is one of the main by-products generated in beet processing, currently valorized through selling it as animal feed with low economic returns. However, the availability and distinctive chemical composition of this pectin-rich waste stream trigger opportunities for alternative valorization strategies through biotechnological processes. Galacturonic acid is one of the main monomers composing beet pulp hydrolysate, which currently represents a technological hurdle for its utilization in fermentative conversion to value-added compounds, despite several metabolic pathways having already been described. Among such metabolic diversity, this thesis is focused on the already proven conversion of galacturonic acid – catalysed by uronate dehydrogenase – to galactaric acid, a compound which has been gaining increased attention for its applications as a platform chemical leading to the synthesis of biodegradable plastics. The conversion of galacturonic acid to galactaric acid in yeast *Saccharomyces cerevisiae* has already been reported in literature, but its proof of principle using sugar beet pulp hydrolysate is yet to be delivered.

In this work, a process for yeast-based production of galactaric acid from sugar beet pulp was proposed and a mass balance model was built using input data from the literature study and DSM internal expertise to estimate possible yields and production costs of main operations like pretreatment, saccharification and fermentation. Moreover, an indicative techno-economic assessment was done through testing different process modifications and genetic engineering strategies using this model, comparing the business cases created through the gross margin percentage. While the pretreatment was not found to be a valuable addition to the process, causing the gross margin to decrease 8% (assuming a reduction of saccharification yields), a so-far hypothetical linking of galactaric acid formation to yeast central carbon metabolism (coupled with reduction of glycerol by-product formation) was demonstrated to be crucial in ensuring an attractive business case, reaching a gross margin of 27%. Therefore, it was concluded that although this valorization strategy shows potential in theory, this process might only be economically feasible if conversion of fermentable sugars like glucose to galactaric acid is achieved. Finally, the sensitivity analysis done supported the conclusions of the techno-economic assessment showing that increasing the galactaric acid yield per unit of feedstock processed is the primary driver for the process profitability.

Keywords: sugar beet pulp, galacturonic acid, galactaric acid, *Saccharomyces cerevisiae*, techno-economic assessment.

Resumo

Desde meados do século XX, o crescente consumo de combustíveis fósseis levou à atual dependência destes mesmos recursos para o abastecimento global de energia. Os preocupantes impactos ambientais desta utilização de combustíveis fósseis apelam a uma urgente transição para uma bioeconomia, na qual os processos biotecnológicos são usados para produzir bioprodutos e bioenergia, sendo a biomassa utilizada como matéria-prima.

A beterraba sacarina é uma planta cultivada para a produção de açúcar, devido às altas concentrações de sacarose presentes na sua raiz. A polpa da beterraba sacarina é um dos principais subprodutos gerados no processamento da beterraba, hoje valorizada pela sua comercialização como ração animal, com um baixo retorno económico. No entanto, a disponibilidade e a distinta composição química deste resíduo rico em pectina, proporcionam oportunidades para estratégias alternativas de valorização através de processos biotecnológicos. O ácido galacturónico é um dos principais monómeros que compõem o hidrolisado da polpa de beterraba, o que atualmente representa uma barreira tecnológica para a sua utilização na conversão fermentativa em compostos de valor acrescentado, apesar de várias vias metabólicas já terem sido descritas. Dentro dessa diversidade metabólica, esta tese foca a já comprovada conversão do ácido galacturónico - catalisado pelo uronato desidrogenase - em ácido galactárico, composto este que tem ganhado atenção pelas suas aplicações como químico de base para a síntese de plásticos biodegradáveis. A conversão do ácido galacturónico em ácido galactárico, usando a levedura *Saccharomyces cerevisiae*, foi já demonstrada na literatura; todavia, ainda não foi realizada a prova de conceito utilizando como substrato o hidrolisado de polpa de beterraba.

Neste trabalho, foi proposto um processo para a produção, através de leveduras, de ácido galactárico a partir da polpa de beterraba e foi construído um modelo de balanço mássico, usando dados *input* provenientes do estudo da literatura e da experiência interna da DSM, a fim de estimar possíveis rendimentos e custos de produção das principais operações. Foi também feita uma análise técnico-económica representativa, testando diferentes modificações processuais e estratégias de engenharia genética usando o modelo referido acima, e foram comparados os vários casos criados, considerando as respetivas margens de lucro. Enquanto que o pré-tratamento não foi considerado uma fase valiosa no processo, fazendo com que a margem de lucro diminuísse 8% (assumindo uma redução dos rendimentos da sacarificação), uma ligação, até ao momento hipotética, da formação de ácido galactárico ao metabolismo de carbono central da levedura (juntamente com a redução da formação do subproduto glicerol) revelou-se crucial para garantir um caso de negócio atraente, atingindo-se uma margem de lucro de 27%. Concluiu-se que, embora esta estratégia de valorização apresente potencial teoricamente, este processo só poderá ser economicamente viável se a conversão de açúcares fermentáveis, como a glicose, em ácido galactárico for conseguida. Finalmente, a análise de sensibilidade feita apoiou as conclusões da análise técnico-económica, demonstrando que o aumento do rendimento do ácido galactárico por unidade de matéria-prima processada é o principal fator motivador da lucratividade do processo.

Palavras-chave: polpa de beterraba, ácido galacturónico, ácido galactárico, *Saccharomyces cerevisiae*, análise técnico-económica.

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List of Abbreviations

AFEX	Ammonia fiber expansion
APD	Ammonia pressurization depressurization
ATP	Adenosine triphosphate
CAPEX	Capital expenditure
CBP	Consolidated Bioprocessing
D-Dha	2- keto-3-deoxy-D-lyxo-heptulosaric acid
DM	Dry matter
DSP	Downstream processing
EU	European Union
FDCA	2,5-furandicarboxylic acid
G3P	Glyeraldehyde-3-phosphate
GaaA	Galacturonate reductase
Gal-UA	Galacturonic acid
Gal-AA	Galactaric acid
GMO	Genetically modified organism
HG	Homogalacturonan
HMF	Hydroxymethylfurfural
KDO	2-keto-3-deoxy-D-manno-octulosonic acid
KDPG	2-keto-3-deoxy-6-phosphogluconate
LPMO	Lytic polysaccharide monooxygenases
MGDA	Methylglycindiacetic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
OPEX	Operational expenditure
PEF	Polyethylene furanoate
PET	Polyethylene terephthalate
PRK	Phosphoribulokinase
R&D	Research and development
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
Rubisco	Ribulose-1,5-bisphosphate carboxylase
SBP	Sugar beet pulp
SSF	Simultaneous saccharification and fermentation
UDH	Uronate dehydrogenase
UxaC	Uronate isomerase
XGA	Xylogalacturonan

1 Introduction

1.1 Background and project presentation

Fossil fuel consumption has been growing considerably in the past decades – registering an eight-fold increase since 1950 and roughly doubled since 1980 (Ritchie & Roser, 2017). In an estimated range of 50-100 years from now, these finite resources will end and thus the ongoing global dependence on oil, gas and coal for both energy and chemicals poses serious short term threats for the future of humanity. In 2019, around 84% of global primary energy came from fossil fuels (Ritchie & Roser, 2017). Besides, petrochemical products are omnipresent in everyday life and their consumption is reaching an unsustainable level. Demand for plastics – the predominant group of petrochemical products – has nearly doubled in the past 20 years and, in 2016, 485 billion units of polyethylene terephthalate (PET) bottles were produced worldwide (Garside, 2019).

This excessive use of fossil fuels is causing severe and irreversible damage to our planet, mainly through the emission of large amounts of carbon dioxide into the air, a gas that contributes significantly to global warming. Consequently, sea level is rising, extreme weather events are occurring more frequently and biodiversity is being lost at the fastest pace ever seen in human history (United Nations Sustainable Development, 2019). Humankind is already experiencing the outcome of having established a dominant fossil-based economy which will certainly become even more harmful in the near future.

Hence, a call for a transition to a more sustainable economic model is urgent. The world needs processes aiming to produce energy and products that are bio-based – wholly or partly derived from sources of biological origin (excluding materials embedded in geological formations and/or fossilized) (European Commission, 2017). As they are obtained from renewable resources, bio-based products offer advantages such as lower toxicity and new product characteristics (e.g. biodegradable materials) (European Commission, 2017). Concluding, it is crucial that this valuable opportunity offered by biotechnology is not neglected, as it allows for a bioeconomic model that contemplates a wider and more ecologic spectrum of feedstocks, as it also gives birth to new and value-added products.

1.2 Company presentation

Koninklijke DSM N.V. (Royal DSM) is a Dutch multinational corporation headquartered in Heerlen, Netherlands. With a workforce of approximately 23,000 employees spread across 47 countries, DSM delivers annual net sales of about €10 billion and is listed in Euronext Amsterdam (DSM, 2019).

DSM started as a mining company when it was founded in 1902 by the Dutch state to mine coal reserves in southern Limburg. Since then, DSM's intervention areas have evolved and diversified over the years. By 1973, when the last mine closed, the company had already shifted their business focus towards commodity chemicals and petrochemicals. Today, DSM is a global, purpose-led, science-based company active in the fields of Nutrition, Health and Sustainable Living. In 2018, DSM's positive social

impact was recognized appearing in the 'Fortune Change the World list' for the third year running and in that same year Sustainalytics named DSM as leader in ESG (environmental, social and governance) in its industry sector.

Regarding DSM's activity in sustainable processes of biomass conversion, it is worth highlight their ongoing cooperative effort with POET focused on the production of cellulosic bioethanol from corn crop residues (Project LIBERTY) and the development of technology for the production of bio-based succinic acid under the joint venture Reverdia, with French company Roquette.

1.3 Main objectives

The main objective of this dissertation is to understand whether the yeast-based production of galactaric acid from sugar beet pulp is a potentially good alternative strategy for the valorization of this by-product stream. In order to achieve this purpose, the work was organized in two different parts.

Firstly, an extensive literature study was carried aiming at thoroughly exploring all the theoretical concepts covered within this topic, while reviewing previous studies reported whenever appropriate. Ultimately, this part of the work intended to gather scattered information and provide a solid story that would clearly express the relevance and opportunity for delivering proof of principle of bio-based conversion and further developing into a full process.

Secondly, a different approach on the theoretical investigation of the theme was done. A biotechnological process for the yeast-based conversion of galacturonic acid in sugar beet pulp hydrolysate to galactaric acid was proposed and a mass balance model was built. In this part, the final aim was to identify the key parameters affecting the profitability of such process and subsequently translate those findings into improvement solutions in terms of process intensification and/or research and development (R&D).

1.4 Thesis outline

This dissertation is organized in four chapters. Chapter 1 introduces the thesis with a contextualization of the project relevance on a wider scope, presentation of the company, definition of the main objectives and description of the work outline.

Chapter 2 provides a literature study divided in six parts. It begins with characterizing the importance and availability of sugar beet as a crop, introducing beet pulp as a by-product of the sugar production process which is characterized as a feedstock and its chemical composition is detailed. The focus is then directed to its biotechnological valorization through fermentation and subsequently the deconstruction of this feedstock is described, as it is crucial to obtain galacturonic acid, the monomer which is converted to galactaric acid. There is one section dedicated to galacturonic acid metabolism and another describing the applications of galactaric acid. Finally, a revision on the so far reported methods for this conversion is presented.

Chapter 3 contains the techno-economic assessment carried for the proposed galactaric acid production process. The assumptions used to build the mass balance model are described, as well as the scenarios created to test process modifications or genetic engineering strategies. The results for each business case generated are discussed and a sensitivity analysis is carried to study the influence of selected individual parameters in the process profitability.

Lastly, chapter 4 states the main conclusions of this thesis and gives recommendations for the work to be developed in the future.

2 Literature study

2.1 Sugar beet

2.1.1 Crop characterization

Sugar beet is the common name for the *Altissima* cultivar group of *Beta vulgaris* spp. *vulgaris*, the primary crop of the genus *Beta* (*Betoideae* subfamily, *Amaranthaceae* family) (Romeiras *et al*., 2016). This subspecies comprises other economically important cultivated forms such as fodder beet (var. *crassa*), Swiss chard (var. *vulgaris*) and red beet (var. *conditiva*) (Graef *et al*., 2010). Sugar beet is a biennial plant growing in temperate regions and ideally in soils rich in nutrients and humus and with a good moisture-holding capacity (Cooke & Scott, 1993). It consists of a root and a rosette of leaves (Figure 1). The root is a conical, white, thick taproot (dominant root from which other roots sprout laterally) with a flat crown, which serves as a storage organ capable of accumulating high sucrose concentrations (Koppert Biological Systems, 2020). The sugar content in sugar beet can vary from 12 to 20% by fresh weight (FAO, 2009; Panella, 2010), making it one of the two predominant plant sources of sucrose – the other being sugar cane – which is thus grown commercially for sugar production (Cooke & Scott, 1993).

Figure 1- Sugar beet (Compo Expert, 2020).

While sugar cane has been grown extensively in tropical regions for many centuries, sugar beet is a relatively new crop which appeared firstly in temperate regions in the nineteenth century and only spread widely in the twentieth century. The history of the sugar beet crop and the global beet sugar industry is detailed in Draycott (2006). One of the first milestones in the history of modern sugar beet industry was set at the end of the eighteenth century when German chemist Franz Carl Achard successfully selected white flesh, high sugar-content beets among fodder beets grown by farmers. This discovery led to the first commercial beet processing and sugar extraction campaign being carried in 1802. However, it was the interest shown by the French - especially Napoleon Bonaparte - which catalysed the development of a flourishing sugar beet industry in Europe as an alternative to cane sugar importation. Since then, several technological developments and favourable government policies have

driven the global expansion of the sugar beet industry, with this crop being cultivated and processed in Europe, North and South America, Asia and Africa.

2.1.2 Global production and future perspectives

Currently, between 250 and 300 million tonnes (ton) of sugar beet are produced per year worldwide (Shahbandeh, 2020), with the European Union (EU) being the leading producer of this crop, accounting for about one half of the global production (Eurostat, 2019; Runneboom *et al*., 2014). In 2018, the EU produced almost 120 million ton of sugar beet with more than one half of this production coming from France (33%) and Germany (22%) together. Other key producer member states include Poland, United Kingdom and The Netherlands (Eurostat, 2019). Outside the EU, countries such as Russia, United States of America and Turkey are also strong players, having featured on the top 5 sugar beet producers worldwide in 2017 (Shahbandeh, 2019).

In recent years, among all sugar beet producers worldwide, one region has stood out for its attractiveness for production and processing of this crop, as pointed out in a study made by Deloitte (Runneboom *et al*., 2014). North-West Europe, and especially The Netherlands, besides having adequate weather conditions for sugar beet cultivation, are also particularly competitive due to low inbound and outbound transportation costs, high sugar yields per hectare of land, large-scale facilities and the ability to supply ample volumes of thick sugar juice on a year-round basis. In fact, data from the 2012/2013 season showed that despite far from being the biggest producer, The Netherlands had the lowest sugar production cost on a global scale (even when comparing with cane sugar producing countries).

Since its appearance in the nineteenth century, sugar beet has had a significant expansion as a crop along with technological developments of its processing industry. Yet, it currently only provides about 20% of the global supply of sugar for human consumption; sugar cane is still the dominant crop in sugar production, providing the remaining 80% (FAO, 2009). However, several factors indicate that sugar beet will be a promising crop in forthcoming years. Firstly, sugar beet is planted every year through seeds, allowing crop improvement by selection of the best breeds; in contrast, new sugar cane is planted only every 5-7 years through cuttings, hence being a much more static crop. Secondly, the continuity of beet cultivation is assured as it will remain a fundamental sugar source, even more since a recent production and import quota lifting in 2017 in the EU allowed higher production volumes of sugar from this crop (Runneboom *et al*., 2014).

Besides sugar production, beet is also starting to be used in the currently emerging fermentation-based industry aiming to produce bio-based alternatives that can compete against fossil-based established products. In fact, in the EU, already 13% of the total sugar beet production is directly used to produce biofuels (Runneboom *et al*., 2014), and more applications are currently under investigation, especially focusing on exploring the potential of beet processing by-products, following a bio-based economy outlook.

2.2 Sugar beet pulp

2.2.1 Formation and current valorization

Sugar beet processing, from harvesting to the formation of sugar crystals, is extensively described by Marzo *et al.* (2019) and is hereby represented in Figure 2.

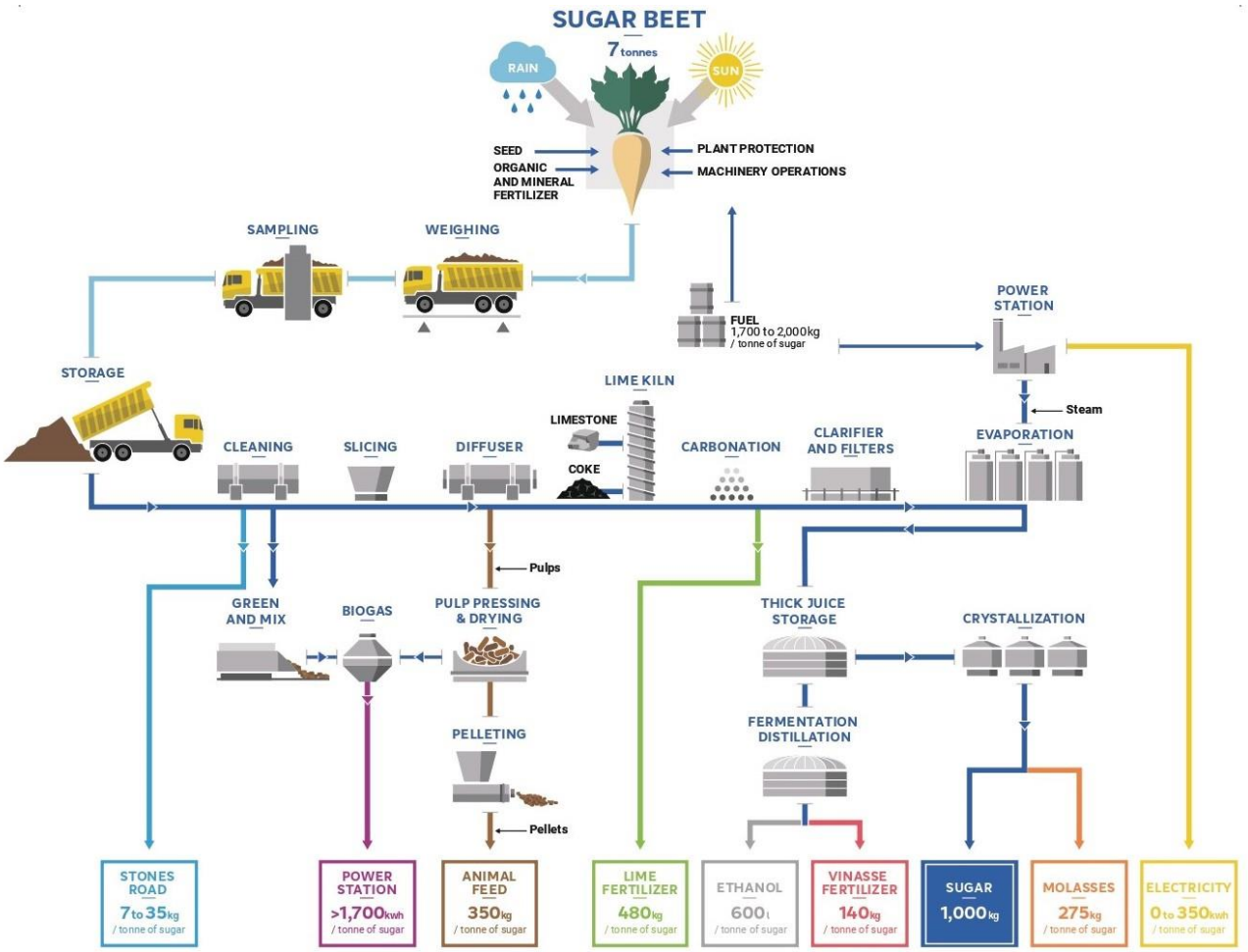

Figure 2 - Sugar beet processing from harvesting to formation of sugar and by-products (Sucden, 2020).

Sugar beet seeds are sown in spring and the roots are harvested in autumn or early winter. In developed countries, harvesting is performed mechanically – a single machine takes the bulbs from the soil and rips the leaves off. The detached stalks and leaves are usually kept on the field while the roots are transported to the processing facility. Transportation of the crop must be done quickly since the sugar content of beets drops rapidly once they are lifted from the soil (FAO, 2009). Upon arrival to the facility, the harvested amount is weighed, sampled for analysis and then stored in piles in a large outdoor area. It is critical to analyze the sugar content of the sugar beets at the moment of arrival in the processing plant since this will determine the sugar extraction rate obtained later in the process. In fact, European norms consider sugar beets to be marketable only if their sugar content is equal to or greater that 14% (FAO, 2009).

Sugar beet processing starts with thorough washing of the roots with a pressurized water jet to remove stones and any adhered soil or remaining leaves. This is followed by slicing into strips called cossettes (resembling thin potato slices). The cossettes are then mixed with hot water (55 °C – 75 °C) in a stainless steel tank (diffuser) where sugar extraction occurs by diffusion. This operation is usually carried in continuous and countercurrent mode, creating a higher concentration gradient which makes the extraction more efficient. As a result, the output stream – called 'raw juice' – contains a sugar concentration between 10% and 15% w/w (weight by weight) and goes forward for the production of sugar crystals. On the other hand (as represented by the brown segment in Figure 2), the spent cossettes form a wet pulp which is firstly mechanically pressed to recover retained juice, then dried in a continuous industrial oven to reduce its water content to lower than 10% and finally compressed into pellets, forming the main by-product of sugar beet industry known as sugar beet pulp (SBP) (British Sugar, 2012). Molasses is another by-product formed at a later stage of sugar production. It is a dark and highly viscous runoff syrup formed in the crystallization step containing the fraction of sugar that is not crystallized and is generally valorized through ethanol production or as a medium for yeast biomass production (FAO, 2009). Both of these by-products are represented in Figure 3.

Figure 3 - Sugar beet processing by-products: pulp, dried (on the top) and pelletized (at the bottom), and molasses (on the right) (Nordzucker, 2019).

On a dry matter basis, 25 ton of sugar beet yield 4 ton of dried pelletized pulp and in Europe, approximately 13 million ton of sugar beet pulp are produced per year (PULP2VALUE, 2015). Currently, the most common strategy for sugar beet pulp valorization is selling it as animal feed. It carries high nutritive value for livestock since it is an excellent digestible energy source and has a high fibre content, placing it in a unique position amongst other feed ingredients (Draycott, 2006). However, selling this waste product results in relatively low economic returns due to the high cost of drying and pelleting (Doran *et al.*, 2000) – which can comprise up to 30% to 40% of the overall energy cost of the waste processing (McGinnis, 1982) - and its low selling price, around 140 €/ton (confidential presentation by Coöperatie Koninklijke Cosun U.A.). In spite of the high cost of drying and pelleting, ensuring the stability of the material is crucial since pulp is produced in campaigns, allowing it to be stored for up to one year

without any adverse effect on its feed value (Berłowska *et al*., 2018). Therefore, the low profitability of sugar beet pulp as an animal feed encourages the creation of new opportunities for the biotechnological valorization of this by-product (Edwards & Doran-Peterson, 2012).

2.2.2 Classification as feedstock and polymeric composition

For the exploration of new strategies to valorize sugar beet pulp, it is essential to know its chemical composition in detail while contextualizing it among other commonly used bio-based feedstocks. One of the most popular classifications for bio-based feedstocks divides them into three 'generations', as described by Larson (2008) and later on by Nigam & Singh (2011). These designations are commonly used when referring to biofuels production, but they can also be applied to other bio-based products. Sugars, grains high in sugar or starch and seeds rich in vegetable oil are known as **first generation** feedstocks. The most well-known first generation process is the production of bioethanol by fermentation of sugar extracted from crop plants and starch contained in corn kernels or other starchy crops. In such cases, the feedstocks require relatively simple processing to obtain the final product. However, their long-term viability is questionable since they compete directly with food supply and lead to reduced land use efficiency - only a small fraction of the total plant biomass is used. These limitations favoured investigation towards the production of bio-based products from non-edible biomass, giving rise to a new feedstock category. **Second generation** feedstocks include all agricultural lignocellulosic biomass, either non-edible residues of food crop production (e.g. corn stover, rice husks) or non-edible whole plant biomass (e.g. energy crops – low cost, low maintenance crops grown solely for energy production – such as switchgrass and giant miscanthus). Despite having appeared later, today there are already several operational commercial scale plants producing second generation bioethanol (Jansen *et al*., 2017). The main advantages associated with second generation feedstocks are the fact that they do not overlap with the demand of resources necessary for food production, while also presenting increased land use efficiency since a greater amount of plant material can be converted on the process. However, they often require a more extensive and complex processing with sophisticated equipment. Finally, **third generation** processes shift their attention past agricultural substrates and focus on using microbes and algae (microalgae and macroalgae) as alternative feedstocks devoid of the drawbacks associated with first and second generation feedstocks. Although they are presented as a promising alternative, there is still significant research to be done as they have their own disadvantages, like the high costs of photobioreactors, fouling problems and algae nutrient requirements.

According to this classification, sugar beet pulp can be considered a second generation feedstock as it consists of non-edible lignocellulosic residue resulting from processing of beets for sugar production. Regarding its content in major carbohydrate polymers, sugar beet pulp has a particularly high pectin fraction, comparable to other waste streams like citrus peel waste and apple pomace; this specific composition makes them commonly known as 'pectin-rich feedstocks' (Huisjes, 2013). Figure 4 presents

a comparison of the polymer dry weight composition of the mentioned pectin-rich feedstocks, a first generation feedstock and common lignocellulosic feedstocks.

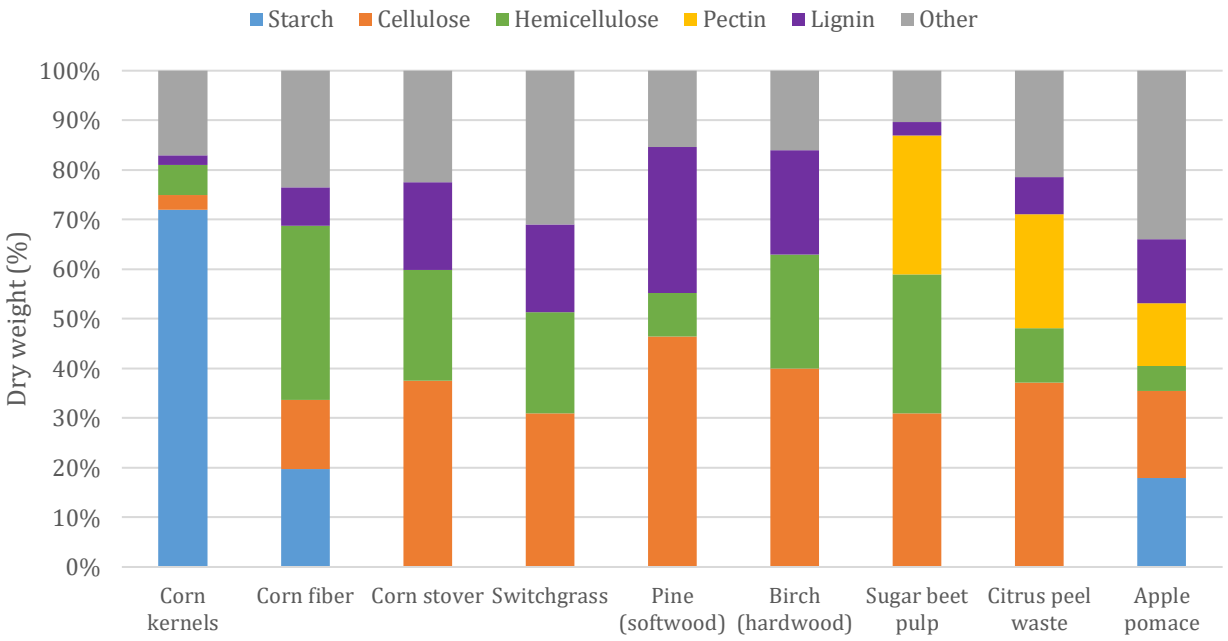

Figure 4 – A comparison of the polymer dry weight composition of pectin-rich feedstocks with other common first and second generation feedstocks. First generation feedstock is corn kernels (Lynd *et al.*, 1999). Second generation feedstocks include corn fiber (Gong *et al.*, 1999), corn stover, switchgrass, pine and birch (Chandel & Singh, 2011). Pectin-rich feedstocks include sugar beet pulp (Berłowska *et al.*, 2018; Leijdekkers, 2015), citrus peel waste (John *et al.*, 2017) and apple pomace (Kennedy *et al.*, 1999).

As lignocellulosic biomass, sugar beet pulp has a significantly higher dry weight cellulose (22-40%) and hemicellulose (24-32%) content when compared to a less complex starchy feedstock like corn kernels (Berłowska *et al.*, 2018). Second generation feedstocks generally do not contain any starch as they are separated from the part of the plant rich in this polymer upon their formation; only corn fiber and apple pomace contain some residual starch from the set of analyzed feedstocks. On the other hand, lignin is present in all lignocellulosic feedstocks. This carbohydrate polymer is an essential component of the plant cell wall, forming strong crosslinked coatings that protect the cellulose and hemicellulose fibers from depolymerizing enzymes, thus correlating inversely with digestibility (Berłowska *et al.*, 2018; Chang & Holtzapple, 2000). While in softwood and hardwood it is present in significant amounts, pectin-rich feedstocks are characterized by low lignin concentrations. In particular, sugar beet pulp is naturally devoid of lignin (approximately 2-3% on a dry weight basis), which makes it highly susceptible to depolymerization and solubilization by mixtures of pectinolytic and cellulolytic enzymes (Grohmann & Bothast, 1994).

2.2.3 Pectin structure and detailed composition

Despite the differences in lignin composition, it is the pectin content that sets sugar beet pulp, citrus peel waste and apple pomace apart from other second generation feedstocks. Pectins are a family of covalently linked D-galacturonic acid-rich cell wall polysaccharides that include homogalacturonan (HG), rhamnogalacturonan I (RG-I), and the substituted galacturonans rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA). In fact, D-galacturonic acid (Gal-UA), a monomer which is neither present in cellulose nor in hemicellulose, forms the backbone of all pectic polysaccharides, comprising approximately 70% of pectin (Mohnen, 2008). Specifically, sugar beet pulp pectin is formed by HG, RG-I and, to a much lesser extent, RG-II regions. Figure 5 shows a schematic representation of the structural elements composing sugar beet pulp pectin.

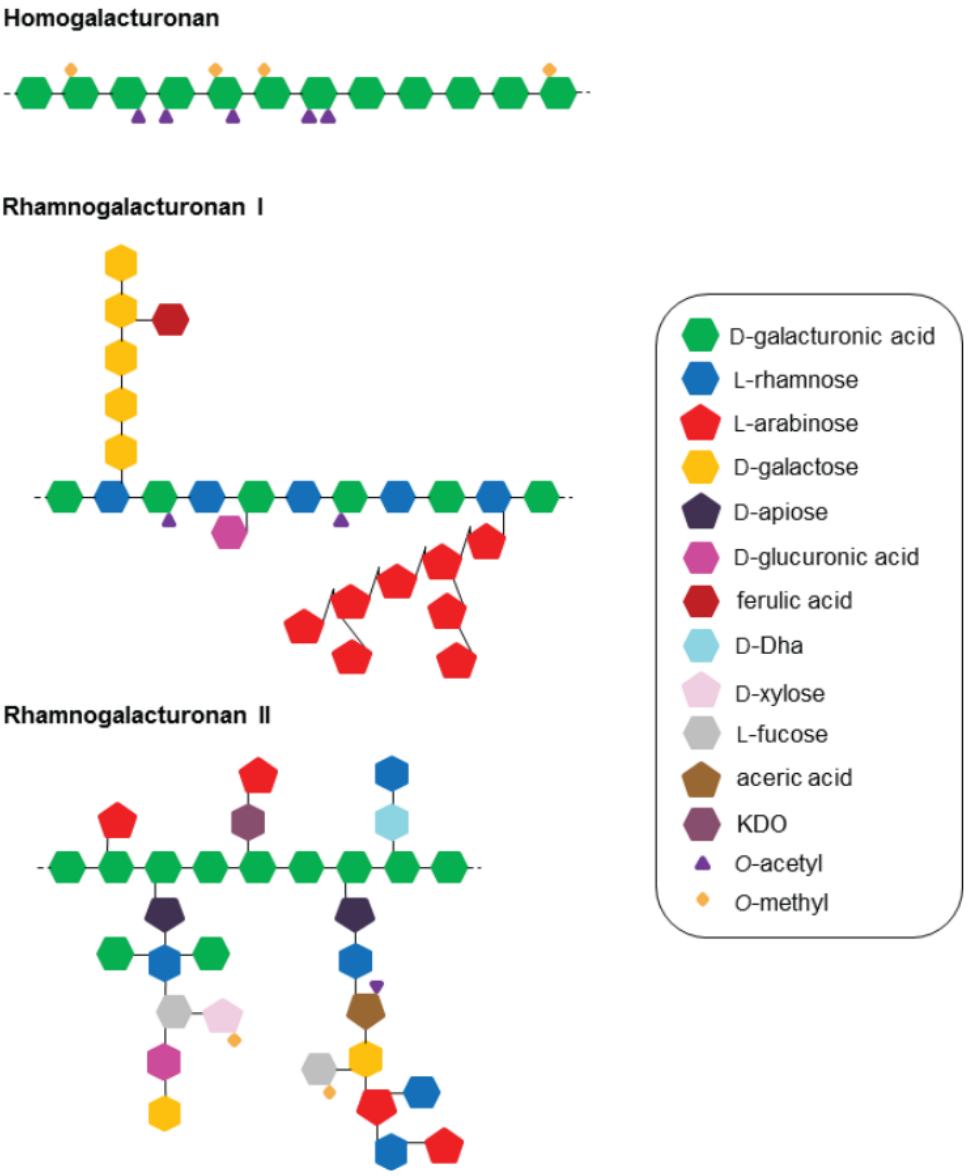

Figure 5 - Schematic representation of the sugar beet pulp pectin substructures HG, RG-I and RG-II (D-Dha: 2- keto-3-deoxy-D-lyxo-heptulosaric acid; KDO: 2-keto-3-deoxy-D-manno-octulosonic acid) (Leijdekkers, 2015).

Sugar beet pulp pectin has been thoroughly characterized by Leijdekkers (2015). HG is a linear homopolymer of α-(1,4)-linked Gal-UA residues, which can be methyl esterified at the C-6 position and acetylated at the *O*-2 or *O*-3 position. RG-I is formed by a backbone of alternating Gal-UA and rhamnose residues. Linear β-(1,4)-linked galactan and highly branched arabinan, composed of α-(1,5)-linked backbones with α-(1,2)- and/or α-(1,3)-arabinofuranosyl substitutions, are side chains of RG I. The galactan and arabinan side chains of sugar beet pectin RG I can be feruloylated. Finally, the most structurally complex substructure only present in minor amounts in sugar beet pulp pectin (RG-II) consists of an HG backbone of at least eight α-(1,4)-linked Gal-UA residues decorated with side branches consisting of 12 different types of sugars in over 20 different linkages (Mohnen, 2008). When compared to the other pectin-rich feedstocks (Figure 4), not only does sugar beet pulp have a higher pectin fraction (24-32% on a dry weight basis), but also its pectin is structurally different from the one in citrus peel waste and apple pomace: the length of HG chains is shorter, the amount of RG-I is higher and richer in arabinan side chains, and its degree of acetylation is also higher as acetyl groups are present in both HG and RG-I (around 30%). The combination of these structural features results in poor gel-forming ability but good emulsification properties due to its relatively hydrophobic nature (Berłowska *et al*., 2018).

In addition to its polymeric composition, the detailed chemical composition of sugar beet pulp is also known. Table 1 presents the relative amounts of cell wall polysaccharide constituents of this feedstock as well as other constituents, on a dry weight basis.

Table 1 - Detailed dry weight composition of sugar beet pulp (Leijdekkers, 2015).

Fraction	**Dry weight (%)**
Glucose±	21.1-24.5
Arabinose±	17.3-23.5
Galactose±	4.3-5.8
Rhamnose±	1.1-2.4
Galacturonic acid±	18.0-22.0
Ferulic acid±	0.5-0.9
Xylose±	1.1-1.7
Mannose±	1.0-1.5
Fucose±	0.1-0.3
Methanol±	0.4-2.3
Acetic acid±	1.6-3.9
Sucrose	2.1-3.0
Protein (N×6.25)	7.0-13.2
Lignin	1.8-3.4
Fat	1.0-2.0
Ash	3.6-8.4

±cell wall polysaccharide constituent

As Table 1 suggests, the monomeric sugars composing beet pulp predominantly consist of D-glucose, L-arabinose and D-galacturonic acid; other monomers like D-xylose, D-galactose and L-rhamnose are present in significantly lower amounts (Grohmann & Bothast, 1994; Micard *et al*., 1996). This rather distinctive and interesting composition prompts new strategies to push valorization of this waste product beyond selling it as animal feed. In fact, the recognition of underexplored value in sugar beet pulp has driven efforts from both sugar producers and research groups. Sugar beet pulp has been studied as feedstock for bioethanol (Edwards & Doran-Peterson, 2012) and biogas production (Ziemiński & Kowalska-Wentel, 2017), while a recent project led by sugar producer Cosun aimed at exploring the applications of microcellulose fibers, arabinose and Gal-UA in sugar beet pulp (PULP2VALUE, 2015).

The present thesis is focused on the valorization of sugar beet pulp through microbial fermentation-based processes with the aim of producing value-added compounds. For this purpose, sugar beet pulp is an attractive feedstock since high concentrations of fermentable sugars and Gal-UA can be obtained through inexpensive enzymatic saccharification of an inexpensive by-product (Protzko *et al*., 2018). Besides its composition, other factors add great value to sugar beet pulp as feedstock: the fact that is originates from the processing of beet to meet sugar demand guarantees a constant and ample supply, and because it is a by-product of an existing industrial process, the material is already stockpiled in large amounts (reducing collection and transportation costs) and partially (hot water) pretreated when exiting the process, facilitating further decomposition of the plant cell wall (Doran *et al*., 2000).

2.3 Sugar beet pulp deconstruction

2.3.1 Pretreatment: purpose and associated drawbacks

In order to be used in microbial fermentation, the cell wall material of lignocellulosic biomass must be decomposed into its simple sugar monomer units. Hydrolysis with exogenous enzymes is the key process in making such monosaccharides available for fermentation, since the most commonly used microorganisms (e.g. *Saccharomyces cerevisiae*, *Escherichia coli*) do not produce the enzymes necessary to degrade cellulose, hemicellulose and pectin (Foster *et al*., 2001; Zheng *et al*., 2012). Figure 6 represents how these polysaccharides are structurally organized within the primary plant cell wall: a scaffold of cellulose microfibrils crosslinked with hemicellulose tethers forms the cellulose-hemicellulose network, which in embedded in a pectin matrix. The other key component of the plant cell wall – lignin - is mostly present in the secondary wall linked in a network to cellulose and hemicellulose, strengthening its structure (Sticklen, 2008).

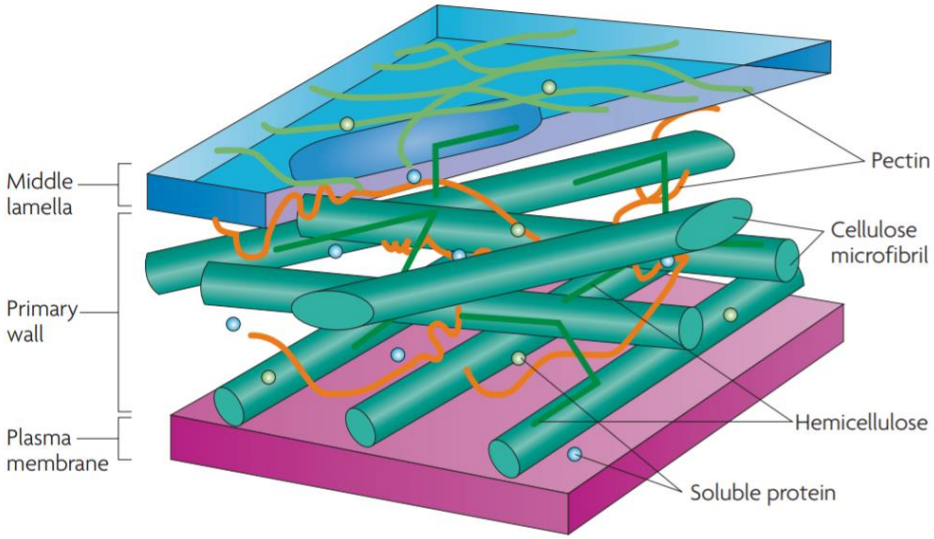

Figure 6 - Plant primary cell wall structure (Sticklen, 2008).

In processes aiming for the biotechnological conversion of lignocellulosic biomass, the feedstocks often undergo a preparation step prior to hydrolysis, commonly known as pretreatment. This is a crucial step in weakening the recalcitrant structure of the material and making it more susceptible to enzymes and chemicals, thus facilitating further processing (Jędrzejczyk *et al*., 2019). Pretreatment methods can be physical, chemical or physicochemical and their main goals include efficient removal of lignin, degradation of hemicellulose, reduction of cellulose crystallinity and increase of surface porosity, while minimizing destruction of fermentable sugars (Jędrzejczyk *et al*., 2019; Kumar *et al*., 2009).

The severity of a pretreatment can be measured by a combined severity factor that takes into account pretreatment time, temperature, acidity (hydrogen ion concentration) and pH after pretreatment (Abatzoglou *et al*., 1992; Chum *et al*., 1990). Overall, a pretreatment has to be strong enough to remove the protective lignin coatings and disrupt the cellulose-hemicellulose network; however, as the severity of the pretreatment increases, more biomass is degraded to non-fermentable products and compounds toxic to fermenting microorganisms (Kühnel *et al*., 2011).

Almeida *et al*. (2007) published a thorough study covering the origin and effects of such compounds, as well as strategies to increase tolerance in yeast *S. cerevisiae*. These inhibitors can be organized in three main groups: furan derivatives (furfural and hydroxymethylfurfural), weak acids (mainly acetic acid, formic acid and levulinic acid) and phenolic compounds. The furan compounds hydroxymethylfurfural (HMF) and furfural are formed by dehydration of hexoses and pentoses, respectively. Regarding weak acids, acetic acid is formed by de-acetylation of hemicelluloses, while formic and levulinic acids are products of HMF breakdown. Phenolics are formed due to lignin breakdown and carbohydrate degradation during acid hydrolysis. Even though these inhibitors are chemically different and thus act by different mechanisms (Figure 7), they all strongly affect the fermentation performance of the fermenting organism, inhibiting growth and reducing product yield and productivity.

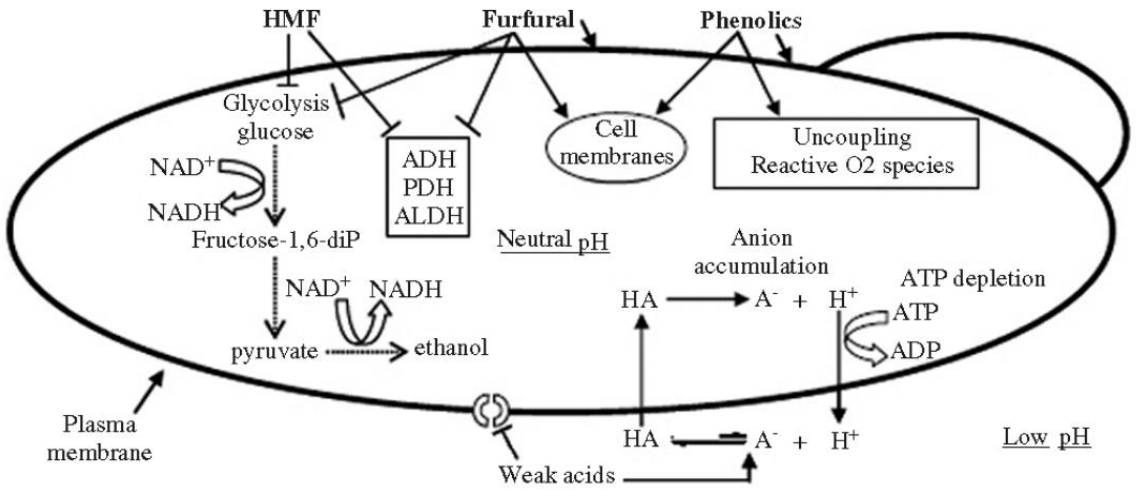

Figure 7 - Schematic representation of the known inhibition mechanisms of furans, weak acids and phenolic compounds in *S. cerevisiae* (Almeida et al., 2007).

As depicted in Figure 7, furan compounds are capable of inhibiting several important enzymes and disrupting cofactor balance, affecting vital pathways like glycolysis. On the other hand, the inhibitory effects of weak acids are caused by the diffusion of their dissociated form across the plasma membrane, dissociating intracellularly and leading to toxic anion accumulation and decrease in cytosolic pH. This ultimately results in ATP depletion as pumping protons out of the cell requires ATP hydrolysis. Finally, although the inhibition mechanisms of phenolic compounds are not completely elucidated in *S. cerevisiae*, it is known that they affect the integrity of the cell membrane while also generating reactive oxygen species.

2.3.2 The need for pretreatment in sugar beet pulp

While for some highly recalcitrant feedstocks like woods and grasses, a harsh pretreatment is a requirement for an efficient hydrolysis (Kumar *et al*., 2009), that is not the case of sugar beet pulp. According to some authors, sugar beet pulp fermentations do not require particle size reduction nor expensive thermochemical processing (Edwards & Doran-Peterson, 2012; Rorick *et al*., 2009). As previously mentioned, its naturally low lignin content makes the polymeric structure easily accessible for enzymatic hydrolysis without prior pretreatment, which is very attractive from a process engineering perspective. This means that sugar beet pulp fermentations generate few inhibitor compounds (especially phenolics), which does not compromise yeast performance. In addition, there is no need to run a pretreatment reactor which usually entails great costs, primarily in energy usage, but also since it must be simultaneously resistant to corrosion, high mechanical abrasiveness and high temperatures, requiring high-grades of steel (Jędrzejczyk *et al*., 2019; Kumar *et al*., 2009).

Despite the low lignin content in this feedstock, numerous authors have tested several different pretreatments in sugar beet pulp conversion studies, aiming at examining their effects in subsequent enzymatic degradability. The most relevant studies thus far in this topic are summarized in Table 2, presenting information regarding the types of pretreatment tested, optimal conditions for sugar release

and the parameter used in each work to express the hydrolysis yield - total reducing sugars yield (mg sugars/g SBP) or total hydrolysis yield (%).

Table 2 - Relevant studies on the influence of pretreatment in enzymatic hydrolysis of sugar beet pulp.

Pretreatment	Optimal conditions	Hydrolysis yield	Reference
Acid	1.1 g H_2SO_4/g SBP T = 80 °C t = 90 min	**After pretreatment** Cellulose = 7.8% Hemicellulose = 86.3%	Chamy *et al*., 1994
Ammonia pressurization depressurization (APD)*	SBP moisture level (w/w) = 50% Ammonia load to SBP = 0.5:1 T = 80 °C t = 5 min	**After enzymatic hydrolysis** Untreated = 786 mg/g APD-treated = 608 mg/g	Foster *et al*., 2001
Hydrothermal and mild acid	Hot water T = 140 °C t = 15 min	**After enzymatic hydrolysis** Untreated = 34% Pretreated = 80%	Kühnel *et al*., 2011
Pressure-thermal and ultrassound	2% (w/w) H_2SO_4 T = 121 °C t = 30 min	**After enzymatic hydrolysis** Untreated = 72% Pretreated = 86%	Berłowska *et al*., 2016
Aqueous ammonia	Solid-to-liquid ratio (m/V) = 1:20 T = 80 °C t = 6 h	**After enzymatic hydrolysis** Untreated = 185.2 mg/g Pretreated = 448.5 mg/g	G. Li *et al*., 2017

Note: T – temperature; t – time.
*modification of the ammonia fiber expansion (AFEX) pretreatment.

Chamy *et al*. (1994) performed one of the first studies in this topic, applying acid pretreatment with hydrochloric or sulphuric acid to selectively solubilize the hemicellulose fraction, yielding a cellulose-rich fraction for further fermentation or enzymatic hydrolysis. Ammonia pressurization depressurization (APD) pretreatment was also tested in another work, but it was found that it only significantly increased the hydrolysis efficiency of the cellulose component. What is more, it was found that this pretreatment seemed to alter the structure of pectin and/or hemicellulose and consequently the enzymatic hydrolysis yields for untreated pulp were higher than for the APD-pretreated pulp when a mixture of hemicellulases and pectinases was added (Foster *et al*., 2001). Later, Kühnel *et al*. (2011) investigated the influence of six mild sulphuric acid or water pretreatments at different temperatures on the enzymatic degradability of sugar beet pulp. Interestingly, one of the less severe pretreatments tested (140°C for 15 minutes, suspended in water) proved to be optimal in solubilizing pectin-associated sugars like arabinose and Gal-UA on its own, whereas higher temperatures destroyed these monomers and promoted the formation of fermentation inhibitors. When comparing the solubilization of the main sugars after enzymatic hydrolysis, the optimal pretreatment was found to largely improve the release of glucose and arabinose, with also an evident but smaller effect in Gal-UA release. These results suggested that an adequate pretreatment can improve the enzymatic degradability of sugar beet pulp. Specifically in pectin hydrolysis, while this study achieved a maximum yield of 75% (140°C for 15 minutes, suspended in 1% w/w H_2SO_4), degradation of this polymer up to 95% has been demonstrated (Leijdekkers, 2015).

Similar results were later obtained by Berłowska *et al*. (2016) using a similar pretreatment (121°C for 30 minutes, suspended in 2% w/w sulphuric acid solution). Among other conditions tested, this pretreatment showed synergy with enzyme action, leading to a final hydrolysis degree of 86%, which corresponded to an increase of approximately 14% when compared to the hydrolysis without pretreatment. In addition, it was found that this pretreatment also significantly improved the solubilization of glucose, xylose, arabinose and Gal-UA, supporting the hypothesis that the enzymatic hydrolysis of cellulose, hemicellulose and pectin in beet pulp is favoured by a previously optimized pretreatment. Finally, G. Li *et al*. (2017) tested a milder ammonia pretreatment and obtained better results with a 2.4-fold higher total reducing sugar yield for the treated pulp.

In general, the track record of previous studies regarding sugar beet pulp pretreatment unarguably indicates that this process can enhance enzymatic hydrolysis, enabling the latter to be carried out within economically feasible time ranges and enzyme concentrations (Kühnel *et al*., 2011). However, past works have not yet converged in claiming a specific pretreatment as the best for this feedstock; what is more, the decisive point in whether to do a pretreatment is to find out if the increased enzymatic hydrolysis yield is worth the investment in such an expensive process and its required equipment, which in turn is very dependent on the value of the final product. An attempt at providing a solution for this debate in the context of the yeast-based conversion of Gal-UA to galactaric acid is presented in section 3.3.1.

2.3.3 Chemical hydrolysis

Although discussing the need for pretreatment is indeed relevant, the central process in any biomass deconstruction is the saccharification. In theory, the breakdown of carbohydrates into their monomeric components can be achieved through chemical or enzymatic hydrolysis reactions. Chemical hydrolysis of lignocellulosic biomass was used long before the enzymatic processes emerged (Lee *et al*., 1999), with sulphuric and hydrochloric acids being the most commonly used catalysts at concentrations of 1-10% w/w (higher concentrations require higher grades of steel) using a moderate temperature (100-150 °C) (Lenihan *et al*., 2010). In this chemical process, the acid plays a dual role: it decrystallizes cellulose (making it accessible to reagents) through disruption of intra and interchain hydrogen bonds, and releases monomeric sugars by catalysing the hydrolysis of glycosidic bonds in cellulose and hemicellulose (Binder & Raines, 2010; Xiang *et al*., 2003). The main advantages of chemical hydrolysis are the low cost of catalyst which can penetrate lignin, reducing the need for pretreatment, its faster hydrolysis rate when compared to the enzymatic process and the fact that it can achieve 80-90% conversion of cellulose and hemicellulose into sugars (Binder & Raines, 2010; Lenihan *et al*., 2010; Taherzadeh & Karimi, 2007), for instance, in rice straw processing (Farone & Cuzens, 1998).

As far as sugar beet pulp is concerned, chemical saccharification has been investigated to a small extent. Schaffeld *et al*. (1987) studied a two-step hydrolysis process consisting of mild acid treatment followed by enzymatic hydrolysis to obtain fermentable sugars from this feedstock. However, the first

stage was only intended to achieve maximum hemicellulose degradation, producing a cellulose-rich fraction which then was treated with cellulases to obtain glucose. In contrast, Spagnuolo *et al*. (1997) subjected sugar beet pulp to complete acid hydrolysis (using higher concentrations) for the purpose of having a comparison term for evaluating enzymatic hydrolysis assays. Hence, the objectives of such studies along with the scarce record of investigation in this chemical process suggest this is not the preferred option for sugar beet pulp hydrolysis.

Other serious drawbacks are associated with chemical hydrolysis. Firstly, the operational conditions of acid hydrolysis are prone to the formation of some the above-mentioned fermentation inhibitors such as furfural, HMF and acetic acid, with their production increasing when higher temperatures and higher acid concentrations are used (Hamelinck *et al*., 2005). Secondly, the hydrolysates obtained must be conditioned before being used as fermentation media. In general, operations of concentration, detoxification, neutralization and supplementation with nutrients are required to eliminate the generated inhibitors and obtain a favorable fermentation media (Lenihan *et al*., 2010). To summarize, chemical hydrolysis is usually an undesired process since it results in high amounts of chemical waste (salts), substantial energy consumption, unspecific polysaccharide degradation and partial breakdown of the monomers released (Leijdekkers, 2015).

2.3.4 Enzymatic hydrolysis

Enzymatic hydrolysis is an alternative to the flawed chemical process. In contrast, the use of enzymes for breakdown of polysaccharides offers several advantages: high specificity of biological catalysts which have the potential for an almost complete conversion, no formation of inhibitory compounds and milder reaction conditions, requiring less energy consumption and no need for corrosion resistant equipment (Taherzadeh & Karimi, 2007; Wahlström & Suurnäkki, 2015). However, the efficiency of enzymatic hydrolysis is strongly dependent on a correct enzyme choice to target the polymer composition of the feedstock being decomposed.

In the case of sugar beet pulp, several enzymes are needed to fully degrade its cell wall polysaccharides due to its diversified composition. Leijdekkers (2015) also described the enzymes needed for the complete saccharification of this feedstock. For the well-known cellulose degradation reactions, three enzymes are needed – this mechanism is illustrated in Figure 8.

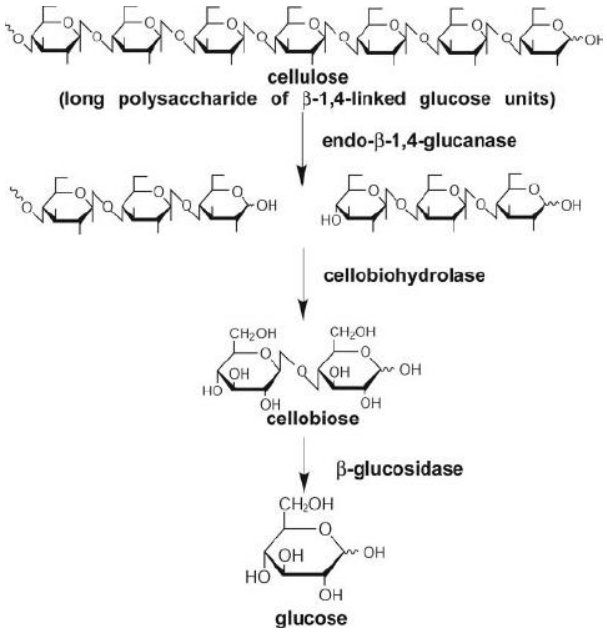

Figure 8 – Cellulose breakdown reactions and the intervenient enzymes (Xie *et al*., 2007).

Cellulase (EC 3.2.1.4) is the first intervenient, catalysing the hydrolysis of β-(1,4)-D-glucosidic linkages in cellulose. Further degradation into disaccharide cellobiose is carried out by cellobiohydrolase (EC 3.2.1.91) and the formation of glucose monomers is due to the action of β-glucosidase (EC 3.2.1.21). Alternatively, the more recently discovered lytic polysaccharide monooxygenases (LPMO) can also be used to catalyse the oxidative cleavage of cellulose (Horn *et al*., 2012), making the crystalline cellulose available for the previously mentioned cellulases.

Pectin hydrolysis is substantially more intricate, with its structural complexity (as described in section 2.2.2) translating into a more complex enzymatic hydrolysis, requiring the use of many different enzymes to assure the release of all its monomeric constituents. The currently known enzymes involved in sugar beet pectin degradation are listed in Table 3.

Table 3 - Overview of enzymes involved in the degradation of sugar beet pulp pectin (Leijdekkers, 2015).

Enzyme name	**Abbreviation**	**EC number**
HG degrading enzymes		
Endo-polygalacturonase	PGA	EC 3.2.1.15
Exo-polygalacturonase	PGX	EC 3.2.1.67
Pectin lyase	PEL	EC 4.2.2.10
Pectate lyase	PYL	EC 4.2.2.2
Pectin methylesterase	PME	EC 3.1.1.11
Pectin acetylesterase	PAE	EC 3.1.1.6
RG-I degrading enzymes		
Rhamnogalacturonan hydrolase	RGH	EC 3.1.1.171
Rhamnogalacturonan lyase	RGL	EC 4.2.2.23/24
Unsaturated rhamnogalacturonyl hydrolase	URH	EC 3.2.1.172
Rhamnogalacturonan rhamnohydrolase	RHA	EC 3.2.1.174
Rhamnogalacturonan galacturonohydrolase	RGX	EC 3.2.1.173

Rhamnogalacturonan acetylesterase	RGAE	EC 3.1.1.86
Endo-galactanase	GAL	EC 3.2.1.89
B-Galactosidase	LAC	EC 3.2.1.23
Endo-arabinase	ABN	EC 3.2.1.99
Exo-arabinase	ABX	EC 3.2.1.-
Arabinofuranosidade	ABF	EC 3.2.1.55
Ferulic acid esterase	FAE	EC 3.1.1.73

Figure 9 represents the structure of HG and signalizes the bonds broken by each of the HG degrading enzymes.

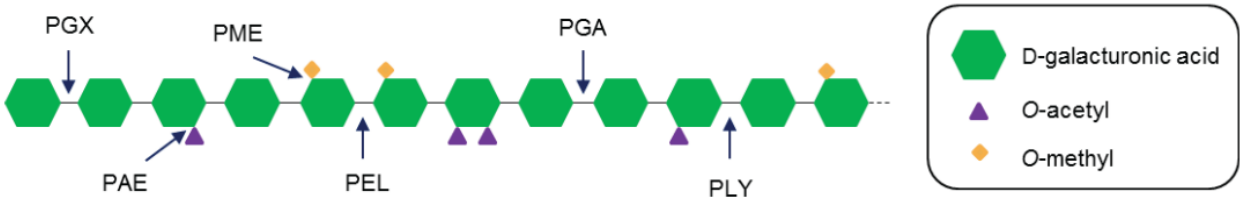

Figure 9 - Schematic representation of HG and the enzymes needed for its degradation (Leijdekkers, 2015).

The Gal-UA backbone of HG is cleaved by endo and exo-polygalacturonases (PGA, PGX). Lyases can also participate in backbone degradation through β- elimination, though their activity depends on the degree of methylesterification – pectin lyases (PEL) prefer a highly esterified HG in contrast to pectate lyases (PYL). De-esterification occurs through the action of pectin methyl esterase (PME) and pectin acetyl esterase (PAE).

Figure 10 represents the structure of RG-I and signalizes the bonds broken by each of the RG-I degrading enzymes.

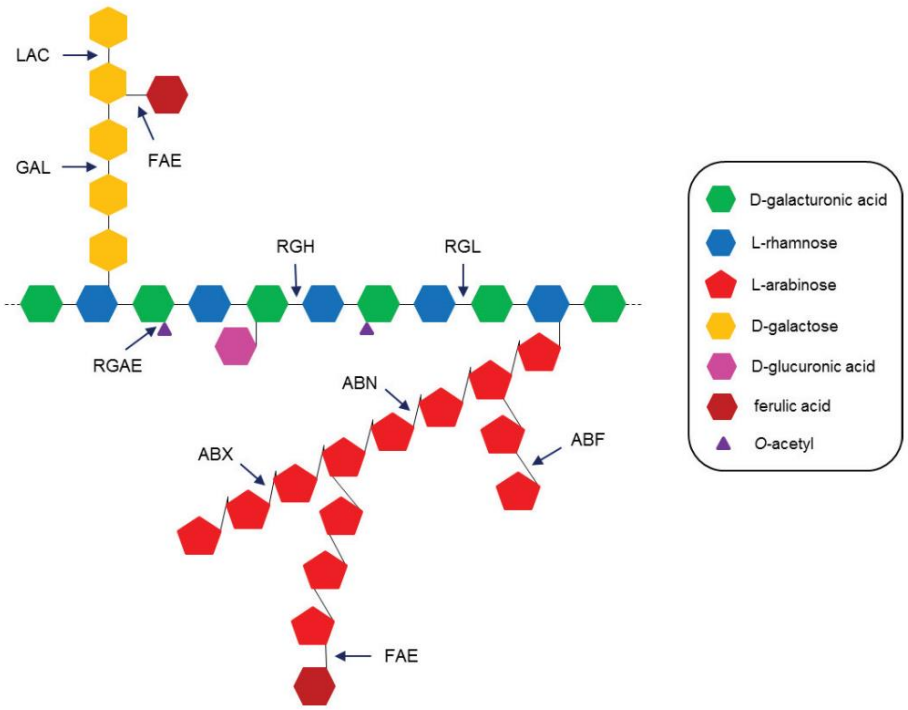

Figure 10 - Schematic representation of RG-I and the enzymes needed for its degradation (Leijdekkers, 2015).

Two types of endo-enzymes catalyse the cleavage of the RG-I backbone: rhamnogalacturonan hydrolase (RGH) and rhamnogalacturonan lyase (RGL). Three other exo-enzymes (not represented in Figure 10) have been identified to act on the main chain: unsaturated rhamnogalacturonyl hydrolase (URH), which acts specifically on unsaturated rhamnogalacturonan (resulting from action of RGL on RG I); rhamnogalacturonan rhamnohydrolase (RHA), which removes terminal rhamnosyl residues; and rhamnogalacturonan galacturonohydrolase (RGX), which removes terminal galacturonosyl residues from the non-reducing end of RG I. De-acetylation in the RG-I backbone is carried out by rhamnogalacturonan acetylesterase (RGAE). Regarding degradation of the side chains, endo-galactanase (GAL) and β-galactosidase (LAC) are needed for galactan degradation, while a group of arabinohydrolases cleaves the heavily branched arabinan chain. Endo-arabinanase (ABN) hydrolyzes the α-(1,5)-linkages in the unsubstituted regions, exo-arabinanase (ABX) attacks the non-reducing end of the backbone and arabinofuranosidase (ABF) is able to release monomeric arabinose from all non-reducing ends of arabinan or arabino-oligosaccharides. Finally, the cleavage of feruloyl groups from the galactan or arabinan side chains is carried out by ferulic acid esterase (FAE). Thus far, no enzymes for the specific degradation of RG-II have been isolated. However, it is thought that some of the enzymes acting on HG and RG-I might also act in RG-II (Bauer *et al*., 2006).

Besides their isolated action, past studies identified a considerable synergy between cellulolytic and pectinolytic activity in sugar beet pulp hydrolysis. It was found that the effectiveness of cellulolytic activity was much higher when pectinases where introduced in the enzyme mixture, indicating that pectic substances create a protection barrier which must be removed to allow efficient attack of cellulases in the other lignocellulosic components (Micard *et al*., 1997; Spagnuolo *et al*., 1997).

Although Leijdekkers (2015) thoroughly describes the enzymes required for hydrolysis of sugar beet pulp cellulose and pectin, it is claimed that this feedstock is almost devoid of xyloglucan and there is no mention regarding enzymes for degradation of hemicellulose. In general, it has been said that an almost total degradation of the primary plant cell wall can be achieved by the use of a mixture of cellulolytic and pectinolytic activities (Micard *et al*., 1997). Specifically in sugar beet pulp, other studies have also assessed the need of hemicellulolytic activity in its hydrolysis: while Spagnuolo *et al*. (1997) observed an absence of significant synergy when hemicellulase was coupled with combined cellulase and pectinase, Zheng *et al*. (2012) tested a hemicellulase preparation with predominant xylanase activity and found that it was not effective in breaking down beet pulp hemicellulose, concluding that the hydrolysis of this feedstock could be conducted only with cellulases and pectinases. Rezic *et al*. (2013) continued to support the theory that hemicellulolytic activity is not crucial by stating that the release of arabinose from beet pulp hemicellulose was due to the activity of pectinases.

In fact, such findings are coherent with the monomeric composition of sugar beet pulp (Table 1). Its minimal xylose and significant arabinose concentrations suggest that beet pulp hemicellulose is indeed low in xylan and xyloglucan but high in arabinan, which explains that enzymes with xylanase activity are not effective whereas pectinases with arabinase activity are effective in both pectin and

hemicellulose. Nevertheless, more experiments with different hemicellulases ought to be carried out in order to fully understand whether these are a valuable addition to enzyme cocktail for sugar beet pulp hydrolysis, especially since different commercial enzymes behave differently, have different optimal reaction conditions are very dependent on factors such as the dry matter content of the suspension being treated (Zheng *et al*., 2012).

2.4 Galacturonic acid

2.4.1 Characterization and underutilization of its fermentation potential

Gal-UA ($C_6H_{10}O_7$) is a weakly acidic and water soluble sugar acid (Figure 11). Sugar acids are monosaccharides with a carboxyl group at one or both ends of its chain, and there are three classes of sugar acids: aldonic, aldaric and uronic acids. More specifically, Gal-UA is an uronic acid derived from galactose - the terminal carbon's hydroxyl group has been oxidized to a carboxylic acid (Mehtiö *et al*., 2016). It is the main component of pectin, in which it exists as the polymer polygalacturonic acid.

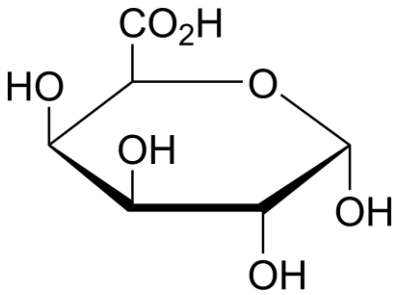

Figure 11 - Chemical structure of galacturonic acid.

Due to its abundance in pectin, Gal-UA is one of the main components of sugar beet pulp hydrolysate, along with glucose and arabinose. Despite being a feedstock with a rich and distinctive composition, some recent studies covering biorefinery concepts for valorization of sugar beet pulp have shown underutilization of its fermentation potential. This trend was motivated by the optimization of pressurized steam pretreatment in sugar beet pulp, which enabled a selective solubilization of pectin (Hamley-Bennett *et al*., 2016). Thereafter, Cárdenas-Fernández *et al*. (2017) proposed a biorefinery concept in which sugar beet pulp was fractionated into soluble pectin and insoluble cellulose fractions. While the cellulose fraction would be readily digested by cellulases to obtain a glucose-rich stream used in yeast fermentation, the pectin fraction would be selectively hydrolyzed to produce arabinose-rich and galacturonic-acid rich streams but these monomers would be converted into value-added compounds (L-gluco-heptulose and 6-amino-2,3,4,5-tetrahydroxyhexanoic acid, respectively) instead of being substrates for fermentation. Subsequent studies followed this idea in the development of biorefinery concepts for the production of succinic acid (Alexandri *et al*., 2019) and lactic acid (Oliveira *et al*., 2020). According to the process proposed in these works, pectin and phenolic compounds would be extracted from sugar beet pulp to be sold, prior to enzymatic hydrolysis. Thus, only cellulose and hemicellulose would be hydrolyzed to yield fermentable sugars.

The fact that Gal-UA is not considered fermentable in such studies suggests that there is a technology gap left to be addressed. Exploring its fermentation potential would strongly increase the potential for fermentative valorization of pectin – which is the polysaccharide that makes sugar beet pulp such a unique feedstock – even if there are already solutions that provide the necessary economical return to conceive a profitable biorefinery. Furthermore, the current neglecting of Gal-UA fermentation in sugar beet pulp biorefineries cannot be justified by a lack of knowledge about this sugar acid metabolism. In fact, Gal-UA can be naturally catabolized by many organisms and there are currently two prokaryotic pathways and one fungal pathway elucidated and reported.

2.4.2 State of the art in galacturonic acid metabolism

Kuivanen *et al*. (2019) and Valk (2020) have recently compiled and described the metabolic pathways of Gal-UA known to this day. In many well-studied bacteria like *Escherichia coli*, *Bacillus subtilis*, *Thermotoga maritima* and *Lactobacillus brevis*, Gal-UA is metabolized via the isomerase pathway (also known as adapted Entner-Doudoroff pathway). This pathway is shown on the left side of Figure 12.

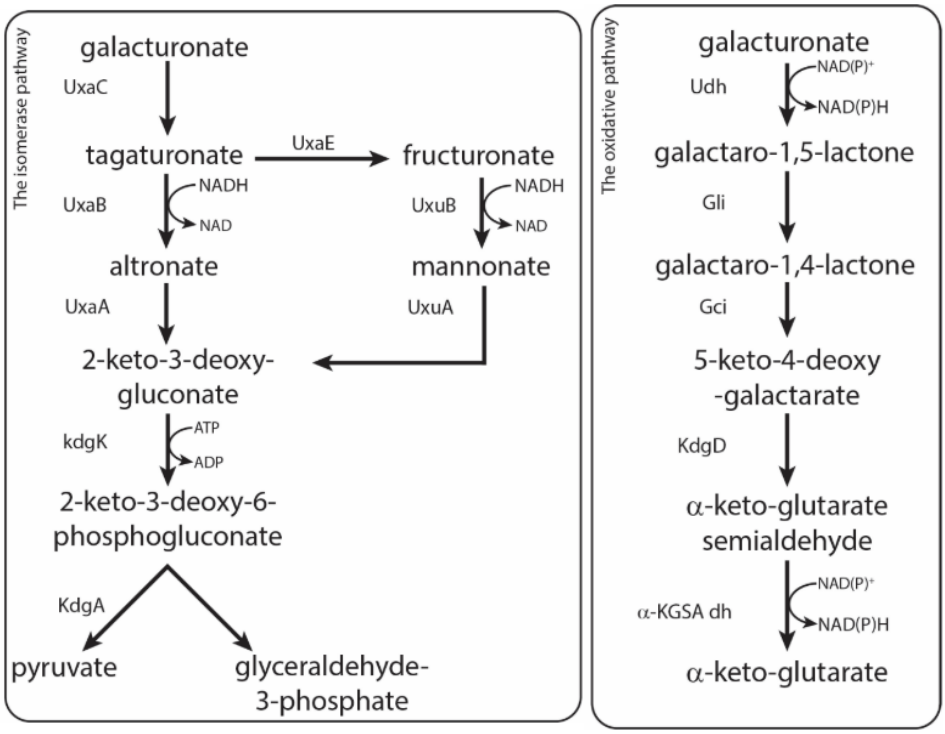

Figure 12 - Known bacterial pathways for galacturonic acid metabolism: isomerase pathway (left) and oxidative pathway (right) (Valk, 2020).

Isomerase pathway

This pathway was first elucidated in *Escherichia coli*. It starts with the isomerization of Gal-UA to tagaturonate by uronate isomerase (UxaC, EC 5.3.1.12) and subsequent reduction and dehydration lead to formation of 2-keto-3-deoxygluconate. An alternative route to form this metabolite was also discovered: conversion of tagaturonate to fructuronate, also followed by reduction and dehydration. From 2-keto-3-deoxygluconate, the isomerase pathway connects with the canonical Entner-Doudoroff pathway for sugar metabolism through the formation of its signature intermediate 2-keto-3-deoxy-6-phosphogluconate (KDPG) by phosphorylation. Finally, KDPG is split into two C3-molecules, pyruvate and glyceraldehyde-3-phosphate (G3P). As shown in Figure 12, in both variants of the pathway, conversion of Gal-UA into pyruvate and glyceraldehyde-3-phosphate requires the input of one ATP and one NA(P)H. Further conversion of G3P to pyruvate via glycolysis produces two ATP and one NADH (Appendix A – Supporting material for yeast metabolism). Thus, the isomerase pathway enables a redox-cofactor-neutral conversion of one mole of Gal-UA to two moles of pyruvate while yielding one mole of ATP (if there is no ATP expense for Gal-UA uptake). This perfect redox-cofactor balance results in a limited product range generated from Gal-UA fermentation since it implies that reactions beyond pyruvate should also be redox-cofactor-neutral. Consequently, acetate is typically found as the main product in Gal-UA anaerobic growth of wild-type microorganisms, as it is formed through redox-cofactor-neutral and ATP-yielding reactions (Valk *et al*., 2020).

Recently, a new study by the same author has elucidated a novel pathway for Gal-UA metabolism that potentially circumvents the limitations inherent to the isomerase pathway (Valk *et al*., 2020). This novel pathway, discovered in *Lactobacillus suebicus* through microbial enrichment cultivations, links the upper part reactions of the canonical isomerase pathway with the phosphoketolase pathway through the concerted action of two enzymes: mannonate kinase and 6-phosphomannonate 2-epimerase. Ultimately, this pathway enables the redox-cofactor neutral conversion of Gal-UA to ribulose-5-phosphate which is part of the non-oxidative phase of the pentose phosphate-pathway, paving the way for new metabolic engineering strategies for high-yield, anaerobic generation of products such as ethanol, isobutanol or lactate.

Oxidative pathway

The other prokaryotic pathway known for catabolizing Gal-UA is the oxidative pathway, represented on the right side of Figure 12. This pathway is present in many respiratory bacteria. It was first observed in a *Pseudomonas* species but is best understood from research on the plant pathogen *Agrobacterium tumefaciens*. It starts with oxidation of Gal-UA to D-galactaro-1,5-lactone by uronate dehydrogenase (UDH, EC 1.1.1.203) using NAD^+ as a cofactor. This is followed by two consecutive isomerization reactions. The last two steps of the pathway are a dehydration and oxidation resulting in formation of the citric acid cycle intermediate α-ketoglutarate. Overall, the oxidative pathway converts one mole of

Gal-UA in one mole each of α-ketoglutarate and carbon dioxide (released in the last reaction), generating two moles of NADH (Figure 12).

Fungal pathway

Finally, the fungal pathway for Gal-UA metabolism was first identified in the mould *Trichoderma reesei* and later also found in the well-studied fungus *Aspergillus niger* and in *Botrys cinerea*. This pathway is represented in Figure 13.

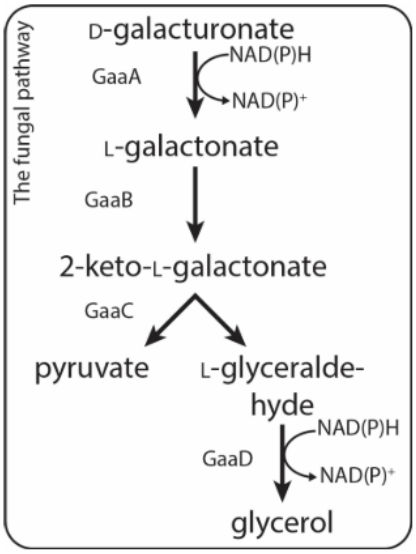

Figure 13 - Fungal pathway for galacturonic acid metabolism (Valk, 2020).

It starts with reduction of Gal-UA to L-galactonate by galacturonate reductase (GaaA, EC 1.1.1.365) using NADPH. Galactonate is then dehydrated to form 2-keto-L-galactonate which is subsequently split into two C3-molecules, pyruvate and L-glyceraldehyde. The latter is then reduced to form glycerol. As in the bacterial isomerase pathway, a C6-intermediate splits to yield two C3-compounds; however, in this case there is no ATP formation by substrate-level phosphorylation. Overall, this pathway converts one mole of Gal-UA in one mole each of pyruvate and glycerol, requiring two moles of NAD(P)H, as demonstrated in Figure 13. More recently, it was found that this pathway for Gal-UA assimilation is also present in the red basidiomycete yeast *Rhodosporidium toruloides*, with particularly high maximal velocities of several involved enzymes and an efficient downstream glycerol metabolism (Protzko *et al.*, 2019).

Of all three pathways presented, only the isomerase pathway allows for anaerobic fermentative growth using Gal-UA as substrate. However, as mentioned above, its neutral redox-cofactor balance constrains the possible range of fermentation products. The oxidative and fungal pathways do not allow for anaerobic growth on Gal-UA due to the absence of substrate-level phosphorylation (respiration would be required for ATP generation) (Valk, 2020).

2.4.3 Engineering galacturonic acid conversion

The reviewed current knowledge on microbial Gal-UA metabolism unfolds new opportunities to engineer the fermentation of this monomer in industrial 'workhorse' organisms such as *S. cerevisiae*. To this date, few studies have successfully achieved that. Regarding the bacterial isomerase pathway, its complete functional expression in yeast is still to be demonstrated (Kuivanen *et al*., 2019), though some of its genes have already been expressed as active proteins (Huisjes *et al*., 2012). In contrast, Biz *et al*. (2016) demonstrated for the first time the complete expression of the fungal pathway in *S. cerevisiae*, enabling consumption of Gal-UA using D-fructose as co-substrate; Protzko *et al*. (2018), while expressing the same pathway, was able to achieve growth exclusively on Gal-UA. More recently, Jeong *et al*. (2020) engineered *S. cerevisiae* for simultaneous consumption of Gal-UA and the pectin-derived pentoses xylose and arabinose, through the expression of the same fungal pathway.

In spite of the progress made in implementing such pathways in engineered yeast, there are still hurdles to overcome in order to achieve a functional expression suitable for an industrial process. Primarily, Gal-UA is more oxidised than the monomeric sugars generally used in fermentation, which inherently make it a more challenging substrate (Kuivanen *et al*., 2019). More importantly, the fact that metabolizing it requires reducing power that is also needed to form products such as ethanol means that further strain engineering and optimization of cultivation conditions are needed to solve this cofactor balancing problem (Biz *et al*., 2016).

The solution explored in this thesis is to metabolize Gal-UA through the bacterial oxidative pathway, but focusing only on the first conversion step carried by UDH (Figure 12), which was already proven to be functional in yeast – as described further on in section 2.6. Besides its role as the starting enzyme of the oxidative pathway, the sole expression of UDH through genetic engineering results in the oxidation of Gal-UA into *meso*-galactaric acid (Gal-AA) (Kuivanen *et al*., 2019). This reaction occurs through the mechanism shown in Figure 14.

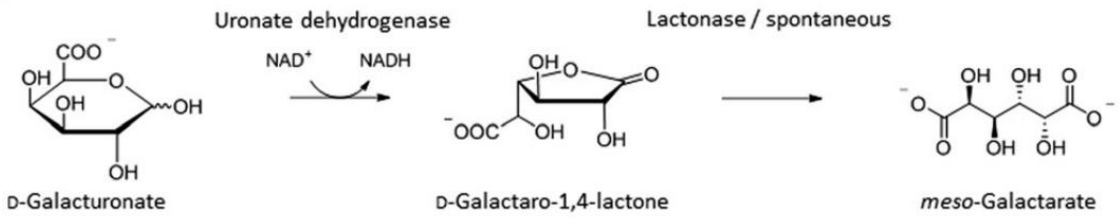

Figure 14 - Conversion of galacturonic acid to galactaric acid catalysed by the initial enzyme of the oxidative pathway - uronate dehydrogenase (Mehtiö *et al*., 2016).

In this case, the initial product of NAD-dependent UDH is D-galactaro-1,5-lactone (not represented in Figure 14), which converts into the more stable D-galactaro-1,4-lactone found in solution (Boer *et al*., 2010). Since the other enzymes of the oxidative pathway are not present, Gal-AA is formed through the opening of galactaro-1,4-lactone; this can be aided by a lactonase or happen spontaneously (Mehtiö *et*

al., 2016; Mojzita *et al*., 2010). However, it was found that this linearization does not occur at a meaningful rate at cytosolic pH, but is rather favoured by slightly basic conditions (Boer *et al*., 2010).

2.5 Galactaric acid

2.5.1 Characterization, applications and commercial interest

Meso-galactaric acid ($C_6H_{10}O_8$), also known as mucic acid, is a poorly soluble (in aqueous solution), optically inactive sugar acid resulting from the formal oxidative ring cleavage of galactose (EMBL-EBI, 2015; HMDB, 2020). Particularly, it integrates the aldaric acids class of sugar acids since both the aldehyde and the terminal hydroxyl groups of the original aldose were oxidized, generating a dicarboxylic acid (Mehtiö *et al*., 2016), as represented in Figure 15.

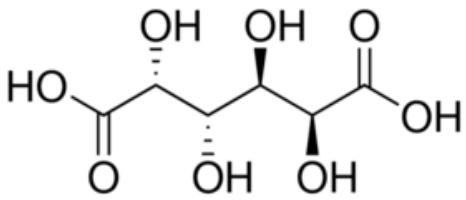

Figure 15 - Chemical structure of galactaric acid.

Recently, Gal-AA has been gaining considerable attention from both academia and industry due to its promising applications. In the food industry, Gal-AA has deserved interest for its weak acid properties – it shares the same applications as L-tartaric acid, so it can be used with carbonate as a leavening agent in self-rising flour (Ortiz-Sanchez *et al*., 2020). Gal-AA is also an interesting compound for the pharmaceutical and cosmetics markets since it can act as a chelator agent. In fact, it offers more effective chelation compared to existing bio-derived chelates such as citrate (Protzko *et al*., 2018), especially in sequestering calcium ions (Abbadi *et al*., 1999). Hence, it is used as an ingredient of skincare products designed for skin protection and anti-aging (Mojzita *et al*., 2010; Ortiz-Sanchez *et al*., 2020).

Finally, and more importantly in the scope of this thesis, Gal-AA holds great value as a platform chemical. High-purity Gal-AA can enable effective one-pot chemical conversions to adipic acid (X. Li *et al*., 2014) and 2,5-furandicarboxylic acid (FDCA) (Taguchi *et al*., 2008). While adipic acid is one of the two monomers required for the production of the high mechanical strength polyamide nylon-6,6, FDCA offers numerous opportunities for the creation of a wide range of polymers including polyesters, polyamides and polyurethanes (Avantium, 2019). In 2004, FDCA earned a distinction as one of the US DOE Top 12 sugar-derived building block chemicals (Werpy & Petersen, 2004). Undoubtedly, one of the most promising applications of FDCA is being the main building block for the synthesis of polyethylene furanoate (PEF). FDCA-based PEF is currently being pursued as a bio-based alternative for conventional, petroleum-based polyethylene terephthalate (PET) (Eerhart *et al*., 2012). Not only does PEF display environmental advantages being a 100% plant-based recyclable bioplastic with a wide range of applications (packaging, textiles, film), but it also demonstrates improved gas barrier and mechanical

properties when compared to PET (Avantium, 2019; Corbion, 2016). A summary of reviewed Gal-AA applications is illustrated in Figure 16.

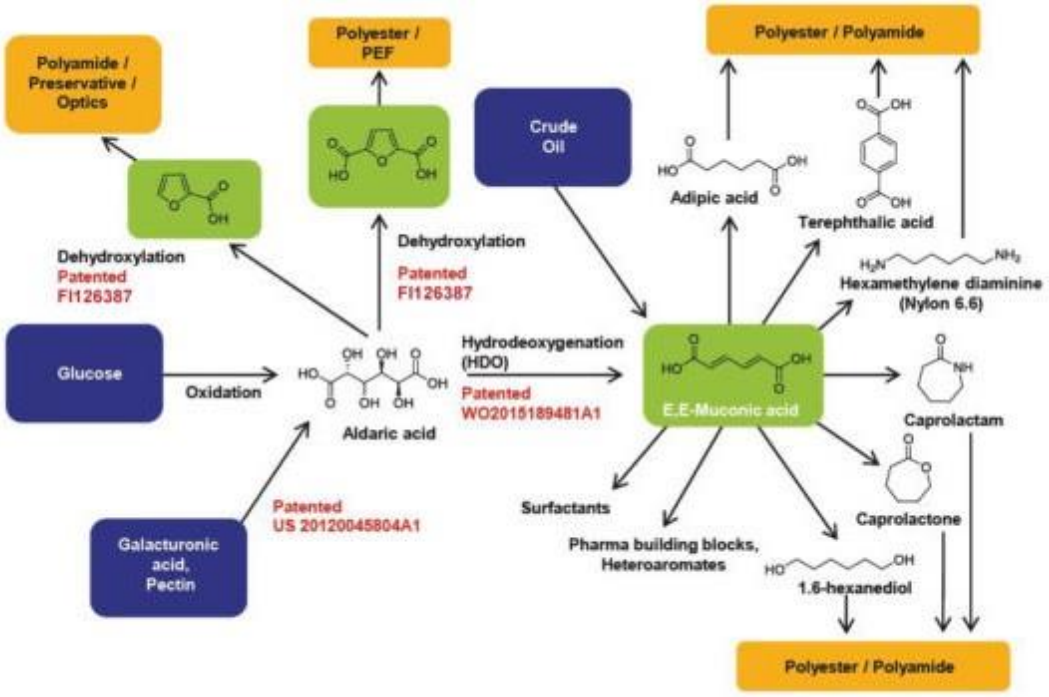

Figure 16 – Schematic representation of the pathways for valorization of galactaric acid. (VTT, 2017).

The distinctive applications of Gal-AA position it as a commercially appealing compound for various industries and markets. Gal-AA sells for up to 100 $ per kilogram (kg) in the cosmetics market (where is its currently applied), with the potential of competing with higher volume biobased chelates such as methylglycindiacetic acid (MGDA), which sells at 3–5 $/kg (Protzko *et al*., 2018). However, to address the chemicals market, Gal-AA would have to sell at a price in the range of other platform chemicals such as adipic acid (2.15 $/kg) and succinic acid (2.94 $/kg) (E4tech, 2015).

2.6 Conversion of galacturonic acid to galactaric acid

2.6.1 Chemical conversion processes

The wide range of applications and the economic value of Gal-AA have led to the creation and development of several technologies to obtain this compound. Currently, there are three chemical processes known for the production of this compound: nitric acid oxidation, electrolytic oxidation and oxidation with gold catalyst.

Nitric acid oxidation is hitherto the most studied chemical method to produce aldaric acids. This method enables the conversion of galactose to Gal-AA (Acree, 1931). The first versions of this process had some drawbacks – the solutions for removal of residual nitric acid after oxidation were inefficient and expensive, and there was generation of a significant amount of environmentally hazardous nitrogen oxide (NO_X) gases as side products which had to be captured and rendered harmless if they could not be recycled within the process (Mehtiö *et al*., 2016). More recently, further developments have improved

the controllability of the process, eliminating issues of thermal control, release of nitrogen into the atmosphere and post-reaction difficulties in removing nitric acid and inorganic nitrates (Kiely & Hash, 2010). Even though some studies claim that this process is currently used for commercial production of Gal-AA (Barth & Wiebe, 2017; Mojzita *et al*., 2010), no product or company were found in literature to be making use of this technology for that purpose.

Electrolytic oxidation uses Gal-UA as a precursor – an aqueous solution of this monomer is placed in the anode compartment of an electrolytic cell in the presence of a redox system (Fauvarque *et al*., 1994). This technology was used by French company Soliance to produce Gal-AA for application in cosmetic products (Mehtiö *et al*., 2016). In 2014, Swiss company Givaudan (a leading company in the flavor and fragrance industries) acquired Soliance (Givaudan, 2014) and started producing a mucic acid powder obtained from fruit-derived pectins and sold for its potent chelating properties (Givaudan, 2020).

The last chemical process for Gal-AA production to be reported was oxidation with gold catalyst. Like electrolytic oxidation, this process converts the pectin-derived uronic acid to the correspondent aldaric acid, but instead uses supported gold catalysts and air as oxidizing agent to achieve a mild, but highly efficient and selective oxidation (Rautiainen *et al*., 2015; van der Klis *et al*., 2013). Although there is still no record of this reaction being used for commercial production of Gal-AA, the technology has been patented (Van Es *et al*., 2015) and its current assignees are producing Gal-AA in a pilot facility, presumably for market development purposes (Cosun Biobased Products, 2018).

2.6.2 State of the art in biotechnological conversion

Table 4 presents a summary of all studies thus far published on the biotechnological conversion of Gal-UA to Gal-AA, indicating the organism used, origin of UDH gene and transporter (when used), process conditions, substrate/feedstock and three fermentation metrics: yield, productivity and rate. The production of Gal-AA from Gal-UA was demonstrated for the first time in fungi *T. reseei*, through overexpression of bacterial UDH and disruption of the fungal catabolic pathway existent in that organism (Mojzita *et al*., 2010). Later, Barth & Wiebe (2017) optimized the same process, reaching titres as high as 20 g/L and a calculated yield higher than theoretically feasible (1.08 g Gal-AA/g Gal-UA), indicating that evaporation and subsequent concentration of both galacturonic and galactaric acids occurred in this cultivation. Lastly, Paasikallio *et al*. (2017) developed this process to use pectin hydrolysate as substrate and scaled up the production to 14 g/L in a 250 L reactor. While in a 10 L reactor it was found that the yield was also higher than the theoretical maximum (probably also because of evaporation), scaling up to 250 L caused a decreased in yield, indicating that more efficient downstream processing (DSP) would be required for a large scale process. Another study explored the potential for Gal-UA conversion in a different fungus – *A. niger* – due to its efficient pectinase production (Kuivanen *et al*., 2016). However, Gal-AA production in this organism required not only expression of UDH gene, but also disruption of the native pathway that catabolised this product. In the end, this strain engineering led to successful production (though in lower titres and significantly lower yield compared

to studies with *T. reseei*) from both lab-grade Gal-UA and orange processing waste, the latter carried in consolidated bioprocessing (CBP) configuration – a single microorganism produces the saccharolytic enzymes and ferments the resulting sugar mixture in the same unit operation (Huisjes, 2013).

E. coli has also been investigated for production of Gal-AA (Zhang *et al*., 2016). Following a similar approach, the oxidative pathway and Gal-AA metabolism were disrupted while the UDH gene was expressed, enabling production at considerable yields, even when using sugar beet pulp hydrolysate as substrate. Finally, production of Gal-AA has also been demonstrated in *S. cerevisiae*. Benz *et al*. (2014) were the first to identify and characterize a novel eukaryotic Gal-UA transporter which, expressed along with UDH gene from *A. tumefaciens*, enabled the production of Gal-AA for the first time in yeast. Later, Protzko *et al*. (2018) not only identified a new and improved transporter for Gal-UA, but also demonstrated production of Gal-AA from citrus peel waste hydrolysate, having obtained higher titre and yield when glucose was used as co-substrate. It is relevant to highlight that this was the only study to this date to express the UDH gene from a different source (*Pseudomonas syringae*).

Table 4 - State of the art in biotechnological conversion of galacturonic acid to galactaric acid.

	Organism	UDH	Transporter	Process	Substrate/ Feedstock	Titre (g L^{-1})	Productivity (g L^{-1} h^{-1})	$Yield_{P/S}$ (g g^{-1})	Reference
Fungi	*Trichoderma reesei*	*Agrobacterium tumefaciens*	-	Batch, pH 6.5	Lab-grade Gal-UA	5.9	0.04	0.87	(Mojzita *et al.*, 2010)
	Aspergillus niger	*Agrobacterium tumefaciens*	-	Batch, pH 5	Lab-grade Gal-UA in YP-medium	4.2	0.035	0.21	(Kuivanen *et al.*, 2016)
				Batch, pH 3	Orange processing waste hydrolysate (CBP)	3.1	0.026	0.31	
	Trichoderma reesei	*Agrobacterium tumefaciens*	-	Fed-batch, pH 4	Lab-grade Gal-UA + lactose (co-subtrate)	20	0.175	1.11	(Barth & Wiebe, 2017)
	Trichoderma reesei	*Agrobacterium tumefaciens*	-	Fed-batch, pH 4, 10 L reactor	Food-grade pectin hydrolysate	21	0.14	1.1	(Paasikallio *et al.*, 2017)
				Fed-batch, pH 4, 250 L reactor	Food-grade pectin hydrolysate	14	0.15	0.8	
Bacteria	*Escherichia coli*	*Agrobacterium tumefaciens*	-	Batch, pH 7	Lab-grade Gal-UA + glucose + arabinose (co-substrates)	10.3	0.215	1.03	(Zhang *et al.*, 2016)
				Batch, pH 7	Sugar beet pulp hydrolysate	6.9	0.144	0.88	
Yeast	*Saccharomyces cerevisiae*	*Agrobacterium tumefaciens*	GAT-1	Batch, pH 6	Lab-grade Gal-UA	n.d.	-	n.d.	(Benz *et al.*, 2014)
	Saccharomyces cerevisiae	*Pseudomonas syringae*	GatA	Batch	Citrus peel waste hydrolysate	3.2	0.04	0.23	(Protzko *et al.*, 2018)
				Fed-batch	Citrus peel waste hydrolysate + lab-grade glucose (co-substrate)	8.0	0.10	0.57	

Notes: Yield is expressed in g Gal-AA/g Gal-UA and was calculated with the concentration of Gal-UA initially present in medium (even when feedstock hydrolysate was used as substrate).
n.d. – not determined.

2.6.3 Envisioned conversion

Provided that proof of principle for conversion of Gal-UA to Gal-AA has been reported for some the main 'workhorse' organisms in industrial biotechnology (Table 4), the novelty and aim of this thesis is directed to placing this conversion mediated by UDH into the context of a full anaerobic fermentation process using sugar beet pulp as feedstock and an engineered *S. cerevisiae* strain as production host, whilst understanding the details of using this fermenting organism in such process. Thus, this section is intended to present some essential considerations of the envisioned conversion for Gal-AA production.

Yeast *S. cerevisiae* is currently one of the most attractive hosts in industrial biotechnology and remains the go-to organism for fermentation processes to produce various products such as bioethanol (Benz *et al*., 2014). Its advantages comprise high tolerance to growth inhibitors from lignocellulosic hydrolysates (Figure 7), the ability to withstand low pH conditions (which prevents outgrowth of bacterial contamination), insensitivity to contamination with phages, fast fermentation kinetics and the suitability for many rounds of recycling (Amorim *et al*., 2011; van Maris *et al*., 2006). In addition, *S. cerevisiae* is able to ferment strictly under anaerobic conditions, which also contributes for lowering risk of bacterial contamination and does not require relatively expensive aerated fermenters.

The main limitation of using yeast in fermentation of pectin-rich feedstocks is that it natively only consumes the C6 sugars (engineered strains also consume xylose and arabinose) but does not metabolize Gal-UA (as seen in section 2.4). In fact, this compound can affect physiology of native yeast strains through weak acid uncoupling, causing lower biomass yields (Huisjes, 2013). This inhibition mechanism is similar to the one occurring with fermentation inhibitors such as acetic acid (represented in Figure 7) – Gal-UA enters the cell and dissociates in the cytosol, causing increased ATP expenditure to counter cytosol acidification. Regarding the influx mechanism of this compound (conversion occurs in cytosol), it has been recently elucidated that yeast transporter Gal2p can indeed mediate Gal-UA uptake at low pH; however, when glucose is present in the same medium, Gal-UA consumption is completely inhibited (Protzko *et al*., 2018).

Such findings prompted the need for a new transport system in order to achieve Gal-UA conversion using sugar beet pulp hydrolysate as substrate. As previously mentioned in this section, Protzko *et al*. (2018) also identified and expressed an improved heterologous transporter from *A. niger* – GatA – which enabled co-consumption of glucose and Gal-UA. GatA was approximately 50 times more active than the first transporter identified (Benz *et al*., 2014) and showed no inhibition by glucose, which make it the most interesting candidate for metabolic engineering strategies.

Finally, another crucial part of implementing this process is isolating the final product. Albeit in literature no information was found regarding the export mechanism of Gal-AA out of the yeast cells, several studies indicate that it is purified from the fermentation medium, taking advantage from its low solubility and subsequent precipitation at low pH (Mojzita *et al*., 2010; Paasikallio *et al*., 2017; Protzko *et al*., 2018; Zhang *et al*., 2016). Hence, while in a culture supernatant at nearly neutral pH values no

precipitate is observed, acidification (using for instance HCl) to pH 2.9 was proven sufficient to precipitate essentially all of galactarate – the salt form of Gal-AA (Mojzita *et al*., 2010). The precipitate can then be recovered by centrifugation and dried (Mojzita *et al*., 2010; Zhang *et al*., 2016).

3 Indicative techno-economic assessment

3.1 Galactaric acid production process

The first step towards the techno-economic assessment of the biotechnological industrial production of Gal-AA was to propose a design for this process. According to the information gathered in the previous chapter, a sequence of unit operations from feedstock to final products was conceived and represented in a process diagram, which was used as a basis for modelling.

This proposal was conceived with a higher level of detail on the unit operations addressed in the literature study, namely the pretreatment, saccharification and fermentation. The conversion of Gal-UA present in sugar beet pulp hydrolysate to Gal-AA is yet to have its proof of concept and thus the hereby presented process is merely indicative and aimed at providing direction towards an optimized, applied process. The proposed process for Gal-AA production is illustrated through the diagram in Figure 17 (Super Pro Designer®, Intelligen Inc.) and its streams are characterized in Table 5.

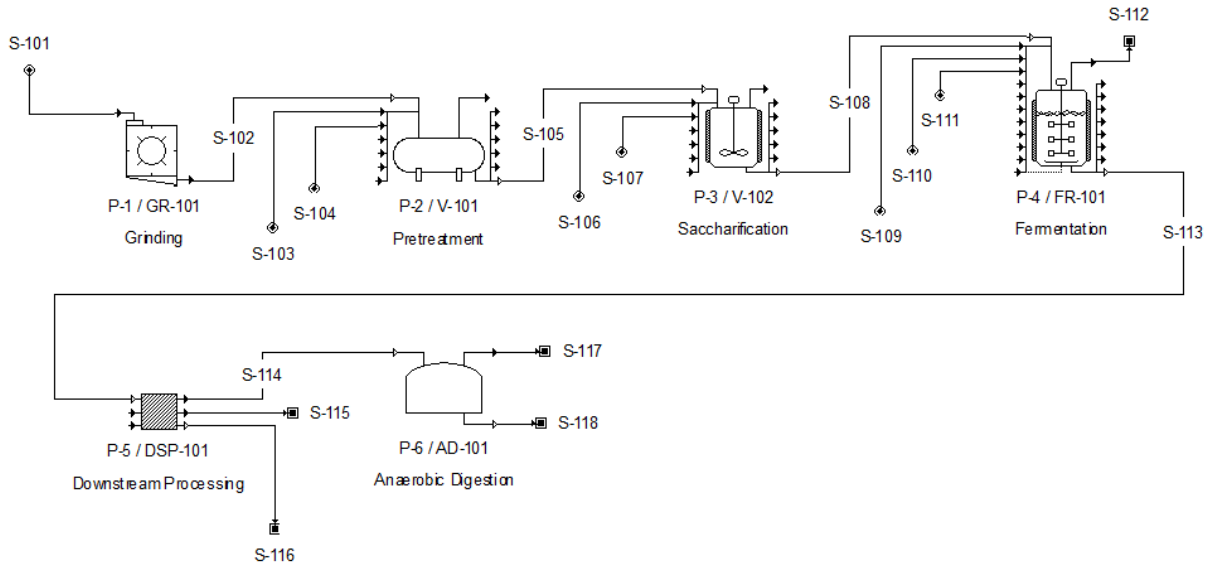

Figure 17 – Process diagram for the envisioned galactaric acid production.

Table 5 - Characterization of the streams present in the envisioned galactaric acid production process.

Stream	Description	Stream characteristics
S-101	Sugar beet pulp	Solid
S-102	Grinded SBP	Solid
S-103	Water	Liquid
S-104	Mild acid solution	Liquid
S-105	Pretreated SBP	Suspension
S-106	Enzyme solution	Liquid
S-107	Titrant solution	Liquid

S-108	SBP hydrolysate	Suspension
S-109	Yeast inoculum	Liquid
S-110	Nitrogen source	Solid
S-111	Base	Solid
S-112	CO_2	Gas
S-113	Fermentation broth	Suspension
S-114	Remaining dry matter	Solid
S-115	Ethanol	Liquid
S-116	Galactaric acid	Solid
S-117	Biogas	Gas
S-118	Digestate	Suspension

The input of the manufacturing facility is sugar beet pulp (S-101), a by-product from sugar production plants. Since the deconstruction of this feedstock and all subsequent steps are carried out in suspension, sugar beet pulp is passed through a solids grinder (GR-101) to reduce the particle size, favouring the preparation of an homogenous suspension and increasing surface area to improve efficiency of the subsequent hydrolysis step. Grinded sugar beet pulp (S-102) then proceeds to the pretreatment reactor (V-101), where water (S-103) or mild acid solution (S-104) is used to obtain a suspension with a desired dry matter content. This is done in continuous mode. The feedstock suspension is then subjected to a pretreatment at high temperature and acidity (in case the mild acid solution is used). The pretreated sugar beet pulp suspension exiting vessel V-101 has its lignocellulosic structure significantly weakened and can be enzymatically hydrolyzed in the saccharification/liquefaction reactor (V-102) after pH adjustment with titrant solution (S-107). The saccharification is carried out in batch mode and hydrolyzes the carbohydrate polymers that constitute sugar beet pulp to their respective monomers, through the action of specific enzymes that are present in the enzyme solution added (S-106). This operation forms the sugar beet hydrolysate (S-108), a stream containing the monomeric sugars that are the substrates for the batch fermentation process occurring in vessel FR-101. Here, three important components are added: a yeast inoculum (S-109), produced in a propagation, or seed-fermentation phase (not represented), provides the initial amount of biomass that will grow and metabolize the monomeric sugars in the feedstock hydrolysate, converting them into the envisioned fermentation products including Gal-AA; a nitrogen source (S-110), essential for yeast growth; and a base (S-111) for titration of the fermentation medium, which is particularly relevant since the Gal-AA produced will otherwise lower the initially set pH. Once this operation is finished, the products of interest are separated and/or purified from the resulting fermentation broth (S-113).

In the proposed process, three fermentation products are intended to be recovered: remaining dry matter (S-114), ethanol (S-115) and galactaric acid (S-116). The first product intends to recover all the organic dry matter present in the fermentation broth (non-hydrolyzed and non-converted SBP, biomass from yeast growth). This organic dry matter will be further processed in an anaerobic digestor (AD-101)

and converted into biogas (S-117) that will be used for production of electrical energy to be reintegrated in the plant.

In this work it was decided to focus on the biotechnological conversion in the initial process steps without proposing a detailed DSP design. As such, the entire DSP section is represented by unit DSP-101, with one output stream and overall recovery yield for each of the products. Even though there is an idea of the main unit operations that would feature the DSP of each product (precipitation for Gal-AA, distillation for ethanol and filtration for the protein by-product), the configuration of this sequence of operations is very dependent on the exact composition and properties of the incoming streams, as well as on the need for the final products to meet specific purity requirements and comply with legislative specifications to enter the market. In fact, from consultation of internal DSM DSP expertise, it is expected that the purification process of commercial Gal-AA is likely far more complex than currently suggested in the literature (Mojzita *et al.*, 2010; Protzko *et al.*, 2018), when comparing with existing DSP operations of similar products like succinic acid.

3.2 Methodology

3.2.1 Assumptions

This high-over techno-economic analysis is designed to take into account the information resulting from an assumed process mass balance, together with raw materials and utility costs, capital expenditure (CAPEX) estimates and other operational expenditures to determine the total cost of goods of the manufactured products, in order to conclude regarding the economic viability of the process at the industrial level. As part of this exercise, frequently the information has to be simplified and many assumptions and estimations were made when this information was not known or not yet available. The most important general assumptions and considerations made in the base case analysis are listed below. To validate the choices made in the selected configuration and identify process improvement options, variations on this base case process are described and discussed in sections 3.2.2 and 3.3.1, respectively.

Feedstock

The sugar beet pulp used as an input in this process was defined to be dried sugar beet pulp, meaning it was dried and compressed into pellets, having a dry matter content of 87-92% w/w (the average value was used in calculations). Since this process was envisioned to run year-round but sugar (and thereby pulp) production is seasonal, dried pellets were considered as they can be stored, preventing spoilage and preserving their fermentation value (Berłowska *et al.*, 2018). The cost of this feedstock was obtained from Coöperatie Koninklijke Cosun U.A. (confidential presentation shared with DSM). Polymer and monomer compositions used are detailed in (Appendix B – Model inputs).

Sugar plant operation

The input of feedstock in the process is directly tied to the production of beet pulp in a sugar production plant. Thus, a daily capacity of such a sugar beet processing plant was assumed based on the capacity of currently existing Cosun plants (confidential presentation shared with DSM), combined with a production of 4 ton of sugar beet pulp per 25 ton of sugar beet (on a dry matter basis) and assuming a sugar beet dry matter content of 25% (FAO, 2009) to calculate the amount of sugar beet pulp produced per sugar production plant (Appendix B – Model inputs).

Galactaric acid plant operation

The envisioned Gal-AA production plant is capable of processing the yearly production of sugar beet pulp of one of Cosun's sugar production plants. It was also assumed that the plant would be operating 333 days per year (2 week scheduled downtime, 95% occupation) and that this plant would be co-located with the sugar production plant. This is convenient as it removes the need for transportation of the by-product and allows for possible sharing of utilities and control of product output (selling pulp as feed or producing Gal-AA and ethanol out of it) depending on market demand.

Pretreatment

The residence time for the suspension in the pretreatment reactor was assumed to be 20 minutes, with water added to sugar beet pulp to form the suspension with a dry matter content of 12% w/w (Berłowska *et al*., 2016), as shown in Appendix B – Model inputs.

Saccharification

As a simplification, it was assumed that the dry matter content of the suspension remains the same after the pretreatment. The saccharification was assumed to run for 48 hours, allowing for significant hydrolysis of cellulose (as indicated by internal DSM expertise). For calculation of the amounts of monomeric sugars released, it was considered that these are exclusively formed in this process step. Thus, hydrolysis yields of pretreatment and saccharification were lump-summed per carbohydrate polymer type (pectin, cellulose, hemicellulose) and used to calculate the amounts of respective monomers formed, taking into the account the increase in molar weight (from polymer unit to monomer unit). The hydrolysis yields considered as well as other information regarding this unit operation are present in Appendix B – Model inputs. For enzyme costs calculation, a singular enzymatic cocktail with all the activities needed was considered, with its dosing set to 1 g enzyme/kg dry matter. Enzyme price was determined with generalized data for production costs in a fungal process based on previous DSM projects (confidential). After hydrolysis, the new dry matter content of the suspension S-108 was calculated considering the formed monomers, non-hydrolyzed polymers, protein and others.

Yeast strain

The yeast strain envisioned to be used in this process is a *S. cerevisiae* engineered for the transport of Gal-UA and its oxidation to Gal-AA through the expression of a heterologous UDH gene. Such genetic engineering would be performed in a DSM second generation bioethanol strain, a robust industrial strain previously engineered for C5 (xylose and arabinose) metabolism.

Fermentation

The duration of the fermentation was assumed to be the same as for saccharification (48 hours), and so equal volumes were assumed for fermentation and saccharification reactors. The stoichiometric yields were calculated for each of the conversions occurring in fermentation, with an assumed yield factor being applied to each conversion as well (Appendix B – Model inputs). The following reactions occur in the envisioned fermentation:

1. $C_6H_{10}O_7 + H_2O + NAD^+ \rightarrow C_6H_{10}O_8 + NADH + H^+$
2. $0.0067\ C_6H_{12}O_6 + 0.0063\ NH_3 + 0.006\ NADPH + 0.011\ NAD^+ + 0.0065\ H^+ \rightarrow X + 0.0065\ NADP^+ + 0.011\ NADH + 0.011\ H^+ + 0.0111\ H_2O$ (Verduyn *et al.*, 1990)
3. $0.5\ C_6H_{12}O_6 + NADH + H^+ + ATP \rightarrow C_3H_6O_3 + NAD^+ + ADP + P_i$
4. $C_6H_{12}O_6 + 2\ ADP + 2\ P_i \rightarrow 2\ C_2H_6O + 2\ CO_2 + 2\ ATP$
5. $6\ C_5H_{10}O_5 \rightarrow 10\ C_2H_5OH + 10\ CO_2 + 10\ ATP$ (Kuyper *et al.*, 2004)

The yeast strain described above is assumed to anaerobically convert Gal-UA to Gal-AA (1). With no direct coupling of yeast central metabolism to Gal-AA being available, metabolism of C5 sugars (arabinose and xylose) results in formation of ethanol (5), while the glucose content of the hydrolysate was assumed to be sufficient to sustain growth (2) and provide carbon for the glycerol production (3) required for balancing the redox cofactor surplus generated in both Gal-AA production (1) and anaerobic yeast growth (2). Remaining glucose was also fermented to ethanol (4). Four fermentation products were considered: Gal-AA, ethanol, glycerol and yeast biomass.

Regarding added nutrients, only nitrogen was included in the analysis, with urea chosen as the source of this nutrient. Base added for titration of fermentation broth was calculated considering potassium hydroxide (KOH) would be used and the fermentation running at pH 5. This amount was overestimated since it was assumed that the suspension had no buffer capacity. For the yeast inoculum, no propagation phase was included in the analysis, instead it was assumed that yeast was added in cream at a concentration of 0.1 kg yeast dry matter/m^3 suspension. After fermentation, the new dry matter content of stream S-113 was calculated considering the fermentation products (except ethanol), non-converted monomers, non -hydrolyzed polymers, protein and others.

Downstream processing (DSP)

Three products were intended to be recovered: Gal-AA and ethanol for commercial purposes, and the remaining non-utilized dry matter for biogas production. Downstream was simplified through

assuming a separate recovery yield for each product: 85% for Gal-AA, 97% for ethanol (distillation) and 95% for the remaining dry matter (recovered through filtration or centrifugation).

Galactaric acid

Despite its current various applications, this analysis considered that the market addressed for Gal-AA sales would be the chemicals market (selling it as platform chemical). Therefore, according to the price of similar compounds also sold as platform chemicals such as succinic acid, which sells at 2.49 €/kg (E4tech, 2015), the assumed price for Gal-AA was 2.5 €/kg. Nevertheless, there is also the possibility of targeting part of the production to address the cosmetics and/or food application markets with a higher selling prices (as described in section 2.5) which would generate greater revenues, provided that DSP is adapted to the degree of purity and legislation imposed by these markets.

Ethanol

The assumed selling price for ethanol was 0.31 €/kg (1.105 $/gal) (Markets Insider, 2020) which is currently very low due to the economic impacts of the COVID-19 pandemic and the overall low oil price and legislative uncertainties (US Renewable Fuel Standard) prior to that.

Biogas

The organic part of the remaining dry matter present (S-114) in the fermentation broth was used for calculating biogas production. Internal DSM biogas experts provided reference values for yield of biogas per kg of organic dry matter processed, however, a 5-fold reduction was applied to such value since it was assumed that biogas production using the recalcitrant part of the feedstock (which resisted saccharification and fermentation) would be substantially lower. The amount of energy produced was calculated using the fraction of methane in biogas and the heat of combustion of that gas. The energy produced was reintegrated in the process, lowering the total electricity consumptions as demonstrated in Appendix B – Model inputs.

Utilities

The utilities included in this analysis are steam and electricity. The estimations considered for the utility consumptions are shown in Appendix B – Model inputs.

Capital expenditure (CAPEX)

CAPEX was calculated using reference data of equipment used in bio-based production and DSP of a typical organic acid platform chemical (confidential). For each operation, a reference capacity and respective price of the equipment were used to determine a price/capacity ratio, which was used to calculate costs associated with the capacity required in the Gal-AA production process. Thus, the final values of the CAPEX estimations (shown in Appendix B – Model inputs) only covered inside battery

limits (ISBL) costs; no outside battery limits (OSBL) costs like utilities or waste water treatment costs were included.

Variable costs calculation

The variable costs considered in this analysis included the costs associated with feedstock, nutrients, chemicals, utilities, enzymes and yeast. Waste disposal and license fees costs were not included in this analysis as they required a higher level of detail in the process in order to make reasonable estimates. The cost model used in this analysis is presented in Appendix D – Total cost of goods and gross margin, showing the values calculated for each component of the variable costs. Production costs were calculated per kg of Gal-AA under the assumption that ethanol is a by-product and thus the incurred costs are only due to the main product.

Fixed costs calculation

The fixed costs considered in this analysis included the costs associated with labor, maintenance, insurance and depreciation. As a simplification, these were calculated applying a percentage to the total CAPEX value, as shown in Appendix B – Model inputs. The values calculated for each of the components of the fixed costs are shown in the cost model present in Appendix D – Total cost of goods and gross margin.

3.2.2 Scenarios

Besides the base case scenario - **Scenario 1 (S1_Base case)** - of the process already described throughout sections 3.1 and 3.2.1, variations on this scenario were defined and assessed through modelling, with the aim of understanding whether imposed changes in particular unit operations or additional genetic engineering in the yeast strain led to a more attractive business case. The scenarios tested are described below.

Scenario 2 - No pretreatment (S2_No Pre)

As explained in section 2.3, even though published studies indicate a positive effect of a pretreatment in enzymatic hydrolysis yields of sugar beet pulp, it is currently not clear whether this compensates the investment in equipment to run this operation. To investigate this, a scenario was created in which the pretreatment operation was removed, causing reduction in CAPEX. In addition, saccharification yields were lowered and enzyme dosage was increased to reflect the absence of this operation (Appendix B – Model inputs). Finally, utility consumption was also lowered since pretreatment consumes both energy and steam.

Scenario 3 - Mild acid pretreatment (S3_Acid Pre)

Another alternative for the pretreatment of sugar beet pulp is to use a mild acid solution instead of water. In fact, some authors have shown that this strategy can be as or more effective at solubilizing

certain monomers in beet pulp than water pretreatment (Berłowska *et al*., 2016; Kühnel *et al*., 2011). To create this scenario, a 5% w/w dose (on a dry matter basis) of sulphuric acid was assumed (Berłowska *et al*., 2016) and the respective amount of acid to add was calculated while its cost was added to the chemicals costs. Hydrolysis yields were not changed, but enzyme dosage was reduced, assuming the action of the acid would lead to higher opening up of the cellulose/hemicellulose structure, increasing accessibility of these substrates to enzyme action and thus allowing a decrease in the amount of enzyme to be added to maintain a hydrolysis yield equal to the base case (Appendix B – Model inputs). In terms of CAPEX, the price for the pretreatment vessel was increased considering that in this scenario it must be resistant to corrosion.

Scenario 4 - SSF implementation (S4_SSF)

Simultaneous Saccharification and Fermentation (SSF) is a process configuration in which enzymatic hydrolysis and fermentation are performed in a single step. Since they occur in the same reactor, yeast is constantly consuming the sugars released by the hydrolase enzymes, which leads to reduced end-product inhibition on the enzymes (Olofsson *et al*., 2008). On the other hand, depending on the donor-organism, (hemi)cellulase hydrolyzing enzymes typically have an optimum temperature (50-60 °C for *A.niger*, 70-75 °C for DSM's *Talaromyces*-based cellulolytic enzymes) that is higher than yeast-fermentation conditions (30-32 °C). To assess the SSF configuration, both effects on enzyme performance were estimated to approximately cancel each other, and hence saccharification yields were not increased assuming that enzymes would not be operating at the ideal conditions of temperature and pH (Appendix B – Model inputs). In addition, hydrolysis and fermentation time was reduced to one 48 hours process, reducing the CAPEX with these operations by half.

Scenario 5 – Redox-cofactor balancing through PRK-Rubisco (S5_PRK-Rubisco)

The conversion of Gal-UA to Gal-AA is NAD^+ cofactor dependent, resulting in formation of NADH (Figure 14). With the envisioned fermentation process being anaerobic, this excess NADH production affects redox-cofactor balancing and product yields in anaerobic fermentation, with glycerol being the major by-product resulting from yeast's mechanism to reestablish cofactor balance - conversion of G3P to glycerol with oxidation of one NADH molecule (Appendix A – Supporting material for yeast metabolism). One possible solution to overcome this problem was reported by Guadalupe-Medina *et al*. (2013), a strategy which consisted in expressing autotrophic Calvin-cycle enzymes in yeast, enabling the use of CO_2 as electron acceptor. The functional expression of phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase (Rubisco) enables the conversion of ribulose-5-P to two molecules of 3-P-glycerate (consuming one CO_2 molecule) which then continue along central carbon metabolism to produce ethanol from pyruvate using 2 NADH molecules, thus alleviating redox-cofactor unbalancing. It is worth noticing that both glycerol formation and PRK-Rubisco as an alternative redox-sink are directly coupled to glycolysis, in contrast to the galacturonic to Gal-AA conversion. Thus, for the

necessary redox balancing, central carbon metabolism must be active. In practical terms, this means that yeast must be consuming sugars (glucose, xylose, arabinose) for the Gal-UA to be produced.

To assess the effect of introducing an alternative redox sink, and thereby decoupling Gal-AA production from glycerol by-product formation, the defined fermentation yield factor of glucose to glycerol (for the base case) was significantly lowered (Appendix B – Model inputs). Consequently, fermentation yields of glucose to ethanol and biomass became higher and there was an increase in CAPEX for ethanol DSP, but also a concomitant increase in ethanol sales.

Scenario 6 - Galactaric acid formation through coupling of central carbon metabolism (S6_Central)

According to the envisioned process, Gal-AA is only formed by the conversion of Gal-UA through an engineered yeast expressing a UDH gene (Figure 14). However, from a product-yield perspective, it would be very beneficial to connect Gal-AA production to central carbon metabolism, representing the first step towards its production from (C6 and C5) sugars present in the beet pulp hydrolysate. Although no such pathway is currently known, a hypothetical scenario was conceived in which Gal-AA could be formed through glucose metabolism at a 1:1 molar ratio. This scenario was built using scenario 5 as a starting point, since it only makes sense that PRK-Rubisco mechanism remains present in the yeast strain to compensate for the even higher amount of NADH that would be generated from such a pathway to form Gal-AA, considering the higher degree of reduction of glucose compared to Gal-UA. To create this scenario, a stoichiometric yield and yield factor of glucose to Gal-AA were added to the fermentation modelling (Appendix B – Model inputs), strongly increasing the Gal-AA yield per ton of beet dry matter and a corresponding increase in CAPEX for DSP of this product.

3.3 Results and discussion

3.3.1 Business case evaluation

The mass balance analysis allowed to determine the yearly consumptions of all materials and utilities required in the Gal-AA production process. Using their respective unit costs, the total production costs were calculated by adding the total variable costs and total fixed costs, expressed in M€/year. Annual revenues of the manufactured products were calculated using their yearly production obtained through the mass balance and their respective selling price. The mass balance summary is presented in Appendix C – Mass balance summary, while the total cost of goods and gross margin percentages calculations are shown in Appendix D – Total cost of goods and gross margin. The gross margin percentage (Equation 1) was the parameter chosen to evaluate the economic attractiveness of the base case and compare each of the scenarios.

$$\text{Gross margin (\%)} = \frac{\text{Total revenues - Total production costs}}{\text{Total revenues}} \times 100 \qquad \text{Equation 1}$$

The total production costs, total revenues and gross margin percentages for all scenarios are presented in Table 6.

Table 6 - Total production costs, total revenues and gross margin percentages for the six studied scenarios.

	S1_Base case	S2_No Pre	S3_Acid Pre	S4_SSF	S5_Rubsico	S6 _Central
Total production costs (M€/year)	150	142	156	146	148	166
Total revenues (M€/year)	169	147	169	169	173	228
Gross margin (%)	12%	4%	8%	14%	15%	27%

Not all positive gross margin percentages correspond to a positive business case. In fact, a positive gross margin only indicates that the process can cover its direct production costs, but there might be additional indirect costs associated like marketing and sales, overhead and R&D costs. According to DSM internal expertise, in this type of business, a gross margin of around 30% suggests a profitable and attractive business case. Therefore, scenario 2 presented a far too low gross margin percentage, the base case and scenarios 4 and 5 most likely are able to cover the additional costs mentioned and only scenario 6 comes close to the profitable business case desired.

However, the limitations of this assessment ought to be carefully taken in consideration when making such statements regarding these final results. As detailed in section 3.2.1, the modelling of Gal-AA production process was subjected to various assumptions which can sometimes be far from the real situation. Among all listed assumptions, it is possible to highlight some which had a major impact in the gross margin results, namely the superficial analysis in the DSP section. Since only the CAPEX of this section was estimated, no OPEX was included; for instance, costs of chemicals used in buffer preparation or membranes for filtrations were not considered in this analysis. In addition, neither waste disposal/wastewater treatment nor license fees were included in the variable costs. A more in-depth cost analysis for the total cost of goods calculation would certainly lead to more realistic gross margin percentages.

Nonetheless, this does not prevent from making a comparative analysis between the base case and modeled scenarios, and concluding whether the tested modifications lead to improvements in the process. Gross margin variation between these scenarios are depicted in Figure 18 to Figure 20. Figure 18 represents the total variable costs and total fixed costs of all scenarios, with its sum (total production costs) represented above the bars. Figure 19 shows the total Gal-AA production costs as the cumulative cost contribution of each component of the variable and fixed costs. Finally, Figure 20 shows the revenues in M€/year of each product sold, its total value represented above the bars and a red line representing the total production costs in the same unit, for better contextualization of such sales values.

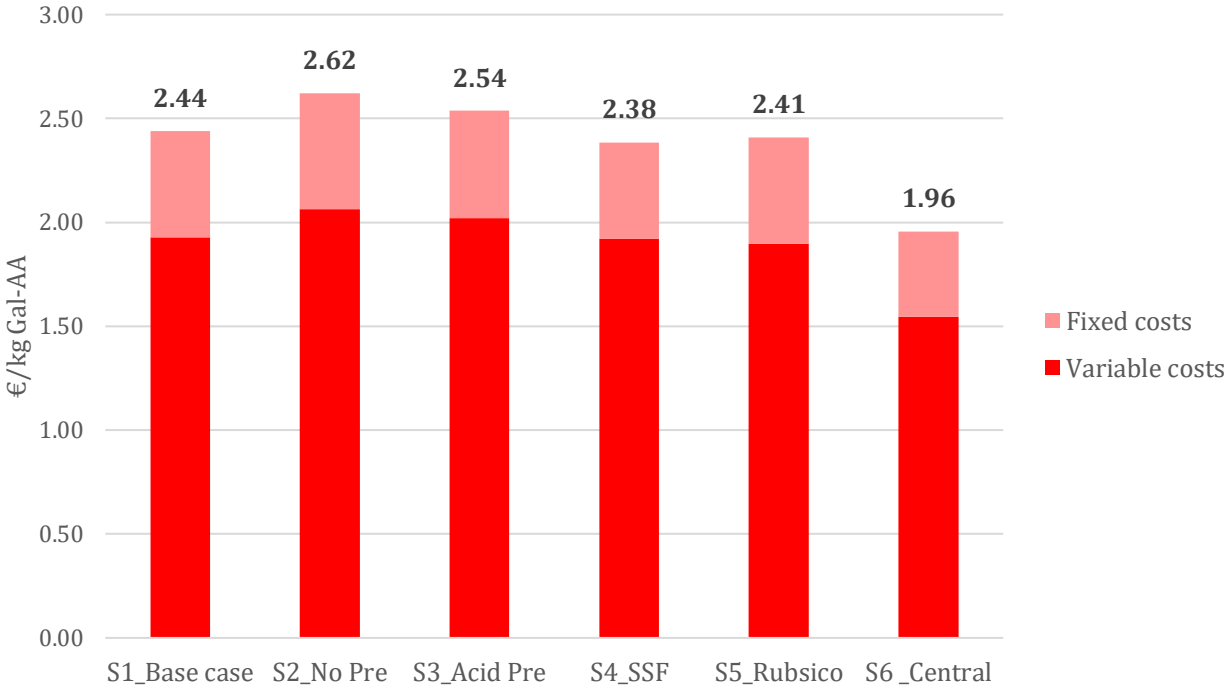

Figure 18 - Total production costs of galactaric acid separated in fixed and variable costs, for all scenarios studied.

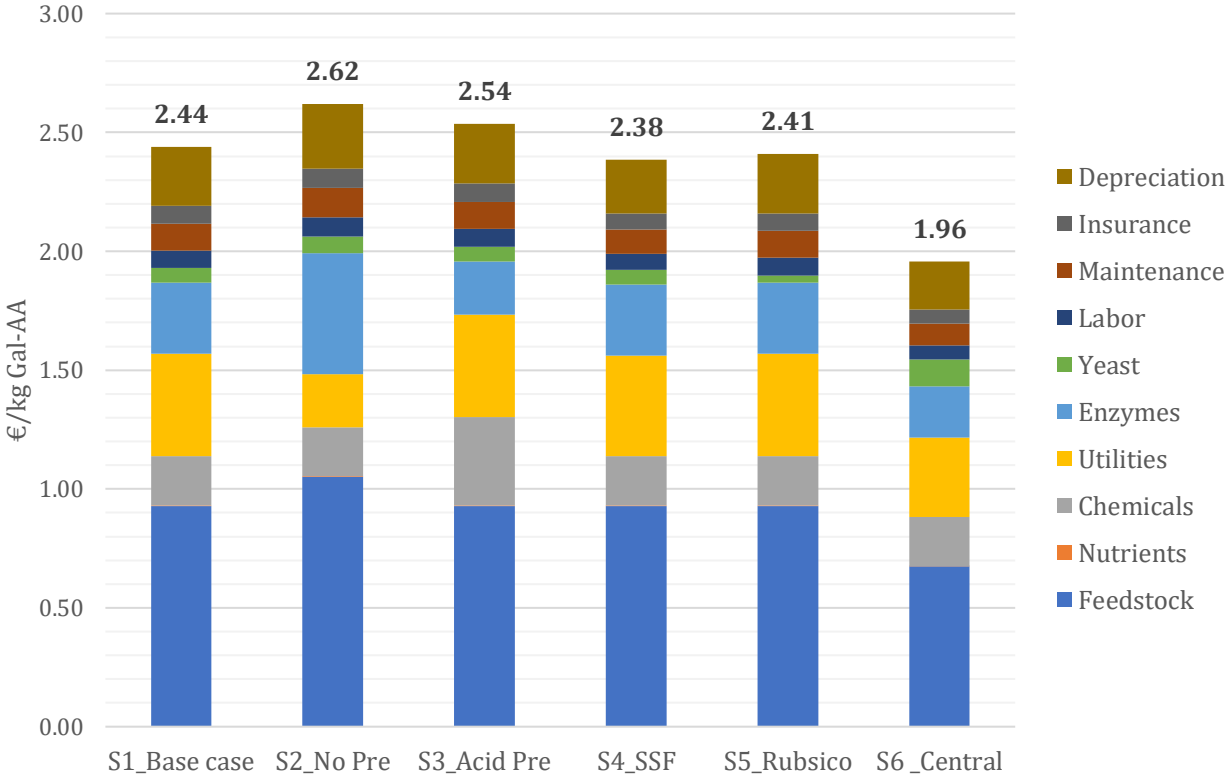

Figure 19 - Cumulative cost contributions for total production costs of galactaric acid, for all scenarios studied.

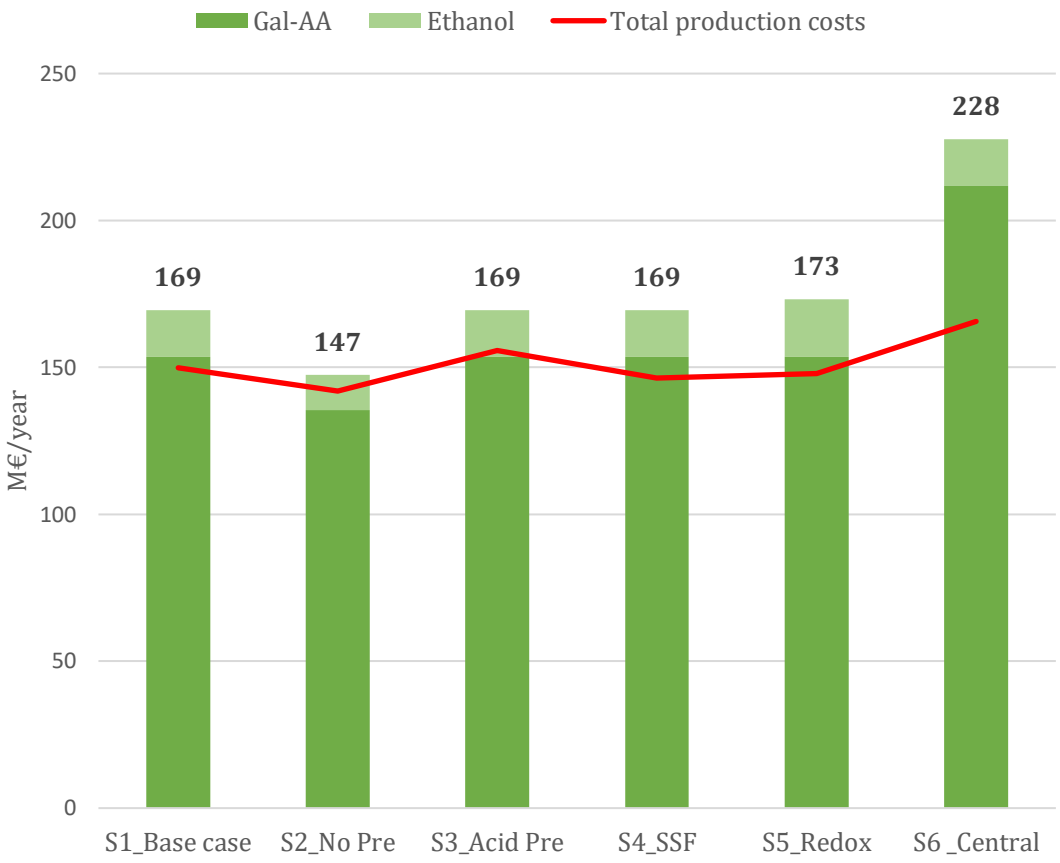

Figure 20 – Total revenues of products sold (separated per product) and total production costs for all studied scenarios. Gross margin is graphically represented through the distance between the top of the bar and the red line.

Scenario 1 - Base case (S1_Base case)

Before comparing the results of each scenario, it is relevant to analyze the results obtained for the process base case, which had a gross margin percentage of 12% (Table 6). Regarding the total cost of goods, Figure 18 shows that 1 kg of Gal-AA costs 2.44 € to produce and the variable costs are the major component of total production costs. Figure 19 reveals that feedstock costs are the major contributors of the total production costs in the base case, followed by utilities, enzymes and chemicals costs, in what concerns the variable costs. It is worth noticing that nutrients costs are not visible as their cost contribution is much smaller when compared to the other cost sources. Regarding fixed costs, depreciation is the portion representing the highest contribution. Through the analysis of Figure 20 it is evident that Gal-AA is the most important product in the total sales, followed by the much smaller contribution of ethanol. Therefore, in general and as expected, it can be stated that the profitability of the base case is largely dependent on the main fermentation product and its respective selling price, as well as on the cost of sugar beet pulp. In this graph, the gross margin is represented by the distance between the top of the sales bar and the red line – the higher the distance, the higher the gross margin. For the base case, this figure shows that the 12% gross margin is actually achieved due to revenue from ethanol produced, meaning that this by-product from yeast fermentation, though much smaller in sales volumes, is essential to ensure a positive business case.

Scenario 2 - No pretreatment (S2_No Pre)

In scenario 2, it was investigated whether not applying a pretreatment to sugar beet pulp would lead to a more attractive business case. According to Table 6, the gross margin percentage of scenario 2 suffers a decrease to 4% as a consequence of removing this unit operation. In fact, despite the reduction in CAPEX and utility consumptions associated with this operation, Figure 18 demonstrates that still the production costs are higher than in the base case, namely due to the higher variable costs. More specifically, Figure 19 clearly shows this increase is attributed to higher feedstock costs and enzyme costs. The explanation for such results relies mainly on the changes imposed in the saccharification yields; according to the studies described in section 2.3.2, it was considered that removing the pretreatment operation caused a reduction in the hydrolysis yields of cellulose, hemicellulose and pectin, as detailed in Appendix B – Model inputs (Berłowska *et al.*, 2016; Kühnel *et al.*, 2011). In particular, a decrease of 10% in the hydrolysis yield of pectin led to a lower Gal-AA production (observed in Figure 20), which caused an increase in feedstock costs since the beet pulp processing input is the same. In addition, the enzyme dosage for saccharification was also increased in 50% to compensate for the lack of pretreatment, which justifies the increase in enzyme costs.

Hence, it can be concluded that, unless Gal-UA release is retained at a similar enzyme dose, it is preferable to have the pretreatment operation included in the process. In the envisioned scenario, the enhancing effect of pretreatment on the production of Gal-AA, combined with lower enzyme dosages used end up compensating the investment in equipment and its associated running costs, generating a more positive scenario.

Scenario 3 - Mild acid pretreatment (S3_Acid Pre)

Scenario 3 was designed to test if using a mild acid solution in the pretreatment operation would be preferable compared to the base case in which water is used, assuming the use of acid would enable lower enzyme dose requirement while maintaining the same saccharification yields (Appendix B – Model inputs). Table 6 shows the gross margin percentage decreases to 8%, suggesting that using a mild acid solution in pretreatment makes the production process less interesting, according to the envisioned scenario. Despite the reduction in the enzyme costs contribution, Figure 18 shows that the total production costs for this scenario were still higher than in the base case. Regarding variable costs, adding sulphuric acid to the initial suspension and subsequently using additional base (KOH) to return pH to the value required in enzymatic hydrolysis caused an increase in the chemicals costs which surpassed the effect of a lower enzyme dosage (Figure 19). On the other hand, the increase in CAPEX due to the need of having corrosion resistant equipment did not lead to a significant increase in the fixed costs. Since the hydrolysis yields were not changed, the production of fermentation products was the same as in the base case, which is evident in the unchanged total sales values (Figure 20).

Thus, using a mild acid solution in pretreatment creates a less positive business case, attributed mainly to the effect of the higher chemicals costs (assuming unchanged hydrolysis yields). Even though

waste disposal was not considered in this analysis, it relevant to highlight that neutralizing the acid added in the pretreatment to reach pH values of saccharification results in higher salt formation which not only complicates DSP, but also increases waste disposal costs.

Scenario 4 - SSF implementation (S4_SSF)

Simultaneous Saccharification and Fermentation (SFF) was implemented in the process model to build scenario 4. The gross margin percentage obtained was slightly higher than in the base case (14%). Having hydrolysis and saccharification occurring in the same vessel allowed a reduction in CAPEX which directly affected the fixed costs (they are determined as percentage of CAPEX). Figure 18 shows this small decrease in total production costs was due to the reduction in fixed costs. On the other hand, total variable costs remained approximately constant since, besides a marginally lower energy use for stirring the vessels, no major changes occur in these cost contributors. Total sales remain the same because the hydrolysis yields of all polymers were equal to the base case (as explained in the description of this scenario). Therefore, implementing this process configuration can bring a marginal increase in the gross margin due to reduced equipment costs, however this is strictly dependent on whether it is possible to find a compromise between the optimum temperature and pH conditions of enzymes and yeast.

One advantageous aspect of implementing SSF that was not included in this analysis is that the reduction in process time lowers the probability and effects of growth of bacterial contaminants, minimizing loss of fermentable sugars and hence maintaining product yields per beet dry matter.

Scenario 5 – Redox-cofactor balancing through PRK-Rubisco (S5_PRK-Rubisco)

Yeast genetic engineering strategies were tested in the latter two scenarios. Scenario 5 aimed to investigate the influence of expressing the heterologous enzymes PRK and Rubisco as a mechanism to alleviate redox-cofactor unbalancing induced by Gal-AA production catalysed by UDH. A gross margin percentage of 15% was obtained in this scenario, also a relatively small increase compared to the base case. Lowering the fermentation yield of glucose to glycerol leads to increased glucose availability to form the other products of anaerobic central carbon metabolism – ethanol and yeast biomass (Appendix B – Model inputs). However, Figure 20 shows this did not have a considerable influence in the total sales as ethanol is a minor contributor for these, while the production of the major contributor (Gal-AA) remained constant. The slight decrease in production costs is attributed to the lower yeast costs (Figure 19); since the reduction of glycerol by-product allows for a higher biomass growth, the initial concentration in the inoculum was lowered. So, despite the substantial effect in reestablishing redox-cofactor balance, reducing the formation of glycerol does not have a great impact in the making the process more profitable as the increase in ethanol production is not enough to raise total revenues significantly.

Scenario 6 - Galactaric acid formation through coupling of central carbon metabolism (S6_Central)

Finally, starting from the changes imposed in the previous scenario, scenario 6 intended to test a so-far hypothetical metabolic network in which Gal-AA formation is linked to central carbon metabolism, in addition to being produced through Gal-UA conversion. From all the studied scenarios, this one had the highest gross margin (27%), making it the most attractive business case, albeit being also the most far-fetched one. Such a high gross margin value is due to the significant increase in Gal-AA yield per beet dry matter. By inputting a conversion of 30% of available glucose to Gal-AA in the process model (Appendix B – Model inputs), although the ethanol production is lower compared to scenario 5, the production of Gal-AA substantially increases and consequently do its revenues (Figure 20). This effect is also preponderant in the total production costs as they are expressed per kg of produced Gal-AA, resulting in the lowest total production cost among all scenarios – 1.96 €/kg Gal-AA. As Figure 19 depicts, the feedstocks, utilities and enzymes costs are lowered just due to the much higher production of Gal-AA. In Figure 20 it can be clearly seen that this scenario yielded the highest gross margin as it presents the highest difference between total sales and total production. Even though this scenario is evidently the most promising one, it is worth reiterating that it is not certain whether is this conversion is actually possible, and aiming to achieve so would require a substantial R&D effort within an extensive development timeline to make this applicable in the production process.

Therefore, having concluded this comparative analysis, it is possible to suggest the process and strain designs which lead to the most attractive business case. The pretreatment was shown to be a valuable operation in the process, assuming it was essential to reach the highest hydrolysis yields possible which then translate into higher total revenues. In its turn, the mild acid pretreatment did not seem to be preferable compared to the water pretreatment, under the assumption that the hydrolysis yields remain the same. While the implementation of SSF resulted in a slight increase in the gross margin, this configuration is strictly dependent on the reaction conditions of enzymes used, and thereby sequential saccharification and fermentation should be prioritized. Regarding strain engineering, while the reduction of glycerol by-product formation does not have a great influence in profitability on its own, it does become an important factor in case Gal-AA is produced from glucose (which strongly increases the need for redox balancing). Ultimately, Gal-AA production yield per beet dry matter is what drives the process towards the highest gross margin, so despite its current strongly hypothetical nature, finding a metabolic pathway to make this conversion possible should be of major interest of any institution looking to start a business in bio-based Gal-AA.

3.3.2 Sensitivity analysis

While the modelling of different scenarios was intended to study which set of modifications in the base case process should be of higher interest towards achieving a more attractive business case, a sensitivity analysis was also carried out with the aim of investigating which individual parameters

predominantly affect the process profitability. Among the numerous variables used in the process model, a thoughtful selection was chosen to integrate this analysis. Performance of fermentation-based processes are commonly characterized using the three metrics of titre, yield and rate. These are parameters typically obtained through experimental work and although there was no empirical data generated to be used as an input in the model, estimations were extrapolated from experience with similar bioprocesses.

As an indirect proxy for titre, the dry matter content of the initial suspension was varied. The yield was defined as kg of Gal-AA obtained per ton of sugar beet pulp dry matter entering the process. Production rate was assessed through combined hydrolysis and fermentation time – process time. Six additional variables were also included in the sensitivity analysis: feedstock costs and enzyme dosage in saccharification, since they were found to be major cost contributors in total production costs (Figure 19); pectin hydrolysis yield, as the results obtained for scenario 2 suggested that this parameter had a great impact in Gal-AA production. Regarding this product, its DSP yield was selected with the purpose of studying the influence of the least detailed part of the process in its profitability, and the impact of inevitable errors in the applied high-over estimations for this operation. The variation in the selling price of this product was also studied. Lastly, CAPEX was also included in the analysis since its estimation was also subjected to several estimations.

The sensitivity analysis was carried out in two parts. Firstly, each of the nine parameters selected were subjected to variations around its base case value and the resulting variation range was used as input in the base case model to show the effect on total production costs, total sales and, consequently, on the absolute gross margin. Plots showing the variation of the three economic parameters were built and are represented in Appendix E – Sensitivity analysis.

Secondly, with the intent of quantifying the sensitivity of associated with the selected parameters, one specific variation was used to determine the consequent effect in the gross margin percentage. However, for this second analysis, only the parameters that can be controlled to improve the gross margin were considered. Such modifications in these six parameters studied had the base case values as their starting point and were carefully designed to reflect a realistic modification according to a what-if rationale, as represented in Table 7.

Table 7 - What-if rationales for the variations in the six parameters selected for the waterfall chart sensitivity analysis.

WHAT would happen to the gross margin percentage IF…
(A) Dry matter content of the initial suspension was increased from 12 to 17% w/w
(B) Enzyme dosage in saccharification was decreased with 25%
(C) Pectin hydrolysis yield was increased from 85 to 90%
(D) Gal-AA yield in fermentation (per ton of beet dry matter) was increased from assumption % of maximum to the theoretical (stoichiometric) maximum
(E) Gal-AA DSP yield was increased from 85 to 90%
(F) CAPEX was decreased with 20%

The increase in the initial suspension (S-105) dry matter content to 17% w/w was chosen due to this being a common upper limit for processing other feedstocks. The higher the dry matter content, the higher the product titre and the lower is the liquid volume required to make the suspension, hence the vessel size is reduced. As mentioned in section 3.2.1, the enzyme dosage used in the base case was 1 g enzyme/kg dry matter, so the reduction considered is a moderate improvement generally achievable by optimization of enzymatic cocktail composition or reaction conditions. While increasing the pectin hydrolysis yield to 90% and purifying Gal-AA at a 90% yield also represented much desired aims which can become possible through extensive R&D efforts, the exact maximum stoichiometric yield (1.08 g Gal-AA/g Gal-UA) is never reached, but it is still interesting to study its effect. Finally, reducing investment in equipment is favourable for any process; considering that, it was decided to investigate the impact of a 20% decrease in CAPEX.

The variations in the base case gross margin resulting of each of the situations created were represented in a waterfall chart, illustrated by Figure 21. The improvements are represented from largest to smallest so that the modifications are ranked in sensitivity.

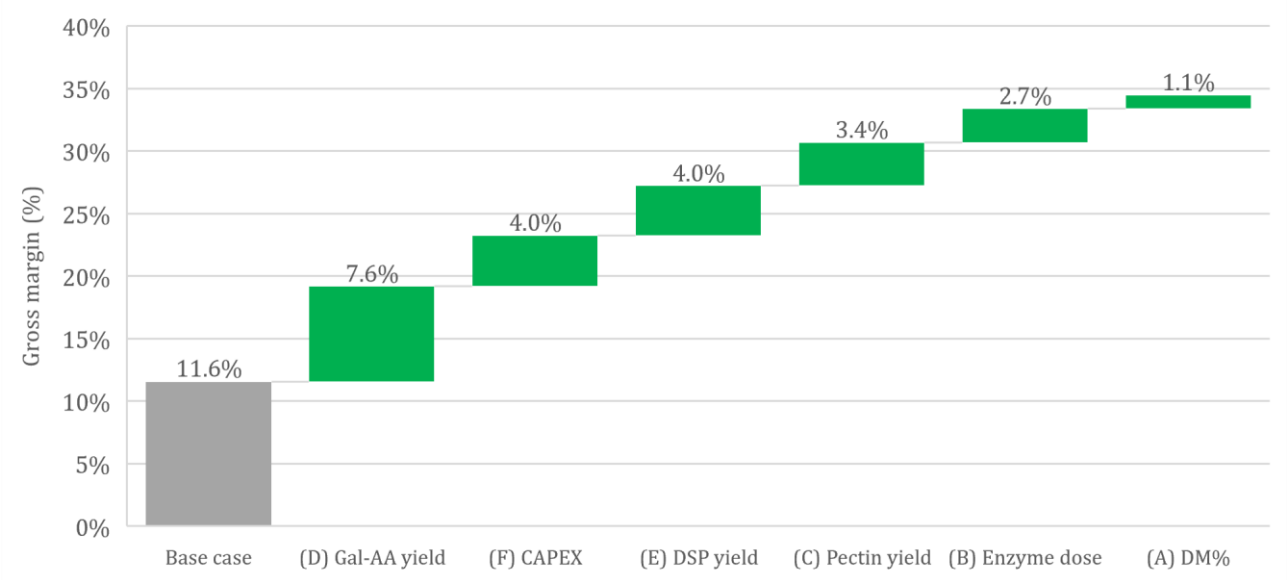

Figure 21 - Waterfall chart for sensitivity analysis.

Through the analysis of Figure 21, it is clear that the modification which led to the highest increase in the gross margin percentage was on the yield of Gal-AA per ton of beet pulp processed. Indeed, increasing the amount of product obtained from the same amount of processed feedstock is largely beneficial since it boosts total revenues and thereby the gross margin is significantly increased. This effect is visible in the sensitivity plot built for this parameter (Appendix E – Sensitivity analysis), as the considerable slope of the gross margin line shows this is quite a sensitive parameter for the overall process financial viability.

Both a 20% reduction in CAPEX and achieving a Gal-AA DSP yield of 90% led to a 4% increase in the gross margin percentage, meaning these modifications had an equivalent effect in the process profitability. Investing in additional equipment and/or R&D efforts to increase recovery of the main

product in purification appears to positively affect total revenues, whereas reducing the investment in equipment lowers the fixed costs which subsequently reduce the total production costs.

An increase of 5% in the pectin hydrolysis yield also proved to have a considerable impact in the gross margin, raising it by 3.4%. Hydrolysing more pectin means that more Gal-UA is available for conversion into Gal-AA which increases product yield per beet pulp dry matter, raising total revenues. In spite of the smaller increase in the gross margin as consequence of reducing the enzyme dosage, this is still a valuable modification as it allows for a reduction in total production costs. Finally, the increase in dry matter content of the initial suspension caused the lowest increase in the gross margin. In fact, due to the way the process model was built, the hourly input of feedstock to be processed is fixed so increasing the dry matter percentage does not translate into higher Gal-AA production. Instead, it only allows for a reduction in vessel sizes, thus the 1.1% increase in the gross margin is actually because of a reduction in CAPEX resulting from smaller hydrolysis vessels, fermenters and DSP sizing requirements.

Ultimately, this sensitivity analysis allows ranking the modifications tested in terms of priority, according to their impact in the gross margin. However, prioritization of activities to increase profitability is not only dependent on the expected outcome, but also on their difficulty, timespan required and probability of success. The results illustrated in Figure 21 suggest that implementation of the envisioned Gal-AA production process should have its major focus in maximizing the yield of this product per ton of beet dry matter processed, yet this is probably the hardest and most time-consuming of the parameters to improve; in fact, the maximum theoretical yield in fermentation is never reached. Though a 20% reduction in CAPEX cause the second highest increase in the gross margin, this modification is significantly limited by the volumes of streams to be processed in the various parts of the process. While improving the DSP of the main product results in an equivalent effect, this also requires significant R&D efforts and a considerable timeline. Likewise, optimizing the hydrolysis yield of pectin to 90% requires much work from R&D but appears to be more achievable than maximizing the fermentation yield, even it that implies using high enzyme dosages (in which a 25% decrease produces one of the lowest increases in the gross margin). Finally, if such a process is implemented with a continuous and fixed beet pulp input coming from a sugar plant (in the same way the model was built), then increasing the dry matter content of the initial suspension is not an improvement that should be prioritized.

4 Conclusions

Although the proof of concept for yeast-based production of Gal-AA from sugar beet pulp hydrolysate has not been reported yet, the hereby developed literature study allows to conclude that there are several factors indicating this a promising path for the valorization of this waste stream. Gal-AA is a valuable sugar acid with applications in the food and cosmetics industries, and has been recently been gaining attention as a platform chemical since it can be converted to building blocks of biodegradable plastics.

However, the potential of this new valorization strategy is not exclusive to its end product. The fact that sugar beet is commercially grown for sugar production ensures the long-term continuity of its cultivation, and hence of pulp availability. The current valorization strategy of sugar beet pulp – selling it as animal feed – not only offers relatively low economic returns but also does not utilize the fermentation potential derived from its rather distinctive chemical composition. The natural absence of lignin in this feedstock make pretreatment merely optional for processing this feedstock, while its rich pectin fraction can be saccharified into high amounts of Gal-UA – a monomer that can be converted to Gal-AA through a single oxidation reaction catalyzed by the enzyme UDH. Previous studies have proven essential in identifying some of the main hurdles to make yeast-based conversion viable, namely Gal-UA tolerance and transport into cytosol, paving the way for the proof of principle using sugar beet pulp hydrolysate.

The model built for the envisioned Gal-AA production process allowed to conclude which process configurations and genetic engineering strategies would result in the most promising business case. In spite of the indicative nature of this analysis – several parameters were determined based on assumptions and estimations – it still provided helpful insight into the design considerations for a fermentative beet pulp-derived galacturonic-to-Gal-AA conversion process. It was found that the pretreatment is not a valuable addition to the process, assuming a 10% reduction in pectin hydrolysis yield occurs without this operation. Using a mild acid solution in pretreatment is not preferable (assuming equal hydrolysis yields) and though implementing SSF seems slightly appealing, it is strictly dependent in finding a compromise between the optimal reaction conditions of enzymes and yeast. Albeit hypothetical at this moment, linking Gal-AA formation to yeast central carbon metabolism was proven vital to reach an attractive business case, raising the gross margin percentage to 27%. In fact, this assessment suggested that, even though in theory this valorization strategy of sugar beet pulp has potential, it might not be feasible unless other fermentable sugars like glucose are converted into Gal-AA. This conclusion was supported by the sensitivity analysis that showed the yield of main product per ton of beet pulp dry matter processed is one of the key parameters affecting the profitability of the envisioned process.

4.1 Recommendations

The recommendations for future work are clear considering the theoretical nature of the present thesis. The following step is to use all knowledge acquired in this investigation and design experiments to achieve the proof of principle of yeast-based conversion on Gal-UA in sugar beet pulp hydrolysate to Gal-AA. Experimental work on genetic engineering of yeast strains expressing UDH genes, sugar beet pulp deconstruction and fermentation on beet pulp hydrolysate will generate data that can be compared with past studies to elucidate some of the still unclear aspects described in the literature study. Furthermore, such data will also be valuable to improve the model built, making it more robust as parameters that were previously input based on assumptions are replaced with realistic experimental results.

If the data from experimental work justifies interest in a full-scale production process, the model developed should be further detailed. For instance, elucidating Gal-AA DSP for better estimations on its recovery yield and equipment costs, specifying CAPEX calculations including outside battery limits costs, introducing waste disposal/wastewater treatment and license fees costs in the cost model. Expanding the business case analysis with the calculation of the net present value (NPV) and internal rate of return (IRR) are also essential improvements to be made in the model.

Another recommendation would be to investigate the degradation of the protein content of sugar beet pulp in enzymatic hydrolysis experiments. In case it is conserved, another by-product of the Gal-AA production process could be envisioned, using the remaining dry matter stream after fermentation along with the yeast biomass formed to create a protein-rich animal feed product. The process model can be used to test whether this alternative is a better option that using that same stream to produce biogas, provided that the GMO-aspect of the yeast protein does not forestall this option.

Finally, but most importantly, the linking of Gal-AA formation to central carbon metabolism seems to be the key to feasibility of a fermentative beet pulp-to-galactaric process. This will, if at all possible, require substantial R&D effort and timespan to make it applicable in a production process. Hence, it would be interesting to approach this aim with support from academic research in a public-private partnership context.

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Appendices

Appendix A – Supporting material for yeast metabolism

This section is intended to provide an overview of the main metabolic pathways involved in yeast central carbon metabolism. Figure A1, besides glycolysis, also presents the heterologous pathways for C5 sugars (xylose and arabinose) metabolism and for reduction of acetic acid to ethanol (in blue). Figure A2 presents a more detailed view on the pentose phosphate pathway, the tricarboxylic acid cycle and glyoxylate cycle correspond to aerobic metabolism and thus are not relevant for this work which focuses on anaerobic fermentation.

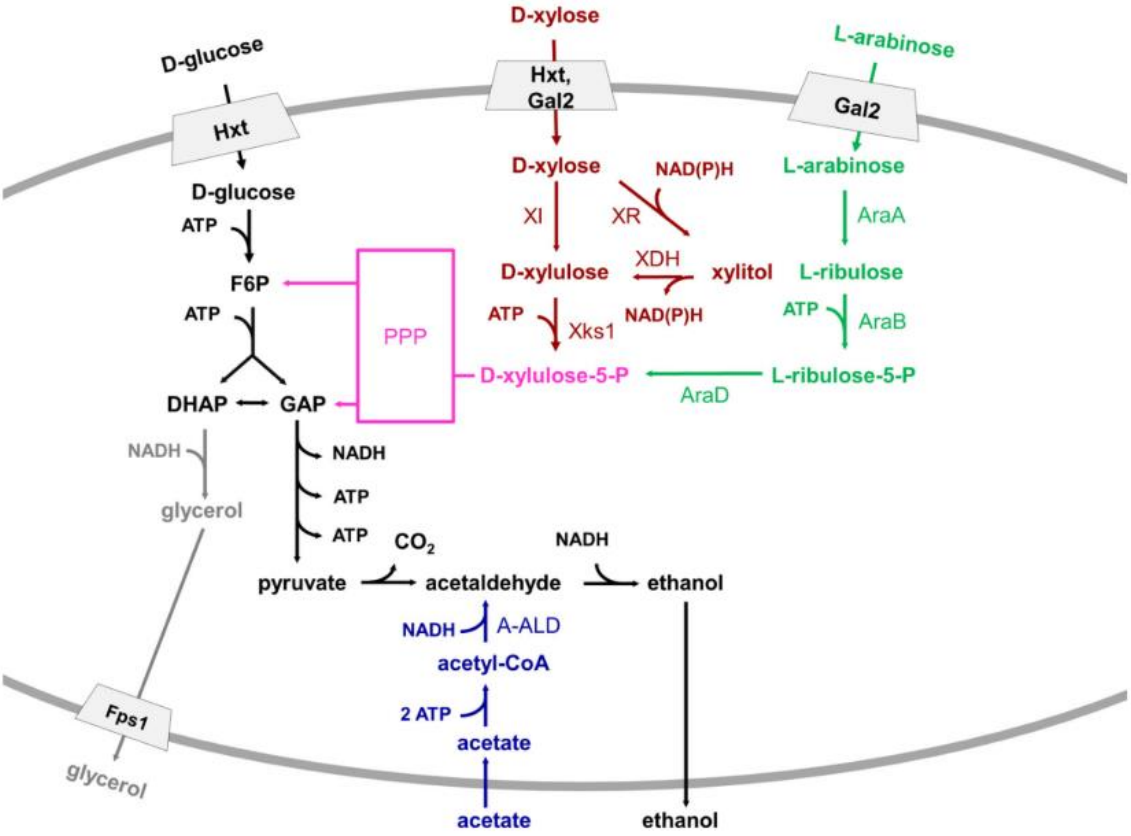

Figure A1 - Key strategies for engineering carbon and redox metabolism in S. cerevisiae strains for alcoholic fermentation of lignocellulosic feedstocks (Jansen *et al.*, 2017).

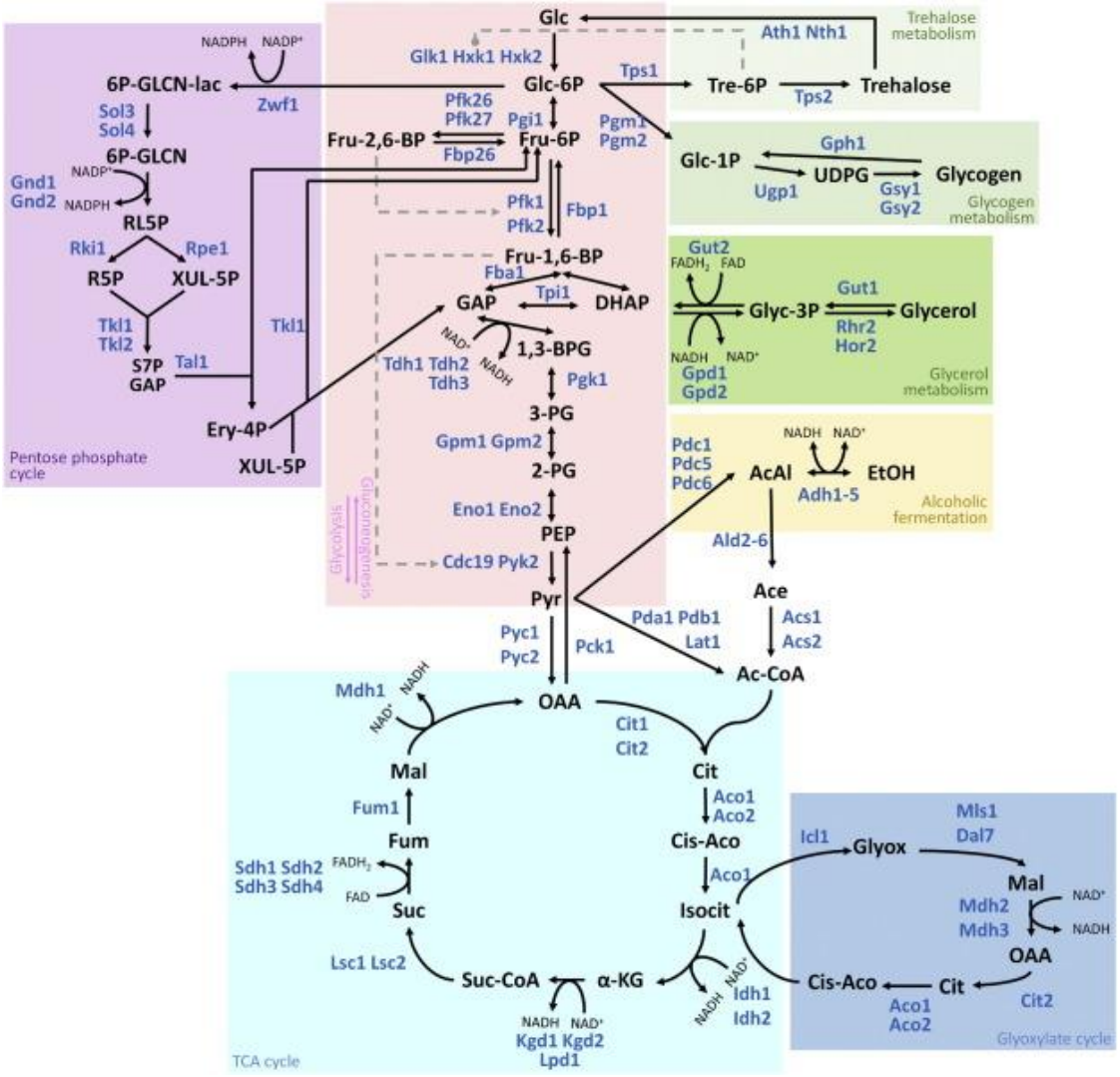

Figure A2 - Metabolic pathways involved in yeast central carbon metabolism (Tripodi *et al.*, 2015).

Appendix B – Model inputs

In this section, parts of the model developed are presented with the purpose on showing calculations done with bigger insight and complementing the information provided in section 3.2.1. The values highlighted in yellow represent data that was input in the model based on references or internal expertise.

Feedstock

Table B1 - Feedstock cost and composition data used in the model.

MASS BALANCE	Scenario no.	1	2	3	4	5	6
	Description	*Base case*	*No pretreatment*	*Mild acid*	*SSF*	*PRK-Rubisco*	*C6 -> Gal-AA*
Parameter	*Unit*	*Value*					
Feedstock: SBP		**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Cost	€/ton	142.00	142.00	142.00	142.00	142.00	142.00
Polymer composition	dry weight						
- Cellulose	wt%	31%	31%	31%	31%	31%	31%
- Hemicellulose	wt%	28%	28%	28%	28%	28%	28%
- Pectin	wt%	28%	28%	28%	28%	28%	28%
- Lignin	wt%	2.6%	2.6%	2.6%	2.6%	2.6%	2.6%
- Protein	wt%	8.2%	8.2%	8.2%	8.2%	8.2%	8.2%
- Other	wt%	2.2%	2.2%	2.2%	2.2%	2.2%	2.2%
TOTAL	wt%	100%	100%	100%	100%	100%	100%
Monomer composition	dry weight						
- Glucose	wt%	24.5%	24.5%	24.5%	24.5%	24.5%	24.5%
- Galactose	wt%	4.5%	4.5%	4.5%	4.5%	4.5%	4.5%
- Mannose	wt%	1.0%	1.0%	1.0%	1.0%	1.0%	1.0%
- Arabinose	wt%	17.9%	17.9%	17.9%	17.9%	17.9%	17.9%
- Xylose	wt%	1.1%	1.1%	1.1%	1.1%	1.1%	1.1%
- Rhamnose	wt%	2.0%	2.0%	2.0%	2.0%	2.0%	2.0%
- Galacturonic acid	wt%	22.0%	22.0%	22.0%	22.0%	22.0%	22.0%

The polymer compositions of sugar beet pulp used in the model are according to the information presented in section 2.2.2. The values for monomer composition used were obtained from Cosun and are within the ranges shown in Table 1.

Sugar plant and galactaric acid plant operation

Table B2 - Sugar plant operation and galactaric acid plant operation data used in the model.

Sugar plant operation - Cosun (2 plants)		**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Average capacity per plant (min.)	ton beet/day	2.70E+04	2.70E+04	2.70E+04	2.70E+04	2.70E+04	2.70E+04
Beet dry matter content	%	25%	25%	25%	25%	25%	25%
Average capacity per plant (min.)	ton beet DM/day	6.75E+03	6.75E+03	6.75E+03	6.75E+03	6.75E+03	6.75E+03
Pulp yield	ton pulp DM/ton beet DM	0.16	0.16	0.16	0.16	0.16	0.16
Pulp DM production	ton pulp DM/year	3.60E+05	3.60E+05	3.60E+05	3.60E+05	3.60E+05	3.60E+05
Processing plant operation - DSM							
Pulp DM input in process	ton pulp DM/year	3.60E+05	3.60E+05	3.60E+05	3.60E+05	3.60E+05	3.60E+05
Dried SPB DM content	%	89.5%	89.5%	89.5%	89.5%	89.5%	89.5%
SBP pellets input in process	ton pulp/year	4.02E+05	4.02E+05	4.02E+05	4.02E+05	4.02E+05	4.02E+05
Cost of feedstock	€/year	5.71E+07	5.71E+07	5.71E+07	5.71E+07	5.71E+07	5.71E+07
Processing plant uptime	days/year	333	333	333	333	333	333
Hourly thoughput	ton pulp/h	50.3	50.3	50.3	50.3	50.3	50.3

Pretreatment

Table B3 - Pretreatment data used in the model.

Pretreatment	#continuous	S1	S2	S3	S4	S5	S6
Residence time	h	0.333	0.000	0.333	0.333	0.333	0.333
Pulp DM mass in reactor	ton DM	15.0	0.0	15.0	15.0	15.0	15.0
Suspension DM content	%	12%	12%	12%	12%	12%	12%
Water mass to add	ton	110.0	0.0	110.0	110.0	110.0	110.0
Water volume	m3	110.0	0.0	110.0	110.0	110.0	110.0
SBP volume	m3	80.6	0.0	80.6	80.6	80.6	80.6
Reactor volume	m3	190.6	0.0	190.6	190.6	190.6	190.6

The pretreatment reactor volume was determined taking into account the volume occupied by both water and feedstock, considering a screw reactor would be the most likely candidate for this operation.

Saccharification

Table B4 - Saccharification data used in the model.

Saccharification	#batch	S1	S2	S3	S4	S5	S6
Yields (pretreatment+hydrolysis)							
- Cellulose (Glu)	%	80%	60%	80%	80%	80%	80%
- Hemicellulose + Pectin (Ara)	%	85%	70%	85%	85%	85%	85%
- Hemicellulose (Xyl)	%	85%	70%	85%	85%	85%	85%
- Pectin (Gal-UA)	%	85%	75%	85%	85%	85%	85%
Molar weight factor							
Glucan -> Glucose		1.11	1.11	1.11	1.11	1.11	1.11
Arabinan -> Arabinose		1.14	1.14	1.14	1.14	1.14	1.14
Xylan -> Xylose		1.14	1.14	1.14	1.14	1.14	1.14
Homogalacturonan -> Gal-UA		1.10	1.10	1.10	1.10	1.10	1.10
Total process time	h	96	96	96	96	96	96
Time	h	48	48	48	48	48	48
SBP DM mass to hydrolyze	ton	2160.0	2160.0	2160.0	2160.0	2160.0	2160.0
Suspension DM content	%	12%	12%	12%	12%	12%	12%
Water mass entering	ton	1.58E+04	1.58E+04	1.58E+04	1.58E+04	1.58E+04	1.58E+04
Suspension mass entering reactor	ton	1.80E+04	1.80E+04	1.80E+04	1.80E+04	1.80E+04	1.80E+04
Water volume	m3	1.58E+04	1.58E+04	1.58E+04	1.58E+04	1.58E+04	1.58E+04
SBP volume	m3	1.16E+04	1.16E+04	1.16E+04	1.16E+04	1.16E+04	1.16E+04
Fill factor	%	80%	80%	80%	80%	80%	80%
Reactor volume	m3	3.43E+04	3.43E+04	3.43E+04	3.43E+04	3.43E+04	3.43E+04

Once again, the determination of the volume of the saccharification vessel included both the volume of water and solids, this time considering a headspace of 20%.

Fermentation

Table B5 - Fermentation data used in the model.

Fermentation (and propagation)	#batch	S1	S2	S3	S4	S5	S6
Stoichiometrical yields							
- Glucose -> EtOH	g/g	0.51	0.51	0.51	0.51	0.51	0.51
- Glucose -> Biomass	g/g	0.83	0.83	0.83	0.83	0.83	0.83
- Glucose -> Glycerol	g/g	1.02	1.02	1.02	1.02	1.02	1.02
- Glucose -> Gal-AA	g/g	1.17	1.17	1.17	1.17	1.17	1.17
- Arabinose -> EtOH	g/g	0.51	0.51	0.51	0.51	0.51	0.51
- Xylose -> EtOH	g/g	0.51	0.51	0.51	0.51	0.51	0.51
- GalUA -> Gal-AA	g/g	1.08	1.08	1.08	1.08	1.08	1.08
- Gal-AA to Glycerol	g/g	0.44	0.44	0.44	0.44	0.44	0.44
Glucose spent to form glycerol (Gal-AA)	kg/ton DM	86.10	75.97	86.10	86.10	10.89	21.78
Yield factors							
- Glucose -> Glycerol (due to Gal-AA)	%	40%	47%	40%	40%	5%	10%
- Glucose -> Gal-AA	%	0%	0%	0%	0%	0%	30%
- Glucose -> Other products	%	60%	53%	60%	60%	95%	60%
== Glucose -> EtOH	%	54%	48%	54%	54%	86%	54%
== Glucose -> Biomass	%	3%	3%	3%	3%	5%	3%
== Glucose -> Glycerol	%	2%	2%	2%	2%	4%	2%
- Arabinose -> EtOH	%	90%	90%	90%	90%	90%	90%
- Xylose -> EtOH	%	90%	90%	90%	90%	90%	90%
- Gal-UA -> Gal-AA	%	90%	90%	90%	90%	90%	90%
Time	h	48	48	48	48	48	48

Utilities and CAPEX

Table B6 - Utilities and CAPEX data used in the model.

Utilities & CapEx							
Utilities		S1	S2	S3	S4	S5	S6
Plant Capacity							
DM entering	ton DM/year	3.60E+05	3.60E+05	3.60E+05	3.60E+05	3.60E+05	3.60E+05
Gal-AA produced	ton/year	6.14E+04	5.42E+04	6.14E+04	6.14E+04	6.14E+04	8.47E+04
Steam consumption							
up to hydrolysis	ton/ton DM entering	2.50	1.00	2.50	2.50	2.50	2.50
DSP	ton/ton Gal-AA	4.00	4.00	4.00	4.00	4.00	4.00
Yearly total	ton/year	1.14E+06	5.76E+05	1.14E+06	1.14E+06	1.14E+06	1.24E+06
Electricity consumption							
up to DSP	kWh/ton DM entering	300.00	250.00	300.00	280.00	300.00	300.00
DSP to product	kWh/ton Gal-AA	1.50	1.50	1.50	1.50	1.50	1.50
Yearly total	kWh/year	1.08E+08	9.00E+07	1.08E+08	1.01E+08	1.08E+08	1.08E+08
Yearly total (using energy from biogas)	kWh/year	5.18E+07	8.35E+06	5.18E+07	4.46E+07	5.11E+07	5.19E+07
Unit Costs							
Steam	€/ton	20.00	20.00	20.00	20.00	20.00	20.00
Electricity	€/kWh	0.07	0.07	0.07	0.07	0.07	0.07
CapEx							
Total installed cost	M€	229.16	221.09	232.65	208.17	229.67	254.75
% CapEx for labor	%/year	2%	2%	2%	2%	2%	2%
% CapEx for maintenance	%/year	3%	3%	3%	3%	3%	3%
% CapEx for insurance	%/year	2%	2%	2%	2%	2%	2%
Depreciation period	years	15	15	15	15	15	15

Steam consumption up to the hydrolysis section was estimated using a reference ratio of ton of steam consumed per ton of dry mater entering the process from second generation bioethanol plants (internal DSM data). Steam consumption of DSP was estimated assuming a ratio of ton of steam consumed per ton of Gal-AA produced.

For estimations of electricity consumptions, the same approach was used. Reference data from bioethanol plants provided a value of kWh consumed up to DSP as a function of dry matter entering the process whereas another value was used for the consumption of electricity in DSP as a function of final product produced.

Appendix C – Mass balance summary

Table C1 - Summary of the mass balance developed for the galactaric acid production process.

MASS BALANCE SUMMARY		Scenario 1	Scenario 2	Scenario 3	Scenario 4	Scenario 5	Scenario 6
Consumptions							
Feedstock	ton/year	4.02E+05	4.02E+05	4.02E+05	4.02E+05	4.02E+05	4.02E+05
Enzyme	ton/year	359.64	539.46	269.73	359.64	359.64	359.64
Urea (nitrogen)	ton/year	259.79	172.36	259.79	259.79	408.16	257.79
Sulphuric acid	ton/year	0.00	0.00	1.80E+04	0.00	0.00	0.00
Potassium hydroxide (base)	ton/year	1.92E+04	1.69E+04	2.94E+04	1.92E+04	1.92E+04	2.64E+04
Yeast	ton/year	1227.39	1230.52	1227.39	1227.39	613.70	3068.48
Steam	ton/year	1.14E+06	5.76E+05	1.14E+06	1.14E+06	1.14E+06	1.24E+06
Electricity	kWh/year	5.18E+07	8.35E+06	5.18E+07	4.46E+07	5.11E+07	5.19E+07
Unit costs							
Feedstock	€/ton	142.00	142.00	142.00	142.00	142.00	142.00
Enzyme	€/ton	5.10E+04	5.10E+04	5.10E+04	5.10E+04	5.10E+04	5.10E+04
Urea (nitrogen)	€/ton	182.24	182.24	182.24	182.24	182.24	182.24
Sulphuric acid	€/ton	182.75	182.75	182.75	182.75	182.75	182.75
Potassium hydroxide (base)	€/ton	663.00	663.00	663.00	663.00	663.00	663.00
Yeast	€/ton	3085.50	3085.50	3085.50	3085.50	3085.50	3085.50
Steam	€/ton	20.00	20.00	20.00	20.00	20.00	20.00
Electricity	€/kWk	0.07	0.07	0.07	0.07	0.07	0.07
Yearly Costs							
Feedstock	M€/year	57.06	57.06	57.06	57.06	57.06	57.06
Enzyme	M€/year	18.34	27.51	13.76	18.34	18.34	18.34
Urea (nitrogen)	M€/year	0.05	0.03	0.05	0.05	0.07	0.05
Sulphuric acid	M€/year	0.00	0.00	3.29	0.00	0.00	0.00
Potassium hydroxide (base)	M€/year	12.70	11.21	19.52	12.70	12.70	17.52
Yeast	M€/year	3.79	3.80	3.79	3.79	1.89	9.47
Steam	M€/year	22.89	11.53	22.89	22.89	22.89	24.76
Electricity	M€/year	3.63	0.58	3.63	3.12	3.58	3.63
TOTAL	M€/year	118.46	111.72	123.98	117.96	116.54	130.83
Production							
Galactaric acid	ton/year	6.14E+04	5.42E+04	6.14E+04	6.14E+04	6.14E+04	8.47E+04
Ethanol	ton/year	5.05E+04	3.82E+04	5.05E+04	5.05E+04	6.25E+04	5.03E+04
Energy from biogas	kWh/year	5.61E+07	8.16E+07	5.61E+07	5.61E+07	5.69E+07	5.61E+07
Sales unit prices							
Galactaric acid	€/ton	2500.00	2500.00	2500.00	2500.00	2500.00	2500.00
Ethanol	€/ton	314.48	314.48	314.48	314.48	314.48	314.48
Energy from biogas	€/kWh	0.040	0.040	0.040	0.040	0.040	0.040
Yearly revenues							
Galactaric acid	M€/year	153.50	135.44	153.50	153.50	153.50	211.74
Ethanol	M€/year	15.87	12.01	15.87	15.87	19.66	15.82
Energy from biogas	M€/year	2.25	3.27	2.25	2.25	2.28	2.25
TOTAL	M€/year	169.37	147.45	169.37	169.37	173.16	227.56

Appendix D – Total cost of goods and gross margin

Table D1 - Total cost of goods and gross margin calculations for the envisioned galactaric acid production process.

COST MODEL							
TOTAL COST OF GOODS		**S1_Base case**	**S2_No Pre**	**S3_Acid Pre**	**S4_SSF**	**S5_Rubsico**	**S6 _Central**
Variable costs							
Feedstock	€/kg Gal-AA	0.929	1.053	0.929	0.929	0.929	0.674
Nutrients	€/kg Gal-AA	0.001	0.001	0.001	0.001	0.001	0.001
Chemicals	€/kg Gal-AA	0.207	0.207	0.371	0.207	0.207	0.207
Utilities	€/kg Gal-AA	0.432	0.224	0.432	0.424	0.431	0.335
Enzymes	€/kg Gal-AA	0.299	0.508	0.224	0.299	0.299	0.217
Yeast	€/kg Gal-AA	0.062	0.070	0.062	0.062	0.031	0.112
License fees	€/kg Gal-AA	0.000	0.000	0.000	0.000	0.000	0.000
Waste disposal	€/kg Gal-AA	0.000	0.000	0.000	0.000	0.000	0.000
Total variable costs	€/kg Gal-AA	1.93	2.06	2.02	1.92	1.90	1.54
Total variable costs	M€/year	118.46	111.72	123.98	117.96	116.54	130.83
Fixed costs							
Labor	€/kg Gal-AA	0.075	0.082	0.076	0.068	0.075	0.060
Maintenance	€/kg Gal-AA	0.112	0.122	0.114	0.102	0.112	0.090
Insurance	€/kg Gal-AA	0.075	0.082	0.076	0.068	0.075	0.060
Depreciation	€/kg Gal-AA	0.249	0.272	0.253	0.226	0.249	0.201
Total fixed costs	€/kg Gal-AA	0.51	0.56	0.52	0.46	0.51	0.41
Total fixed costs	M€/year	31.32	30.21	31.79	28.45	31.39	34.82
Gross margin							
Total sales	M€/year	169.37	147.45	169.37	169.37	173.16	227.56
Total production costs	€/kg Gal-AA	2.44	2.62	2.54	2.38	2.41	1.96
Total production costs	M€/year	149.78	141.93	155.77	146.41	147.93	165.64
Gross Margin	M€/year	19.59	5.52	13.60	22.97	25.23	61.92
Gross Margin	%	12%	4%	8%	14%	15%	27%
		Base case	*No pretreatment*	*Mild acid*	*SSF*	*PRK-Rubisco*	*C6 -> Gal-AA*

Appendix E – Sensitivity analysis

This section presents all the plots built for the nine parameters selected to integrate this sensitivity analysis. For the variations of each parameter, the effects in the total production costs, total revenues and gross margin (in M€/year) are represented, and the base case value for each parameter is also marked in the graph.

Dry matter content

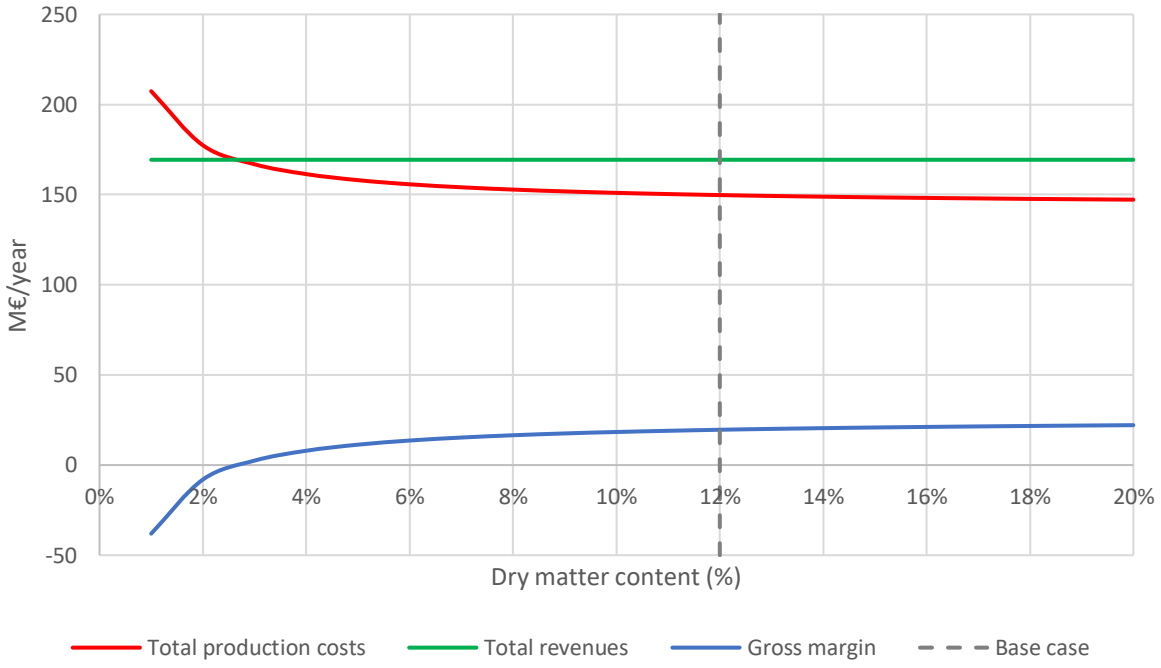

Figure E1 - Sensitivity plot for dry matter content.

Galactaric acid yield

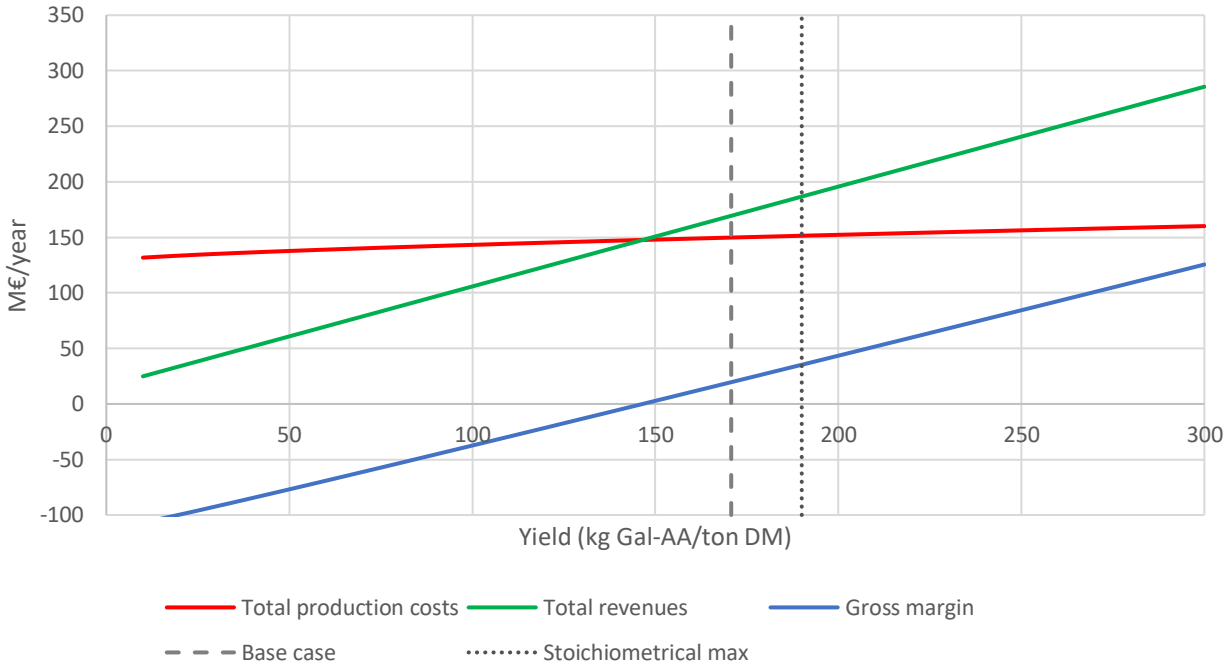

Figure E2 - Sensitivity plot for galactaric acid yield per ton of feedstock processed.

Process time

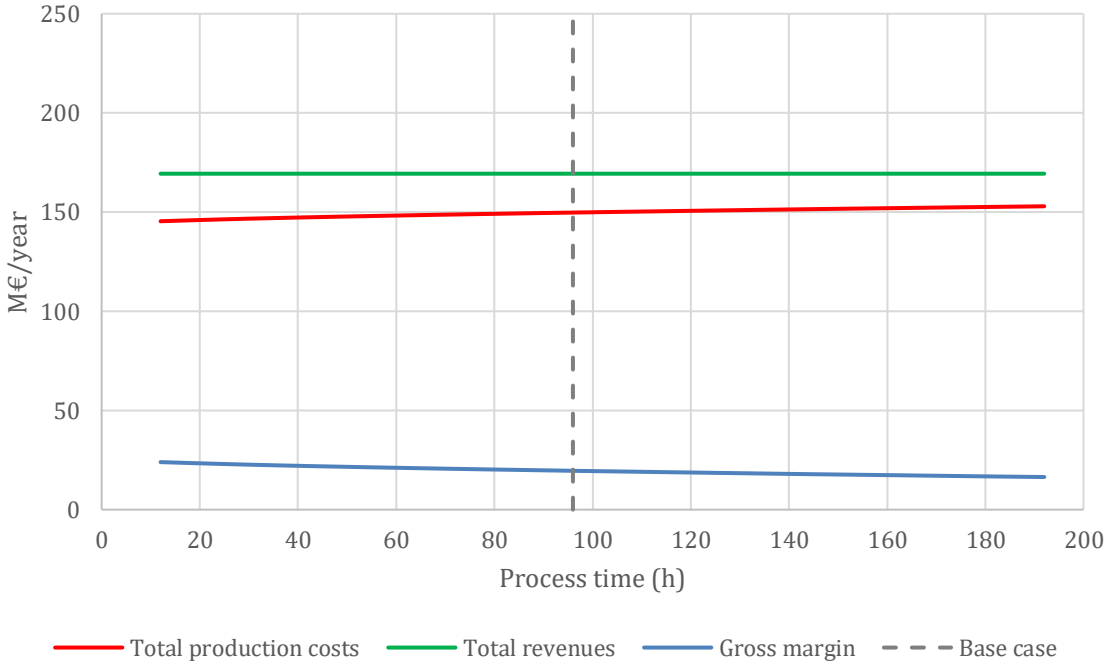

Figure E3 - Sensitivity plot for process time.

Feedstock cost

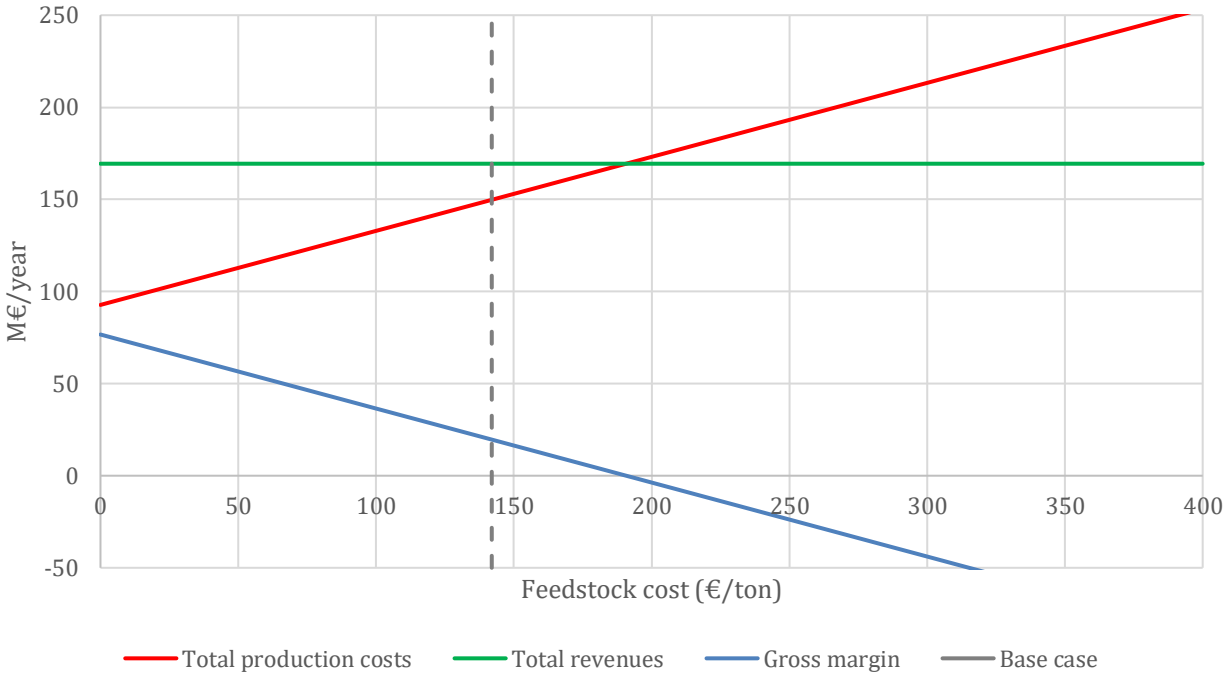

Figure E4 - Sensitivity plot for feedstock cost.

Enzyme dosage

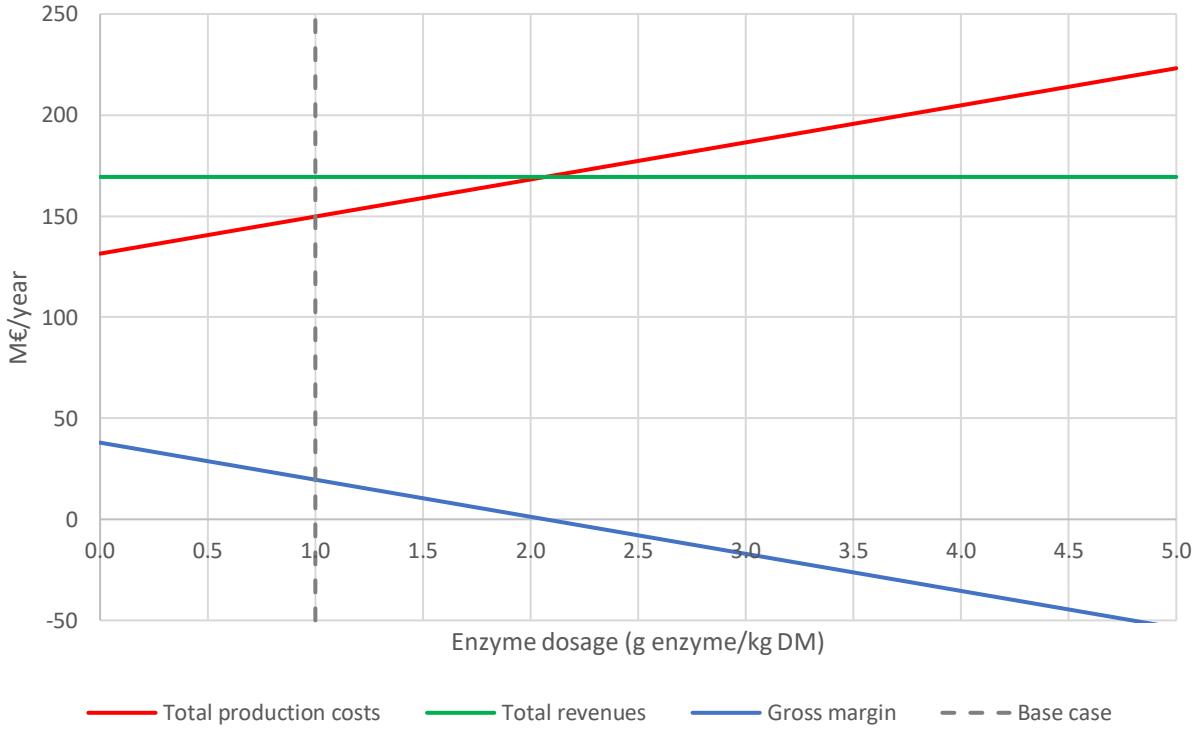

Figure E5 - Sensitivity plot for enzyme dosage.

Pectin hydrolysis yield

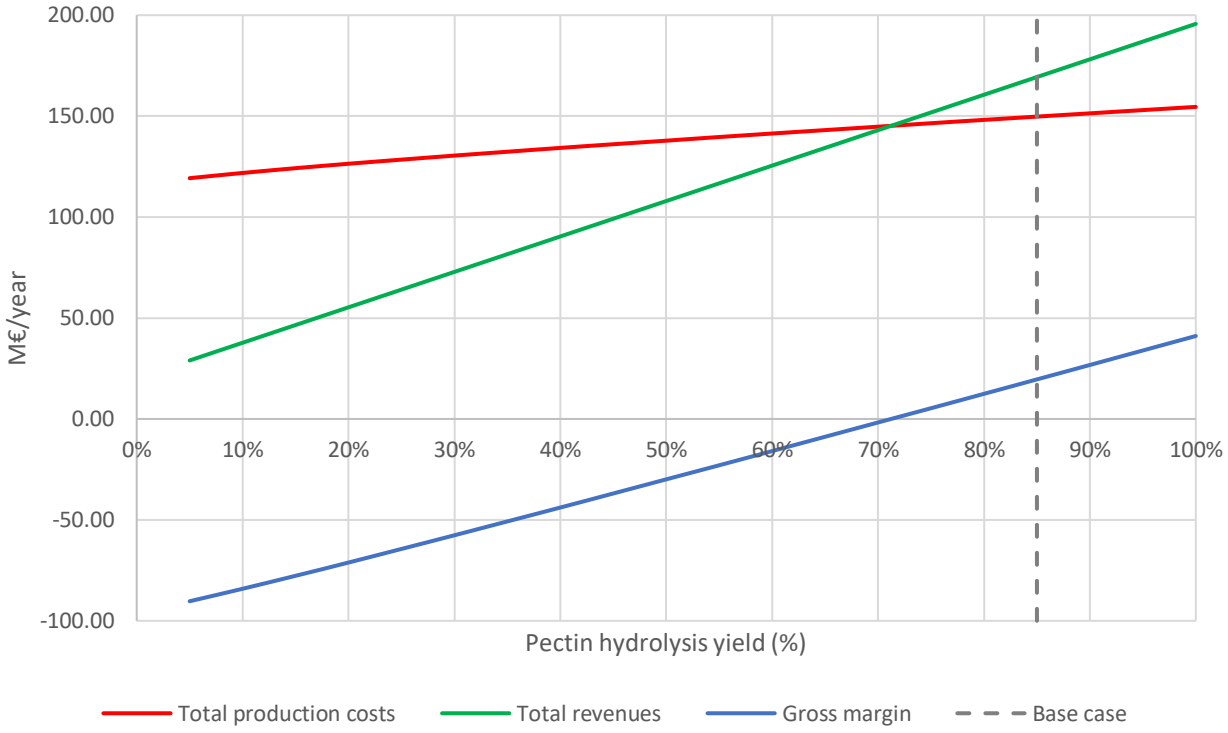

Figure E6 - Sensitivity plot for pectin hydrolysis yield.

Galactaric acid DSP yield

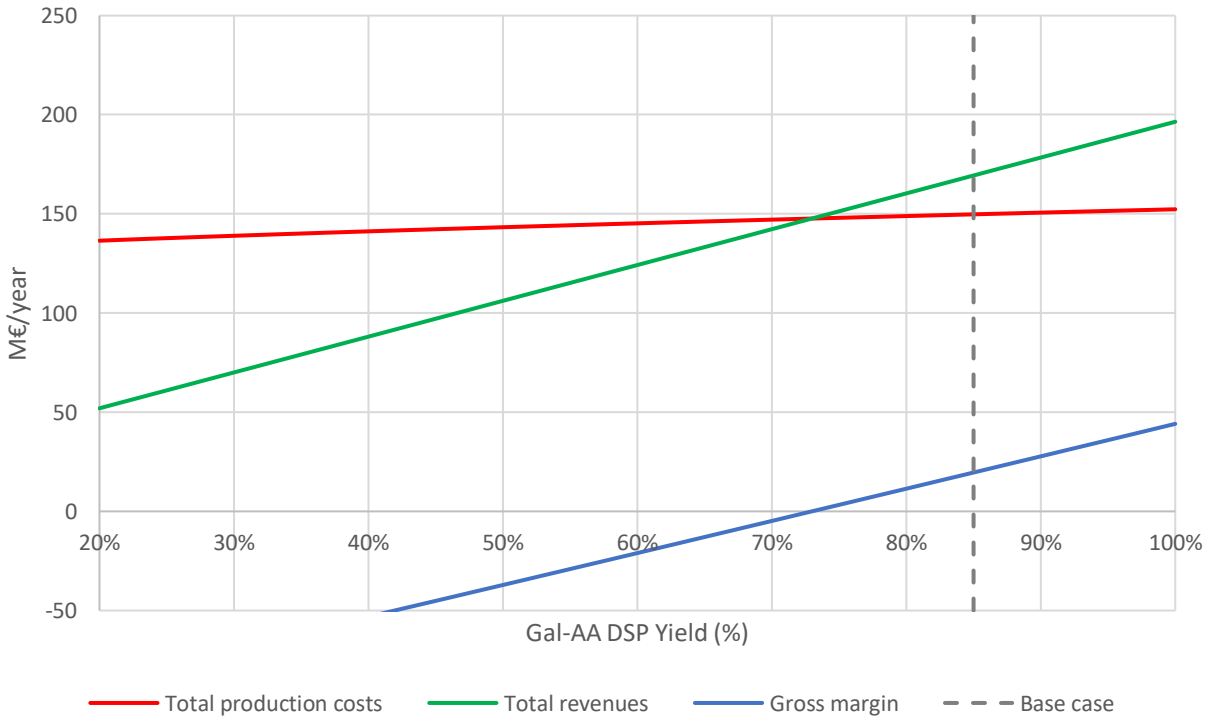

Figure E7 - Sensitivity plot for galactaric acid DSP yield.

Galactaric acid price

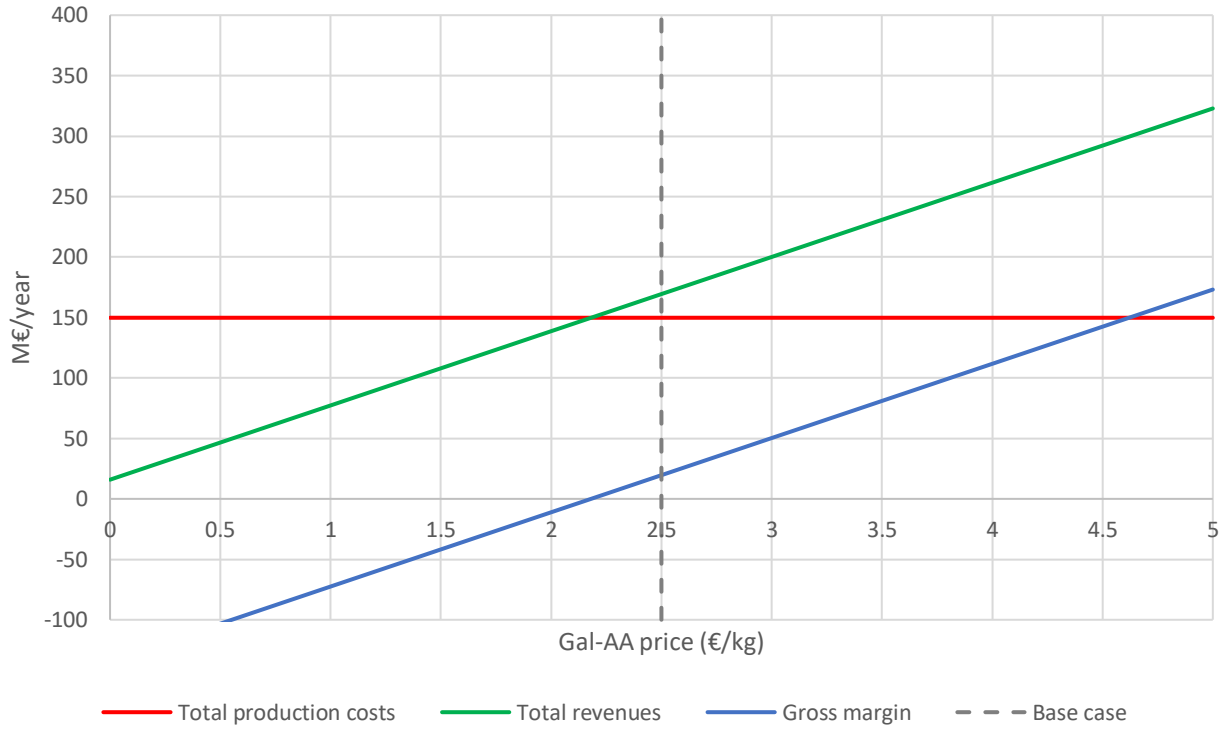

Figure E8 - Sensitivity plot for galactaric acid price.

CAPEX

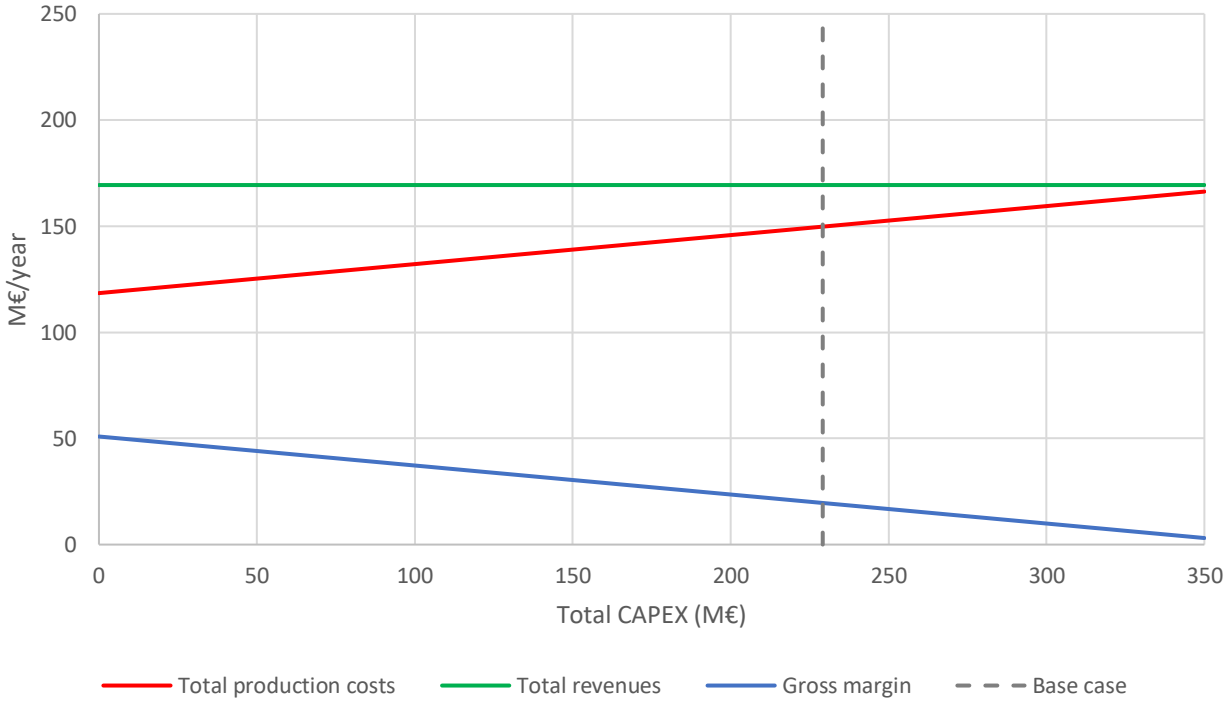

Figure E9 - Sensitivity plot for CAPEX.