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Coutinho Campos, Joana

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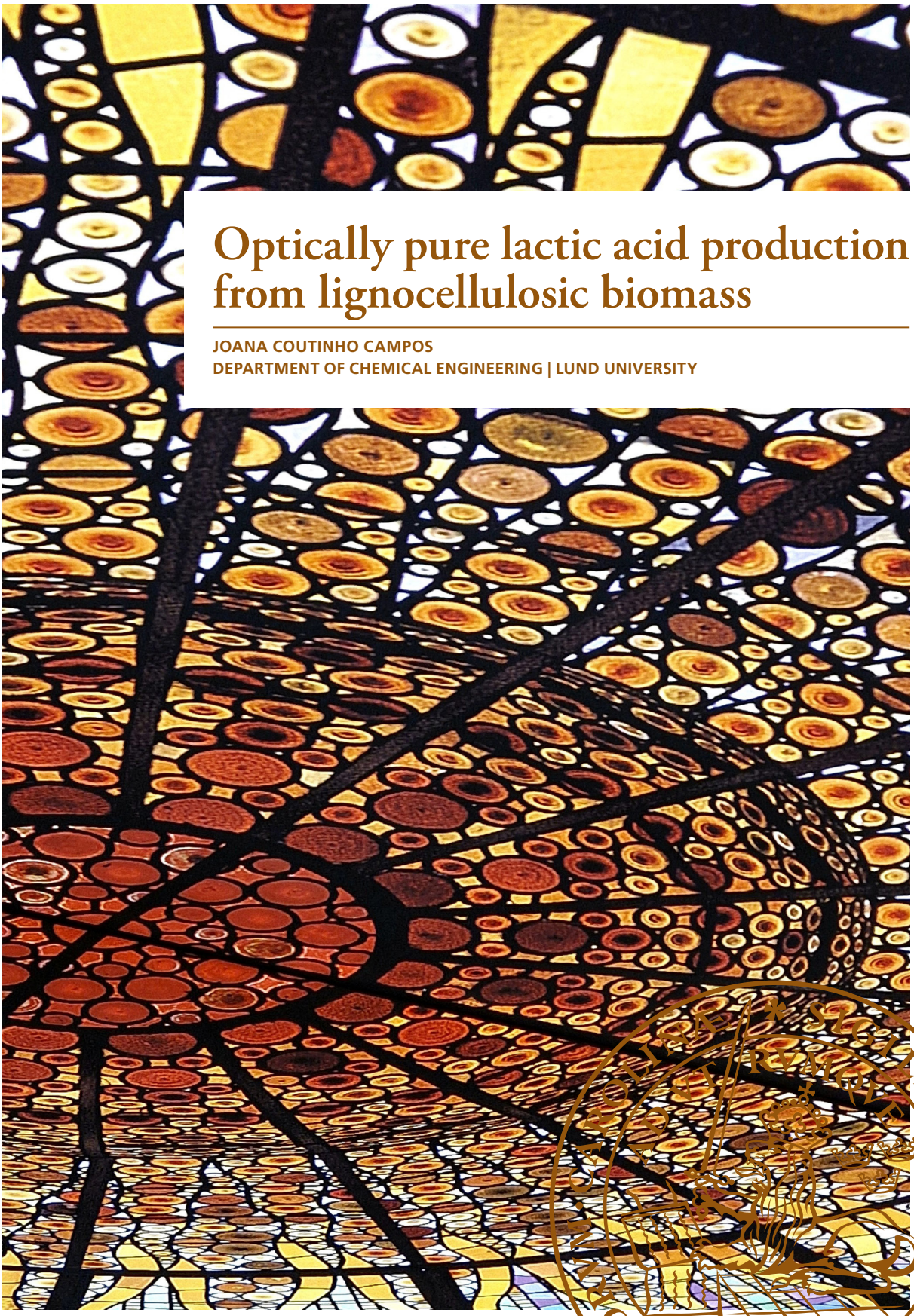

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DOCTORAL DISSERTATION

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Abstract:

Lactic acid is a platform chemical that can be used for many applications. More recently, optically pure lactic acid—L- or D-lactic acid enantiomers—have been used for the polymerisation to poly-lactic acid (PLA). The possibility of tailoring the properties of this bioplastic by adjusting the ratio of each enantiomer might help to accelerate the substitution of petroleum-based plastics with bio-based and compostable alternatives. Today, PLA is produced primarily from raw materials that can be used as food—sugarcane and corn. To decrease the utilisation of food crops for producing bioplastics, alternative processes using lignocellulosic biomass and organic waste are under development. In this thesis, optically pure lactic acid production from lignocellulosic biomass was examined using softwood (spruce) and post-consumer waste viscose.

Softwood was subjected to a SO_2-catalysed steam explosion pretreatment to saccharify hemicellulose and open the solid structure of cellulose and lignin. The pretreated softwood was separated into two fractions (liquid and solid). The hemicellulose hydrolysate (liquid fraction) was used without further treatment as a carbon source in the bacterial cultivations, whereas the solid fraction of pretreated softwood and the waste viscose (a man-made cellulosic fibre) had to undergo enzymatic hydrolysis before use in cultivation studies.

Two strains of *Pediococcus acidilactici* were used in this work. Each strain was genetically modified to produce only one enantiomer: *P. acidilactici* TY112 generates L-lactic acid, and *P. acidilactici* ZP26 produces D-lactic acid. The two strains were used to produce the respective enantiomers from the liquid fraction of softwood, with similar results. Subsequently, their resistance to the inhibitory compounds generated during the pretreatment was evaluated. A fed-batch strategy for co-consumption of glucose and mannose and conversion of hydroxymethylfurfural (HMF) and furfural was studied and reported. Simultaneous saccharification and fermentation (SSF) was used in cultivations with the softwood solid fraction as the carbon source. Despite initial results of a long lag phase of 48 h under several conditions, this adaptation phase was decreased by starting the cultivation with monomeric sugars before the addition of the solid fraction and enzymatic blend for SSF. *P. acidilactici* ZP26 also showed promising results in cultivations using saccharified waste viscose, despite the presence of dyes and likely other additives and contaminants in the media.

These results add knowledge to a broader research project that aims to use *P. acidilactici* to produce optically pure lactic acid and high-quality PLA from several sources of lignocellulosic biomass. These modified bacteria synthesise their respective enantiomers with high yields, even in the presence of possibly toxic compounds in softwood and waste viscose.

Keywords:

Lactic acid, enantiomers, softwood, waste viscose, fermentation, *Pediococcus acidilactici*

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I used to think much of this was wasted time.
However, it was part of the process.

Belinda Kirk, Explorers Connect

Contents

Abstract

Lactic acid is a platform chemical that can be used for many applications. More recently, optically pure lactic acid—L- or D-lactic acid enantiomers—have been used for the polymerisation to poly-lactic acid (PLA). The possibility of tailoring the properties of this bioplastic by adjusting the ratio of each enantiomer might help to accelerate the substitution of petroleum-based plastics with bio-based and compostable alternatives. Today, PLA is produced primarily from raw materials that can be used as food—sugarcane and corn. To decrease the utilisation of food crops for producing bioplastics, alternative processes using lignocellulosic biomass and organic waste are under development. In this thesis, optically pure lactic acid production from lignocellulosic biomass was examined using softwood (spruce) and post-consumer waste viscose.

Softwood was subjected to a SO_2-catalysed steam explosion pretreatment to saccharify hemicellulose and open the solid structure of cellulose and lignin. The pretreated softwood was separated into two fractions (liquid and solid). The hemicellulose hydrolysate (liquid fraction) was used without further treatment as a carbon source in the bacterial cultivations, whereas the solid fraction of pretreated softwood and the waste viscose (a man-made cellulosic fibre) had to undergo enzymatic hydrolysis before use in cultivation studies.

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Scientific Summary

This thesis concerns lactic acid production by bacteria, using components from spruce wood chips or viscose, a man-made plant-based textile, as raw materials.

Lactic acid is a versatile chemical that is used in many sectors: food and beverage, pharmaceutics and cosmetics, agriculture and other industrial processes. In products for human and animal consumption, the raw materials and processes must adhere to high standards. However, wastes and agricultural residues can be used as raw materials for bio-based plastic. One example is poly-lactic acid (PLA), which has many applications, including 3D printing.

Despite its many potential benefits, it is challenging to use lignocellulosic biomass, particularly if one wishes to use wood residues from sawmills, as in this thesis. The oldest spruce in the world is Swedish—old Tjikko is over 9560 years old and inhabits Fulufjället National Park, in the Dalarna region. To survive for this long, trees require a complex and sturdy structure. Thus, the first step in using wood chips is a harsh physicochemical pretreatment to open this recalcitrant structure. The high temperature and acidic conditions used in the pretreatment effect the formation of some compounds that are toxic to bacteria. To design a process for the utilisation of softwood sugar, part of this thesis focused on studying how these inhibitors affect lactic acid production by the chosen microorganisms.

In this thesis, two variants of the bacterial species *Pediococcus acidilactici* were found to be able to use the sugars in lignocellulosic biomass (e.g., softwood and cellulosic waste textile) to produce lactic acid. These bacteria have been modified from a parent organism grown in pretreated corn stover, a carbon source that also has the afore mentioned inhibitors. Consequently, the bacteria used in this thesis also tolerate these harmful compounds and have demonstrated good potential for use in equally adverse media.

Waste viscose is degraded to sugars more easily than softwood, but textiles have many additives, including dyes, that are toxic to the environment. These chemicals differ from the inhibitors in the softwood process, but our experiments show that the bacteria are not severely affected, and the results on lactic acid production are promising.

This work is part of a larger endeavour: using *Pediococcus acidilactici* to produce lactic acid from several types of plant-based residues, with the requisite purity for generating PLA sustainably. If successful, this approach could replace the current use of food crops to produce these materials.

This technology is far from ready, legislation can still be improved to support the production of bio-based materials and the world needs to learn how to use natural resources better. Nonetheless, every step is progress! 🍃

Populärvetenskaplig Sammanfattning

Denna avhandling handlar om produktion av mjölksyra från granflis eller återvunnen viskos, en konstgjord växtbaserad textil, med hjälp av bakterier.

Mjölksyra är en mångsidig kemikalie med många användningsområden. Den finns i livsmedel och drycker, används vid framställning av läkemedel och kosmetika, i jordbruket och i flera andra industriella processer. Ett exempel är för framställning av bioplasten poly-mjölksyra, PLA, som kan användas till exempel i 3D-skrivare. När det gäller produkter för direkt användning av människor och djur, tex i livsmedel, måste råvaror och processer uppfylla mycket höga krav. Avfall och restprodukter från jord- och skogsbruk, kan dock användas som råmaterial för biobaserad plast.

Trots många potentiella fördelar med att använda restprodukter för framställning av mjölksyra är det ofta utmanande. Detta gäller i synnerhet om man vill använda restprodukter såsom träflis från skogsråvara, vilket görs i denna avhandling. Den äldsta granen i världen är svensk – gamla Tjikko växer i Fulufjällets Nationalpark i Dalarna och är mer än 9560 år gammal! För att ett träd ska kunna överleva så länge behöver det ha en robust struktur, som ofta är komplext uppbyggd. Det första steget för att kunna använda träflisen är därför en rätt tuff fysikalisk-kemisk process för att öppna den motsträviga och ganska komplexa strukturen. Den höga temperaturen och de sura förhållandena leder till att olika nedbrytningsföreningar från träet bildas, av vilka vissa är giftiga för bakterier - så kallade inhibitorer. För att utforma en process för användning av socker från barrträd måste man förstå hur dessa inhibitorer påverkar mjölksyraproduktionen i mikroorganismer.

I denna avhandling har två varianter av bakteriearten *Pediococcus acidilactici* studerats. Varianterna kommer från en värdstam som kan växa i termokemiskt i bakterie behandlad majsstubb, vilket visar en god tolerans mot inhibitorer och troligen en god potential för stammen att användas för processer med också andra typer av råvaror t.ex. barrträd och cellulosabaserat textilavfall. Avhandlingsarbetet har visat att bakterien kan omvandla de huvudsakliga sockerarterna i barrträd till mjölksyra med en lämpligt vald process.

Viskosavfall bryts lättare ned till socker än barrträd, men textilier har många tillsatser, inte minst färgämnen, som har visat sig vara giftiga för miljön. Kemikalierna skiljer sig från inhibitorerna i barrträdsprocessen, men studier i denna avhandling visade att bakterierna inte påverkades menligt och resultaten för mjölksyraproduktion var mycket lovande.

Detta arbete är en del av ett större koncept: att kunna använda *Pediococcus acidilactici* för att producera mjölksyra från flera typer av växtbaserade restprodukter, med den (höga) renhet som krävs för att användas för PLA-

produktion i en hållbar process. Om detta lyckas skulle det kunna ersätta den nuvarande användningen av livsmedelsgrödor för att producera dessa material.

Tekniken är långt ifrån färdig, lagstiftningen kan fortfarande förbättras för att stödja produktionen av biobaserade material, och världen måste lära sig att använda naturresurser på ett smart sätt. Men varje steg framåt är ett framsteg! 🍃

Resumo de Divulgação Científica

Esta tese é sobre a produção de ácido lático com bactérias, utilizando lascas de madeira e viscose, um tecido vegetal sintético, como matérias-primas.

O ácido lático é um químico muito versátil utilizado em diversos setores: alimentação, farmacêutica e cosmética, agricultura e outros processos industriais. Em produtos para consumo humano ou animal, as matérias-primas e processos têm de obedecer a normas de qualidade muito restritas. No entanto, desperdícios e resíduos agrícolas podem ser utilizados como matérias-primas na produção de bioplásticos. Um dos exemplos é o poli-ácido lático, *polylactic acid* – PLA, que pode ser usado em aplicações como por exemplo impressão 3D.

Apesar de todos os potenciais benefícios, usar biomassa linhocelulósica é bastante desafiante. Mais ainda no caso da utilização de resíduos de madeira, vindos de serralharias, como é o caso nesta tese. O abeto mais antigo do mundo é Sueco – o velho Tjikko tem mais de 9560 anos, e vive no Parque Nacional Fulufjället, na região de Dalarna. Para conseguirem sobreviver tanto tempo, as árvores precisam de ter uma estrutura complexa e robusta! Portanto, o primeiro passo para usar lascas de madeira é submeter a biomassa a um tratamento físico-químico severo para abrir a estrutura recalcitrante. As altas temperaturas e condições ácidas levam à formação de compostos que podem ser tóxicos para alguns organismos. Para planear um processo para a utilização dos açúcares em madeira macia (abeto), parte do trabalho desta tese consistiu em estudar como é que estes inibidores afetam a produção de ácido lático nos micro-organismos escolhidos.

Nesta tese foi verificado que duas variantes da espécie bacteriana *Pediococcus acidilactici* são capazes de usar os açúcares obtidos a partir de abeto e resíduos de viscose para produzir ácido lático. Estas bactérias foram modificadas a partir de uma bactéria-mãe capaz de crescer em palha de milho pré-tratada, e portanto tolerante a inibidores, e potencial para ser usada noutros meios de cultura igualmente adversos.

Os resíduos de viscose são reduzidos a açúcares mais facilmente do que a madeira, mas os têxteis têm aditivos, como por exemplo corantes, que já foram identificados como tóxicos para o ambiente. Estes químicos e os inibidores do pré-tratamento da madeira são diferentes, mas experiências em laboratório demonstraram que as bactérias não foram severamente afetadas por estes corantes e aditivos, e os resultados da produção de ácido lático foram muito promissores.

Este trabalho faz parte de um conceito maior: utilizar *Pediococcus acidilactici* para produzir ácido lático com vários tipos de resíduos vegetais, com a (elevada) pureza necessária para ser usado na produção de PLA com um processo sustentável. Se isto for bem-sucedido, este processo pode substituir a atual utilização de produtos alimentícios para a produção destes materiais.

A tecnologia ainda não está finalizada, ainda é possível melhorar a legislação para apoiar a produção biológica de plásticos, e o mundo ainda precisa de aprender ser inteligente na utilização dos seus recursos naturais. Ainda assim, todos os resultados contam para o progresso! 🍃

List of Papers

Paper I

Campos, J., Bao, J. & Lidén, G. (2021). Optically pure lactic acid production from softwood-derived mannose by *Pediococcus acidilactici*. *Journal of Biotechnology*, vol. 335, p. 1-8, doi: 10.1016/j.jbiotec.2021.06.007

Paper II

Campos, J., Tejada, L. G., Bao, J. & Lidén, G. (2023). Fed-batch strategies for biodetoxification in production of optically pure lactic acid from softwood hydrolysate using *Pediococcus acidilactici*. *Process Biochemistry*, vol. 125, p. 162-170, doi: 10.1016/j.procbio.2022.12.027

Paper III

Campos, J., Almqvist, H., Bao, J., Wallberg, O. & Lidén, G. (2023). Overcoming extended lag phase on optically pure lactic acid production from pretreated softwood solids. *Frontiers in Bioengineering and Biotechnology*, vol. 11, doi: 10.3389/fbioe.2023.1248441

Paper IV

Campos, J., Bågenholm-Ruuth, E., Sanchis-Sebastiá, M., Bao, J. & Wallberg, O. (2023). Waste viscose for optically pure lactic acid production. *[Manuscript]*

Author's contribution to the papers

Paper I

I planned the study with my co-authors and performed the experimental work and data evaluation. I wrote the manuscript.

Paper II

I planned the study with my co-authors and performed the experimental work and data evaluation. I wrote the manuscript.

Paper III

I planned the study with my co-authors and performed the experimental work and data evaluation. I wrote the manuscript.

Paper IV

I planned the study with my co-authors and performed the experimental work and data evaluation involving microorganisms. I wrote the manuscript with my co-authors.

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During my interview for this PhD position, I was reminded that it would be a very lonely journey. Although it was true for some moments in 2020 and 2021, I am incredibly grateful that I had so many people around me during these four and a half years.

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When I was younger I said I did not want to become a teacher, but life has a way to change your plans. Teaching and mentoring are now a couple of my callings, and I have my students in Biochemical Reaction Engineering to thank for that. As one of those examples, thank you Rishi—you will be great. Together with teaching, I was also a student representative for DOKT, the PhD student union at LTH, at the

Research Board. Thank you to everyone on the board and to my student representative colleagues and friends, Ellen, Henrik, Josef and Ilayda. Also very early in my PhD, I decided that the Renewable Resources and Biorefineries (RRB) Conference was the right place to show the advancement of my work. I thank all the people I met through this conference, especially my mentors from Universidade de Aveiro – obrigada Professora Luísa Serafim e Professora Ana Xavier.

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And lastly, and quoting Calvin Cordozar Broadus Jr., a.k.a. Snoop Dog,

I want to thank me for believing in me.
I want to thank me for doing all this hard work.
I want to thank me for having no days off.
I want to thank me for never quitting.
I want to thank me for always being a giver, and trying to give more than I receive.
I want to thank me for doing more right than wrong.
I want to thank me for just being me at all times.

1 Introduction

1.1 Current state of the transition from fossil-based plastics to more sustainable alternatives

Synthetic plastics were invented in the 19th century primarily as a substitute for animal-derived materials from endangered species, such as ivory from elephants and carapaces from turtles. In the 20th century, they evolved into practically irreplaceable materials, with applications in virtually every area of human life. Petroleum and chemical industries developed processes and produced highly useful and durable materials.

However, neither they nor society considered the consequences of the enormous use of these resilient polymers. The issues that arose included greenhouse gas emissions, the use of finite resources during production and the complicated disposal of these materials, many of which require thousands of years to degrade in a landfill. The 21st century, however, has introduced a more conscientious approach to waste and residues, associated with such buzzwords as reduce, reuse and recycle.

Despite the improvements that have been made, the use of synthetic plastics continues to be a social and political problem that is far from being solved (Science Museum Group UK, 2019). More alternatives must be found to replace these materials while ensuring that such replacement polymers and their production and disposal methods close the natural carbon cycle and do not leave a large environmental imprint. Although it is unrealistic to aim for the total replacement of petroleum-based plastics (Rosenboom et al., 2022), a reduction in their consumption and improvements in their separation for recycling are imperative.

There is no law in the European Union specifically for bio-based, biodegradable and compostable plastics (European Commission, 2022). Yet, policymakers are making strides in this area. For example, the Directorate-General for Environment of the European Commission has recently issued communication on the policy framework for bio-based, biodegradable and compostable plastics. In brief, this document compiles information on the labelling, sourcing, use and disposal of bioplastics (Directorate-General for Environment of the European Commission, 2022). The use of this communiqué as the basis for laws that enforce the application of scientifically accurate and clear information on bio-based, biodegradable and compostable

plastics would promote the incorporation of bioplastics and ensure their proper disposal, contributing to a circular and carbon-neutral economy.

The terms *biodegradable* and *compostable* are sometimes misused, which may impede the application of certain biopolymers in commercial products. Both terms refer to materials than can be degraded by biological activity to carbon dioxide, water, new microbial biomass, minerals and, under anaerobic conditions, methane (Directorate-General for Environment of the European Commission, 2022). These processes occur under controlled environmental conditions (e.g., defined temperature, humidity and active microbial population) or in open, uncontrolled environments.

Many polymers are biodegradable. However, for a product to be certified as *compostable* (EN 13432/14995/17427 standards), it must pass a series of standardised tests under specified conditions and times until it is fully degraded (European Bioplastics e.V., n.d.). Some examples of biodegradable and potentially compostable materials are starch blends, cellulose blends, protein- and lipid-based biopolymers, polylactic acid (PLA), polyhydroxyalkanoate (PHA) and polybutylene succinate (PBS). If they are integrated with the production of other bio-based compounds, these biopolymers can constitute part of a biorefinery portfolio (Kamm and Kamm, 2004; Ali et al., 2023a).

Despite the need for new policies that encourage the use of bioplastics, there is current legislation that supports the use of some biopolymers for specific uses, including Directive (EU) 2019/904 of 5 June 2019 on the reduction of the impact of certain plastic products on the environment. This directive specifically addresses single-use plastics and indicates that they should be produced only from unmodified natural polymers, which excludes bioplastics that are manufactured from non-naturally occurring bio-based raw materials, such as PLA. Although this directive is well intended, it may introduce a bias against the use of bioplastics if they are bio-based but unavailable as such in nature (e.g., PLA). This tendency is unfortunate, because these materials also have the potential to replace fossil-based virgin materials (European Bioplastics e.V., 2023). Studies such as those in this thesis aim to demonstrate the potential of bio-based chemicals and downstream materials as clear substitutes for fossil-based materials.

1.2 Bioplastics in biorefineries

A bio-based polymer, commonly acknowledged as a bioplastic or biopolymer, is a compound that is derived from biomass (living matter) or from monomers that are produced from biomass; bio-based polymers can be processed into finished products by thermal reshaping (thermoplastics) or polymerisation into a tougher desired shape (thermosets) (Vert et al., 2012; Rosenboom et al., 2022). The global market for bioplastics in 2022 was estimated to be 11.6 billion USD, with an expected growth of 18.8% between 2023 and 2030. Bioplastics are mainly applied in packaging (62.0%). However, only 52.0% of the market value of bioplastics corresponds to biodegradable biopolymers (Grand View Research, 2023a).

Figure 1. Scheme of a biorefinery concept in a circular economy. (1) Lignocellulosic biomass is used in construction, manufacturing and consumer goods, generating waste and residues. (2) These compounds and other raw materials are used in physicochemical and biological processes. (3) New bio-based value-added goods are produced, and any side streams proceed through waste management to be recycled, reused or composted. (4) The cycle is closed when recycled, reused and composted materials are used to feed the growth of new biomass or are included in new production processes.

Biorefineries form the core of a circular bio-based economy, aligned with the European Green Deal of the European Commission (European Commission, 2019a;

European Commission et al., 2021). The concept of *biorefinery* emerged from the goal of replacing petroleum-based processes and products with plant-based counterparts. To shift to a plant-based economy and products in a timely manner, synergies must be created between existing disciplines, such as biology, physics, chemistry and other technical fields, and integrated into a sustainable network of processes that transform biological raw materials into a wide range of products (Kamm and Kamm, 2004). Moreover, a biorefinery should incorporate communication and social principles that potentiate the growth and information of the society in which it is placed, therefore directly influencing the success of the transition from petroleum-based to bio-based processes and products. The biorefinery concept was referenced in the 1990s (Kamm et al., 1998), but a more recent definition by the International Energy Agency (IEA) Bioenergy Task 42 (de Jong et al., 2020) states that:

> *Biorefinery is the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat).*

Examples of bio-based products that can be integrated into a biorefinery concept, as illustrated in Figure 1, include lactic acid and its polymeric version, PLA. To highlight the importance of the production of lactic acid and PLA in Europe, including its drivers and barriers, an entire chapter of a recent independent expert report for the European Union has been dedicated to this building block and respective biopolymer (European Commission et al., 2021).

One of the barriers that was signalled by this report is the need for information on the composition and utilisation of agricultural residues, wastes and by-products for combined and predictable lactic acid production. This thesis targets some of the gaps in this subject, especially regarding the methods for and results on optically pure lactic acid production with residual softwood and waste cellulosic fibres and the characterisation of the feedstocks.

1.3 PLA: applications, demand and opportunities

PLA is a thermoplastic aliphatic polyester that is synthesised from the esterification of lactic acid monomers (Drumright et al., 2000; Casalini et al., 2019; Yu et al., 2023). It was discovered in 1932 by Carothers, Dorough and Natta, when Carothers was the director of research at DuPont (Carothers et al., 1932).

PLA is a bio-based, biodegradable, industrially compostable and biocompatible polymer (Figure 2) that has the potential to replace some petroleum-based plastics. The possibility of forming PLA co-polymers and blends with other biopolymers allows various materials with a wide range of physical properties (e.g., melting temperature, density, tensile strength, elastic modulus, elongation) to be created (Clarinval and Halleux, 2005; Freeland et al., 2022). As a result, PLA can supplant the petroleum-based plastics, such as polyethylene (PE), polystyrene (PS) and polyethylene terephthalate (PET), in, for example, plastic containers, packaging material, coatings and fibres (Jamshidian et al., 2010; Augustiniene et al., 2022; Costa et al., 2023).

PLA is used primarily in packaging, with growing markets in fibres, biomedicine, cosmetics and the automotive industry (Oliveira et al., 2022; Costa et al., 2023; Grand View Research, 2023c). Total Corbion PLA (NL) surpassed Nature Works LLC (USA) as the leading producer of PLA and lactide, with its plant in Thailand producing 75 000 tons PLA/year; it plans to build a second plant in France (Oliveira et al., 2022; TotalEnergies Corbion, 2023).

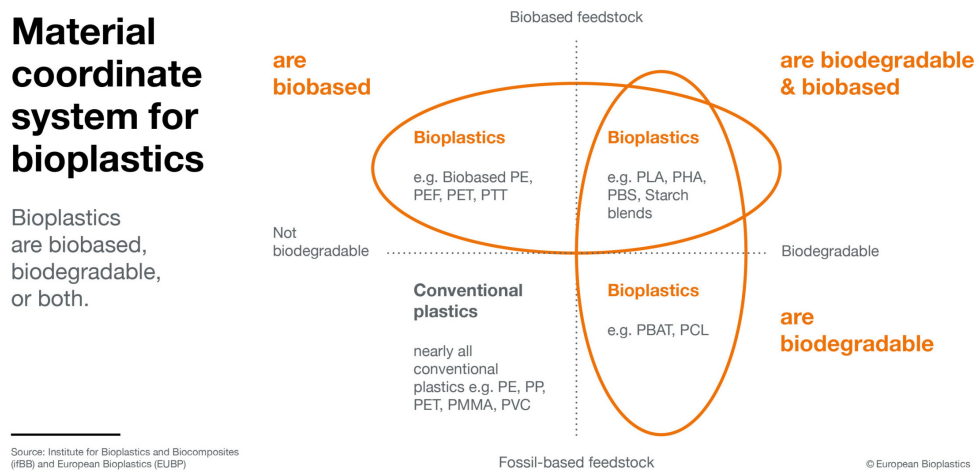

Figure 2. Coordinate system for the classification of bioplastics, according to their biodegradability and production feedstock (European Bioplastics e.V., 2020).

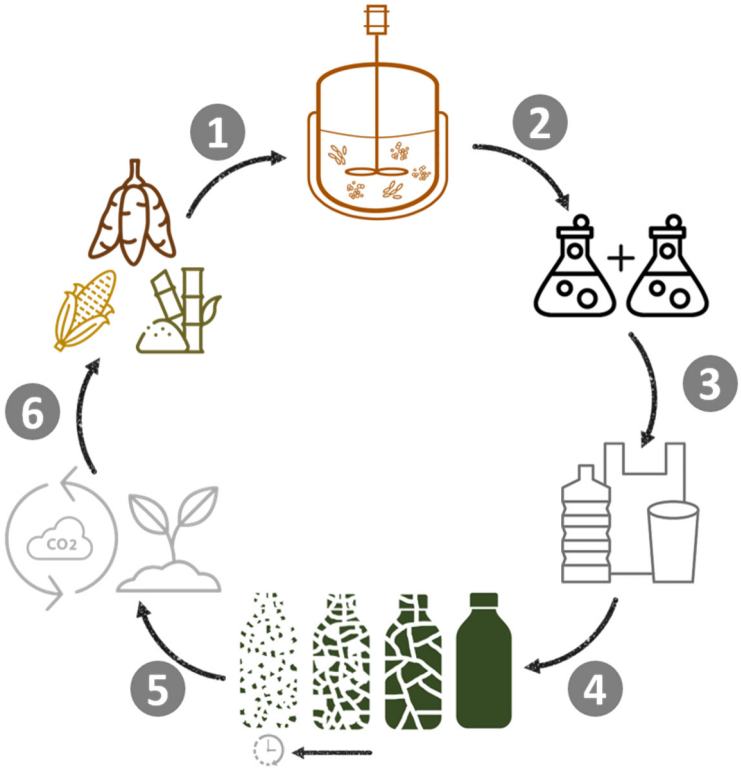

Figure 3. Schematic of the life cycle of PLA. (1) Bio-based raw materials used as carbon source for microorganisms in a lactic acid bioprocess. (2) After purification and extraction, PLA is produced by chemical synthesis and (3) moulded into materials and objects for several applications. (4) Industrial composting can then be applied to decompose PLA into carbon and minerals for integration in (5) the carbon cycle and (6) new feedstock production.

The life cycle of PLA (Figure 3) starts with the production of monomers of it from starch-based or sugar-containing biomass through fermentation. There are several alternatives for their subsequent polymerisation, the choice of which ultimately depends on the characteristics, disposal and reuse of the intended final product. Regardless, lactic acid is first polymerised through polycondensation to low-quality PLA, with a broad range of low molecular weights (1000-5000 Da) and low polydispersity. This low-quality PLA is then depolymerised into lactide, a cyclic ester that is formed from two lactic acid molecules. Through several possible ring-opening polymerisation methods, lactide molecules can then be coupled to generate high-quality PLA. There are two enantiomers of lactic acid, resulting in three possible variants of PLA: amorphous, which is obtained from a racemic mixture of lactic acid enantiomers, and crystalline alternatives in which the PLA is composed of only one type of enantiomer, poly-D-lactic acid (PDLA) or poly-L-lactic acid (PLLA). PDLA and PLLA have higher crystallinities and melting points compared with the racemic polymer (Van Wouwe et al., 2016; Casalini et al., 2019; Oliveira

et al., 2022; Ali et al., 2023a; Taib et al., 2023). These optically active polymers can then be co-polymerised into tailored stereocomplexes to overcome some of the less desirable characteristics of a racemic polymer, such as its (low) crystallinity—property which influences a number of other features such as the hardness, tensile strength, stiffness and melting point of any polymer (Tsuji, 2005; Tsuji et al., 2021; Costa et al., 2023; Yu et al., 2023). Alternatively, PLA composites with several structural modifications (e.g., co-polymerisation and blending with other biopolymers) are being examined to design biopolymers with physical properties that are more comparable with those of petroleum-based plastics, as discussed (Jacobsen and Fritz, 1996; Murariu and Dubois, 2016; Saini et al., 2016; Castillejos et al., 2018; Khosravi et al., 2020; Ali et al., 2023b; Yu et al., 2023).

PLA is one of the most highly produced bio-based polymers, constituting 20.7% of global bioplastics production in 2022 (European Bioplastics e.V., 2022). The estimated PLA market was 625 million USD in 2022, with expected growth of 21.4% until 2030 (Grand View Research, 2023c). As introduced, the potential of bio-based bioplastics, such as PLA, can be enhanced with supportive policies that include, for example, production specifications, collection and composting strategies and targets for replacing conventional plastics (European Commission, 2022; Rosenboom et al., 2022; Ali et al., 2023a).

In parallel with legislation, the production capacity for lactic acid and lactide should be increased (European Commission et al., 2021; Oliveira et al., 2022). Two such strategies to sustainably expand lactic acid production are the utilisation of alternative non-food feedstocks and the engineering of highly productive and robust genetically modified organisms (Upadhyaya et al., 2014; Ali et al., 2023a). These issues are addressed in this thesis, which assessed two bacterial strains, modified from the same parent strain to produce L- or D-lactic acid, with regard to their ability to convert softwood and waste viscose in a fermentation process.

1.4 Lactic acid as a platform chemical

Lactic acid (2-hydroxypropionic acid) was discovered in 1780 by Karl Wilhelm Scheele (1742–1786), a German-Swedish chemist who, during his early career, worked in Malmö and established ties with academia through a chemistry professor at Lund University, Anders Jahan Retzius (Carl Wilhelm Scheele | Biography, Discoveries, & Facts, 2023). During the 19th century, Gay-Lussac and Pelouze examined and analysed lactic acid production to produce lactic acid in sufficient amounts to further their research. Consequently, Pelouze asked one of his students, Frémy, to study a new method for synthesising lactic acid from sugar—i.e., fermentation. Frémy and his father-in-law, Boutron, were the first to establish a method for lactic acid fermentation, extraction and purification on a larger scale than it was possible with a laboratory setup. This advance led to the rise of its industrial production, and its use as a preservative and flavour enhancer (Benninga, 1990).

Although lactic acid can be produced through a chemical route, this approach is neither economically nor technically preferable, and it is limited to generating a racemic mixture of both lactic acid enantiomers (Juodeikiene et al., 2015). Thus, at the start of the 21st century, 90% of the lactic acid worldwide was produced through fermentation. This rate is likely to increase further, given the advantages of this bioprocess (Hofvendahl and Hahn-Hägerdal, 2000).

The global market for lactic acid in 2022 was estimated to be 3.1 billion USD and is predicted to grow by 8.0% until 2030 (Grand View Research, 2023b). This organic acid is used widely in the food, cosmetics, pharmaceutical, medical and chemical industries, experiencing its most recent upsurge in the production of PLA, as discussed (Ojo and de Smidt, 2023). This use and other applications are shown in Figure 4.

Of the two enantiomers, L-lactic acid is preferred for biomedical and food applications, because in contrast to D-lactic acid, it can be metabolised by animal cells—enzymes that metabolise D-lactic acid are not part of the machinery of animal cells, and the accumulation of D-lactic acid might cause acidosis in humans (Hudson et al., 1990; Pohanka, 2020). Certain bacteria and fungi, however, can produce and metabolise D-lactic acid and are thus potentially compelling L- and D-lactic acid producers (Abdel-Rahman et al., 2013; Abedi and Hashemi, 2020).

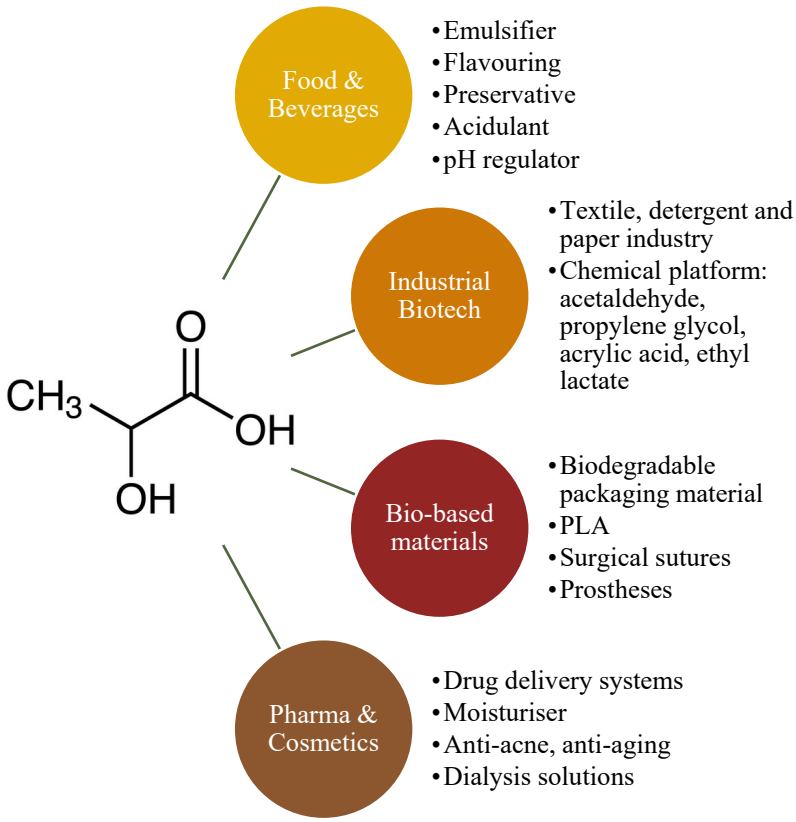

Figure 4. Applications of lactic acid (Adapted from Ojo & de Smidt, 2023).

Two challenges in lactic acid bioproduction are the acidic character of lactic acid in aqueous solutions and feedstock utilization. Acid production can effect product inhibition and high osmotic pressure due to the addition of base to control pH (Gonçalves et al., 1997; Boonmee et al., 2016; He et al., 2021). Further, the costs for feedstock can amount to 70% of total production outlays for the fermentation process (Tejayadi and Cheryan, 1995; Oliveira et al., 2018), most of which is spent on the glucose and starch-based materials that serve as the carbon source (Abdel-Rahman et al., 2011).

Lignocellulosic biomass waste has been identified as a widely available and potentially less expensive alternative raw material that can lower lactic acid production costs, consequently increasing its demand (Augustiniene et al., 2022). This constitutes a good opportunity to integrate lactic acid as a product in a biomass-based biorefinery. However, the utilisation of lignocellulose materials or waste materials is associated with many challenges.

1.5 Scope and outline of this thesis

The production of lactic acid by fermentation has been extensively explored. This thesis concerns a small but important subfield: the use of softwood and waste viscose for producing optically pure lactic acid. Two genetically modified strains, *Pediococcus acidilactici* TY112 and *Pediococcus acidilactici* ZP26, were used in this work to study and report the:

- Metabolism of mannose and glucose that were derived from pretreated softwood hydrolysate for L- and D-lactic acid production
- Inhibition of *P. acidilactici* TY112 by compounds that formed during softwood pretreatment and potential strategies to overcome it
- Elimination of a long lag phase that was caused by the addition of pretreated softwood solids to simultaneous saccharification and fermentation (SSF) cultivations of *P. acidilactici* ZP26
- D-lactic acid production from post-consumer white and coloured waste viscose

Paper I examined the consumption of mannose, which is more prevalent in softwood than in other lignocellulosic materials, by both strains. Also, with regard to the utilisation of the hemicellulose-rich liquid fraction, batch and fed-batch cultivations were established to study hexose consumption and lactic acid production by *P. acidilactici* TY112 in a medium with the inhibitors that are typically in pretreated biomass (e.g., HMF and furfural). Cultivations were performed with synthetic media and the liquid fraction from pretreated softwood to observe possible matrix effects; the conclusions were published in **Paper II**.

An extensive study was performed and documented in **Paper III**, which suggested an approach for overcoming an unexpectedly long lag phase in the SSF bioreactors. Although more studies are necessary to fully understand why the presence of pretreated softwood solids delays the start in production, several hypotheses were presented, as was a method to circumvent this delay.

A novel alternative substrate, waste viscose, was tested regarding the production of optically pure lactic acid in *P. acidilactici* ZP26. Waste viscose from post-consumer textiles is a rich source of cellulosic glucose. However, solutions that are based on the hydrolysis of this material retain soluble additives and dyes that might inhibit microorganisms and harm the environment, as signalled by environmental agencies and policymakers. The methods and results are discussed in this thesis and **Paper IV**.

2 Background: Lactic Acid-Producing Organisms and Carbon Sources

This chapter provides background information on the work that was developed in this thesis. After the introduction of information on microbial production of lactic acid, several examples of lactic acid organisms that have been used in recent studies with lignocellulosic biomass are presented in this chapter. The microorganisms and feedstocks that were used in the current work are also detailed, with information on strain improvements and the preparation of carbon sources.

As discussed, although there are chemical routes for the production of lactic acid, microbial fermentation is clearly the best means of obtaining optically pure lactic acid (Juodeikiene et al., 2015). Metabolically, microbial lactate is formed from pyruvate, following glycolysis. The reduction of pyruvate to lactate is reversible, but lactate dehydrogenase has a higher affinity for pyruvate. Moreover, lactic acid bacteria harbour NAD-dependent lactate dehydrogenases that catalyse the reduction of pyruvate to lactate, important to regenerate NAD^+ from the NADH formed during glycolysis (Takenaka and Schwert, 1956; Holbrook and Gutfreund, 1973; Garvie, 1980). *In vivo*, pyruvate is thus converted efficiently to lactate.

Genetic engineering has been used to improve several steps of the lactic acid production process (Upadhyaya et al., 2014; Qiu et al., 2017, 2017, 2020a, 2020b; Yankov, 2022). In particular, inactivating one of the lactate dehydrogenases (L-lactate dehydrogenase [EC 1.1.1.27] or D-lactate dehydrogenase [EC 1.1.1.28]) is useful to generate only one of the enantiomers (Figure 5) per microorganism used. Wild-type and genetically modified organisms have been used to produce a specific enantiomer. However, there is one case in which two strains were engineered from the same parental organism to synthesise each of the enantiomers separately. Thus, similar cultivation conditions can be applied for the independent production of each enantiomer, which is beneficial for process scale-up. In the next section, this advance and other developments in these engineered strains are described.

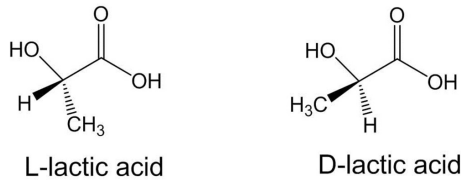

Figure 5. Lactic acid enantiomers (Casalini et al., 2019)

2.1 Lactic acid from lignocellulosic biomass

As addressed in Chapter 1, lactic acid can be integrated with other value-added products in a biorefinery. The production of lactic acid from lignocellulosic biomass entails several steps: pretreatment of the raw material, enzymatic hydrolysis of cellulose and hemicellulose, fermentation of sugars into lactic acid, and product separation and purification.

Pretreatment is essential for rendering cellulosic and hemicellulosic sugars accessible for subsequent steps in the process. Pretreatment methods can be biological, physical, chemical and physicochemical, and often more than one method is applied to increase the accessibility of cellulose to enzymes, maximising sugar concentrations (Galbe and Zacchi, 2012).

Enzymatic hydrolysis is the preferred method for depolymerising cellulose and hemicellulose into single sugars that can be metabolised by microorganisms. The efficiency of enzymatic hydrolysis is influenced by several factors, such as the crystallinity of cellulose, lignin content, sugar concentration and the presence of inhibitory compounds that bind to or alter the active sites of enzymes (Kang et al., 2013; Zhai et al., 2018; Chu et al., 2021; Huang et al., 2022). Enzyme blends also constitute a significant percentage of the cost that is associated with the bioprocess. Although enzyme recycling has been examined to render the process more sustainable, the characterisation and pretreatment of biomass are important for optimising the entire procedure (Laca et al., 2019).

Fermentation of lignocellulosic sugars into lactic acid can be achieved by several types of microorganisms, under controlled conditions and in nature. The fermentation process can be combined with enzymatic hydrolysis, for example, to limit inhibition by high sugar concentrations in the broth. This practice is termed simultaneous saccharification and fermentation (SSF), in contrast to separate hydrolysis and fermentation (SHF). The main disadvantage of SSF is that the optimal conditions differ between enzymatic hydrolysis and fermentation (i.e., with regard to pH and temperature), complicating the optimisation of both processes. However, it has several advantages over SHF, such as its faster processing time, lower enzyme loadings and reduced inhibition of enzymatic hydrolysis due to the

accumulation of the products obtained in this step (Abdel-Rahman et al., 2011). Nevertheless, each approach has distinct challenges, prompting more studies to resort to hybrid processes that combine SHF and SSF (Yi et al., 2016; Cassells et al., 2017).

Product separation and purification is an essential part of a biorefinery and is affected by the processes above and the desired application of the product(s). Separation and purification techniques can include several steps of chemical reaction, precipitation, filtration, chromatography and distillation (Abdel-Rahman et al., 2011; Din et al., 2021; He et al., 2022; Kim et al., 2022).

Strategies for addressing the challenges using softwood—a recalcitrant lignocellulosic resource—for producing lactic acid will be discussed throughout this thesis.

2.2 Lactic acid-producing organisms

Several organisms can synthesise lactic acid, of which lactic acid bacteria are the best wild-type producers, because they cannot produce ATP through respiration and thus must rely on lactic acid metabolism to generate energy carriers for their needs (Augustiniene et al., 2022). Some examples include organisms in the genera *Lactobacillus*, *Lactococcus*, *Corynebacterium* and *Pediococcus* (Carr et al., 2002; Abedi and Hashemi, 2020).

Generally, lactic acid bacteria are facultative anaerobes, non-motile and do not form spores. Their optimal growth temperature and pH vary between 20°C and 45°C and 5.5 and 6.5, respectively, and they require complex media, such as extracts containing amino acids and vitamins, which increases production costs. Lactic acid bacteria can be classified as homofermentative if they produce only lactic acid or heterofermentative if other products are synthesised (e.g., acetic acid and ethanol) (Kandler, 1983). Homofermentative bacteria have the highest theoretical lactic acid yield from sugar, producing 2 moles of lactic acid per 1 mole of converted glucose (or 1 gram of lactic acid per gram of glucose). The actual yield is lower because some of the carbon is used for cell growth.

Genetic engineering has enabled researchers to modify lactic acid bacteria to improve their thermotolerance, osmotolerance and resistance to inhibitory compounds. It has also allowed model organisms that do not synthesise lactic acid naturally, such as *Saccharomyces cerevisiae*, *Escherichia coli* and *Candida* spp., to be used as industrial producers (Grabar et al., 2006; Liu et al., 2022). Table 1 lists some microorganisms that have been used to produce lactic acid with various raw materials and cultivation conditions, in efforts to solve the challenges of using lignocellulosic biomass as substrate.

Table 1. Compilation of studies on lactic acid production with various organisms, carbon sources and process operation modes. [1]Specifications on concentrations and yields are detailed in the respective reference. [2]No data/not determined.

Organism	Optical purity	Carbon source	Mode of operation	Concentration (g/L)[1]	Yield[1]	Improvement	Reference
Bacillus coagulans	L(+) (99%)	Pasta waste	SHF	47.7	0.81	Demonstrated on pilot scale	(López-Gómez et al., 2022)
Bacillus coagulans	L(+) (99.8%)	Coffee mucilage	Batch	45.3	0.77	Alternative raw material, and demonstrated on pilot scale	(Neu et al., 2016)
Bacillus coagulans	ND[2]	Sugar beet pulp	Continuous	36.92	0.71	Biorefinery process simulation	(Oliveira et al., 2020b)
Bacillus coagulans	L(+) (>99.4%)	Sugarcane bagasse hydrolysate	Batch	56.0	0.87	Capable of biodetoxification of HMF and furfural	(Oliveira et al., 2019)
Bacillus coagulans	L(+) (99%)	Rye straw	SSF	18.3	0.97	Complete utilisation of lignocellulosic biomass	(Schroedter et al., 2021)
Bacillus coagulans	L(+) (ND)	Red macroalgae cellulosic residue	SSF	ND	0.41	Third-generation biorefinery concept	(Wong et al., 2022)
***Bacillus coagulans* LA-15-2**	L(+) (ND)	White rice bran and corn steep liquor	SSF	117	0.99	Unsterilised SSF	(Wang et al., 2015)
***Bacillus coagulans* L-LA 1507**	L(+) (ND)	Corn stover	Fed-batch SSF	78.0 – 97.5	0.33 – 0.41	Thermotolerant strain (50°C), fed-batch to optimise cellulase utilisation	(Chen et al., 2020b)
***Enterococcus faecalis* SI**	L(+) (>99%)	Waste plywood chips	SHF	59.8 – 102.4	0.95 – 1.04	High solid loading of pretreated plywood on pilot scale	(Yuan et al., 2018)
***Enterococcus hirae* BoM 1-2**	L(+) (99.7%)	Glucose	Fed-batch	109.9	0.96	Alkaliphilic (pH 9.0)	(Abdel-Rahman et al., 2019)
***Klebsiella oxytoca* KMS004**	D(-) (ND)	Cassava starch	Fed-batch	133	0.98	Strain engineering for improved production	(In et al., 2020)
Lactobacillus brevis	ND	Cottonseed cake/ wheat straw/ sugarcane bagasse	SSF	ND	0.22/ 0.49/ 0.52	Pretreatment with ionic liquids, immobilised cellulase	(Grewal and Khare, 2018)
***Lactobacillus casei* Shirota**	ND	Food waste	Batch	82.6 – 94.0	0.92 – 0.94	Use of different types of alternative raw materials	(Kwan et al., 2016)
Lactobacillus delbrueckii	D(-) (97.66%)	Molasses	Fed-batch	162	0.81	Substrate does not require pretreatment	(Beitel et al., 2020)
Lactobacillus delbrueckii	D(-) (99.5%)	Rice straw	Continuous	46.6	0.92	Vitamin B supplementation and membrane-integrated process	(Ma et al., 2022)

Lactobacillus delbrueckii* spp. *delbrueckii	D(-) (ND)	Orange peel waste and corn steep liquor	Batch	5	0.88	Alternative raw material	(Torre et al., 2018)
Lactobacillus delbrueckii* spp. *bulgaricus	D(-) (>90%)	Beechwood	SSF	62	0.69	Utilisation of recalcitrant forest biomass	(Karnaouri et al., 2020)
Lactobacillus plantarum	ND	Rice straw	SSF	65.6	0.69	Inhibitor-resistant new isolate	(Tu et al., 2019)
***Lactobacillus plantarum* NCIMB 8826**	D(-) (99.6%)	Brown rice	SSF	117.1	0.93	Without nutrient supplementation	(Okano et al., 2017)
Lactobacillus rhamnosus	L(+) (98%)	Mixture of lignocellulosic biomass	SSF	61.7	0.97	Multi-supply biorefinery concept	(Pontes et al., 2021)
Lactobacillus rhamnosus	ND	Softwood hydrolysate and paper mill sludge	SSF	60	0.83	Integration of lactic acid production in a paper mill	(Shi et al., 2015)
***Leuconostoc* sp. (A250 isolate)**	D(-) (>99%)	Nanofiltered sugar beet pulp	Batch	21.7 – 40.0	0.45 – 0.78	Novel isolate (wild-type) grown in nanofiltered medium	(Alexandri et al., 2022)
Mixed culture (i.e., *Aerococcus* sp., *Enterococcus* sp. and *Trichococcus* sp.)	L(+) (100%)	Food waste	Batch	12.05	0.48	Sewage sludge supplement and intermittent alkaline fermentation	(Li et al., 2015)
Mixed culture of *Bacillus coagulans* and *Lactobacillus rhamnosus*	ND	Cassava bagasse	SSF	112.5	0.88	Mixed culture used for co-consumption of hexoses and pentoses	(Chen et al., 2020a)
***Pediococcus acidilactici* KTU05-7**	L(+) (>90%)	Agro-industrial wastes	Batch	11.3 – 93.0	0.33 – 1.45	Screening of several organisms and raw materials	(Juodeikiene et al., 2016)
Pediococcus pentosaceus	L(+) (66%)	Rice husk residue	Fed-batch	29.8 – 46.2	0.82 – 0.99	Novel pretreatment to avoid hydrolysis and detoxification	(Liu et al., 2021)
***Saccharomyces cerevisiae* BTCC3**	L(+) (ND)	Sugarcane bagasse	Batch	25.3 – 53.7	0.24 – 0.51	Non-detoxified substrate, osmotolerant organism	(Pangestu et al., 2022)
Sporolactobacillus inulinus	D(-) (>90%)	Glucose	Fed-batch	222	0.87	Osmotolerant organism	(Klotz et al., 2017)
***Sporolactobacillus inulinus* NBRC 13595**	D(-) (>98%)	Palmyra palm jaggery and whey protein hydrolysate	Batch	189	0.94	Alternative raw materials	(Tadi et al., 2017)
***Streptococcus* sp.**	ND	Food waste	SHF	66.5	0.33	Integrated production of lactic acid and biogas	(Demichelis et al., 2017)
***Thermoanaerobacterium* sp.**	L(+) (ND)	Food waste	Batch	78.4	0.85	Unsterilised SSF	(Yang et al., 2015)
***Trichoderma viride* R16**	ND	Corncob	Fed-batch SSF	30.3 – 65.0	0.37 – 0.54	Organism capable of degrading phenolic inhibitors	(Liu et al., 2020)

The information in Table 1 and other reviews (Alexandri et al., 2019; Augustiniene et al., 2022; Yankov, 2022) confirms that much effort has been devoted to studying the production of lactic acid in a second-generation biorefinery (non-edible biomass).

Based on a literature review and our understanding of the challenges of producing lactic acid in biorefineries, especially PLA, there are many areas that merit further study:

- utilisation of microorganisms that can be used under non-sterile conditions, such as bacteriocin producers and extremophiles
- the test of blends of feedstocks to evaluate the flexibility of a process, rendering it more sustainable
- integration of lactic acid and PLA production with other processes for full utilisation of biomass
- development of prediction models for scaled-up processes
- collaborations with lactic acid production companies and the promotion of more pilot studies
- sustainability studies and techno-economical and life-cycle analyses (LCAs) to encourage the production of lactic acid and PLA from second-generation feedstocks

The lactic acid bacteria in this thesis have previously been grown in non-sterile processes with various types of lignocellulosic biomass, addressing some of the endeavours above. Their development with regard to the production of optically pure lactic acid and, consequently, PLA is presented in the next section.

2.2.1 Two modified strains of *Pediococcus acidilactici*

A research group at the State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology (Shanghai), has been engineering *Pediococcus acidilactici* to produce optically pure lactic acid from lignocellulosic biomass. *P. acidilactici* is a Gram-positive and catalase-negative coccus, non-sporulating, facultative anaerobic and homofermentative organism that synthesises a mixture of D- and L-lactic acid (Wilhelm H. Holzapfel et al., 2006). Figure 6 shows improvements that have been made to it since the isolation of the strain *P. acidilactici* DQ2 from corn stover hydrolysate (Zhao et al., 2013).

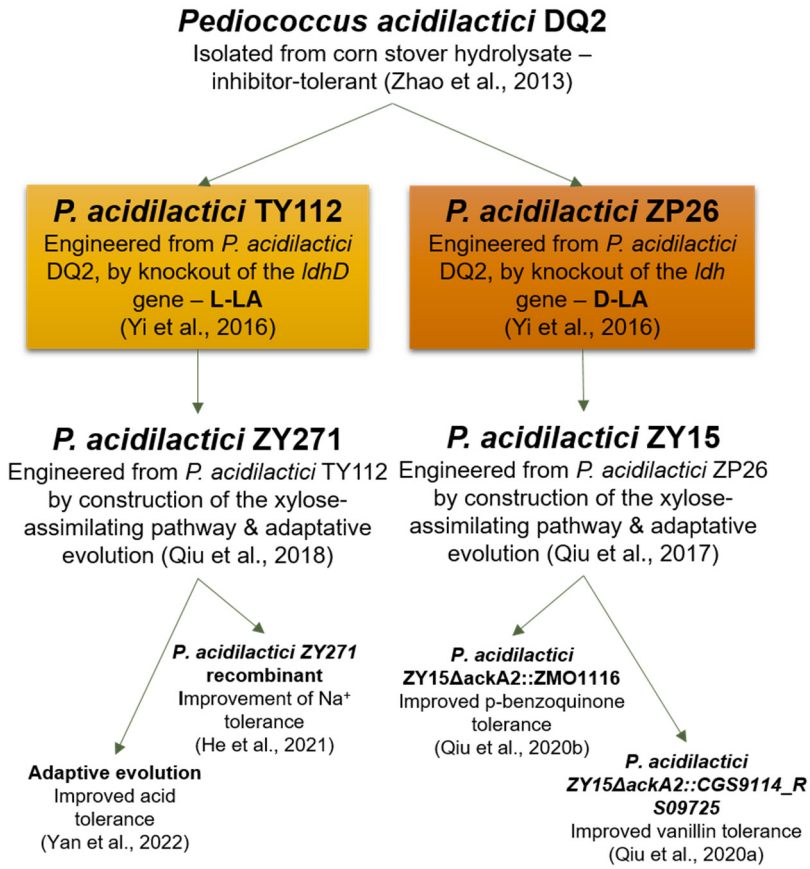

Figure 6. Pediococcus acidilactici strains that have been isolated and engineered by the Bao research group at State Key Laboratory of Bioreactor Engineering, ECUST (Shanghai, China)

Compared with other *Pediococcus* species, this isolate has better resistance to common inhibitory compounds that are formed during the pretreatment of lignocellulosic biomass (Liu et al., 2013). It also produces high concentrations of lactic acid from lignocellulosic biomass (Dao et al., 2013; Zhao et al., 2013). The two strains that were used in this thesis were constructed from this isolate: *P. acidilactici* TY112, a L-lactic acid producer, was obtained by knockout of the gene that encodes for D-lactate dehydrogenase, whereas knockout of the L-lactate dehydrogenase gene resulted in *P. acidilactici* ZP26, a D-lactic acid producer (Yi et al., 2016). Optical purity can be assessed using several techniques, including enzymatic tests and liquid chromatography with a chiral column, as presented in Figure 7. The two strains above have been used in several studies (Li et al., 2015; Qureshi et al., 2017; Han et al., 2021) and were the strains studied in this thesis.

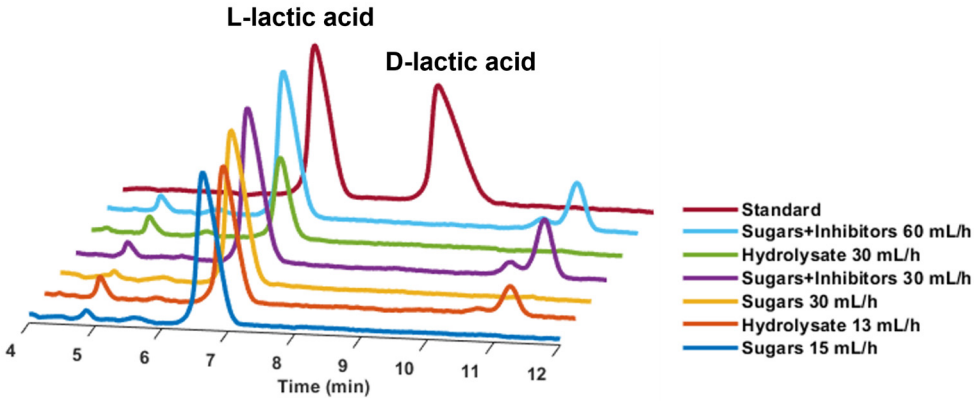

Figure 7. HPLC analysis with a chiral column of D- and L-lactic acid monomers from fed-batch bioreactor cultivations with synthetic sugars and softwood hydrolysate, at various feeding rates (**Paper II**).

The conversion of xylose was not a central question in the current study. However, a metabolic pathway for xylose consumption has been included and enhanced in *P. acidilactici* TY112 and ZP26 by adaptive evolution, generating the strains ZY271 (Qiu et al., 2018) and ZY15 (Qiu et al., 2017), respectively. Other improvements have been made to these strains (Figure 6) to increase their osmotolerance (He et al., 2021) and their resistance to process inhibitors, such as its own product—lactic acid (Yan et al., 2022), and inhibitors in the feedstock, including vanillin (Qiu et al., 2020a) and p-benzoquinone (Qiu et al., 2020b). Industrial processes pose other challenges that have been examined in recent studies, such as pH control (Wei et al., 2018), supplementation with nutrients (Han et al., 2019; Zhang et al., 2022b), resistance to contamination (Qureshi et al., 2017) and mode of operation (Zhang et al., 2022a). Notably, these strains were used in the production of optically pure lactide (He et al., 2022, 2023).

Thorough and detailed projects, like those that have been undertaken for these strains, are essential for developing industrial PLA production processes. Understanding the utilisation of different sources of lignocellulosic biomass sugars will encourage their use in biorefineries. The present thesis supplemented the knowledge acquired in the studies presented in Figure 6 by examining challenges that are encountered in the utilisation of softwood (**Paper I**, **Paper II** and **Paper III**) and waste viscose (**Paper IV**) as carbon sources for *P. acidilactici* TY112 and *P. acidilactici* ZP26.

2.3 Softwood as a raw material

Lactic acid is, today, produced mainly using sugar-rich edible crops (first-generation feedstocks), such as corn and sugarcane. The transition to the use of non-edible biomass (second-generation), such as agricultural and forestry residues, will accelerate the scale-up and sustainability of the production of PLA and other biopolymers (Balakrishnan et al., 2020).

Lignocellulosic biomass from the forest industry is an abundant and economically valuable resource, especially in Nordic countries. The Nordic Forest Statistics report has stated that, in 2020, Sweden and Finland had the largest forest areas among all countries in Europe, with over two-thirds of their land covered by woods, in which softwoods, such as pine and spruce, account for 78% of the growing stock (Ekström and Hannerz, 2021). Softwood is one of the most recalcitrant types of lignocellulosic biomass and thus is not usually the first choice as a raw material for bioprocesses. Nonetheless, after wood is collected from forests for its primary applications (e.g., construction, furniture, pulp and paper), new valorisation strategies should be implemented to treat branches, bark, wood chips and other unused parts of the tree.

2.3.1 Composition

Spruce is composed of glucan (42% to 50%), other carbohydrates (17% to 26%), lignin (27% to 35%), extractives (>7%) and ashes (>1%) (Söderström et al., 2002; Frankó et al., 2015, 2019; Wang et al., 2018). The carbohydrate fraction is divided between cellulose and hemicellulose, intertwined with lignin (Figure 8).

Cellulose is a linear polymer of repeating D-glucose units that are linked by β-(1→4)-glycosidic bonds. Hemicellulose, in contrast, is a branched heteropolymer that comprises hexoses, pentoses and uronic acids, linked by various types of α- and β-(1→2, 1→3, 1→4, 1→6) bonds. Mannose is the most predominant sugar in softwood hemicellulose and thus must be converted completely if softwood is used as a feedstock for bioprocesses. Mannose consumption by two *P. acidilactici* strains (TY112 and ZP26) was the subject of **Paper I**. The third major macromolecule, lignin, is a complex compound, the composition of which varies by plant or tree species. In softwood, it is formed primarily from coniferyl alcohol (G unit) with low amounts of p-coumarin alcohol (H unit) and sinapyl alcohol (S unit) (Gellerstedt and Henriksson, 2008). Although it was not studied in this thesis, lignin has large commercial potential in such applications as fuels, syngas, macromolecules, phenols, oxidised products and hydrocarbons (Holladay et al., 2007).

In lesser amounts, certain compounds are analysed as ashes and extractives. Extractives are non-structural components of wood that give it its colour, scent and durability. Categories of extractives include fats, fatty acids, fatty alcohols, phenols, terpenes, steroids, resins and waxes (Rowell, 2005).

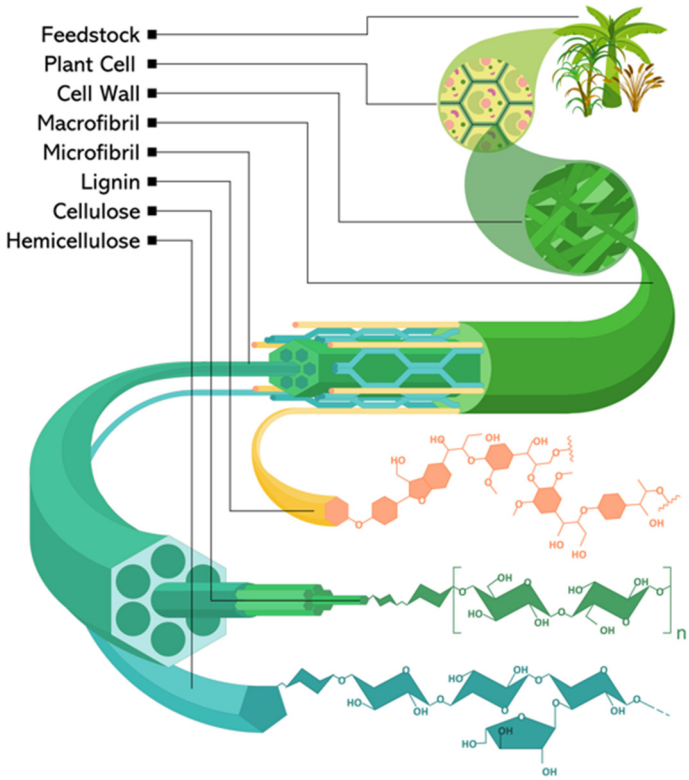

Figure 8. Schematic of the structure of lignocellulosic biomass (Magalhães Jr. et al., 2019).

2.3.2 Pretreatment

Lignocellulosic biomass from wood comprises a tight weave of cellulose, hemicellulose and lignin, which gives plants their robust structure. However, to be able to use the sugars in cellulose and hemicellulose, this structure must be opened, and the polymers must be depolymerised. One of the first steps in the utilisation of lignocellulosic material is the pretreatment, the primary purpose of which is to open the structure and facilitate subsequent depolymerisation. There is no universal pretreatment method, and several options exist, the selection of which depends on the type of material that is depolymerised, the desired products and the technologies that are available. In **Papers I to III**, SO_2-catalysed steam explosion (STEX) was chosen as the pretreatment due to its efficacy on softwood (Palmqvist et al., 1996; Tengborg et al., 1998; Frankó et al., 2015, 2019). Dilute-acid pretreatments, such as SO_2-catalysed pretreatment, primarily effect hydrolytic cleavage of hemicellulose (Galbe and Wallberg, 2019), leading to the solubilisation of hemicellulosic sugars in the liquid fraction, and a solid fraction that is composed of a more porous cellulose-lignin structure, typically coupled with some remaining hemicellulose.

During the pretreatment step, compounds that are toxic to microorganisms are also formed. Carboxylic acids, furans and phenolic compounds are generated by the degradation of cellulose, hemicellulose and lignin under the harsh conditions that are needed to open the wooden structure—in this case, an acidic environment, high temperature and high pressure. One of the most prominent acids that form is acetic acid, from the acetyl groups in hemicellulose (Galbe and Wallberg, 2019). HMF originates from the degradation of hexoses and can decompose into levulinic and formic acids. Furfural is formed from pentoses and is thus related to the degradation of hemicellulose. Phenolic compounds emerge from degradation of the complex lignin structure; although their levels are lower than those above, they are the most inhibitory toward *P. acidilactici* (Qiu et al., 2020a, 2020b). Because it is difficult to remove inhibitory compounds, the microorganism in use must be able to tolerate—or convert—them, as addressed in **Paper II**.

2.3.3 Saccharification

Enzymatic hydrolysis is typically applied to physicochemically pretreated material to generate monomeric sugars for consumption by microorganisms. Given the diversity of polymers and chemical bonds between them, enzyme blends are usually preferred, with cellulases and hemicellulases adapted to the composition of the lignocellulosic biomass yielding better results (Laca et al., 2019). Lytic polysaccharide monooxygenases (LPMOs), a class of proteins that were recently identified to enhance the decomposition of polysaccharides, have emerged as a significant advance in the industrial saccharification of lignocellulosic biomass and can be added to enzyme blends (Vaaje-Kolstad et al., 2010; Moon et al., 2022; Caputo et al., 2023). Despite the work of the scientific community and established firms, such as Novozymes, in the development of enzymes and respective application methods, the enzymatic hydrolysis step remains a considerable cost in the utilisation of lignocellulosic biomass (Jørgensen et al., 2007).

As discussed, the saccharification of hemicellulose and cellulose is necessary in order to use lignocellulosic biomass as a carbon source in any fermentation process. The saccharification of hemicellulose sugars during steam explosion pretreatment generated the water-soluble sugars that were used for the experiments in **Paper I** and **Paper II**. For the SSF reactors in **Paper III,** an enzyme blend was added to the pretreated softwood solid so that the cellulose could be depolymerised and used to produce lactic acid.

2.3.4 Downstream processing

The complex nature of the cultivation media in this type of bioprocess requires a multi-step downstream operation to obtain lactic acid with sufficiently high purity for polymerisation. Although the downstream process was not explored in this

thesis, it is described briefly here. The downstream process usually consists of separation, purification, and final recovery of the intended product—in this case, optically pure lactic acid. This practice is a critical step in the application of this platform chemical and can amount to up to 50% of the operating costs of the whole process (Oliveira et al., 2020a). Downstream processing of first-generation lactic acid (i.e., from sugars or starchy materials) is a well-researched area. Traditionally, lactic acid that is produced industrially using fermentative processes has been separated from the complex broth by precipitation with calcium hydroxide. Conversely, second-generation lactic acid, produced from lignocellulosic biomass, requires further examination (Oliveira et al., 2020a).

Because other compounds precipitate with lactic acid, precipitation alone might not necessarily be the ideal separation process when lignocellulose is used as a feedstock. Nevertheless, He et al. (2022, 2023) used precipitation to obtain sufficiently pure cyclic lactide from wheat straw, after several purification steps. In their study, after pretreatment, the fungus *A. resinae* ZN1 was added to pretreated lignocellulosic biomass for biodetoxification. *A. resinae* ZN1 removed the inhibitory compounds that formed during pretreatment completely (furan aldehydes and organic acids) or decreased their concentration to minimal levels (phenolic aldehydes) while maintaining the concentrations of fermentable sugars. After fermentation, lactic acid was purified by solid-liquid separation, decolourization, crystallisation, acidification and cation exchange. Then, the lactic acid enantiomers were polymerised to their respective cyclic lactides, with high chemical purity (He et al., 2022, 2023).

Oliveira et al. (2020a) compiled and reviewed separation and purification methods for second-generation lactic acid, including neutralisation and precipitation, solvent and reactive extraction, affinity-based processes (e.g., adsorption, ion exchange chromatography), membrane processes, salting-out and molecular distillation (Oliveira et al., 2020a). As the demand for optically pure lactic acid increases, so will research and development with regard to its industrial production. Nevertheless, even after lignocellulose-based processes have been established, the need for continuous process improvements will remain.

Biorefineries add complexity to this process, because their aim is to produce several value-added products from the entirety of lignocellulosic raw materials, applying the three pillars of sustainability: social, economic and environmental (Purvis et al., 2019).

2.4 A cellulose-based raw material: waste viscose

Viscose, also known as rayon, is manufactured through a multi-step process (Figure 9), using cellulose from such plants as eucalyptus, beech, pine, sugarcane, bamboo and soy (Asia Pacific Rayon Ltd., 2022; Quynh Nguyen, no date). The first patent for the production of viscose was granted to Cross and Bevan (England) in 1893, and viscose remains one of the most versatile man-made fibres (Woodings, 2001). In this thesis, lactic acid production from coloured and white viscose waste was compared.

2.4.1 Viscose manufacturing

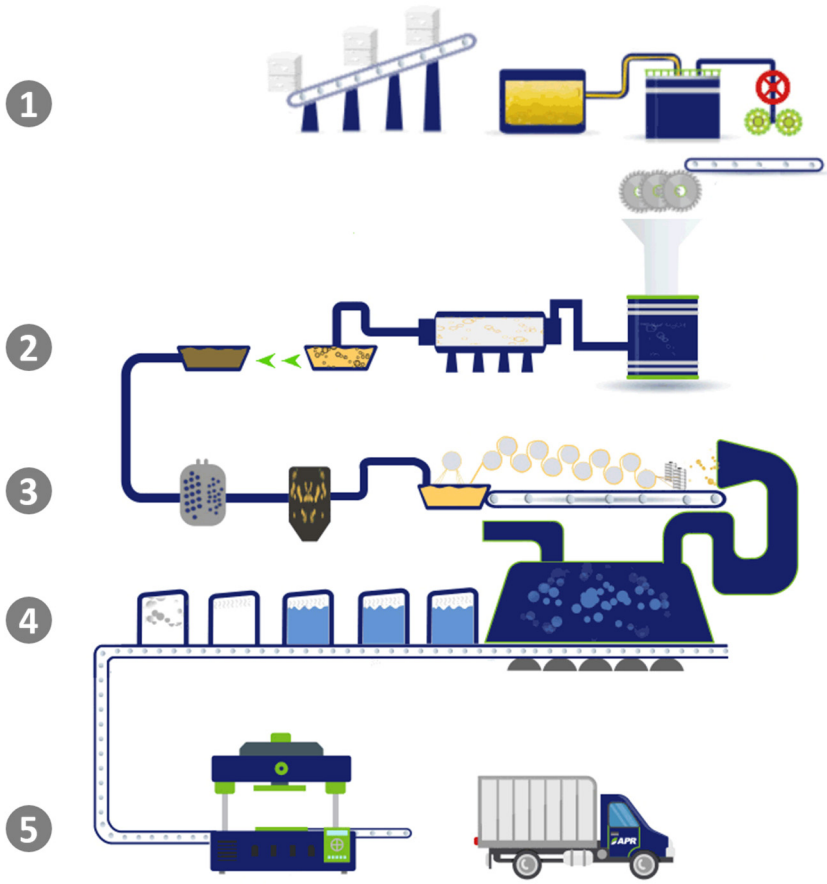

Figure 9. Viscose production process. Wood pulp is mixed with sodium hydroxide to solubilise lignin and retrieve cellulose fibres (1). The cellulose reacts with carbon disulphide and is left to mature (2) until it is ready to be spun into filaments (3). The new filaments are washed to remove impurities (4) and dried for transport to fabric manufacturers (5) (Adapted from Asia Pacific Rayon Ltd., 2022).

The viscose production process is presented in Figure 9 and begins with the extraction of cellulose from lignocellulosic biomass. Because the objective is to obtain cellulose fibres, alkaline pretreatment is preferred, in contrast to the acid pretreatment method for softwood presented in the previous section. The most frequently applied alkaline process is kraft pulping, which uses sodium hydroxide, sulphide, and bisulphite to separate cellulose fibres from lignin, hemicellulose and extractives, which are discarded as "black liquor". After cellulose extraction from the lignocellulosic material, cellulose fibres can be bleached to remove residual lignin, hemicellulose and extractives from the cellulose fibres (Woodings, 2001).

Next, the cellulose fibres undergo depolymerisation and repolymerisation for transformation into viscose. Initially, carbon disulphide is added to the alkali cellulose to form xanthate, which is dissolved in sodium hydroxide solution (Alter and Mast, 2022; Asia Pacific Rayon Ltd., 2022). In the next step, solid particles and trapped air are removed from the solution before it is spun into regenerated cellulose fibres, which is performed by forcing the liquid through small holes into a bath of sulphuric acid, sodium sulphate and zinc sulphate (Alter and Mast, 2022). The fibres are then washed, cut and dried before being packed for distribution to fabric companies (Asia Pacific Rayon Ltd., 2022).

The properties of viscose, such as its smooth texture, absorptive capacity and fibre strength, render it a suitable material for many applications, ranging from clothing to hygiene products (Re_fashion, 2021; Textile Exchange, 2023). Approximately 5.8 million tonnes of viscose was produced in 2021, making it the most commonly used man-made cellulosic fibre (Textile Exchange, 2022). It is often blended with other synthetic fibres, including PET, nylon and polyamide, to improve the characteristics of the fabric, posing an additional challenge in the recycling process.

2.4.2 Textile additives

Chemical additives can be applied to fabric to enhance its quality or adjust the characteristics of the textile for future uses. They can be included during fabric pretreatment, colouring and finishing, and many chemicals can be added at each stage. Chemical additives are classified according to their solubility, chemistry, and affinity to certain fibres. A book chapter written by Darbra et al. on additives in the textile industry (Darbra et al., 2012) compiles many chemicals that are used as additives in textiles discusses their potential negative effects on human health and the environment.

These chemicals have been estimated to account for approximately 20% of the pollution of clean water worldwide (European Parliament, 2023). The landfilling of textiles and the consequent washout of these additives by rainwater into the soil are also causes of environmental pollution. The number, variety and low concentrations

of possible additives that are used in a single piece of garment make it challenging to identify and remove the pollutants.

The impact of these additives in textile recycling and reutilisation of the fibres must be considered. This thesis compared the results with regard to lactic acid production from coloured and white viscose waste. Dyes and additives can possibly inhibit lactic acid bacteria, as they are known to be toxic for organisms in aquatic environments. The results of the work are shown in **Paper IV,** and results are presented in Chapter 3.

2.4.3 Textile waste handling

The rise of fast fashion has led to several environmental problems for which solutions are being sought. With regard to the disposal of clothing items, most is burnt or buried in landfills (73%), whereas less than 13% is recycled into new textiles or other, lower-quality materials. Fibre-to-fibre recycling is preferred, although this practice is applied to less than 1% of fibres. Thus, it is important to increase this percentage by promoting educational campaigns on the fate of discarded textiles, increasing the access to fabric collection points and recycling, and by supporting technological recycling innovations (Ellen MacArthur Foundation, 2017).

The potential for optically pure lactic acid production by *P. acidilactici* with an underrated cellulose-based residue—post-consumer waste viscose—was evaluated. The waste viscose in **Paper IV** was sorted among used textiles from large-scale textile sorting facilities in Sweden (SIPTex) and Finland (REISKAtex) (REISKA, 2017; Sysav, 2022). These facilities use near-infrared (NIR) spectroscopy to separate by textile colour and type, so that each fabric is recycled appropriately, as shown in Figure 10.

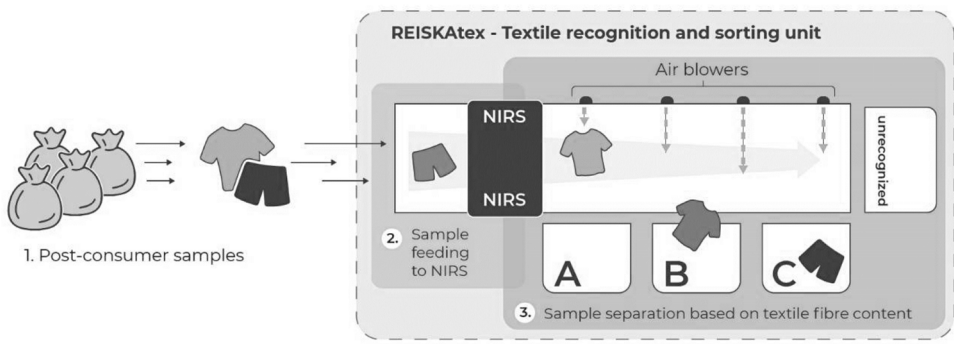

Figure 10. REISKAtex textile recognition and sorting unit (Cura et al., 2021).

Textile recycling is divided into high-quality and low-quality recycling. In high-quality recycling, waste textiles are transformed into new fibres (fibre-to-fibre). Low-value recycling, also called downcycling, converts waste fibres into lower-quality products, such as insulation material and upholstery. There are technological challenges that are associated with fibre-to-fibre recycling, because it depends on achieving sufficient polymerisation for high-quality fibres (Wedin et al., 2019). Thus, a significant proportion of waste textile is downcycled or destroyed (European Commission, 2019b). Another proposal for decreasing the amount of textiles in landfills entails transforming the structural components of the fibres into other value-added biochemicals through fermentation, such as optically pure lactic acid.

2.4.4 Viscose saccharification

After the waste viscose was sorted, as discussed in the previous subsection, all non-viscose elements were removed, and the textiles were shredded. The feedstock was thus considered to be composed only of viscose, which is made entirely of cellulose. Because cellulose is readily available for enzymatic hydrolysis, harsh pretreatments, such as that for softwood, are unnecessary. However, the quality of waste textile can vary by fabric, pre-production, and post-production addition of additives to the garment and its level of use. For this thesis, only post-consumer viscose waste textiles were used. Post-consumer textiles have other contaminants from daily use, such as stains, ingredients in personal care products, sebum and sweat, that render post-consumer waste textiles more challenging to treat than virgin textiles.

Cellulase and β-glucosidase have been used individually and in blends (such as the commercially available enzyme blends Cellic CTec2 or Cellic CTec3) to hydrolyse cellulose fibres. The use of waste textiles as feedstock for producing value-added chemicals has garnered recent attention from researchers, and consequently, there are few studies on optimising the saccharification of textile fibres and the utilisation of sugar. Nevertheless, these studies have shown promising results on the saccharification and microbial utilisation of sugars from waste textile, as mono-fibres or blends, with additives and dyes (Jeihanipour et al., 2010; Safartalab et al., 2014; Mihalyi et al., 2023).

In the current work, the hydrolysates that were obtained after enzymatic hydrolysis of the white and coloured waste viscose were used to produce D-lactic acid. These results are presented at the end of Chapter 3.

3 Current Work – Overcoming Obstacles for the Utilisation of Lignocellulosic Biomass by *P. acidilactici*

As discussed in the previous chapters, the use of alternative raw materials, such as lignocellulosic biomass, can decrease that of food crops to produce bio-based chemicals and promote a circular economy that is based on the utilisation of residues as substrates.

Despite these benefits, industrial producers are reluctant to use lignocellulosic biomass and other residues as raw materials. Two of the obstacles found are the release of sugars from the complex chemical structure of the biomass and the conversion of sugars, despite inhibition by any pretreatment degradation products, fermentation products or additives. This chapter presents the studies that were performed during this thesis, in which some of these challenges were examined and overcome in the production of optically pure lactic acid by *P. acidilactici* TY112 and *P. acidilactici* ZP26.

3.1 Utilisation of softwood sugars

Softwood was the main feedstock used in this work, based on its availability and prior process knowledge, as described in Subchapter 2.2. It was necessary to subject the softwood to a physicochemical pretreatment step to access the sugars in its cellulose and hemicellulose components. When hydrolysed completely, the cellulose provides glucose, whereas the hemicellulose in softwood consists of several linked monosaccharides—primarily, the hexoses glucose and mannose and the pentose xylose, with minor amounts of the hexose galactose and the pentose arabinose.

The strains that were used in this thesis were obtained by knockout of a lactate dehydrogenase (L- or D-) in *P. acidilactici* DQ2, with the remaining genome intact (Yi et al., 2016). Thus, these strains were expected to be able to use the

predominating hexoses in softwood biomass (glucose and mannose). However, the strains used did not utilise xylose.

The procedure and results of the softwood pretreatment are described below, whereas the utilisation of hemicellulosic sugars and the challenges that were posed by inhibitory compounds are discussed later.

3.1.1 Softwood pretreatment

Softwood chips were pretreated using a steam explosion method, catalysed by gaseous sulphur dioxide (SO_2). The equipment that was used for the steam explosion pretreatment was first described by Palmqvist et al. (1996), and the optimised addition of SO_2 as a catalyst was reported by Stenberg et al. (Stenberg et al., 1998). This method has been applied for a wide range of lignocellulosic materials, such as corn stover, wheat straw and various wood types, effecting hydrolysis yields above 60% (Alvira et al., 2010; Yu et al., 2022).

Table 2. Composition of the solid fraction of spruce after pretreatment [analysed according to Sluiter et al. (2008a, 2008c, 2012)].

Total solids [TS]	Water-insoluble solids [WIS]	Glucan	Mannan	Acid-insoluble lignin	Ash
(% biomass)		(% dry matter)			
41.6	38.4	88.2	1.4	25.5	0.1

Table 3. Composition of the liquid fraction of the pretreated spruce, in concentration (g/L), immediately after pretreatment [analysed according to Sluiter et al. (2008b)].

Glucose	Mannose	Xylose	Arabinose	Galactose
24.1	29.4	9.2	3.1	6.7
HMF	**Furfural**	**Formic acid**	**Acetic acid**	**Levulinic acid**
3.4	1.1	1.4	6.8	1.0

Steam explosion is an established pretreatment method for lignocellulosic biomass, in which wood is exposed to high-pressure saturated steam, at temperatures between 110°C and 265°C for several minutes, followed by rapid expansion to atmospheric pressure. This treatment increases the porosity of the wood by opening the structure of the fibres (Figure 8), making them accessible to hydrolytic enzymes that release monomeric sugars from the polymers. The characteristics of the raw material, such as the presence of bark, its relative content of macromolecules and chip size, affect the pretreatment efficiency (Galbe and Wallberg, 2019). In the current study, SO_2 was added to the pretreatment (2.5% w/w, based on the water content of the wood)

to promote deacetylation, thus aiding in the hydrolysis of hemicellulose and cellulose (Alvira et al., 2010; Liu et al., 2012). The composition of the solid and liquid fractions after pretreatment is presented in Tables 2 and 3.

The disadvantages of harsh steam explosion conditions (210°C, 5 min) and SO_2 as a catalyst are the degradation of sugars that are released from the solid fraction, the re-aggregation of cellulose fibres after the removal of hemicellulose, the formation of inhibitors of microbial fermentation (e.g., through thermochemical degradation of sugars) and high energy consumption. Considering the development of enzyme blends for hydrolysing lignocellulosic biomass, studies are underway to re-evaluate the optimal conditions for the saccharification of softwood for microbial use (Caputo et al., 2022).

Nevertheless, steam pretreatment with a catalyst remains the preferred pretreatment method for recalcitrant softwood material (Alvira et al., 2010; Yu et al., 2022).

3.1.2 Mannose consumption

Mannose is the second-most abundant sugar in spruce, constituting about 10% of its dry matter (DM). Other hexoses, such as galactose, are present at low levels (< 1.5% DM). Thus, mannose is a considerable carbon source in softwood; the conversion of mannose to lactic acid by *P. acidilactici* was the topic of **Paper I**.

Shake flask and bioreactor experiments were conducted using synthetic media and the pretreated softwood liquid fraction (termed "hydrolysate"). *P. acidilactici* TY112 and *P. acidilactici* ZP26 consumed glucose and mannose and produced lactic acid with high optical purity (>99%). The consumption of mannose was, however, slower than that of glucose and did not always reach completion within a reasonable time (see **Paper I**).

A simplified metabolic map of the production of lactic acid from glucose and mannose is presented in Figure 11. The utilisation of sugars begins with their phosphorylation, followed by the formation of pyruvate through the catabolic Embden-Meyerhof-Parnas (EMP) pathway (Figure 11), because *P. acidilactici* is a microaerophilic homofermentative bacterium. Lactate dehydrogenases then convert pyruvate into lactic acid, which is transported out of the cell via passive diffusion or carrier-mediated transport (facilitated diffusion).

In an earlier master's thesis (Tejada, 2021), sugar consumption and lactic acid production profiles were studied under aerobic conditions (1 vvm air). Compared with anaerobic reactors, mannose consumption rates increased. Acetic acid also formed during these cultivations, suggesting that under aerobic conditions, *P. acidilactici* TY112 adapts its metabolism to the presence of oxygen. Because *P. acidilactici* is a strict homofermentative organism (Carr et al., 2002), it is unlikely that its metabolism shifts to being heterofermentative. However, lactic acid bacteria

have been reported to use oxygen (and hydrogen peroxide, formed from oxygen) as an electron acceptor during sugar metabolism, altering their consumption and production (Condon, 1987). This unusual metabolism of lactic acid bacteria can increase mannose consumption and promote the formation of acetyl-phosphate, which can then be dephosphorylated to acetate.

P. acidilactici TY112 was cultivated under aerobic conditions to test the hypothesis that oxygen supplementation increases the robustness of bacterial cells, as proposed for *Lactococcus lactis* by Johanson et al. (Johanson et al., 2020), possibly improving the volumetric rate of lactic acid production. Our results on cell robustness were inconclusive. However, broth oxygenation led to acetic acid production, deviating the sugars consumed by the bacteria away from lactic acid production without a significant increase in bacterial cell growth. This conclusion prompted us to refocus on feedstock composition instead of aeration. Further, because *P. acidilactici* does not require completely aerobic or anaerobic conditions for lactic acid production, most challenges that are related to gas-liquid transfer and foaming can be avoided by not sparging the media, benefitting scale-up processes.

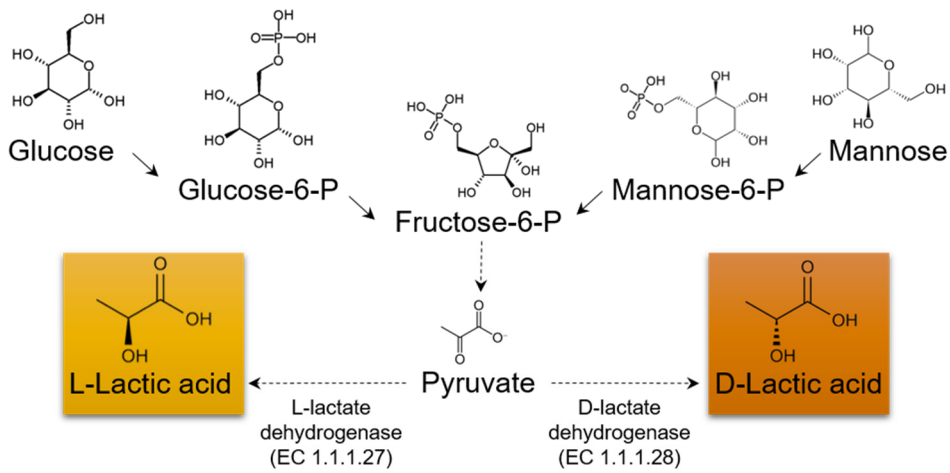

Figure 11. Schematic of metabolic pathways of lactic acid production from glucose and mannose through the Embden-Meyerhof-Parnas (EMP) pathway.

The co-consumption of glucose and mannose was observed in **Paper I** but was more evident in the pulse feed cultivations in **Paper II**. As expected, both *Pediococcus* strains preferred glucose over mannose, showing higher consumption rates for this hexose as the sole carbon source or in mixtures with mannose. This well-documented regulatory mechanism, called carbon catabolite repression, inhibits the use of secondary carbon sources when a preferred carbon source is present (Magasanik, 1961; Görke and Stülke, 2008).

In recent studies, He et al. reported the simultaneous conversion of glucose and other lignocellulosic sugars by *P. acidilactici* ZY271 and *P. acidilactici* ZY15 in the production of L- and D-lactide, respectively (He et al., 2022, 2023). These engineered strains were subjected to adaptive evolution to perform simultaneous consumption of hexoses and pentoses from wheat straw (glucose, xylose, arabinose, mannose and galactose), with minimal residual sugars left at the end of the cultivation.

A fed-batch strategy was used in **Paper II** to study the fermentation characteristics of *P. acidilactici* in media with inhibitors from the degradation of lignocellulosic sugar (e.g., HMF, furfural and acetic acid). In this work, the total volume of the liquid fraction that was fed to the reactors and thus the total amount of inhibitors that was added could be increased compared to previous batch experiments.

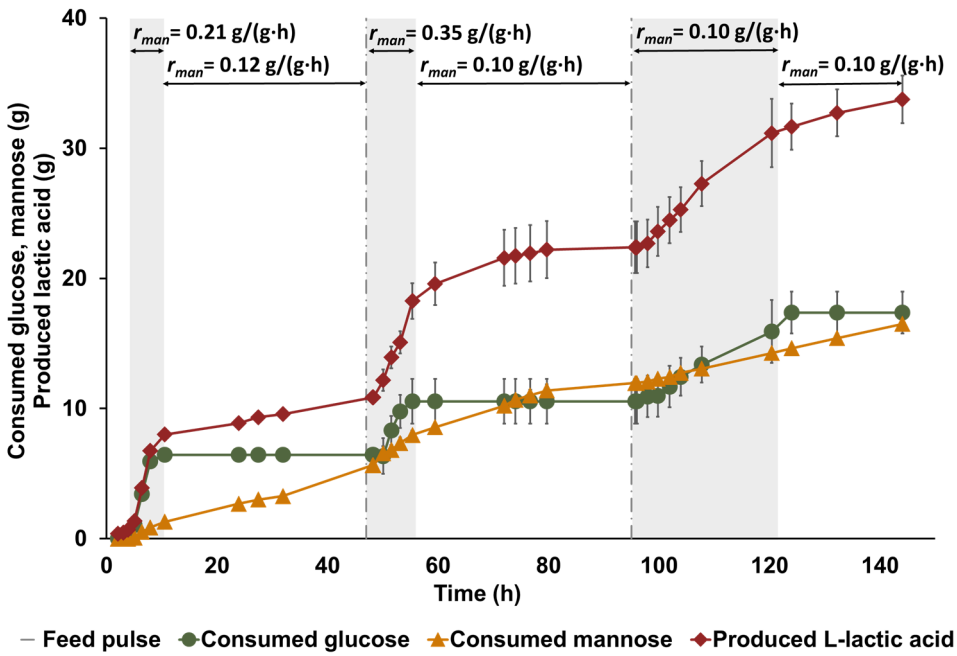

Figure 12. Calculated consumed mass of glucose and mannose and produced mass of lactic acid in a fed-batch cultivation with pulsed additions of a mixture of glucose and mannose.

As is possible to observe in Figure 12, mannose was consumed simultaneously with glucose. Notably, after the depletion of glucose, mannose consumption rates decreased (Figure 12), possibly related to the shift in the uptake of the sugars. Several transport systems have been described for *Pediococcus sp.* and the annotated *P. acidilactici* ZPA017 genome (Liu and Ji, 2016). Genes that encode for glucose-specific and mannose-specific phosphotransferase systems (PTSs), which are active transport membrane structures, were identified in the annotated genome.

Other identified transporters included permeases and ABC transporters. Possible transport systems for glucose and mannose in the cellular membrane are illustrated in Figure 13.

The transport systems that mediate the uptake of sugars by the two *P. acidilactici* strains in this thesis have not been identified. However, if glucose transport occurs by facilitated diffusion, through permeases, it is possible that glucose uptake aids in the production of energy, through glycolysis, required for the active transport of mannose. This model supports the hypothesis that, in a mixture of substrates, the presence of glucose facilitates the uptake of other sugars.

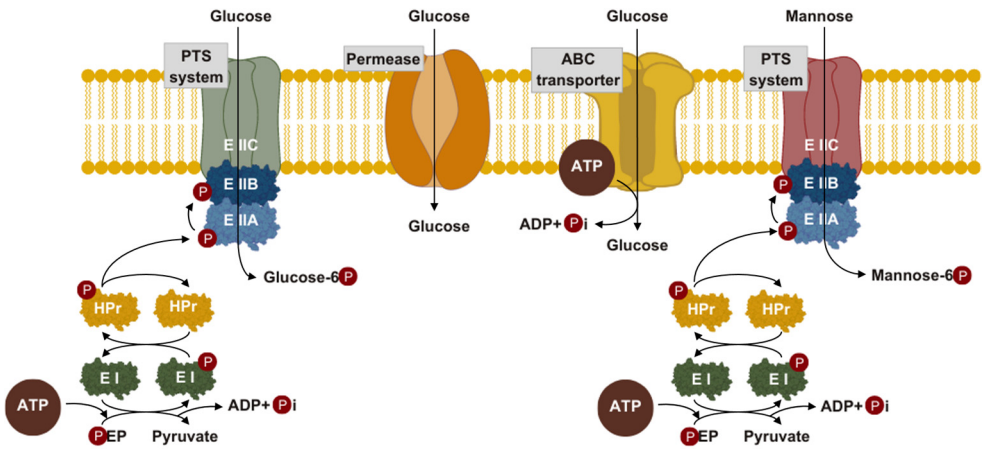

***Figure 13**. Putative transport systems for glucose and mannose in the cellular membrane of* P. acidilactici *(based on Liu and Ji (2016)).*

Simultaneous uptake of sugars was also examined in continuous feeding experiments in **Paper II** with regard to the use of the liquid fraction of pretreated softwood by *P. acidilactici*. The objective was to hold lactic acid production rates constant by maintaining glucose and mannose co-consumption throughout the cultivation. This was achieved in the continuous fed-batch reactors in **Paper II** that were fed a synthetic carbon source at 15 mL/min and in the reactors that were fed softwood hydrolysate at 13 and 30 mL/min. These feed rates thus allowed mannose uptake rates to approximate those of glucose. In cultivations with a higher feed rate, however, glucose was taken up faster than mannose. In reactors with a 30-mL/min feed rate of hydrolysate, substrate uptake was likely impeded by inhibitors in the complex carbon source, decreasing glucose uptake rates to those of mannose. This information is important in achieving complete consumption of mannose (and other hexoses) before glucose is exhausted; thus, full utilisation of hexose should be targeted.

Further, this study aimed to determine whether it was possible to adapt the cultivation to the inhibitory compounds in the liquid fraction that originated from

the degradation of sugar during the pretreatment. The resistance of *P. acidilactici* to this inhibition will be discussed further in Subchapter 3.2.

3.1.3 Exopolysaccharide production

The formation of extracellular polysaccharides, or exopolysaccharides (EPSs), is a common feature of many lactic acid bacteria. The abbreviation EPS is sometimes used for extracellular polymeric substances in general, but here, it is restricted to mean extracellular polysaccharides.

Organisms produce EPSs to protect the integrity of the cell against adverse conditions (i.e., dehydration, osmotic stress, toxic compounds and pathogens). EPSs are also involved in the colonization of natural habitats, biofilm formation and cell recognition. EPS polymers differ in sugar composition, constituent subunits, length, charge, sugar linkages and degree of branching. In lactic acid bacteria, they are usually heteropolysaccharides that are formed by two or more types of sugar monomers. Based on their wide variety of compositions and structures, EPSs have several notable applications, including sweeteners, thickeners, emulsifiers, and flocculants. Despite the abundance of these polymers, their production and mechanisms of utilisation are strain-dependent and incompletely understood (Kumar et al., 2020; Korcz and Varga, 2021; Poulin and Kuperman, 2021).

In the current work, EPS formation potentially influenced lactic acid production because of the consequent formation of flocs. Biomass flocculation in *P. acidilactici* TY112 has been prevented by the addition of amyloglucosidase (or glucoamylase) to the cultivations (Liu et al., 2015). Even from initial cultivations for this thesis, it was observed that both strains formed a similar type of agglomerate, which affected the sampling and quantification of biomass. To be able to assess biomass growth, amyloglucosidase was added to some of the cultivations in **Paper I**.

In **Paper I**, the agglomerates were submitted to enzymatic hydrolysis to determine their sugar composition. It was mainly possible to determine that the EPSs that formed during cultivations with mannose were heteropolymers of glucose and mannose. Since EPSs were not subject to investigation in this thesis, the composition of these polysaccharides was not evaluated for other cultivation conditions, but it is possible that it was influenced by the sugar composition of the carbon source.

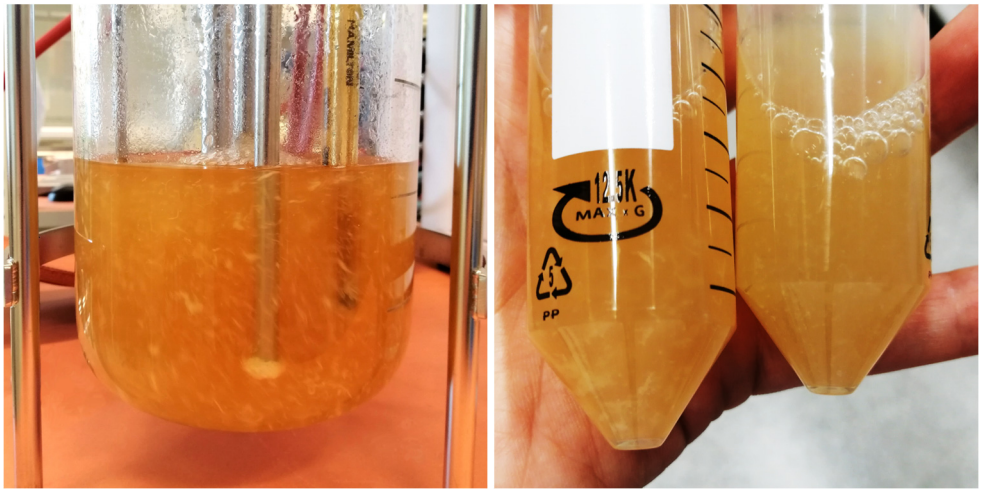

Figure 14. Visual assessment of biomass flocculation of P. acidilactici in bioreactor cultivations.

Amyloglucosidase (used in **Paper I** and **Paper IV**) and Cellic CTec3 (used in **Paper II** and **Paper III**) were able to hydrolyse the flocs or prevent their formation. Whereas amyloglucosidase hydrolyses α-1,4 and branching α-1,6 linkages between glucose units, Cellic CTec3 is a blend of cellulases, hemicellulases and other compounds that aid in the enzymatic hydrolysis of lignocellulosic biomass, the monomers of which are linked with various types of β bonds. *Pediococcus parvulus* produces β-glucans (Velasco et al., 2007), and *P. acidilactici* NCDC 252 synthesises α-glucans (Kumar et al., 2020), illustrating the variety of EPSs in the genus *Pediococcus*.

In recent studies on further developed variants of *P. acidilactici* DQ2, EPS formation was not reported in the production of optically pure lactic acid and posterior polymerisation to lactide (He et al., 2022, 2023). Minute amounts of residual sugars and proteins were detected in the cultivation broth, which were eliminated by an activated carbon adsorption step (He et al., 2022, 2023). The absence of EPS production in the strains used by He et al. might have resulted from modifications to and adaptive evolution of the two strains in the studies or differences in the raw materials and methods.

The formation of EPSs by *P. acidilactici* TY112 and *P. acidilactici* ZP26 has practical significance, leading to flocculation, thus influencing the experimental method, and potential cell surface interactions with pretreated softwood solids. This possibility is hypothesised in **Paper III** and discussed in the Chapter 4.

3.2 Overcoming inhibition by compounds formed during pretreatment

One important objective of this work was to determine the effects of inhibition by the substrate and to identify strategies for overcoming it.

Microbial growth can be inhibited by many factors—for example, the substrate itself, by-products in the substrate, metabolic products, or cultivation conditions. The main product of the microorganisms used in this thesis, lactic acid, might also become a toxin if its concentration in the broth surpasses the intrinsic resistance limit of a given strain. A decrease in pH in the media caused by the increase of the concentration of a dissociated acid leads to acidification of the cytoplasm and disruption of the ion-motive force within the microorganism (Gonçalves et al., 1997). A strain that was derived from *P. acidilactici* TY112 has been subjected to several tests to increase its tolerance to low pH (Yan et al., 2022). Although the overexpression of genes that increase tolerance to acid did not have the intended results in the study by Yan et al. (2022), adaptive evolution at low pH resulted in clear improvements in cultivations at pH 4 compared with the non-adapted strain. Obtaining resilience mechanisms at low pH would be beneficial for the scale-up of the bioprocesses because the need for the addition of base during fermentation could potentially decrease. Parallelly, several studies have reported high final lactic acid concentrations (>100 g/L) with *P. acidilactici* (Zhao et al., 2013; Liu et al., 2015; Zhang et al., 2022a; He et al., 2023). Product inhibition has been studied extensively but was not the most significant type of inhibition in the current study—inhibitory compounds originated from lignocellulosic sugars are.

As discussed in Section 2.3, there are several degradation products that form during lignocellulosic biomass pretreatment that are detrimental to many microbes, including *P. acidilactici*. As it was mentioned in the previous chapter, *P. acidilactici* DQ2 was isolated from corn stover hydrolysate and compared with other *Pediococcus* strains (Liu et al., 2013; Zhao et al., 2013). Those studies concluded that the isolate had high resistance to inhibitory compounds such as HMF and furfural. A thorough investigation with the strains in this thesis was also conducted with synthetic media that was supplemented with several concentrations of various degradation products of sugars (furfural, HMF, acetate) and lignin (4-hydroxybenzaldehyde, syringaldehyde, vanillin) (Yi et al., 2016). In the referenced study, *P. acidilactici* TY112 and *P. acidilactici* ZP26 showed good tolerance to the inhibitors (maintaining lactic acid production until 6 g/L of HMF or furfural), except for syringaldehyde and vanillin, which inhibited production at 0.3 g/L. Based on these findings, these strains were deemed as suitable for pretreated softwood biomass utilization, having shown adequate tolerance to some of the inhibitors that are found in pretreated softwood (Yi et al., 2016).

Studies on variants of *P. acidilactici* DQ2 (Figure 6) that utilise wheat straw and corn stover used a "biodetoxification strategy" to mitigate the negative effects of these and other sugar and lignin degradation products. The fungus *Amorphotheca resinae* ZN1 was also isolated from pretreated corn stover (Zhang et al., 2010) and can remove furfural, HMF, acetic acid, vanillin, syringaldehyde and 4-hydroxybenzaldehyde within 10 days of cultivation, without consuming the sugars that can be used later for lactic acid fermentation (Yi et al., 2016). *A. resinae* ZN1 cannot biodetoxify the media of phenolic compounds, and thus, strain improvements have been made to render *P. acidilactici* more resistant to these and other inhibitors (Qiu et al., 2020a, 2020b). Although biodetoxification with *A. resinae* ZN1 yielded good results, this step was not included in the softwood processes that were evaluated in this thesis.

As an initial step, the behaviour of *P. acidilactici* in the presence of inhibitors in pretreated softwood was characterised and is presented below.

3.2.1 Batch cultivations with softwood liquid fraction

The results for the cultivations that used only the liquid fraction from the pretreatment of softwood (also termed "hydrolysate") are discussed in **Paper I** and **Paper II**. This fraction contains primarily sugars from hydrolysed hemicellulose—i.e., it has a higher content of mannose than glucose. Shake flask cultivations were started with 10%, 20% and 50% (v/v) liquid fraction, supplemented with the adequate amount of glucose to minimise any performance discrepancies that might arise from different sugar concentration. The shake flasks with 10% and 20% liquid fraction yielded similar results, but no bacterial growth, and consequently no lactic acid production, were observed for the flasks with 50% liquid fraction. Thus, the first bioreactor cultivations were conducted as batch reactors with 20% (v/v) liquid fraction, in simplified MRS media (Yi et al., 2016), without additional sugar supplementation (Figure 15).

These cultivations confirmed the findings on sugar consumption in Section 3.1.2:

- Optically pure lactic acid is produced by *P. acidilactici* TY112 and *P. acidilactici* ZP26 from hexoses, with pentoses remaining in the media
- Glucose is preferred over mannose as the carbon source
- Mannose consumption rates are higher when glucose is present

The findings also showed that the degradation compounds in the 20% (v/v) liquid fraction did not negatively impact sugar consumption or lactic acid production profiles. However, it was necessary to identify strategies to increase the concentration of sugars that were fed to the microorganisms.

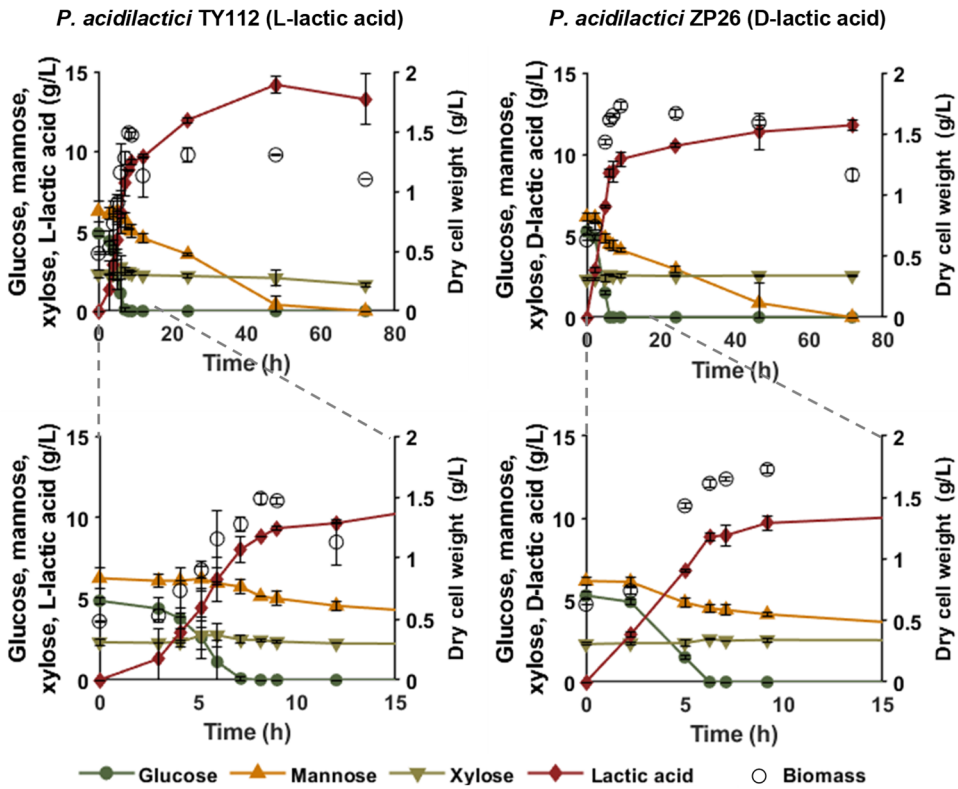

Figure 15. Batch cultivation with 20% (v/v) pretreated softwood liquid fraction using both P. acidilactici strains, magnified for the first 15 h of cultivation in the lower panels.

Later, batch cultivations were conducted with the 50% (v/v) liquid fraction, as well as with synthetic media with glucose, mannose, HMF and furfural at the concentrations in the reactor with the liquid fraction (Figure 16). Lactic acid production was possible with 50% hydrolysate when the cultivation times were prolonged. Production began after a long period of cultivation adaptation, in which HMF and furfural concentrations decreased. This effect was observed for the reactor with the liquid fraction and for the reactor with synthetic media. The conversion of HMF and furfural by *P. acidilactici* had not been previously reported, because *A. resinae* ZN1 was used in the first biodetoxification step (Yi et al., 2016). The capacity for HMF and furfural conversion by *P. acidilactici* was reported in **Paper II** and is detailed in the next subsection. This newly reported ability might obviate the need for a separate biodetoxification process when the concentration of other inhibitors is not harmful to *P. acidilactici*.

It was also possible to observe a matrix effect in the results in **Figure 16**. The synthetic media with inhibitors contained HMF, furfural, acetic acid, formic acid

and levulinic acid but does not include all possible inhibitors that form during the pretreatment of lignocellulosic biomass. The longer lag phase and lower lactic acid production rate in Figure 16A (versus Figure 16B) demonstrate that other inhibitory compounds affect lactic acid production and HMF and furfural removal in *P. acidilactici* TY112. These compounds are just two of many toxic compounds that are solubilised in the liquid fraction, and synergistic effects between inhibitors have been observed (Liu et al., 2013).

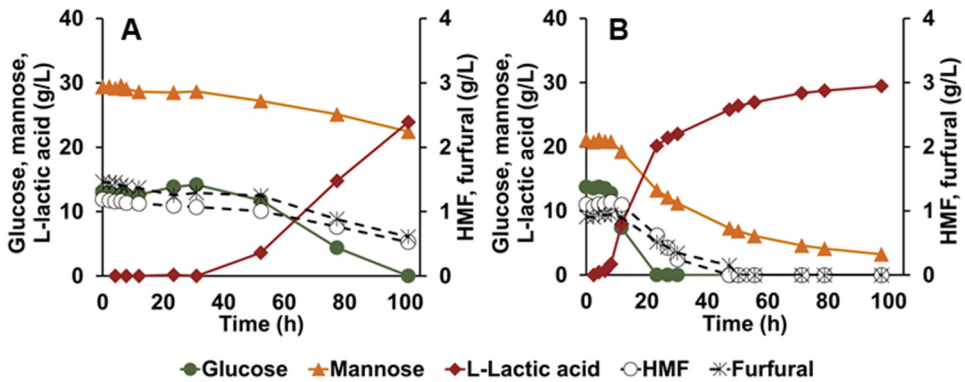

Figure 16. Batch cultivation of P. acidilactici TY112 with (A) 50% (v/v) pretreated softwood liquid fraction and (B) synthetic media with inhibitors.

A follow-up study was conducted to evaluate fed-batch strategies for favouring biomass adaptation to the inhibitors in the liquid fraction (**Paper II),** which is discussed next.

3.2.2 Fed-batch strategies to promote hexose co-consumption and biomass adaptation to the inhibitory media

The addition of pretreated softwood liquid fraction in pulses was studied first (Figure 17). Pulses of 200 mL of liquid fraction were added to the reactor at the start of the cultivation, at approximately 48 h and at 96 h (with 800 mL of nutrient media as the initial volume). The goal was to promote complete consumption of mannose before the subsequent addition to elicit adaptation of the cultivation in the reactor.

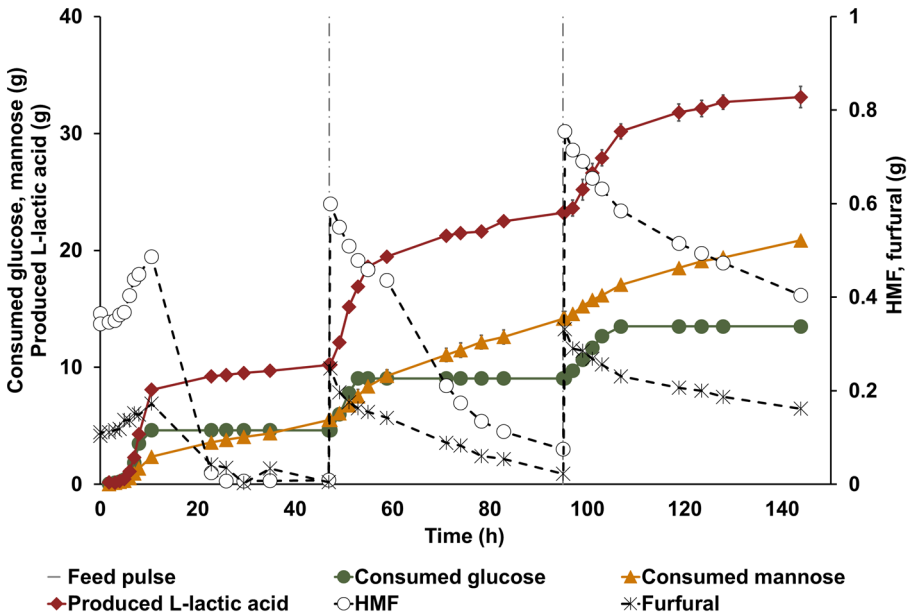

Figure 17. Calculated consumed mass of glucose and mannose, produced mass of lactic acid, and HMF and furfural in the broth in a fed-batch cultivation of P. acidilactici TY112 with pulsed additions of pretreated softwood liquid fraction ("hydrolysate").

It was possible to increase the volume of the liquid fraction that was fed to the reactor (from 200 mL in Figure 15 to 500 mL in Figure 16A and to 600 mL in Figure 17) while maintaining high lactic acid production rates (see **Paper II**). However, in the pulse fed-batch reactors represented in Figure 17, the lower mannose consumption rate after glucose depletion was not conducive to complete the consumption of mannose before each new pulse, and there was no clear sign of biomass adaptation after such a short number of cycles.

The strategy then shifted to continuously feed glucose with mannose. Experiments in which the liquid fraction was fed at 13 mL/min or 30 mL/min demonstrated well-maintained lactic acid production and co-consumption of the sugars throughout the fermentation (Figure 18). As mentioned before, the novel results of this study showed that *P. acidilactici* can convert HMF and furfural. This activity has been reported in other bacteria (Wierckx et al., 2011)—specifically, lactic acid bacteria (van Niel et al., 2012; Giacon et al., 2022)—to allow for their growth on hydrolysed lignocellulosic biomass, provided that the concentrations of these and other inhibitors are not excessive. It remains unknown which metabolic pathways are involved in the conversion of HMF and furfural during anaerobic growth of lactic acid bacteria, but similarities might exist with the mechanisms that have been proposed for *Saccharomyces cerevisiae*, which reduces furfural and HMF to their respective alcohols using NADH as a cofactor (Almeida et al., 2009).

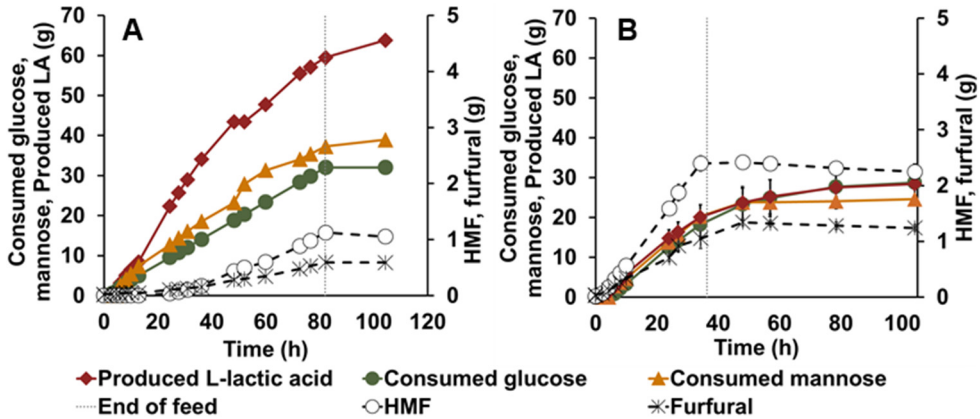

Figure 18. Calculated consumed mass of glucose and mannose, produced mass of lactic acid, and HMF and furfural amount in the broth in a fed-batch cultivation of P. acidilactici TY112 with continuous addition of pretreated softwood liquid fraction ("hydrolysate") at (A) 13 mL/min and (B) 30 mL/min.

To determine whether strategies such as fed-batch cultivations and adaptation of *P. acidilactici* are more effective than an additional detoxification step—for example, biodetoxification with *A. resinae* ZN1—techno-economic evaluations of the entire process must be performed.

3.3 Interaction between *P. acidilactici* and pretreated softwood solids

The final study with spruce in this thesis aimed to use the solid and liquid fractions of pretreated softwood in a SSF, SHF or hybrid process to produce optically pure lactic acid. However, during operation of the SSF reactors with the solid fraction, a long lag phase arose—in some cases, over 48 h. This delay was unexpected. Long lag phases are problematic for industrial bioprocesses and must be addressed. The aim of the final softwood study (**Paper III**) was thus shifted to significantly decrease or eliminate the lag phase of SSF cultivations with pretreated softwood solids and hypothesise about the cause of this phenomenon is.

In previous cultivations with the liquid fraction of pretreated softwood, the lag phase was less than 4 h, even with only 1% (v/v) seed inoculum (see **Paper I** and **Paper II**). An increase in seed inoculum volume to 10% (v/v) and additional prehydrolysis of the solids for 12 h led to a slight increase in the lactic acid concentration at the start, although the exponential phase of production was not detected until after over 40 h of fermentation (**Paper III**).

For this study, the lag phase was defined as the time between inoculation and the exponential production of D-lactic acid. For some of the cultivations, it was not possible to determine exactly where this shift began. The point at which maximum volumetric production occurred, however, was easier to determine; thus, this criterion was used.

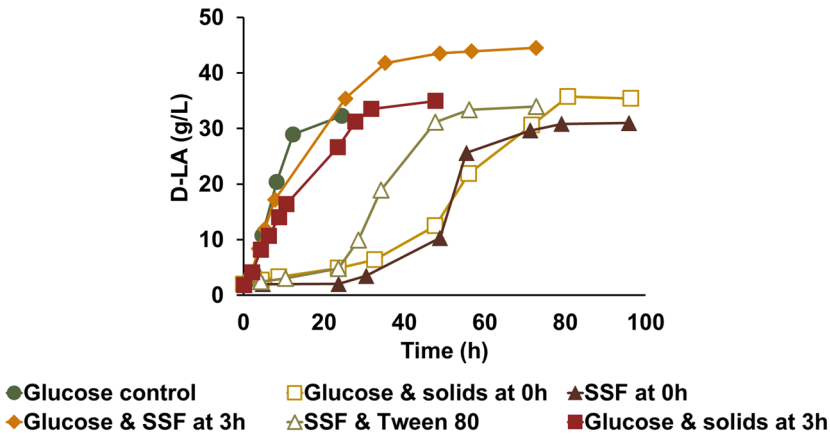

Figure 19. D-lactic acid production by P. acidilactici ZP26.

SSF cultivations that started with fresh inoculum, pretreated softwood solids and enzyme blend (Figure 19, ▲**SSF at 0h**) had a lag phase of approximately 40 h. A similar profile to the later condition was obtained when *P. acidilactici* ZP26 was grown in synthetic media with glucose in the presence of pretreated softwood solids (Figure 19, □**Glucose & solids at 0h**). This finding shows that the delayed exponential production was not caused by a lack of monomeric glucose in the media. The concentrations of inhibitory compounds that formed during pretreatment (e.g., HMF, furfural, formic acid, acetic acid) were analysed, confirming that their levels were too low (for example, compared with the cultivations in Figure 16A) to prolong the lag phase. Cultivations with synthetic glucose as the carbon source (Figure 19, ●**Glucose control**) were established to exclude the effects of a poor inoculum; fermentation began immediately in these controls. Thus, we attributed the delayed start of the lactic acid production to the presence of solid material instead of an inhibitory compound in solution.

Further tests were performed, in which softwood sawdust, or a mixture of Avicel cellulose and Kraft lignin was added to synthetic media with glucose (**Paper III**, Supplemental Material), but none effected a lag phase in the production of D-lactic acid. Thus, the delay was likely caused by the pretreated solids, which might have interacted with the microbial biomass.

In order to find ways to eliminate the lag phase in the presence of pretreated softwood solids. In subsequent cultivations, pretreated solids were added to actively growing cells in synthetic media with glucose as the carbon source. Pretreated softwood solids were added after 3 h (Figure 19, ■**Glucose & solids at 3h**) to determine whether they affected the metabolism of the microorganisms. In this case, there was no interruption in D-lactic acid production and no lag phase, yielding the same profile as the glucose control (see Figure 19). Similar cultivations were established with the addition of the enzyme blend; thus, the pretreated solids could also be used as a carbon source for D-lactic acid production (Figure 19, ◆**Glucose & SSF at 3h**). Although this process was not optimised for lignocellulosic sugar utilisation, the lag phase was eliminated.

It was not possible to determine the cause of the extended lag phase from these experiments alone. Nevertheless, potential interactions between *P. acidilactici* ZP26 and the pretreated softwood solids are discussed in the sections below.

3.3.1 Possible cell-solid adhesion mechanisms underlying the extended lag phase

Previous studies have shown that certain cellulolytic enzymes lose their catalytic activity on binding to lignin (Pan et al., 2005). It was later found that the addition of surfactants, such as Tween, limits the adsorption of enzyme to pretreated biomass, counteracting this effect (Eriksson et al., 2002). Since it had been determined that the delayed production of lactic acid was not due simply to the presence of solid particles or an underperforming inoculum, SSF experiments were conducted with Tween 80 in the media (Figure 19, △**SSF & Tween 80**). As a result, the lag phase decreased to approximately 24 h, prompting us to speculate that pretreated solids might interacted with microorganisms in a manner that was detrimental to D-lactic acid production.

Possible mechanisms for the interaction between the surface of microorganisms and other solid surfaces are presented in Figure 20. These interactions are influenced by the characteristics of the cells and of the solid material, including the surface charge, which can promote ionic and electrostatic bonds; the topography, roughness and geometry of the surfaces, which can engender physical bonds between the cell and material; non-bonding interactions, such as van der Waals forces and hydrophobic effects; and stronger covalent bonds between chemicals on the surfaces of the cells and of the solid material (Kreve and Reis, 2021).

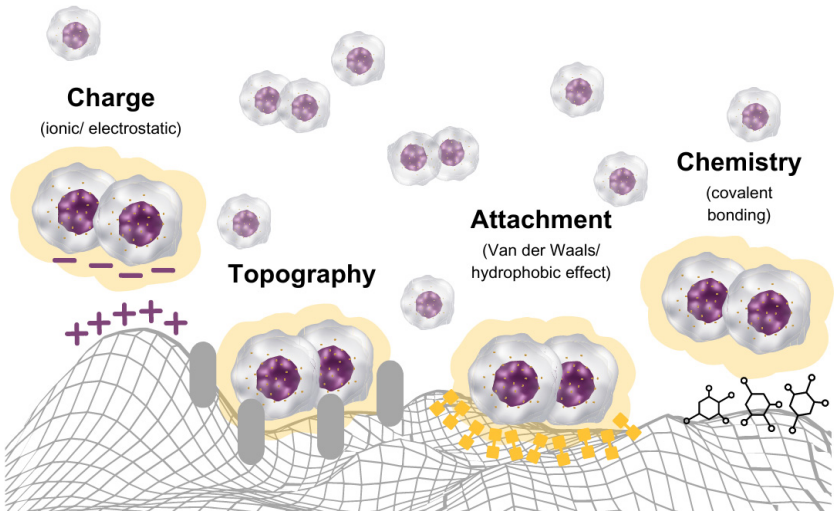

Figure 20. Substrate and cell surface characteristics that influence adhesion (Adapted from Kreve & Reis, 2021).

Adhesion mechanisms between materials and lactic acid bacteria are usually associated with biofilm formation (Alp and Kuleaşan, 2019), especially in organisms that produce extracellular matrices, such as EPSs (see Section 3.1.3). Bacterial adhesion to the surface of a material involves physicochemical interactions that are specific to each bacteria-substrate pair and depends on the strength of the bond between the cell and material, properties of the bacteria (such as physiological state), available nutrients and bacterial cell density. Methods for analysing this type of interaction have been developed recently—for example, atomic force microscopy—but the sheer diversity and number of variables have impeded our understanding of cell-surface interactions (Kreve and Reis, 2021; Santore, 2022).

3.3.2 How might the D-lactic acid production be resumed after the extended lag phase?

In **Paper III**, after the long lag phase that was caused by pretreated softwood solids, all cultivations entered the exponential production phase, after which they showed no other signs of inhibition. This finding indicates that cell-solids interactions changed, in some cases, after 48 h of fermentation, with or without enzymatic hydrolysis. Saccharification studies with pretreated lignocellulosic biomass are rarely performed solely with the solid fraction, due to the considerable amount of hemicellulosic sugars in the liquid fraction. Researchers usually attribute cell inhibition to toxic compounds that form during the pretreatment of lignocellulosic biomass. However, because they reside primarily in the liquid fraction, their

influence on *P. acidilactici* ZP26 cultivations with only the solid fraction was considered to be minimal. Thus, more studies are needed to fully explain the cause for this extended lag phase.

As discussed, the addition of Cellic CTec3 also contributed to dissolving the biomass flocs (see Section 3.1.3). In the cultivations in **Paper III**, a long lag phase was observed regardless of whether the enzyme blend was added. This finding indicates that the interaction that inhibits lactic acid production is unrelated to EPS production alone or that the removal of EPSs by the enzyme blend was incomplete.

Another explanation for this change in the cultivation is the release of planktonic cells from biofilm that might have formed by EPS-producing *P. acidilactici* on the surface of the pretreated softwood solids. A typical cycle of biofilm formation (Figure 21) begins with the initial adhesion of free cells (planktonic form) to a surface, through the interactions in Figure 20. Then, a biofilm develops accompanied by the production of a polymeric matrix, perhaps comprising EPSs and other compounds, and cell division happens in the biofilm. Mature biofilms possess dormant cells in their interior, due to nutrient limitation and low mass transfer in the biofilm. Dispersion of the biofilm occurs when cells leave the biofilm to return to their planktonic form (Kreve and Reis, 2021).

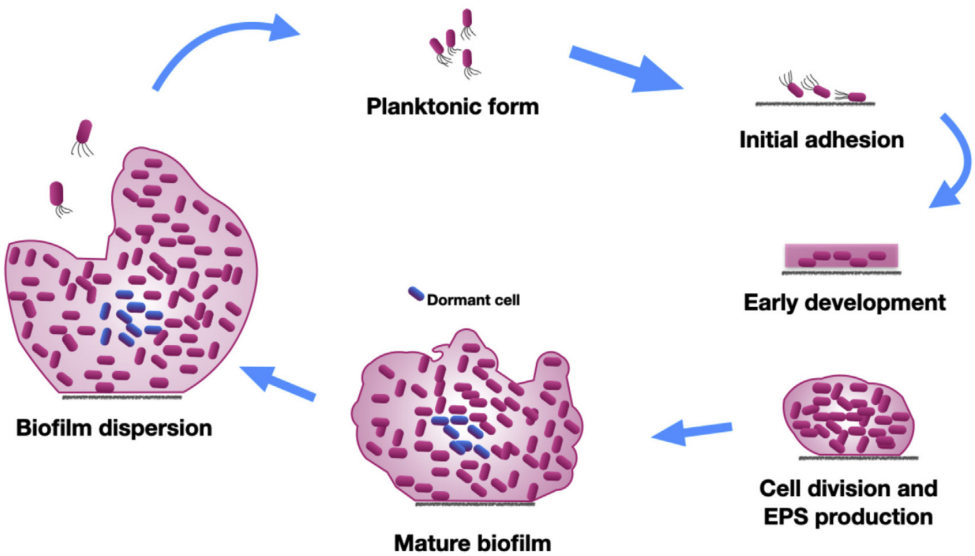

Figure 21. Stages of biofilm formation on material surfaces (Kreve and Reis, 2021).

The inocula in this thesis were left to grow until the cultivation reached late exponential phase, to maximise cell density for the inoculation of the reactor. As observed in the cultivations in **Paper I**, the EPS production that caused the

flocculation of biomass started in the late exponential phase of growth. Biomass flocculation was also observed in the seed before inoculation of the reactor. These cells, enveloped by EPSs, are likely to have interacted with pretreated solids immediately after inoculation. Biofilm formed on the solids during the long lag phase, and only after the start of biofilm dispersion was it possible to observe lactic acid production by the released cells. Subsequently, the free cells divided in the nutrient-rich medium and produced lactic acid at similar rates as those observed for the controls without solids.

3.3.3 Other considerations in the utilisation of pretreated softwood solids for SSF

The optimisation of SSF processes for lignocellulosic biomass is labour-intensive and time-consuming and depends on several variables (e.g., raw material, microorganism, deleted processes and desired products and their respective characteristics,). Some considerations that were derived from the study on pretreated softwood solids for SSF are presented below.

As mentioned in **Paper III**, a low solids loading (7% WIS) was used in this study. This was chosen to avoid the negative effects of high solids loadings (>15% WIS) in the mixing of the broth, and the thereby associated decrease in efficiency of the enzymatic hydrolysis of spruce (Kadić et al., 2014). This reduction also decreased the mixing power input that was required for complete fluid motion. Palmqvist et al. (2011) concluded that impeller speeds of 300 to 400 rpm are required to achieve complete fluid motion for enzymatic hydrolysis of whole slurry of pretreated spruce (pretreated by SO_2-catalysed steam explosion and not separated into liquid and solid fractions) (Palmqvist et al., 2011). For the study in **Paper III**, the pretreated softwood solids were mixed at 150 rpm, which was sufficient to effect adequate mixing of the material, based on visual inspection. This difference between the stirring speeds that were required for complete mixing might be associated with the different viscosities of the slurries.

Although viscosity was not measured in the current study, it is an important parameter in the scale-up of lactic acid production with lignocellulosic biomass. The potentially higher viscosity of the pretreated softwood slurry in Palmqvist et al. (2011) might be related to the water constraint that is created by higher concentrations of free monosaccharides in solution (Roberts et al., 2011; da Silva et al., 2020). On separation of the two fractions, the free monosaccharide content at the start of the cultivation declined, which might be linked to a decrease in the viscosity of the SSF broth that was examined in this thesis. Because a lower stirring rate was sufficient to effect complete mixing in **Paper III**, separation of the solid and liquid fractions might have been advantageous with regard to decreasing the

energy that is needed for the process (stirring power). In turn, this separation step could increase the initial solids content in an SSF process.

It is possible to apply the knowledge from this thesis in considering a hybrid SSF process for the utilisation of separate liquid and solid fractions of pretreated softwood for producing optically pure lactic acid. The cultivation could be initiated in a fed-batch process using a low concentration of liquid fraction, rich in hemicellulose monomers, to spur the growth of *P. acidilactici* and lactic acid production. Once exponential phase is initiated, pretreated solids and enzymes could be added to proceed with the SSF. The liquid and solid fractions could be fed to the reactor in fed-batch mode, to account for any substrate-related inhibition. Process optimisation would need to be examined carefully with regard to limitations due to microorganism viability, increases in the concentrations of toxic compounds, retention of insoluble solids in the media and product inhibition.

Although more work is needed to design the entire process, the potential for applying *P. acidilactici* strains in the industrial production of PLA from lignocellulosic biomass in a biorefinery setting is evident.

3.4 Post-consumer waste viscose as a carbon source

In addition to pretreated spruce biomass, waste viscose was studied as an alternative feedstock for *P. acidilactici*, given that it is less complex than wood, and contains cellulose as the only macromolecule. However, viscose fibres are modified in various ways for commercial applications and thus cannot be regarded simply as "cellulose". For its demonstrated resistance to inhibitory compounds formed during lignocellulosic biomass pretreatment, *P. acidilactici* was used to test optically pure lactic acid production with a glucose-rich solution obtained from the enzymatic hydrolysis of white and coloured waste viscose, forming the study in **Paper IV**. A simple proof-of-concept study was conducted and is presented below.

3.4.1 Enzymatic hydrolysis of waste viscose

Waste textile quality can vary according to fabric, pre-production and post-production additives added to the garment and level of use. The experimental work in this thesis used post-consumer waste viscose textiles. Post-consumer textiles contain other contaminants from daily life, such as various stains, remnants of ingredients in personal care products, sebum and sweat, that render post-consumer waste textile more challenging to use compared with virgin textile. Waste textile was sorted before hydrolysis by type of fibre and colour, all non-viscose elements were removed, and the textile was shredded.

Textile dyes and other additives are added to treat, colour, and finish fabrics. Many compounds that have long been used for clothing products are hazardous to the environment and organisms (Darbra et al., 2012). Campaigns, such as *Detox My Fashion* (Greenpeace International, 2018), have been lobbying for the elimination of these toxic chemicals from the textile industry, and policymakers have been publicising tools for the substitution of certain chemicals with safer alternatives (European Chemicals Agency, 2016). However, there remain many dyes and additives in discarded textiles that can hinder their reutilization. Moreover, post-consumer use textiles contain layers of stains and contaminants from daily use (e.g., food, oils, and sebum) that may interfere with the hydrolysis of the fibres. In the case study in this thesis, the production of optically pure lactic acid was evaluated in batch and fed-batch fermentations to determine whether the metabolism of *P. acidilactici* ZP26 is inhibited by white or coloured waste viscose solutions.

Figure 22 shows the colour difference between a control flask with glucose, flasks that were supplemented with three concentrations of white viscose waste solution (golden and brown media) and three concentrations of coloured viscose waste solution (black media). Instinctively, the dyes that give the darker colour to the flasks on the right were hypothesised to hinder lactic acid production by *P. acidilactici* ZP26. The preparation of these solutions is documented in **Paper IV**.

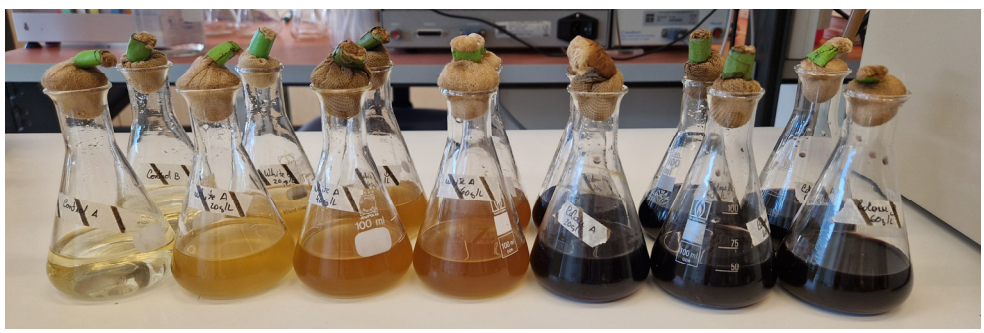

Figure 22. Shake flask trial with a glucose control and 20%, 40% and 60% waste viscose solution added, for white and coloured waste (from left to right).

In shake flask experiments, media that was buffered with calcium carbonate was used. The lactic acid yields and volumetric production rates for white and coloured waste viscose solutions were higher than in the control, and there was no evidence of inhibition (see **Paper IV**). Subsequent bioreactor cultivations were run with the highest concentration of both waste viscose solution (white and coloured), translating into a starting glucose concentration of approximately 50 g/L. The lactic acid production in these bioreactors is shown in Figure 23.

The results of the bioreactors with the two waste viscose solutions (white and coloured) were similar to those of the glucose control (Figure 23, A and C for batch

reactors and B and D for fed-batch reactors). As detailed in **Paper IV**, the glucose uptake rate and, consequently, lactic acid production rate decreased after the first 12 h of cultivation for all bioreactors. Nevertheless, yields exceeded 0.9 g D-lactic acid/g glucose for the batch reactors, and fed-batch reactors were run to assess production patterns with the addition of the respective carbon source after 12 h. Because the main objective was to evaluate lactic acid production, the only concern was to provide ample carbon for the organism. Thus, the decrease in glucose uptake rate was accompanied by a surplus of carbon source in the fermenter (residual glucose in Figure 23D).

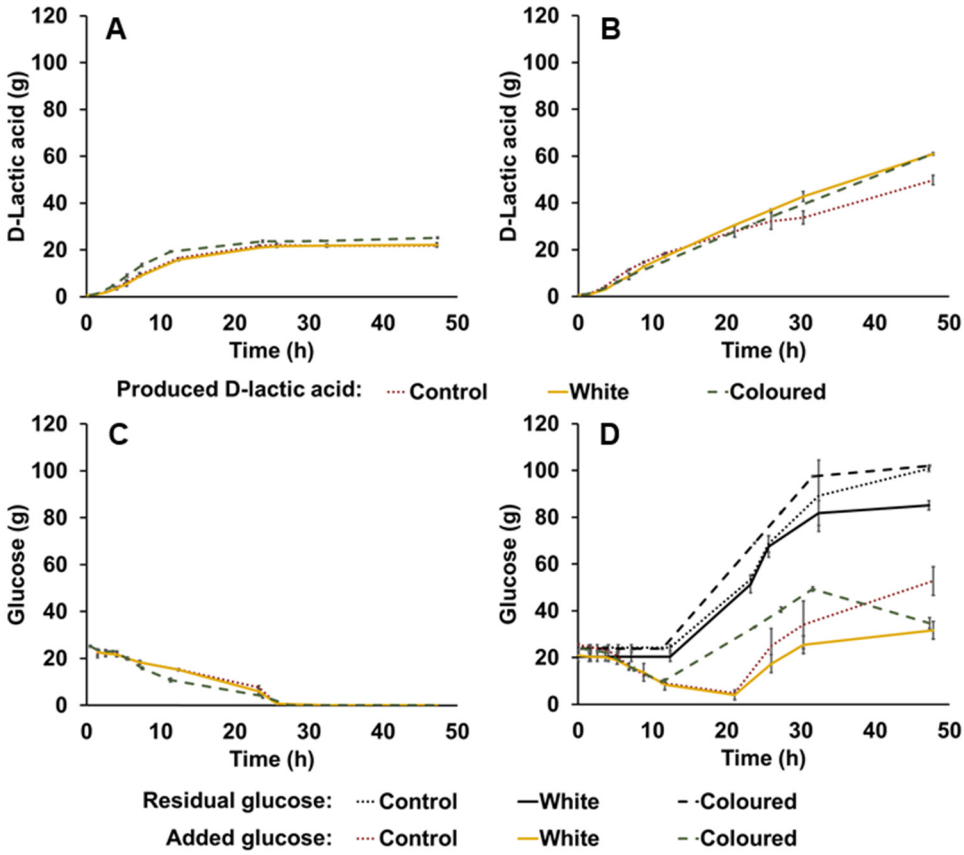

Figure 23. (A) D-lactic acid and (C) residual glucose amounts in batch reactors with white and coloured waste viscose hydrolysates against control experiments; (B) D-lactic acid and (D) residual and added glucose amounts in fed-batch reactors with white and coloured waste viscose hydrolysates against control experiments.

Notably, considering the dyes, additives and possible contaminants in solution, the results of the bioreactors were consistent with those of the shake flask experiments. Lactic acid production with the viscose solutions was slightly better compared with the control. The batch reactor with coloured viscose had the highest lactic acid volumetric production rate at 6.0 g/(L·h)—24% higher than that of the control. It is unknown what caused this result and if this finding is an isolated case for the viscose solutions in this study. The type and concentration of additives in solution will vary between batches of recycled textiles, and more hydrolysis and cultivation studies are necessary to achieve a representative average result for optically pure lactic acid production from post-consumer waste viscose. Nevertheless, these findings are positive with regard to the hydrolysis and utilisation of sugars from waste textiles, which should prompt continued research in this field.

4 Conclusions

This thesis examined two genetically modified strains, *Pediococcus acidilactici* TY112 and *Pediococcus acidilactici* ZP26, regarding their production of L- and D-LA, respectively, from softwood and waste viscose, two lignocellulosic raw materials. The work was driven by the desire to identify easily applicable solutions for the challenges encountered during the development of this thesis, to assist a possible scale-up of these processes for the industrial production of optically pure lactic acid.

After softwood pretreatment, the slurry was separated into a liquid and a solid fraction to study any possible sources of inhibition independently. This simple additional filtration step allowed the use of a fed-batch strategy for each fraction separately, to address the various challenges of each fraction. With the liquid fraction, the conversion of HMF and furfural by *P. acidilactici* TY112 improved L-lactic acid production. Moreover, a continuous feed maintained a constant, albeit limited, supply of glucose, which in turn increased the consumption of mannose. The liquid fraction is a complex medium, and the presence of inhibitors and an overall intricate matrix lowered the production of lactic acid. This inhibitory effect of the matrix did not arise during the SSF of the solid fraction, leading to the conclusion that the long lag phase in the cultivations was caused by solids-cell interactions. An inoculation with a limited amount of monomeric glucose was sufficient to spur the exponential phase of the cultivation, to which solids and hydrolytic enzymes were added without any detrimental effect on the lactic acid production. Ideally, monomeric glucose should be supplied as a non-inhibitory amount of liquid hydrolysate, for utilisation of the liquid and solid fractions in the cultivation.

Waste viscose was assessed as a new substrate for the production of optically pure lactic acid. The low degree of polymerisation of its cellulosic fibres renders viscose as the most suitable textile for fermentation as an option in propelling the carbon cycle of this material. No inhibition was observed for white or coloured textiles, constituting an important proof of concept that should encourage more explorative studies to produce PLA from recycled textiles.

Overall, this thesis has demonstrated that it is possible to use softwood and waste viscose to produce both lactic acid enantiomers separately. The potential of using two variants of the same parental bacterial strain for the production of two different

monomers can bring sizable advantages in process engineering and planning at large scale.

Knowledge on the two strains in this thesis, as well as improved strains, has expanded during the development of this thesis. Today, it is possible to produce and purify cyclic L-lactide and cyclic D-lactide, the building blocks for high-quality PLA, from lignocellulosic biomass, reinforcing the potential of these organisms as part of the production of PLA in a biorefinery.

5 Future Prospects

The production of PLA from various classes of lignocellulosic biomass has long been the subject of intensive research. Despite the knowledge that has been acquired and the many alternatives at every stage of this process, it has not yet been deemed as industrially feasible. There is always potential for optimisation, but it will require concrete actions for the application of these technologies in profitable industrial settings. Nevertheless, several suggestions are presented below for future research and development.

The project with *P. acidilactici*, of which this thesis is a part, has undergone several phases of strain improvement, that included evolutionary adaptation. This technique builds the metabolic machinery for specific conditions that can be indispensable for a well-established process. However, desirable strain characteristics might disappear after growth in less selective conditions, diluting confidence in strain performance. A long-term study on strain robustness and the procedures for maintaining peak performance would support the utilisation of adapted microorganisms in industrial settings.

As addressed in this thesis, unusual process engineering strategies such as separate feeding of the liquid fraction, rich in monomeric sugars and inhibitory compounds, and the solid fraction, rich in cellulosic glucose, might aid to the full utilisation of the lignocellulosic sugars. Hybrid processes like this are sometimes unconventional alternatives that might bring a competitive advantage in comparison with, for example, traditional SSF or SHF.

An important subject that was not studied in this thesis is downstream processing. The optical purity of lactic acid is only part of the solution for high-quality PLA, because a high-quality polymer also requires high chemical purity. Efforts are underway to develop a feasible purification process. As performed for the fermentation, the downstream process must be evaluated with various lignocellulosic materials and improved accordingly so that it can be scaled up.

Nevertheless, and perhaps most importantly, LCA or hotspot analysis of PLA production from several classes of lignocellulosic biomass should be performed. As a tool for assessing sustainability, an LCA should demonstrate the feasibility of optically pure lactic acid production processes and help define a course of action. These analyses are laborious yet fundamental for their application on a commercial scale.

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Optically pure lactic acid production from lignocellulosic biomass

This thesis is a culmination of 15 years in Biotechnology, contributing to the industrial production of biomass and biochemicals, and the operation of bioreactors. Industrial biotechnology is a valuable tool for more environmentally friendly production strategies. And I can only aim to continue to work hard, lending my knowledge and passion to make the world a better place.

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