

Perennial ryegrass as feedstock for the bio-based production of chemicals

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A mio padre

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Abstract

The economic and environmental challenges are driving the search for alternative and sustainable energy resources. In this scenario, lignocellulosic biomasses are gaining more attraction due to their high abundance, renewability, and low cost, and can therefore contribute to the development of a bio-based economy. Grass materials from dedicated crops or crop residues can be utilized as feedstock in green biorefinery platforms. In these platforms, the biomass is firstly fractionated into a press-cake and a press-juice. Then, further technologies are applied including the extraction of valuable compounds from the press-juice, and the utilization of the press-cake in feed, material, pulp and paper, and fuel applications. This thesis outlines the development of an innovative green biorefinery platform using the perennial ryegrass *Lolium perenne* for the production of chemicals. To accomplish this, all the conversion steps—including biomass pretreatment, enzymatic hydrolysis, and fermentation—were carried out to promote the utilization of both the press-cake and the press-juice. Mechanical pretreatment was identified as the most efficient method to get sugars from enzymatic hydrolysis, and to separate the press-cake and the press-juice. Different bioprocesses were investigated to convert the press-cake and the press-juice into the final chemicals. The press-cake was utilized as substrate in a Consolidated Bioprocess where *Aspergillus niger* was used as a biocatalyst for citric acid production. The nutrient-rich press-juice was exploited in ethanol and lactic acid fermentation. For ethanol production, short-term adaptation was applied to enhance the fermentation capacities of *Saccharomyces cerevisiae* in pure press-juice. Lactic acid production was ensured by optimizing the press-juice as a fermentation medium at bench- and reactor-scale. Overall, the main findings of this thesis demonstrate that, despite their limitations, lignocellulosic biomasses—including the perennial ryegrass—are valuable feedstocks for the bio-based production of chemicals, fuels, and materials.

Zusammenfassung

Die wirtschaftlichen und ökologischen Herausforderungen treiben die Suche nach alternativen und nachhaltigen Energieressourcen voran. In diesem Szenario gewinnen lignozellulosehaltige Biomassen aufgrund ihres großen Vorkommens, ihrer Erneuerbarkeit und ihrer geringen Kosten zunehmend an Attraktivität und können daher zur Entwicklung einer biobasierten Wirtschaft beitragen. Grasmaterial aus bestimmten Kulturen oder Ernterückständen kann als Ausgangsmaterial in grünen Bioraffinerie-Plattformen verwendet werden. In diesen Plattformen wird die Biomasse zunächst in einen Presskuchen und einen Presssaft fraktioniert. Anschließend werden weitere Technologien angewandt, darunter die Extraktion wertvoller Verbindungen aus dem Presssaft und die Nutzung des Presskuchens als Futtermittel, Material, Zellstoff und Papier sowie als Brennstoff. In dieser Arbeit wird die Entwicklung einer innovativen grünen Bioraffinerieplattform unter Verwendung des mehrjährigen Weidelgrases *Lolium perenne* für die Herstellung von Chemikalien beschrieben. Zu diesem Zweck wurden alle Umwandlungsschritte - einschließlich der Vorbehandlung der Biomasse, der enzymatischen Hydrolyse und der Fermentation - durchgeführt, um die Verwertung sowohl des Presskuchens als auch des Presssaftes zu ermöglichen. Die mechanische Vorbehandlung erwies sich als die effizienteste Methode zur Gewinnung von Zuckern aus der enzymatischen Hydrolyse und zur Trennung von Presskuchen und Presssaft. Zur Umwandlung des Presskuchens und des Presssaftes in die endgültigen Chemikalien wurden verschiedene Bioprozesse untersucht. Der Presskuchen wurde als Substrat in einem konsolidierten Bioprozess verwendet, bei dem *Aspergillus niger* als Biokatalysator für die Herstellung von Zitronensäure eingesetzt wurde. Der nährstoffreiche Presssaft wurde für die Ethanol- und Milchsäuregärung genutzt. Für die Ethanolproduktion wurde eine kurzfristige Anpassungsstrategie angewandt. Dies führte zu einer Verbesserung der Fermentationskapazitäten von *Saccharomyces cerevisiae*, die in reinem Presssaft kultiviert wurde. Die Milchsäureproduktion wurde durch Optimierung des Presssaftes als Fermentationsmedium im Labor- und Reaktormaßstab sichergestellt. Insgesamt zeigen die wichtigsten Ergebnisse dieser Arbeit, dass lignozellulosehaltige Biomassen - einschließlich des mehrjährigen Weidelgrases - trotz ihrer Einschränkungen wertvolle Ausgangsstoffe für die biobasierte Produktion von Chemikalien, Kraftstoffen und Materialien sind.

List of Publications

- I. **L. Varriale** and **R. Ulber**; Fungal-based biorefinery: from Renewable Resources to Organic Acids; *ChemBioEng. Rev* (2023), 10, 3 (1-22). DOI: 10.1002/cben.202200059.



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- II. **L. Varriale***, **M. Volkmar***, **J. Weirmuller**, **R. Ulber**; Effects of Pretreatment on the Biocatalysis of Renewable Resources; *Chem. Ing. Tech* (2022), 94 (11). DOI: 10.1002/cite.202200137.



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- III. **L. Varriale**, **K. Kuka**, **N. Tippkötter**, **L. Olsson**, **R. Ulber**; Development of a Consolidated Bioprocess for the Production of Citric Acid using *Aspergillus niger* as biocatalyst; (2024)
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- IV. **L. Varriale**, **D. Geib**, **R. Ulber**; Short-term adaptation as a tool to improve bioethanol production using grass press-juice as fermentation medium; *Appl Microbiol Biotechnol* (2024), 108: 393. DOI: 10.1007/s00253-024-13224-0.



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- V. **L. Varriale**, **J-N. Hengsbach**, **T. Guo**, **K. Kuka**, **N. Tippkötter**, **R. Ulber**; Sustainable Production of Lactic Acid Using a Perennial Ryegrass as Feedstock – A Comparative Study of Fermentation at the Bench- and Reactor-Scale, and Ensiling; *Sustainability* (2024), 16(18), 8054. DOI: 10.3390/su16188054.



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List of Abbreviations

1G: first-generation biorefinery or first-generation biomass

2G: second-generation biorefinery or second-generation biomass

ALE: Adaptive Laboratory Evolution

BBCH: phenological development stage of plants

CA: Citric Acid

CBP: Consolidated Bioprocessing

CF: Crude Fibre

CDW: Cell Dry Weight

CGQ: Cell Growth Quantifier

CP: Crude Protein

DM: Dry Matter

E: Enzyme

ES: Enzyme-Substrate

FA: Fumaric Acid

FM: Fresh Mass

GA: Gluconic Acid

GB: Green Biorefinery

GH: Glycosidic Hydrolase

GRAS: Generally Recognized As Safe

HPLC: High Performance Liquid Chromatography

IA: Itaconic Acid

IC: Ion Chromatography

LA: Lactic Acid

LAB: Lactic Acid Bacteria

LCA: Life-Cycle Assessment

LCB: Lignocellulose Biomass

LHW: Liquid Hot Water

LMPO: Lytic Polysaccharide Monooxygenase

MA: Malic Acid

MRS: De Man – Rogosa – Sharpe medium

NIRS: Near Infrared Spectrophotometry

NREL: National Renewable Energy Laboratory

OD: Optical Density

OPA: Ortho-PhtAldehyde

OS: OrganoSolv

PLA: Polylactic Acid

PTFE: Polytetrafluorethylene

S: Substrate

SA: Succinic Acid

SDG: Sustainable Development Goals

SmF: Submerged Fermentation

SSF: Solid-State Fermentation or Simultaneous Saccharification and Fermentation

SSCF: Simultaneous Saccharification and Co-Fermentation

TEA: Techno-Economic Analysis

USD: United States Dollar

UV: Ultraviolet

YPD: Yeast Peptone Dextrose medium

WSC: Water Soluble Carbohydrates

Chapter I – Introduction

1 Facing the problems of the 21st century: lignocellulosic biomasses as a solution

The 21st century has been characterized by rapid globalization and industrialization driven by the consumption of different energy sources [1]. As a result, the world population has increased and it is estimated to reach 9.7 billion people by 2050 [2]. To support this growing population, finite fossil resources have been largely overexploited causing severe environmental concerns including pollution, climate change, waste disposal, and natural resources reduction [2]. In order to overcome these problems, the replacement of conventional fossil resources such as coal, oil, and natural gas with more sustainable sources has become imperative.

Sustainable energies include wind, solar, hydro, tidal, and biomass [3,4]. Among them, biomass is considered one of the most promising feedstocks due to the high availability, versatility, renewability, and carbon neutrality [5]. The economy based on the utilization of biomass as feedstock for the production of chemicals, fuels, and materials is defined as a bio-based economy (or bioeconomy) [6]. To accelerate its development, the European Union has presented several policies including the European Green Deal and the Bioeconomy Strategy to reach the carbon-neutrality by 2050 and to contribute to the Sustainable Development Goals (SDGs), respectively [7–9].

It is reported that over 200 billion tons of biomass are produced every year [3], accounting for 9.4% of the global energy supply and 10% of chemical feedstock [10]. Currently, non-edible resources such as lignocellulosic biomasses (LCBs) are gaining significant attention for bio-based production. LCBs are the most abundant resources on earth supplied from agriculture residues (dedicated crops and crops residues), forestry, industry (process residues and wastes), domestic activities (organic wastes), and aquaculture (algae and seaweed) [11]. To convert LCBs into the final product(s), several technological processes can be applied using a biorefinery approach [12].

1.1 Biorefinery concept, distribution, and classification

Biorefinery is considered one of the key strategies facilitating the bio-based economy, which enables to close the loop of raw materials, water, minerals, and carbon [2]. Thus, biorefining is defined as the sustainable conversion of biomasses and their side-streams into a spectrum of marketable products and energies [13]. According to this, the biorefinery chain includes different conversion pathways (thermochemical, biochemical, microbial, etc.) to separate the biomass resources into their building blocks (carbohydrates, lignin, proteins, fibres, triglycerides, etc.) that can be converted into platform or commodity chemicals, fuels and high-value products (**Fig. 1**) [12]. In this scenario, carbon sequestration, bioenergy generation, and the production of diverse products are the main advantages of using biomass as a renewable carbon source [14]. One critical point that has to be considered is the integrated utilization of biomass that aims to maximise the use of the feedstock— including the wastes generated during the conversion pathways— to produce valuable product-streams [14,15]. According to this, multiple products can be extracted from the same feedstock [14].

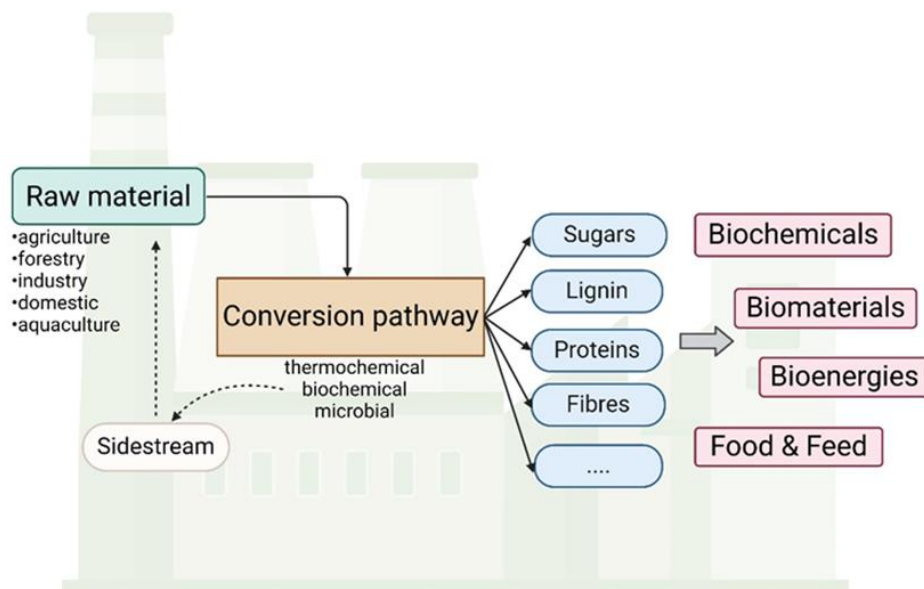


Fig. 1: Schematic representation of a biorefinery: raw materials can be supplied from agriculture, forestry, industry, domestic residues, and aquaculture. The raw material is processed using different conversion pathways to release its building blocks which can be transformed into different bioproducts.

Overall, these characteristics highlight how biorefining synergistically supports different SDGs, including access to sustainable energy, responsible production and consumption, and contrast climate change [16].

Currently, Europe accounts for approximately 2362 bio-based industry and biorefinery facilities, out of which 2186 are at commercial scale (**Fig. 2**). Among them, only 33% are product-energy integrated plants, and they are concentrated mainly in Germany (594), France (356), Sweden (302), and Italy (222).

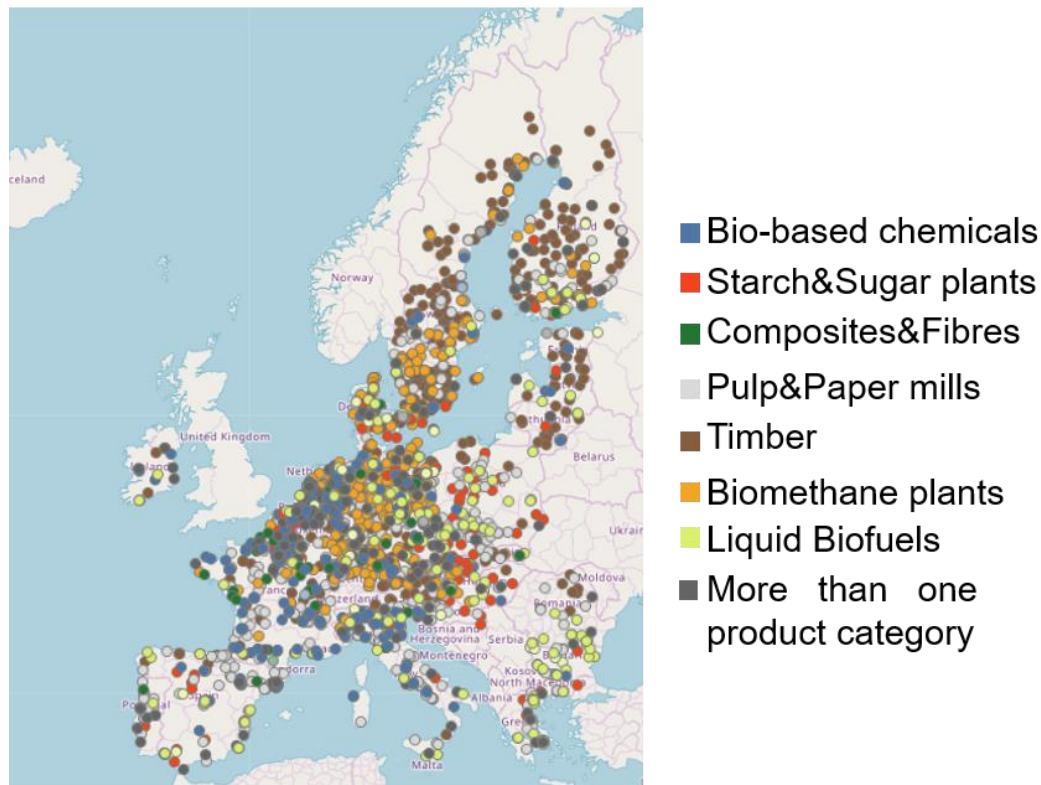


Fig. 2: Bio-based industry and biorefinery facilities across Europe. The figure was adapted from [17].

A general classification of biorefineries can be done based on the feedstocks, platforms, products, and conversion processes used [13]. Based on feedstocks used, biorefineries can be divided into conventional biorefineries (sugar, starch, and vegetable oils), lignocellulosic biorefineries (woody biomasses), green biorefineries (green grass, green crops and cereals), and marine biorefineries (micro and macroalgae) [18].

1.2 Green Biorefinery

Among the different biorefinery categories, Green Biorefinery (GB) has emerged as a multiproduct system that utilises green biomass [19,20]. The core concept of GB is to fully exploit the green raw material to generate a panel of different products, either valuable on their own or serving as input for other production lines. [19]. Generally, GB consists of a primary processing step that involves a mechanical wet fractionation to convert the biomass into a liquid (press-juice) and a solid fraction (press-cake) [20,21]. Both fractions have an economic value and can be further refined in a secondary processing step [19]. The juice fraction contains several compounds including sugars, proteins, lipids, organic acids, vitamins, minerals, enzymes, and dyes that are extracted and used for animal or human consumption [20, 22]. In some cases, it was reported the utilization of the juice as bio-fertiliser and bio-stimulant, or in anaerobic digestion for biogas production [23,24]. The press-cake is a fibre-rich fraction that can be used in several applications such as for animal feed (ensiling), insulation material, fibre board, biocomposites, packaging material, pulp and paper, and bioenergy [20,25,26]. Some examples of GBs in Europe are documented in literature and are reported in Table 1.

Table 1. Current Green Biorefineries in operation across Europe

Facility	Country	Feedstock	Products	Scale	URL
Upper	Austria	Ensiled grass and legumes	Lactic acid, amino acids, biogas	Demonstration	https://www.botres.com/
Biorefinica	Germany	Grass	Proteins, fermentation media, biogas	Demonstration	http://www.biorefinica.de/
Newfoss	The Netherlands	Grass	Materials, fibers	Commercial	https://newfoss.com/en/
Grassa	The Netherlands	Grass	Proteins, prebiotic sugars, bio-fertilizers	Commercial	https://grassa.nl/en/
Gramitherm	Belgium	Grass	Materials	Commercial	https://gramitherm.eu/
Biowert	Germany	Grass	Material, fertilizer	Commercial	https://biowert.com/en/
Biorefinery Glas	Ireland	Grass	Proteins, fertilizer, biogas	Demonstration	https://biorefineryglas.eu/

1.2.1 Green biomass

Green biomasses include a wide range of raw materials supplied from dedicated crops and crop residues including grasses, green crops, and immature cereals [20,22]. These lignocellulosic materials primarily consist of structural carbohydrates (40-60% cellulose, 20-40% hemicellulose, and 10-25% lignin), along with pectin, proteins, acids, salts, minerals, dyes, hormones, amino acids, and enzymes [22,27]. The overall

lignocellulose structure is summarised in **Fig.3**. The qualitative and quantitative composition of carbohydrates varies depending on the plant variety, its developmental stage, and environmental conditions [27,28]. For instance, with the maturity of the plant, the structural carbohydrates increase and the other cell components tend to decrease [27,29].

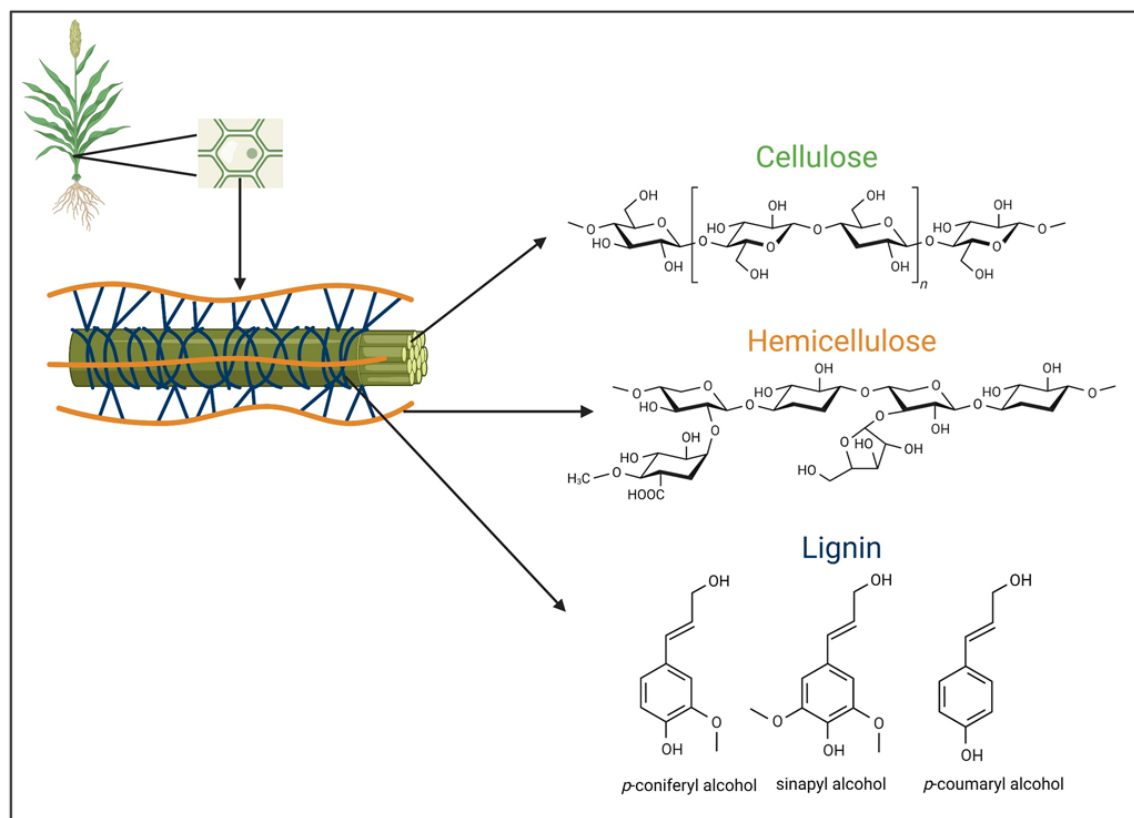


Fig. 3: Lignocellulose biomass structure. Cellulose (green) is made up of repeating D-glucose units linked covalently by β -(1,4) glycosidic bonds; hemicellulose (orange) is a heteropolymer of hexoses and pentoses, lignin (blue) is a heterogeneous polymer with a different number of three aromatic alcohols: p-coumaryl, coniferyl, and synapyl alcohols. The figure has been created on BioRender.com.

Cellulose is the major structural homopolymer that consists of linear chains of repeating D-glucose units linked covalently by β -(1,4) glycosidic bonds [30,31]. The degree of polymerization of cellulose differs among the plants and it ranges from 500 to 15'000 units [32]. The cellulosic chains are interconnected through hydrogen bonds to form microfibrils [32,33]. Hemicellulose is a branched heteropolysaccharide. Depending on the source and type of plants, hemicellulose contains pentoses (D-xylose, D-arabinose) and/or hexoses (D-glucose, D-mannose, D-galactose), and, in

small proportion, uronic acids and acetyl groups [32,34]. Lignin is a highly heterogeneous biopolymer insoluble in water with a different number of three aromatic compounds: p-coumaryl, coniferyl, and synapyl alcohols. It links the cellulose and hemicellulose by covalent, ester, and hydrogen bonds leading to increased mechanical strength, rigidity, and impermeability of cell walls [32,34]. Another important polymer is pectin which is found in the primary wall of the plant cell walls. It can be described as a complex family of heteropolysaccharides that contains approximately 70% galacturonic acid [34].

Overall, due to the high biomass yield (8-150 t/hectare), broad adaptability, greater ability to capture carbon, and high carbohydrate content (two-thirds of cell wall dry matter) [19], green biomasses are interesting candidates for bio-based production.

1.3 Aim of the thesis

It is estimated that more than one-third of the total agricultural area in Europe is covered by grasslands. In Germany alone, permanent grasslands represent about 28% of the total land area, highlighting their critical role in the country's agriculture scene [35,36]. Due to the high availability and productivity, grass raw materials have emerged as ideal feedstocks for the development of GB. GB can be directly integrated into pre-existing farming systems, thus supporting a more sustainable primary production sector, providing new business models in rural areas, and valorising green waste streams [21]. In addition, GB ensures the integrated utilization of the biomass for the sustainable production of food, feed, chemicals, materials, and fuels.

The aim of this thesis was to develop an innovative Green Biorefinery concept. Unlike the conventional GBs, the driving idea was to promote the utilization of both the solid and the liquid fractions as substrates in different biochemical processes. In particular, the German perennial ryegrass *Lolium perenne* was used as raw material for the production of chemicals (citric acid, ethanol, and lactic acid) (**Fig.4**). *Lolium perenne* was chosen due to the low water and fertilizer requirements, the high and stable biomass yield, and the strong resistance to diseases [30]. Firstly, different pretreatment methods were explored to break down the lignocellulose structure and to reduce the biomass recalcitrance to enzyme digestion. Mechanical pretreatments were performed to separate the press-cake and the press-juice, and were even combined with harsher pretreatment methods. The resulting cake residues were

enzymatically hydrolysed to assess which pretreatment method resulted in the highest sugar release. The results of these investigations are reported in **Chapter III**. Another possible utilization of the press-cake fraction is discussed in **Chapter IV** where a Consolidated Bioprocess was developed to produce citric acid. *Aspergillus niger* was chosen as a biocatalyst due to its ability to produce citric acid and to secrete hydrolytic enzymes. Both the acid titer and the enzymatic activity were quantified. Finally, Confocal Laser Scanning Microscopy analyses were performed to evaluate the interaction between the fungus and the press-cake and to assess the fungal morphology. The microscopy analyses were performed at Chalmers University (Sweden) under the supervision of Prof. Lisbeth Olsson and Dr. Vera Novy.

The utilization of the press-juice is discussed afterward. In **Chapter V**, *Saccharomyces cerevisiae* was selected as a microorganism for the production of ethanol. In this work, it was initially investigated the ability of the yeast to produce bioethanol in pure press-juice. Then, the fermentation performances and efficiencies of the yeast were improved using the short-term adaptation strategy. Several physiological parameters were evaluated and calculated in both the parental and adapted strains, and the results were compared. In **Chapter VI**, lactic acid was produced using the homofermentative bacteria *Lactobacillus delbrueckii* subsp. *lactis*. In this section, the press-juice used as a fermentation medium was optimized according to previous studies. The resulting press-juice medium was tested firstly at a bench-scale using a pH-control system, and then at a reactor-scale. The results were compared with those obtained using the conventional complex medium. As state-of-the-art, biorefineries based on the use of filamentous fungi as main microorganisms are discussed in **Chapter II**. The aim was to highlight the potential of filamentous fungi in biorefinery platforms for the production of organic acids.

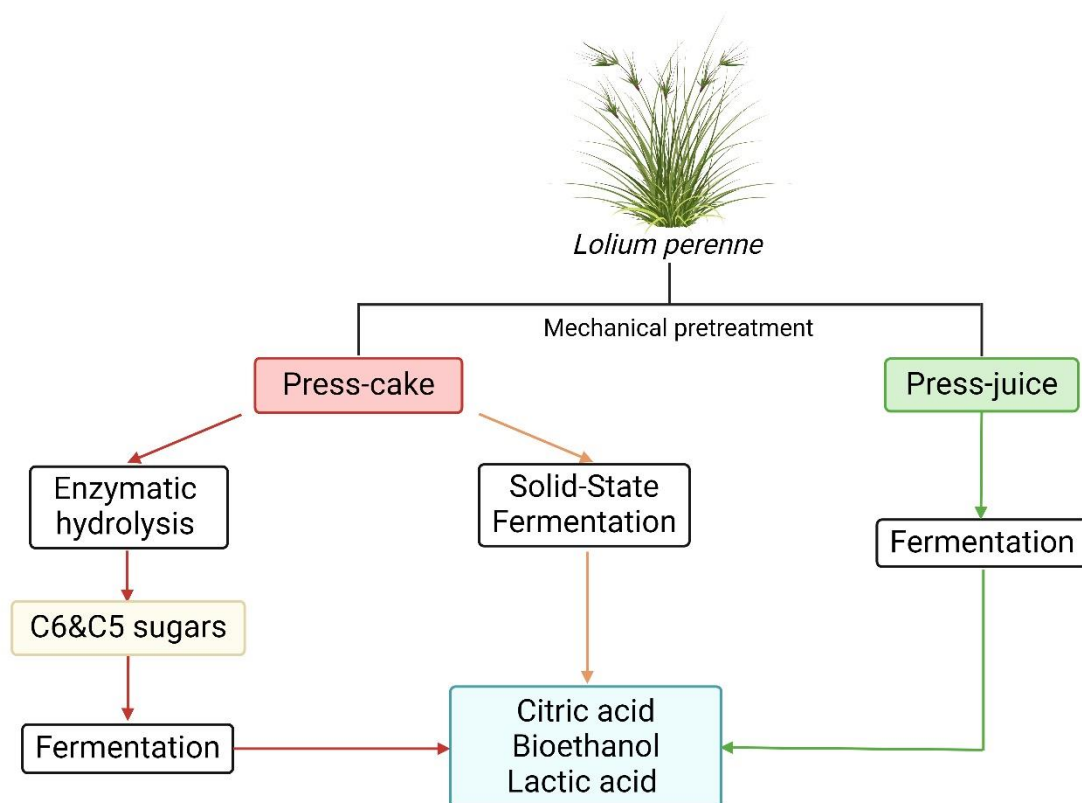


Fig. 4: Schematic representation of the Green Biorefinery concept of this thesis. *Lolium perenne* is mechanically pretreated to separate the press-cake (red box) and the press-juice (green box). The utilization of the press-cake is discussed in Chapter III and Chapter IV for the production of C5 and C& sugars (yellow box) and citric acid (blue box), respectively. The use of press-juice as fermentation media is examined in Chapter V and Chapter VI for ethanol and lactic acid production (both in blue box). The figure was generated using BioRender.com.

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Chapter II – Paper I

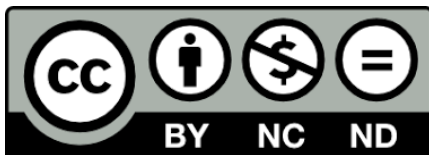
Fungal-Based Biorefinery: From Renewable Resources to Organic Acids

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Authorship contribution:

L. Varriale: investigation, conceptualization, writing – original draft, visualization

R. Ulber: supervision, writing – review and editing, project administration, funding acquisition

Keywords: Bioeconomy, Biorefinery, Filamentous fungi, Lignocellulosic biomass, Organic acids.

Abstract

Biorefineries are facilities in which lignocellulosic biomasses are converted in a wide range of bioproducts facilitating the transition from the use of petrochemical resources to renewable ones. Organic acids are considered very attractive for their utilization in different industrial areas as building blocks or as final bioproducts leading to a considerable market growth. They are metabolites which are naturally produced by microbials. The production of these molecules by filamentous fungi are attracting more attention due to their ability to hydrolyze lignocellulosic biomasses and to contextually produce different organic acids. Contrarily to a lot of other microorganisms, fungi have the ability to ferment pentoses, broadening the substrate utilization. The integrated use of lignocellulosic biomasses as material input and fungi as biocatalyst can contribute to make biorefineries more successful. This review gives an overview about the lignocellulosic biomass structure and hydrolysis, fungal morphology and how they are connected. Further, it describes some relevant organic acids with regards to their processes, biocatalysts, industrial applications and market considerations.

1 Introduction

In the recent years, the over-production of energy, chemicals and synthetic materials from petroleum-based resources lead to several environmental issues. In particular, pollutions, climate change, global warming, waste disposal and natural resource reduction have reached devastating levels [1]. Nowadays, all the countries are gaining deep attention to energy security, environmental concerns as well as to the development of alternative sources of chemicals [2]. It is recognized that the use of fossil resources has several disadvantages, such as an unequal distribution on Earth, price vacillation, and not renewability. Taking together, all these characteristics make the use of fossil resources unsustainable for the future [3,4]. In order to overcome the present and the future needs of humanity and to contrast the recent energy pandemic, biomass is emerging as an alternative renewable energy source for the production of fuels and chemicals [5]. In this view, the use of the renewable raw materials and/or key components to produce different bioproduct(s) (bioenergy, biochemicals, biomaterials) was defined bio-based economy or bioeconomy. This new and greener economy model aims to replace fossil-based products by using the full potential of all types of sustainable sourced biomass due to its versatility, renewability and its carbon-

neutral character [1,6,7]. The exploitation of renewables results in the development of biorefineries. To be economically relevant, several and different bioproducts (high and low value products) must be produced at the same time. Among the others, lignocellulosic biomasses (LCBs) are the most abundant natural carbon resource on Earth. They are typically supplied from agriculture (dedicated crops and crop residues), forestry (woody materials), industry (residues and wastes) and domestic activities (organic residues), aquaculture (algae, seaweed) [8]. Because of the different sources, these feedstocks have different composition and proportion in terms of basic components: cellulose (30-50% w/w), hemicellulose (20-40% w/w) and lignin (15-25% w/w) [9,10]. Cellulose is a linear homopolymer consisting of glucose subunits, hemicelluloses are short-branched heteropolymers of pentose and hexose sugars, and lignin is a cross-linked polymer composed of three different substituted aromatic phenols[11]. In order to convert biomass resources to biofuels and biochemicals, they are separated in their building blocks (i.e. carbohydrates) (**Fig. 1**) [8]. As mentioned above, biorefineries are able to transform feedstocks into bio-fuels as well as chemicals. Among the latter, organic acids are key products that can be produced by microbial processes either as natural products or as natural intermediates [12]. Depending on the specific acid, many applications (e.g., food, pharmaceutical, cosmetic, and textile industry) can be proposed [13]. Several attempts have been made to produce organic acids using raw material as substrate by either bacteria or fungi. Akerman et al., reported the production of acetic acid by *Cellulomonas uda* using brewers' spent grain as feedstock [14]. Lactic acid was produced by *Lactobacillus* spp. on several raw materials, such as brewers' spent grain [15,16], Corn Stover and Corncobs [17]. Further, succinic acid was produced by *Actinobacillus* spp. using municipal solid waste [18], sugar beet pulp [19], and foot waste [20], among the others. Traditionally, bacteria and yeasts have been employed as cell factories to produce bioproducts. However, filamentous fungi can be considered more suitable as biocatalysts in biorefineries. In particular, filamentous fungi are able to grow on low cost substrate due to their ability to secrete a wide range of hydrolytic enzymes. Furthermore, they can ferment pentoses (mainly xyloses and arabinose) and are well recognized as major organic acid producers. Another aspect to take in consideration is the easy separation of the fungal biomass from the fermentation media, which makes the downstream processing effortless. The recovered biomass of filamentous fungi

can be furthered exploited due to the proteins and lipids contained, thus increasing the circularity of the process. All these statements enable to consider filamentous fungi suitable candidate in biorefineries [21–24]. Nonetheless, some limitations related to the use of filamentous fungi have to be mentioned. Firstly, the morphological complexity which causes problems during the fermentation step, especially for the mass and oxygen transfer limitation and the viscosity of the fermentation broth [25–28]. According to this, scale-up processing results to be complicated [29]. Furthermore, filamentous fungi have to observe some production process requirements. The biocatalyst has to be compelled to growth and to convert the biomass of interest. It has to produce the final bioproduct with high titer (50-100 g/L), high yield (more than 0.5 g/g) and high productivity (1.3 g/L h) [13,30]. Lastly, the final bioproduct should be cheap to compete with the petroleum-based counterpart (about € 2 per kg) [29,31]. It is clear that wild-type microorganisms might be not able to ensure these assumptions. To overcome all these issues, genetic modifications in the biocatalyst as well as the development of a consolidated bioprocess (CBP) may represent possible solutions [13,29,32]. CBP integrates enzyme production, biomass hydrolysis and sugars fermentation in a single step [33,34]. According to this, CBP requires an efficient biocatalyst capable of producing efficient hydrolytic enzymes, consuming both pentoses and and tolerating lignin-derived inhibitors [33,35].

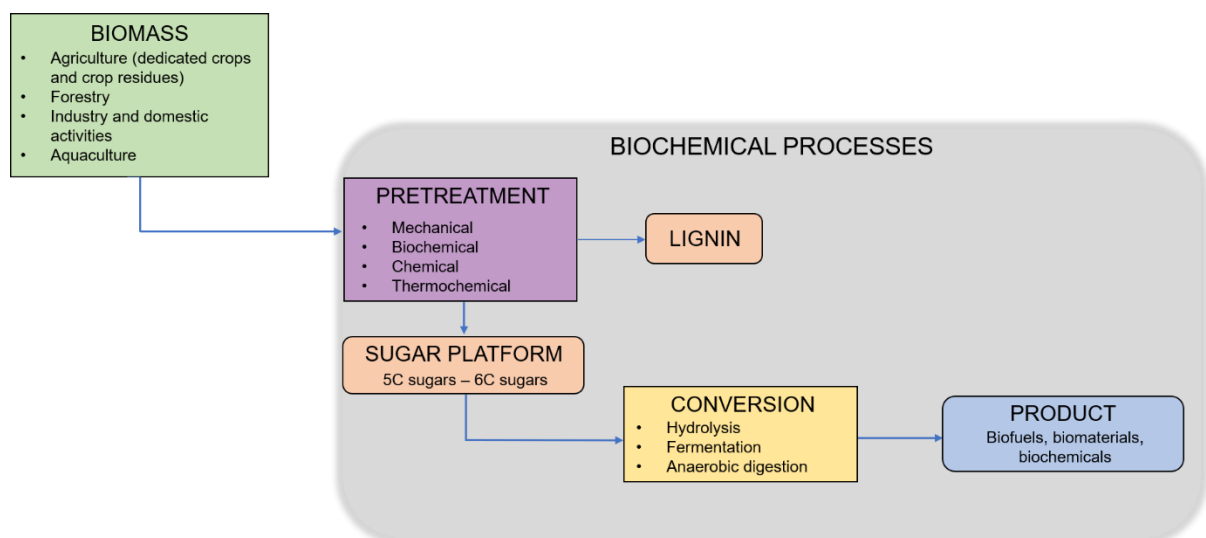


Fig. 1: Biochemical conversion process: from renewable resources to final product(s). Biomasses can be transformed using biochemical technologies. Firstly, pretreatments

are carried out to remove lignin from cellulose and hemicellulose. Then, the obtained sugar platforms are converted into final bioproducts.

Several works about the biomasses, organic acid production and recovery are available in literature [12,13,29,36–40]. However, the present review considers all the processes involved within a biorefinery platform. In particular, the aim of this review is to assess the production progress on the common organic acids by using filamentous fungi. Firstly, a short overview on the fungal morphology as well as the factors that influence it is discussed. Considering the general composition of LCB, the saccharification process and the advantages of using fungi on biomasses will be introduced. Afterwards, it will be presented the biotechnological production routes of the most relevant organic acids, emphasizing the advantages and the features of these acids with respect to their industrial applications. Downstream strategies for organic acids recovery and refining are briefly discussed. Finally, techno-economic aspects, current bottlenecks in the commercial production of organic acids and future perspectives are examined.

2 Fungi: features and morphologies

Fungi are eukaryotic organisms that naturally are able to (bio)degrade organic material. Filamentous fungi are structurally characterized by units called hyphae, which in turn can aggregate to form a mycelium [41]. At this point, mycelia can be further differentiated into micro- and macroscopic morphology [42]. The micromorphological fungal growth was described by Krull et al., who stated that the process starts from spores. Spores are quiescent structures which are not able to further moved to other morphologies until the nutrients necessary for activation are supplied. Germination is the subsequent phase and it consists in the extension of the spores due to a swelling process. Finally, a singular germination tube emerges [43]. In submerged cultivations, the final macromorphology is affected by strain properties and cultivation conditions (e.g., inoculum properties, pH, cultivation temperature, and medium composition). All these parameters influence the final macroscopic morphology that can vary from freely dispersed mycelium, mycelial clumps and pellets (**Fig. 2**) [43,44]. Pellets are spherical, branched agglomerates of several hyphae [43,45]. Two different types of formation processes are known: the coagulative and

the non-coagulative. In the coagulative formation (e.g., *Aspergillus* spp.), the electrostatic and salt bridging between the growth media and the polysaccharides caused the thickening of the spores. At the end, the spores of the coagulative type turn in pellets. On the contrary, spores of the non-coagulative type (e.g., *Rhizopus* spp.) germinate before pellet formation. Some fungi, including *Penicillium chrysogenum*, exhibit characteristics of both the coagulative and non-coagulative type: different hyphae are combined to form hyphal clumps which can further agglomerate to pellets [46,47]. Cairns et al., reported a hypothetic strategy for the formation of loose clumps. It could be possible that clumps are formed due to the culture conditions which disrupt or inhibit agglomeration [47].

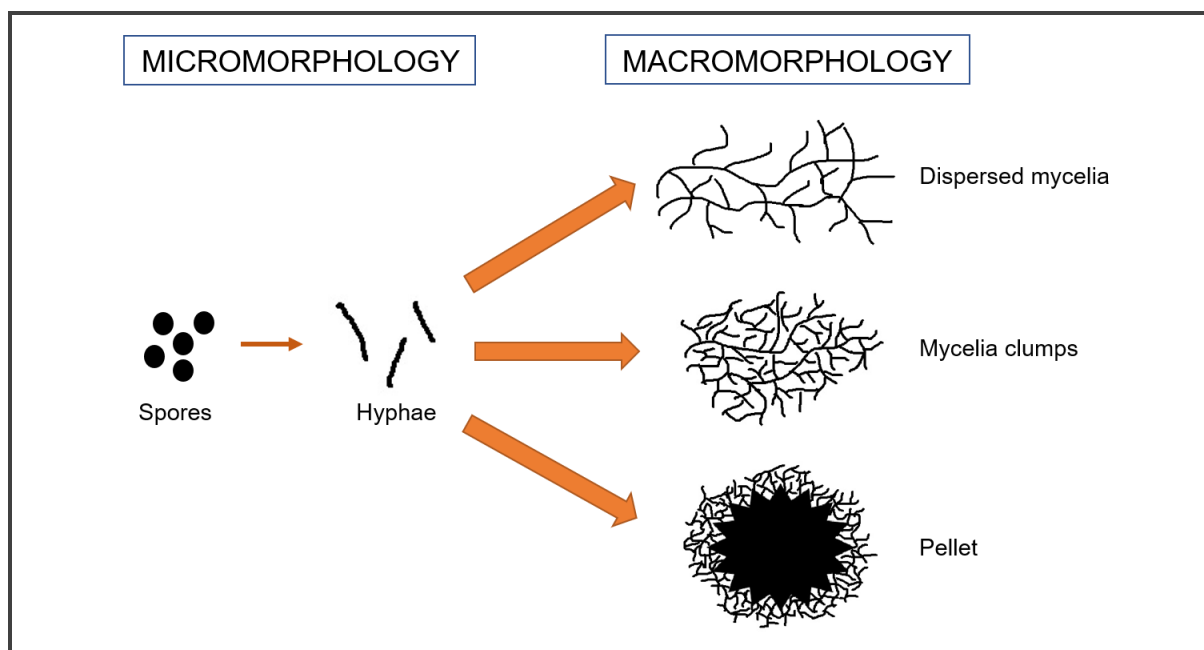


Fig. 2: Schematic representation of micro- and macromorphologies of filamentous fungi. The micromorphologies consist of spores that germinate to hyphae when the environmental conditions are opportune. Macromorphologies include dispersed mycelia, mycelia clumps and pellets.

It is recognized that the mycelial macromorphology is unpredictable since several factors affect this morphology [25]. Among the others, important factors include the carbon source and its concentration [48], nitrogen and phosphate limitation [49], pH [49], agitation [49,50], spore inoculum level [51], oxygen enrichment [52], and osmolarity [53].

Among the different filamentous fungi groups, *Zygomycetes* and *Ascomycetes* spp. have been employed in a wide range of industrial sectors [23]. For instance, *Ascomycetes* have been used in white biotechnology sectors for the production of antibiotics (*Penicillium chrysogenum*) [54], enzymes (*Aspergillus* spp. and *Trichoderma* spp.) [55], pigments (*Monascus* spp.), and human food products (*Fusarium* spp. and *Neurospora* spp.) [56]. Particularly, *Aspergillus* spp. are exploited for the commercial production of organic acids, which are considered relevant for the big market potential [23]. Nowadays, due to the transition to the use of renewable biomasses, several feedstocks such as industrial wastes and lignocelluloses are employed for this purpose. In this regard, ascomycetes are considered central biocatalyst for their ability to produce hydrolytic enzymes that can break down the recalcitrant structure of lignocellulosic biomass [23,55].

3 Biomasses as renewable resources

Plants are mainly made up of lignocellulose that play an important role from a structural point of view [57]. Several parameters affect the chemical composition of LCB, such as plant species, maturity, climate and environmental conditions [58,59]. In general, lignocellulose is composed of three main polymers: cellulose (40-50%), hemicellulose (25-30%) and lignin (15-25%) (**Fig. 3**) [9,10]. They are usually associated with other minor components, such as pectin, proteins, extractables and ash [60]. Cellulose is a linear polymer organized in *D*-glucose subunits, which are linked by β -1,4-glycosidic bonds [59,61,62]. Cellulose is characterized by both crystalline and amorphous regions. Inter- and intramolecular hydrogen bonds and Van der Waals forces are found in the crystalline cellulose regions. On the contrary, amorphous cellulose is identified by a more casual structure due to weak bond linkages [63,64]. Hemicellulose is the second most abundant polysaccharide presented in plants [63]. It is characterized by less ordered and less resistant structure than cellulose [59,62]. Various polymers can be found in the hemicellulose, particularly pentoses (especially *D*-xylose and *L*-arabinose) and/or hexoses (*D*-mannose, *D*-glucose, and *D*-galactose) [63,65]. Hydrogen and covalent bonds are the typical linkages in the hemicellulose. Further, hemicellulose is closely connected to both lignin by aromatic esters, and to cellulose by hydrogen bonds, thus forming a bridge between cellulose and lignin [63]. Lignin is an amorphous heteropolymer insoluble in water composed of three aromatic alcohols:

p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Each plant species has a different amount of these three units [63,66–69]. Furthermore, covalent, ester and hydrogen bonds link the lignin to cellulose and/or hemicellulose [63,70]. All the structural components described above are responsible for the recalcitrant feature of LCB [63].

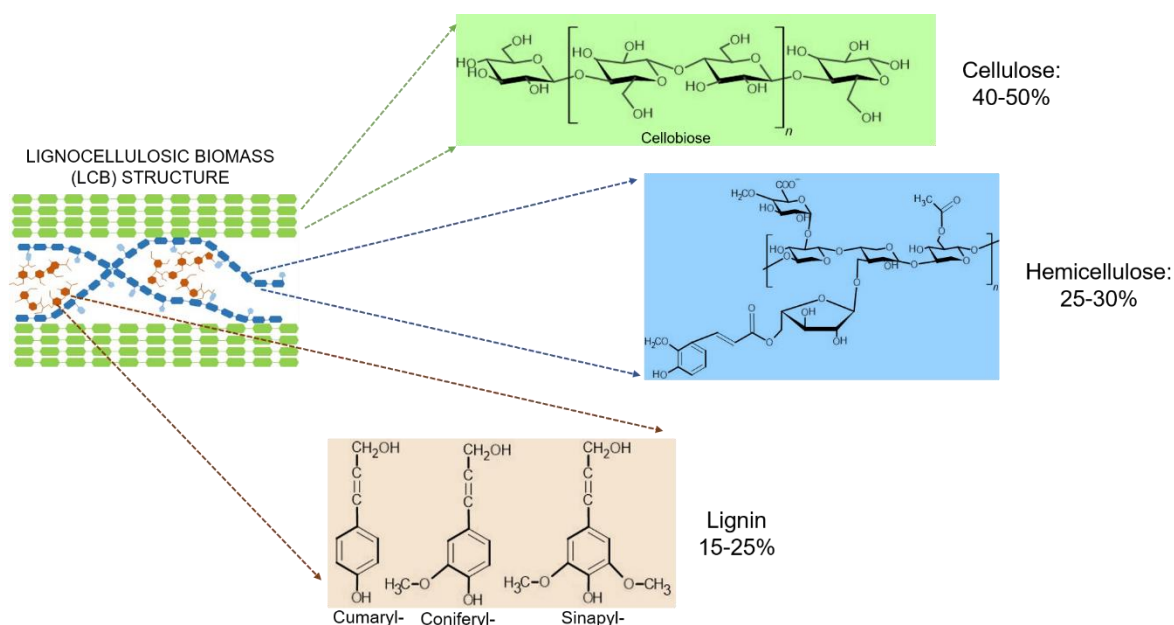


Fig. 3: Lignocellulosic biomass structure. It contains cellulose (40-50%), hemicellulose (25-30%) and lignin (15-25%) in different proportions. Cellulose consists of a linear chain of D-glucose units linked by β -(1,4) glycosidic bonds. Hemicellulose is a branched, substituted polymer of different sugars (pentoses and hexoses). It is characterized by different glycoside linkages and side-chain composition. Lignin is a complex heteropolymer composed of aromatic units (*p*-coumaryl, coniferyl and sinapyl alcohol).

Filamentous ascomycetes such as *Aspergillus* spp., *Fusarium* spp., *Monascus* spp. and *Neurospora* spp. are interesting and versatile biocatalysts due to their ability to grow in a wide range of different substrates [23]. In particular, filamentous fungi can consume carbohydrates released during the saccharification of lignocellulosic materials, such as hexoses and pentoses, and disaccharides such as cellobiose and sucrose [23,71–73]. Furthermore, the ascomycetes can also grow on xylan, arabinan and glucan [23,56]. Due to the secretion of different hydrolytic enzymes, filamentous fungi can be considered very flexible and adaptable. Indeed, the pattern of hydrolytic enzymes secreted depends on the substrate used [23]. For instance, it was proved

that filamentous ascomycetes can use agricultural feedstocks such as wheat [74] and corn straw [75], wheat bran [72], rice hulls, sugarcane bagasse [76] or corn cobs [74]. Further, filamentous ascomycetes could also be used for valorization of agro-industrial waste streams including banana, orange, pineapple, carrots, anions and potato peels [23,72], empty fruit bunches, or pomace [23,77]. Several metabolites such as ethanol and organic acids have been produced by filamentous using lignocellulosic feedstocks. Although the commercial production of organic acids is carried out by using refined sugars, several attempts are currently in progress to use LCBs. The use of renewables from different sources represents an economically alternative that contributes to the development of circular economy [23,55,78]. Consequently, as mentioned by Ferreira et al., the possibility to produce organic acids by using LCBs would lower the overall production costs with simultaneous increase in the range of possible applications [23]. Table 1 shows the feedstock used as raw material to produce the most prevalent organic acids by filamentous fungi.

Table 1. Organic acids produced by filamentous fungi

Organic acid	Substrate	Microorganism	Fermentation type	Yield	Operation scale	Reference
Citric acid	Apple pomace	<i>Aspergillus niger</i> NRRL 567	SSF	90 g/kg (DM)	Shaking flask	[77]
	Banana peel	<i>Aspergillus niger</i> UABN 210	SSF	82 g/kg (DM)	Shaking flak	[298]
	Corncob	<i>Aspergillus niger</i> NRRL 2001	SSF	254 g/kg (DM)	Shaking flask	[299]
	Orange peel	<i>Aspergillus niger</i> NRRL 599	SSF	193 g/kg (DM)	Shaking flask	[300]
	Pomegranate peel waste	<i>Aspergillus niger</i> B60	SSF	351.5 g/kg (DM)	Shaking flaks	[301]
Fumaric acid	Wheat straw	<i>Aspergillus niger</i> ATCC 9142	SSF	60.5 g/kg (DM)	Shaking flask	[302]
	Corn powder and citric acid effluent	<i>Aspergillus niger</i> (industrial strain)	SmF	-	Pilot scale	[103]
	Liquefied cassava chips	<i>Aspergillus niger</i> 831	SmF ^a	0.82 g/g	Shaking flask	[104]
	Woody substrate hydrolysate	<i>Rhizopus delemar</i> DSM 905; <i>Aspergillus oryzae</i> DSMZ 1863	SmF	0.39 g/g 0.58 g/g	Shaking flask	[39]
	Dairy manure	<i>Rhizopus oryzae</i> ATCC 20344	SmF	0.31 g/g	Shaking flask	[303]
	Lignocellulosic syrup	<i>Rhizopus oryzae</i> ATCC 20344	SmF	0.43 g/g	Shaking flask	[304]
	Corncob	<i>Rhizopus oryzae</i> CICC 40351	Fed-batch SSF ^b	0.21 g/g	Shaking flask	[305]

Gluconic acid	Waste paper	<i>Aspergillus niger</i> IAM 2094	SmF	0.92 g/g	Shaking flask	[306]
	Corn Stover	<i>Aspergillus niger</i> SIIM M276	SmF	0.94 g/g	Bioreactor	[188]
Itaconic acid	Beech wood	<i>Aspergillus terreus</i> DSM 826	SmF	0.3 g/g	Shaking flask	[307]
	Cellulose pulp	<i>Aspergillus terreus</i> NRRL 1960	SmF	0.62 g/g	Shaking flask	[308]
	Food waste	<i>Aspergillus terreus</i> BD	SmF	0.27 g/g	Shaking flask	[309]
Lactic acid	Food waste	<i>Rhizopus arrhizus</i> 2062	SmF	0.97 g/g	Shaking flask	[173]
Malic acid	Thin stillage	<i>Aspergillus niger</i> ATCC 9142	SmF	0.8 g/g	Shaking flask	[310]
	Corn starch	<i>Aspergillus oryzae</i> engineered	SmF	0.82 g/g	Shaking flask	[202]
	Crude glycerol	<i>Aspergillus niger</i> MTCC 281 mutant	SmF	-	Shaking flask	[204]
Succinic acid ^{c)}	Birchwood chips and soybean hull	<i>Aspergillus niger</i> Y-78	SSF	32.5 g/kg (DM)	Shaking flask	[23]
		<i>Phanaerochaete chrysosporium</i> A-381				
		<i>Trichoderma reesei</i> RUT-C30				

DM: dry matter; SmF: Sub-merged Fermentation; SSF: Solid-State Fermentation.

a) Repeated-fed batch with cell immobilization

b) Fed-batch-SSF combined with Separate hydrolysis and Fermentation

c) co-culture

3.1 Saccharification of LCBs

Several hydrolytic enzymes are required to break down the LCB structure, such as cellulolytic enzymes (cellulases), hemicellulytic enzymes (hemicellulases) and delignification enzymes (lignases) [23,79]. The conversion of cellulose into glucose is lead to the action of enzymes belonging to glycoside hydrolase (GH) family with complementary activities [80]. The complete hydrolysis of the cellulose requires three main group of enzymes which work synergistically and concurrently [63,81–83]. Endoglucanases randomly cut β -1,4 glycosydic linkages in the less ordered regions of the cellulose thus releasing reducing and nonreducing chain ends; exoglucanases (also known as cellobiohydrolases) work at the end of the resulting chains producing cellobiose. The accumulation of cellobiose can lead to the inhibition of the cellobiohydrolases that, in turn, results in a lower cellulose conversion [84]. In order to complete the cellulose hydrolysis and to avoid cellobiohydrolases' inhibition, β -glucosidases are necessary. This last group of enzymes cleaves cellobiose into glucose units. More recently, it has been proved that the auxiliary activity of other enzymes is essential for improving the degradation of the cellulose. Among these enzymes, lytic polysaccharide monooxygenases (LPMOs) play an important role in boosting the cellulose hydrolysis [85]. LPMOs are believed to aid cellulose saccharification by creating nicks in the glucan chain on the amorphous regions of the cellulose microfibril. These nicks provide new binding sites for the cellulolytic enzymes [24,80,86]. The hydrolysis of the hemicellulose involves the action of several groups of enzymes due to the heterogenous nature and the presence of different side chains accordingly to the raw material [87]. Xylan is the most abundant constituent of hemicellulose characterized by β -1,4 linkages [88]. The complete hydrolysis into xylose monomers is performed by xylanolytic enzymes which include both endo- and exo-xylanase (also known as β -xylosidase). Endo-xylanase act on internal glycosidic bonds which results in the release of long and short xylooligosaccharides; exo-xylanase cleave the same linkages at the end of short xylooligosaccharides producing xylose monomers [9,89,90]. Mannan is made up of mannose residues or of a combination of mannose and glucose residues link together by β -1,4 bonds [87]. β -mannanase catalyze the random hydrolysis of these linkages producing shorter chain of manno-oligomers with reducing and nonreducing ends. The activity of β -mannanase is sustained by β -mannosidase (exo-mannosidase) that work on the nonreducing ends

to release mannose [80,91]. Due to the heterogeneity of the hemicellulose, other hemicellulases are required to completely hydrolyze the polymer. In particular, side chains can be cut from the backbone due to the activity of accessory enzymes such as α -arabinofuranosidase, α -glucouronidase, α -galactosidase and several esterase [91]. Lignin degradation is a more complex process which occurs *via* an oxidative process. Several enzymes are involved, that are laccases, manganese-dependent peroxidase, lignin peroxidases, and versatile peroxidases [80,92–94].

4 Organic acids production and their applications

Organic acids are considered key building blocks chemicals with a large market. They are characterized by low molecular weight and one or more carboxyl groups (**Fig. 4**) [95]. As reported by Mazzoli, the industrial application of organic acids produced by microbial processes strongly depends on their price, which represent the first limitation to their use [13]. To expand the market of these organic compounds, the development of economically competitive bioprocesses is becoming imperative [13,96]. In this view, the production of bioproducts will ensure the development of a more sustainable economy, which is considered as a priority by the European Commission [13][97].

4.1 Citric acid

Citric acid (CA) has been the first organic acid industrially produced by wild type and engineered recombinant fungi [95]. CA is characterized by three carboxylic groups and it is naturally found in citrus fruits. Although it can be produced by purely chemical reactions, microbial fermentations became the preference route of commercial CA production [98,99]. Currently, the global CA market is expected to reach USD 3.2 billion by 2023 and the global production is estimated to be around 736,000 tones/year [100]. As stated by Show et al., due to low price and vast areas of application, it is evident that the demand of CA will be higher than the actual supply [99]. For this reason, several efforts need to be carried out to optimize CA production, such as looking for alternative resources that are more economically, environmentally and productively convenient than current methods [99].

During the years, it has been demonstrated that the filamentous fungus *Aspergillus niger* is preferred with respect to other microorganisms for the commercial production of CA. The choice of *Aspergillus niger* is essentially due to higher production yield and to its ability to consume cheap raw materials [99]. The presence of some genes

encoding for extracellular enzymes (invertase, glucoamylase, α -glucosidase and α -amylase), make *Aspergillus niger* able to use various LCBs with different polymeric composition [29]. Nowadays, all industrial CA production is carried out via fermentation [101]. Different fermentation processes have been developed: submerged fermentation (SmF), surface fermentation, and solid-state fermentation (SSF) [102]. SmF is the most widely used fermentation strategy, especially in tank and bubble-column reactors [29]. It ensures higher yields, higher productivity, less risk of contamination, and process standardization [29,101,102]. Wang B. et al., reported the production of 157.3 g/L of CA in a batch reactor using corn powder and citric acid affluent as feedstock [103]. Yu B., et al., produced 162.7 g/L of CA from liquefied cassava with a repeated-fed-batch strategy [104]. Surface fermentation was the first industrial technique used for CA production [104]. The process is performed in trays assembled vertically (total capacity of 50-100 L) with temperature and humidity control. After the germination of the spores, the fungus starts to grow on the surface of the media as mycelial mat. Surface fermentation has several benefits due to the less energy costs, easy installation and operation processes [29,101]. Therefore, surface fermentation results to fit better in small and medium-scale industries [105]. Generally, refined or crude sucrose, cane syrup and beet molasses are the carbon sources employed [29,101]. SSF involves the utilization of a solid insoluble support that works either as source of nutrients or as physical support in a water-free environment [29,101]. It can be performed in different bioreactor configurations, i.e. flasks, trays, horizontally drums and glass columns [101,102,106]. LCB materials can be valorized in SSFs, as they work as perfect substrates for fungal cultivations [105]. Vandenberghe L. et al., produced 347 g/kgDM of CA using gelatinized cassava bagasse in flasks. Replacing flasks with column bioreactors, the authors obtained 309 g/kgDM of CA [107,108]. Soccol C. et al., reported the production of CA in SSF using different raw materials, such as agro-industrial wastes, corncob and wheat bran [105]. Less consumptions in term of energy and water are among the main advantages of SSFs [102,106]. However, the process is not yet performed at industrial scale because of the difficulties in the standardization and longer time for CA recovery [29,101]. As reported by Di Lorenzo R. et al., several companies are carrying out CA biotechnological production using glucose and/or saccharose-derivate carbohydrates, such as Archer Daniels Midland-ADM (USA), Cargill (USA), COFCO Biochemicals

(China), FoodChem (China), and Tate and Lyle (UK) [29]. Due to its biodegradability, its status of “GRAS” (“Generally Recognized As Safe”), its versatility and its economic potential, CA has wide range of applications [102]. Mores et al, reported the application of CA in the food and beverages industry, mainly as food acidulant to avoid the loss of the organoleptic properties (with a purity of 99.5%) [102]. Cirimina et al., describe the antioxidant ability since CA can chelate metal ions [109]. In the pharmaceutical industry, CA is used as ingredient for effervescent tablets, as a palatability enhancer and as a part of films composition [102,109–112]. Because of its low pH, CA is used also in the cosmetic industry in astringent lotions. CA finds applications in the chemical industry because of its ability to form complexes at neutral or low pH. Particularly, it is used in electroplating, leather tanning, and metal oxides remover [102,105,111]. Innovative areas of applications are emerging by year, such as in biodegradable packaging, antiseptic and extracting, and for (bio)remediation purposes [102,109].

4.2 Fumaric acid

Fumaric acid (FA) is a naturally occurring organic acid that is usually produced by involving chemical reactions. The chemical route requires butane, which is transformed to maleic anhydride. However, because of the increase of petroleum prices, the cost of the chemical precursor has increased as well. Further, the production of harmful by-products such as greenhouse gas increased the research of alternative processes [113,114]. Traditionally, the FA production has occurred via SmF and lead to 4000 tons/year [29,113,115,116]. Later, the chemical synthesis prevailed and it is still the primary choice since it became economically advantageous and more efficient (111% w/w respect to 85% w/w using microbial production from glucose) [114]. However, due to the above mentioned economic and environmental concerns, the fermentation-based production is gaining new attraction [29,114]. The global market of FA has reported to be of 660.9 million USD and is projected to increase annually by 5.5% (period 2021-2026) [117]. Filamentous fungi from *Rhizopus* spp. are mainly involved in the production of FA, as they are able to produce FA under aerobic and anaerobic conditions [113,118–122]. SmF is the preferred fermentation strategy and it usually performed using glucose as carbon source [116]. The production is carried out in batch reactors, although other configurations involving microorganism immobilization strategy are reported [116]. Particularly, stirred tank reactors are the

most used at industrial level, as they are easily to control and have low operation costs [116,123]. The utilization of a batch reactor started with Du Pont patent who reported a production of 121 g/L of FA [124]. Roa Engel C.A. et al., produced FA in tank reactor with oxygen and pH control reaching a yield of 0.28 g of FA per g of glucose [113]. Kang S.W. et al., reported the production of 32.1 g/L of FA by *Rhizopus oryzae* in a batch reactor [125]. Liu H., et al., used a batch slurry reactor to immobilize *Rhizopus arrhizus* on loofah fibers to produce FA from glucose [123]. The production of FA from renewables are still at lab scale investigations. Das R.K. et al., studied the production of FA in SmF using two different substrates: apple pomace ultrafiltration sludge [126] and brewery wastewater [127]. The authors obtained 25.2 g/L and 31.3 g/L respectively [126,127]. LCBs are also used as substrate in SSFs. Liu X. et al., used food waste as feedstock in a fed-batch-SSF combined with a previous separate hydrolysis and fermentation strategy to produce FA [128]. In 2012, Xu Q. et al., reported that two Chinese companies Changmao Biochemistry and Jiangsu Jiecheng Bioengineering were focusing on the biotechnological production of FA at industrial level. However, Xu Q. and co-authors stated that the fermentation process for FA production is still on development, as it results to be less efficient in terms of titer (30-50 g/L) and productivity (0.4-1.0 g/L h) respect to the microbial production of CA, LA and SA [115].

The industrial applications of FA are related to its structure: it has two carboxylic acid groups and a double bond between two carbon atoms [113]. As reported by Yang et al., and Das et al., FA is mostly used in the food and feed industries as an antimicrobial agent, as a nutritional additive and acidulant agent due to its safety feature [129] [130]. Further, the hydrophobic nature of FA results in persistent, long-lasting sourness and flavor impact. Das et al., mentioned the application of FA in food products to buffer the pH, as it requires less amount compared to other organic acids due to its strength. Thus, the use of FA has the advantage to reduce the costs per unit weight. In the resin industry, FA is used to assembly resins with different chemical nature [130]. In particular, due to its chemical structure, FA is suitable for polymerization and esterification reactions [113,130]. As reported by Das et al., FA resins have the advantages to be more resistance to chemical corrosions (resulting in a more durability), and to be biodegradable and biocompatible, thus outcoming safer for environmental exposures [130]. FA is also employed to produce biodegradable

polymers, plasticizers and carboxylating agent for rubber. Novel applications of FA esters are in the pharmaceutical industry for the treatment of psoriasis and sclerosis [29,113,131,132].

4.3 Itaconic acid

Itaconic acid (IA) is an interesting organic acid because of the presence of a methylene groups conjugated double bonds, which give important chemical properties. Particularly, these groups allow polymerization through condensation and esterification with distinct co-monomers by carboxylic groups. According to this, IA is defined an innovative platform chemical suitable for monomer or during monomer synthesis [78,133,134]. Nowadays, IA has a restricted market mainly because of to the limited assimilation of IA products and the presence of substitutes for those. The price of IA has changes during the years and it is now stable in the range of 1.5-2.5 USD per kg [13,135]. However, as reported by Cunha Da Cruz et al., the possibility to decrease the production costs may contribute to the growth of IA market [136]. Nevertheless, the US Department of Energy has included IA in the leading 12 building block chemicals produced from renewable biomass [78,137], with a market potential of 260 million USD in 2025 [138]. Traditionally, the industrial production of IA occurred using different chemical routes: pyrolysis of CA, decarboxylation of aconitic acid, and oxidation of isoprene, among the others [135,137]. Because of the long time, the low efficiency and the high costs, the chemical synthesis is losing interest [135,139]. According to this, biotechnological rout gained more attraction due to higher yield, production rate and sustainability [135,140,141]. Cunha Da Cruz et al., listed some of the main advantages of IA-related products respect to other chemicals from fossil resources, such as its biodegradability (as homopolymer), non-toxicity, and the variety of possible derivates, among the others [136]. IA is mainly produced by the fungus *Aspergillus terreus* due to its tolerance to low pH, high yield, and titer of product [29]. More recently, the natural producer basidiomycete *Ustilago maydis*, gained attention as an alternative biocatalyst for large scale production [29,133,142–144]. Nowadays, the whole industrial production of IA is carried out *via* fermentation using glucose or simple sugars or starch as carbon source reaching 41,400 tons/year [13,29,145,146]. De Carvalho J. and co-authors reported that the utilization of glucose as carbon source ensured a theoretical yield of 72% with a final concentration of 86-129 g/L and with a productivity of 05-1.14 g/L h [147]. SmF is the fermentation strategy widely diffuse

either in laboratory or pilot scale using stir tank reactor and *Aspergillus terreus* as main producer [145,148]. Kuenz A. et al., used a 15L tank reactor and obtained 86.2 g/L of IA after 7 days. The production yield reached 0.62 g of IA per g of glucose employed [149]. Hevekerl A. et al., cultivated *A. terreus* in a 1.5L stir tank bioreactor. They obtained 129 g/L of IA from glucose after almost 5 days of cultivation [150]. Molnár Á. et al., reported the production of IA using a 9L stirred reactor with glucose as carbon source. At the end of the fermentation, the process yielded 0.85 g of IA per g of glucose [151]. Kolláth I.S. et al., demonstrated the possibility to produce IA from xylose in a batch fermentation mode [152]. Because of the high shear stress caused by mechanical agitation which lead to cell damage, tank reactors might be not ideal for filamentous fungi [145]. To overcome this issue, air lift reactors represent a suitable alternative for IA production, as it provides good mixing and oxygen transfer rates due to the liquid recirculation [145][153]. Using glucose as carbon source, Yahiro K. et al., produced 63.7 g/L of IA in an air lift reactor after 7 days of cultivation [154]. Other fermentation strategies are reported in literature, for instance a fed-batch cultivation as reported by Krull S. et al., [155]. Although glucose ensures the highest yield of IA, the development of process with the utilization of renewables is in progress. At the moment, the use of lignocellulosic materials is still at lab scale level [156–158]. One of the major limitations is the presence of impurities that inhibit the IA production capacity of the fungi [29,159]. Recent studies are promoting continuous fermentations to improve the productivity [145]. Several advantages are led to continuous fermentation process: the possibility to maintain the optimal conditions during the whole fermentation period, the continuous product stream which simplify the downstream process, and the circumvention of product's inhibition [145,148]. The industrial production of IA is concentrated in China, USA and India [29,139]. In their recent review, Cunha da Cruz J. et al., listed the name of the companies currently involved in IA production [136].

Contrary to most of the other organic acids, IA is employed in non-food applications [95]. It is relevant as building block for adhesives, unsaturated polyester resins, finishing agents, detergent and cleaners, superabsorbent polymer and dispersants [95,136]. Because of its trifunctional structure, IA allows the production of multiple novel biopolymers. These biopolymers are employed in different materials like

intelligent food packaging, drug delivery, elastomers, hydrogels in water treatment, antibacterial biofilms, among the others [78][160].

4.4 Lactic acid

Lactic acid (LA) is an organic acid widely diffuse in nature. It exists in two enantiomeric forms because of the chiral carbon atom [38][161]. It is estimated that LA market will reach 9.8 billion USD by 2025, with an annual growth rate of almost 19% [162–164]. LA can be produced by either chemical synthesis or microbial fermentation. The main disadvantage of the chemical route is related to the production of a racemic mixture of the isomers L- and D-. Moreover, this process involves the use of strong acids and lactonitrile hydrolyzed. In this view, biotechnological processes offer several advantages such as the production of optically pure L- or D-lactic acid (depending on the strain used), and the use of alternative raw materials as substrates [165]. Actually, more than 90% of LA is produced *via* fermentation processes with a yield of 90-95% w/w (according to the concentration of sugar used) [29,37,166,167]. Refined sugars such as glucose and fructose have been the traditionally employed for LA production [13,166]. These carbon sources were substituted with first- generation biomasses (such as cassava, corn, molasses and sugarcane) [13,166,168]. However, their utilization has long debated as these feedstocks are in competition with the food/feed industry, thus causing ethical and economic issues [13,37,169]. During the last years, several attempts have been made to shift the production of LA towards the utilization of nonfood sources, such as LCBs [166,169]. According to this, the use of low-cost raw materials would promote the development of competitive processes [161]. Although both fungi and bacteria are widely employed for LA production, the industrial production is actually ensured by lactic acid bacteria [29,38,170]. Nonetheless, filamentous fungi have the main advantages to directly hydrolyze LCBs due to their ability to release saccharolytic enzymes, to tolerate low pH, and to simplify the separation of the fungal biomass from the fermentation broth [38,169,171]. Fungi belonging to the genus *Rhizopus* attracted more attraction for their ability to produce the L(+) isomer of LA [161,172–174]. Among the others, *Rhizopus oryzae* is widely employed at lab scale level by using immobilization techniques [168]. Efremenko E.N. et al., studied the production of LA using semi-batch conditions and with the immobilization on poly (vinyl alcohol)-cryogel [175]. Tay A. and Yang S-T., immobilized *R. oryzae* on cotton cloth using a repeated batch and fed-batch approach. The authors

obtained 127 g/L of LA from starch and 226 g/L of LA from glucose [176]. Sun Y. et al., reported the immobilization of *R. oryzae* on polyurethane foam cubes in air lift bioreactor [177]. Nowadays, it is estimated that the LA price was around 1.3-4.0 USD per kg and can vary according to the feedstock price and the final application (for instance, pharma and food required higher purity and this means more purification and refining steps) [13,37,178]. The main companies involved in LA supply are Cargill (USA), Galactia (Belgium), Corbion-Purac (The Netherlands), Fkura (Germany) and Shimadzu (Japan) [29,37,170,179].

LA possess a strong versatility as basic unit, which allow the use of LA in many applications such as precursor of small (propylene glycol) or large (acrylic polymers) compounds [38,165]. The latter products possess important characteristics such as biodegradability and biocompatibility. According to this, they are largely employed for labeling and packaging purposes. They can also be used in the medical industry for manufacturing sutures and devices [38,180]. As reported by Du et al., most of the LA produced (about two-thirds) is used in the food industry to make yogurt and cheese [38]. L-lactic acid is the most common commercial form because the human body can better assimilate it. With regard to LA, one of the most crucial processes is the polymerization to a highly crystalline PLA (polylactic acid). PLA was one of the earliest renewable chemicals presented on the market, particularly as biodegradable plastics, suitable for commercial use, in fiber, film production and food wrap [165,181,182]. Also, PLA started to be present in hygiene and esthetic cosmetic products due to its moisturizing, antimicrobial, and rejuvenating action on skin [38].

4.5 Other valuable organic acids

Gluconic acid (GA) is an organic acid produced from glucose through a dehydrogenation reaction conducted by the enzyme glucose oxidase. GA can be also produced by chemical process, however the fermentation process is the most used [183]. Currently, the annual global market is between 50-80 million USD, and its industrial utilization will expect to pass 120,000 tons by 2024 [184]. The restricted market respect to other organic acids is due to the high price of GA obtained through microbial production (about 1.20-8.50 USD per kg) [185]. The conversion of glucose to GA is usually conducted by the filamentous fungus *Aspergillus niger*. As reported by Ramachandran et al., *Aspergillus niger* is able to produce all the enzymes involved in the transformation of glucose into GA, including glucose oxidase, catalase,

lactonase and mutarotase [183]. SmF is the most widely process employed for GA production at industrial scale as it results to be highly efficient [184,186]. Glucose is the main carbon sources used and it is required at high concentrations [187]. Since the fungal growth and the product formation are not related each other [187], SmFs are carried out in batch reactors as reported by Zhang H. et al., [188], and by Lu F. et al., [27]. Although continuous fed-batch process is generally used at industrial scale, it is not a suitable for GA production by *A. niger* because of the high glucose concentration [184]. Indeed, at high concentration of glucose, *A. niger* forms an excess of mycelia which in turns caused an increased viscosity of the media and a reduced aeration [183,184,189]. In the recent years, new fermentation strategies with cells and enzymes immobilization are emerging. With regards to SSFs, only few studies have been reported. Roukas T. stated the production of 685 g/kgDM of GA from fig [190]. Sharma A. et al., used sugarcane molasses as media and tea waste as solid support for the production of GA. After the optimization, the authors obtained 82.2 g/L of GA [191]. Singh O.V. et al., tested different fermentation strategies (SmF, surface fermentation and SSF) to produce GA from sugarcane bagasse. The authors found out that SSF gave the higher GA concentration (106.5 g/L) under the optimum time period [192]. Among the others, Jilian Biotech in India and Jungbunzlauer in Switzerland were found to produce GA via fermentation.

GA is noncorrosive, nonvolatile, nontoxic acid and it is a chelator agent at high pH values. Further, GA can be employed as plasticizer with good biodegradable properties (98% at 48 h). Due to its properties, GA finds application especially in the food industry as a refreshing sour taster in wine and fruit juices. In the same sector, it can be employed also as component in meat and dairy products preparation. Moreover, as reported by Ramachandran et al., various salt forms of GA have various applications based on their features. For example, sodium salt of GA acts as an anti-limescale on different surfaces. It is also used in the textile industry due to its ability to sequester iron at different pH values. On the other hand, calcium salt form of GA finds application in the pharmaceutical industry as a nutritional supplement of calcium, and in feed industry for animal nutrition [183].

Malic acid (MA) is four-carbon dicarboxylic acid that can be produced by chemical, enzymatic and biotechnological methods, where the first two routes are well established. In the chemical synthesis, MA is produced from maleic anhydride, and

the resulting product is a racemic mixture of D- and L-malic acid. Since maleic anhydride is produced exclusively from fossil resources, the need for greener way is imperative. The current global MA production is around 80,000-100,000 tons/year, while the annual market demand is estimated to be more than 200,000 tons [12,193,194]. Two main advantages are reported for the microbial MA production: first, only the L-form of MA is produced and, secondly, microbial fermentations are performed by using a waste range of renewable substrate [195]. MA is mainly produced by filamentous fungi belonging to *Aspergillus* spp. [196]. The price of bio-based MA was evaluated to range from 1.80 USD to 4.4 USD per kg, but it is considered too high to be competitive with the petrochemical production [13,28,196]. The production of MA *via* fermentation has not reach industrial production, thus only small-scale batch processes have been performed so far [195]. Glucose is the most used substrate which is generally needed at high concentration [197]. For instance, Knuf C. et al., stated the production of 30.3 g/L of MA in 2.7L bioreactor using glucose [198]. Recently, Schmitt V. et al., produced MA using a repeated batch cultivation using *Aspergillus oryzae* [199]. By using this fermentation approach, Schmitt V. and co-authors obtained almost 178 g/L of MA [199]. However, it was noted that the utilization of high concentration of glucose for MA production may cause the formation of the carcinogenic aflatoxins [197,200]. In addition, the utilization of glucose as carbon source make the microbial production unsuitable to replace the chemical route production due to the high costs [195]. Hence, the utilization of LCBs may represent a valid alternative [193,195,201]. Several studies reported the production of MA from renewables at lab scale. Liu J. et al., produced MA in SmF using shaking flasks co-fermenting glucose and corn starch. The authors obtained a yield of 0.82 g of MA per g of total carbon sources by employing an engineered strain of *Aspergillus oryzae* [202]. Ochsenreither K. and co-authors obtained almost 40 g/L of MA from xylose in shaking flasks [203]. Iyyappan J. et al., reported the production of MA from crude glycerol. In particular, the authors modified a strain of *Aspergillus niger* that was able to produce 77.4 g/L of MA after 192h of fermentation in flasks [204]. The current application of MA is in the food and beverage industry where it is used as acidulant agent, often in combination with CA [195,205,206]. MA is furthermore used in cosmetic industry in personal care products as well as in cleaning formulations due to its buffering and chelating properties [207]. Other applications are in the pharmaceutical

sector [195], in the semiconductor fabrication [207,208], and as animal feed additive [209]. Because of the presence of two carboxylic groups, MA represents an interesting building block compound for the synthesis of homo- and heteropolymers [210].

Succinic acid (SA) is characterized by two carboxylic acid groups. It has been traditionally produced from fossil-based resources, although many efforts are being made to use biotechnological processes. The total SA market is estimated to reach 94,000 tons by 2025, with an annual growth rate of 6.5% [211]. However, the SA production through fermentation processes is not competitive with the petrol-chemical route: the cost for SA produced using petrochemical route is 2.5 USD per kg while for the biotechnological production is 2.94 USD per kg [29]. Generally, SA is produced by bacteria isolated from rumen, such as *Actinobacillus succinogenes* [212], *Anaerobiospirillum succiniproducens* [213], *Mannheimia succiniciproducens* [214], and *Basfia succiniproducens* [215]. The production of SA from fungi is rarely described in literature. Nonetheless, some optimized fungal production systems are known to produce and to excrete the acid, such as *Fusarium* [216], *Aspergillus* [217], and *Penicillium* spp. [218]. Moreover, literatures also reported the possibility to exploiting co-culture of fungi and bacteria to obtain SA [219–221]. In this latter case, SSFs were selected as fermentation strategy [20,219]. According to the biorefinery concept, few companies are able to produce SA at commercial scale, such as Myriant (USA), Reverdia (Italy), Succinity (Spain), and BioAmber (France) [29,222–225].

As an important building block, there are four major applications of SA. It is used as a surfactant/detergent/extender and foaming agent. Further, SA is applied in food industry as acidulant/pH modifier, as a flavoring agent as well as antimicrobial agent [226]. Finally, SA can be employed in the area of biodegradable plastics due to the possibility to be the precursor of a biodegradable polyester (polyethylene succinate) [12,13,227].

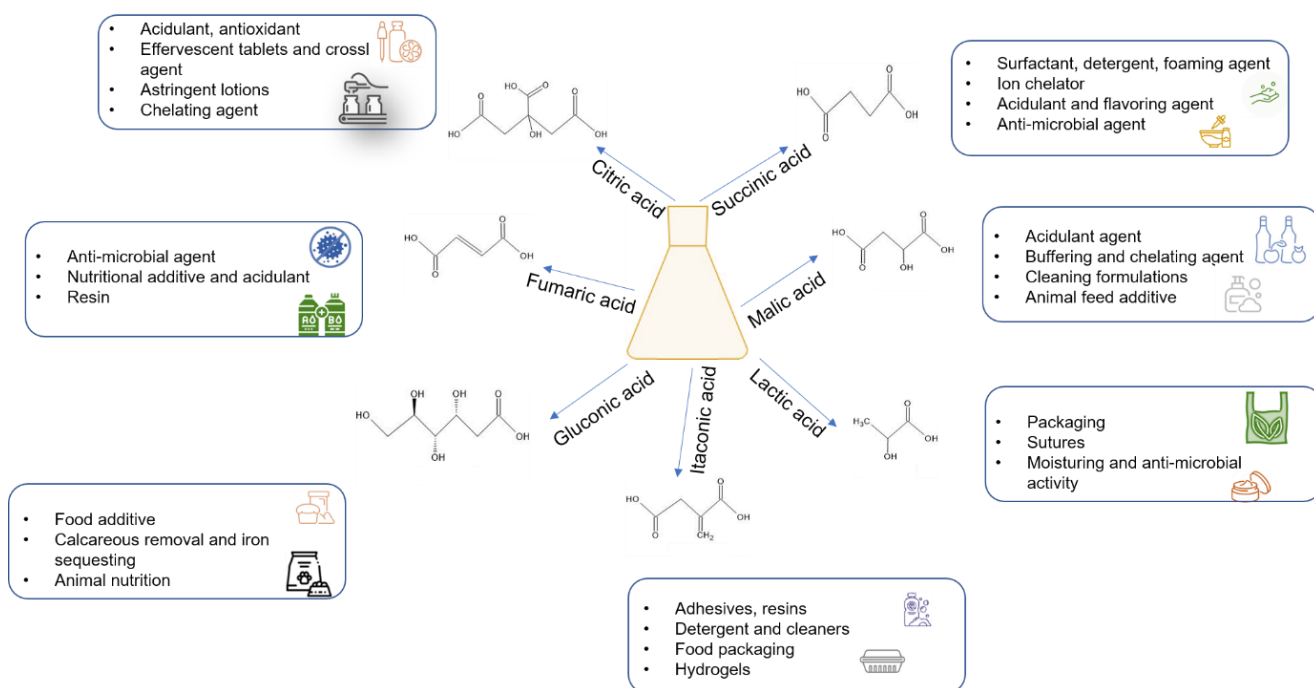


Fig. 4: Applications of the most industrially relevant organic acids. Nowadays, organic acids find application in several industrial areas such as food and beverages industry, pharmaceutical industry, resins industry, cosmetic industry, among the others.

5 Downstream strategies

The downstream of organic acids is one of the main limiting steps in the bio-chemicals production [228]. It is estimated that 50-70% of the total cost comes from separation/purification process(s) [229,230]. Low acid concentration in the fermentation broth, difficult recovery technologies, utilization of harmful solvents, low purity and selectivity, and byproducts formation are the most diffuse downstream problems [228,231]. Furthermore, common difficulties are related to the complexity of the fermentation broth [232]. A wide range of different methods have been developed to recovery organic acids in fermentation broths such as precipitation [145,233], membrane separation [230,234,235], and extraction [236,237]. Other technologies such as distillation [228,238], crystallization [239–241], and chromatography [241] are often used as a further purification step to refine the final acid [228]. Nowadays, precipitation is the most used recovery method at industrial scale, thus indicating its economic advantage respect to the other strategies [228,232]. Thus, further efforts

need to be done in order to decrease the recovery costs and, therefore, the total costs of organic acids production in a biorefinery platform [237].

5.1 Precipitation

Precipitation has been the conventional method to recover organic acids from aqueous solution [237]. It usually involves the use of calcium hydroxide at high temperature (up to 90°C) to precipitate the calcium salt of the acid. The obtained insoluble salt form of the acid is separated from the liquid using a filtration step and then it is acidified with sulfuric acid [102,228,237]. Finally, the acid can undergo to a further refining processes, for instance crystallization [228,232]. Precipitation is especially used for the recovery of CA [102], FA [242,243], GA [244], IA [239], LA [245], and SA [224]. Several advantages can be mentioned, such as higher selectivity, no phase transition, and high purity [228]. However, major limitations are the formation of a considerable amount of solid waste and the production of calcium sulfate as side-product [102,237,239].

5.2 Membrane separation

In the recent years, membrane separation technology has attracted more attraction due to the high selectivity and adaptability [228]. It involves the use of a membrane barrier to transport the compound of interest based on its permeability and gradient [228,229,239]. Membrane separation can be pressure-driven or non-pressure-driven depending on whether a pressure is applied or not [234]. In particular, based on the pressure levels, pressure-driven processes include microfiltration, ultrafiltration or nanofiltration [234,246]. These techniques have the great advantage of working continuously [229]. Membrane separation methods without any pressure embrace inverse osmosis, pervaporation and, more recently, electrodialysis [234,247]. This latter, in particular, permits the recover with high yields and without solvent addition [230]. Disadvantages of membrane separation processes are mainly due to the membrane fouling and clogging, high energy consumption, and high costs [229,248]. CA, FA, GA, IA, LA, MA, and SA are generally recovered via membrane separation techniques [242–244,249–253].

5.3 Extraction

Extraction methods are based on the diverse solubility of the acid between two different phases [229]. One of the most diffuse technique is the liquid-liquid extraction

in which two immiscible liquids are used [229,254]. Typical organic solvent extractants are butyl alcohol, tributyl phosphate and aliphatic amines [229]. These extracts have incredible properties, such as the possibility to change the distribution coefficient, high efficiency, in situ process, low energy consumption, large scale of operation and low cost due to the regeneration of the solvent [228–230,254]. However, they are not eco-friendly and have a limited use in the chemical industry due to the environmental standards [231]. In the last years, green alternatives have emerged for extraction techniques, i.e. bio-based solvents and ionic liquid solvents [231,236,255]. Ionic liquid solvents involve the use of organic salts such as imidazolium, quaternary phosphate or quaternary ammonium salts [228,256–258]. Several benefits can be mentioned, such as chemical and thermal stability, low viscosity, non-volatile and non-flammable nature [228,259,260]. Further, they are often considered as “designer solvents” as they can be adapted to different applications by mixing different ions. A wide range of organic acids can be recovered through extraction procedures such as CA, FA, LA and SA [102,116,259,261–264].

5.4 Distillation

Distillation is often used as refining step or in combination with other recovery methods (i.e. crystallization). Distillation acts by exploiting the differences in compound boiling points. By applying the heat, a partial vaporization of the fermentation broth takes place. This in turns forms two phases (liquid and vapor) with different composition because of the diverse volatility of the components in the liquid phase [265]. Although distillation is an efficient recover technology since years, high costs are necessary to perform the process. Several studies have reported the application of distillation to separate LA from fermentative broth [238,245,262,266,267].

5.5 Crystallization

Crystallization is a typical purification step to produce a dry, stable and solid organic acid [268]. It can be performed under different conditions to obtain the final product in form of a crystals with different sizes and morphologies [269]. One main advantage is the possibility to recycle the reagent used for the crystallization, although it needs further chemicals and harsh conditions (low pH and high temperature) [229]. Moreover, this technology needs high energy input as well as a preliminary step to remove impurities [232]. For these reasons, crystallization is a suitable method for

larger amount of fermentation broth. CA, IA, and SA are generally purified via crystallization [239,270,271].

5.6 Liquid Chromatography

Liquid chromatography technique is used during as refining process [228]. It requires the use of an adsorptive resin (stationary phase) which must be stable and insoluble in the solvents [228,229]. Ion-exchange resins are the most used to separate salts, sugars and other organic molecules. The separation is based on the different affinity of the compounds to the resins. In particular, higher affinity means slower migration of the compounds along the resin bed [263,272–274]. Advantages are associated to the good selectivity, low energy requirements, no co-product formation and higher yields. However, the management of the fermentative broth as well as the decrease of the exchange capacity are the main disadvantages [228,275]. This purification method is especially suitable for FA, IA, LA, MA and SA [241,243,263,272,276].

6 Techno-economic aspects

In 2017, 224 active biorefineries were counted in Europe and the construction of new ones was already planned. About 181 of these operating biorefineries belong to the “first-generation” (1G) which employed sugar and starch-containing feedstocks, and only 43 biorefineries are “second-generation” (2G) biorefineries that work with lignocellulosic feedstock [277–279]. Although LCBs are highly available, some challenges might be addressed to the supply and to the logistic [277,280]. Particularly, lignocellulosic feedstocks are subject to quality and quantity variability: since biorefineries operate all over the year, it is of a great importance to ensure a continuous supply [277,280,281]. After the harvesting, LCBs undergo to compaction processes with the aim to homogenize the material in terms of sizes and weights [277]. This step contributes to an efficient transportation and storage operation [277,280]. To overcome the challenges in the whole supply chain, the development of an integrated and centralized biorefinery facility represents a successful strategy [277,282–284]. This strategy is expected to reduce the costs related to the logistics and, consequently, the total production costs of a biorefinery [277,284,285]. Taking in consideration a biorefinery scheme, four other steps could be identified: a pretreatment to make the feedstock more accessible to the enzymes, the saccharification to hydrolyze the polysaccharides into sugar monomers, the fermentation to convert the monomers

into final product(s), and the recovery [286–288]. With regard to the pretreatment, several technologies have been developed [278]. The choice of the pretreatment should take into consideration the efficacy, the scalability, the costs and the environmental impact [277,280]. High energy requirement, large scale feasibility, and formation of inhibitors effect the pretreatment costs as well as the whole conversion process [280]. For instance, the formation of inhibitors directly influences the fermentation step, as they can inhibit the growth of the selected microorganism [278]. To overcome this issue, a detoxification step might be required with an increase in the total costs [278,289,290]. The enzymatic hydrolysis has a huge impact on a biorefinery chain. Generally, the utilization of enzymes at industrial scale is limited due to low availability since enzyme-producing microorganisms have low production yield of enzymes [280]. Further, several saccharolytic enzymes are required to completely hydrolyze the biomass. A different panel of solutions can be mentioned, such the “in-house” production [280,284,291], the recyclability through enzyme immobilization [280,292–294]. and the consolidated bioprocessing (CBP) [33,280,295]. As reported above, filamentous fungi are exceptional biocatalysts for the simultaneous production of enzymes and organic acids [21]. In the next step of the conversion process of lignocellulosic feedstock to the final product, all the sugars produced during the enzymatic hydrolysis must be consumed [211,280]. Actually, one of the major limitations is the consumption of both exoses and pentoses [278]. Thus, recent studies are towards the research of wild-type or engineered microorganisms able to ferment all the sugars produced and the possibility to employ a *consortium* of several microorganisms with different nutritional preferences [220,278,295]. The recovery and the purification of the bioproduct(s) from the fermentation broth can be a very complex process that involves several technologies [280]. Furthermore, to commercialize the final product, it must have a certain purity (especially for food and pharmaceutical applications) [29]. Hence, the cost related to the downstream processing is associated to the kind of the product recovered and to its final application [13].

Generally, most are the number of the steps involved in a biorefinery scheme, higher are the costs related to the facility [280,296]. One possible way to reduce the costs is the development of an integrated biorefinery in which multiple bioproducts are co-produced from different feedstocks [280]. According to this, integrated biorefinery not only can generate both high-added value products and biofuels at the same time, but

it can use the product of one feedstock as energy source for the other processes [280,297].

7 Conclusion

Organic acids are important compounds with application as building blocks as well as final products in several industrial areas, such as food and feed, pharma, cosmetic and polymers. Due to the wide range of uses, it is estimated that the worldwide organic acids market will reach about 35 billion EUR in 2027 [29]. Until now, the fossil-based production has significantly contributed to the rapid expansion of their market because of the low prices, the high conversion yields and the high productivity. Nonetheless, critical concerns about the environmental, social and economic impact might be assessed. A concrete solution is represented by the bio-based economy in which renewable resources replace the fossil ones to produce the same goods or services. The overall idea of the bio-based economy is to promote the sustainability at all levels: a better quality of life in a conserved environment without any economic damage. The development of integrated biorefineries based on the use of different biomasses to produce a wide portfolio of bioproducts and able to be energy self-sufficient might be a key solution for the development of a circular bioeconomy in the next future. In this review, the organic acids production by filamentous fungi has been analyzed for its potential to support the development of a bio-based economy. Some organic acids produced from first-generation biomasses are already on market, however speculations on their sustainability can arise. On the contrary, biochemicals from second-generation biomasses are not yet marketable. At the moment, the main limitations regard the conspicuous number of steps for the conversion of LCB into the final acid, the capability of the biocatalyst to metabolize complex substrates, the robustness of the biocatalyst, and the scale-up processing. Genetic modifications to improve the biocatalysts' performances, either in the metabolization of the substrate or in the production of the final acid, are possible solutions. This is a common strategy adopted by the companies which generally work with genetically engineering strains. Moreover, until now, any discovered microorganism is able to ferment LCB to the final bioproduct in one step. Many efforts have been made to develop a single-step process (the so-called consolidated bioprocessing), in which the LCB is converted by the same organism. This novel process can potentially ensure an important reduction (about 40-

77%) on the total costs of the process [13]. Taking into consideration all the above-mentioned elements, more research toward the biotechnological production (from the upstream to the downstream processing) of organic acids must be carried out.

8 References

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Chapter III – Paper II

Effect of pretreatment on the biocatalysis of renewable resources

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Keywords: Biocatalysis, Lignocellulosic biomass, Pretreatment, Valorization

Abstract

Within a biorefinery platform several conversion steps such as pretreatment, saccharification, fermentation and downstream processing are necessary to obtain the final bio-based product(s) from lignocellulosic biomass. The structural composition of the biomass, especially the lignin content, determines the necessary pretreatment steps. To obtain sugar monomers, the hydrolysis of lignocellulosic biomass is an essential step. This work examines the impact of different pretreatments on the sugar release during biocatalysis. Even without prior pretreatment the biocatalysis of low lignin biomass achieves glucose yields of up to 93 %, the biocatalysis of high lignin biomass requires an upstream hydrothermal procedure to achieve a glucose yield of 74 %.

1 Introduction

During the past decades the uncontrolled consumption of fossil-based resources has caused several environmental issues, such as greenhouse gas emissions, climate change and the reduction of natural resources [1]. Further, at present, the energy and organic chemical consumptions are growing incessantly due to the rapid increase of the world's population with improved standards of living [2]. As a consequence, the exploration of new renewable resources to produce energy and chemicals is becoming imperative. In this scenario, lignocellulosic biomasses have been recognized as emerging and sustainable alternative resources because of their relatively low cost, great abundance, and sustainable supply [3]. In addition, an important element in the transition towards a bio-based economy is the biorefinery facility. The International Energy Agency defines biorefining as the conversion of renewable raw materials into a spectrum of marketable products and bioenergy [4]. Among others, lignocellulosic biomasses (LCB) such as municipal green waste and herbaceous materials are particularly attractive as they are one of the major underused waste streams occurring in urban and agricultural areas respectively [5]. In order to use LCB in a biorefinery concept, physical, chemical and catalytic pre-treatment steps followed by (bio)catalytic conversion steps are necessary. The first ones have great influence on the efficiency of the (bio)catalytic conversion steps.

The chemical composition of lignocellulosic biomass determines their potential as biorefinery feedstocks, even if it differs depending on the species, its maturity, and

environmental conditions [5, 6]. Lignocellulose is mainly composed of cellulose (30-50%), hemicellulose (20-35%) and lignin (15-20%) [7]. Cellulose is a linear polymer of *D*-glucose linked to each other by β -1,4 glycosidic bonds [8]. Because of its crystalline structure, cellulose is highly resistant to hydrolysis thus impeding efficient conversion of this polymer during biorefining processes [2]. Hemicellulose is an amorphous and branched polymer of five carbon (xylose and arabinose) and six carbon sugars (galactose, glucose and mannose) linked by β -1,4 and β -1,3 glycosidic bonds [2]. Due to their amorphous branched structure and low molecular weight, hemicellulose can be readily hydrolyzed [8]. Lignin is an amorphous heteropolymer network of three-dimensional polymers composed of three different methoxylated phenylpropane units (coniferyl alcohol, sinapyl alcohol and coumaryl alcohol) that are bonded together by different kinds of linkages [3]. It provides structural support and acts as natural, impermeable barrier to microbial attacks and oxidative stress on plant tissues [3, 8]. As reported by Langsdorf et al., slight differences can be observed with regards to the biomass type [5]. In particular, stems and leaves in municipal waste contain a lower amount of cellulose and hemicellulose and higher concentration of lignin compared to grassy material [5]. In the current study, two different lignocellulosic biomasses were considered for a high-lignin content biomass (municipal green waste) and low-lignin content biomass (perennial ryegrass). The overall structure made up of cellulose, hemicellulose, and lignin exist in complex carbohydrates linkages formed by hydrophobic and covalent interactions between lignin and carbohydrates. All the structure components in biomass cellulose-hemicellulose-lignin are responsible for the recalcitrant character of lignocellulose [7].

To overcome biomass recalcitrance, pretreatment is used for the isolation of cellulosic and hemicellulosic polysaccharides [7]. The main objectives of this process are the breakdown of cell wall structures, the reduction of crystallinity, particle size or degree of polymerization, the increase of porosity and accessibility, as well as the delignification in order to make the cellulose and hemicellulose more accessible to hydrolytic enzymes. Factors such as energy consumption and economics of a pretreatment process are also a key criterion for defining the viability of the process [9]. A generalized classification of pretreatment methods groups them into physical, chemical, biological or combinatorial pretreatment [3, 10].

The production of lignocellulosic value-added products is mainly divided into three steps: pretreatment, hydrolysis (also known as biocatalysis), and fermentation. Enzymatic hydrolysis [11, 12] is one of the most important unit operations, in which polysaccharides are hydrolyzed into monosaccharides that can be further converted to bioproducts. The enzymatic hydrolysis is the result of the synergistic action of multiple enzyme components having different mechanisms of action. The main enzymes employed are cellulases (endoglucanases, exoglucanases and β -glucosidases) and hemicellulases (xylanase, mannanase, arabinose, galactosidase etc.). In the first step of biocatalysis, the binding of the enzymes to the substrate takes place. The bound fraction of endo- and exoglucanases converts cellulose to cellobiose. On the contrary, the unbonded fraction of β -glucosidases converts cellobiose to glucose. The binding step is followed by a hydrolysis reaction which is inhibited by hydrolysis products like cellobiose and glucose [13]. Nevertheless, the biocatalysis process is affected by several substrate related factors, enzymes related factors, presence of inhibitors and feedback inhibition [14].

The aims of this work are to compare different pretreatment methods and conditions on two biomasses with different lignin content and to assess the effects of the procedures on the following biocatalysis step. For this purpose, a physical pressing step and a chemical organosolv as well as a liquid hot water method was chosen for the pretreatment of municipal green waste and perennial ryegrass. The present work demonstrates that by using different pretreatment procedures on different lignin-content biomass, differences in sugars released can be observed. As expected, the higher sugar amount was obtained by treating the biomasses with more severe processes. Finally, a kinetic study on the low-lignin biomass was performed in order to assess the rate of the polymers degradation over time.

2 Materials and methods

2.1 Material

Grass cutting from German ryegrass (*Lolium perenne*) was used as representative example for herbaceous lignocellulosic biomass with a low lignin content and was kindly provided by Julius Kühn-Institute, Braunschweig, Germany. A mixture of beech and pine wood chips, originating from local gardens, was used exemplary for recalcitrant lignocellulosic material with a high lignin content.

2.2 Mechanical pretreatment

Biomass with a low lignin content was mechanically pretreated using either a tincture press (high pressure tincture press HP2H, Fischer Maschinenfabrik GmbH, Neuss, Germany) or screw press (Angel Juicer 7500, Luba GmbH, Bad Homburg, Germany). The tincture press operates at $p=440$ bar for 20 minutes and the screw press, which is made up of two rotating cylinders, at 82 rpm. The wood chips as an example for biomass with high lignin content were shredded into particles with a size of about 5 mm and further milled to a particle size of about 200 μm . Biomass was dried in a drying cabinet (Memmert GmbH & Co. KG, Schwabach, Germany).

2.3 Hydrothermal pretreatment

Biomass was pretreated hydrothermally in a high-pressure reactor BR-500 (Berghof Products + Instruments GmbH, Eningen, Germany). The reaction vessel consisted of Polytetrafluorethylene (PTFE) and had a volume of 0.5 L. The solid:liquor ratio of all experiments was 1:10. Demineralized water was used as solvent for LHW (Liquid-Hot-Water) and 50% (w/w) ethanol for organosolv pretreatments. The reactor was heated using an electric heating jacket (Berghof Products + Instruments GmbH) with a holding time of 15 minutes at 180 °C. An overpressure of 5 bar N_2 was applied to the reactor vessel and the reaction mixture was stirred at 600 rpm. Solid and liquid fraction of the reaction mixture were separated via centrifugation. The residue was washed three times with demineralized water to remove the solvent and possible by-products of the hydrothermal pretreatment.

2.4 Biocatalysis

The pretreated low lignin content biomass was enzymatically hydrolyzed in 0.1 mM sodium-acetate buffer. To prevent microbial contamination, 0.02% (w/v) sodium-azide was added during hydrolysis for analytical purposes. If the hydrolysate is used for subsequent fermentations, it needs to be omitted and the substrate autoclaved instead. The reaction was conducted at 50 °C and 50 rpm. Two different enzyme mixtures were employed. Ultraflo® Core and Ultraflo® Max (Novozymes A/S, Bagsvaerd, Denmark) were used in a ratio of 50:50 with an enzyme loading of 0.1 $\text{g}_{\text{enzyme solution}} \text{g}_{\text{biomass}}^{-1}$ and a solid content of 50 $\text{g} \cdot \text{L}^{-1}$. Xylanase 2x and Pectinase L-40 (both ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) were used in a ratio

xylanase: pectinase of 60:40. Xylanase 2x contained a mixture of endo-1,4-β-D-xylanases and endo-1,3-β-D-xylanase, Pectinase L-40 consisted of polygalacturonase. Enzyme loading was based on the protein concentration in the stock solutions and they were tested using the Bradford (Coomassie Bradford Protein Assay Kit, ThermoFisher Scientific) assay. The protein concentrations were found to be 23.5 mg mL⁻¹ for xylanase and 4.2 mg mL⁻¹ for pectinase. The enzyme loading was set at 0.16 g_{enzyme} g_{biomass}⁻¹, with a biomass loading of 10% (w/w). The hydrolysate samples were taken at 0, 2, 4, 8, 24, 48, 72 and 96 h and were thermally inactivated at 100°C for 7 minutes, centrifuged and filtered. All the enzymatic hydrolysis were performed at higher enzyme loadings in order to get comparable results between the different biomasses. Thus, the experiments focused on the comparability without considering the optimization process as well as the economic evaluations. The enzymatic hydrolysis was carried out in duplicate and the results have been analyzed as average values and corresponding standard deviation.

2.5 Analytical methods and data processing

The carbohydrate composition of the pretreated materials was performed according to the protocol [15]. Sugar monomers were analyzed using a High Performance Liquid Chromatography system (ESA Inc. 542 autosampler (Chelmsford, Massachusetts, USA), Azura pump P 6.1 L (Knauer GmbH, Berlin, Germany)) equipped with a refractive index detector and a BioRad Aminex HPX- 87H column (300 x 7.8 mm) (Hercules, California, USA). In the chromatographic analysis, the samples were diluted with deionized water, filtered, and thus 20 μL of each sample was injected into the chromatograph under the conditions of a column temperature (80°C), and 2.5 mM H₂SO₄ as mobile phase at a flow rate of 0.6 mL min⁻¹. The yield of the enzymatic hydrolysis was calculated as the amount of sugar released over the total carbohydrate content in the biomass.

The percentage was calculated as follow:

$$yield [\%] = \frac{C_S - C_{S0}}{\alpha_s C_{i0} M_{P0}} * 100 \quad (1)$$

Where C_s is the concentration [g L^{-1}] of solubilized sugar in the supernatant, C_{s0} is the initial sugar concentration [g L^{-1}], α_s is the molecular weight of glucose to glucan monomer ($\alpha_s = 1.11$), C_{i0} is the initial concentration [g L^{-1}] of insoluble solids, and M_{P0} is the initial mass fraction of the polymer in the insoluble solids.

3 Results and discussion

Pretreatment of lignocellulosic material is necessary to obtain carbohydrates which can be applied for further uses such as carbon source for fermentations. Due to its varying composition, different strategies are necessary for different biomass types. As lignin plays an important role in the digestibility of lignocellulosic biomass, the lignin content can serve as a decision criterion to select an appropriate pretreatment method.

3.1 Pretreatment of biomass with low lignin content (grass biomass)

Herbaceous material is one example for a biomass with low lignin content. Therefore, the lignocellulosic structure is less recalcitrant. Due to a higher moisture content, the biomass can be pressed, resulting in a liquid and a solid fraction [16]. The pressing process can be carried out in decentralized facilities (e.g. on a farm) to obtain a solid fraction with reduced water content, resulting in a better shelf life and lower transport costs due to the lower weight. However, the press juice must be used immediately, e.g. as an additive for biogas fermentation. The solid press cake must be further pretreated, for example in a central biorefinery.

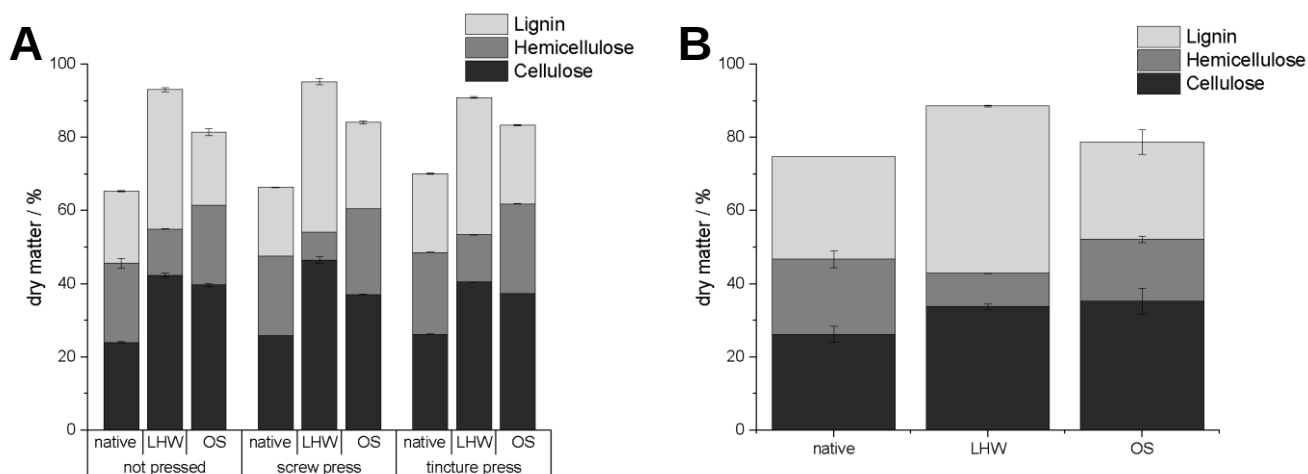


Fig. 1: Influence of different pretreatment procedures on the composition of the dried biomass of grass cutting (A) and mixed wood chips (B). Native: no further pretreatment.

Grass cutting was pressed using either a screw press or a tincture press. This was followed by a hydrothermal pretreatment as described in chapter 2.3 and [17] using water (LHW) or 50% (w/w) ethanol (OS) as solvent. As can be seen in **Fig. 1**, the influence of a pressing step on the composition of the residue can be neglected. Neither for the native grass, which was not further pretreated after pressing, nor for the hydrothermally processed grass there is a significant difference in the composition of the dried biomass between substrates which were pressed by screw or tincture press or not pressed at all. Compared to the native composition, the hydrothermal procedure increases the share of cellulose in the residue. This effect is stronger for LHW pretreated grass, with an increase of up to 70%, compared to an increase of up to 50% for the organosolv pretreatment. At the same time, the LHW procedure reduces the share of hemicellulose in average by 50%, while it does not change significantly during organosolv pretreatment. The percentage increase of lignin during the LHW pretreatment procedure can be explained by the removal of other soluble compounds during the pretreatment, leading to lignin to account for a bigger share. Dimos et al. report a 58% increase of the lignin share during hydrothermal pretreatment of cotton stalks, and a 74% decrease during an organosolv process using formic acid [18]. This increase of lignin does not occur during organosolv pretreatment with ethanol due to the increased solubility of lignin in this solvent compared to the solubility in water [19].

3.2 Pretreatment of biomass with high lignin content (woody biomass)

Biomass with a high lignin content such as wood chips usually is more solid and also has a lower moisture content than grass cuttings. Consequently, a pressing step is either mechanically not possible (e.g. for the screw press) or does not yield a notable amount of press juice and was thus neglected in this work. However, the surface area of the material was increased by crushing and grinding to achieve a better yield in the processes used.

The effects of applying a hydrothermal procedure on biomass with a low lignin content could be confirmed for woody biomass with a lignin content of 30%. In accordance with the findings for grass cutting, the hydrothermal pretreatment of wood chips increased the cellulose content in the residue by up to 35%. In contrast to the previous shown results, there is no significant difference in the increase of cellulose content between LHW and organosolv pretreated biomass. The hemicellulose content decreases by 55% and 18% during the LHW and organosolv pretreatment, respectively. Kabir et al. report a hemicellulose decrease of 28.5% during an organosolv process of forest residues [20]. The percentage increase of lignin previously described can also be observed, with an increase of 63% for LHW pretreated wood chips. In contrast, during the organosolv procedure, the lignin content decreases by 5%. Again, this can be explained by the solubility of lignin in ethanol [19].

3.3 Biocatalysis of biomasses with different lignin contents

Grass cutting was digested enzymatically in a native condition as well as after pressing with either a screw or a tincture press. All biomass samples were saccharified both right after cutting or pressing, respectively, and after drying to constant weight. Similar to commonly used enzyme preparations like Cellic CTec the enzyme mixture of Ultraflo® Max and Ultraflo® Core consists of β -glucanases, xylanases and cellulases and is therefore promising for the degradation of lignocellulosic biomass. Weiermueller achieved a glucose yield of 87% from grass cuttings with these enzymes [21].

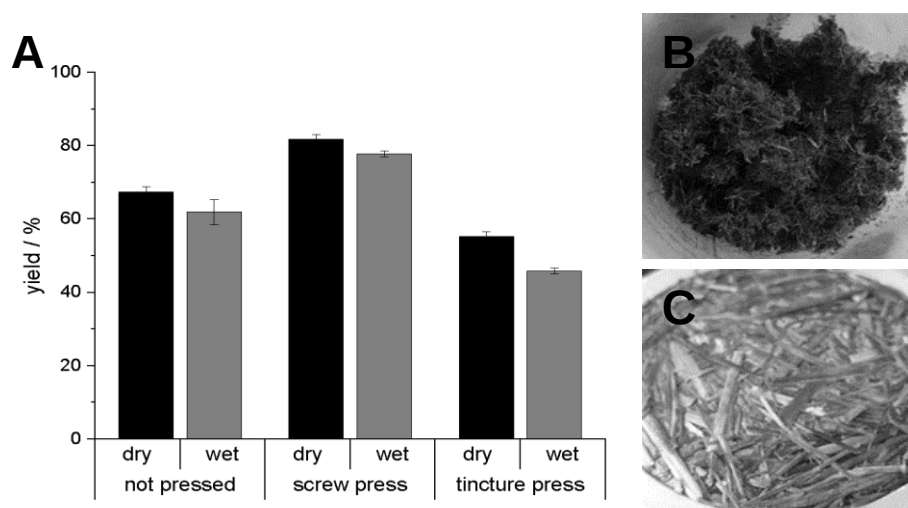


Fig. 2: Conversion of cellulose during the biocatalysis step of untreated and pressed grass cuttings in wet or dry conditions (A). Press cake after pressing by screw press (B) or tincture press (C). Enzymatic hydrolysis was carried out with Ultraflo® Max (50%) and Ultraflo® Core (50%).

As can be seen in **Fig. 2**, slightly higher conversion rates were achieved when the biomass was dried to constant weight at 50°C prior to the enzymatic hydrolysis. The best result was obtained from screw pressed grass with a conversion rate of 82% of the total amount of glucose based on the NREL-protocol, followed by native grass with 67%. Biocatalysis of grass pretreated by tincture press yielded 55% cellulose conversion. This can be explained by the influence of the pressing mechanism. The tincture press applies pressure of up to 440 bar, resulting in a strong compression of the biomass. Thus, the produced press cake is very dense and compact as can be seen in **Fig. 2C**. In the screw press however, the material is crushed between two rotating cylinders. The result is a very fluffy grass cut (**Fig. 2A**). The screw press thereby changes the integrity of the grass cuttings. Konan et al. also discuss the possibility of extrusion, breaking up the lignocellulose structure by the shearing forces of rotating screws, as a potential pretreatment procedure for lignocellulosic biomass [22]. The fibers are shredded and partially broken up. This results in a significantly enlarged surface. The pretreatment step aims to disrupt the cell wall as well as to reduce the particle size in order to make the cellulose and hemicellulose available to hydrolytic enzymes. According to this, the press cake obtained from the screw press

is more accessible to the enzymes than the compact press cake from the tincture press.

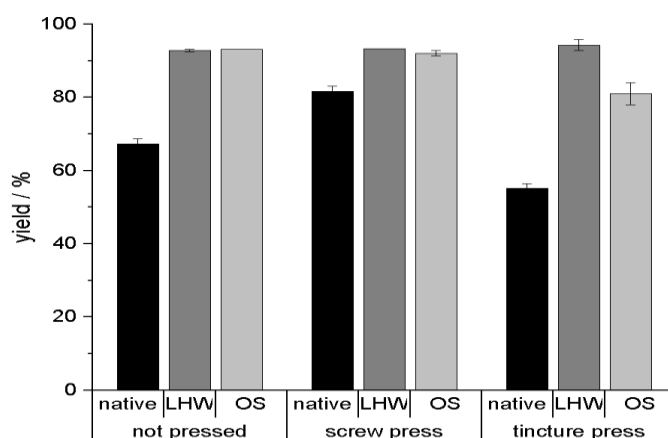


Fig.3: Conversion rate of cellulose after the biocatalysis step of differently pressed and hydrothermally pretreated grass cutting. Native: no further pretreatment. Enzymatic hydrolysis was carried out with Ultraflo® Max (50%) and Ultraflo® Core (50%).

Fig. 3 shows the influence of different pretreatment methods on the cellulose conversion during the biocatalysis process. A hydrothermal process step increases the cellulose conversion rate both for pressed and not pressed biomass. Regardless of the prior pretreatment method, the conversion rate of the pretreated biomass is on average 93% with no significant difference between different pressing or hydrothermal procedures. Only the enzymatic hydrolysis of organosolv pretreated grass, which was pressed via tincture press, yields a lower amount of 81%. The hydrothermal procedure increases the glucose yield for grass which was not pressed by 38%, while the yield for screw pressed grass only increased by 13% as the conversion rate for this pressing method was already at 81% without an additional hydrothermal step. The hydrothermal process is a time- and energy-consuming procedure. Thus, the small increase of the glucose yield does not justify the expenditure. A pressing step with a screw press, which mechanically breaks up the structure of the biomass, is a sufficient pretreatment method for herbaceous biomass with a low lignin content of up to 20%. A further hydrothermal procedure is economically not worthwhile.

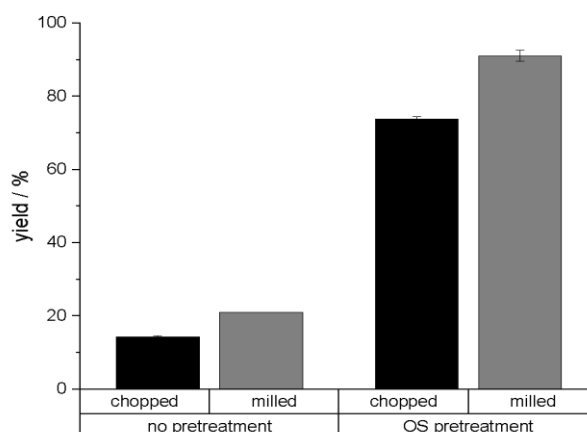


Fig. 4: Conversion rate of cellulose after the biocatalysis step of differently pretreated wood chips. Enzymatic hydrolysis was carried out with Xylanase 2x (60%) and Pectinase L-40 (40%).

For more recalcitrant biomass with a higher lignin content of around 30% however, the situation is different. Kirui et al. observed about twice as much lignin-cellulose interactions in woody biomass than in grasses. This leads to the mechanical strength and also the reduced accessibility for enzymes of biomass with a higher lignin content [23]. The cellulose conversion rate of chopped wood chips is only 14%. This can be increased by 5% if the biomass is milled prior to the enzymatic hydrolysis. The smaller particle size facilitates the access of saccharolytic enzymes to cellulose. Csiszar et al. examined the hydrolysis of linen and cotton powders by a cellulase after different ultrasound pretreatments. The authors demonstrated a preferential hydrolysis of smaller particles by cellulases as well as a higher saccharification rate of substrates with a smaller particle size [24]. Fernandes et al. found an increase in glucose yield during enzymatic hydrolysis of sugarcane bagasse of 76% with particles smaller than 60 mesh compared to native particle size [25]. However, the achieved glucose yield of milled biomass particles is still unsatisfactory. An organosolv pretreatment of wood chips increases the glucose yield by 414%, reaching a cellulose conversion rate of 74%. This can be further increased to a final conversion rate of 91% by milling the organosolv pretreated residue prior to the enzymatic hydrolysis to improve the cellulose accessibility (**Fig. 4**). An overview of different sugar yields obtained from various lignocellulosic feedstocks using different pretreatment processes and biocatalysis steps is given in table 1.

Table 1: Comparison of sugar yields obtained from different lignocellulosic feedstocks after pretreatment and biocatalysis using varying enzymes

Substrate	Pretreatment	Biocatalysis	Yield	Ref.
Eucalyptus wood chips	LHW and extrusion	CelliCTec2 solid loading: 10% (w/v) enzyme loading: 20 mg g _{cellulose} ⁻¹	79.6 %	[26]
Sugarcane bagasse	LHW	Cellulase, Xylanase solid loading: 5% (w/v) enzyme loading: 15 FPU g _{substrate} ⁻¹	55.6 %	[27]
German ryegrass	LHW	Ultraflo Max, Ultraflo Core 1:1 solid loading: 50 g·L ⁻¹ enzyme loading: 0.1 g g _{dry matter} ⁻¹	87 % glucose	[21]
Sorghum sweet biomass	Organosolv	CelliCTec2 solid loading: 27.7 mg g _{solid} ⁻¹ enzyme loading: 10 FPU g _{dry matter} ⁻¹	42.1 % total sugars	[28]
Poplar sawdust	Organosolv	CelliCTec2 solid loading: 2% (w/v) enzyme loading: 20 FPU g _{cellulose} ⁻¹	78 % total sugars	[29]
Perennial ryegrass	LHW Organosolv	Ultraflo Max, Ultraflo Core 1:1 solid loading: 50 g·L ⁻¹ enzyme loading: 0.1 g g _{dry matter} ⁻¹	93 % glucose	this work
Mixed beech and pine wood chips	Organosolv	Xylanase 2x, Pectinase L-40 3:2 solid loading: 10 % (w/w) enzyme loading: 0.16 g g _{dry matter} ⁻¹	74 % glucose	this work
Spruce and birch chips	Organosolv	CelliCTec2 solid loading: 5% (w/v) enzyme loading: 10 mg g _{solids} ⁻¹	69.1%	[30]
Energy crop	Organosolv	Cellulase solid loading: 5% (w/v) enzyme loading: 20 FPU g _{dry matter} ⁻¹	87.5%	[31]

3.4 Kinetic of the biocatalysis of low-lignin content biomass

The enzymatic hydrolysis of low-lignin content biomass was conducted in order to study the sugar release over time. **Fig. 5** reports the yield of glucose and xylose released as a function of the time. Sugars were released fast at the beginning of the hydrolysis and progressively reached a steady-state. In particular, the glucose released changed little after 24 h, suggesting that the hydrolysis of glucan was almost completed. At the same time, the amount of xylose was still increasing thus indicating that the xylanase needs more time to completely hydrolyze the xylan. The results are shown in **Fig. 5**.

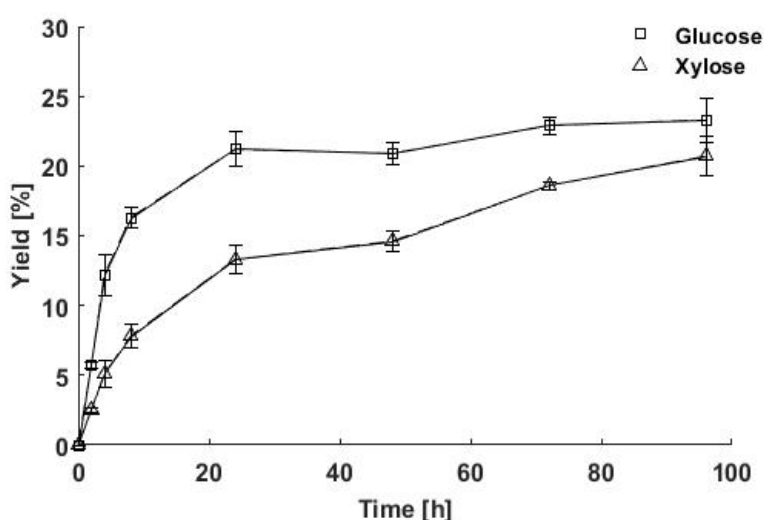


Fig. 5: Glucose and xylose yield from mechanical pre-treated low-lignin content biomass over time during biocatalysis. Enzymatic hydrolysis was carried out with Xylanases (60%) and Pectinases (40%). All experiments were performed in duplicates.

The overall results were expected as the raw material was pretreated using a weak procedure, thus not completely disrupting the recalcitrant structure of lignocellulosic biomass and reducing the efficiency of the hydrolysis process. As mentioned above, the main goals of the pretreatment are the breakdown of the cell wall and the increase of accessibility for the enzymes. Harsh process methods with chemicals (acids, bases, organic solvents) are extensively used in industrial production and ensure higher sugar release during the enzymatic hydrolysis step. However, the use of strong conditions caused the formation of undesirable products which inhibit the fermentation process. For all these reasons, the physical and chemical pretreatment procedures are usually

combined because no pretreatment technique alone can ensure the goals mentioned above [7].

Another explanation for the present result could be the high biomass loading. As reported by Niglio et al., the increase of biomass loading causes high viscosity that makes it more difficult for the solution to access and wet the biomass. Moreover, enzyme inactivation and product inhibition could hinder the enzymatic hydrolysis process [32].

In the usual single-substrate-enzyme-catalyzed reactions (Eq. 2), the relationship between the initial reaction rate and the substrate concentration assumes the form of a saturation curve (**Fig. 5**) [33]. A mathematical model to describe the kinetic is the Michaelis-Menten equation (Eq. 3):



$$v = \frac{V_{max} [S]}{K_M + [S]} \quad (3)$$

where v is the product formation rate, V_{max} is the maximum velocity achieved at the maximum substrate concentrations, and K_M is the substrate concentration at which the velocity is 50% of the V_{max} .

The initial rate of reaction is calculated by correlating the substrate concentration or product concentration change and the reaction period insofar as their time course is estimated as a linear relationship [33]. In this work, as showed in **Fig. 6**, the linear range was found within the first 8 h. To experimentally define the kinetic parameters V_{max} and K_M , the Michaelis-Menten equation is modified using the integration method. The integration method was applied by assuming that the reverse reaction is negligible and that the product does not affect the reaction rate [33].

According to this, the it is possible to integrate the Michaelis-Menten equation (Eq.2):

$$\int_{S_0}^{S_t} \frac{[S] + K_M}{[S]} = - \int_0^t V_{Max} dt \quad (4)$$

$$\frac{1}{t} \ln \ln \frac{S_0}{S_t} = -\frac{1}{K_M} \frac{(S_0 - S_t)}{t} + \frac{V_{Max}}{K_M} \quad (5)$$

Equation 4 can be recognized as an equation of a line in which the data are plotted as $S_0 - S_t/t$ versus $(1/t)\ln(S_0/S_t)$.

Since the calculation is based on the decreasing substrate concentration over time, the substrate concentration was calculated as:

$$S_{(t)} = \text{Total carbohydrate content according to NREL} - c(t)_{glu} - c(t)_{xyl} \quad (6)$$

The resulting curve (**Fig. 6**) is a line with a negative slope. From equation 3, V_{max} and K_M result to be $0.6 \text{ mmol min}^{-1}$ and 93.4 mmol L^{-1} , respectively. The spectrum of experimentally determined K_M values of enzymes varies depending on the experimental setup and the substrate. Typical K_M values of cellulases range from 0.45 to 6.7 mmol L^{-1} for the model substrate carboxymethyl cellulose [34, 35]. For xylanases, K_M values between 0.03 mmol L^{-1} for beechwood xylan [36] and 7.7 mmol L^{-1} for birchwood xylan [36, 37] are reported. Polygalacturonases display a slightly lower range of K_M values from 0.02 mmol L^{-1} to 0.6 mmol L^{-1} [38, 39]. The K_M value of the enzyme mixture of Xylanase 2x and Pectinase L-40 used in the present experiments is in the same order of magnitude as the reported literature data for these kinds of enzymes. The slightly higher K_M value, signifying a lower affinity between enzymes and substrate, can be explained by the different substrates used for the experiments.

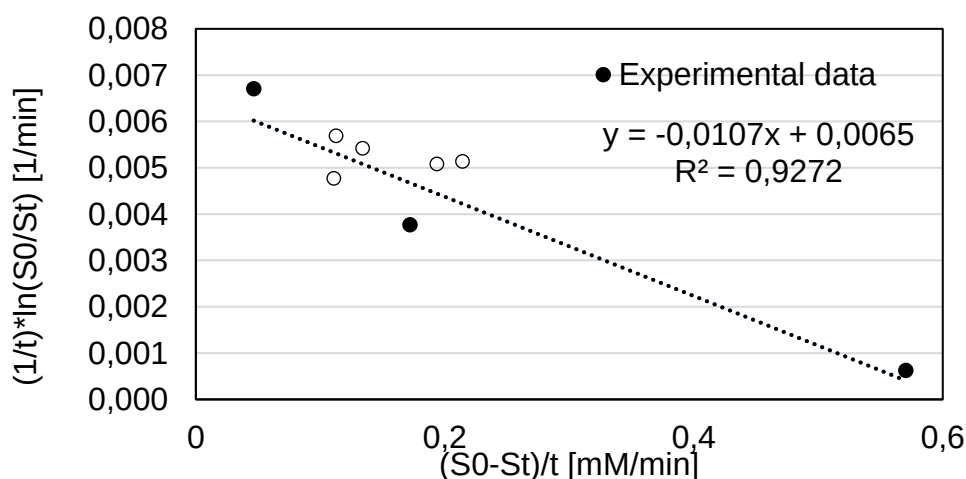


Fig. 6: Integrated Michaelis-Menten method to determine the kinetic parameters V_{max} and K_M . Full circles: experimental data; empty circles: predicted data.

4 Conclusion

Lignocellulosic biomass is a potential carbon source for various purposes. However, a pretreatment procedure is indispensable prior to its use. Complex carbohydrates are broken down into fermentable monosaccharides by saccharolytic enzymes. Depending on the lignin content of the biomass, an additional hydrothermal process is necessary to break up the recalcitrant structures. Cellulose from grass biomass can be converted with an efficiency of 93% without prior hydrothermal pretreatment. In contrast to this, the cellulose conversion rate of woodier biomass such as wood chips without a hydrothermal processing is 14%, whereas an organosolv procedure increases the cellulose conversion rate to 74%. The overall results demonstrated that the saccharification step depends on the pretreatment process used, as it aims to disrupt the biomass structure in order to make the biomass more accessible for the hydrolytic enzymes. To make a biorefinery process more efficient, lignocellulosic biomass should be separated according to its lignin content. In this way, the energy consuming hydrothermal pretreatment is only applied to biomass which requires this procedure for an effective saccharification. Finally, kinetic studies were carried out on the grass material over time. With this regard, the time-course of the enzymatic hydrolysis revealed that after 24 h the hydrolysis reached the steady-state. In order to study the kinetic parameters, the Michaelis-Menten equation was integrated by considering the time window where product concentration and the reaction time had a

linear relationship. The parameters of V_{max} and K_M were estimated to be 0.6 mmol min⁻¹ and 93.4 mmol L⁻¹ respectively, which are in line with the data reported in literature.

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Chapter IV – Paper III

Development of a Consolidated Bioprocess for the production of citric acid using *Aspergillus niger* as biocatalyst

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Keywords: lignocellulosic biomass, *Aspergillus niger*, Consolidated bioprocessing, citric acid, cellulases, CLSM

Abstract

Industrial production of citric acid, an ingredient in beverages, pharmaceuticals, and cosmetics, is based on microbial fermentation of glucose or sucrose. Given the elevated cost of these sugars, lignocellulosic biomass is emerging as a cost-effective and environmentally friendly feedstock for sustainable bioprocesses. However, fermentation of lignocellulosic materials requires that they are first broken down enzymatically. This can be achieved by the filamentous fungus *Aspergillus niger*, that has the ability to secrete hydrolytic enzymes and to produce citric acid. Here, we investigated the production of citric acid using a consolidated bioprocess, in which all conversion steps—from the solid substrate to the final product—occurred in a single process stage. The press-cake derived from a perennial ryegrass (*Lolium perenne*) was used as substrate and glucose or the remaining press-juice were utilized as additional carbon source. *A. niger* successfully produced citrate only when the press-cake was supplemented with press-juice and especially glucose. Confocal laser scanning microscopy revealed differences in fungal mycelia based on the carbon source supplemented. Overall, the results indicate the successful implementation of solid-state fermentation for the sustainable production of citric acid by *A. niger* fed on press-cake.

1 Introduction

Lignocellulosic biomass (LCB) is the most abundant renewable resource on Earth and is attracting increasing interest for the sustainable production of chemicals, materials, and fuels (Mazzoli, 2021). Given its widespread availability, low cost, and carbon neutral footprint, LCB could overcome environmental and economic concerns related to the exploitation of fossil-based resources (Grewal and Khare, 2018). Global annual production of LCB has been estimated at 181.5 billion tons and its output is expected to grow if we are to reach net zero carbon dioxide emissions by 2050 (Mujtaba et al., 2023). LCB includes residues from agriculture, forestry, industry, and household activities (Cherubini et al., 2009). Conversion from LCB to the targeted product involves several steps, such as pretreatment, saccharification, and fermentation, which can be executed using different process layouts (Ali et al., 2016). In Separate Hydrolysis and Fermentation (SHF), enzymatic treatment, saccharification, and

fermentation are carried out separately under optimal pH and temperature. However, SHF might not function at industrial scale because enzymes may be inhibited by the release of certain sugars (Perez et al., 2023). In addition, SHF requires distinct vessels for hydrolysis and fermentation, along with additional heating and cooling steps, which drastically increase operational costs (Paulova et al., 2015). To overcome these limitations, Simultaneous Saccharification and Fermentation has emerged as an alternative, combining enzymatic hydrolysis and fermentation into a single step. Despite its advantages, this process requires the addition of expensive hydrolytic enzyme mixtures. At 4–20 USD/kg, the cost of cellulases and hemicellulases significantly impacts overall production expenses (Liu et al., 2016; Troiano et al., 2020). An emerging strategy is Consolidated Bioprocessing (CBP), whereby the same microorganism accomplishes the production of hydrolytic enzymes, biomass hydrolysis, and fermentation of the released sugars into target product(s), all within a single process step (Rajak and Banerjee, 2020; Banner et al., 2021; Perez et al., 2023). This strategy is considered a low-cost, material- and energy-efficient configuration due to the reduced number of processing steps (Liu et al., 2020). Filamentous fungi are particularly well-suited for CBP, as they produce large amounts of hydrolytic enzymes and can achieve high yields of target secondary metabolites (Troiano et al., 2020). One of the latter is citric acid, which has a market potential projected to reach 3.2 billion USD by 2027, due to its low cost and wide range of industrial applications, including food, beverages, pharmaceuticals, and cosmetics (Tong et al., 2019; Cairns et al., 2021). To satisfy the increasing demand for citric acid, an innovative biotechnological approach termed Solid-State Fermentation (SSF) has been proposed. In SSF, microorganisms are grown on a solid matrix (inert support or insoluble substrate) in the complete or nearly complete absence of free water (Singhania et al., 2010; Lizardi-Jiménez and Hernández-Martínez, 2017). SSF has several advantages, including low water and energy demand, utilization of unexploited LCB, and resistance to catabolic repression by excessive substrate (Singhania et al., 2010; Yoon et al., 2014; Lizardi-Jiménez and Hernández-Martínez, 2017). These advantages are estimated to result in 10-fold lower production costs compared to conventional liquid fermentation (Yoon et al., 2014).

The present study aimed to assess whether the press-cake from a perennial ryegrass (*Lolium perenne*) obtained via mechanical pretreatment could be employed as

substrate for the production of citric acid using SSF (**Fig. 1**). The filamentous fungus *Aspergillus niger* was selected as biocatalyst owing to its ability to grow on different LCB and to produce large volumes of citric acid. Initially, the grass press-cake was intended as the only source of nutrients, but was then supplemented with glucose or press-juice to provide additional carbon, along with nitrogen, phosphate, potassium, and methanol. To determine whether *A. niger* used the press-cake as solid support or as insoluble substrate, β -glucosidase activity measurements were used as an indicator of cellulolytic hydrolysis of the press-cake. Interactions between the press-cake and the fungus, along with fungal morphology, were evaluated by confocal laser scanning microscopy (CLSM) at both micro- and macroscopic level.

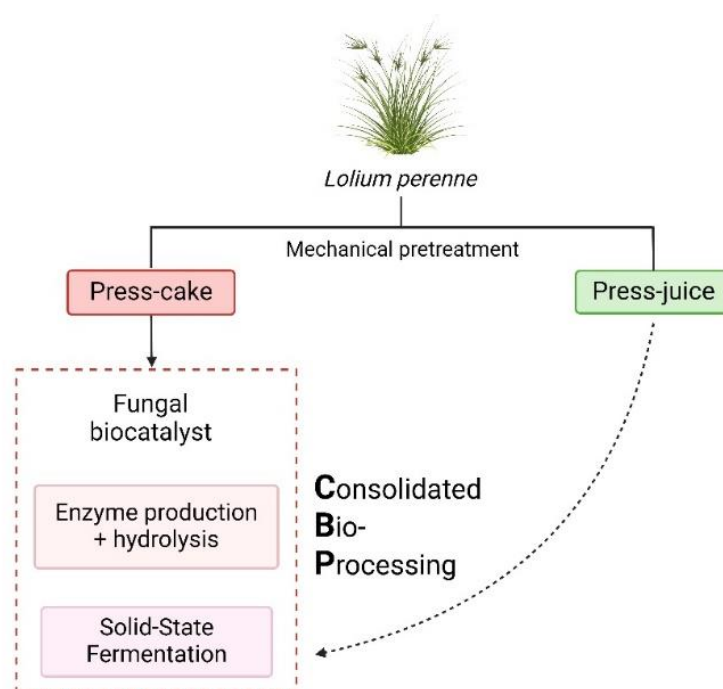


Fig. 1: Overall process for citric acid production investigated in the present study. *L. perenne* was mechanically pretreated using a screw-press to separate the press-cake (solid fraction) and press-juice (liquid fraction). The press-cake was used as substrate in CBP for citric acid production. The fungal biocatalyst *A. niger* secreted the enzymes necessary to hydrolyse the solid substrate and then fermented it to citric acid via SSF. The remaining press-juice was used as additional carbon source.

2 Materials and methods

2.1 Production of the grass press-cake

The perennial ryegrass *L. perenne* was provided by the Julius-Kühne Institute (Braunschweig, Germany). The conditions used for pretreatment of the raw material can be found elsewhere (Varriale et al., 2022). Briefly, mechanical pretreatment using a screw-press was carried out to separate the press-cake (solid fraction) from the press-juice (liquid fraction). The resulting press-cake was sealed in vacuum bags and stored at -20°C until further use.

2.2 Microorganism and spore solution preparation

The filamentous fungus *Aspergillus niger* ATCC 1015 was obtained from LGC Standards (Teddington, UK) and was re-activated following the supplier's instructions. Spore solution agar plates were prepared with 24 g/L potato dextrose broth and 15 g/L agar (Carl Roth GmbH + Co KG, Karlsruhe, Germany). After approximately 7 days of incubation at 32°C, spores were harvested using 10 mL sterile 0.9% (w/v) NaCl solution and scraped from the plate with a sterile spatula. To determine their concentration, spores were counted under a light microscope (B1 Series, Motic, Hong Kong, China) using an improved Neubauer chamber. The spore concentration was calculated as the ratio between the average number of counted spores and the volume of the counting chamber.

2.3 Solid-state fermentation

SSF was carried out in 500-mL Erlenmeyer flasks. In all experiments, the total reaction mass was 10 g, and moisture content was adjusted to 75% (w/w) prior to inoculation with sterile MilliQ water. In the experiments with nutrient addition, the press-cake was supplemented with 25% (v/w) of either one of the following mixtures: 1) glucose, ammonium sulphate, potassium dihydrogen phosphate or 2) press-juice, ammonium sulphate, potassium dihydrogen phosphate. All chemicals were purchased from Carl Roth GmbH + Co KG, except glucose, which was purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). In all cultivations, 4% (w/v) methanol was added, and the inoculum contained 1×10^7 spores/g_{DM} (dry matter). Growth was carried out at 32°C and 140 rpm in a shaking incubator (Ecotron, Infors AG, Bottmingen, Switzerland).

2.4 Downstream process

After 5 days of cultivation, 10% (w/w) MilliQ water was added to the flasks and cultures were incubated for 1 h at 32°C and 140 rpm. Then, the solid and liquid fractions were separated by centrifugation at 4500 rpm for 20 min (Z38K, Hermle Labortechnik GmbH, Wehingen, Germany). The supernatant was filtered to remove any remaining fungal mycelia using Whatman® filter paper (grade 4; Sigma-Aldrich), and then stored at -20°C for further analyses.

2.5 Analytical methods

The dry mass content of the press-cake was measured in triplicate as dry weight after overnight incubation in an oven at 105°C (Memmert GmbH, Schwabach, Germany). The pH of the supernatant was measured using a pH meter (Microprocessor pH 211, Hanna Instruments GmbH, Vöhringen, Germany). The citric acid concentration was determined by high-performance liquid chromatography (HPLC) [Merck Hitachi L-6200 pump (Merck KGaA, Darmstadt, Germany), Midas cool autosampler (Spark Holland B.V., Emmen, Netherlands), and an RI 101 refractive index detector (Shodex, Kawasaki, Japan)]. The HPLC was equipped with an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) at 80°C. The mobile phase was 2.5 mM H₂SO₄ at a flow rate of 0.6 mL/min. The instrument and data evaluation were managed with Clarity software (Data Apex, Prague, Czech Republic). Protein content was determined using the Bradford assay (Pierce® Coomassie Bradford Protein Assay kit, Thermo Scientific, Waltham, MA, USA) and bovine serum albumin as internal standard. Absorbance was measured at 595 nm using a Cary 60UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

2.6 β -glucosidase activity assay

The β -glucosidase activity was determined as described previously (Takashima et al., 1999), with adjustments according to Caputo et al. (2022). Briefly, 50 μ L of supernatant was mixed with 50 μ L of 2.0 mM para-nitrophenyl- β -D-glucopyranoside (in 50 mM sodium acetate buffer, pH 5.0). The assay was performed in 96-well plates with 200 μ L reaction volume at 50°C. The samples were incubated for 10 min at pH 5 using 50 mM sodium acetate buffer. The reaction was stopped with 1 M Na₂CO₃ and absorbance of both the calibration curve and samples was measured at 405 nm. One

unit of enzyme activity (U) corresponded to 1 μ mol of para-nitrophenol produced per minute.

2.7 Confocal laser scanning microscopy

Samples for microscopy analysis were cut in small pieces and fixed in 10% (v/v) formaldehyde (100 g/L). Then, they were washed with 50 mM sodium carbonate-bicarbonate buffer (pH 9.2), immersed in 0.01 g/L Calcofluor White solution (Sigma-Aldrich), and incubated for 5 min. Images were captured with a Nikon A1 (Nikon Instrument Inc., Melville, NY, USA) using a 40 $\times$ /0.75NA objective, at 414 nm excitation and 420–460 nm emission.

3 Results and discussion

3.1 Citric acid production in SSF

Citric acid was produced using the press-cake obtained from mechanical pretreatment of *L. perenne* with a screw-press. First, the possibility to produce citric acid using the press-cake as sole nutrient source was assessed. However, after 10 days of cultivation, no fungal growth or citric acid were detected (data not shown). The lack of growth could be explained by the complex nutrient requirements of *A. niger*, and the specific cultivation conditions required.

Several factors influence citric acid production, including medium pH, morphology of the microorganism, aeration, phosphate and nitrogen limitation, and the concentration and type of carbon source (Show et al., 2015). Only sugars that are rapidly metabolized by the fungus can contribute to good citric acid yields, which means that non-hydrolysed polysaccharides do not support high conversion rates (Papagianni, 2007). To overcome such limitations, the press-cake was supplemented with a 25% (v/w) mixture of glucose or press-juice from perennial ryegrass as carbon source, along with ammonium sulphate as nitrogen source, potassium dihydrogen phosphate as potassium source, and methanol. The latter was added based on previous reports of its positive effect on citric acid production. Dhillon et al., (2011) demonstrated that supplementing apple pomace waste with methanol resulted in 113 g of citric acid per kg of dry apple pomace. When using pomegranate peel waste as substrate, addition of up to 3% (w/w) methanol correlated positively with citric acid yields (Roukas and Kotzekidou, 2020). Similarly, addition of methanol to molasses nearly doubled citric

acid titres, possibly because methanol increased cell permeability (Pazouki et al., 2000). *A. niger* supplemented with two different carbon sources and nutrients was cultivated for 5 days, after which pH and citric acid production were determined (Table 1).

Table 1: Production of citric acid by *A. niger* after 5 days of SSF cultivation. In all the experiments, the total mass of reaction was 10 g, the moisture content was set at 75% (w/w). The press-cake was supplemented with a 25% (v/w) mixture of either glucose or press-juice, plus ammonium sulphate, potassium dihydrogen phosphate, and 4% (w/v) methanol. The spore inoculum was 1×10^7 spores/g_{DM}. The SSFs were carried out at 32°C, 140 rpm.

	pH	Citric acid [g/kg _{DM}]
Press-cake + 25% (v/w) glucose	2.3	84.7 ± 0.3
Press-cake + 25% (v/w) press-juice	3.1	2.1 ± 0.0

Values represent the mean ± standard deviation of two replicates.

The pH is a crucial parameter in citric acid production. Spores germinate at pH ~5; however, during citric acid production, the pH drops as a result of ammonia uptake and the consequent release of protons by the spores (Papagianni, 2007). The lower pH of the culture broth favours citric acid production (Show et al., 2015), reduces the risk of contamination, and inhibits the production of side-products, such as gluconic and oxalic acids (Papagianni, 2007; Show et al., 2015). Here, the starting pH was 4.4 and 4.9, when glucose and press-juice were supplemented, respectively. During cultivation, the pH dropped to 2.3 and 3.1, respectively. This decrease in pH could be explained by increased citric acid production, which reached 84.7 g/kg_{DM}, and was a direct consequence of more fungal cells. Their greater numbers would be favoured by the addition of glucose, but would also result in greater ammonium sulphate consumption. In line with the above hypothesis, experiments performed with press-juice, yielded only 2.1 g/kg_{DM} citric acid. The press-juice may have contained compounds that limited citrate production. For instance, an excess of heavy metals

and alkaline metals can inhibit citric acid production (Papagianni, 2007; Behera, 2020). Yet, 0.3 ppm zinc and 1.3 ppm iron promote citric acid output (Shu and Johnson, 1948). Matthey and Bowes, (1978) reported that 10 mg/L manganese reduced citric acid production by 50%. Supporting this possibility, various ions have been detected in press-juice (Boakye-Boaten et al., 2016; Varriale et al., 2024).

3.2 Protein concentration and enzymatic activity

Filamentous fungi play a critical role in the degradation of LCB thanks to their ability to secrete a wide range of extracellular enzymes, which break down polysaccharides into sugar monomers. The hydrolysis of cellulose requires three different cellulases, which act synergistically and concurrently (Ilić et al., 2023; Varriale and Ulber, 2023; Okeke et al., 2024). *Trichoderma reesei* is known for its high production of exo- and endoglucanase, while *A. niger* is particularly effective in producing β -glucosidase (Le Traon-Masson and Pellerin, 1998; Kang et al., 1999; Lima et al., 2013; Oh et al., 2018; Srivastava et al., 2019). This enzyme is crucial for the complete hydrolysis of cellulose, as it converts cellobiose and celooligomers to glucose monomers. Removal of cellobiose is important as it prevents product inhibition on other cellulolytic enzymes. In this work, β -glucosidase activity was used as an indicator of cellulolytic hydrolysis of the press-cake.

To obtain an insight on protein production during cultivation, the supernatant resulting after downstream processing was tested for protein content (**Fig. 2**). The protein concentration was twice as high when the press-cake was supplemented with glucose ($10.51 \pm 2.49 \mu\text{g/mL}$) compared to press-juice ($5.04 \pm 0.99 \mu\text{g/mL}$). In contrast, β -glucosidase activity was highest following supplementation with press-juice ($1.15 \pm 0.07 \text{ U/g}_{\text{DM}}$) as opposed to glucose ($0.89 \pm 0.02 \text{ U/g}_{\text{DM}}$) (**Fig. 2**). This finding suggested that *A. niger* might have been compelled to hydrolyse the press-cake to access sugars. Accordingly, growth conditions and/or cultivation parameters might influence the ability to produce enzymes necessary to hydrolyse the press-cake. Indeed, biomass composition, pH, temperature, dissolved oxygen, and salts have been shown to influence production of cellulolytic enzymes (Zhang et al., 2024). Both oligosaccharides and cellulose analogues can induce cellulase production (Znameroski et al., 2012); whereas nitrogen limitation has the opposite effect (Callow et al., 2016). So far, β -glucosidase activity has been shown to be affected more by

acidic than alkaline pH (El-Ghonemy, 2021). The presence of metal ions can also influence β -glucosidase. In particular, Mn^{2+} , Pb^{2+} , Hg^{2+} , Cu^{2+} and Co^{+} have a negative effect on its activity (Le Traon-Masson and Pellerin, 1998; Kang et al., 1999; El-Ghonemy, 2021; Osaro-Matthew et al., 2023).

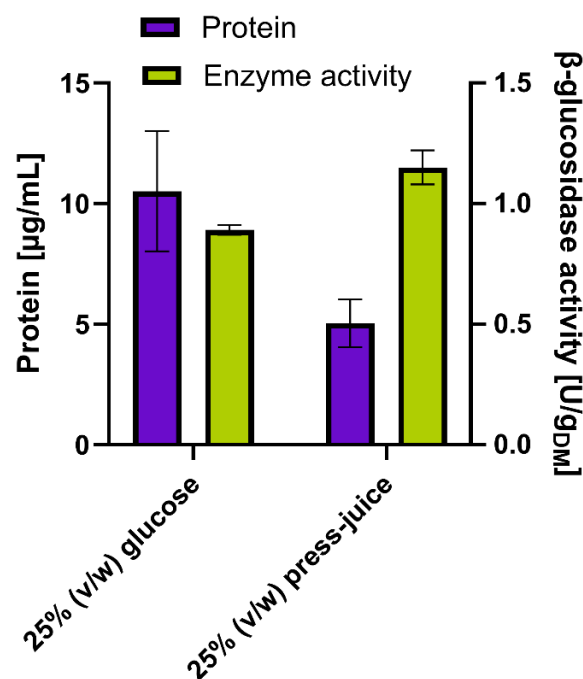


Fig. 2: Protein concentration and β -glucosidase activity of the supernatant after SSFs supplemented with either 25% (v/w) glucose or press-juice. One unit of enzyme activity (U) corresponded to 1 μ mol of para-nitrophenol produced per minute. Error bars represent the average value of two replicates.

3.3 Morphological analysis

The morphology of filamentous fungi such as *A. niger* varies significantly depending on the desired product and the bioprocess used. In submerged cultivation, filamentous fungi can grow in dispersed or pelletized form (Papagianni, 2004). In SSF, a dispersed mycelium is the most frequent morphological type observed. The dispersed morphology can be described as a branched pattern where a hypha emerges from the spore and elongates at the tip by creating new branches. The process continues until a three-dimensional network of hyphae called mycelium is formed. The mycelium is particularly suitable for growth on solid substrates, as it allows filamentous fungi to colonize and penetrate such environments in search of nutrients. At present, SSF has

been applied for the production of enzymes, antibiotics, organic acids, biosurfactants, and aroma compounds (Lizardi-Jiménez and Hernández-Martínez, 2017).

To understand the interaction between *A. niger* and the press-cake, cultivations were imaged by CLSM. A close interaction between *A. niger* and the insoluble substrate was observed in cultures supplemented with either glucose (**Fig. 3A–C**) or press-juice (**Fig. 3D–F**). In both cases, the mycelial hyphae were growing in and around the biomass surface (**Fig. 3A, C, and D**). However, the choice of carbon source led to differences at the microscopic level. When *A. niger* was cultivated on the press-cake supplemented with glucose, the degree of ramification increased, suggesting higher enzyme production (**Fig. 3B**). This result is in accordance with the findings by Novy et al., (2021), who reported that a higher degree of branching boosted protein production in *T. reesei*. Furthermore, intact, robust, and thick hyphae could be observed, thus evidencing a favourable growth condition for the fungus.

Instead, when the press-cake was supplemented with press-juice, the hyphae appeared thinner, fragmentated, and less compact (**Fig. 3E**). As marked in **Fig. 3F** (yellow circle), in some spots, a bulbous cell growth was observed. This growth is generally associated with nutrient deficiency. It might be possible that the scarcity of sugar in press-juice and/or the presence of inhibitory compounds such as metal ions had a negative effect on fungal growth.

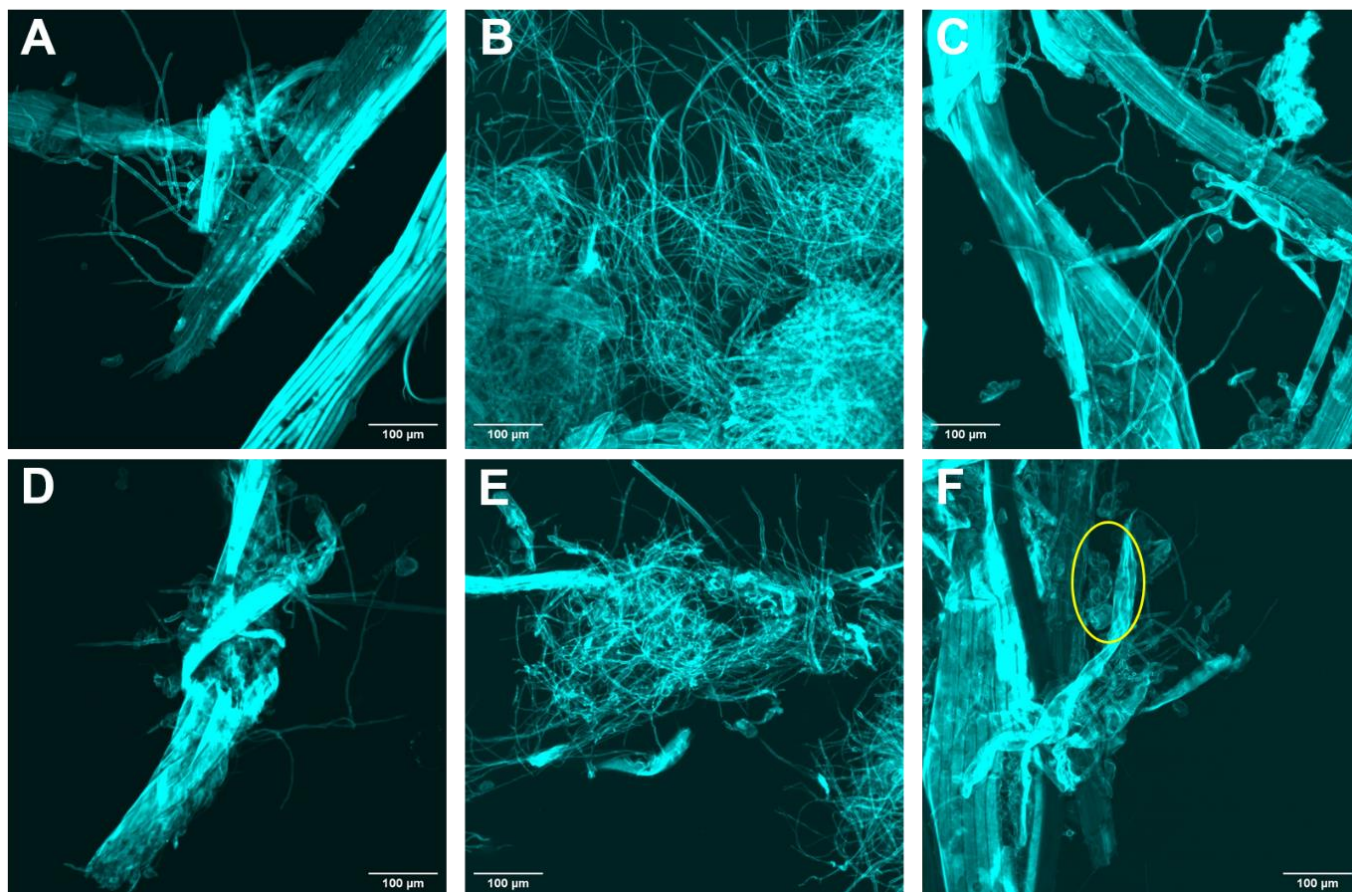


Fig. 3: CLSM micrographs of *A. niger* after SSF using glucose (A–C) or press-juice (D–F) as additional carbon source. The figure depicts the fungal mycelia growing on the press-cake (A, C, D), branching degree of fungal mycelia (B, E), and the presence of bulbous cells (yellow circle in F). Staining was performed with Calcofluor White. Colour was assigned arbitrarily.

4 Conclusion

In the present study, we explored the use of grass press-cake for the production of citric acid by *A. niger* in a CBP. When the press-cake was used as the sole source of nutrients, neither fungal growth nor citric acid production were observed. However, supplementing the press-cake with additional carbon source in the form of glucose or press-juice, along with nitrogen, phosphate, potassium, and methanol, led to successful citric acid production. These findings highlight the importance of optimizing process conditions such as nutrient availability in SSF processes. Interestingly, β -glucosidase activity was higher when press-juice was used as additional carbon source, suggesting that the lower sugar content of this supplement prompted *A. niger* to hydrolyse the press-cake to access sugar. Qualitative information about the

interaction between the fungus and the press-cake was obtained by CLSM analysis. At a macroscopic level, close interaction between *A. niger* and the press-cake was observed, regardless of the carbon source. At a microscopic level, differences in mycelial appearance and degree of ramification could be observed with respect to carbon source. Overall, these findings reveal that filamentous fungi such as *A. niger* are particularly suitable for developing CBP, in which production of hydrolytic enzymes, biomass hydrolysis, and fermentation occur simultaneously in a single system.

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Chapter V – Paper IV

Short-term adaptation as tool to improve bioethanol production using grass press-juice as fermentation medium

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L. Varriale: conceptualization, methodology, formal analysis and investigation, writing- original draft preparation, review and editing, data curation and visualization

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Keyword: Lignocellulosic biomass, bioethanol, *Saccharomyces cerevisiae*, fermentation, short-term adaptation

Abstract

Grass raw materials collected from grasslands cover more than 30% of Europe's agricultural area. They are considered very attractive for the production of different biochemicals and biofuels due to the high availability and renewability. In this study, a perennial ryegrass (*Lolium perenne*) was exploited for second-generation bioethanol production. Grass press-cake and grass press-juice were separated using mechanical pretreatment, and the obtained juice was used as a fermentation medium. In this work, *Saccharomyces cerevisiae* was utilized for bioethanol production using the grass press-juice as the sole fermentation medium. The yeast was able to release about 11 g/L of ethanol in 72 h, with a total production yield of $0.38 \pm 0.2 \text{ g}_{\text{Ethanol}}/\text{g}_{\text{sugars}}$. It was assessed to improve the fermentation ability of *Saccharomyces cerevisiae* by using the short-term adaptation. For this purpose, the yeast was initially propagated in increasing concentration of press-juice. Then, the yeast cells were re-cultivated in 100 % (v/v) fresh juice to verify if it had improved the fermentation efficiency. The fructose conversion increased from 79% to 90%, the ethanol titers reached 18 g/L resulting in a final yield of $0.50 \pm 0.06 \text{ g}_{\text{Ethanol}}/\text{g}_{\text{sugars}}$ with a volumetric productivity of $0.44 \pm 0.00 \text{ g/Lh}$. The overall results proved that short-term adaptation was successfully used to improve bioethanol production with *S. cerevisiae* using grass press-juice as fermentation medium.

1 Introduction

Nowadays, our society is dealing with important challenges such as environmental destruction, climate change and insufficient fossil resources. In order to overcome these problems, the transition towards a bio-based economy has gained more attraction. Lignocellulosic biomasses, also known as second-generation feedstocks (2G), are emerging due to the high availability, no-competition with the food/feed industry and renewability. They are supplied from different resources such as agriculture and forestry residues, industrial and domestic wastes, and aquaculture (Cherubini et al. 2009).

In Europe, grassland and field grass cover one third of the agriculture land in Europe (Kamm et al. 2016). Grass raw materials from herbaceous crops are very attractive due to the multiple growth cycles per year (Kongkeitkajorn et al. 2021), high biomass yield and productivity (Takara and Khanal 2015; Tsai et al. 2018), and low energy

input (Ask et al. 2012). Further, the high content of structural polysaccharides, such as cellulose and hemicellulose, makes grass materials a potential substrate for energy and biochemicals production (Prasertwasu et al. 2014; Tsai et al. 2018; Kongkeitkajorn et al. 2020). The general scheme to process green biomass is to separate the fiber-cake fraction and the juice fraction using mechanical pretreatments (Sieker et al. 2011). These pretreatments methods help to reduce the water content thus decreasing the costs for the transportations (Varriale et al. 2022). Moreover, mechanical pretreatments avoid the formation of inhibitory compounds which affect the microbial growth (Nielsen et al. 2015). For all the reasons mentioned above, perennial ryegrass can have an important role in the lignocellulose-to-ethanol process.

In 2017, petroleum resources provide 94% of total energy in the transportation sector, which accounts for 15% of the total pollutants emissions (International Energy Agency 2020; Sharma et al. 2020; Dobrescu et al. 2021). In this view, the Renewable Energy Directive Recast fixed a minimum target on the use of renewables to produce bioethanol of 14% by 2030 (European Parliament 2018; Dobrescu et al. 2021). According to this, it is expected that the ethanol bioproduction will surpass 130 billion liters/year worldwide by 2030, with the United States and Brazil as main producers (OECD/FAO 2021; Tse et al. 2021). The baker's yeast *Saccharomyces cerevisiae* is the conventional microorganism for bioethanol production. It has several appealing industrial qualities, such as fast growth, high production yields, and high tolerance to environmental stress factors. During the last years, several strategies have been used to improve its fermentative ability in lignocelluloses, such as rational engineering (mutagenesis) and evolutionary engineering (Adaptive Laboratory Evolution (ALE) and the short-term adaptation) (Ask 2013). Evolutionary engineering is performed by applying a selective pressure for the desired phenotype in order to let the microorganism adapt to the condition(s) by natural evolution (Wang et al. 2023). Whereas by ALE the changes are incorporated into the genome, short-term adaptation results in the selection of the phenotype(s) which is more adapted to the specific environmental factor (Nielsen et al. 2015; Nielsen 2016). The critical step in the short-term adaptation is the culture's propagation which led to get cells with high fermentative efficiency and performance (Tomás-Pejó and Olsson 2015). Short-term adaptation has been used to obtain *S. cerevisiae* strains tolerant to inhibitors (Wallace-Salinas and Gorwa-Grauslund 2013; Gu et al. 2014; van Dijk et al. 2020; Almeida et

al. 2023) or capable to co-ferment glucose and xylose (Klimacek et al. 2014; Nielsen et al. 2015; Dobrescu et al. 2021).

The aim of this work was to test the capability of *S. cerevisiae* to produce bioethanol using the press-juice from a perennial ryegrass (*Lolium perenne*) as fermentation medium. It was firstly examined the juice composition to verify the nutrients content. Then, it was investigated the possibility to improve the fermentation performance of *S. cerevisiae* adopting the short-term adaptation tool. For this purpose, the yeast was propagated in increasing concentration of press-juice. All the cultivations were evaluated in terms of sugars consumption, ethanol and by-products production, and biomass growth. Finally, physiological parameters were calculated and used to compare the fermentation performance of *S. cerevisiae* before, during and after the adaptation.

2 Materials and Methods

2.1 Raw material and juice preparation

The perennial ryegrass (*Lolium perenne*) was kindly provided by the Julius Kühn-Institute (Braunschweig, Germany). The production of the press-juice is described elsewhere (Varriale et al. 2022). The resulting juice was further processed before the use as fermentation medium. In particular, the juice was centrifuged twice at 4500 rpm for 20 min (Z38K, Hermle Labortechnik GmbH, Wehingen, Germany) to separate the slurry portion. To remove the remaining solid particles, the supernatant was filtered using filter paper (Macherey-Nagel, 185mm). To prevent the loss of important nutrients, the sterilization process was carried out using a sterile filtration (Stericup®, 0.2 µm pore size, MerckMillipore, Massachusetts, USA). The obtained press-juice was stored at -20°C until further use.

2.2 Microorganism and medium

The yeast *Saccharomyces cerevisiae* 3799 was obtained from the German Collection of Microorganisms and Cell cultures (DSMZ GmbH, Braunschweig, Germany). Preculture and control experiments were carried out in Yeast Peptone Dextrose (YPD) medium with the following composition: yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L (pH 6.2). Unless otherwise stated, all the chemicals were purchased from Carl Roth + Co KG (Karlsruhe, Germany), except dextrose which was purchase from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.3 Preculture and main culture preparation

Precultures were started from stock cryo-culture stored in 25% (v/v) glycerol at -80°C. They were grown aerobically in 100 mL baffled shake flasks for 24 h until the late-exponential phase was reached. The main cultures were inoculated to 0.1 OD (optical density) without pH adjustment. The main cultivations were performed anaerobically in 100 mL glass bottles with 50 mL working volume, tightly sealed with rubber septa. The bottles were sparged with pure nitrogen prior to inoculation. Both the precultures and the main cultures were incubated at 32°C and 120 rpm (Ecotron, Infors AG, Bottmingen, Switzerland).

2.4 Sampling and cell growth determination

Samples were regularly taken for the OD measurement. One mL of each sample was further centrifuged (14000 rpm, room temperature, 7 min; Eppendorf, Hamburg, Germany) and the supernatant was stored at -20°C for HPLC (High-Performance Liquid Chromatography) analysis. The cell dry weight (CDW) was determined in a separate experiment using YPD medium and aerobic condition for the generation of an OD-CDW correlation. The experiment was performed as follows: a 5 mL of cell suspension was harvested by centrifugation (4500 rpm, 10 min, 4°C, Z38K, Hermle Labortechnik GmbH, Wehingen, Germany). The cell pellet was washed with 5 mL and resuspended in 5 mL NaCl solution (9 g/L). Finally, the cell suspension was transferred to 15 mL tubes previously dried and then dried (both at 50°C, 48 h) in the oven (Mettler GmbH, Schwabach, Germany) until a constant weight was reached. The CDW/OD₆₀₀ correlation was determined to be 0.32 ± 0.02 and it was established in triplicates. The maximum specific growth rate (μ_{MAX}) was determined in separate experiments in duplicates. Both the not adapted and adapted *S. cerevisiae* strains were cultivated in 100 % (v/v) juice anaerobically. 1 mL sample was harvested and centrifuged (14000 rpm, 7 min, room temperature, Eppendorf, Hamburg, Germany). The pellet was washed and resuspended in distilled water. The maximum specific growth rates were estimated from the slope of the linear regression between $\ln(\text{OD})$ and time.

2.5 Analysis of press-juice components and metabolites

The analysis of the press-juice was carried out with regard to pH, protein content, sugars, amino acids, cations and anions. Before any analysis, the samples were

filtered through a 0.22 µm pore size nylon filter (KX Syringe Filter Nylon, Cole-Parmer GmbH, Wertheim, Germany). The pH value was measured using a pH meter (Microprocessor pH 211, Hanna Instruments Deutschland GmbH, Vöhringen, Germany). The protein concentration was determined using Bradford assay (Pierce® Coomassie Bradford Protein Assay kit, Thermo Scientific, Massachusetts, United States) and the Bovine serum albumin as internal standard (Thermo Fisher Scientific, Massachusetts, United States). The absorbances of the calibration curve and of the samples were measured at 595 nm (Cary 60 UV-Vis, Agilent Technologies, USA). Sugars and products concentrations were analyzed by HPLC [Autosampler AS 6.1L (Knauer GmbH, Berlin, Germany), Azura pump P 6.1L (Knauer GmbH, Berlin, Germany)] and a refractive index detector (RI 101 Shodex, Kawasaki, Japan). The HPLC was equipped with an Aminex HPX-87H column (Bio-Rad, 300x7.8 mm, Hercules, California, USA) at 80°C. The mobile phase was 2.5 mM H₂SO₄ at a flow rate of 0.6 mL/min. The instrument control and data evaluation were carried out with a Clarity software system (Data Apex, Prague, Czech Republic). Amino acids were separated using a resolve C18 column (150x3.9 mm, Waters Corporation, Milford, USA) with a SecurityGuard Cartridge (C18, 4 x 3.0 mm ID, Phenomenex, Torrance, California, USA) placed in a column heater CT 2.1 (Knauer GmbH, Berlin, Germany) set at 30 °C. The detection was performed using Azura photodiode-array detector DAD 2.1L at 230 nm (Knauer GmbH, Berlin, Germany). The analysis included a precolumn derivatization with ortho-Phthaldialdehyde (OPA). The derivatization process was automated using the Azura AS 6.1L autosampler (Knauer GmbH, Berlin, Germany) using the own “mix method” option. The OPA reagent was prepared by weighting 270 mg OPA in a 50 mL volumetric flask. The reagent was dissolved in 5 mL ethanol. Then, 200 µL 2-mercapto-ethanol were added and the final volume was filled up with 0.4 M borate buffer. The mobile phase consisted of solvent A (0.025 M sodium-acetate anhydrous and 0.025 M NaH₂PO₄ monohydrate) and solvent B (50% methanol). The pH value of solvent A was adjusted to 7 with 10 M NaOH, then 21 mL of both tetrahydrofuran and methanol were added. The gradient elution program was as follows: from 0 to 50 min, solvent B changed linearly from 0% to 100%; from 50 to 55 min, solvent B was set as isocratic at 100%; from 55 to 60 min, solvent B changed linearly from 100% to 0%; from 60 to 67 min, solvent B was set as isocratic at 0%. The flow rate was 1.0 mL/min. To analyze the samples, they must be protein and particles-

free. For this reason, the proteins were firstly precipitated by adding 4 parts of ice-cold methanol and 1 part of the sample. The sample was placed at -20°C overnight. Then, the sample was centrifuged for 10 min at 10000 rpm (Eppendorf, Hamburg, Germany). Finally, the sample was diluted with 0.4 M borate buffer. A dilution of at least of 1:1 is necessary as the sample has to have a pH 10 for the derivatization. The derivatization was performed by adding 80 µL of the sample or the standard and 50 µL of OPA reagent. After 1 min, 40 µL were injected for measurement. Cations and anions were analyzed by ion chromatography (IC) (930 Compact IC Flex, Metrohm GmbH & Co. KG, Germany) with an inline system for dialysis (930 Compact IC Flex, Metrohm, Filderstadt, Germany) and an IC Conductivity Detector (Metrohm, Filderstadt, Germany). Cations were measured with a cation column (Metrosep C6-250/4.0, Metrohm) using 4 mM HNO₃ and 0.7 mM dipicolinic acid as mobile phase at a flow rate of 0.9 mL/min. Anions were measured with an anion column (Metrosep A Supp5-250/4.0, Metrohm) using 1 mM NaHCO₃ and 3.2 mM Na₂CO₃ as mobile phase at a flow rate of 0.7 mL/min. The oven temperature in both cases was 35°C. For each analysis, the samples were diluted to a concentration inside the external calibration range (using 2 mM HNO₃ for cation determination).

2.6 Propagation

Propagation was performed in sequential anaerobic processes (**Fig. 1**). *S. cerevisiae* was cultivated in increasing concentration of grass press-juice (10 %(v/v), 25 %(v/v), 30 %(v/v) 50 %(v/v), 60 %(v/v), 75 %(v/v), 100 %(v/v)). 10 %(v/v) was used as starting point and not considered in bioethanol production. The cells were transferred to the next juice concentration after 24 h, except from the passage to 60 %(v/v) which occurred after 48 h due to the lower yeast growth. After the cultivation in 100 %(v/v), the adapted cells were harvested to prepare cryo-cultures. They were used to recultivate the cells in 100 %(v/v) fresh juice to analyze the physiological parameters and fermentation capacity. The results were compared with those obtained from the not adapted *S. cerevisiae* strain. In every passage of the propagation, the juice was inoculated to 0.1 OD using the cells from the previous concentration.

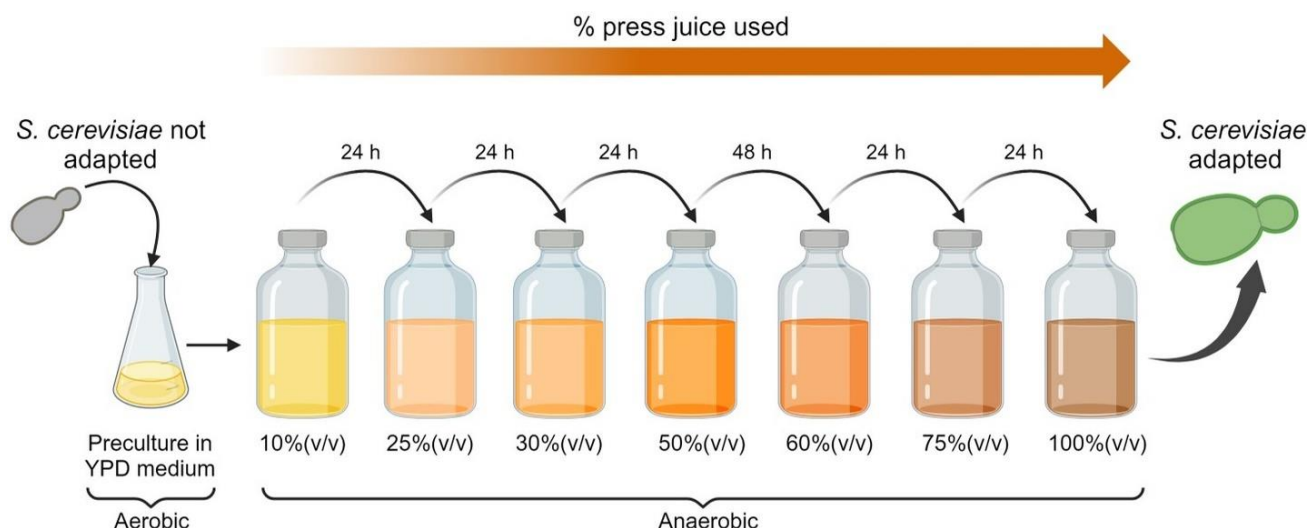


Fig. 1: Propagation scheme of *S. cerevisiae* during short-term adaptation. The figure was generated using BioRender program

2.7 Data processing and evaluations

The percentage of sugars utilization (S_u) was calculated according to equation (1):

$$S_u = \frac{S_0 - S_t}{S_0} * 100 \quad (1)$$

where S_0 is the starting sugar concentration, S_t is the sugar at the time.

Substrate consumption rates (r_s) were calculated using the following equation:

$$r_s = \frac{S_t - S_0}{t} \quad (2)$$

where S_t is the substrate concentration at specific time, S_0 is the substrate concentration at the beginning of the fermentation, and t is the time. Accordingly, the sugar consumption rate was characterized by a negative value as it described the decrease of sugar over time. The volumetric ethanol productivity was expressed in g/Lh (Q_{EtOH}) and was estimated from the ratio between the ethanol concentration and the time at the end of the fermentations. The yields were calculated considering the g of product per g of total sugars consumed (glucose and fructose). Carbon balances included both biomass and metabolite yields, and it was assumed that 1 mol CO_2 was formed per mol ethanol and acetate (Novy et al. 2017). For biomass yields, a C-molar weight of 26.4 g/Cmol was used (Lange and Heijnen 2001).

3 Results

3.1 Analysis of press-juice

In order to use the press-juice as fermentation medium it is important to know its composition in terms of sugars, proteins, amino acids, ions, and pH. The composition of the juice is reported in Table 1. The analysis of the sugars revealed that the juice was mainly composed by fructose (over 30 g/L) and small quantities of glucose (< 4 g/L). Moreover, since *S. cerevisiae* can metabolize only hexoses, no further sugars analysis was performed. With regard to the nitrogen content, both proteins and amino acids contributed to the total amount with 1008 mg/L and 480 mg/L, respectively. In particular, high concentrations of arginine, lysine and leucine were found in the juice (**Fig. 2**). The analysis of the ions revealed that the elements present at higher concentrations were potassium (6.31 g/L), calcium (0.57 g/L) and magnesium (0.23 g/L) as cations, and chloride (4.23 g/L), phosphate (2.75 g/L) and sulphate (1.42 g/L) as anions.

Table 1: Press-juice composition from the perennial ryegrass *Lolium perenne*. The pH value was 5.3

Sugars [g/L]	
Glucose	3.7
Fructose	36.4
Total	40.1
Protein [mg/L]	
Total	1008
Amino acids [mg/L]	
Total	479.2
Cation [g/L]	
Sodium	0.14
Ammonium	0.066
Potassium	6.31
Magnesium	0.23
Calcium	0.57
Total	7.32
Anion [g/L]	
Chloride	4.23
Nitrite	< 0.01
Nitrate	0.03
Phosphate	2.75
Sulphate	1.42
Total	8.43

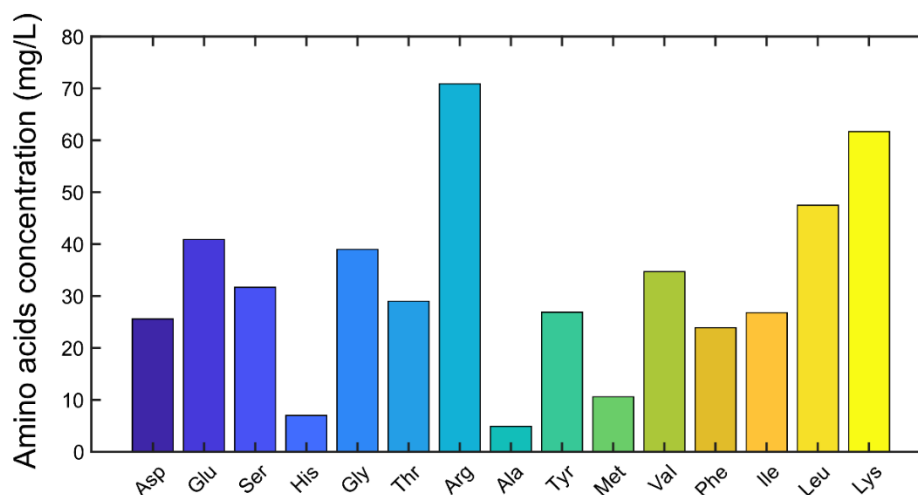


Fig. 2: Amino-acids pattern in grass press-juice analyzed by HPLC.

3.2 Time-course of bioethanol production using the press-juice as fermentation medium

To evaluate the potential of grass press-juice as fermentation medium, it was assessed whether *S. cerevisiae* was capable to grow and to produce bioethanol in 100 % (v/v) press-juice. The cultivation of the yeast lasted for 72 h to ensure almost complete sugars consumption. The growth was monitored using the OD₆₀₀ measurement. The growth profile showed that *S. cerevisiae* cultivated in press-juice as sole fermentation medium exhibited a lag-phase of about 6 h (**Fig. 3a**). This was further confirmed by the fact that no sugars were yet consumed (**Fig. 3b**). The lag-phase was followed by the exponential-phase, which lasted for about 18 h. During the exponential-growth phase, glucose and fructose strongly decreased of about 41% and 35% respectively, and the ethanol started to be produced. After 24 h of fermentation, *S. cerevisiae* continued to growth slower until the end of the cultivation. At this point, the glucose was completely depleted, 7.6 g/L fructose remained, and 11.5 ± 3.4 g/L of bioethanol were produced corresponding to a production yield of 0.38 g_{Ethanol}/g_{sugars} (glucose and fructose).

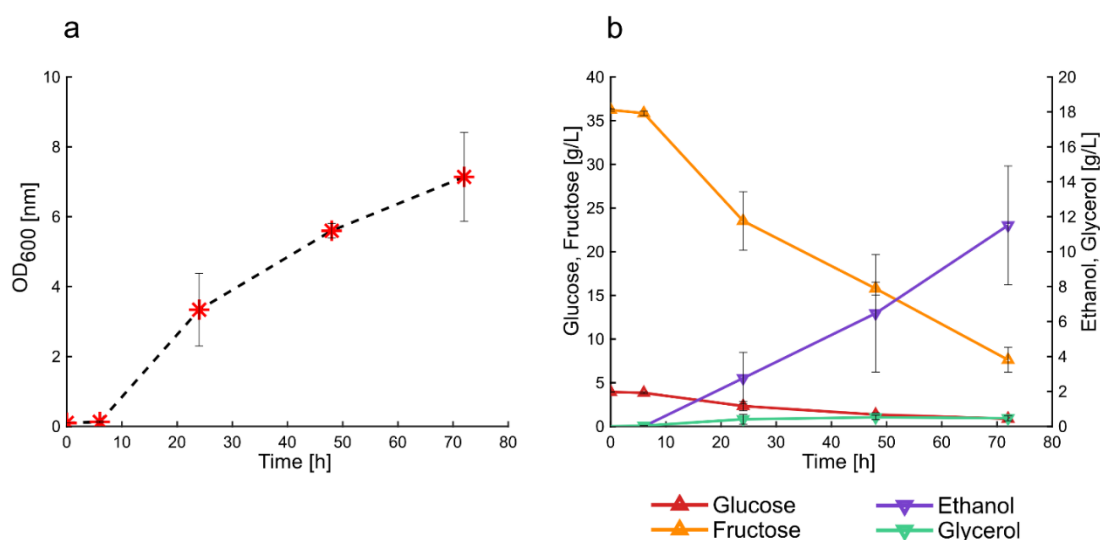


Fig. 3: Cultivation of *S. cerevisiae* in 100% (v/v) press-juice. Growth curve (a), sugars consumption and products formation (b) in a 72h fermentation. Data and error bars represent mean values and the standard deviations of triplicates. Growth conditions: 120 rpm, 32°C, pH 5.3.

3.3 Growth profile during short-term adaptation

Once it was proved that bioethanol could be produced using press-juice as a fermentation medium, it was investigated the possibility of increasing the fermentation performance (in terms of sugars consumption and ethanol production) through short-term adaptation. For this purpose, the cells were propagated in increasing concentration of press-juice (25, 30, 50, 60, 75 and 100 %(v/v)) in order to possibly obtain an adapted-*S. cerevisiae* strain with improved fermentation capabilities. The growth profiles of *S. cerevisiae* did not change depending on the percentage of juice used (**Fig. 4**). In all cases the lag-phase last about 6 h, followed by the exponential-phase. At 24 h, the highest cell density was recorded at 50 %(v/v) of juice, followed by 60 %(v/v), 25 %(v/v) and 30 %(v/v). Nonetheless, small differences could be observed in the beginning of the specific growth-phase. In some juice percentages, the stationary-phase started at 24 h and ended at 48 h, such as for 60 and 30 %(v/v) press-juice. In other cases (25 and 50% (v/v)), the plateau was not distinct because of the few samplings, while at 75 %(v/v) of press-juice the exponential-phase last until 48 h of cultivation. In all cases except at 100% (v/v), the cell densities started to decrease between 48 and 72 h of cultivation, thus meaning that the death-phase took place. However, when the juice was used as sole fermentation medium, the exponential-growth phase lasted until 72 h.

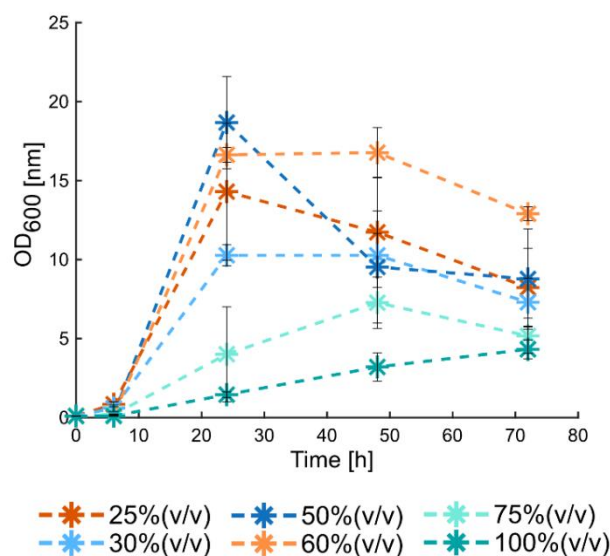


Fig. 4: Growth profile of *S. cerevisiae* cultivated in different juice percentages during the propagation. Data and error bars represent mean values and the standard deviations of triplicates.

3.4 Sugars consumption and ethanol production during the short-term adaptation

The time-course fermentations during the short-term adaptation strategy are illustrated in **Fig. 5 (a-f)**. In all the percentages used for the propagation, glucose was almost totally consumed at the end of the fermentations. At 25 %(v/v) and 30 %(v/v) juice concentration, glucose was the predominant sugar in the medium and was depleted within the first 24 h (Fig. 5 a-b). In the cultivation with 50 %(v/v), glucose and fructose represented 56.8% and 43.2% of the total sugars, respectively. In this case, after 72 h the glucose was almost totally consumed (0.3 g/L) and 2.01 g/L of fructose remained. The highest juice concentrations corresponded to higher fructose concentrations and the sugar utilization was dependent on the percentage of juice used. In particular, between 25-60 %(v/v) juice the remaining fructose did not exceed 16% of the initial fructose concentration. A further increase in the juice percentage to 75 %(v/v) and 100 %(v/v) resulted in higher percentage of remaining fructose (21.6% and 30.5% respectively). The concentration of fructose remained at the end of the fermentation in each percentage is reported in Table 2. Regarding the products formation, ethanol was the primary product detected in the alcoholic fermentations (**Fig. 5 a-f**). The titers

and the yields for each juice percentages are reported in Table 2. In all the cultivations, its production started after 6 h according to the beginning of sugars consumption. Between 6 h and 24 h, the solvent was rapidly produced. More than 78% of the total bioethanol was released within the first 24 h of fermentations at 25, 30, 60 % (v/v) of juice, corresponding to 7.51 ± 0.34 g/L, 8.02 ± 0.32 g/L, and 8.21 ± 0.14 g/L, respectively. Extending the time to 48 h resulted in the formation of 83.4%, 65.5% and 57.5% of the total bioethanol when using 50, 75 and 100 % (v/v) of juice concentrations, respectively. These results corresponded to 3.8-, 2.6- and 3.6-fold product increase respect to the first 24 h of fermentation. The ethanol production reached a plateau after 24 h of fermentation when 25-50 %(v/v) were used. In the other conditions, it was still produced until the end of the fermentations due to the high sugars' availability in the media. After 72 h, the solvent production increased by increasing the juice percentage, up to 75 %(v/v). In terms of production yields, the highest values were recorded using 50%(v/v) of press-juice (0.41 g/g), followed by 30%, 60% and 25% (v/v) of juice (Table 2). These results corresponded to 80% and 70% of the maximum theoretical ethanol yield, respectively. Aside from ethanol, glycerol and acetate are the main byproducts in the alcoholic fermentation. However, in this study side-products (biomass, acetate and glycerol) were detected only in small amount.

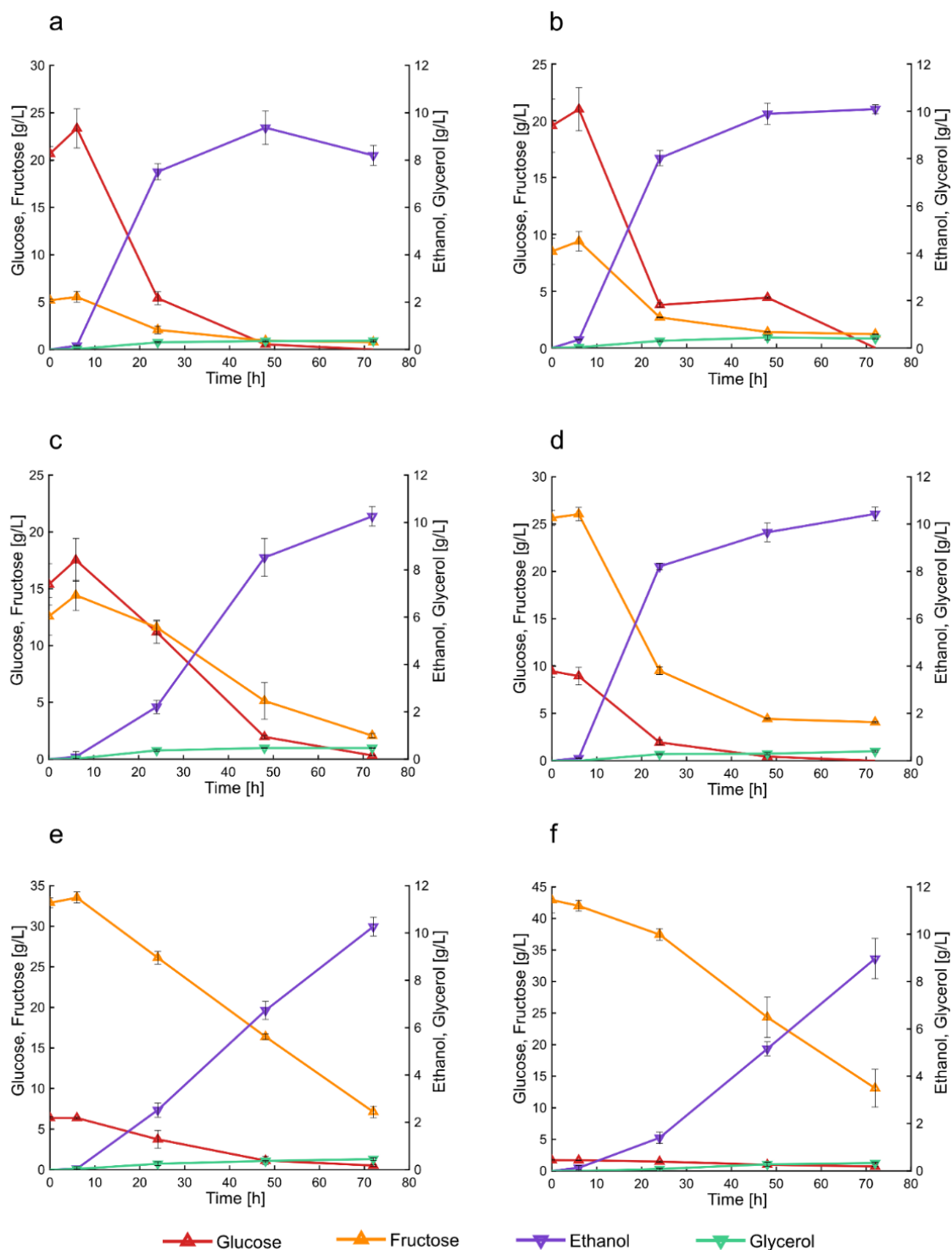


Fig. 5: Cultivation of *S. cerevisiae* in different percentages of juice during short term adaptation. Cultivation in 25 % (v/v) press-juice (a), cultivation in 30 % (v/v) press-juice (b), cultivation in 50 % (v/v) press-juice (c), cultivation in 60 % (v/v) press-juice (d), cultivation in 75 % (v/v) press-juice (e), cultivation in 100 % (v/v) press-juice (f). Data and error bars represent mean values and the standard deviations of triplicates. Growth conditions: 120 rpm, 32°C, pH 5.9, 5.8, 5.6, 5.5, 5.3 respectively.

Table 2: Physiological parameters of the fermentations with *S. cerevisiae* in different percentage of press-juice. r_{s1} and r_{s2} are the glucose and the fructose consumption rate, respectively. C_{EtOH} is the ethanol production at the end of the fermentations. Q_{EtOH} is the volumetric ethanol productivity. The yields were calculated considering the total sugar content.

	25 %(v/v)	30 %(v/v)	50 %(v/v)	60 %(v/v)	75 %(v/v)	100 %(v/v)
Fructose remained (g/L)	0.78±0.06	1.21±0.02	2.07±0.19	4.07±0.09	7.12±0.72	13.10±2.99
r_{s1} (g/Lh)	-0.42±0.02 ^a	-0.40±0.05 ^a	-0.28±0.04 ^a	-0.22±0.04 ^a	-0.11±0.01 ^a	-0.02±0.00 ^a
r_{s2} (g/Lh)	-0.09±0.01 ^a	-0.15±0.03 ^a	-0.16±0.03 ^a	-0.44±0.01 ^a	-0.42±0.08 ^a	-0.39±0.05 ^a
C_{EtOH} (g/L)	8.20±0.42	10.09±0.19	10.26±0.41	10.43±0.29	10.27±0.40	8.97±0.85
Q_{EtOH} (g/Lh)	0.12±0.02	0.14±0.00	0.14±0.01	0.14±0.00	0.14±0.01	0.12±0.00
Y_{EtOH} (g/g)	0.36±0.06	0.38±0.04	0.41±0.06	0.36±0.05	0.22±0.01	0.29±0.02
$Y_{Glycerol}$ (g/g)	0.014±0.003	0.014±0.000	0.018±0.002	0.014±0.000	0.015±0.001	0.011±0.000
$Y_{AceticAcid}$ (g/g)	n.d	n.d	n.d	n.d	n.d	n.d
$Y_{Biomass}$ (g/g)	0.20±0.06 ^a	0.13±0.03 ^a	0.15±0.07 ^a	0.19±0.04 ^a	0.09±0.01 ^a	0.05±0.01 ^a

n.d., not detectable

^aParameters were determined at 48 h

3.5 Sugars consumption and ethanol production by the adapted strain of *S. cerevisiae*

After the adaptation, the cells cultivated in the last step of propagation were recovered and re-cultivated in fresh 100 % (v/v) press-juice. The experiment was performed to assess if the yeast adapted to the set environmental conditions. According to this, cell density, sugars utilization and products formation were evaluated (**Fig. 6 a-b**).

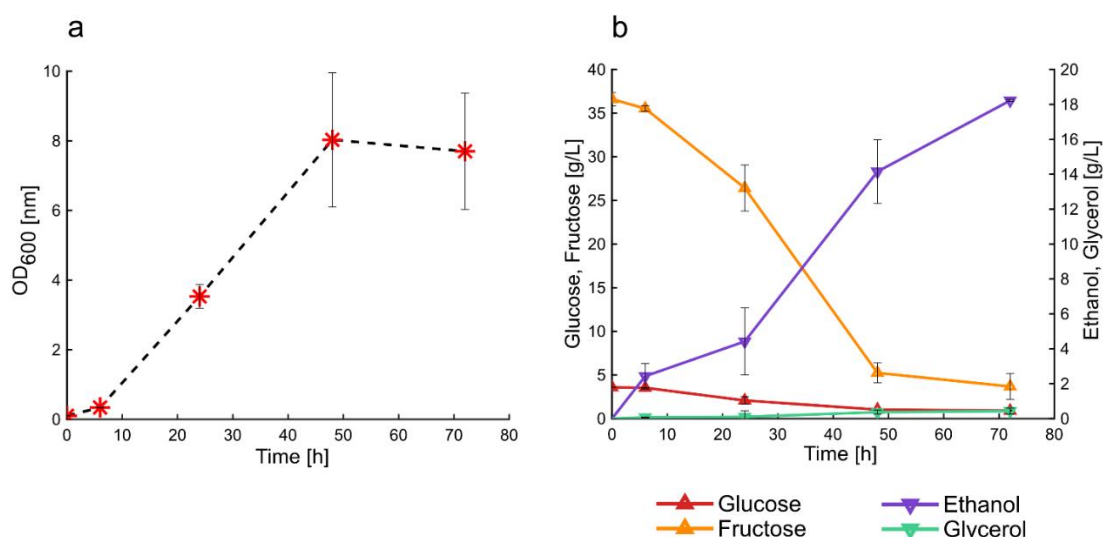


Fig. 6: Cultivation of adapted *S. cerevisiae* in 100 % (v/v) press-juice. Growth curve (a), sugars consumption and products formation (b) during 72 h fermentation. Data and error bars represent mean values and the standard deviations of triplicates. Growth conditions: 120 rpm, 32°C, pH 5.3.

In contrast to the not adapted *S. cerevisiae*, the growth of the adapted *S. cerevisiae* started immediately (**Fig. 6a**). This was also supported by the initial utilization of sugars, especially fructose. In the first 24 h of cultivation, approximately 13 g/L of fructose were metabolized. The fructose consumption led to a bioethanol production of 4.42 ± 1.92 g/L. However, the exponential-growth phase lasted until 48 h, resulting in further fructose consumption (about 20 g/L) and increased solvent production (14.15 ± 1.82 g/L). Although notable differences between the not adapted and the adapted strains were observed in the case of ethanol production, the titers and the yields for glycerol and biomass were almost identical (Table 3). **Fig. 7a** shows the percentages of fructose conversion in the not adapted and adapted *S. cerevisiae* strains. At the end of the cultivations, the not adapted strain consumed about 79% of the total fructose

and the adapted strain converted about 90%. The faster and broadly fructose consumption resulted in higher product titer (Table 3), as well as higher product yields and productivities (**Fig. 7b**), reaching almost the maximum theoretical ethanol yield of 0.51 g_{Ethanol}/g_{Sugar(s)} (Koppram et al., 2013). At 24 h, the bioethanol productivities between the two strains did not differ considerably, reaching about 0.74 g/Lh. However, in both cases the productivities decreased overtime, and the adapted strain showed higher values for all the time-points considered (**Fig. 7b**). To better compare the fermentation ability of both strains, the fermentation efficiency was calculated by dividing the bioethanol yield for the theoretical yield. The results are shown in **Fig. 7c**. In particular, at 24 h the fermentation efficiencies were 45% for the not adapted strain and 78% for the adapted strain. The fermentation efficiencies were higher in the adapted strain than in the not adapted at the other time points. In particular, for the adapted strain the values reached 85% at 48 h and 97% at 72 h.

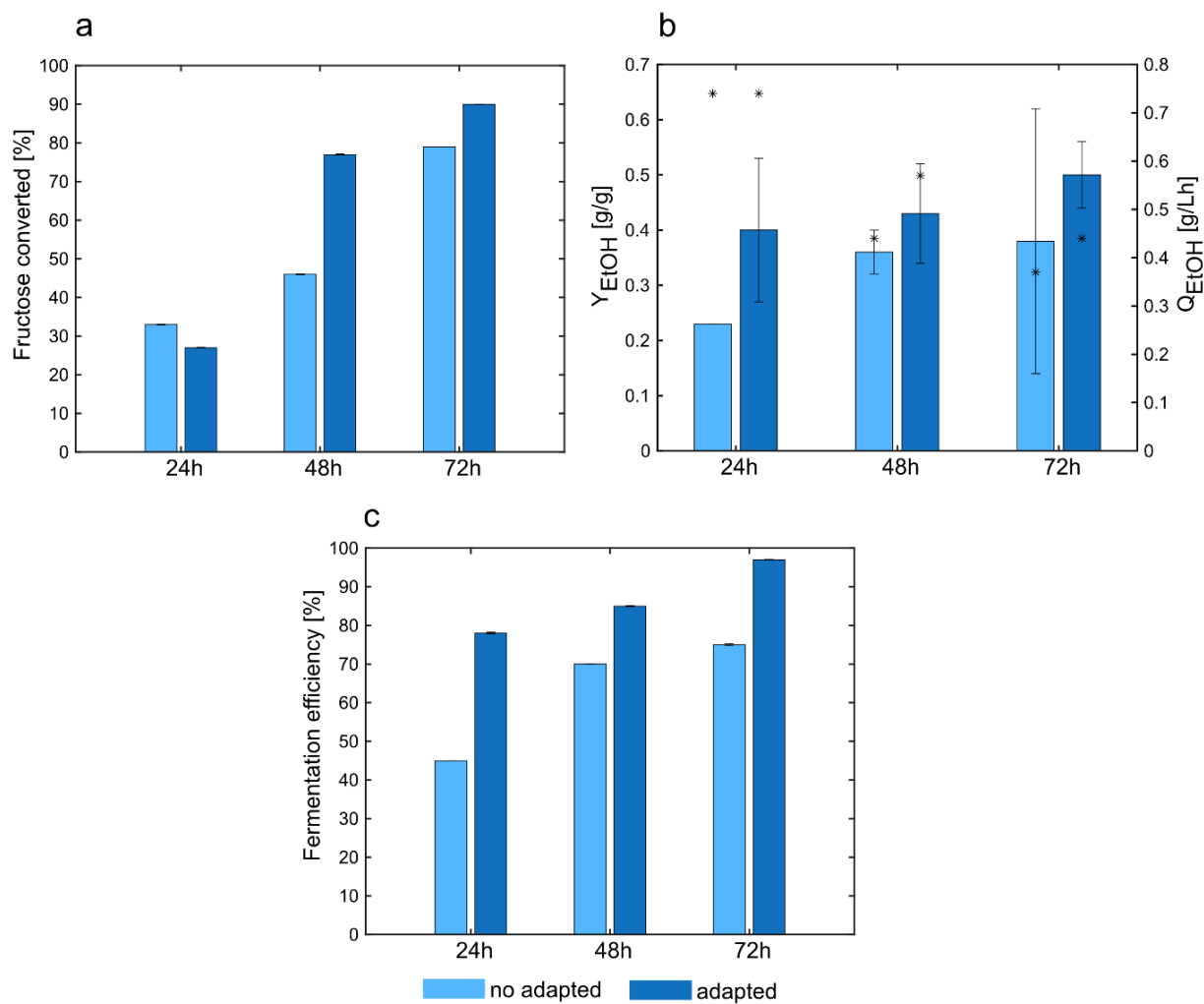


Fig. 7: percentages of fructose consumed with respect to the initial concentration (a), ethanol yields (bars) and productivities (stars) (b), fermentation efficiencies at 24h, 48h and 72 h of cultivation (c). The percentage of fructose consumed was calculated as mentioned in Eq. 2.

Table 3: Physiological parameters of the not adapted and the adapted *S. cerevisiae* strain. r_{s1} and r_{s2} are the glucose and the fructose consumption rate, respectively. C_{EtOH} is the ethanol production at the end of the fermentations. Q_{EtOH} is the volumetric ethanol productivity. The yields are calculated considering the total sugars content.

	<i>S. cerevisiae</i> not adapted	<i>S. cerevisiae</i> adapted
Fructose remained (g/L)	7.62±1.43	3.68±1.48
μ_{MAX} (h ⁻¹)	0.30±0.00	0.26±0.03
r_{s1} (g/Lh) ^a	-0.05±0.00	-0.05±0.00
r_{s2} (g/Lh) ^a	-0.36±0.06	-0.58±0.1
C_{EtOH} (g/L)	11.5±3.4	18.2±0.06
Q_{EtOH} (g/Lh)	0.37±0.04	0.44±0.00
Y_{EtOH} (g/g)	0.38±0.2	0.50±0.06
$Y_{Glycerol}$ (g/g)	0.014±0.00	0.013±0.00
$Y_{AceticAcid}$ (g/g)	n.d	n.d
$Y_{Biomass}$ (g/g) ^a	0.09±0.01	0.08±0.01
C-recovery (%) ^a	72%	95%

n.d., not detectable

^aParameters were determined at 48 h

4 Discussion

The grass raw material was mechanically pretreated to separate the solid fraction (press-cake) and the liquid fraction (press-juice). Mechanical pretreatment was chosen to avoid the formation of inhibitory compounds that can reduce the growth capacity of the yeast in lignocellulosic-derived media. In a biorefinery concept, the integrated utilization of both the solid and the liquid fractions would be beneficial. Several examples of the utilization of the press-cake fraction can be mentioned such as for animal feed (Damborg et al. 2020), for biogas production (Damborg et al. 2020), and as substrate in enzymatic hydrolysis and in solid-state fermentation steps (Kongkeitkajorn et al. 2020; Taufikurahman et al. 2020). In this work, the utilization of the press-juice as medium in fermentation processes was investigated. Firstly, the

nutritional potential of the juice was evaluated. For this reason, the analyses of sugars, proteins, amino acids and ions were carried out.

The higher concentration of fructose compared to glucose was found also by Sieker et al., and Volkmar et al., Putra et al., and Sipos et al., (Sipos et al. 2009; Sieker et al. 2011; Putra et al. 2017; Volkmar et al. 2023). The nitrogen source was provided by organic nitrogen compounds, i.e., proteins and amino acids and both are required for yeast metabolism and growth (Takagi 2019; Rojas et al. 2021). In particular, amino acids are essential components in liquid fermentation processes and they are usually supplied by yeast extract in high amount (Tomé 2021). However, yeast extract is expensive, and its addition to standard fermentation media increases the overall process costs. Among the amino acids, arginine and leucine were quantified at higher amounts. Arginine is known to work as a cryoprotector which acts by suppressing the protein aggregation (Takagi 2019). Leucine belongs to the branched-chain amino acids family. It plays an important role as building block for protein synthesis and in the metabolism of fatty acids (Zhang et al. 2017; Takagi 2019). Ions are also essential for cellular metabolism as they act as cofactors for many enzymes (Cao and Liu 2013). Similar trends were found out by Boakye-Boaten et al., 2016 (Boakye-Boaten et al. 2016). The authors reported a concentration of 1968.2 ppm of calcium, 93083.3 ppm of potassium, 19658.3 of magnesium (corresponding to 1.97 g/L, 93.08 g/L and 19.66 g/L, respectively) (Boakye-Boaten et al. 2016). On the contrary, Cao et al., 2013 found out in sweet sorghum juice lower concentration of calcium (0.35 g/L) and potassium (3.23 g/L) (Cao and Liu 2013). On the other hand, the authors got higher concentration of other elements such as sodium (1.01 g/L), while almost the same concentration of potassium was detected (6.16 g/L) (Cao and Liu 2013). Discrepancies in grass juice composition can be attributed to the different in grass species, location, climate, and maturation stage (Prasertwasu et al. 2014; Takara and Khanal 2015). According to these findings, it is possible to state the potential of the grass press-juice as fermentation medium, since it contains many components usually present in the standard cultivation media.

The growth of *S. cerevisiae* in the pressed juice was monitored by measuring the OD. The resulting growth curve revealed that the yeast had a longer lag-phase compared to the growth in standard complex medium (**Fig. S1**). This could be justified by the fact the preculture used was grown in YPD medium, so the yeast would need more time

to adapt to the juice. During the propagation of *S. cerevisiae* in increasing concentration of press-juice, the growth profile was monitored. It was possible to state that the lower the juice concentration, the higher was the cell density. These results were probably associated to a better nutrients balance between the components in the press-juice and those in the YPD medium. These results are consistent with the study of Tan et al., who found that maximum bioethanol production was reached at 80 %(v/v) in comparison to 100 %(v/v) banana juice (Tan et al. 2019). The authors suggested that this was likely due to the presence of heavy metals which cause a detrimental effect on the yeast growth (Tan et al. 2019). However, a study of Boakye-Boaten et al., showed that at 90 %(v/v) miscanthus juice, both the cell concentration and growth rate were higher compared to the lower juice percentage of 50 %(v/v) (Boakye-Boaten et al. 2016). The authors supposed that this was due to the higher availability of minerals and other compounds which stimulate the growth (Boakye-Boaten et al. 2016). Discrepancies with these results may be related to the type of grass, yeast strain used, and growth conditions. Nevertheless, in our study, the cells were continuously propagated, meaning that the cells from the previous juice percentage were used as preculture for the next one. It is highly possible that not all the cells passed were viable; therefore, further experiments regarding cell viability should have been performed. For instance, Nielsen et al., carried out experiments on cell quantification and viability using cell counting and methylene blue staining, respectively (Nielsen et al. 2015). The authors found out that the number of cells decrease with the increase of hydrolysate liquor percentage. However, when the authors assessed the viability, they found out that number of viable cells increased by increasing the amount of hydrolysate liquor. These results were explained by assuming that these cells might be more tolerant to those conditions (Nielsen et al. 2015).

During the propagation, sugars consumption and ethanol production were evaluated too. The sugar consumption rates were calculated according to Eq. 2 and resulted in negative values. The negative values expressed the decrease of sugar concentration in the fermentation environment due to substrate metabolization by the yeast cells. In all the percentages, the residual glucose was around 8% of the initial concentration, except when 100 %(v/v) juice was used (40% remained). This could be attributed to the low initial glucose concentration (1.7 g/L) in the press-juice, which may be

insufficient for its metabolization (Meijer et al. 1998). At 50% (v/v) press-juice, the concentration of glucose and fructose were nearly the same; however, the residual fructose was 16.4% respect of the initial concentration. Nonetheless, with increasing juice percentage, more fructose remained at the end of fermentations. This result confirms that glucose is the preferred substrate, although *S. cerevisiae* is able to consume both glucose and fructose. Similar results were reported by Putra et al., who cultivated *S. cerevisiae* in a 1L reactor using date fruit syrup as fermentation medium containing 142.5 g/L total sugars (glucose and fructose). The authors pointed out that 83.5% of the fructose remained after 96 h of fermentation (Putra et al. 2017). The amount of fructose not metabolized can be explained by the fact that during the last phase of the fermentations, the yeast is overstressed due to nitrogen starvation and/or to the increased ethanol concentration (Berthels et al. 2008; Tronchoni et al. 2009). These conditions, combined with the high fructose-to-glucose ratio, may led to stuck fermentations (Berthels et al. 2004). Another reason could be related to the diverse sugars internalization. Guillaume et al., found out that glucose and fructose have different consumption trends due to differences in the transportation across the membrane (Guillaume et al. 2007). Berthels et al., asserted that the dissimilarity was associated to differences in the hexose phosphorylation (Berthels et al. 2008).

Sugars consumption is related to the bioethanol production, which is the primary product of yeast fermentation. During the propagation, the bioethanol production started after the end of the lag-phase (6 h). However, when *S. cerevisiae* was cultivated in YPD medium, the ethanol production started after 4 h (**Fig. S2**). Therefore, when press-juice was used as fermentation medium, the yeast required a longer phase to adapt its metabolism. The highest yield value of 0.41 g_{Ethanol}/g_{sugars} was obtained when 50 %(v/v) was used, which corresponded to ethanol production of 10.26 ± 0.41 g/L. This ethanol yield corresponded to 80% of the maximum theoretical ethanol yield. Gomez-Florez et al., achieved a yield of 0.27 g_{Ethanol}/g_{Carbohydrates} using juice sugar corn supplemented with 3 g/L yeast extract (Gomez-Flores et al. 2018). Tan et al., reached a yield of 0.33 g_{Ethanol}/g_{sugar} using banana frond juice in a 2L bioreactor (Tan et al. 2019). Bautista et al., utilized corn stalk juice and immobilized *S. cerevisiae* to produce ethanol, reaching 0.45 g_{Ethanol}/g_{sugar} (Bautista et al. 2022). These findings demonstrate that all the percentages used for propagation were optimal for ethanol production.

Once propagated, the yeast cells were re-cultivated in 100 % (v/v) to assess if they acquired enhanced fermentation capabilities. So far, short-term adaptation has been used as a strategy to develop a strain with novel characteristics which reflect its adaptation to a specific condition. However, in this study, the potential of short-term adaptation as a tool to improve the fermentation performance of *S. cerevisiae* in grass press-juice was explored. The sugars consumption and the bioethanol production before and after the adaptation were compared to evaluate the yeast's performance. The improvements in the adapted strain were remarkable, resulting in a higher ethanol titer and a higher ethanol yield (0.50 g_{Ethanol}/g_{sugars}). So far, reaching a production yield close to the theoretical one in unconventional media has not been obvious. The overall results indicate that short-term adaptation can be exploited to enhance fermentation processes.

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Supplementary

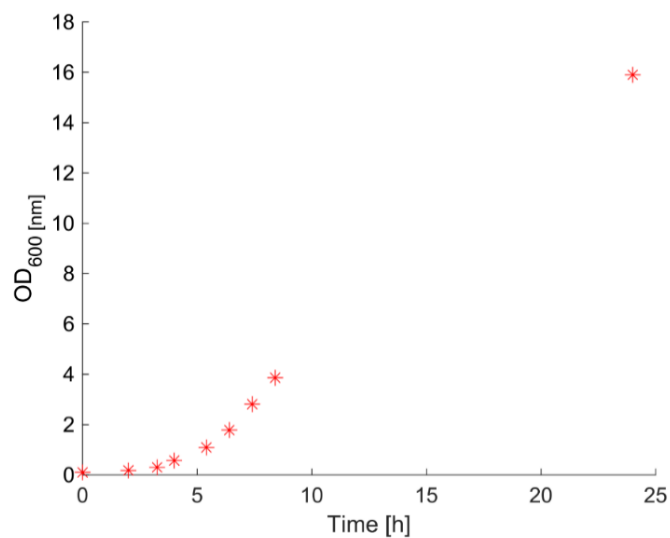


Fig. S1: Growth curve of *Saccharomyces cerevisiae* in YPD medium. Growth conditions: 120 rpm, 32°C, pH 6.2.

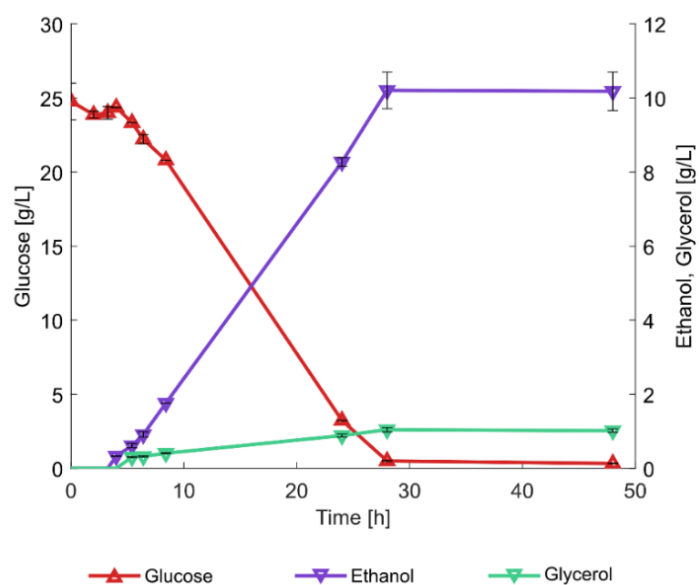


Fig. S2: Glucose consumption and bioethanol production in YPD medium. Growth conditions: 120 rpm, 32°C, pH 6.2

Chapter VI – Paper V

Sustainable Production of Lactic Acid Using a Perennial Ryegrass as Feedstock – A Comparative Study of Fermentation at the Bench- and Reactor-Scale, and Ensiling

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L. Varriale: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing- original draft preparation, visualization

J-N. Hengsbach: methodology, writing- original draft preparation

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K. Kuka: methodology, writing- review and editing, project administration, funding acquisition

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Keyword: bio-based economy, renewable resources, perennial ryegrass, fermentation, ensiling, lactic acid, *Lactobacillus*

Abstract

Perennial ryegrass (*Lolium perenne*) is an underutilized lignocellulosic biomass that has several benefits such as high availability, renewability, and biomass yield. The grass press-juice obtained from the mechanical pretreatment can be used for the bio-based production of chemicals. Lactic acid is a platform chemical that has attracted consideration due to its broad area of applications. For this reason, the more sustainable production of lactic acid is expected to increase. In this work, lactic acid was produced using complex medium at the bench- and reactor scale, and the results were compared to those obtained using an optimized press-juice medium. Bench-scale fermentations were carried out in a pH-control system and lactic acid production reached approximately 21.84 ± 0.95 g/L in complex medium, and 26.61 ± 1.2 g/L in press-juice medium. In the bioreactor, the production yield was 0.91 ± 0.07 g/g, corresponding to a 1.4-fold increase with respect to the complex medium with fructose. As a comparison to the traditional ensiling process, the ensiling of whole grass fractions of different varieties harvested in summer and autumn was performed. Ensiling showed variations in lactic acid yields, with a yield up to 15.2% dry mass for the late-harvested samples, surpassing typical silage yields of 6–10% dry mass.

1 Introduction

In the past years, fossil resources have been used for energy and material production purposes without considering their limited reserves [1]. Their overuse has caused severe concerns such as global warming and reductions in natural resources. Thus, there is an urgent need to identify alternative resources. Renewable organic materials, such as biomasses, have attracted significant attention for the sustainable production of chemicals, fuels, and materials, offering important economic, environmental, and social benefits [2]. It is estimated that 1 kg of biomass raw material can be transformed into 6 MJ of energy or 0.8 kg of chemicals [2,3]. Lignocellulosic biomass from agriculture, forestry, and urban waste has huge potential due to its high abundance, renewability, and low cost. It has been assessed that more than 200 value-added compounds can be produced from lignocelluloses and some even at the industrial scale through microbial fermentation. In particular, these compounds include platform chemicals such as lactic acid, succinic acid, itaconic acid, glycerol, and sorbitol [4]. Among the others, perennial ryegrasses are considered an important biomass

resource due to their high and stable biomass yield, which accounts for approximately 2–20 ton (dry matter)/ha [5].

As mentioned above, lactic acid (LA) is an important platform chemical with a wide range of applications, especially in food, chemical, cosmetic, and pharmaceutical markets. More recently, LA has reached the plastic sector for the production of biodegradable and biocompatible polylactic acid (PLA) polymers [6,7]. Because of the broad application areas, the corresponding market is estimated to reach 1960 kt in 2025 [8,9]. The production of LA using microbial fermentation accounts for more than 90% of the total output with high yields of up to 90–95 wt% [9,10]. The cost of LA depends on its final application and ranges between 1.38 USD/kg for the lowest purity and 1.59 USD/kg for the highest purity [11]. However, feedstock costs account for the 40–70% of total production costs [8,11]. Therefore, the exploitation of underutilized materials such as lignocelluloses can contribute to lowering the final price. Several attempts have been made to use lignocellulosic biomass for LA production. Table 1 summarizes the main findings over the last years.

Traditionally, lactic acid bacteria (LAB) are the microorganism of choice for LA production, as they produce LA as major product using homo- or heterofermentation, and are recognized as safe [12]. Homolactic bacteria such as *Lactobacillus*, *Lactococcus*, and *Enterococcus* produce two molecules of LA per mol of sugar with a theoretical yield of 1 g/g (**Fig. 1**), whereas heterolactic bacteria yield 0.5–0.6 g of LA per g of sugar [13]. Some LAB are defined as facultative heterolactic since they metabolize hexoses and pentoses to produce LA along with by-products (acetic acid, ethanol, and formic acid) [14]. Since homolactic bacteria can reach yields close to the maximum theoretical one, they are more suited for industrial production [12].

Table 1. Microorganism, feedstock, and type of cultivation used for LA production over the last years. SSF indicates Simultaneous Saccharification and Fermentation, and SSCF indicates Simultaneous Saccharification and Co-Fermentation

Microorganism	Feedstock	Cultivation	Yield [g/g]	Ref.
<i>L. rhamnosus</i> LA-04-01 ^a	Defatted rice brain hydrolysate	Batch	0.95	[15]
		Continuous	0.98	
<i>L. paracasei</i> 7BL ^a	Wood chips	Fed-Batch	0.96	[16]
	Rice Straw		0.97	
<i>L. casei</i> G-02 ^a	Jerusalem artichoke	Fed-Batch (SSF)	0.96	[17]
<i>L. agilis</i> LPB 56 ^b	Soybean vinasse	Batch	0.85	[18]
<i>L. plantarum</i> NCIMB 8826 ^a	Delignified hardwood pulp	Batch (SSF)	0.88	[19]
<i>L. paracasei</i> LA104 ^a	Curcuma longa waste	Batch (SSCF)	0.69	[20]
<i>L. coryniformis</i> ATCC 25600 ^b			0.65	
<i>L. pentosus</i> FL0421 ^a	Corn Stover	Fed-Batch (SSF)	0.66	[21]
<i>L. pentosus</i> DSM20314 ^a	Wheat bran	Batch	0.73	[22]
<i>L. delbrueckii</i> NBRC 3203 ^b	Cassava fibrous waste	Batch	0.50	[23]
<i>L. ramosus</i> ATCC 7469 ^a	Lignocellulosic mixture	Batch (SSF)	0.97	[24]
<i>L. delbrueckii</i> subsp. <i>bulgarius</i> ATCC 11842 ^b	Beechwood hydrolysate	Batch (SSF)	0.69	[25]
	Pine hydrolysate		0.40	
<i>L. delbrueckii</i> DSM 20074 ^b	Household bio-waste	Batch	0.65	[26]
<i>L. casei</i> DSM 20011 ^a	Agro-industrial waste	Batch	0.78	[27]
<i>L. delbrueckii</i> subsp. <i>lactis</i> DSMZ 20072 ^b	Brewers' spent grain	Batch	0.89	[28]
	Cottonseed cake		0.22	
<i>L. brevis</i> MTCC 4460 ^c	Wheat Straw	Batch (SSCF)	0.49	[29]
	Sugarcane bagasse		0.52	
<i>L. buchneri</i> NRRL B-30929 ^c	Elephant grass liquor	Batch	0.50	[30]
<i>B. coagulans</i> A107 ^b	Tapioca starch hydrolysate	Continuous	0.80	[8]
<i>B. coagulans</i> AD ^b	Corn Stover hydrolysate	Continuous	0.95	[31]
<i>B. coagulans</i> LA204 ^b	Corn cob	Fed-Batch (SSF)	0.77	[32]
<i>B. coagulans</i> LA1507 ^b	Sweet sorghum bagasse	Open Fed-Batch (SSF)	0.44	[31]
<i>E. faecalis</i> RKY1 ^b	Molasses	Batch	0.95	[33]
<i>E. faecium</i> WH51-1 ^b	Corn steep water effluent	Batch	0.89	[34]

^a facultative heterofermentative LAB. ^b homofermentative LAB. ^c heterofermentative

Several key parameters influence LA production, including sugar concentration, nutrient availability, pH, temperature, and acid stress [13]. Specifically, LA release can lead to product inhibition due to pH reduction, which in turn affects membrane potential. Therefore, maintaining an optimal pH in the fermentation medium is essential [13]. Regarding the nutrients, LAB have complex nutrients requirement, as they have a limited capacity to synthesize molecules for their growth. For instance, nitrogen must be supplemented in the fermentation media as yeast extract, peptone, or meat extract since LAB are unable to synthesize essential nitrogen-based substances such as amino acids, purines, pyrimidines, and co-factors [35,36]. Both mineral elements and vitamins are crucial in many enzymatic reactions, in membrane transport processes, and in molecules and structural complexes [36–38]. However, the addition of those elements increases the overall cost of LA production.

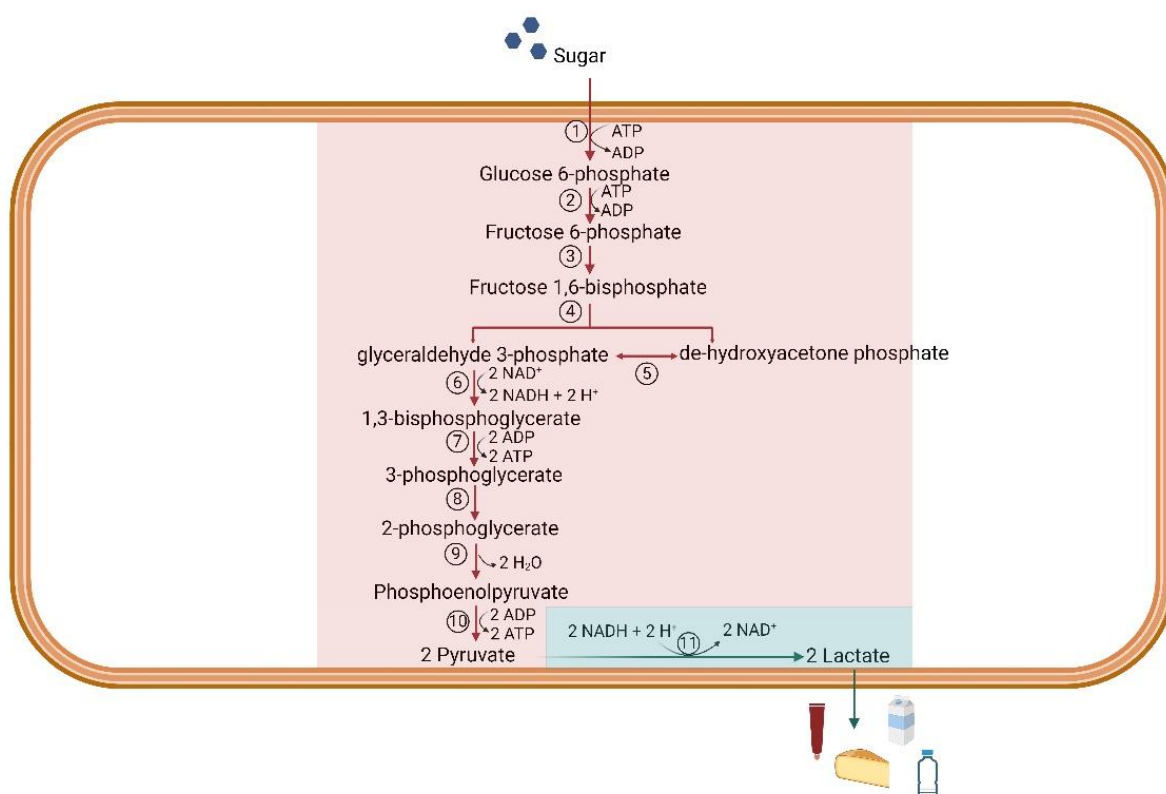


Fig. 1: Schematic representation of the LA homo-fermentation pathway from sugar to LA. The red box represents glycolysis, the green box homolactic fermentation. The numbers in the circles are the enzymes involved in that step: (1) hexokinase, (2) phosphoglucose isomerase, (3) phosphofructokinase, (4) aldolase, (5) triose phosphate isomerase, (6) glyceraldehyde 3-phosphate dehydrogenase, (7) phosphoglycerate kinase, (8) phosphoglyceromutase, (9) enolase, (10) pyruvate kinase, (11) lactate dehydrogenase. The figure is adapted from Pessione A. et al., [39] and it was created on BioRender.com.

The aim of this work was to compare the ability of the homofermentive *Lactobacillus delbrueckii* subsp. *lactis* to produce LA in conventional complex medium (De Man—Rogosa—Sharpe (MRS) medium) and in press-juice from a perennial ryegrass (*Lolium perenne*). *Lolium perenne* was selected as feedstock since it has several appealing traits including fast growth, low water and fertilizer requirements, and an abundance of above-ground biomass (leaves, stalks, and nodes) which can be harvested and used in biorefineries [40]. The entire production process was designed to enhance sustainability. Generally, lignocellulosic biomasses undergo physical pretreatments prior to any other methods to facilitate enzymatic hydrolysis [42]. In this study, a mechanical pretreatment using a screw press was used as the sole method [41].

Mechanical methods are usually considered more eco-friendly as they avoid the utilization of chemicals or heat [46,47]. Additionally, in a general biorefinery scheme, the monomeric sugars obtained after saccharification must be recovered. However, the recovery process requires additional energy and power [43]. The use of screw-pressing bypassed the need for an additional recovery step, as the sugars are already contained in the press-juice and can be immediately used in the fermentation process. The remaining solid fraction, also known as press-cake, is generally used as a substrate in enzymatic hydrolysis and in solid-state fermentation [44,45] for animal feed and for biogas production [46].

Different juice percentages were tested for online growth measurements to determine the optimal juice concentration for bacterial growth. Then, based on previous findings, the selected press-juice concentration was optimized with glucose, sodium-acetate, and Tween 80 to ensure LA production [28]. The experiments in both complex medium and in press-juice medium were firstly performed at bench-scale using a pH-control system to counteract the pH drops in the cultivations. This system aimed to eliminate the addition of neutralizing agents that make the downstream process difficult. Then, the experiments in both media were scaled up in a bioreactor and various fermentation parameters including sugar uptake and sugar consumption rate, production yield, productivity, and selectivity were compared. Finally, a preliminary study on the ensiling process on different *Lolium perenne* varieties was carried out.

2 Materials and Methods

2.1 Raw material

At the Julius Kühn Institute (Braunschweig, Germany), a field experiment was conducted to produce fresh biomass for investigating its use as a raw material in a biorefinery. The study site is located in Braunschweig at an altitude of 80 m above sea level. The region has an annual precipitation of 617 mm and an average temperature of 9.1°C. The soil is characterized as silty sand with a topsoil depth of 30 cm. Selected varieties of *Lolium perenne* (Table 2) were cultivated in large-scale plots to produce sufficient biomass for subsequent analysis. In order to obtain representative samples for Near Infrared Spectroscopy (NIRS) quality determination, chopped samples of at least 500 g were collected from each plot for dry matter (DM) determination and subsequent grinding. These samples were dried in a drying oven (Memmert GmbH,

Schwabach, Germany) at 60 °C for 48 h and milled uniformly with a Brabender cutting mill (Anton Paar TorqueTec GmbH, Duisburg, Germany). Quality parameters including crude protein (CP), water soluble carbohydrates (WSCs), and crude fibre (CF) were analyzed using a Foss NIRSystem 5000 spectrometer (Foss GmbH, Hamburg, Germany) with a wavelength range of 1100 to 2498 nm at a resolution of 2 nm. For the biorefinery application, fresh biomass samples were harvested, vacuum sealed, and immediately cooled for shipment.

Table 2: *Lolium perenne* varieties used in this study with information on ploidy and breeder.

Variety	Ploidy	Breeder
Agaska	2x	DLF (Roskilde, Denmark)
Honroso	2x	DSV (Asendorf, Germany)
Arvicola	4x	Feldsaaten Freudenberger (Krefeld, Germany)
Barmigo	4x	Barenbrug (Nijmegen, The Netherlands)
Explosion	4x	DSV (Asendorf, Germany)

These varieties were selected to encompass a range of genetic diversity and breeding backgrounds. The evaluation aimed to assess biomass yield and quality characteristics under the specified conditions, critical for their potential use in biorefinery processes. The implementation of standardized procedures for sample collection, drying, and analysis ensures the reliability and comparability of the results across different varieties.

2.2 Pretreatment and Preparation of Sterile Press-Juice

The pretreatment of the samples and the production of the press-juice can be found elsewhere [41]. Briefly, the raw material was mechanically pretreated using a screw press (Angel Juicer 7500, Luba GmbH, Bad Homburg, Germany). The liquid fraction (press-juice) was centrifuged (4500 rpm, 20 min; Z38K, Hermle Labortechnik GmbH, Wehingen, Germany) to remove the remaining solid particles, and filter sterilized (Stericup®, 0.2 µm pore size, MerckMillipore, Burlington, MA, USA). The amount of press-juice obtained was approximately 52% of the total mass of the raw material. The

pressed juice (pH 5.3, Microprocessor pH 211, Hanna Instruments Deutschland GmbH, Wertheim, Germany) was analyzed in terms of sugars (approximately 40 g/L), organic nitrogen (approximately 14.87 g/L), and ions (15.75 g/L, including cations and anions). A detailed description of the composition can be found elsewhere [47].

2.3 Microorganism, Preculture, and Complex Medium Preparation

The bacteria *Lactobacillus delbrueckii* subsp. *lactis* DSM 20729 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Precultures and the experiments in complex media were carried out using MRS medium with the following composition: casein peptone 10 g/L, meat extract 10 g/L, yeast extract 5 g/L, Tween-80 1 g/L, K₂HPO₄ 2 g/L, sodium-acetate 5 g/L, (NH₄)₃ citrate 2 g/L, MgSO₄ × 7 H₂O 0.20 g/L, MnSO₄ × H₂O 0.05 g/L, glucose or fructose carbon source approximately 20 g/L. The pH of the medium was adjusted to 6.2 ± 0.1 (Microprocessor pH 211, Hanna Instruments Deutschland GmbH, Wertheim, Germany) using 2.5 M NaOH. In all the experiments, the carbon source (glucose or fructose) was prepared and autoclaved (Systec V-150, Systec GmbH & Co. KG, Linden, Germany) separately to avoid degradation. All the chemicals were purchased from Carl Roth + Co KG (Karlsruhe, Germany), except glucose and fructose (Sigma Aldrich, Merck KGaA, Darmstadt, Germany). Precultures were anaerobically grown in 150 mL glass bottles with 100 mL working volumes. The bottles were tightly sealed with rubber septa and incubated at 37 °C, 150 rpm for approximately 24 h (Ecotron, Infors, AG, Bottmingen, Switzerland).

2.4 Cultivation in Different Percentages of Press-Juice

The cultivations in different percentages [10, 25, 30, 50, 60, 75%(v/v) (volume per volume)] of press-juice were carried out in 100 mL serum bottles with 50 mL working volumes, which were tightly sealed with rubber septa. The remaining percentages were filled with MRS medium. Before the inoculation (0.1 OD (optical density)), the bottles were sparged with pure nitrogen. Incubation was carried out at 37°C and 150 rpm in an incubator shaker (Ecotron, Infors AG, Bottmingen, Switzerland) equipped with a Cell Growth Quantifier (Scientific Bioprocessing, Aquila BioLAB GmbH, Baesweiler, Germany). The system recorded the growth of the bacteria by taking scattered light measurements and the values were directly used to develop growth curves.

2.5 Bench-Scale Fermentation in Schott Bottles

Bench-scale fermentations were carried out in 150 mL Schott bottles with 100 mL working volumes. Before the inoculation (0.1 OD), the bottles were sparged with pure nitrogen. The pH values were constantly measured using pHenomenal® pH-electrodes (IDP 721, VWR, Darmstadt, Germany) and the Profilux 3.1T aquarium computer with six pH modules (GHL Advanced Technology GmbH & Co. KG, Kaiserslautern, Germany). During the cultivations, the pH value was regulated to 6.8 by automatically adding 2.5 M NaOH as a titrant correction agent (accuracy ± 0.1). A heating and stirring plate (MULTI-HS 6, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was used for temperature control and for mixing of the Schott bottles. The medium temperature in the bottles was set to 37 °C, and mixing was carried out at 200 rpm using cylindrical magnetic stirrer bars with a diameter of 8 mm and a length of 20 mm (ROTILABO, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The experiments in complex medium and the experiments in press-juice medium were conducted over 25 and 28 h, respectively.

2.6 Scaled-Up Fermentation in the Bioreactor

Scaled-up fermentations were carried out in a 0.5 L BIO-STAT® Qplus controlled bioreactor system (Sartorius AG, Göttingen, Germany). In the experiments performed with complex media, the bioreactors were filled with all the medium components and autoclaved (121°C, 15 min), except for the sugar. Pure nitrogen was sparged overnight to ensure anaerobic conditions. Afterward, the sterilized sugar was added aseptically to the reactors, and nitrogen was sparged for one hour to remove any remaining oxygen. In the experiments with 75 % (v/v) press-juice, the bioreactors were sterilized (121°C, 15 min) before the addition of the press-juice. After sterilization, the press-juice was aseptically filled in, and pure nitrogen was sparged overnight. The optimized nutrient mixture was added afterward, and nitrogen was sparged for one hour. In all the experiments, the temperature was set at 37 °C, the stirring speed at 150 rpm, and the pH at 6.5 (EasyFermBio HB K8 120, Hamiltorn, Hamburg, Germany) by the automatic addition of 2.5 M NaOH with peristaltic pumps. In all the experiments, the reactors were inoculated to 0.1 OD using preculture previously prepared.

2.7 Ensiling of Different Varieties of *Lolium perenne*

To obtain lactic acid from the ensiling of *Lolium perenne* fresh grass, the grass was cut into small pieces. Before ensiling, dry matter was determined using a DBS 60 3 moisture analyzer from KERN (Balingen-Frommern, Germany). For each setup, a vacuum bag was filled with 50 g of fresh mass (FM) of the chosen variety, and 10 mL of a silage additive (diluted with deionized water 1:500) was added. This additive, sourced from Lactosan (Kapfenberg, Austria), consisted of a mixture of the strains *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Pediococcus pentosaceus*. After the addition, the mixture was briefly mixed, and the bag was sealed using a VMKH-300 vacuum sealer (Gmgastro, Ochtrup, Germany). The prepared setups were placed in plastic buckets, purged with nitrogen gas, and sealed. Ensiling was conducted at room temperature for 75 days. To maintain the nitrogen environment inside the buckets, nitrogen was renewed every three days. For sampling, the designated bags were removed from the buckets. To measure the ensiling products, complete sample-bags were removed from the ensiling and pressed. The press juice obtained was centrifuged at 16,100× rcf for 15 min using a centrifuge (5415d Centrifuge, Eppendorf, Hamburg, Germany). Afterward, the samples were diluted to a concentration within the calibration range and filtered through a 0.22 µm pore size polyethersulfone filter.

2.8 Analytical Methods

The analyses of the press-juices were carried out with regard to sugars, protein, amino acids, cations, and anions. Protein concentrations were determined using the Bradford assay (Pierce® Coomassie Bradford Protein Assay kit, Thermo Scientific, Waltham, MA, USA) and bovine serum albumin as an internal standard (Thermo Fisher Scientific, Waltham, MA, United States). The absorbances of the calibration curve and of the samples were measured at 595 nm (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). Sugars and lactic acid were analyzed by HPLC (High Performance Liquid Chromatography) [Autosempler AS 6.1L and Azura pump P 6.1L (Kneur GmbH, Berlin, Germany)]. The HPLC was equipped with an Aminex HPX-87H column at 80°C (Bio-Rad, 300 mm × 7.8 mm, Hercules, CA, USA) and a refractive index detector (RI 101 Shodex, Kawasaki, Japan). The mobile phase was 2.5 mM H₂SO₄ and the flow rate was set at 0.6 mL/min. The instrument control and the data evaluation were carried out with the Clarity Software system (Data Apex, Prague,

Czech Republic). Before the analysis, the samples were diluted to a concentration inside the calibration range using external standards, and then filtered through a 0.22 µm pore size nylon filter (KX Syringe Filter Nylon, Cole Parmer GmbH, Wertheim, Germany). Amino acids were separated using a resolve C18 column (150 mm × 3.9 mm, Waters Corporation, Milford, CT, USA) with a SecurityGuard Cartridge (C18, 4 mm × 3.0 mm ID, Phenomenex, Torrance, CA, USA) set at 30°C. The detection was performed using an Azura photodiode-array detector DAD 2.1L at 230 nm (Knauer GmbH, Berlin, Germany). The analysis included a precolumn derivatization with ortho-phthaldialdehyde (OPA). The derivatization process is described in the Supplementary Materials. The mobile phase consisted of solvent A (0.025 M sodium-acetate anhydrous and 0.025 M NaH₂PO₄ monohydrate) and solvent B (50% methanol). The pH value of solvent A was adjusted to 7 with 10 M NaOH, then 21 mL of both tetrahydrofuran and methanol were added. The gradient elution program was as follows: from 0 to 50 min, solvent B changed linearly from 0% to 100%; from 50 to 55 min, solvent B was set as isocratic at 100%; from 55 to 60 min, solvent B changed linearly from 100% to 0%; from 60 to 67 min, solvent B was set as isocratic at 0%. The flow rate was 1.0 mL/min. Sample preparation is described in the Supplementary Materials. Cations and anions were analyzed by ion chromatography (IC) (930 Compact IC Flex, Metrohm GmbH & Co. KG, Filderstadt, Germany) with an inline system for dialysis (930 Compact IC Flex, Metrohm, Filderstadt, Germany) and an IC Conductivity Detector (Metrohm, Filderstadt, Germany). Cations were measured with a cation column (Metrosep C6-250/4.0, Metrohm) using 4 mM HNO₃ and 0.7 mM dipicolinic acid as the mobile phase at a flow rate of 0.9 mL/min. Anions were measured with an anion column (Metrosep A Supp5-250/4.0, Metrohm) using 1 mM NaHCO₃ and 3.2 mM Na₂CO₃ as the mobile phase at a flow rate of 0.7 mL/min. The oven temperature in both cases was 35°C. For each analysis, the samples were diluted to a concentration inside the external calibration range (using 2 mM HNO₃ for cation determination). Cell growth was monitored by optical density measurements at 600 nm (UV spectrophotometer LAMBA Bio+, Perkin Elmer Corporation, Waltham, MA, USA). The samples collected during the fermentations were centrifuged at 18407× *g* for 7 min (Eppendorf, Hamburg, Germany) and the supernatant stored at -20 °C for metabolite analysis.

2.9 Data Processing

The percentage of sugar uptake (S_u) and the sugar consumption rate (r_s) were calculated at the time the cells were growing exponentially and followed Equations (1) and (2), respectively:

$$S_u = \frac{S_i - S_t}{S_i} \times 100 \quad (1)$$

$$r_s = \frac{S_t - S_i}{t} \quad (2)$$

where S_i is the initial sugar(s) concentration(s) and S_t is the sugar(s) concentration(s) at time t .

The selectivity (S) was calculated following Equation (3):

$$S = \frac{LA_f}{S_i - S_t} \quad (3)$$

The production yield ($Y_{P/S}$) of LA was expressed as Equation (4):

$$Y_{P/S} = \frac{LA_f - LA_i}{S_i - S_f} \quad (4)$$

where LA_f indicates the final LA concentration and LA_i is the starting LA concentration.

The volumetric LA productivity (Q_{LA}) was estimated as (5):

$$Q_{LA} = \frac{C_{LA}}{t} \quad (5)$$

C_{LA} represents the concentration of LA at the end of the fermentation.

The biomass production yield ($Y_{X/S}$) followed Equation (6):

$$Y_{X/S} = \frac{X_f - X_i}{S_i - S_f} \quad (6)$$

where X_f indicates the final biomass concentration and X_i is the initial biomass concentration.

The volumetric cell productivity (Q_{CELL}) was expressed as the ratio between the final biomass concentration (C_{CELL}) and the time t , according to (7):

$$Q_{CELL} = \frac{C_{CELL}}{t} \quad (7)$$

3 Results and Discussion

3.1 Influence of Raw Material

The 2021 harvests were analyzed in terms of yield and quality parameters in order to diversify their use in a biorefinery context. In particular, DM, CF, CP, and WSCs were evaluated and the results are reported in Table 3. Agaska exhibited the highest DM yield, followed by Barmigo and Arvicola (Table 3). This suggests that Agaska is the most productive variety for the second cut of the year in terms of biomass yield, which is beneficial for processes requiring large quantities of raw material, such as bioenergy production. However, it is important to note that the first cut of the year typically produces more DM than the second cut. In terms of CF content, Agaska also had the highest amount, indicating a robust cell wall structure. This high CF content can be advantageous for producing materials that require high fiber content. Barmigo and Arvicola had slightly lower CF contents, with Arvicola having the lowest, which might make it more suitable for processes requiring lower fiber content and higher digestibility. Arvicola had the highest CP content, making it potentially more valuable for animal feed applications where protein content is critical. Barmigo and Agaska had lower CP contents, suggesting that while Arvicola is less productive in terms of biomass yield, its higher protein content could offer advantages in feed applications. WSCs are crucial for fermentation processes. Arvicola again led with the highest WSC content, followed by Barmigo and Agaska. The high WSC content in Arvicola highlights its potential suitability for processes like chemical and fuel production, where a high sugar content is desirable. Separately, the comparison between the Explosion and Honroso varieties harvested in 2022 revealed some differences. Both varieties had similar DM yields, with that of Honroso slightly lower than that of Explosion. The CF content was comparable between the two, indicating a robust structure for both varieties. However, Honroso had a higher CP content compared to Explosion, while the WSC content was similar between the two, making both varieties viable options for biorefinery processes. For lactic acid production, both the Explosion and Honroso

varieties were particularly suitable due to their high DM yields and adequate carbohydrate contents. The large volume of juice required for LA production was obtained by utilizing both replicates.

Table 2: Yield and quality parameters of *Lolium perenne* varieties. BBCH Stage: phenological development stage of plants; DM: dry matter (in tons per hectare); CF: crude fibre; CP: crude protein; WSCs: water soluble carbohydrates.

Variety	Cut of the year	Harvested Date	BBCH Stage	DM (t/ha)	CF (%)	CP (%)	WSCs (%)
Agaska ^a	2	07/07/2021	49	2.5 ± 0.4	25.2 ± 1.4	11.6 ± 1.1	16.2 ± 0.6
Arvicola ^a	2	07/07/2021	31	2.5 ± 0.5	21.8 ± 1.4	10.9 ± 0.6	22.2 ± 0.9
Barmigo ^a	2	07/07/2021	45	2.5 ± 0.4	24.8 ± 0.2	10.5 ± 0.6	18.0 ± 0.3
Explosion ^b	1	03/06/2022	61	5.2 ± 0.0	25.9 ± 0.3	5.8 ± 0.7	22.6 ± 3.0
Honroso ^b	1	03/06/2022	59	4.3 ± 0.0	25.3 ± 1.6	7.9 ± 1.3	20.4 ± 0.3

^aData and standard deviations represent mean values of triplicates. ^bData and standard deviations represent mean values of duplicate

3.2 Press-Juice Analysis

LAB have high and complex nutrient requirements to grow and to produce LA. To evaluate the suitability of different press-juices, their nutrient contents including sugars, proteins, amino acids, and ions were analyzed. The results are reported in Tables 4 and 5.

Table 4. Analysis of the nutrient contents of different varieties of grass press-juices after mechanical pretreatment (grass samples from 2021). The data presented in the table were obtained from one measurement.

	Arvicola	Agaska	Barmigo
Total sugars [g/L]	23.8	39.5	28.3
Protein [mg/L]	559.4	682.9	641.7
Amino acids [mg/L]	n.a	3130.5	2560.9
Cations [g/L]	9.3	8.1	8.2
Anions	6.0	5.9	4.8

n.a. not analyzed.

Table 5. Analysis of the available monosaccharides (glucose and fructose) in grass press-juices (grass samples from 2022).

	Glucose Content [% (m/m)]	Deviation	Fructose Content [% (m/m)]	Deviation
Honroso	2.93%	0.41%	5.32%	1.20%
Explosion	5.09%	0.79%	7.56%	3.00%

3.3 Bench-Scale Fermentation in Complex Medium

To assess the ability of *L. delbrueckii* to grow and to produce LA, bench-scale fermentations in MRS medium were initially performed under pH-controlled conditions using NaOH addition. During LA fermentation, the pH of the broth drops below the LA-pKa value (3.8), which inhibits the metabolic functions of LAB [13,48]. Neutralizing agents such as Ca(OH)₂ or CaCO₃ are typically used to maintain the pH within the optimal range (pH 5–7) [36,48]. However, these agents have some disadvantages

including limited LA accumulation and the formation of calcium lactate, which can increase the costs of the downstream processing [48]. Therefore, pH control is critical for achieving successful sustainable LA production. The fermentations in MRS medium lasted for 25 h (**Fig. 2**). Bacterial growth exhibited a lag-phase of approximately 6 h, during which glucose levels remained constant and no LA was produced. The lag-phase was followed by the exponential growth-phase (6–21 h), during which glucose was rapidly metabolized (87% with respect to the initial glucose concentration) and LA was produced (17.6 ± 0.9 g/L). By the end of the cultivation (25 h), the cell density decreased, the glucose was completely depleted, and LA accumulation reached approximately 22 g/L (**Fig. 2**). Mussatto et al. demonstrated that pH-controlled experiments yield better results compared to those without pH control. Using MRS medium with an initial glucose concentration of 50 g/L, they achieved approximately 23.5 g/L of LA [49]. The authors noted that glucose consumption was positively affected by the pH. Si et al. investigated different pH values and found that maintaining a pH of 6 during LA production in corn straw hydrolysate led to the highest LA production [50].

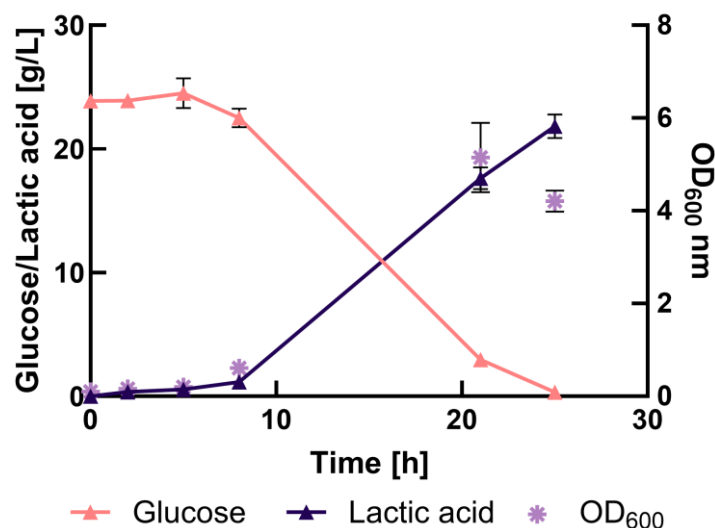


Fig. 2: Bench-scale fermentation in MRS medium with a pH-regulated system. Culture conditions: 200 rpm, 37 °C, pH = 6.8 ± 1 (2.5 M NaOH buffer). Error bars indicate standard deviations of the mean (n = 3).

3.4 Cultivation in Different Percentages of Press-Juice

To evaluate if *L. delbrueckii* could grow in press-juice, cultivations in different juice percentages (10, 25, 30, 50, 75% (v/v)) were carried out. Since the experiments aimed

to assess bacterial growth, online measurements with CGQ and without pH control were performed. The results revealed that *L. delbrueckii* could grow in all the juice percentages, although in different manners (**Fig. 3**). At lower juice concentrations, the latency phase was shorter than in the other cases. This was likely due to the fact more than 50% of the medium at these concentrations consisted of complex media components. Since the preculture was grown in MRS medium, it is possible to state that the bacteria were not significantly perturbed and could adapt rapidly. At higher juice percentages, the lag-phase was extended due to the change of the medium. As depicted in **Fig. 3**, the higher the juice concentration, the longer the lag-phase lasted. This might be attributed to the differences in nutrient availability. Indeed, LAB have elaborated nutrient requirements because of their limited capability to synthesize vitamins and amino acids from inorganic nitrogen sources [36]. Therefore, an optimal fermentation medium should provide all the compounds required to support bacterial growth and metabolic activity. For instance, MRS complex medium includes multiple nitrogen sources (casein peptone, meat extract, and yeast extract) that supply several amino acids, peptides, minerals, and vitamins crucial for LAB growth [28,51]. In contrast, press-juice primarily contains nitrogen sources in the form of amino acids and proteins at a usually lower concentration than the complex medium. Furthermore, proteins in the press-juice can only be used as a nutrient source when proteolysis takes place [52]. According to this, the nutrients in the press-juice are not immediately available for LAB growth and activity.

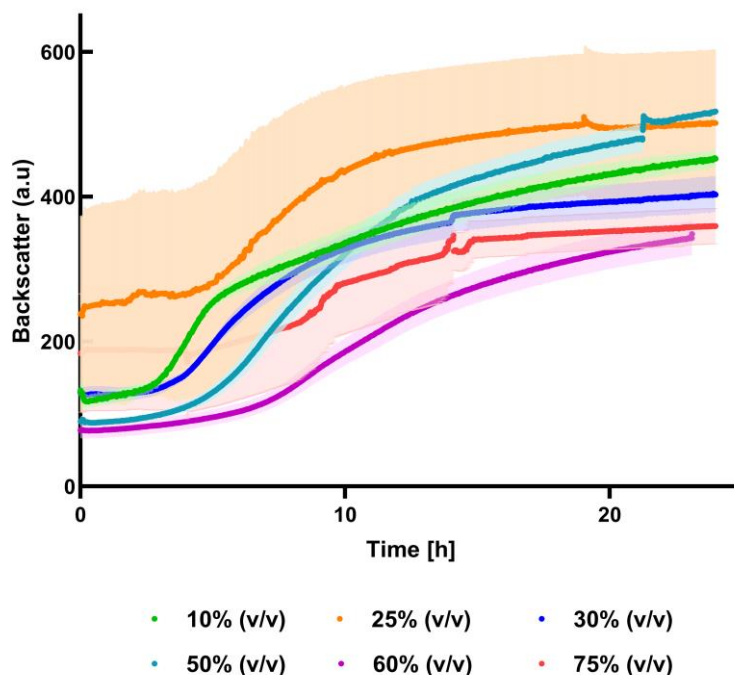


Fig. 3: Cultivation of *L. delbrueckii* in different juice percentages. The experiments were performed using the CGQ to measure online the bacterial growth (37 °C, 150 rpm). The cultivations were carried out in triplicates and the standard deviations are shown as the corresponding color shade.

Despite these challenges, the experiments aimed to establish whether *L. delbrueckii* could grow in the tested juice percentages to replace as much of the expensive complex media components as possible. Based on these findings, 75% (v/v) was selected as the juice percentage for the further experiments.

3.5 Bench-Scale Fermentation Using the Press-Juice

A concentration of 75% (v/v) of pressed juice was selected for LA production, aiming to replace expensive complex media components such as yeast extract. Previous studies have demonstrated the high nutrient content of juice from grass raw materials [53,54], suggesting that supplementation of only the essential nutrients could be economically advantageous. Previous studies investigated the optimization of the fermentation medium using brewers' spent grain liquor. The effect strength of each MRS medium component on LA production was analyzed, and it was found out that sodium-acetate and Tween 80 had the highest impacts [28]. It is reported that sodium-acetate enhances both final cell density and LA concentration, while Tween 80

supplies long-chain unsaturated fatty acids that increase cell membrane permeability [55–57]. Based on these results, 75% (v/v) press-juice was supplemented with a mixture containing 25% (v/v) glucose, sodium-acetate, and Tween 80. Preliminary experiments were conducted in Schott bottles equipped with a pH-control system to evaluate the potential of this optimized juice medium for LA production.

The results revealed that both glucose and fructose were consumed during the fermentation, with no discernible preference between the sugars (**Fig. 4**). This finding was in line with previous investigations; Mousavi et al. observed similar sugar utilization patterns in pomegranate juice by different *Lactobacilli* strains [58]. Costa et al. used a mixture of pear residues and ricotta cheese whey to produce LA, revealing that fructose and glucose were completely metabolized by *L. casei* within 24 h. The LA reached 43 g/L after 48 h with a yield of 50.6% with regards to total sugars [27]. Volkmar et al. found out that *L. delbrueckii* can consume glucose and fructose contained in municipal residues, producing approximately 17 g/L of LA [59]. Although the cell growth was not monitored due to the brownish colour of the medium, bacterial growth can be inferred from LA release. During the first 6 h of cultivation, the sugars were not metabolized and no LA was produced. Subsequently, exponential LA production was observed over the next 22 h supported by rapid sugars consumption. By the end of the fermentations, approximately 88% of sugars were consumed and 26.6 ± 1.2 g/L of LA was produced. The results corresponded to a production yield of 0.78 ± 0.03 g_{Sugars}/g_{Lactate}. Boakye-Boaten N. et al. produced LA in 90% (v/v) miscanthus press-juice using *L. platarum*, *L. brevis* and the combination of both in serum bottles. The results gave 11.7, 10.3, and 10.2 g/L of LA after 48 h of fermentation, respectively [60]. Santamaria-Fernandez M. et al. produced up to 22 g/L of LA from grass clover using *L. salivarius* [61]. Si et al. used a pH-control system to produce LA from corn straw hydrolysate. The authors obtained 99.8 g/L of LA, which corresponded to a production yield of 0.67 g_{LA}/g_{Sugars} [50].

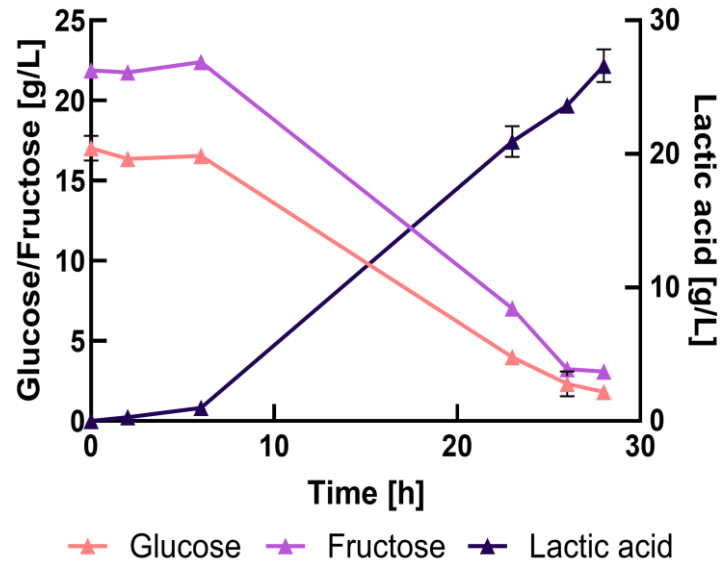


Fig. 4: Bench-scale fermentation in 75% (v/v) press-juice and a 25% (v/v) mixture of glucose/sodium-acetate/Tween-80 (mixture component concentration: glucose 10 g/L, sodium-acetate 5 g/L, Tween-80 1 g/L). The cultivations were performed in Schott bottles with a pH-regulated system. Culture conditions: 200 rpm, 37 °C, pH = 6.8 ± 1 (2.5 M NaOH buffer). Error bars indicate standard deviations of the mean (n = 2).

3.6 Comparison between Complex Medium and Press-Juice in the Bioreactor

After the evaluation of LA production in a pH-control system using either MRS medium or press-juice, both the experiments were scaled-up in stirred tank bioreactors. In MRS medium, fermentations were carried out using either glucose or fructose as a carbon source (**Fig. 5a, b**). Fructose was included to provide a further comparative analysis since it is the primary sugar in the press-juice. The experiments with the press-juice were performed as previously described (75% (v/v) press-juice and 25% (v/v) nutrients mixture) (**Fig. 5c**).

The choice of the carbon source significantly influenced the bacterial growth in MRS media. When glucose was used as the carbon source, the lag-phase lasted approximately 2 h (**Fig. 5a**). In contrast, the lag-phase was extended to approximately 4 h when fructose was used as the carbon source (**Fig. 5b**). For the press-juice experiments, OD measurements were not collected due to the brownish colour of the medium. However, as for the experiments in Schott bottles, information regarding the growth can be deduced from the LA release. In this case, the lag-phase lasted 8 h, which was longer than in complex media (**Fig. 5c**). During this phase, the cells turned

on their metabolic activity, such as the activation of signaling pathways, upregulation of protein assembly, nucleotide metabolism, and other processes necessary for differentiation and duplication. All these activities result in cell division, which is fully activated during the exponential-growth phase [62,63]. The prolonged lag-phase observed in press-juice may stress the bacteria due to suboptimal or insufficient nutrient concentrations. According to Kim J. et al., the nitrogen-to-carbon ratio significantly impacts sugar conversion to LA [36]. Akermann et al. [28] reported that sodium-acetate and Tween 80 were the medium components that most affected the cultivation of *L. delbrueckii*. Hebert E. et al. studied the nutritional requirements of *L. delbrueckii* subsp. *lactis* and they found out that MnSO_4 and FeSO_4 are essential for the growth, since they act as a cofactor in several enzymatic reactions [36,51]. It might be that the formulation of the press-juice medium may not be optimal for bacterial growth. Additionally, the transition from the complex medium used in the preculture to the press-juice could have affected the growth. It is also reported that the microbial lag-phase is an adjustment period before active growth begins, and its duration can vary based on environmental conditions [63].

The exponential-growth phase lasted until 10 h and 18 h when glucose and fructose were used, respectively (**Fig. 5a, b**). Throughout the cultivations, higher cell density was observed with glucose as the carbon source. The same result was obtained by Petrut S. et al., who cultivated *L. rhamnosus* on different sugars. The authors found out that glucose gave a higher cell density with respect to other sugars such as fructose, sucrose, mannose, and arabinose [64]. Chen H. et al. cultivated *L. acidophilus* on maltose, glucose, lactose, and whey powder. The authors obtained higher cells concentration (in Colony Forming Units/mL) when the bacteria were grown on glucose and whey powder [65]. During the exponential growth phase, most of the glucose and the fructose were consumed when the complex media were used. In particular, approximately 22.3 ± 3.6 g/L of glucose and approximately 16.5 ± 0.5 g/L of fructose were metabolized, which corresponded to $94.6 \pm 4.5\%$ and $77.2 \pm 1.4\%$ of sugar utilization (Table 3). The biomass yield was higher when glucose was used as the carbon source (0.17 ± 0.02 g_{Biomass}/g_{Glucose}) than with fructose (0.12 ± 0.01 g_{Biomass}/g_{Fructose}), indicating that glucose stimulated more cell growth than LA production. When the 75% (v/v) press-juice was used as the fermentation medium, the exponential-phase lasted until 28 h (**Fig. 5c**), likely due to the higher sugar

availability. During this phase, the glucose was totally metabolized, and approximately $86.8 \pm 0.6\%$ of the fructose was consumed. The corresponding LA concentrations were 14.1 ± 0.8 g/L in the experiment in MRS with glucose, 18.3 ± 2.2 g/L in the case of MRS with fructose, and 25.6 ± 3.4 g/L in pressed juice. Extending the fermentation time in complex media led to LA degradation.

The production yield and the productivity are crucial parameters in fermentation processes, and they are summarized in Table 6. The highest production yield was achieved in MRS with fructose as the carbon source, reaching the maximum theoretical yield of 1 g/g [13]. However, when 75% (v/v) press-juice was used, the production yield was slightly lower (0.91 ± 0.07 g_{LA}/g_{Sugars}). This result is comparable to those of Chen C. et al., who obtained a yield of 0.93 g_{LA}/g_{Sugars} with *L. plantarum* 23 using microalgae hydrolysate [66]. Similarly, LA produced from orange peel wastes using *L. delbrueckii* subsp. *delbrueckii* CECT286 reached a production yield of 0.95 g_{LA}/g_{Sugars} [67]. Thus, the results of this experiments are consistent with those reported in the literature.

In terms of productivity (Q_{LA}), the highest value was obtained when 75% (v/v) press-juice was used (0.87 ± 0.10 g/Lh), outperforming other studies. For instance, Pontes et al. obtained an LA productivity of 0.81 g/Lh using forest and marginal lands lignocellulosic biomass and *L. rhamnosus* ATCC 7469 [68,69].

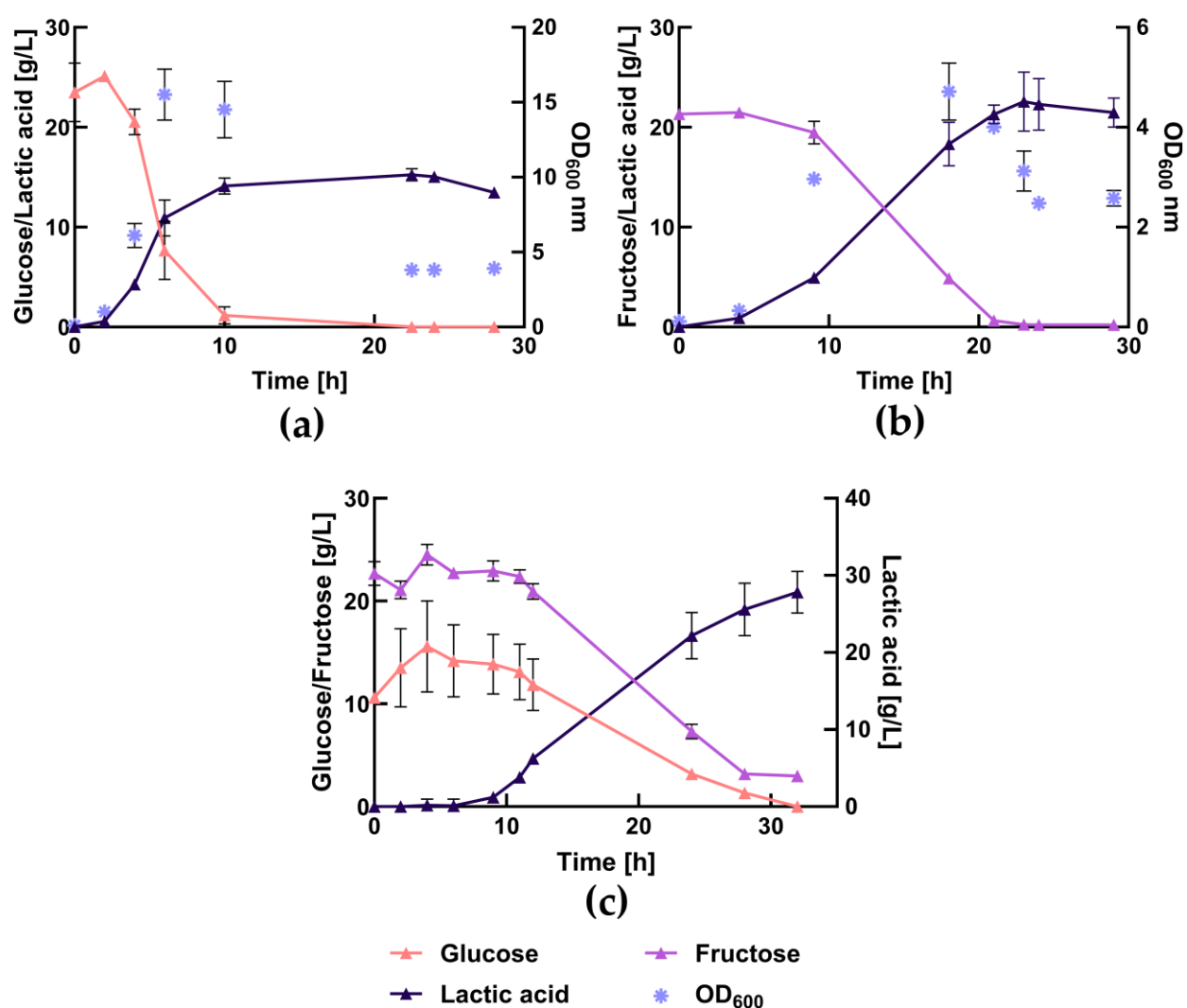


Fig. 5: Bioreactor fermentations in MRS medium with glucose (a) ($n = 3$), MRS medium with fructose (b) ($n = 2$), and 75% (v/v) press-juice (c) ($n = 2$). In all the experiments, the cultivations were carried out anaerobically, at 37 °C, 150 rpm, pH 6.5.

3.7 Lactic Acid Yield from Ensiling Whole Grass Fractions of Different Varieties of *Lolium perenne*

As a preliminary study to assess the influence of different varieties on ensiling, a classical whole grass ensiling process was conducted for the first project crops in the summer and autumn of 2020. LA yields after ensiling the whole grass fraction of *Lolium perenne* varieties harvested in 2020 are shown in **Fig. 6a**. The pH values dropped rapidly within the first 15 days, stabilizing around pH 4, which is indicative of a

successful fermentation process. The maximum LA yield was reached after 2–3 weeks of ensiling. Samples harvested in October showed substantially higher LA yields of up to 15.2% of the grass dry mass after ensiling compared to those harvested in May and June. Among the October-harvested samples, Agaska had the highest LA yield, while Honroso had the lowest with 12.8% DM. For the samples harvested in May and June, Explosion had the highest LA yield at 11.8% DM, while Arvicola had the lowest at 8.6% DM. Among the different *Lolium perenne* varieties, Agaska showed the least impact, with a 24% decrease in lactic acid yield when harvested in May/June compared to October, while Barmigo exhibited the greatest reduction, with a 41.7% decrease. High-quality silages exhibit LA yields of 6–10% based on dry matter. This range was achieved across all varieties and experiments [70]. Notably, the grasses harvested in October even exceeded 10%. The yields obtained in this study are consistent with values from the literature, with the 15.2% yield of Agaska harvested in October surpassing them. This superior yield positions Agaska as a highly efficient variety for LA production through ensiling. Johnson et al. reported yields of up to approximately 6.5% DM in 16 days with silages in vacuum bags without the addition of a silage additive [71]. Haag et al. achieved yields of 7% DM for untreated silages and silages with a biological silage additive, and up to 13% DM for silages with both biological and chemical additives [72]. Kung et al. reported yields of around 14% DM for silages with a biological additive [70]. Fazzino et al. reached 54.5 g/kg of total solids of LA after 8 weeks of ensiling using orange peel waste [73].

The WSC values for the 2020 crops could not be assessed as precisely as in the following years. Given that the WSC contents are assumed to be relatively similar across the varieties, the differences in LA yields are likely due to the inherent genetic variability among the *Lolium perenne* varieties, impacting their fermentation efficiency. The WSC content may include oligosaccharides that cannot be converted into LA by microorganisms. Factors such as microbial efficiency, nutrient availability, and specific conditions during ensiling also significantly influence LA yields.

The average proportions of the by-product acetic acid during the ensiling are shown in **Fig. 6b**. In high-quality silages, the acetic acid content should be between 1% and 3% DM [70]. The upper limit for acetic acid content was maintained across all varieties. The highest acetic acid content was found in the variety Agaska (October) with 2.0% DM, while the lowest was in the variety Agaska May/June at 0.2% DM. Low acetic acid content is desirable in silage because it increases aerobic stability. However, since this work primarily focuses on lactic acid production, falling below the lower limit is not considered negative.

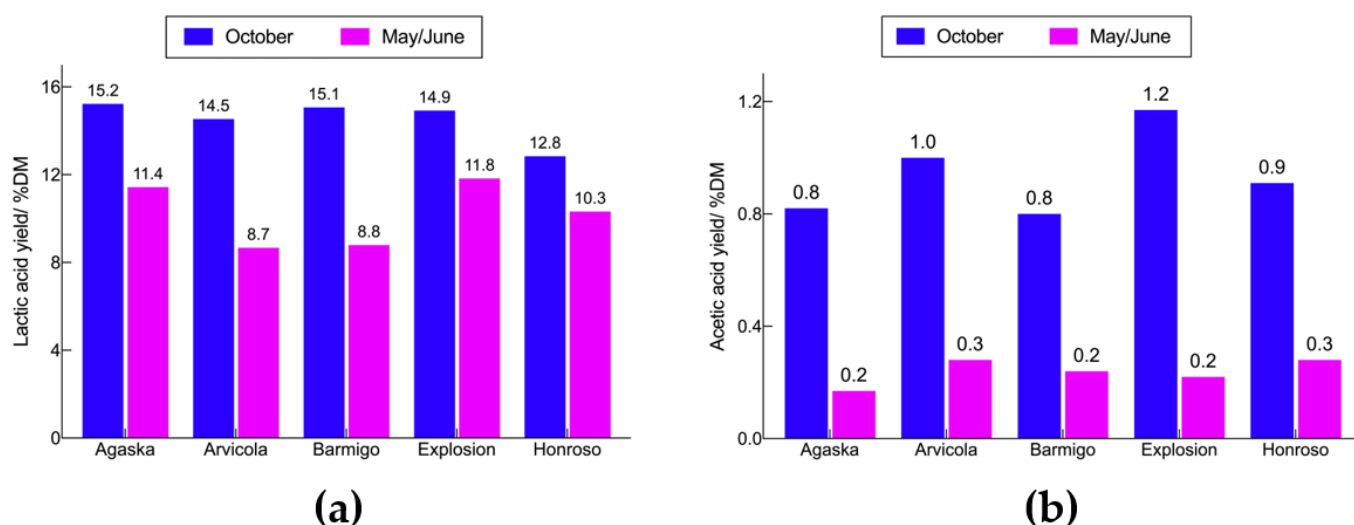


Fig. 6: Lactic acid (a) and acetic acid (b) yields from ensiling different varieties of *Lolium perenne* (room temperature, 14 days).

4 Conclusions

In this study, it was demonstrated that press-juice from perennial ryegrass can effectively replace or partially substitute expensive media components that are generally used in fermentation processes. The experiments performed in Schott bottles confirmed that the pH-control system is a fundamental parameter to control in LA production. This strategy not only eliminates the addition of neutralizing agents which complicate the downstream process, but it also reduces the total costs. The fermentation in bioreactors proved that the optimized press-juice medium allowed the production of more than 27 g/L of LA, which corresponded to a production yield of 0.91 ± 0.07 g_{LA}/g_{Sugars}. Overall, the use of press-juice as a fermentation medium not only allowed us to approach the maximum theoretical yield but also provided high

productivity, making it a promising and economically viable substitute to traditional complex media for LA production.

The whole grass fraction of *Lolium perenne* also showed significant potential for lactic acid production. The Agaska variety, in particular, achieved a lactic acid yield of 15.2% dry mass (DM) when harvested in October, significantly surpassing typical silage yields of 6–10% DM. This study highlights the significant potential of certain *Lolium perenne* varieties, particularly Agaska and Explosion, for high-efficiency lactic acid production through ensiling. Barmigo and Arvicola also show reasonable efficiency, while Honroso appears less suitable for maximizing lactic acid yields. These findings suggest that selecting appropriate grass varieties is critical for optimizing lactic acid yields of whole grass ensiling. Additionally, the impact of harvest time on ensiling outcomes cannot be overlooked, as autumn harvests tend to produce better lactic acid yields.

5 References

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Supplementary

Amino acids analysis

The derivatization process was automated using the Azura AS 6.1L autosampler (Knauer GmbH, Berlin, Germany) using the own “mix method” option. The OPA reagent was prepared by weighting 270 mg OPA in a 50 mL volumetric flask. The reagent was dissolved in 5 mL ethanol. Then, 200 μ L 2-mercapto-ethanol were added and the final volume was filled up with 0.4 M borate buffer. To analyze the samples, they must be protein and particles-free. For this reason, the proteins were firstly precipitated by adding 4 parts of ice-cold methanol and 1 part of the sample. The sample was placed at -20°C overnight. Then, the sample was centrifuged for 10 min at 10000 rpm (Eppendorf, Hamburg, Germany). Finally, the sample was diluted with 0.4 M borate buffer. A dilution of at least of 1:1 is necessary as the sample has to have a pH 10 for the derivatization. The derivatization was performed by adding 80 μ L of the sample or the standard and 50 μ L of OPA reagent. After 1 min, 40 μ L were injected for measurement.

Workflow

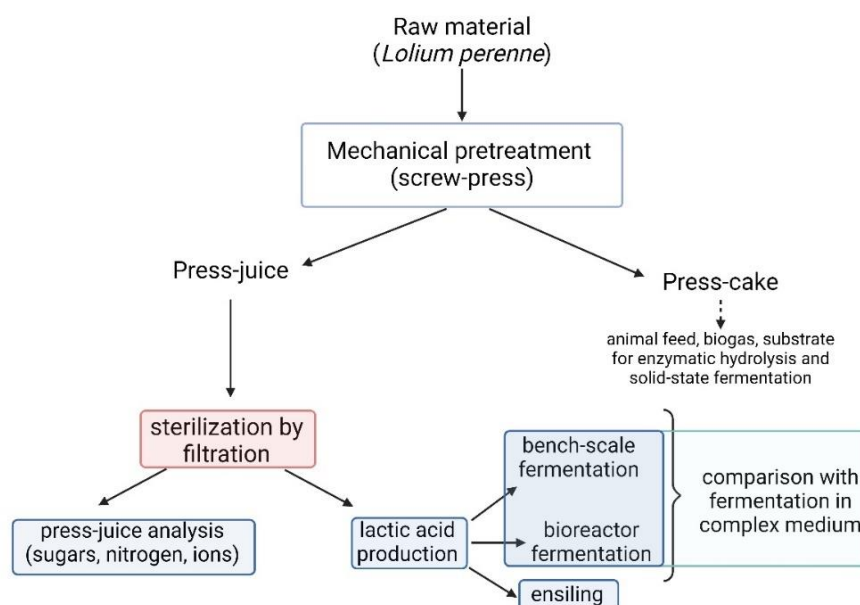


Fig. S1: Workflow of the experiments performed

Chapter VII – General Discussion and Conclusion

In this chapter, the main bottlenecks in the use of LCB are identified. It will be discussed how this thesis overcame the drawbacks of biomass processing— from the pretreatment to the proposal of novel strategies— with the scope to design a successful Green Biorefinery platform.

1 Pretreatment and enzymatic hydrolysis of lignocellulosic biomass

The utilization of lignocellulosic biomass offers important advantages over finite fossil resources, especially in environmental and economic aspects. However, LCB has some bottlenecks for the commercial viability of bio-based products. Firstly, LCB is characterized by a complex structure. The three structural polymers (cellulose, hemicellulose, and lignin) are tightly interconnected, resulting in a highly recalcitrant structure resistant to depolymerisation [2]. Consequently, processing LCBs using a biorefinery approach is challenging and expensive. The first step in the LCB deconstruction and conversion to monomeric sugars is the pretreatment, which accounts for approximately 40% of the total costs [3][4]. Therefore, the choice of an appropriate pretreatment method impacts the sustainability of the process. The selection of the pretreatment method must consider the conversion rate, the energy consumption, the formation of degradation products, the accumulation of undesired by-products, and the overall costs [5].

In this thesis, the impact of different pretreatment methods on enzymatic hydrolysis was investigated (**Chapter III**). It was found that the lignin and the moisture content of the biomass can guide the choice of the pretreatment method. Grass materials, including *Lolium perenne*, are considered low in lignin and high in moisture compared to woody materials. As a result, weak pretreatments, such as mechanical pretreatments, were sufficient to deconstruct the lignocellulosic structure and to enable the conversion of polysaccharides into monomers during the enzymatic hydrolysis. Enzymatic hydrolysis is also an expensive process in a biorefinery chain, with an estimated cost of 20-30% of the total production costs [2]. Therefore, the

saccharification might be optimised in terms of processing time and amount of enzyme. One way to achieve this is to perform kinetic studies (**Chapter III**). In this thesis, the integrated Michaelis-Menten model was applied to calculate key parameters such as the maximum reaction rate at which the enzyme is saturated with the substrate ($V_{\max}$), and the affinity of the enzyme for the substrate (K_M). The estimation of these kinetic parameters can accurately quantify the biomass conversion, and the hydrolysis rate, and can determine the causes of rate reduction during the enzymatic process [6].

2 Use of an integrated biorefinery approach to produce chemicals

Unlike single-product facilities, integrated biorefineries are considered highly versatile as they enable the production of multiple products [7]. According to this, several fermentation strategies can be applied depending on the substrate used. In this thesis, the screw pressing enabled the separation of the press-cake and the press-juice, both of which were employed as substrates in different bioprocesses.

2.1 Utilization of the press-cake as substrate

In a general biorefinery chain, harsh pretreatment methods release both sugars and inhibitory compounds from the LCB. For this reason, detoxification procedures are required with a further increase in operational and production costs [8]. In order to design a more economically viable process, the press-cake obtained after the screw pressing was directly utilized as substrate in a Solid-State Fermentation (SSF) process (**Chapter IV**). Other fermentation strategies including Simultaneous Saccharification and Fermentation have been widely applied. However, they need the addition of costly enzyme mixtures to convert cellulose and hemicellulose into simple sugars [9]. In this thesis, a Consolidated Bioprocess was outlined to ensure the production of enzymes, the biomass hydrolysis, and the fermentation by one microorganism. In this scenario, filamentous fungi are considered the ideal candidates compared to bacteria and yeasts. In **Chapter IV**, it was discussed the cultivation of *Aspergillus niger* in a Solid-State Fermentation process, highlighting its ability to secrete cellulases and to produce citric acid. The production of citric acid is a complex process influenced by several cultivation conditions including pH, type and concentration of the carbon source, limitation of other nutrients, and, in the case of fungi, even fungal morphology [10]. In this work, it was found that the addition of the carbon source (glucose or press-juice)

along with nitrogen, phosphate, potassium, and methanol led to a successful citric acid production. In particular, the type of carbon source supplemented had a major impact on the final titer. CLSM analysis revealed that, regardless of the carbon source added, *A. niger* and the press-cake were closely associated. Nonetheless, differences were observed in the fungal mycelia depending on the carbon source used, suggesting that the presence or the absence of certain nutrients may influence the morphology of *A. niger*.

2.1.1 Use of filamentous fungi as biocatalyst in biorefineries

Bacteria and yeast have been widely used as cell factories to produce a variety of bioproducts such as native metabolites, heterologous products, and combinations of heterologous proteins [11]. Despite the advantages of these microorganisms—including shorter fermentation time, easy cultivation systems, and abundant manipulation tools—filamentous fungi may be considered superior biocatalysts [12]. One of the main advantages of these microorganisms is the ability to assimilate an array of different substrates, including pentoses, hexoses, and disaccharides [2]. In addition, filamentous fungi can grow on lignocellulose polysaccharides such as glucan, xylan, and arabinan, so they can play an important role in the valorisation of lignocellulosic materials [13]. The growth versatility of filamentous fungi is a crucial trait in biorefinery platforms, and it is correlated to their ability to secrete hydrolytic enzymes based on the substrates they grow on [13]. Besides enzymes, filamentous fungi have been extensively used for the production of metabolites including antibiotics, pigments, food products, and organic acids [14,15]. Back in the past, citric acid was the first organic acid to be produced via fungal fermentation using conventional carbon sources [16]. However, at present, considerations about sustainability must be taken into account. In **Chapter II**, the advantages of the use of filamentous fungi as biocatalysts for the production of organic acids within a biorefinery approach are discussed. Along with the morphological traits of filamentous fungi, the main fermentation processes that led to the production of different organic acids are described. The market size and the industrial application of these compounds were also reviewed, and it was highlighted the urgent need to develop economically competitive bioprocesses in order to reduce the production costs.

2.2 Utilization of press-juice as fermentation media

2.2.1 Production of ethanol

Some of the sugars released during the enzymatic hydrolysis are not fermentable, especially pentoses. Over the past years, bacteria and yeast strains have been improved *via* rational engineering (mutagenesis) to assemble new metabolic pathways. An example is the yeast *Saccharomyces cerevisiae* that has been widely engineered to metabolize xylose [17–23]. However, rational engineering techniques are labor-intensive, time-consuming, and might be unsuccessful due to the lack of knowledge [24,25]. Additionally, the resulting strains are often not suitable for industrial application because of lower stress tolerance [18]. To overcome the limitations of rational design, novel approaches such as evolutionary engineering have emerged. Evolutionary engineering can be defined as a method that mimics Darwinian selection where the improved strains are obtained by applying a selective pressure for the desired phenotypes [26,27]. Evolutionary engineering has been widely used to get strains tolerant to lignocellulosic inhibitors or able to ferment unconventional sugars. In **Chapter IV**, another application of evolutionary engineering was demonstrated. In particular, the press-juice was used as a fermentation medium for ethanol production using the yeast *Saccharomyces cerevisiae*. For this purpose, the press-juice was analysed in terms of nutrient content such as sugars, organic nitrogen, cations, and anions. It was demonstrated that *S. cerevisiae* could either grow or produce ethanol in pure press-juice, suggesting that press-juice contained all the necessary nutrients for both yeast growth and ethanol production. Despite the yeast's ability to consume hexoses, fructose—the main sugar contained in the press-juice—was not completely metabolized, resulting in a low ethanol yield. For this reason, short-term adaptation was applied as a strategy to improve the fermentation performances and efficiencies of *S. cerevisiae*. After the adaptation, the strain enhanced its fermentation capacity, including the consumption of sugars which led to almost the maximum theoretical ethanol yield.

2.2.2 Production of lactic acid

Saccharomyces cerevisiae is one of the most versatile microorganisms due to its ability to adapt to various media conditions. In contrast, other microorganisms, such as Lactic Acid Bacteria (LAB), have complex nutrient requirements. For instance, LAB

can not synthesize nitrogen-based substances, which must therefore be supplemented in the media [28]. To meet these requirements, complex fermentation media have been formulated to include all the necessary nutrients for bacterial growth. However, certain components, such as yeast extract, peptone, trace elements, and vitamins are expensive and significantly increase the overall production costs [28,29]. In **Chapter VI**, the press-juice was used as part of fermentation media to produce lactic acid using *Lactobacillus delbrueckii* subsp. *lactis*. This study aimed to replace expensive complex media elements to reduce lactic acid production costs. Based on previous studies [30], the press-juice was supplemented with glucose, sodium-acetate, and Tween-80 to ensure successful acid production. The optimized press-juice was tested either at bench-scale using a pH-control system or in bioreactors. The idea behind the utilization of a pH-control system was to maintain the optimal pH to prevent the product inhibition process. Yet, it eliminated the addition of neutralizing agents which would make the downstream process hard and expensive. Fermentation parameters such as sugar uptake, yield, productivity, and efficiency revealed that cultivations in press-juice were comparable to those obtained in the standard complex media.

3 Conclusion

In this thesis, the production of different biochemicals through innovative and cost-effective bioprocesses was investigated. Due to the numerous industrial applications and significant market potential, citric acid, ethanol, and lactic acid were selected as target bioproducts.

The first process in a biorefinery chain is the pretreatment, which aims to open the lignocellulose structure to make the carbohydrates more accessible to enzymatic digestion. However, selecting the appropriate method is challenging, requiring a balance between maximizing the recovery of structural polymers and minimizing the formation of inhibitory compounds. In **Chapter III**, the effects of different pretreatment methods on the enzymatic hydrolysis were evaluated. Besides the perennial ryegrass, municipal wastes were used as an example of woody biomass for comparative purposes. For the perennial ryegrass, mechanical pretreatment *via* screw pressing alone resulted in a glucose release of more than 80% during enzymatic hydrolysis. The addition of the hydrothermal pretreatment further increased the glucose yield by

13%. In contrast, for woody materials, the sole chopping or milling led to a significantly lower glucose release (14% and 19%, respectively). The inclusion of the Organosolv increased significantly the glucose yield to 75% and to 91% after chopping and milling, respectively. These findings suggested that for grass materials, screw pressing was a sufficient method to achieve a satisfying glucose release. The use of energy-intensive pretreatment methods should be applied only to biomass that requires harsher processes to ensure an efficient saccharification. Overall, this study highlighted the importance of tailoring the pretreatment approach to the specific characteristics of the biomass, such as the lignin and the moisture content. In **Chapter III**, kinetic analyses were also performed. The time-course hydrolysis on the screw-pressed perennial ryegrass revealed that the steady-state was reached within 24 h. Using the integrated Michaelis-Menten model throughout the steady-state, the kinetic parameters V_{MAX} and K_M were calculated, and were estimated to be 0.6 mmol/min and 93.4 mmol/L, respectively. This investigation would help to optimize the process by keeping the hydrolysis going until the maximum rate of reaction is reached.

Despite advances in biorefinery technologies, the saccharification step is still an expensive step due to the high cost of the enzymes. Therefore, the search for alternative strategies to circumvent costly processes is a discussed topic. One example is the Consolidated Bioprocess explored in **Chapter IV**. In this study, the ability of *A. niger* to simultaneously produce citric acid and secrete cellulolytic enzymes was investigated. For this purpose, the press-cake derived from the screw pressing was used as a substrate in the Solid-State Fermentation process. The addition of glucose or the remaining press-juice led to a successful citric acid production, reaching 84.7 g/kg_{DM} and 2.1 g/kg_{DM} of citric acid, respectively. To have an insight into the hydrolytic activity of *A. niger*, the β -glucosidase activity was used as an indicator. When supplemented with glucose or press-juice, the resulting β -glucosidase activities were 0.89 ± 0.02 U/g_{DM} and 1.15 ± 0.07 U/g_{DM}, respectively. These results indicated that *A. niger* was forced to hydrolyse the press-cake to access the sugars essential for its growth. To further evaluate the interaction between the fungus and the press-cake, as well as to observe fungal morphology, CLSM analyses were performed. Regardless of the carbon source, a close interaction between the fungus and the press-cake was observed; in contrast, differences in the fungal mycelia were noticed based on the carbon source. When glucose was supplemented, the fungal mycelia

appeared more compact and robust. Yet, a higher degree of ramification, which is related to higher enzyme production, was observed. At present, although citric acid is mainly produced via microbial fermentation, the use of lignocellulosic biomass as substrate is still relatively limited. Thus, the process proposed in **Chapter IV** aimed to enhance the economic viability of the bio-based production by utilizing a low-cost substrate like the press-cake.

In **Chapter V**, a novel bioprocess was developed for ethanol production. Short-term adaptation was used as a strategy to enhance the fermentation of *S. cerevisiae* in pure press-juice. In the parental yeast strain, the ethanol yield reached 0.38 ± 0.2 g/g, and the amount of fructose converted was 79%. After a period of adaptation wherein yeast cells were progressively cultivated in increasing concentrations of press-juice, the adapted strain achieved 0.50 ± 0.06 g/g and a fructose conversion of about 90%. While short-term adaptation has been traditionally applied to develop strains with novel characteristics (such as the ability to metabolize xylose), this study highlights its potential for optimizing existing bioprocesses. The remarkable improvements in the adapted strain emphasize the possibility of applying evolutionary engineering to enhance industrial bioprocesses.

From a bio-based economy perspective, the development of a bioprocess must not only focus on the technical feasibility but also assess economic viability. For instance, although 90% of the total lactic acid production is carried out via microbial fermentation, the associated costs remain substantially high. In **Chapter VI**, the production of lactic acid using the press-juice was investigated. *L. delbrueckii* subsp. *lactis* was cultivated in different press-juice amounts (10, 25, 30, 50, 60, 75% (v/v)), and it was found that all the juice percentages supported the bacterial growth. Therefore, to replace as much as possible complex medium components, 75% (v/v) press-juice was selected as the starting medium formulation. Previous studies have shown that LAB have complex nutrient requirements, so medium optimization is often required. In this study, 75% (v/v) press-juice was supplemented with glucose, sodium-acetate, and Tween-80, as they were identified as key factors in lactic acid production. Initially, fermentations were conducted at bench-scale to evaluate the medium optimization, and later scaled-up in bioreactors. In those systems, the lactic acid yield reached 0.91 ± 0.07 g/g and only 2.9 g/L of residual fructose remained at the end of the fermentation. These results were comparable to those achieved with

a conventional complex medium containing fructose, where the lactic acid yield was 1.02 ± 0.08 g/g with a full fructose consumption.

Overall, this thesis demonstrated that the integrated utilization of the perennial ryegrass *Lolium perenne* is an effective strategy for developing a multi-product green biorefinery based on citric acid, ethanol, and lactic acid production. Although the experimental outcomes of this work are promising, they are insufficient for the concrete realization of a green biorefinery. Therefore, to improve the environmental performance and to translate the scientific data into economically viable processes, life-cycle assessment (LCA) and techno-economic analysis (TEA) must be performed. These evaluations are necessary to identify the environmental and economic hotspots and to provide insights for scaling up the proposed bioprocesses.

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Appendix A

A1 Supervised students

- Elizabeth Morgan: Organic acids production from a perennial ryegrass (2023). Praktikum
- Jovanna Kruger: Enzymes in Biorefineries (2022). Presentation for Biotransformation and Biokatalyse Kurs
- Hatice Bektas: Fermentative production of organic acids (2022). Bechelorarbeit BCI
- Alexander von Lochow: Enzymatic hydrolysis of a German ryegrass (2021). Forschungsarbeit Master Wirtschaftschemie

A2 Conference Contributions

L. Varriale, K. Kuka, N. Tippkötter, R. Ulber; Cascade use of a German ryegrass for the production of valuable bioproducts with a view to a biorefinery; Himmelfahrtstagungen (2022); Mainz, Germany (Poster)

L. Varriale, R. Ulber; Production of organic acids from a perennial ryegrass with a Biorefinery view; Renewable Resources and Biorefineries (RRB) (2022); Bruges, Belgium (Lecture)

L. Varriale, K. Kuka, N. Tippkötter, R. Ulber; Use of a green biomass in a biorefinry platform; (Bio)Porcess Engineering – a Key to a Sustainable Development (2022); Aachen, Germany (Lecture)

L. Varriale, K. Kuka, N. Tippkötter, R. Ulber; Perennial ryegrass: a suitable lignocellulosic biomass for the development of a biorefinery platform; Renewable Resources and Biorefineries (RRB) (2023); Riga, Lettland (Lecture)

L. Varriale, K. Kuka, N. Tippkötter, R. Ulber; Exploiting different fermentation strategies to produce biochemicals from a green lignocellulosic biomass; 46th Symposium on Biomaterials, Fuels and Chemicals (2024); Alexandria, USA (Poster)

L. Varriale, K. Kuka, N. Tippkötter, R. Ulber; Valorisation of a green lignocellulosic biomass fot the bio-based chemicals production in a biorefinery-like concept; Renewable Resources and Biorefineries (RRB) (2024); Bruxelles, Belgium (Lecture)

A3 Curriculum vitae

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Academic career

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Statutory declaration

I, Ludovica Varriale, hereby confirm that I have prepared this thesis independently, have not used any aids other than those permitted, and I have cited all the references. I also confirm that this thesis has not been submitted for any other examination procedure in this or a similar form.

City, Date

Ludovica Varriale