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PRODUCTION OF LACTIC ACID FROM FOOD PROCESSING RESIDUES

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Submitted by
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Abstract

This dissertation focuses on the production of lactic acid as a bio-based building block from which a wide platform of value-added derivatives can be obtained. Current industrial production of lactic acid, pulled by a market in continuous growth, is based on the fermentation of first generation biomass feedstock like starch and refined sugars. In contrast to this, this work aimed to use residues from food processing as a resource in order to reduce waste, valorize residual streams, create value and avoid competition with food production. The primary interest of this dissertation is to gather knowledge about the risks and technical constraints in the upstream production of lactic acid from starch-rich and lignocellulosic residues, develop alternative process set-ups, and evaluate concrete solutions to facilitate their implementation in current and future industrial plants.

This work articulates into three main studies. The first one offers a valuable insight into the wheat bran biorefinery concept, with focus on lactic acid fermentation. The lignocellulosic nature of the substrate offered interesting issues to deal with. After the separation of the starch fraction, the residual cake was treated hydrothermally and enzymatically (cellulases, xylanases and β-glucanases) to release pentose and hexose sugars (21 g/L overall). The resulting liquid hydrolysate – containing also 1.8 g/L furfural – was used as feedstock for *L. pentosus* DSM 20314, that produced lactic acid with a yield of 73% after 52 h. When the initial sugar concentration in the hydrolysate was almost triplicated through nanofiltration, a maximum lactic acid concentration of 39.2 g/L was obtained. In both cases yields were limited by a poor conversion of the pentose fraction and co-production of acetic acid.

Within the second study, higher product concentrations (102 g/L) and yields (92%) were obtained with *Lactobacillus casei* on starch-rich residues from the potato processing. This viscous mash of puree and peels was hydrolyzed with α- and glucoamylases to obtain a liquid glucose syrup that was supplemented with yeast extract (3 g/L). Compared to the lignocellulosic fraction of wheat bran, this substrate offered the following advantages: milder extraction conditions, higher sugar availability and less inhibitors. Moreover, the lactic acid produced (92% L-isomer, impaired by uncontrolled fermentation during transport and storage) is of suitable quality for numerous technical applications after purification. Results indicate that it is possible to produce 54 kg of technical grade lactic acid (90% w/w) from 103 kg of fresh potato residues. Even if there is still potential for improvements regarding productivities and yields, it was clearly demonstrated that the developed process is promising for large-scale biorefinery applications.

The third study aimed to provide scientific innovation using a thermophilic strain that directly converts starch into lactic acid. Working at high temperatures reduces operating costs

connected with sterilization and cooling, where the latter is needed to compensate the metabolic heat generated by the exothermic formation of lactic acid. Moreover, the use of an amylolytic strain avoids costs for enzymatic formulations for starch hydrolysis. Starting from 50 g/L raw potato starch, *Geobacillus stearothermophilus* yielded 37 g/L optically pure (98%) L- lactic acid in 20 h. The observed productivity was high in comparison to similar processes described in the literature. As co-metabolites, smaller amounts of acetic acid, formic acid and ethanol were formed. The inhibitory effect of high concentrations of lactic acid and of the co-metabolites was considered responsible for limiting the maximum lactic acid concentration achievable. However, yields and product concentration could be increased to 81% and 59 g/L respectively when potato residues were used as a substrate instead of raw starch. It was clearly proven, that stable processes can be conducted operating in non-sterile conditions and without sterilization of the medium.

Within these studies, a number of difficulties connected with the chemical and physical nature of the residues were remarked: lack of homogeneity, high water content, logistics of conservation and presence of inhibitors. From the microbiology aspect, instead, desirable characteristics for application in a modern biorefinery were identified, such as: efficient use of pentose sugars, direct conversion of starch, performance under thermophilic conditions and in presence of inhibitors.

Finally it was concluded that, in order to be competitive, fermentative production of lactic acid from food industry residues must be implemented into a wider biorefinery context. The future vision is that of an integrated biorefinery where differentiated local raw materials are fully processed into a variety of products (and energy forms), among which lactic acid has an important function and market.

Kurzfassung

Diese Dissertation befasst sich mit der Herstellung von Milchsäure als bio-basiertem Grundbaustein, aus dem eine breite Plattform von hochwertigen Derivaten gewonnen werden kann. Die aktuelle, aufgrund steigender Nachfrage wachsende, industrielle Milchsäureproduktion basiert auf der Fermentation von Biomasse-Rohstoffen der ersten Generation, wie Stärke und raffiniertem Zucker. Ziel dieser Arbeit ist, im Gegensatz dazu, die Nutzung von Nebenprodukten der Lebensmittelindustrie als Ressource, um Abfälle zu reduzieren, Restströme aufzuwerten, Wert zu schöpfen und Konkurrenz zur Nahrungsmittelproduktion zu vermeiden. Das primäre Interesse dieser Dissertation ist es, sowohl grundlegendes Wissen bezüglich der Risiken und technischen Grenzen bei der Gewinnung von Milchsäure aus stärkereichen und lignozellulosehaltigen Reststoffen zu sammeln, als auch neue Verfahren zu entwickeln und Lösungen für deren Umsetzung in bestehenden und künftigen industriellen Anlagen zu evaluieren.

Diese Arbeit gliedert sich in drei Hauptstudien. Der erste Teil gibt einen Einblick in das Konzept einer Weizenkleie-Bioraffinerie, wobei die Milchsäurefermentation im Fokus steht. Die Verarbeitung des lignocellulosehaltigen Substrats stellt eine interessante Herausforderung dar. Nach der Abtrennung der stärkehaltigen Fraktion, wurde das Substrat einer hydrothermischen und enzymatischen (Cellulasen, Xylanasen und β-Glucanasen) Vorbehandlung unterzogen, um Pentosen und Hexosen verfügbar zu machen (21 g/L insgesamt). Das so erzeugte flüssige Hydrolysat, inklusive 1.8 g/L Furfural, wurde als Fermentationssubstrat zur Milchsäureproduktion durch *L. pentosus* DSM 20314 verwendet. Es wurde nach 52 Stunden eine Milchsäureausbeute von 73% erhalten. Nach annähernd dreifacher Aufkonzentrierung der im Hydrolysat enthaltenen Zucker durch Nanofiltration wurde eine maximale Milchsäurekonzentration von 39.2 g/L erreicht. In beiden Fällen wurde die Milchsäureausbeute durch die schlechte Umwandlung der Pentosen und die Koproduktion von Essigsäure eingeschränkt.

Höhere Milchsäurekonzentrationen (102 g/L) und Ausbeuten (92%) wurden im Rahmen der zweiten Studie mit *Lactobacillus casei* aus stärkereicheren Nebenprodukten der Kartoffel verarbeitenden Industrie erreicht. Die dickflüssige Mischung aus Kartoffelschälbrei und -püree wurde mit α- und Glucoamylasen aufgelöst. Der so gewonnene flüssige Zuckersaft wurde mit Hefeextrakt (3 g/L) versetzt. Verglichen mit der lignocellulosehaltigen Fraktion der Weizenkleie, bietet dieses Substrat folgende Vorteile: Schonendere Extraktionsbedingungen, leichtere Zugänglichkeit des Zuckers und geringer Anteil an Hemmstoffen. Die so erzeugte Milchsäure (92% L-Isomer, 8% D-Isomer entstehen durch die unkontrollierte Fermentation während des Transports und der Lagerung) ist für zahlreiche technische Anwendungen nach entsprechender Aufreinigung geeignet. Die Ergebnisse zeigen, dass 54 kg Milchsäure

technischer Qualität (90% w/w) aus 103 kg frischen Kartoffelresten gewonnen werden können. Wenn auch noch Verbesserungspotential hinsichtlich der Produktivität und Ausbeute besteht, wurde eindeutig nachgewiesen, dass der entwickelte Prozess große Chancen für den Einsatz in einer biobasierten Raffinerie im industriellen Maßstab birgt.

Die dritte Studie zielt darauf ab, ein innovatives Verfahren zu entwickeln, das mittels thermophiler Bakterien Stärke unter autoselektiven Bedingungen direkt zu Milchsäure umwandelt. Bei hohen Prozesstemperaturen ist zu erwarten, dass operative Kosten für Sterilisation und Kühlung während der Fermentation reduziert werden. Letztere sorgt für den Ausgleich der Wärmeentstehung bei der exothermen Milchsäurebildung. Darüber hinaus werden Kosten für enzymatische Formulierungen zur Stärkehydrolyse vermieden. Aus 50 g/L Kartoffelstärke erzeugte *Geobacillus stearothermophilus* in 20 Stunden 37 g/L optisch reine (98%) L-Milchsäure. Verglichen mit ähnlichen in der Literatur beschriebenen Verfahren war die gemessene Produktivität hoch. Als Co-Metaboliten wurden geringe Mengen an Essig-, Ameisensäure und Ethanol gebildet. Die Ergebnisse legen nahe, dass die Hemmwirkung der hohen Milchsäure- und der Co-Metabolitenkonzentration die maximale erreichbare Milchsäurekonzentration beeinträchtigt. Nichtdestotrotz konnten Produktausbeuten und -konzentrationen jeweils bis zu 81% und 59 g/L erhöht werden, wenn Kartoffelreste anstatt Kartoffelstärke als Fermentationssubstrat eingesetzt wurden. Es wurde eindeutig bewiesen, dass auch bei unsterilen Prozessbedingungen und ohne Mediensterilisation stabile Verfahren durchgeführt werden können.

Im Lauf dieser Studien wurden einige Schwierigkeiten im Zusammenhang mit den chemischen und physikalischen Eigenschaften der Reststoffe festgestellt: Mangel an Homogenität, hoher Wassergehalt, Logistik der Aufbewahrung und Anwesenheit von Hemmstoffen. Aus der mikrobiologischen Sicht wurden wünschenswerte Eigenschaften der eingesetzten Produktionsstämme für den Einsatz in der modernen Bioraffinerie definiert, unter anderem: Effiziente Nutzung von Pentosen, direkte Stärkeumwandlung, Wachstum unter thermophilen Bedingungen und geringe Empfindlichkeit gegenüber Inhibitoren.

All dies legt die Schlussfolgerung nahe, dass die fermentative Milchsäureerzeugung aus Nebenprodukten der Lebensmittelindustrie in einen breiten Bioraffineriekontext implementiert werden muss, um kompetitiv zu sein. Die zukünftige Vision ist die, einer integrierten biobasierten Raffinerie, wo vielfältige und lokal vorhandene nachwachsende Rohstoffe in ein ganzes Spektrum aus Produkten und Energieträger umgewandelt werden unter denen Milchsäure als zentrales Produkt eine wichtige Rolle spielt.

Keywords

Biorefinery, lactic acid, fermentation, wheat bran, potato residues, lignocellulose, starch, enzymatic hydrolysis, *Lactobacillus pentosus*, *Lactobacillus casei*, *Geobacillus stearothermophilus*, thermophiles, amylolytic bacteria.

1. General

This dissertation consists of a series of studies for the valorization of agro-industrial residues to produce green chemicals, with special focus on lactic acid as a prominent building block. In fact, residual biomasses are a highly available and valuable resource and can be converted in a cost-effective way, not only to different forms of energy and fuels, but also to green chemicals.

The data presented here provide valuable information for the development of robust and sustainable industrial routes from residual biomasses to green lactic acid in a biorefinery context. In contrast to current conventional processes, the aim is to avoid competition with food production, reduce consumption of fossil energies and generate value from residual streams.

The outcome of this work has been presented in one oral and six poster presentations. Moreover, these studies also resulted in three original research articles published in Science Citation Index (SCI) Journals.

Parallel to this, the author was involved in several contributions to a second PhD Project, carried out by the colleague Cornelia Haas on a closely connected topic: the microbial production of PHB from agro-residues. This mutual collaboration consisted in the joint development of methods, experimental design, process set-up and interpretation of results. The outcome was presented in further publications, poster and oral presentations and will not be described in further details within this work.

This framework collects the most relevant findings on lactic acid production in four years of research at the Institute for Environmental Biotechnology (Department of Agrobiotechnology, IFA-Tulln).

2. Chemicals from renewables

Global society is threatened by severe political and social issues like climate change, unstable oil prices, depletion of fossil resources and an increasing world population. This is driving governments and industries to develop more sustainable industrial processes and energy systems based on renewable resources. The key role of renewables for a sustainable progress was remarked also at the United Nations Climate Conference held in Paris in 2015 (IRENA, 2016).

Scientific and industrial research have the important role to develop efficient technologies to make our economy shift to a rational use of bio-based resources for the production of “green” chemical building blocks. The latter are molecules with multiple functional groups that can be converted into a wide range of marketable fine and specialty chemicals, intermediates and polymers using combinations of biological, thermal and chemical processes. “Green” building blocks are derived from renewables like agricultural and forestry residues, oil and energy crops, aquatic biomass and other organic residues. Moreover, these are not necessarily the same intermediates occurring in the traditional oil-based routes, but are often different, more reactive compounds (Jang et al., 2012; Sauer et al., 2008).

There are several reasons to promote a change from fossil to bio-based resources. Firstly, in contrast to petroleum, bio-based resources are renewable and intrinsically more CO_2 neutral (Ragauskas et al., 2006), even if carbon neutrality of some applications became recently a matter for debate (Johnson, 2009). Therefore, in the long run, it is expected to reduce greenhouse emissions. Secondly, the molecules extracted from different types of biomass (e.g.: carbohydrates, terpenes, lignocellulosic material) contain functional groups that are in fewer steps transformed into added-value chemicals (Sauer et al., 2008). Moreover, some fine chemicals (flavors, dyes, bioactive molecules) are even readily available after extraction (Gallezot, 2007). Finally, biotechnological processes – mostly used for the conversion of bio-based molecules into primary building blocks – operate at less severe conditions in terms of temperature, pressure and pH, compared to the traditional chemical routes required in the petroleum-based industry.

It might be argued that bio-based processes are often not yet as efficient as the traditional petrochemical ones. However, the fuel and chemical industry from hydrocarbons has more than a century of history, in which the petroleum refinery has been extensively optimized to minimize losses and give the highest yields. Following this example, the new-born bio-based industry is striving for an increase of yields and efficiencies to meet the industrial requirements, also fueled by American and European policies (e.g. FP7, Horizon 2020) that promote scientific research in this direction (Gallezot, 2007). Research has the fascinating

critical role of providing the technical background to support global society moving towards a more sustainable chemical industry.

2.1. Bio-based chemical building blocks and derivatives

In the petrochemical industry, a few key building blocks dominate the primary output. These are: methanol, ethylene, propylene, butadiene, benzene, toluene and xylene, which are further processed to fine and specialty chemicals and polymers (de Jong et al., 2012).

As an alternative, a large variety of C2–C6 building blocks can be obtained from renewable substrates mainly via microbial fermentation to be then further processed to added-value chemicals and polymers, mainly through chemical pathways (Werpy & Petersen, 2004). The manufacture of finished goods from renewable raw materials has been progressing rapidly in recent years, gaining competitiveness over traditional processes. Nevertheless, “green” building blocks are currently at different stages of industrial development and market. For instance, citric acid, lactic acid and ethanol – with even decades of history in industrial production – and 1,3-propanediol are already well established on the market. Succinic acid, 1,4-butanediol, isobutanol, acetic acid and isoprene have only recently accessed industrial production. Other products like levulinic acid, acrylic acid, isopropanol and caprolactam (see also Table 1) are still on a pilot or demonstration stage of development (Jang et al., 2012).

Besides the production of so-called “drop-in” intermediates, biotechnological routes from biomass can deliver compounds that otherwise could not be taken into consideration as building block materials since their chemical synthesis from oil-based resources is economically inconvenient and/or technically difficult (Harmsen et al., 2014). For instance, in addition to current direct applications, succinic acid has also a great potential to replace oil-based maleic anhydride as a building block for large volume production of polyesters, polyamides and environmentally friendly solvents (Table 1) (Beauprez et al., 2010). Thus, in the last years several efforts have been made to establish a competitive microbial process for its production (Li et al., 2011; Raab et al., 2010). Another example is the production of optically pure D- and L-lactic acid – indispensable for polymer applications – that is feasible only via microbial fermentation, as usual chemical pathways only lead to racemic mixtures (Narayanan et al., 2004).

Besides succinic and lactic acid, other organic acids have been identified as interesting building blocks in the last decade, especially owing to their bi-functional groups (Werpy & Petersen, 2004). In particular, acids with keto- or hydroxyl-groups are favorable for polyesters, while dicarboxylic acids are for polyamides (Becker et al., 2015). There are,

however, not only organic acids among the most promising building blocks currently on the market or in the pipeline. Table 1 gives an overview thereof inclusive of present production size (figures collected from the websites of the respective companies) and derivatives (Biddy et al., 2016; Bozell & Petersen, 2010; de Jong et al., 2012). Current global production of chemicals and polymers from crude oil resources is estimated to be around $3.30 \cdot 10^{11}$ kg, while the bio-based fraction accounts for $5.00 \cdot 10^{10}$ kg, namely ca. 13% of the total. The strongest growth is expected in the field of bioplastics, especially with regard to poly (lactic acid) (PLA) and polyethylene (PE) (de Jong et al., 2012). In recent years the available technologies and resources allowed the development of a number of marketable synthetic plastics from "green" building blocks: these are reported in Table 2. By definition, the list excludes natural polysaccharides, bacterial polyhydroxyalkanoates and derivatives thereof (e.g. starch blends, cellulose nanomaterials).

Two aspects related to bioplastics are worth to note. Firstly, there is not yet a standard threshold value that fixes the minimum biobased content for a plastic material to be defined biobased. In general, the biobased content of a material or product can be determined as percent of mass weight of the total organic carbon with standard test methods. An example is the ASTM D 6366 from the American Society for Testing and Materials (ASTM), based on the radioactive ^{14}C signature of non-fossil substrates (versus ^{12}C) (Song et al., 2009).

Secondly, biobased plastics are not necessarily biodegradable. Biobased plastics may be either designed to be durable (e.g. polyurethanes for the automotive industry) or biodegradable (e.g. single-use, short-life disposable packaging). Biodegradable plastic (of fossil or bio-based origin) is degraded to fragments by biotic or abiotic factors and then assimilated by naturally occurring microorganisms to yield water, CO_2, CH_4, biomass and inorganic residues in a defined period of time. If a plastic material reflects given characteristics of desintegrability, biodegradability and safety under certain environmental conditions (composting), it is certified as compostable according to official standards, e.g. EN 13432, ASTM D6400, ISO 14855.

Table 1: Bio-based production of chemical building blocks: derivatives, production size and main producers

Building block	Derivatives	Annual bio-based production size $\cdot 10^3$ kg	Main producers
C2			
Ethylene	polyethylene: HDPE, LDPE, LLDPE, PET; polyvinyl chloride (PVC), ethylene oxide	$2.00 \cdot 10^5$	Braskem, DOW/Mitsui, Songyuan Ji´an Biochemical
Ethanol	ethylene; ethylene glycol; propylene	$8.60 \cdot 10^7$	DuPont, POET, Abengoa, ZeaChem, Beta Renewables Inc., Chemtex, Tereos, CropEnergies, Agrana, Pannonia Ethanol, etc.
Glycolic acid	polyglycolate (PGA)	$4.00 \cdot 10^4$	METabolic EXplorer
Ethylene glycol	polyethylene terephthalate (PET)	$2.75 \cdot 10^5$	India Glycols, Greencol Taiwan Corp
Acetic acid	vinyl acetate; acetic anhydride; cellulose acetate; ethyl acetate	$1.40 \cdot 10^6$	Wacker Chemie
C3			
1,2-Propanediol (propylene glycol)	unsaturated polyester resins, applications as coolants, antifreeze, aircraft deicing fluid, moisturizer in cosmetics, food/feed additive	$2.00 \cdot 10^5$	ADM, BASF, Oleon, Global Bio-Chem, METabolic Explorer
1,3-Propanediol	polytrimethylene terephthalate (PTT) polymers	$1.28 \cdot 10^5$	DuPont/Tate & Lyle (JV)
Lactic acid	PLA; ethyl lactate; acrilic acid; pyruvic acid; propylene glycol; 2,3-pentanedione; 1,2 propanediol	$4.72 \cdot 10^5$	NatureWorks, Galactic, Corbion Purac, Cellulac, Henan Jindan, ThyssenKrupp, etc.
Acrylic acid	acrylates, polyacrylates	Pipeline	Cargill, Perstorp, Genomatica, Arkema
Glycerol	3-Hydroxypropionic acid; 1,3 propanediol; glyceric acid; propylene glycol; propanol; glycerates; polyesters; nylons	$1.50 \cdot 10^6$	ADM, Renewable Energy Group Inc., etc.
3-Hydroxypropionic acid	acrylic acid; acrylonitrile; acrylamide; 1,3-propanediol; methyl acrilate; propiolactone; etc.	Pipeline	Cargill
Propylene	polypropilene (PP); propylene oxide; acrylonitrile; acrylic acid; butanol	$3.00 \cdot 10^4$	Braskem/Toyota Tsusho, Mitsubishi Chemical, Mitsuhi Chemicals
Epichlorohydrin	epoxy resins	$2.00 \cdot 10^5$	Solvay, DOW
n-Propanol	Propylene	Pipeline	Braskem
Ethyl lactate	industrial solvents	$1.20 \cdot 10^6$	ADM, Galactic, Cargill Dow, Vertec Biosolvents
Isopropanol	Propylene	Pipeline	Genomatica, Mitsuhi Chemicals

Building block	Derivatives	Annual bio-based production size $\cdot 10^3$ kg	Main producers
C4			
1,4 Butanediol	γ-butyrolactone (GBL); tetrahydrofuran (THF); polybutilene terephthalate; N-methylpyrrolidone (NMP); N-ethylpyrrolidone (NEP); 2-pyrrolidone; polyurethanes; polyesters; polycarbonates	$3.00 \cdot 10^3$	Genomatica/M&G, Genomatica/Mitsubishi, Genomatica/Tate & Lyle
n-Butanol	propylene, polypropylene, iso-butene	$5.90 \cdot 10^5$	Kathai Industrial Biotech, Butamax, Butalco, Cobalt/Rhodia
iso-Butanol	propylene, polypropylene	$1.05 \cdot 10^5$	Butamax, Gevo
iso-Butene		Pipeline	Gevo/Lanxess
Methyl methacrylate	polymethyl methacrylate	Pipeline	Lucive/Mitsubishi Rayon, Evonik/Arkema
Succinic acid	1,4 Butanediol; tetrahydrofuran; 2-pyrrolidone; γ-butyrolactone; n-Methyl-2-Pyrrolidone	$5.50 \cdot 10^4$	BioAmber (DNP Green Technology/ARD/Mitsui & Co.), Myriant, Succinity (BASF SE/Corbion Purac), Reverdia (DSM/Roquette), PTT Chem/Mitsubishi CC
C5			
Furfural	furfuryl alcohol, tetrahydrofurfuryl alcohol, tetrahydrofuran, 2-methyltetrahydrofuran, furan, methylfuran, acetylfuran, furfurylamine, etc.	$3.00 \cdot 10^5$	Xing Tai Chunlei Furfural Alcohol Ltd, Tieling North Furfural Group Co., Central Romana Corporation, Illovo Sugar, etc.
Itaconic acid	3-methyl THF; 3- and 4-methyl-γ-butirolactone; 2-methyl-1,4-butanediol; 2-methyl-1,4-butanediamine; 3-methylpyrrolidine; itaconic diamide	$4.10 \cdot 10^4$	Qingdao Kehai Biochemistry Co, Itaconix
Xylitol	xylaric acid; propylene glycol; ethylene glycol; glycerol; lactic acid; hydroxyfurans	$1.60 \cdot 10^5$	Danisco/Lenzig, Xylitol Canada
Isoprene	polyisoprene rubber; styrenic thermoplastic elastomer block copolymers; butyl rubber	Pipeline	Goodyear/Genencor, GlycosBio
Glutamic acid	glutaminol; normoline; 5-amino-1-butanol; 1-5 pentandiol; glucaric acid; proline; prolinol; etc.	$2.50 \cdot 10^6$	Global Biotech, Meihua, Fufeng, Juhua
Levulinic acid	2-methyl-THF; γ-valerolactone; 1-4 pentanediol; diphenolic acid	Pipeline	Maine BioProducts,Avantium, Segetis, Circa Group

Building block	Derivatives	Annual bio-based production size $\cdot 10^3$ kg	Main producers
C6			
Sorbitol	isosorbide; ethylene glycol; propylene glycol; glycerol; 1,4 sorbitan; 2,5-anhydrosugars; lactic acid	$1.64 \cdot 10^5$	Roquette, ADM
Adipic acid	polyamides (Nylon-6,6)	$8.00 \cdot 10^1$	Verdezyne, Rennovia, Bioamber/CELEXION, Genomatica
Lysine	caprolactam; diamino alcohols; 1,5-diaminopentane	$1.50 \cdot 10^6$	Global Biotech, Ajinomoto, Evonik/RusBiotech, BBCA, Draths,
2,5 Furan dicarboxylic acid (FDCA)	2,5-dihydroxymethyltetrahydrofuran; 2,5-dihydroxymethylfuran; 2,5-bis(aminomethyl)tetrahydrofuran; succinic acid	Pipeline	Avantium
Isosorbide	polyesters, polycarbonates	$5.00 \cdot 10^3$	Roquette
Glucaric acid	glucarodilactone; glucaro-γ-lactone; glucaro-δ-lactone; polyhydroxypolyamides; esters; α-ketoglucarates	Pipeline	Rivertop Renewables
Citric acid	applications as food additive	$1.60 \cdot 10^6$	Cargill, DSM, BBCA, Ensign, TTCA, RZBC
Caprolactam	polyamides (Nylon-6)	Pipeline	Genomatica, BioAmber
Cn			
Para-xylene	terephthalic acid, dimethyl terephthalate for PET	Pipeline	Gevo Inc., Micromidas, Virent, Anellotech, Biochemtex S.p.A.

Table 2: Synthetic plastics from "green" building blocks

Polyesters	Polyamides	Other	Vinyl Polymers	Synthetic rubbers
Poly (ethylene terephthalate) (PET)	Polyamide 6,6	Polyurethanes	Polyethylene (PE)	Polyisobutylene
Poly (trimethylene terephthalate) (PPT)	Polyamide 11	Epoxy resins	Polypropylene (PP)	Polybutadiene
Poly (butylene furandicarboxylate) (PBF)	Polyamide 6		Poly (vinyl chloride) (PVC)	
Poly (lactic acid) (PLA)	Polyamide 4,6		Polystyrene (PS)	
Poly (butylene terephthalate) (PBT)	Polyamide 6,10		Poly (vinyl acetate) (PVA)	
Poly (butylene succinate) (PBS)	Polyamide 10,10		Poly (methyl methacrylate) (PMMA)	
Poly (ethylene furandicarboxylate) (PEF)	Polyamide 4,10		Poly (acrylic acid) (PAA)	

2.2. Biorefineries

In analogy to crude oil refineries, the aim of bio-based refineries is to process abundant biomass resources in a sustainable and efficient way to provide a wide range of intermediates and marketable final products, including transportation fuels and direct energy. The fraction of biomass that is globally harvested and used for food and non-food applications (3% of total on earth) consists of 75% carbohydrates, 20% lignin and 5% oil, fat, proteins and terpenes on a dry basis (Sheldon, 2014). Biomass also contains small amounts of minerals. Within the carbohydrate portion it is possible to distinguish between structural polysaccharides (i.e. cellulose, hemicellulose, chitin) and storage carbohydrates (i.e. starch, inulin, sucrose). Lignocellulose, that constitutes the plant cell wall, is particularly abundant but requires tailored thermochemical or enzymatic hydrolytic treatments to be further processed (Sheldon, 2014).

Figure 1: Raw material for biorefineries (de Jong et al., 2012)

Photos: https://upload.wikimedia.org http://www.terrain.org; http://www.usnews.com.

A sustainable and cost-effective provision of raw materials can be obtained through plant cultivation, forestry, animal production and algaculture, as well as from biogenic residues and waste materials. For the commercial operation of a biorefinery it is crucial to procure raw materials in suitable and consistent amount and quality all over the year. Moreover, manufacturing sites should develop in locations with high biomass availability or at a close distance to them, in order to minimize transportation costs. An overview of the most common raw materials is given in Figure 1. Compared to agricultural biomass, the use of algal

biomass is still limited. Figures from 2010 report an overall dry algal biomass production of $3 \cdot 10^6$ kg, mainly focused on the extraction of glycerol, fatty acids and other lipophilic ingredients such as carotenoids (Wijffels & Barbosa, 2010). Further research on algal production and utilization is required for the development of technologically and economically efficient processes (Wagemann et al., 2012).

After collection, storage and transport, raw materials are ready for utilization. This articulates into primary and secondary refining. In primary refining, biomass undergoes preconditioning and pretreatment steps and it is then separated into its main raw components (cellulose, lignin, vegetable oil, sugar, starch etc.) that also identify the biorefinery platforms (Figure 2). In secondary refining, biomass components from the different platforms are further processed to intermediates (including building blocks) and finally converted to finished (added-value) products that enter the market. Along the biorefining value chain several processes are typically used for the pretreatment and conversion of biomass, as shown in Figure 3.

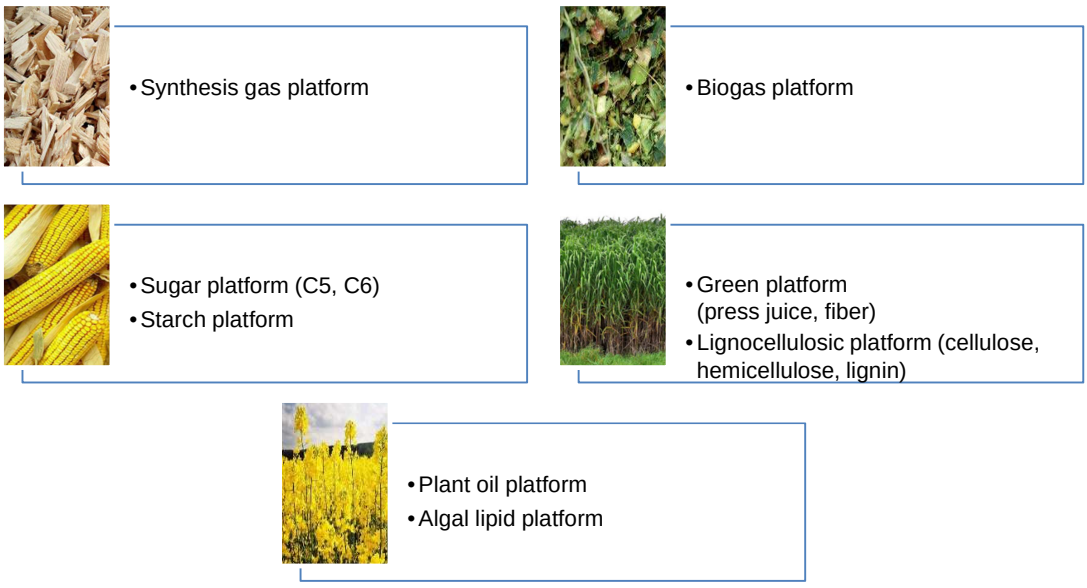

Figure 2: Five most promising biorefinery concepts and their platforms (Wagemann et al., 2012).

Photos: http://www.aet-biomass.com; http://southeastfarmpress.com; http://c.miragro.com

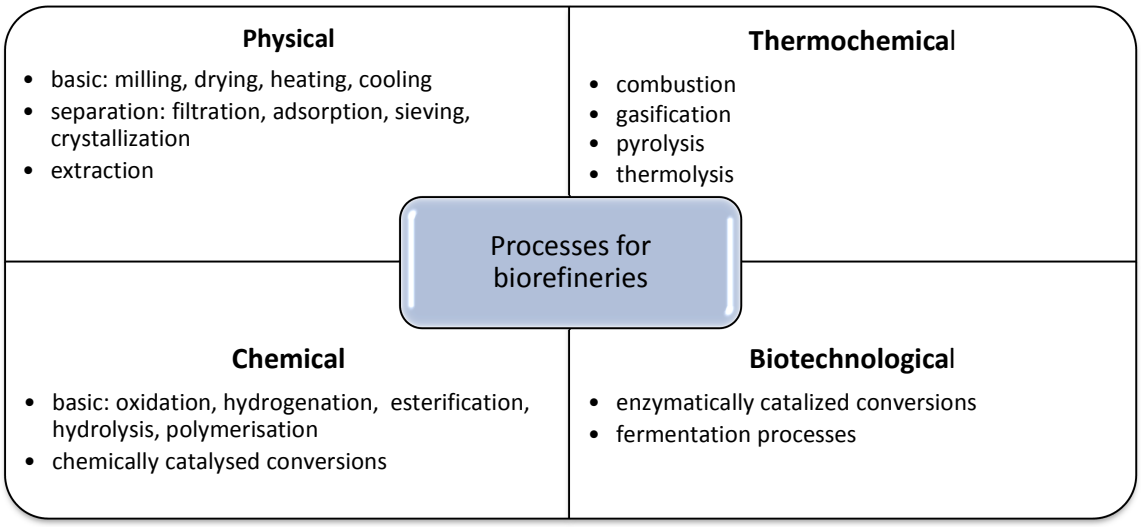

Figure 3: Processes used in the pretreatment and conversion of biomasses within biorefineries (de Jong et al., 2012)

Some aspects contribute significantly to sustainability and economical benefits and must therefore be taken in high consideration:

- Full utilization of all biomass components
- Flexible use of raw materials for primary refining
- Parallel and linked processing of raw materials
- Linking of primary and secondary refining
- Integration of biorefineries into existing value chains and infrastructures

Evidence has shown that biorefineries are often either material-driven – aiming primarily to the production of lower volumes of highly added value chemicals and materials – or energy-driven – oriented to the production of huge volumes of one or more bioenergy carriers: fuels, electricity and/or heat (Bell et al., 2014). In both cases, residues and co-products from processing are also used for food and feed purposes. The integrated processing of biomass for both energy and material purposes, together with a multifunctional approach combining product and processing lines, offers instead major advantages, also of a synergistic nature. Benefits include: reduction of production costs through common process operations, materials and equipment, joint water-, wastewater- and waste-management, use of economies of scale.

All these factors positively impact on the overall profitability and productivity of biorefineries and thus contribute to make them competitive on the market and attractive for investors (Wagemann et al., 2012).

2.2.1. Examples of biorefineries

Two examples of biorefinery facilities are presented and discussed below. The first one reflects particularly well the aim of this dissertation, describing a demonstration plant for the production of lactic acid from different residual biomasses. The second one is an Austrian example of a successful commercial-scale biorefinery plant that uses several raw materials and adequate recovering systems to obtain diverse outputs, including ethanol.

Production of lactic acid from multiple second generation raw material

Goal: Develop a process technology to improve the profitability of existing bioethanol plants by maximizing energy efficiency and product recovery, while minimizing water consumption and waste. The demonstration plant in Dundalk (Ireland) should prove whether the developed technology leads to a feasible and profitable lactic acid production from residual biomasses (mainly DGGS). Sixty people are foreseen to run the plant at a scale of 10^9 kg.

Type of biorefinery: C5, C6 and lignin for the production of biochemicals.

Feedstock: Lactose whey permeate, wheat straw, brewers´ spent grains from beer production and distillers grains from ethanol production.

Output: Optically pure L- and D-lactic acid, ethyl lactate and sodium lactate.

Advantages: Maximization of energy efficiency and product recovery; full utilization of raw materials; reduction of chemicals and water consumption; minimization of waste.

Cellulac (Ireland - UK), a small company founded in 2009, claims the development of proprietary technology for the production of optically pure lactic acid, ethyl lactate and sodium lactate from multiple second generation feedstock (Figure 4). The stated aim is to valorize locally available raw materials and increase profitability of already existing fermentation plants, in good agreement with the scope of this dissertation. The company addresses for instance the inefficiency of ethanol production plants. In fact, these suffer from poor carbon yield of ethanol fermentation (in contrast to lactic acid) due to CO_2 formation and high energy costs connected to the drying of distillers grains recovered after ethanol fermentation and that are sold as DDGS (distillers dry grain with solubles), like in the next example. The idea within this project is to use distillers wet grains (together with other locally available agro and industrial food residues) as feedstock for sodium lactate production and

recover CO_2. The lignocellulosic raw material is pretreated using a cavitation technology that also takes advantage of the recovered CO_2 to open up the lignocellulosic structure, which is subsequently treated with enzymatic cocktails. Lactic acid fermentation is done with non-GMO *Lactobacillus* species using NaOH as a neutralizing agent, like in the studies presented in this framework. Cellulac claims that both C5 (arabinose and xylose) and C6 sugars and even disaccharides are efficiently converted (> 95%) to optically pure L- or D-lactic acid. In the end, ethanol, CO_2 and sodium lactate are combined in a single step process to produce ethyl lactate. Alternatively, high purity lactic acid can be further polymerized to polylactic acid. The company expects the abovementioned pretreatment and downstream processing technologies to drive 40% cost savings over conventional processes. If this process turns out to be effectively practicable and profitable – which has still to be demonstrated –, this would represent a consistent result towards a more sustainable industrial production of lactic acid.

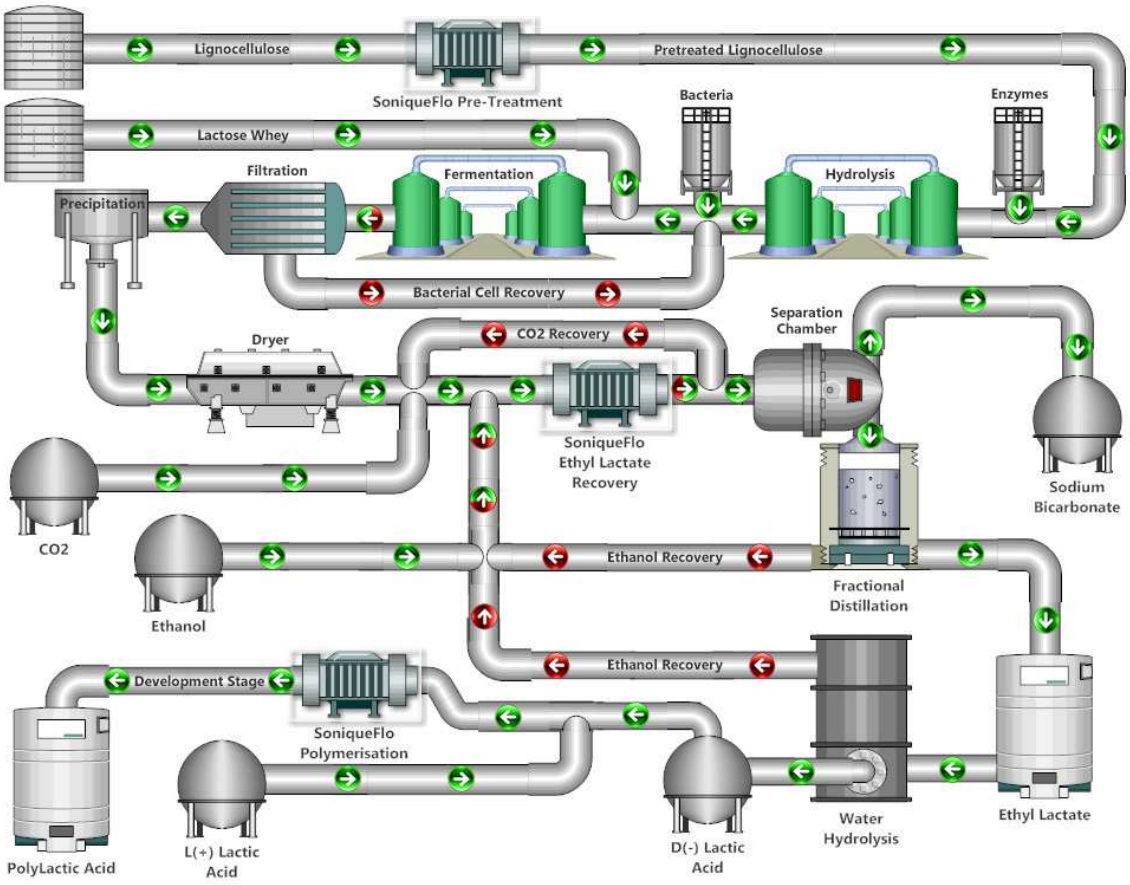

Figure 4: Production of ethyl lactate, L- and D-lactic acid and polylactic acid from lignocellulosic raw materials and lactose whey.

Photo: http://cellulac.co.uk

Bioethanol Plant Pischelsdorf (Austria)

Goal: Produce and sell ethanol, wheat starch, gluten, and related co-products. The integrated wheat starch and bioethanol facility in Pischelsdorf (Lower Austria) is a commercial factory that employs 80 people.

Type of biorefinery: C5 and C6 sugars, starch.

Feedstock: corn and wheat (up to $5.0 \cdot 10^5$ kg/year), concentrated sugar beet juice

Output: Ethanol ($2.4 \cdot 10^5$ m^2/year), wheat starch (10^8 kg/year), wheat gluten ($2.4 \cdot 10^7$ kg/year), DDGS sold as animal feed under the commercial name Actiprot ($1.8 \cdot 10^8$ kg/year) and CO_2 (10^8 kg/year).

Advantages: Full utilization of raw materials and valorization of co-products from processing. Increased carbon yield of ethanol fermentation through the recovery of CO_2.

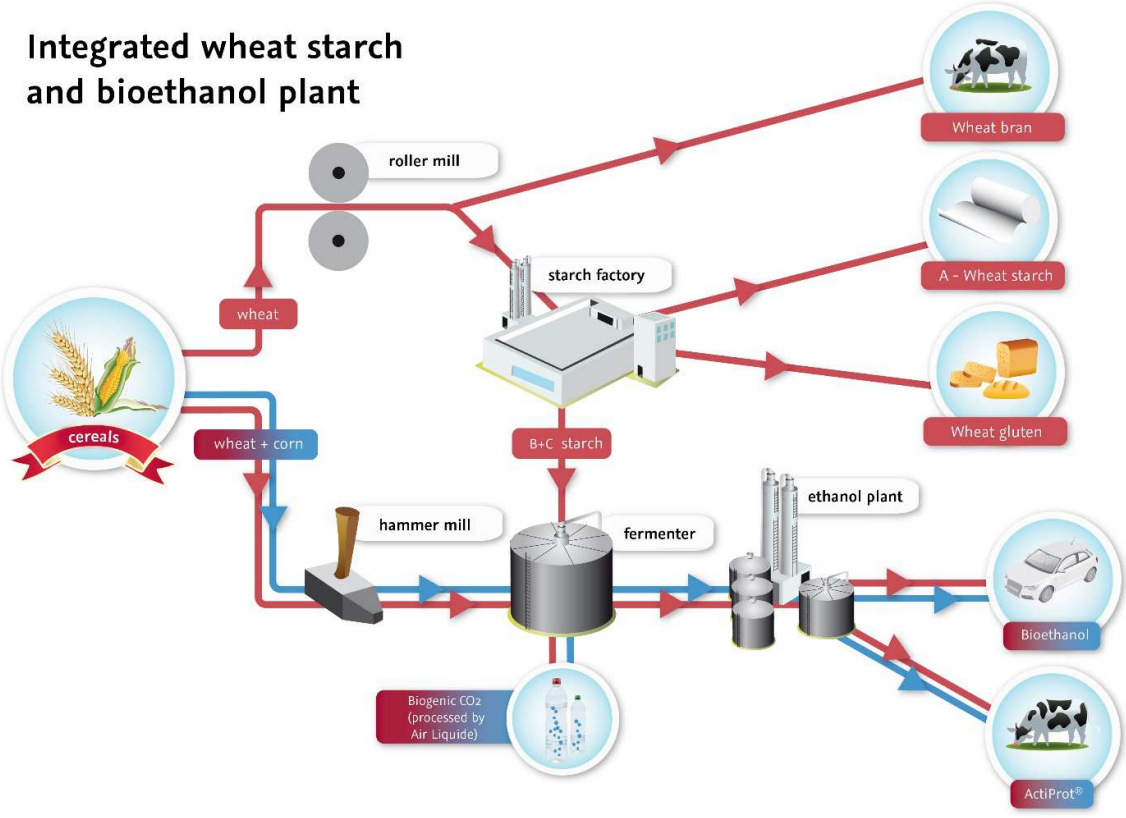

Figure 5: Full utilization of wheat and corn as raw materials for the integrated production of wheat starch, wheat gluten, bioethanol, animal feed and high-purity CO_2.

Photo: http://www.agrana.com

The bioethanol plant in Pischelsdorf (Agrana Stärke GmbH, Austria) is active since 2008 and has been using since then wheat and corn as raw materials. In 2013 the production has been extended to wheat starch and gluten. The process articulates in two production lines (Figure 5): one aims to deliver wheat starch, gluten and wheat bran, while the second one operates in parallel to produce bioethanol from corn and wheat together with residues from the first line. Residual DDGS after ethanol production is then sold as protein-rich animal feed, as anticipated in the previous example. In addition, the partner company Airliquide recovers pure CO_2 from ethanol fermentation (Agrana, 2016). Recovery of CO_2 is a great achievement in the overall process economy, since it boosts the intrinsically impaired carbon yield (50%) of biomass to ethanol (Porro et al., 2014). As seen in the previous example, this process set-up may still be susceptible of improvements, in that more valuable products than DDGS can be obtained, for instance proteins for human nutrition and other biochemicals like lactic acid and derivatives.

Nevertheless, this example of a commercial bioethanol plant illustrates well how the profitability of bio-based chemicals and fuels is strongly dependent on the full utilization of raw materials and valorization of co-products from processing.

2.3. Valorization of residues from the food supply chain

The use of first generation biomass feedstock (e.g. corn, sugarcane and edible oil seeds) is no longer perceived as a sustainable option for the production of bio-based chemicals, materials and fuels since it competes with food production. Alternatively, second and third generation products can be derived from fast growing, inedible, dedicated crops or residues thereof. Considerable attention is gained especially by residues from the food supply chain that are generated during agricultural production and industrial processing as well as at a retail and consumer level (Gustavsson et al., 2011; Luque & Clark, 2013). Studies conducted by the FAO estimate that food losses amount to $1.8 \cdot 10^{12}$ kg per year, namely one third of the entire food production (Gustavsson et al., 2011). Residues originate at various levels of the food value chain (Figure 6).

While awareness has to be raised among retailers and consumers to avoid food losses at the last two stages, it has to be accepted that the accumulation of residues along the whole chain is unavoidable to a certain extent. Therefore, there is a high interest in using food value chain residues in the most beneficial and efficient way.

The valorization of residues represents also a great opportunity to improve waste management systems. For instance, the load of waste in landfills will be reduced and also accidental organic spillages and uncontrolled greenhouse emissions will be avoided.

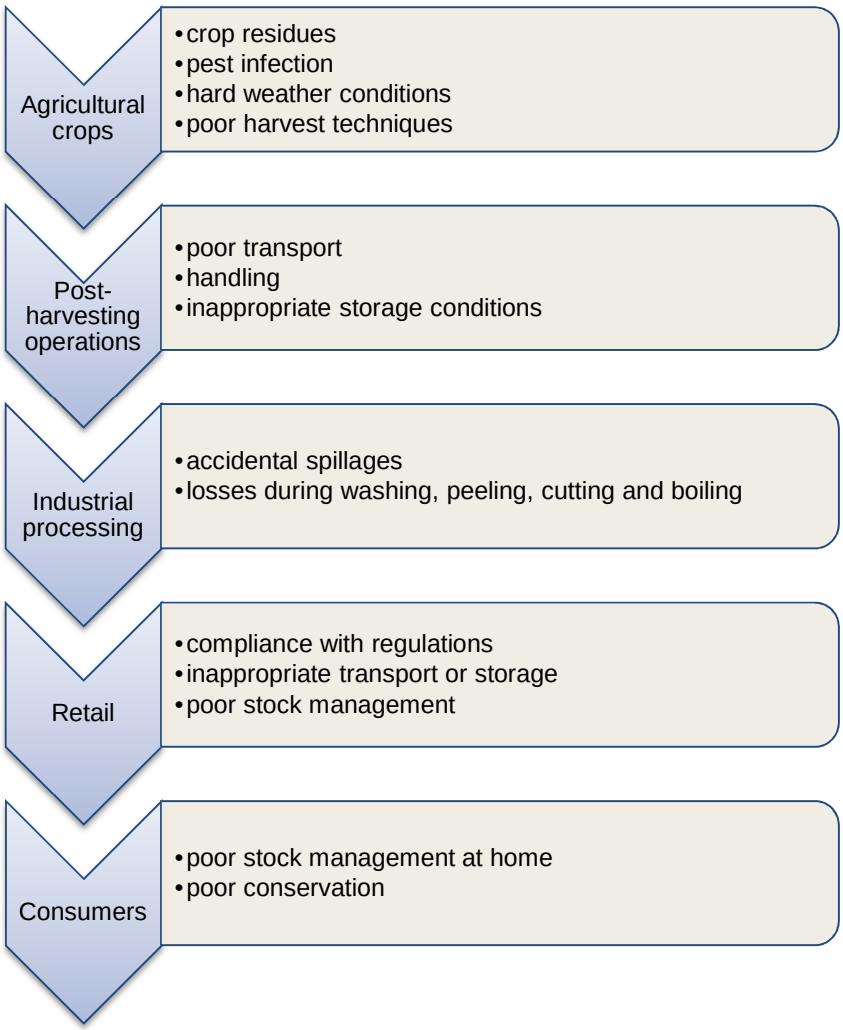

Figure 6: Origin of residues at various levels of the food value chain

From a logistic point of view, it is easier to collect and valorize residues from agricultural and industrial processing as opposed to domestic wastes, since the former is generated in a more concentrated manner. Some challenges, however, are connected with the valorization of such residues, for instance:

- Varying pH and chemical composition due to:
    - seasonal variations
    - different processing techniques
    - storage conditions
- Tendency to bacterial contamination and spoilage
- Transport costs due to high water content.

Conventional practices for the valorization of food value chain residues include – among others – anaerobic digestion, composting, animal feed and incineration. However, these are not always applicable or convenient. For instance, incineration for energy recovery is not economically reasonable in case of wet residues. Similarly, bacterial contaminations and/or poor nutritive value exclude feed applications from the possible options (Russ & Meyer-Pittroff, 2004). More value can be recovered by using the characteristic complex chemical moieties of these substrates for large scale production of green chemical building blocks (Kiran et al., 2015; Lin et al., 2013).

3. Industrial production of lactic acid

3.1. Chemical properties, market and applications

Lactic acid is a α-hydroxy carboxylic acid and exists in two chiral forms: L- and D-lactic acid. The double functionality gives the molecule great chemical reaction properties, making lactic acid one of the top bio-based chemical building blocks (Becker et al., 2015).

Lactic acid has a solid established market of $4.00 \cdot 10^8$ kg in 2013 (Biddy et al., 2016; Harmsen et al., 2014). Prices vary depending on the purity and typically range from 1.30 to 2.30 USD per kg (Biddy et al., 2016). Generally, they also follow the price of commodity starch and sugar raw materials. Lactic acid finds its main application as a precursor of PLA (or polylactide), whose demand in 2013 was $3.26 \cdot 10^8$ kg. PLA is a versatile compostable thermoplastic polyester with a broad range of applications: from the textile industry (fibers), over packaging (e.g. for vegetables and fruits in supermarkets) and consumables (e.g. cups and wares) to the automotive sector. Therefore, PLA has the potential to replace petroleum based plastics such as PET and polystyrene. Moreover, PLA is widely used as a biocompatible material in the medical sector for orthopedic implants, resorbable bone grafting, sutures and controlled drug release (Castillo Martinez et al., 2013; Corbion, 2017; Litchfield & Schaechter, 2009; Makadia & Siegel, 2011; Narayanan et al., 2004).

PLA is obtained either by direct polycondensation of the monomer lactic acid or, more conveniently, by ring opening polymerization of the cyclic lactide dimer. Degradability and physical properties of PLA can be modulated by varying its molecular weight and composition (Narayanan et al., 2004). The stereoisomerism of PLA affects its physico-chemical and mechanical properties, thus the importance of having chirally-pure LA (>99%) as a starting monomer (Miller et al., 2011; Reddy et al., 2013).

Besides the biopolymer market, LA and its salts are used in numerous other applications. In the food industry, which represents the second largest market, food grade L-LA is used as a mild acidulant (e.g. in beverages, meat, candies, sauces), as a preservative against bacterial spoilage, as a flavoring agent (lactate esters) and as an emulsifying agent in bakery goods (lactylated fatty acid esters) (Datta & Henry, 2006; John et al., 2007). Non-food applications of food and technical grade LA are diverse and include leather tanning, textile finishing, cosmetic products (moisturizing and anti-acne lotions, oral hygiene products), pharmaceutical products (e.g. sodium lactate in dialysis and parenteral solutions, calcium lactate in the treatment of calcium deficiency), adhesive formulations and lithographic printing. Lactate esters and especially ethyl lactate are used as “green” solvents in several sectors, comprising also precision metal cleaning for electronics (Litchfield & Schaechter, 2009; Narayanan et al., 2004).

3.2. Fermentative versus chemical routes

Lactic acid can be produced either via fermentation or via chemical synthesis. However, chemocatalytic routes always generate racemic mixtures, independent of the process. The commercial synthetic process is based on the catalytic reaction of acetaldehyde with hydrogen cyanide to give lactonitrile, which is then hydrolyzed to lactic acid (Narayanan et al., 2004). There are also research activities to develop innovative homogeneous and heterogeneous catalytic routes to produce lactic acid from renewable compounds like glycerol and sugars (Dusselier et al., 2013). Anyway, since chemical routes have not been economically feasible so far (Gao et al., 2011), more than 90% of the current commercial production is fermentative (Dusselier et al., 2013; Hofvendahl & Hahn-Hagerdal, 2000). Fermentative pathways exist since decades, but have been extensively researched and improved in the last years to contribute to and support the market expansion of PLA and ethyl lactate. The great advantage of biochemical processes over chemical syntheses is that suitable microbial strains enable the production of optically pure lactic acid (more than 99% optical purity) and also the fact that cheap raw materials like sugarcane molasses, whey permeate and other agro-industrial residues can be used (Assavasirijinda et al., 2016; Jiang et al., 2013; Kuo et al., 2015; Sun et al., 2015; Wang et al., 2013).

3.3. Microbiology of lactic acid fermentations

3.3.1. Lactic acid bacteria

Lactic acid bacteria produce lactic acid as the main or only product of fermentation from sugars. They include about 20 genera of the phylum *Firmicutes*: among others *Lactococcus, Streptococcus, Enterococcus, Pediococcus, Leuconostoc,* and *Lactobacillus* (Reddy et al., 2008).

Most lactic acid bacteria are aereotolerant anaerobes: they are protected against toxic forms of oxygen by-products (e.g. H_2O_2) by peroxidases, superoxidase dismutases and NADH oxidases. Lactic acid bacteria lack catalases, do not sporulate and can tolerate pH lower than 5, which gives them an important advantage over others. The optimum growth temperature ranges from 20 to 45 ℃ (Hofvendahl & H ahn-Hagerdal, 2000). Even if some properties may be affected by specific environmental conditions, they are distinguished by their Gram-positive cell wall characteristics and the inability to synthetize porphyrin groups and the resulting incapability to produce cytochromes, unless heme is provided (Reddy et al., 2008). Since most of them obviously do not carry out phosphorylative oxidation, lactic acid bacteria only obtain energy from the fermentation of sugars, which therefore have to be abundantly available. Distribution of ^{14}C-labeled substrates indicate that only 5% of the glucose carbon consumed is converted into biomass, while the rest is converted into

metabolic end products (Moat et al., 2003). Due to their limited biosynthetic capabilities, lactic acid bacteria generally need to introduce amino acids, vitamins especially of the B group, nucleotide bases and minerals from external sources (Madigan et al., 2015). Therefore, these compounds have to be supplied in industrial processes for lactic acid production in order to promote the synthesis of cellular biomass and/or activate enzymatic reactions.

From a metabolic point of view, homofermentative lactic acid bacteria can produce exclusively lactic acid from glucose and are for this reason more interesting for industrial processes. From 1 mol glucose they generate 2 mol lactic acid and a net yield of 2 mol ATP, following the Embden-Meyerhof-Parnas (EMP) pathway, also known as glycolysis.

Heterofermentative bacteria instead lack aldolase, a key enzyme in EMP, and therefore divert the carbon flow through the phosphoketolase pathway. In this way, they gain equimolar amounts (1 mol) of lactic acid, ethanol or acetic acid, and CO_2 per mol of glucose. The ratio of ethanol and acetic acid depends on the redox potential of the system: anaerobic conditions favor the formation of ethanol. Since in this pathway only 1 ATP is formed, energy yields and growth rates are lower. Facultative heterofermentative lactic acid bacteria, e.g. *L. casei*, possess the enzyme transketolase and can utilize this pathway under aerobic conditions or for the degradation of pentoses (Litchfield & Schaechter, 2009). A more efficient way to convert pentoses to lactic acid is through the pentose phosphate pathway, since only lactic acid and no acetic acid is formed.

At altered conditions, homofermentative bacteria can show a mix acid fermentation. In this case, after pyruvate formation the EMP pathway is diverted to the formation of formate, acetate, ethanol and CO_2 besides lactate. Moreover, in presence of oxygen, CO_2 rather than formate is produced. This phenomenon is concomitant of environmental alterations like for example: glucose limitation, growth on sugars different from glucose (e.g. maltose, lactose, galactose), alkaline pH values or temperature below the optimum (Hofvendahl & Hahn-Hagerdal, 2000; Moat et al., 2003).

Finally, some lactic acid bacteria possess amylolytic enzymes that enable the direct conversion of starch to lactic acid. Examples thereof are strains of *Lactobacillus plantarum, L. manihotivorans, L. fermentum, L. amylophilus, L. amylovorous, Streptococcus bovis* (Altaf et al., 2006; Litchfield & Schaechter, 2009; Naveena et al., 2005; Panda & Ray, 2008; Reddy et al., 2008; Vishnu et al., 2002).

3.3.2. Other microorganisms producing lactic acid

Apart from lactic acid bacteria, the biotechnological production of lactic acid can be based on a variety of other microorganisms, often already in use in industrial processes:

- Other gram-positive bacteria, especially *Bacillus* spp. (e.g. *Bacillus coagulans*)
- Filamentous fungi e.g. *Rhizopus* spp.
- Metabolically engineered yeasts

The advantage of the above-mentioned microorganisms over lactic acid bacteria is that they – in most cases – do not have complex nutritional requirements. Hence, lactic acid fermentations can be conducted on synthetic media, rendering the process of recovery and purification of lactic acid simpler than in presence of complex organic nitrogen sources.

Moreover, filamentous fungi and yeasts can tolerate acidic pH values, which allows to reduce costs for chemicals used for pH-regulation during fermentation and also to limit the production of waste streams (gypsum), possibly generated in the recovery of lactic acid (see paragraph 3.4.3). *Rhizopus* spp. are particularly interesting for industrial purposes, since they natively produce only the L- chiral form of lactic acid and *R. oryzae* in particular can also directly utilize starch as a substrate for lactic acid production (Huang et al., 2005; Koutinas et al., 2007; Park et al., 2004). However, *R. oryzae* strains suffer from a number of limitations. Undesired by-products like ethanol, fumaric acid and glycerol can lower yields in lactic acid and pH values must be kept anyway above 4.5 for efficient lactic acid production. In contrast to bacterial strains, *Rhizopus* spp. require aeration and it has been observed that the production of lactic acid is enhanced at higher oxygen transfer rates (Miller et al., 2011). The filamentous nature of the fungi, however, hinders mass transfer and also creates mixing issues.

Yeasts do not naturally produce lactic acid or at least not in relevant amounts, but can survive at pH values as low as 1.5, ferment a variety of sugars and grow on simple chemically defined media. Genetic modifications of the genera *Saccharomyces, Pichia, Klyveromyces, Candida*, etc. enabled the development of strains that produce lactic acid as primary fermentation product instead of ethanol (Branduardi et al., 2006; Ishida et al., 2006; Sauer et al., 2010; Turner et al., 2015). Cargill, one of the main producers of lactic acid for the PLA market, developed and implemented an engineered yeast strain for the commercial production of lactic acid from glucose at pH lower than three (Miller et al., 2011).

3.4. Conventional fermentative production of lactic acid

3.4.1. Substrate

Conventional commercial processes for the production of lactic acid are based on the use of glucose or sucrose as a carbon source. Depending on the geographical availability and based on economic considerations, the preferred raw material in Europe and South America is sucrose from sugar beet or sugarcane, while in North America glucose is more likely obtained from the hydrolysis of corn starch. The latter is carried out with commercial enzyme formulations in two steps. The first one, liquefaction, is industrially conducted at temperatures between 90 and 110 ℃ for a few minutes (5–10 min). During liquefaction α-amylase randomly breaks 1-4 α-bonds in the starch molecule and releases maltodextrins. In the second step, saccharification, glucoamylase is responsible for the release of glucose units. This last step takes place at milder temperatures (typically 60 ℃) and lasts several hours (up to 100). Simultaneous saccharification and fermentation is an option to reduce the total duration and prevent substrate inhibition of the microbial strain (John et al., 2009): as starch is gradually hydrolysed to glucose units, these are converted to lactic acid, avoiding high substrate concentrations at a time.

So-obtained glucose syrups are supplemented with sources of amino acids, proteins and vitamins like corn steep liquor from the corn wet milling process, protein hydrolysates, yeast extract or a mixture thereof. Initial C-source concentrations in the fermentation medium typically range from 150 to 200 g/L.

The use of microbial strains that can directly convert starch to lactic acid represents an interesting alternative to reduce costs. However, such strains have not been implemented in commercial-scale production so far, since substrate utilization and yields in lactic acid are not yet satisfactory (Litchfield & Schaechter, 2009; Reddy et al., 2008).

3.4.2. Commercial considerations

Important criteria for selecting microbial strains for commercial production have been summarized and discussed by several authors (Abdel-Rahman et al., 2013; Litchfield & Schaechter, 2009; Sauer et al., 2008) and include:

- Lactic acid yield higher than 90%. This also implies that no co-metabolites should be produced.
- Final lactic acid concentration higher than 100 g/L and thus, tolerance to high product concentrations, which otherwise inhibit growth and further lactic acid production. Low product concentrations increase water removal costs during downstream processing.

- Productivity: generally 2–6 g/L h
- Stereospecificity: ideally the microbial strain produces exclusively L- or D- lactic acid. Typical stereochemical purities for commercial 88% lactic acid formulations (i.e. after purification) are min 97%. For PLA applications this value increases to min. 99%.
- Tolerance to acidic pH values: for the purification of lactic acid it is desirable to run the fermentation process at pH values below the pK_a of 3.86, where lactic acid is mainly present in its undissociated form, rather than as lactate salt.
- Tolerance to undissociated lactic acid: in this form lactic acid can pass through the lipidic membrane and enter the cell, where it has generally a toxic effect. In the ionized (dissociated) form lactic acid cannot enter the cell.
- Temperature optimum: strains that have their temperature optimum above 45 ℃ are less subjected to contamination and the process for the production of lactic acid is therefore more stable (autoselective process).
- Strain performance can be further improved through acclimatization, mutagenesis and genetic engineering and process optimization approaches (Miller et al., 2011).

3.4.3. Process set-up and control

Lactic acid fermentations can be run in different modes. Batch fermentations with pH-control are easy to operate and bring to high product concentrations, reportedly up to 192 g/L (Moon et al., 2012). However, productivities are generally below 4 g/L h (Jiang et al., 2013) and microbial strains often suffer from product and/or substrate inhibition (Abdel-Rahman et al., 2013; Li et al., 2012). The last effect can be mitigated operating in fed-batch mode, even if process complexity increases with regards to the feeding method (continuous, exponential, pulse, etc.). In this case, even higher product concentration up to 226 g/L can be reached (Wang et al., 2011), while reported productivities are below 5 g/L h (Ge et al., 2010). Volumetric productivities can be highly increased in continuous fermentations, since in this case end-product inhibition is avoided. However, due to low product concentrations, low cell densities and unconverted sugars, more complex set-ups than the classical chemostat are required. Possible techniques to mitigate these drawbacks are cell immobilization and membrane cell recycling. However, in most studies reported lactic acid concentrations are still below 60 g/L, making continuous fermentation unfavorable for commercial processes (Abdel-Rahman et al., 2013).

Independently on the technical set-up, pH control is a critical factor for maximizing lactic acid concentrations. Generally, $Ca(OH)_2$ (lime) or alternatively NaOH, KOH and NH_4OH are used

to neutralize lactic acid as it is formed. In the former case, however, gypsum ($CaSO_4$) is produced when H_2SO_4 is added to the concentrated broth to obtain free lactic acid during product recovery. The amount of gypsum formed – 1 kg per kg of lactic acid produced (Bozell & Petersen, 2010) – which is merely a waste stream, is obviously dependent on the pH at which the fermentation is run. Employing low pH values, therefore, allows to save chemicals for pH control during fermentation and to reduce the amount of gypsum formed in the recovery steps (Miller et al., 2011). Alternatively to base titration, the pH value can be controlled through continuous product recovery by electrodialysis, adsorption and solvent extraction (Abdel-Rahman et al., 2013; Hofvendahl & Hahn-Hagerdal, 2000).

4. Objectives

Most of this work was conducted within the LEAD-ERA Project CARBIO, with the aim of valorizing residues collected from local food industries in the region of Navarra (Spain). The agro-industrial residues provided by the Spanish company TRASA (Tratamiento Subproductos Agroalimentarios s.l.) were: potatoes, chicory roots and corn cobs. After chemical characterization, these products were evaluated for their suitability as feedstock for lactic acid fermentation. Another part of the work was carried out in collaboration with the Department of Food Science and Technology (BOKU, Vienna) and Christian Doppler Research Laboratory for Innovative Bran Refinery (Vienna). The main aim of this collaboration was to study the fermentability of the residual lignocellulosic fraction of wheat bran after hydrolysis of starch.

Overall, the objectives of this PhD project can be summarized as follows:

Screening of bacterial strains for the production of lactic acid from hexoses, pentoses and starch: yield, productivity, optical purity of lactic acid. Evaluation of microbial growth and production of other metabolites.

Chemical characterization of industrial residues from the food processing: potato residues, chichory roots and corn cobs. Selection of the most convenient substrate for lactic acid fermentation.

Hydrolysis of potato residues: selection of suitable commercial enyzmes and choice of appropriate process parameters for liquefaction and saccharification.

Medium optimization: supplementation of the potato hydrolysate with cheap nutrients that provide organic nitrogen to promote lactic acid production.

Process set-up (500 mL – 3 L) and comparison of different configurations (batch, fed-batch).

Operation in sterile and non-sterile conditions with thermophilic bacteria.

Critical evaluation of the suitability of the developed processes for commercial applications: yield, productivity. Considerations about the potential fields of application of the produced lactic acid after purification.

5. Results and discussion

5.1. Conventional lactic acid fermentation from alternative carbon sources: wheat bran and potato residues

The use of refined sugars as a carbon source for lactic acid fermentation is the major element in the cost of lactic acid fermentation, also because they need more nitrogen supplementation then complex substrates. Even if refined substrates make product purification easier and more economic, overall costs can be significantly reduced by introducing cheaper raw materials (John et al., 2009). These include especially starchy and cellulosic material from agro-industrial residues and food wastes but also lignocellulosic biomass, glycerol and microalgae (Abdel-Rahman et al., 2013; Hofvendahl & Hahn-Hagerdal, 2000; John et al., 2009; Yadav et al., 2011).

Research in this work focused on the evaluation of substrates and processes for the production of lactic acid from residues from the food industry. A number of residues were made available for this purpose: corn cobs, potato residues (peels and puree), chicory roots and wheat bran hydrolysate. These were characterized in terms of water, carbohydrates, starch, lignin, protein and ashes. In particular, wheat bran hydrolysate and potato residues were selected for further investigations. Potato residues were regarded as optimal for the intended purpose due to their high content in starch, which is easily accessible. Wheat bran hydrolysate was considered interesting and challenging as a substrate for its lignocellulosic nature. Both substrates were pretreated (thermochemical extraction and/or enzymatic hydrolysis accordingly) in order to make structural carbohydrates available and accessible for the bacterial strains used in the following fermentation process.

Wheat bran biorefinery – An insight into the process chain for the production of lactic acid

In the first study, the whole wheat bran biorefinery concept has been object of evaluation, in which the author took care especially of the data concerning lactic acid fermentation. Wheat bran is namely an abundant and cheap side product from the milling industry. About 45% w/w of wheat bran is suitable for human nutrition since it consists of starch, proteins and minerals, while the rest is lignocellulose. The biorefinery articulated into three major processing steps. During pre-extraction – consisting of lipid extraction and starch hydrolysis – the main nutritive fractions were separated, recovering 32% of dry mass. This portion was not used for fermentation. The residual cake was then subjected to hydrothermal pretreatment and hydrolysis by an enzyme mix containing cellulases, xylanases and β-glucanases. The so-obtained liquid hydrolysate, with an overall sugar concentration of 21 g/l (glucose, arabinose, xylose), was finally used as a substrate for lactic acid fermentation.

L. pentosus DSM 20314 could grow on the wheat bran hydrolysate without supplementation of additional nutrients or growth factors, and even in presence of low amounts of inhibitors like furfural and acetic acid (ca. 4 g/L in total). Final lactic acid yields were 73% after 52 h of fermentation, with a final lactic acid concentration of 18.6 g/L. Increasing the initial sugar concentration by concentrating the hydrolysate through nanofiltration allowed to reach a lactic acid concentration of 39.2 g/L after 120 h.

The results presented here show that the following targets must be set to improve process convenience: higher productivity, more efficient pentose conversion to lactic acid, elimination of acetic acid as a co-metabolite and higher stereospecificity of the product. Overall, the study offers a valuable insight into the process chain and suggests critical measures for yields to approach the theoretical maximum.

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Wheat bran biorefinery – An insight into the process chain for the production of lactic acid

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HIGHLIGHTS

- A wheat bran biorefinery concept including the major processing steps was studied.
- A pre-extract containing 32% of the total dry mass was obtained.
- Fermentation of lignocellulosic fraction yielded 0.73 g lactic acid/g substrate.
- An overall product efficiency of 47% was achieved experimentally.
- A maximum product yield of 80% could be estimated theoretically.

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ABSTRACT

The present study investigates a wheat bran biorefinery including major processing steps pre-extraction, pre-treatment and lactic acid fermentation. Concerning the dry mass distribution, during the pre-extraction 32% of the feedstock was recovered, offering some perspectives for applications in food area. The pre-treatment (hydrothermal/enzymatic hydrolysis) of the remaining cake solubilized 34% of dry mass and led to a fermentable sugar concentration of 21 g/L. The fermentation resulted in a lactic acid yield of 0.73 g/g substrate. Concentrating the fermentation feed via nanofiltration did not improve the lactic acid productivity. Taking into account that *Lactobacillus pentosus*, a heterofermentative microorganism was used, the dry mass balance revealed a product yield of 47% (32% extract, 15% lactic acid). Based on a theoretical consideration involving a cellulolytic enzyme production (10% feedstock allocation) and lignin utilization, under optimized conditions a maximum product yield of around 80% (35% extract, 39% lactic acid, 6% lignin) could be expected.

1. Introduction

The growing interest in renewable materials as an alternative to fossil resources for the production of commodity chemicals and energy has already been widely acknowledged. In addition to the fact that the total amount of fossil fuel on earth is limited, the urge to replace crude oil also arises from the desire to lessen the dependence on it (Kamble et al., 2013). Over the last decades, the utilization of some commonly used renewable materials, such as corn, sugar beet, wheat and potato as source of glucose for the production of energy commodities and chemicals has been partially industrialized (Gupta and Kumar, 2007; Lipnizki, 2010; Palmarola-Adrados et al., 2005; Vink et al., 2003). However, both the concern on the sustainability of food crops and the necessity for the market competition requiring cheaper raw materials, favor the concept of so-called second generation biomass utilization. Therefore, research and development bodies show an intense interest on lignocellulosic feedstocks as raw materials for future biorefineries (Abdel-Rahman et al., 2011). In this respect, main problems arise from the recalcitrant nature of lignocellulose and from various challenges on developing corresponding technologies for its utilization. For instance, the severe conditions required to disintegrate the lignocellulose, often result in the formation of undesired compounds, such as furfural, hydroxymethylfurfural and levulinic acid. Those compounds do not only affect the

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microbial activity in a subsequent fermentation step, but also refer to a decrease in sugar yields. Additionally, the hydrolysation of hemicellulose, a constituent of the lignocellulose, mainly leads to the formation of pentose sugars, which represents a challenge for an effective fermentative utilization. Consequently, lignocellulosic biorefinery systems aiming at a zero-waste concept face difficulties of minimizing the side-streams.

As far as the feedstock is concerned, wheat bran, the side product of the milling industry, can be suggested as a raw material for a future biorefinery (Favaro et al., 2012; Reisinger et al., 2013; Palmarola-Adrados et al., 2005; Tirpanalan et al., 2014). However, the composition of wheat bran differs from the usual lignocellulosic feedstocks such as wood, straw, corn stover, etc. While around 55% of wheat bran is lignocellulose, the rest can be considered as a resource for human nutrition, as it is mainly composed of starch, protein and minerals. In this relation, a future wheat bran biorefinery involving fermentation routes may follow two main technological pathways. One of them follows a so-called pre-treatment of wheat bran as a whole under relatively severe conditions (e.g., above 180 °C at low pH values), which introduces relatively complex slurries containing sugar monomers and oligomers, proteins and amino acids, minerals, lipids, organic acids and degradation products (Reisinger et al., 2013, 2014). Following this, complicated separation technologies need to be considered for the subsequent processing steps. The second one involves a pre-extraction of solubles under relatively mild conditions and a pre-treatment of the residual wheat bran. By doing so, some main extractables, i.e., along with the starch fraction, water soluble fractions of the proteins, minerals and hemicellulose, can be pre-separated without being exposed to severe conditions. Thus they can be more readily used for food purposes and the aforementioned formation of the complex slurry can be partially circumvented. Additionally, the loss of valuables such as sugars and proteins can be reduced to some extent and the formation of inhibitory substances caused by hexose degradation can be limited (Tirpanalan et al., 2014). In principle, the latter possibility appears superior over the former.

Irrespective of the applied technological strategy, there are various chemicals that can be produced following the biochemical conversion of the disintegrated lignocellulose. Succinic acid, adipic acid, itaconic acid, ethanol, butanol, 3-hydroxypropionic acid or polyhydroxybutyrate represent some of the various possibilities. However, up-to-date it is not easy to specify the building blocks, which can commercially replace the fossil based chemicals. Lactic acid is also a well-known candidate, which has gained an incremental interest as the monomer of polylactic acid.

The present study investigates a future wheat bran biorefinery involving the following major processing steps: pre-extraction, pre-treatment and lactic acid fermentation. Pre-extraction and some pre-treatment methods of wheat bran were investigated in detail in previous studies (Reisinger et al., 2013, 2014; Tirpanalan et al., 2014). In this work the lactic acid fermentation has been combined with pilot scale pre-extraction and pre-treatment to investigate the processing chain. The fermentation was carried out with the hydrolyzed lignocellulose, without any nutritional supplementation. Particular interest was shown in the mass distribution of the raw material within the process flow providing an insight into the overall product efficiency. The process scheme illustrated in Fig. 1 gives an overview of the treatments applied to the previously defatted wheat bran.

2. Methods

2.1. Raw material

Wheat bran used in this study was provided by GoodMills GmbH (Schwechat, Austria). A lipid extraction was performed by employing hexane as organic solvent. The defatted wheat bran contained 90% of dry mass and was composed of 67% carbohydrates in its dry mass. Total carbohydrates (monomeric and polymeric form) comprised 33.4% glucose, 19.0% xylose, 12.3% arabinose and 2.3% galactose. The starch content of the wheat bran corresponded to 10.9% of the bran dry mass. The crude protein, lignin and ash fractions of the bran dry mass were 17.0%, 6.5%, 6.5%, respectively. Additionally, 2% of the bran dry mass consisted of acetic acid.

2.2. Liquefaction and saccharification

An amount of 3000 g of defatted wheat bran was mixed with deionized water (DW) at a ratio of 1:5. Starch degrading enzymes, α-amylase from *Bacillus licheniformis* (500 unit/mg protein) and amyloglucosidase from *Aspergillus niger* (300 unit/mL) were purchased from Sigma–Aldrich Co., Germany. The hydrolysis was carried out in a 20 L double-jacketed pressure-tight agitator vessel heated by thermal oil (Kiloclave type 3, Büchi Glas Uster, Switzerland). Heat stable α-amylase (up to 90 °C) was added to the slurry (2 mL) and the reaction was carried out for 3 h at 85 °C, pH 6.3. After adjusting the pH to 5.5 using H_2SO_4 (2 g/L) and the temperature to 55 °C, amyloglucosidase was added (3 mL) and the slurry was treated for 18 h. The slurry was continuously stirred at 400 rpm throughout the process.

2.3. Solid–liquid separation

Subsequently to the saccharification, the bran–water suspension was separated into its solid and liquid fractions with a manually operated filter press aided with a double cheese cloth. The dry mass content of the solid fraction after pressing was 38%. This solid fraction was washed with a water amount required to wash out 95% of dissolved substances. The washing was mixed with the filtrate and centrifuged for 15 min at an rcf of 7878*g* (6000 rpm) (Sorvall evolution RC, Rotor SLC 6000). The pellet was returned to the solid fraction.

2.4. Hydrothermal and enzymatic treatment

The solid fraction obtained from the above described liquefaction and saccharification steps was subjected to hydrothermal treatment carried out in the 20 L double jacketed pressure-tight agitator reactor (Kiloclave type 3, Büchi Glas Uster, Switzerland). Demineralized water was added to the solid fraction in an amount resulting in a solid-to-water ratio of 1:7. The reactor was sealed, heated to 180 °C and the reaction time was set to 10 min. The slurry in the reactor was continuously stirred at 400 rpm throughout the treatment.

To the hydrothermally treated bran slurry, 18 g of enzyme mix comprising cellulases, β-glucanases, xylanase, Cel-P and X100-P was added. For the purpose, following enzymes were applied: cellulase (0.017 units/mg), endo-1,3(4)-β-glucanase (0.017 units/mg) and endo-1,4-β-xylanase (1 unit/mg) from *Trichoderma longibrachiatum* (Sigma–Aldrich Co., Germany), Tegazyme Cel-P, Tegazyme X100P (enzymes for lignocellulose disintegration) (Tegaferm GmbH, Austria), Bioglucanase (2000 units/mg) from *Trichoderma reesei* and Biocellulase (8750 units/g) from *A. niger* (Kerry Inc., Ireland). The enzyme mix was prepared in high dose to ensure a high degree of carbohydrate hydrolysis. The process was carried out at 45 °C for 41 h under continuous stirring at 400 rpm. At the end of the process the slurry was centrifuged at an rcf of 1969*g* (3000 rpm) for 10 min (Sorvall evolution RC, Rotor SLC 6000) and the pellet was washed with a water amount required to wash out 95% of dissolved substances. The washing was mixed with the supernatant and labeled as wheat bran hydrolysate (WBH).

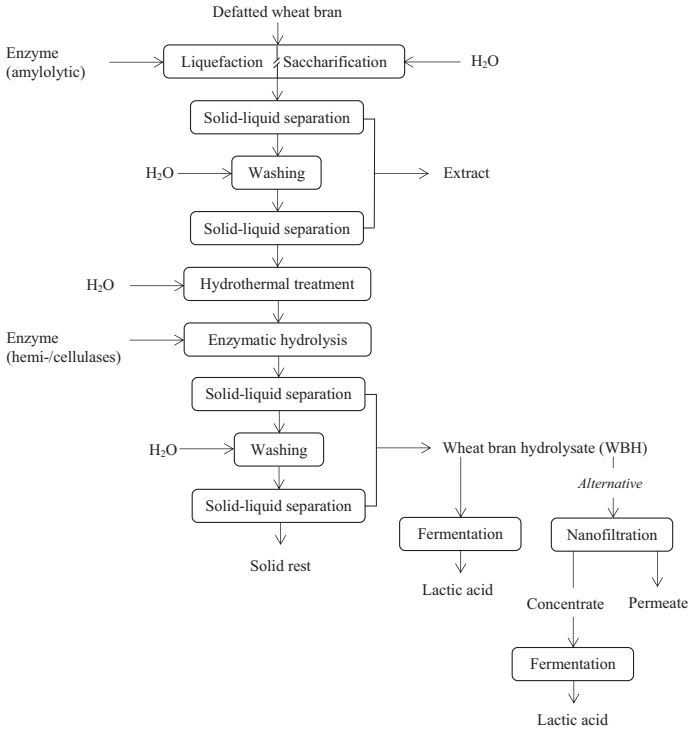

Fig. 1. Flow chart of the featured wheat bran biorefinery consisting of the steps pre-extraction, pre-treatment, lactic acid fermentation.

The remaining solid fraction at the end of the process had a total solid content of 17.6%.

2.5. Membrane filtration

A nanofiltration (NF) process was carried out in order to concentrate the WBH. For this purpose a stirred membrane filtration cell with 2 L hold-up volume and a filtration area of 0.017 m^2 was employed and the frequency of rotation of the magnetic stirrer (Midi MR1 digital, Ika Labortechnik) was set at 500 rpm. The NF was performed with an acid/base stable flat sheet membrane (Sel-RO® MPF-34) having a molecular weight cut off (MWCO) of 200 Da (Koch Membrane Systems Inc., Germany). The NF was performed at a pressure of 3 MPa. Throughout the process the temperature was maintained at 60 °C by using an external water circulating heat exchanger (Julabo-F12, -ED, Germany).

Necessary calculations used in this study regarding the membrane filtration were carried out according to following equations:

$$\text{Retention} = 1 - (cp/cf) \cdot 100 \quad (\%)$$

$$\text{Volumetric concentration factor} = Vf/(Vf - Vp(t)) \quad (\%)$$

where the *cp* and *cf* represent concentrations of compounds of interest in the permeate and in the feed and the *Vp*, *Vf* represent volume of permeate (at time t) and of feed, respectively.

2.6. Fermentation

The inoculated microorganism, *Lactobacillus pentosus* strain DSM 20314, was purchased from the Leibnitz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. Cells were maintained for long term storage at −80 °C in 1 mL aliquots of MRS broth (Sigma Aldrich, Austria) containing 10% v/v glycerol. A pre-culture was prepared by inoculating cells into MRS broth (50 mL) containing glucose, xylose and arabinose in concentrations equal to those found in the WBH. The enrichment was carried out for 15 h at 30 °C, on a continuous shaker (80 rpm). The cell density of the pre-culture reached $2.6 \cdot 10^9$ cells/mL. The fermentation substrates (WBH, concentrated WBH) were first pre-filtered through 110 mm diameter filter paper circles #595 (Schleicher & Schuell GmbH, Dassel, Germany) in order to separate particles and thereafter sterile-filtered through a 45 μm pore sized filter membrane in a 500 mL bottle top filter unit (Semadeni AG, Ostermundigen, Switzerland). Batch fermentations were conducted under anaerobic conditions by continuously stirring (100 rpm) in computer aided parallel bioreactors (1 L reactor volume with 0.7 L max. operation volume) (DASGIP Information and Process Technology GmbH, Jülich, Germany). The pH was maintained at 6.3 by automatized addition of NaOH (10 M) controlled by the system. The initial working volumes were 400 mL, of which 10% (v/v) consisted of the pre-culture.

2.7. Analytical methods

Samples were subjected to analyses of dry matter, ash content, sugar (monomeric and polymeric glucose, xylose, arabinose), degradation products and organic acids (furfural, hydroxymethylfurfural, levulinic acid, lactic acid and acetic acid). The Standard Laboratory Analytical Procedures (LAP) for biomass analysis provided by the National Renewable Energy Laboratory (NREL) were followed. The (Klason-) lignin content of the defatted wheat bran was determined likewise (Sluiter and Sluiter, 2011a,b). The starch

content of the defatted wheat bran was examined using the Megazyme total starch (AA/AMG) enzyme assay kit and according to AOAC 996.11 (AOAC, 2010). The Kjeldahl method was followed for the determination of protein content using the conversion factor of 5.26 (Tkachuk, 1969). Sugars and above mentioned organic acids and degradation products were quantified by means of high performance liquid chromatography (HPLC). A Phenomenex Rezex RPM-Monosaccharide column was employed for the sugar separation, while for the organic acid and degradation product separation a Phenomenex Rezex ROA-Organic Acid column was used. Densities of solutions were measured with a densitometer (Densito 30P, Mettler-Toledo GmbH, Analytical). Dry matter contents of cakes after pressing were assessed using a moisture analyzer (AND MX-50).

Cell densities were measured by means of a spectrophotometer (DR3900, Hach Lange GmbH, Austria) at a wavelength 600 nm. The biomass dry weight in the fermentation broth was determined after 2 cycles of washings with bi-distilled water. The optical purity of the lactic acid (fraction of L- and D-isomers) was determined by an enzymatic assay kit (K-DLATE 06/08, Megazyme).

The accuracy of data was within the range of ±10%.

3. Results and discussion

3.1. Liquefaction and saccharification (pre-extraction)

In a previous work, a laboratory scale glucose extraction from the starch portion of the wheat bran was described (Tirpanalan et al., 2014). In the current study the process was carried out at pilot scale and with defatted bran as the input material. Compositional details of the extract and yields are given in Table 1. Results obtained are comparable with the previous study, except the solubilized ash content. While at lab scale the extracted ash contents were around 45% of the total ash, at pilot scale with the defatted wheat bran it reached around 70%.

In total, 32% of the dry matter of defatted wheat bran, which could be envisaged for food applications, was extracted through the amylolytic enzymatic hydrolysis.

3.2. Hydrothermal and enzymatic treatment after pre-extraction (pre-treatment)

The solid residue of the pre-extraction step (cake) was treated hydrothermally and enzymatically. According to earlier findings (Reisinger et al., 2013), the optimum conditions of hydrothermal treatment are 180 °C involving 10–20 min reaction time. This approach was also applied in the present study.

The liquid fraction of the hydrothermal/enzymatic treatment (WBH) contained 34% of the dry matter of the initial defatted wheat bran. The compositional details of the WBH are given in Table 2 together with the results of a NF trial. The yields of total glucose, xylose, arabinose and the ash contents based on the corresponding fractions of the initial defatted wheat bran were 29.7%, 48.7%, 14.9% and 25.2%, respectively. The solid rest was mainly composed of carbohydrates, i.e., 23% of glucose, 21% of xylose and 24% of arabinose as well as approximately 19% of lignin.

Table 2
Compositional details of the pre-treatment and nanofiltration end-process streams.

Analyte	Feed (WBH)	Concentrate (C. WBH)	Permeate
Dry matter (%)	4.1	11.1	0.1
Ash (%)	0.2	0.7	<0.1
Fermentable sugar (%)	2.1	5.6	
Free glucose (g/L)	11. 20	32.02	<0.2
Free xylose (g/L)	7.14	18.13	<0.2
Free arabinose (g/L)	2.64	7.61	<0.2
Degradation and by-products (%)	0.4	0.9	0.2
Hydroxymethylfurfural (g/L)	0.15	0.38	<0.05
Furfural (g/L)	1.78	2.74	1.21
Levulinic acid (g/L)	0.26	0.72	<0.05
Acetic acid (g/L)	2.27	4.94	0.89

WBH: wheat bran hydrolysate.
C.WBH: concentrated wheat bran hydrolysate.
(%) signifies the mass fraction.

3.3. Lactic acid production (fermentation)

3.3.1. Nanofiltration of the WBH

As one of the main factors influencing the product recovery cost is the concentration of the product, corresponding high substrate concentrations in the fermentation medium are preferred. To increase the fermentable sugar concentration and also to investigate the fermentation performance of the concentrated WBH (C.WBH), a NF (MWCO of 200 Da) step was performed. The employed membrane was selected according to a previous study, in which the sugar monomers were successfully concentrated (Tirpanalan et al., 2014). Other than conventional concentration processes, NF is also of interest with regard to detoxification and water recycling purposes. In this context, results presented here (without involving an optimization study) deliver some information of interest.

The time-dependence of the permeate flux during the NF is illustrated in Fig. 2. The WBH to be treated was fed to the cell in two steps because of the limited capacity of the filtration cell. The average permeate mass flow of the process was around 15 kg h^{-1} m^{-2}.

With a final volumetric concentration factor of around 3, the fermentable sugar concentration (glucose, arabinose, xylose) reached 57.8 g/L, while the fermentable sugar concentrations in the permeate were close to the limit of quantification (0.1 g/L).

Table 1
Compositional details of the liquid fractions obtained through a solid/liquid separation subsequent to the pre-extraction.

Analyte	Extract (liquid fraction of the pre-extraction)		
	Mass fraction (%) concentration (g/L)	g dry analyte/100 g dry matter of extract	Extraction yield* (%)
Dry matter	4.16		31.6
Free glucose	1.61 (16.35)	38.7	101.2**
Total glucose	2.00 (20.38)	48.1	45.6
Total xylose	0.30 (3.02)	7.2	11.9
Total arabinose	0.25 (2.53)	6.0	15.4
Ash	0.59	14.2	69.4
Organic rest	1.02	24.5	26.1

* Based on the corresponding fraction of initial bran.
** Based on the starch fraction, calculated with a conversion factor of 0.9 from glucose to anhydrosugar.

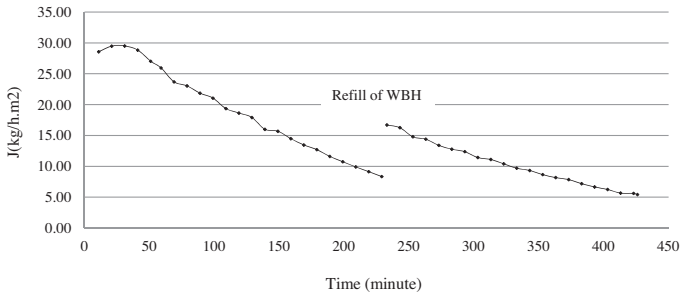

Fig. 2. Time-dependent permeate mass flow during the nanofiltration (200 Da, 60 °C, 3 MPa, 500 rpm) of the wheat bran hydrolysate (WBH).

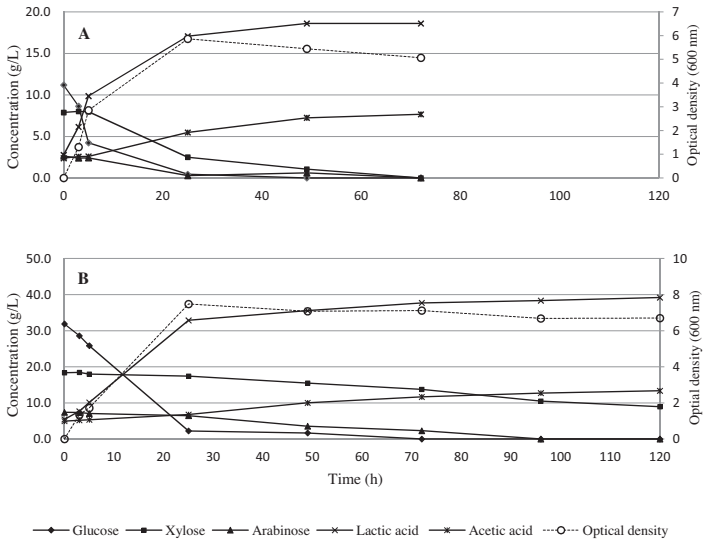

Fig. 3. Fermentation performances of (A) wheat bran hydrolysate (WBH), (B) concentrated wheat bran hydrolysate (C.WBH) for the lactic acid production.

The total dry matter, ash and fermentable sugar contents as well as degradation and by-product concentrations are given in Table 2.

As expected, the NF process provided the opportunity for the reduction of inhibitory compounds. For instance, as only 33% of furfural was retained by the membrane a significant amount of furfural could be removed from the substrate. Owing to the low dry matter content in the permeate, the process may also facilitate further water recycling.

3.3.2. Fermentation performance of WBH and concentrated WBH for the lactic acid production

In order to investigate the fermentability of the produced hydrolysate for the production of lactic acid, fermentation runs were carried out using the WBH as well as the C.WBH as substrates.

One of the purposes was to clarify whether the microbial growth occurs under the presence of the given concentrations of inhibitory compounds. The total degradation product concentrations (HMF, furfural and levulinic acid) were 22 and 38 mM in the WBH and C.WBH, respectively. Additionally, the pentose conversion was of great relevance, as the pentose fraction in the hydrolysate corresponds to around 45% of the total fermentable sugar. Herein, while lactic acid fermentation from the starch fraction of wheat bran was already dealt with in literature (John et al., 2006; Naveena et al., 2004; Yun et al., 2004), there is only very limited research available dealing with the lignocellulosic portion of wheat bran for lactic acid production (Givry et al., 2008). One of the distinguishing properties of the present study is that the fermentation was carried out with the de-starched wheat bran hydrolysate without any additional nutritional supplementation or growth factors.

Fermentation of the WBH was completed after approximately 52 h when the monomeric sugars (monomeric glucose, xylose and arabinose) were quantitatively consumed. Regarding the C. WBH, fermentation was ceased after 120 h due to a low conversion rate and around 9 g/L xylose monomers remained non-utilized in the fermentation batch. Fig. 3 illustrates the sugar consumption, the lactic acid and acetic acid production as well as the time-dependent optical density (600 nm) values corresponding to the cell growth in the fermentation batches with both media, WBH and C.WBH. In the batch of WBH the consumption of pentoses became noticeable after marked reduction of the glucose content, however, in the C.WBH the rate of pentose consumption was rather low even after considerable glucose depletion. Expectedly, the production of acetic acid was concomitant with pentose consumption, as a result of the facultatively heterofermentative behavior of the

Table 3
Fermentation performances using wheat bran hydrolysate (WBH) and concentrated WBH (C. WBH) as substrates.

	WBH (52 h)	C.WBH (120 h)
Total lactic acid yield (g/g consumed substrate)	0.73	0.69
Average lactic acid productivity ($g\,L^{-1}\,h^{-1}$)	0.30	0.28
Final lactic acid concentration (g/L)	18.6	39.2
Final lactic acid purity (%)	45.3	33.9
Total acetic acid production (g/g consumed substrate)	0.24	0.17
Final acetic acid concentration (g/L)	7.7	13.4

WBH: wheat bran hydrolysate.
C.WBH: concentrated wheat bran hydrolysate.

microorganism used (Zanoni et al., 1987). Although the rate of glucose consumption was relatively high, the rate of pentose conversion stood for the limiting factor impairing the average lactic acid productivity, which was around 0.3 $g\,L^{-1}\,h^{-1}$ for both batches. Table 3 depicts some results of interest concerning the fermentation performances.

It is noteworthy that furfural was depleted in both fermentation batches. In order to reduce the toxic effect of furfural, some microorganisms are capable of modifying it if it exists at low concentrations. Reportedly, a few microbial transformations of furfural are known (Boopathy, 2009; Veeravalli et al., 2013).

In the present study, concerning the lactic acid fermentation of the lignocellulosic portion of wheat bran, fermentation took place and a high degree of pentose utilization was obtained, despite the inhibitor concentrations. It could be shown that the lactic acid bacteria were able to grow on the WBH and C.WBH without supplementation of additional nutrients or growth factors. Certainly, through the development of a wheat bran biorefinery producing lactic acid, further research toward the investigation on a process using homofermentative microorganisms with the property of producing optically pure lactic acid (present case 50% L-lactic acid) is required. Additionally, the fermentation productivity, in particular concerning the pentose fraction needs to be substantially improved.

Irrespectively, the pre-concentration of the substrate and concomitant increase in the reducing sugar content did not result in an improvement of the fermentation performance.

3.4. Evaluating the overall process based on the mass balance

As the future face of the biorefinery aims at a zero waste concept (Star-COLIBRI, 2011), an in-depth consideration of the side-streams of biorefinery systems is required. In this study the major side-streams, namely the solid residue of the hydrothermal treatment and the fraction related to the fermentation medium, are considered. Fig. 4 demonstrates the dry mass distribution of the feedstock within the process flow involving both experimentally obtained results and estimated values based on assumed optimal scenarios.

The solid rest (cake) obtained subsequent to the hydrothermal/enzymatic treatment contained 34% of the initial dry bran mass. By modifying the pre-treatment parameters or the method the amount could be decreased to some extent. However, a cost effective technology accomplishing an optimal solubilization of the biomass while limiting the formation of degradation products

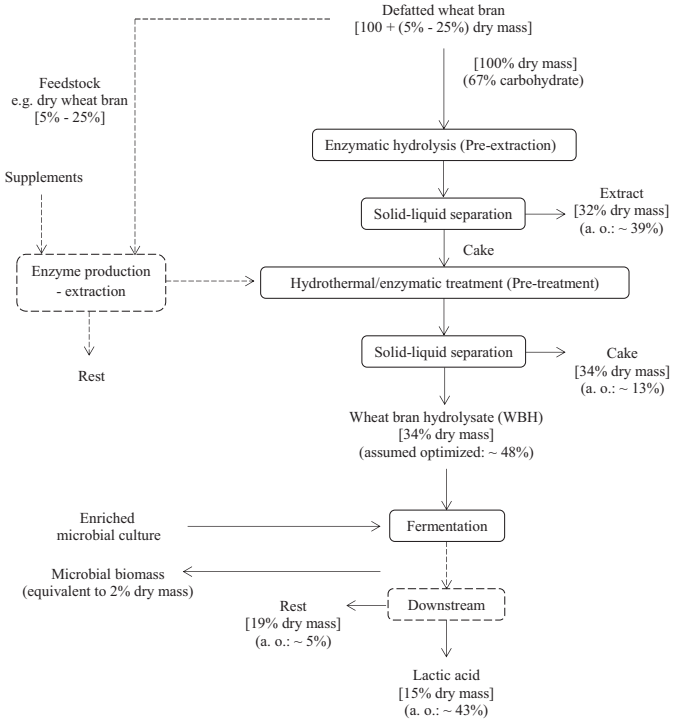

Fig. 4. The distribution of the dry mass within the basic process flow of a wheat bran biorefinery including experimentally obtained results (squared brackets) and assumed optimal (a.o.) values based on a best-case scenario (- - - not experimented).

Table 4
Yields of possible product streams (g product output/100 g dry bran mass input) obtained experimentally and estimated theoretically for their maximum values.

Yield (g product output/100 g dry wheat bran)				
	Extract	Lactic acid	Lignin fraction	Total (including lignin fraction)
Experimentally obtained	32	15		47
Theoretical maximum[a]	39	43	6.5	82 (88.5)
Theoretical maximum[a,b]	35	39	6	74 (80)

[a] *Assumptions:* improved protein recovery during the pre-extraction, homofermentative lactic acid fermentation with a quantitative sugar consumption.
[b] Involving an integrated cellulolytic enzyme production with a 10% dry feedstock allocation.

to acceptable levels has not emerged yet (Reisinger et al., 2013, 2014). In the case of wheat bran an educated estimation for an optimized process indicates a limit of approximately 13% of solid rest remaining as a cake, which mainly consists of lignin, some proportion of protein and cellulose. This fraction is a subject that needs further investigation for an integrated utilization strategy, while lignin (6.5%) has already some suggested utilization possibilities (Bozell et al., 2007).

Regarding the fermentation process, 19% of the initial dry bran mass represented the rest material in the medium, which mainly consists of proteins, total xylose and acetic acid as well as of some minerals. Assuming a homofermentative lactic acid production, a quantitative conversion of total sugars and an improved protein pre-extraction, this fraction could theoretically be reduced down to 5% mainly comprising proteins as well as some acetic acid and minerals.

At the end of the fermentation, the dry cell mass content of the fermentation medium, which was separated by centrifugation, was 0.2%. This value is equivalent to 2% of dry mass input.

Another point of discussion in the realization of a lignocellulosic biorefinery is the necessary amount of feedstock required to provide the cellulolytic enzymes. In many studies the optimization of the cellulases production from agro-industrial by-products have been investigated, yet the portion of the feedstock required to produce those enzymes has been only scarcely examined (Lever et al., 2010). For the present feedstock, a literature based optimistic estimation indicates a range between 5% and 25% (Fig. 4) (Liming and Xueliang, 2004; Singhania et al., 2007; Wooley et al., 1999).

Regarding the fermentation product, 15% of initial dry mass was experimentally obtained as lactic acid. Following abovementioned assumptions (optimized pre-treatment and fermentation) a theoretical maximum could be around 43%. Additionally, with an improved protein recovery during the pre-extraction step e.g., an integrated alkaline extraction, the protein fraction of the extract could be enhanced (Idris et al., 2003; Jones and Gersdorff, 1923). Given that the extract is also a product containing (in an assumed optimized case) approximately 39% of initial dry mass, a total product yield of 82% (88.5% including lignin) could be an appreciable portion.

Taking into account a cellulolytic enzyme production with an allocated feedstock input of 10%, a maximum overall efficiency (product output/feedstock input) of 74% (80% including lignin) could finally be expected.

Abovementioned product yields, both the experimentally obtained and theoretically estimated maximum values are summarized in Table 4.

4. Conclusions

This study exemplarily demonstrates the main steps of a wheat bran biorefinery and attempts to elucidate overall product efficiency. Under assumed optimized conditions the product efficiency of this concept could theoretically reach around 80%. In this context, the feedstock allocation to produce cellulases was suggested as 10%. It is noteworthy that this approach so far has rarely been discussed and requires more detailed investigations. Concerning lactic acid fermentation, the productivity of the process is largely determined by pentose utilization. Apart from the optimizations required, the pronounced zero waste target will certainly further increase the complexity of a biorefinery system.

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Valorization of Potato-processing Residues for the Production of Lactic Acid

The second study aimed to develop a process for the production of lactic acid from potato residues with final product titers and yields high enough to guarantee profitability (i.e. min 100 g/L and 90 % respectively; see 3.4.2). Residues from potato processing are generated in large amounts and can therefore sustain large-scale lactic acid production. They provide starch as a carbon source and additional proteins, vitamins and minerals that can supply at least part of the nutrients required for microbial fermentation. A suitable process for the enzymatic hydrolysis of the starch fraction was developed. This step differs from established industrial hydrolysis processes, since the substrate here was not refined starch, but a viscous mash of puree and peels, containing also fibers and impurities. This represented an exciting challenge to face.

The enzymatic hydrolysate obtained from potato residues (46 % w/w dry mass starch) was used as a glucose-rich substrate in batch and fed-batch fermentations with *Lactobacillus casei*. From the liquid hydrolysate, lactic acid was produced in reasonably high concentrations (102 g/L) and yields (92%), thus meeting the goal set above. In this case, the hydrolysate was supplemented with yeast extract to sustain bacterial growth and glucose conversion to lactic acid. The lactic acid produced (92% L-isomer) is of suitable quality for numerous technical applications after purification. Results indicate that it is possible to produce 54 kg of technical grade lactic acid (90% w/w) from 103 kg of fresh potato residues. This study offers a critical evaluation of the effectiveness and limitations of the investigated process. For instance, uncontrolled fermentation during transport and storage resulted in the formation of both D- and L- lactic acid that impaired overall chiral purity. Even if the productivities and product yields achieved indicate that there is still potential for improvements, it was clearly demonstrated that the developed process is promising for large-scale biorefinery applications.

Valorization of Potato-processing Residues for the Production of Lactic Acid

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Every year large amounts of residues accumulate from potato processing. The aim of this study is to develop a process to valorize these residues as feedstock for lactic acid fermentation in a way that is suitable for industrial production. An enzymatic hydrolysate obtained from potato residues (46 % w/w dry mass starch) was used as a substrate in batch and fed-batch fermentations with *Lactobacillus casei*. Supplementation of yeast extract (3 g L^{-1}) was required to improve productivities. Despite the inhibitory effect exerted by phenolic compounds in the medium, reasonably high lactic acid concentrations (102 g L^{-1}) and yields (92 %) were obtained, with 92 % (L-isomer) optical purity. It was clearly demonstrated that potato residues are suitable as feedstock for large-scale production of technical grade lactic acid and that the developed process is promising for large-scale biorefinery applications.

Key words:
biorefinery, enzymatic hydrolysis, lactic acid fermentation, *Lactobacillus casei*, potato residues

Introduction

Losses along the food production chain are of great concern to the food industry as well as to the consumers, raising both economic and ethical issues. The worldwide production of potatoes in 2013 was $3.68 \cdot 10^{11}$ kg[1]. Two-thirds of the entire production is utilized for human nutrition and is consumed as fresh or processed product[2]. About 10–15 % of the total amount of roots and tubers entering industrial food production are lost during processing and packaging[3]. This corresponds to a worldwide loss of $2.30 \cdot 10^{10}$ kg potatoes from the food processing industry every year[4].

Losses of potatoes at the processing level occur due to sorting, washing, peeling, size reduction or during process interruptions or accidental spillages[3], resulting in residual streams with a high organic load. Such residues mainly consist of potato peels and, to a lesser extent, of pulp and slurry. The typical water content of potato peel residues is 77–85 % w/w[5,6] and the composition on a dry mass (DM) basis is as follows: 47–52 % w/w starch, 8–16 % w/w protein, ca. 7 % w/w ash, up to 1 % w/w soluble sugars, 0.5–2.6 % w/w fat, while the rest is fibers[6,7].

An overview on current practices and potential applications of potato residues is available in the literature[5,8]. It is possible to use these materials as animal feed, however, the nutritive value is limited, and they undergo rapid microbial spoilage. Thus, in many cases, the residues are treated as waste. Because of the high water content, incineration is not appropriate, and therefore, biological treatment processes, such as composting and anaerobic digestion, are more suitable.

An alternative use for this kind of residue is as feedstock for industrial fermentation processes. The valorization of potato residues as starch-rich feedstock for the production of lactic acid (LA) within an integrated biorefinery represents an appealing option. The market for LA is constantly growing and will exceed 10^9 kg $year^{-1}$ by 2020[9]. Therefore, the effective production capacity must be extended rapidly. Currently, this appears to be challenging regarding the availability and costs of raw materials[9]. Ninety-five percent of the LA on the market is produced by microbial fermentation from cane or beet sugar or hydrolyzed cornstarch[10]. However, social and political concerns promote the development of sustainable production processes that do not interfere with the food supply chain[11].

The major advantage of potato residues as an alternative substrate for LA production is the high starch content. In addition, potatoes also contain proteins, vitamins and minerals[12,13] that can supply at least part of the nutrients required for the microbial fermentation. For applications requiring high purity LA (e.g. polymer-grade), residues are not a

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suitable resource. Therefore, the LA obtained from potato residues should rather be used for technical applications, like textile dyeing and finishing, leather softening, descaling and cleaning in the oil industry[14]. Furthermore, there is a demand for the derived lactate esters, which can be utilized as solvents and cleaning agents in the paint and ink industry or in microelectronics. The market volume of lactate esters in 2013 was $6.82 \cdot 10^8$ kg[15]. Food and cosmetic applications are also possible, provided that a chiral purity of 95–97 % L-LA can be guaranteed.

Research studies on the use of potato residues as feedstock for LA fermentation have been published recently[16–21]. They address the production of LA with different microbial strains and at different working conditions. In particular, Liang *et al.*[16–17] describe the production of LA with a mixed undefined microbial inoculum in batch and sequencing batch culture. The study conducted by Bilanovic *et al.*[18] evaluates the benefits of using potato residues as an alternative carbon source for LA production with *Lactobacillus* spp.. In another work, up to 10 g L^{-1} LA were produced from sweet potato residues with *Lactobacillus* spp. after enzymatic hydrolysis[19]. Finally, direct fermentation of unhydrolysed potato residues with *Rhizopus oryzae* in immobilized form[20] and *Geobacillus stearothermophilus*[21] has been described. However, the LA concentrations reached in the abovementioned studies are limited to 10–60 g L^{-1}, which is not comparable with conventional production processes. The approach for this study is to develop a process that reaches titers higher than 100 g L^{-1} and yields higher than 90 %, as crucial requirements for process economy[14].

The aim of this study was the valorization of potato residues as feedstock for the fermentative production of LA with *Lactobacillus casei*. The characterization of potato residues, the set-up of a suitable process to use them as a substrate to produce technical grade LA in accordance with the current industrial and economic requirements, and finally, the evaluation of the effectiveness and/or potential limitations of such a process were investigated.

Materials and methods

Potato residues: Physical pretreatment and characterization

Potato residues were obtained from two potato-processing facilities in Navarra (Spain) that together produce $2.00 \cdot 10^6$ kg $year^{-1}$ of solid residues – a viscous mash of puree and peels. In order to preserve the product from degradation during transportation and long-term storage, the material was dried in an industrial drum dryer to decrease the water content from 86.7 ± 1.4 % to 13.0 ± 1.4 %. The particle size was 4–15 mm in diameter.

Enzymatic hydrolysis of starch

The enzymatic hydrolysis of starch in the potato residues was performed in a 5-L stainless steel vessel (id 190 mm), equipped with an overhead stirrer (model RW 20, IKA®-Werke GmbH & Co KG, Germany) and a Teflon lid to avoid evaporation losses. Two Rushton impellers were mounted on the stirrer. A hotplate and a temperature sensor were installed for temperature regulation. For liquefaction, boiling water was added to the dry biomass in order to have a dry solid content in the mash of 20–26 % w/w. Liquefaction was run at 70 °C and pH 6. The pH-value was adjusted from an initial value of 4.0 ± 0.1 (due to the presence of LA in the residues) to 6.0 ± 0.2 by the addition of 10 M NaOH. An amount of 2 g kg^{-1} dry solids of α-amylase BAN 240 L (Novozymes, Denmark; ≥ 250 units g^{-1}) was added to the slurry together with $CaCl_2$ (0.5 g L^{-1}) to generate maltodextrins of differing chain lengths and oligosaccharides. After 2-h reaction time, the pH-value was adjusted to 4.3 ± 0.2 with 10 M HCl. This was done to provide optimal pH conditions for saccharification and, at the same time, to enhance the deactivation of BAN 240 L. α-Amylase was deactivated at 88 ± 3 °C for 15 min. Once the temperature in the mash reached 60 °C, saccharification was initiated by the addition of 1.2 mL kg^{-1} dry starch of glucoamylase AMG 300 L (Novozymes, Denmark; ≥ 300 units mL^{-1}) to generate glucose units from dextrins and oligosaccharides. After 20 h, the mash was collected, weighed, and centrifuged (30 min, 20 °C, 16000 g; centrifuge: J2-MI, rotor JA-10, Beckman, USA). The glucose-rich liquid fraction was used as a substrate for LA fermentations – after appropriate dilution with water – and was freshly prepared for each experiment.

Chemicals

MRS broth (de Man, Rogosa, Sharpe) and corn steep liquor (CSL) were purchased from Sigma-Aldrich (Austria). Yeast extract (YE) type CMRT was obtained from Ohly GmbH (Germany), and glucose (Meritose) from Tereos Syral (France).

Microorganism

The bacterial strain used in this study was initially obtained from a dairy. The species was confirmed as *Lactobacillus casei* by sequencing of 16S rDNA performed by LGC Genomics (Teddington, UK) and by BLAST similarity search analysis. We selected this strain because of its good LA production abilities and its specificity for L-LA. *L. ca-*

sei cultures grown in MRS broth for 14–16 h were preserved in 1-mL aliquots (10 % v/v glycerol) at –80 °C.

Analytical methods

The total starch in the dry biomass and the amylose fraction were determined with enzymatic assay kits (K-TSTA 07/11 and K-AMYL 06/15, respectively, Megazyme, Ireland). Total Kjeldahl nitrogen (TKN) and NH_4-N in the potato residues were determined with an AutoKjeldahl Unit K-370 (Buechi, Switzerland). The crude protein content was estimated from the TKN, using a conversion factor of 6.25. Low MW water-soluble organic compounds were determined by incubating 1 g of finely milled dry potato residues in 50 mL of bi-distilled water for 30 min at 60 °C, and analyzed via HPLC. Inductively Coupled Plasma optical emission spectrometry (ICP-OES) analyses were performed in an ULTIMA 2 device (HORIBA Jobin Yvon GmbH, Germany) to determine the concentration of trace elements in the potato residues (Table 1b). Phenolic compounds were determined with the Folin-Ciocalteu phenol reagent[22]. Determination of acid insoluble lignin was performed via gravimetric analysis as described in Sluiter *et al.*[23]

Table 1a – *Dry mass composition of potato residues (water content: 86.7 ± 0.3 g per 100 g)*

	(g/100 g) DM
Starch	45.77 ± 3.48
of which amylose	22.99 ± 1.29
Protein	9.36 ± 0.18
NH_4-N	0.25 ± 0.03
Lignin	5.15 ± 0.71
Ash	5.70 ± 0.88
Low MW water-soluble organic compounds	
Lactic acid	5.01 ± 1.60
Ethanol	0.69 ± 0.01
Succinic acid	0.32 ± 0.04
Maltose	0.26 ± 0.05
Fructose	0.08 ± 0.01
Acetic acid	0.07 ± 0.02
Glucose	0.03 ± 0.02

Table 1b – *Mineral composition of the potato residues as determined with ICP-OES*

Minerals	(mg/100 g) DM
K	2368.28 ± 89.09
P	298.81 ± 0.09
Ca	141.68 ± 44.60
Mg	104.21 ± 7.95
Na	62.99 ± 5.37
Fe	13.76 ± 1.28
Al	9.04 ± 1.09
Zn	3.44 ± 0.27
Cu	0.82 ± 0.06
Mn	0.80 ± 0.08

Sugars and organic acids were determined via HPLC with a refractive index detector (Agilent Series 1100). An ion exclusion column ION 300 (Transgenomic) was used at 45 °C with 0.005 M H_2SO_4 at a flow rate of 0.325 mL min^{-1} as mobile phase. The L- and D- optical isomers of LA were quantified by using an enzymatic assay kit (K-DLATE 06/08, Megazyme, Ireland).

All analyses were performed in duplicate.

Fermentations in shake flasks

Preliminary lab-scale fermentations were run in 100-mL shake flasks (Schott Duran, Germany) with a working volume of 50 mL. The liquid potato residue hydrolysate obtained after centrifugation was used as a source of glucose (70 g L^{-1}) after dilution with water. A multilevel factorial design (2^3) with two factors (CSL, YE) defined on three levels (concentrations) was developed (Table 2). The aim was to study the effect of CSL and YE as N-rich supplements on LA production. In addition, two controls with 70 g L^{-1} refined glucose instead of the hydrolysate were included: one in MRS medium, the other in YE 6 g L^{-1} – CSL 5 % v/v. All treatments were run in triplicate; results are expressed as mean values and relative standard deviations of the LA production after 48 h (response). Differences between the treatments were evaluated with analysis of variance (ANOVA) and multiple range tests at the 95 % confidence level.

Each group of replicates was inoculated with 5 mL of a separately prepared 14-h-old preculture of *Lactobacillus casei* IFA 32 (3.4 ± 0.3 10^9 cells per mL, OD 2.6 ± 0.3) grown in MRS broth. As a buffering agent, 3.3 g of $CaCO_3$ were added to the medium to neutralize the LA formed throughout the fermentation and keep the pH-value between 5 and 6. Cultures were incubated for 144 h at 37 °C and 90 rpm in an Infors HT Multitron rotary shaker.

Fermentations in bioreactors

Batch and fed-batch fermentations were run in parallel in DASGIP 1-L bioreactors (DASGIP Information and Process Technology GmbH, Germa-

Table 2 – *Production of LA at 48 h (n=3) in shake flasks from the potato residue hydrolysate, at different CSL-YE concentrations. Initial LA concentrations in the medium were subtracted. Treatments with different indicators (a–e) have significantly different means (p > 0.05). In all treatments: final 63.02 ± 2.68 g L^{-1} LA reached within 144 h of fermentation (70 g L^{-1} initial glucose).*

Run	Factors		Response
	CSL (% v/v)	YE (g L^{-1})	LA at 48 h (g L^{-1})
1	0.0	0.0	27.87^{a} ± 0.55
2	0.0	3.0	43.17^{c} ± 1.63
3	0.0	6.0	44.50^{c} ± 1.12
4	2.5	0.0	38.98^{b} ± 0.80
5	2.5	3.0	45.16^{c} ± 1.38
6	2.5	6.0	44.44^{c} ± 1.92
7	5.0	0.0	39.22^{b} ± 0.80
8	5.0	3.0	43.07^{c} ± 0.99
9	5.0	6.0	43.56^{c} ± 0.83
10	Control 1*		61.35^{e} ± 1.86
11	Control 2**		54.20^{d} ± 0.38

*Control 1: MRS medium, refined glucose.

**Control 2: CSL 5.0 % v/v – YE 6.0 g L^{-1}, refined glucose.

ny). Agitation (100 rpm) was provided by two six-bladed Rushton turbine impellers (od 46 mm). The initial glucose concentration was 136.9 ± 0.8 g L^{-1} for batch fermentations (tot 540 mL). In the fed-batch mode instead, this value was limited to 78.0 ± 1.6 g L^{-1} by dilution with water (tot 200 mL); the rest of the sugar was provided in two subsequent additions of hydrolysate (120 + 220 mL) as soon as the glucose concentration in the culture dropped to 20 g L^{-1}, to bring it again to 80 g L^{-1}. In the end, working volumes and total glucose were comparable in the two set-ups (Table 3).

Additional batch fermentations with a higher working volume (3 L) were carried out in a Biostat ED 5-L fermenter (Braun Biotech International GmbH, Germany). A flat-six-bladed-disc-turbine impeller (od 70 mm) was mounted on the stirrer shaft and the agitation speed was set to 150 rpm.

In all cases, the liquid potato residue hydrolysate – used as a source of glucose – and YE were sterilized separately at 121 °C for 20 min before being mixed in the reactor. A 10 % v/v 16 h-old preculture was used as the inoculum. Throughout the process, temperature was regulated to 37 °C and pH-value to 5.5 with the automatic addition of 10 M NaOH. Dissolved oxygen (DO) in the culture was monitored but not regulated during the process. DO levels dropped to zero during the exponential growth phase of *L. casei*, which was sufficient to ensure anaerobic conditions in the culture. All experiments were performed in duplicate; results are expressed as mean values and relative standard deviations.

Results and discussion

Characterization of the potato residues

The composition of the potato residues is reported in Table 1a-b. Values are expressed on a DM base and are relative to two batches, one collected in 2012, and the other in 2013. The data presented here are in good agreement with those available in the literature on potato-peel residues[6,7].

On a fresh mass basis (100 g), potato residues before drying consisted of 86.7 ± 0.3 g water, 6.1 ± 0.5 g starch, 1.3 ± 0.0 g proteins, and 0.7 ± 0.2 g LA. An amount of 62.3 ± 1.1 % of LA was present as D-LA, and the pH-value was 4.0 ± 0.1. In comparison to the residues, fresh potatoes consist of 62.7–87.0 g water, 9.1–22.6 g starch, and 0.9–4.2 g proteins per 100 g[12].

The composition of potato residues may vary from one batch to another, depending on factors like potato variety, processing, and storage conditions (duration, temperature, humidity, etc.) before drying and/or use. The relatively high water content is an important factor affecting transportation costs, as well as the microbiological stability of the residues over time[5]. Due to the activity of indigenous microbial species, considerable amounts of LA can already be formed during storage (Table 1a).

Because they are collected after processing, potato residues contain less starch than the fresh crop. Nevertheless, the starch content is still considerable, which – together with the amounts of proteins, ammonium and minerals – makes potato residues an excellent substrate for fermentations.

Table 3 – *Comparison of lactic acid fermentations on the potato hydrolysate as a C-source with 3 g L^{-1} YE*

Reactor	Mode	Culture volume (mL)	LA yield (%)	Productivity (g L^{-1} h^{-1})	Optical purity (% L-LA)	Reference
DASGIP	batch	540	75.15 ± 0.17	0.56 ± 0.01	90.12 ± 0.01	Figure 2
DASGIP	fed-batch	540*	67.16 ± 0.34	0.53 ± 0.01	89.35 ± 0.19	Figure 3
Biostat	batch	3000	92.21 ± 0.15	1.44 ± 0.00**	92.37 ± 0.13	Figure 4

*Final volume

**Productivity at 62 h.

Enzymatic hydrolysis of the potato residues

Potato residues were treated enzymatically to obtain a glucose-rich hydrolysate from starch (Figure 1). This approach was preferred to acidic treatments in order to limit the formation of undesired inhibitory by-products like hydroxymethylfurfural[24].

Unlike refined starch used in traditional industrial processes, the starch fraction in the potato residues constitutes scarcely 50 % of the DM. The rest of the solids, however, contributes to the viscosity of the slurry. Still, it is necessary to operate at a high solids load to reach high glucose concentrations in the hydrolysate. At the conditions described earlier, the highest possible solids load was 26 % w/w, resulting in a maximum final glucose concentration of 200 g L^{-1}.

It was decided to insert a centrifugation step before fermentation in order to avoid complications due to the solids in the bioreactor. After centrifugation, 51 % w/w of the total mash was recovered as a liquid fraction. The discarded solids (32 % w/w DM) also contained a considerable amount of glucose, and therefore, the total recovery of sugar in the liquid was limited to 63.5 ± 1.7 %. In an integrated biorefinery approach, the solid fraction may still be used for biogas production, composting or as animal feed, especially if mixed with the microbial biomass as a protein source[25] and dried.

Fermentations in shake flasks

The performance of the potato residue hydrolysate as a substrate for LA fermentation was evaluated in multilevel factorial design tests in shake flasks. The aim was to determine how well the liquid hydrolysate works as a sole C- and N-source for *L. casei,* and if additional nutrients were necessary, e.g., to increase the productivity. LA-producing microbial strains are usually supplemented with YE as an effective (but expensive) source of amino acids and essential vitamins[13]. YE and CSL were selected as N-rich nutrients[26] to be tested. The results are presented in Table 2.

The LA concentration after 48 h was chosen as the response, since differences in LA production among the different treatments were most evident at this stage (Table 2). Five homogeneous groups with significantly different means ($p > 0.05$) were identified. The highest LA concentrations at 48 h were obtained with controls 1 (MRS) and 2 (CSL 5 % v/v – YE 6 g L^{-1}), where refined glucose was used as a C-source instead of the hydrolysate. These results suggest that the hydrolysate may contain substances that inhibit the growth of LA bacteria.

It could be demonstrated that the potato residue hydrolysate can serve as a substrate for LA fermentation without further supplements. However, the conversion is significantly faster ($p > 0.05$) if supplements like CSL and YE are added. In particular, YE showed a stronger positive effect than CSL: Glucose (70 g L^{-1}) was completely consumed and converted to LA in 48–72 h, except for the treatments without YE (1, 4 and 7), where the fermentation process lasted longer. The results show that a concentration of 3 g L^{-1} of YE in the hydrolysate is sufficient to boost the production of LA by *L. casei*, and higher amounts of YE or the addition of CSL will not improve the process. Based on these find-

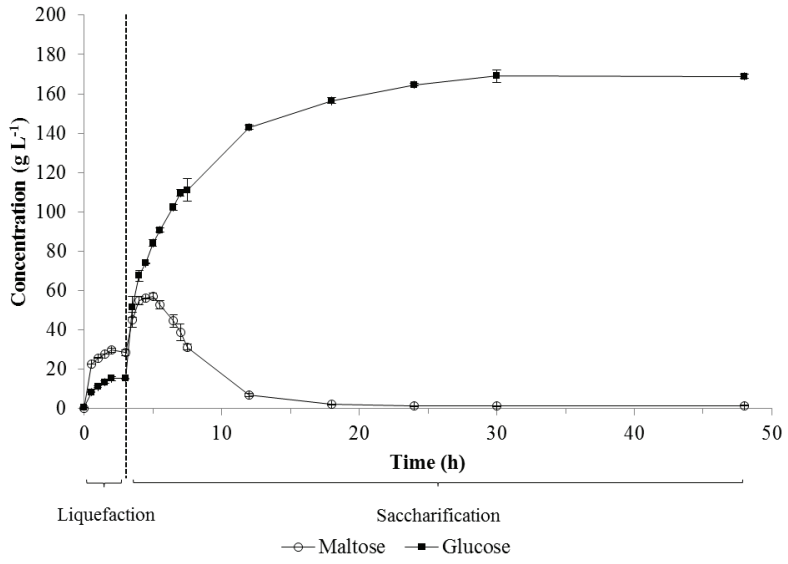

Fig. 1 – *Time-course profile of the enzymatic hydrolysis of potato residues (20 % w/w solids)*

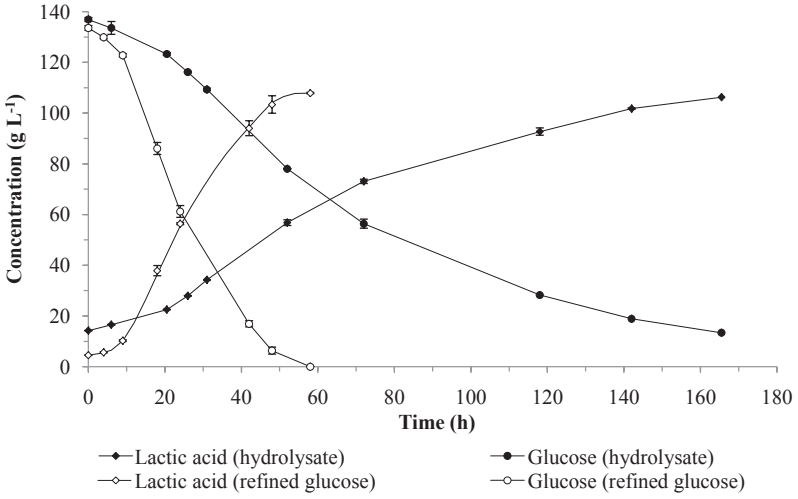

Fig. 2 – *Time-course profile of LA batch fermentation (37 °C, pH 5.5) on:*
– Potato residue hydrolysate as a C-source and 3 g L^{-1} YE (black bullets)
– CSL-YE medium and refined glucose as a C-source (white bullets)

ings, it was decided to supplement 3 g L^{-1} of YE to the liquid potato residue hydrolysate for further experiments in lab-scale bioreactors.

Batch and fed-batch lactic acid fermentation of potato residue hydrolysate

For the production of LA in bioreactors, a batch and a fed-batch configuration were compared. The aim was to develop a process that can reach high LA concentrations (more than 100 g L^{-1}) and productivities. In the batch process, an initial glucose concentration of 140 g L^{-1} was set, and 106 g L^{-1} of LA were obtained after 166 h (Figure 2).

The fed-batch process started at an initial glucose concentration of 80 g L^{-1} in order to avoid substrate inhibition[27]. Consequently, the production of LA started faster and with a higher productivity compared to the batch case. However, the productivity dropped notably after each addition of the hydrolysate: In the end, the overall productivity of the fed-batch process was reduced, and a lower concentration of LA (97 g L^{-1}) was reached after 166 h, when the process was stopped (Figure 3).

In both configurations, there were residual unconverted sugars in the broth after almost one week of fermentation. This suggests that elevated hydrolysate concentrations and an increasing LA titer exert an inhibiting effect on the strain and delay further LA production. Previous experiments (data not shown) demonstrated that *L. casei* IFA 32 is able to tolerate glucose concentrations higher than 250 g L^{-1} and produce LA up to 150 g L^{-1} in MRS medium. Therefore, the sugar and LA titers alone do not explain the observed delay. Moreover, when *L. casei* was tested with refined glucose as a C-source in 5 % v/v CSL-6 g L^{-1} YE, the sugar was completely converted within 58 h (Figure 2). It is known that potatoes – and peels in particular – contain phenolic compounds like chlorogenic and caffeic acid[12,28]. The content of phenolic compounds in the fermentation broth was 4.40 ± 0.10 g L^{-1} of vanillin equivalent. Studies on the effect of phenolic compounds on the growth of *L. plantarum* show that caffeic acid, at a concentration higher than 1 g L^{-1}, inhibits growth and reduces bacterial counts[29]. It can be expected that the inhibitory effect exerted by phenolic compounds will also affect other cell functions, e.g. LA production. Hence, it was decided to reduce the hydrolysate concentration in the fermentation medium, thus lowering the glucose concentration and the phenolic content.

Batch lactic acid fermentations at a reduced hydrolysate concentration

It was demonstrated that diluting the hydrolysate from 140 g L^{-1} glucose to 100 g L^{-1} shortens the total duration of the batch process to 62 h (Figure 4). This can mainly be attributed to the dilution of the phenolic compounds (and other potential inhibitors). The chosen hydrolysate concentration still allows fulfilling the industrial production requirements (titer, yields). A final product concentration of 101.7 ± 0.3 g L^{-1} was obtained, with yields (92 %, Figure 4, Table 3) comparable to those obtained on refined glucose as a C-source (91 %, Figure 2). However, a lower total productivity was observed (1.44 g L^{-1} h^{-1} instead of 1.78 g L^{-1} h^{-1}), which most likely results from the relatively high initial LA concentration and the presence of growth inhibitors

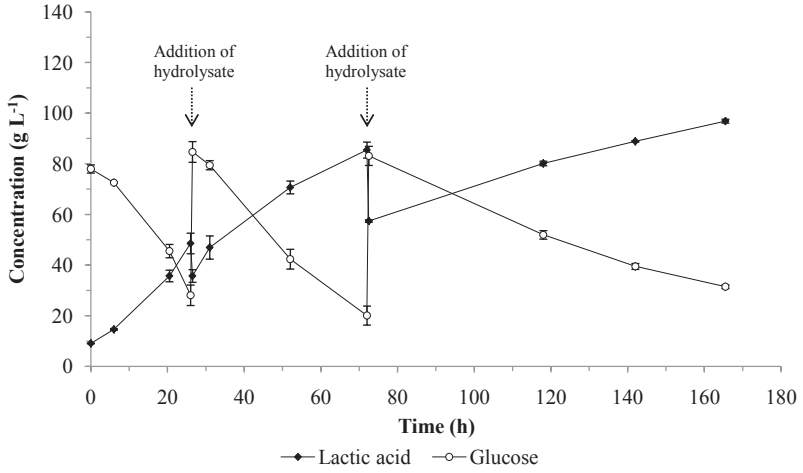

Fig. 3 – *Time-course profile of LA fed-batch fermentation (37 °C, pH 5.5) on the potato residue hydrolysate as a C-source, with 3 g L^{-1} YE*

(e.g. phenolic compounds) in the potato residue hydrolysate. A practicable strategy to limit the inhibitory effect exerted by phenolic compounds is to treat the hydrolysate with laccase formulations for detoxification. For instance, oxidative coupling and oligomerization/polymerization of polyphenols catalyzed by laccases is already exploited in wine production for stabilization[30]. Another way to achieve higher productivities is to run simultaneous saccharification and fermentation, with the advantage of limiting substrate inhibition effects due to glucose accumulation[31]. This implies, however, that fermentations have to be carried out in the presence of solids, and at temperature and pH conditions suitable for both the LA-producing microorganism and the amylolytic enzymes. The abovementioned strategies can be implemented to obtain even higher product concentrations and productivities, thus increasing the suitability of the process for industrial applications.

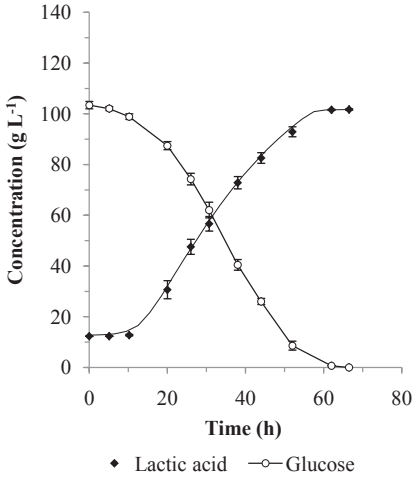

Fig. 4 – *Time-course profile of LA fermentation on the potato residue hydrolysate as a C-source with 3 g L^{-1} YE. In comparison to Figure 2, the medium has been diluted to limit the initial glucose concentration to 103 g L^{-1}.*

Due to the initial amount of LA already present in the potato residue hydrolysate (12.3 ± 0.1 g L^{-1} with 53 % D-LA), the optical purity of the LA in the fermentation broth was 92 % L-LA. This is still practical for many non-polymer applications, while an optical purity of minimum 99 % is required for PLA production[32–33], which makes refined substrates more convenient for the fermentation of polymer grade LA. Hence, it is expected that LA produced from potato residues will be suitable for technical applications after purification. According to the results obtained in this study, it is possible to produce 54 kg of technical grade LA (90 % w/w) from 10^3 kg of fresh potato residues.

Conclusion

Residues from potato processing are generated in large amounts, and are a suitable substrate for large-scale lactic acid production. They provide starch as a C-source and additional nutrients for microbial growth. Supplementing the enzymatic hydrolysate with yeast extract (3 g L^{-1}) significantly increases LA productivities. Inhibitory effects of phenolic compounds on *Lactobacillus casei* were mitigated successfully by limiting glucose concentrations to 100 g L^{-1}. The developed process meets the industrial requirements of reasonably high LA concentrations (101.7 g L^{-1}) and yields (92 %). The lactic acid produced (92 % L-isomer) is of suitable quality for numerous technical applications.

ACKNOWLEDGMENTS

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Abbreviations

LA – lactic acid
MRS – de Man, Rogosa, Sharpe
CSL – corn steep liquor
YE – yeast extract
ICP-OES – inductively coupled plasma optical emission spectrometry
DO – dissolved oxygen
DM – dry mass
id – inside diameter
od – outside diameter
tot – total

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5.2. Alternative process for lactic acid production: direct conversion of starch at non-sterile conditions

The use of residues from the food processing as cheap renewable substrates as an alternative to refined substrates was not the only effort in this work. It is well known that besides raw materials, the other factor impacting the economy of the biotechnological production of lactic acid is high operating costs, first of all the energy input for sterilization (Gao et al., 2011). Therefore, a possible way to improve cost competitiveness is to develop a process that does not require sterilization. For this purpose, it was decided to use thermophilic microorganisms capable of growing and producing lactic acid at high temperatures (i.e. above 50℃), where the risk of c ontaminations is minor. Unique features of thermophile fermentation are that the sterilization of the medium is not required and the whole process can be run in non-sterile conditions, leading to obvious economic benefits. In addition to saving time and energy for sterilization, working at high temperatures also allows to reduce the cooling demand to compensate the metabolic heat released during the microbial conversion of the C-source to lactic acid, which is an exothermic reaction (Abdel-Rahman et al., 2013; Zhao et al., 2010). Moreover, the dissolved oxygen concentration is inversely proportional to temperature, thus anaerobic conditions can be better maintained throughout the process (Taylor et al., 2009). In the literature some examples of suitable lactic acid-producing thermophilic microorganisms are reported (Ma et al., 2014; Ouyang et al., 2013; Zhao et al., 2010). None of the cited works, however, considered the use of amylolytic thermophilic strains for the direct conversion of starch to lactic acid.

Direct fermentation of potato starch and potato residues to lactic acid by *Geobacillus stearothermophilus* under non-sterile conditions

The third study presented in this dissertation focused on the development of an innovative process for the production of lactic acid from starchy feedstock. The aim was to use a microorganism capable of direct hydrolysis of starch and of its conversion to lactic acid under non-sterile conditions. *Geobacillus stearothermophilus* was identified as a suitable species for this purpose, as a known producer of α-amylase and due to its optimum growth temperature between 50 and 60 ℃. The strain DSM 49 4 was selected among other candidates due to its lower production of by-products. The developed process yielded 37 g/L optically pure (98%) L-lactic acid in 20 h from 50 g/L raw potato starch. The observed productivity was high in comparison to similar processes described in the literature. Smaller amounts of acetic acid, formic acid and ethanol were formed as co-metabolites. The inhibitory effect of high concentrations of lactic acid and the co-metabolites was considered responsible for limiting the maximum lactic acid concentration achievable. However, yields and product concentration could be increased to 81% and 59 g/L respectively when potato

residues were used as a substrate instead of raw starch. It was proven that stable processes could be conducted operating in non-sterile conditions and without sterilization of the medium.

Research Article

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Direct fermentation of potato starch and potato residues to lactic acid by *Geobacillus stearothermophilus* under non-sterile conditions

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Abstract

BACKGROUND: Lactic acid is an important biorefinery platform chemical. The use of thermophilic amylolytic microorganisms to produce lactic acid by fermentation constitutes an efficient strategy to reduce operating costs, including raw materials and sterilization costs.

RESULTS: A process for the thermophilic production of lactic acid by *Geobacillus stearothermophilus* directly from potato starch was characterized and optimized. *Geobacillus stearothermophilus* DSM 494 was selected out of 12 strains screened for amylolytic activity and the ability to form lactic acid as the major product of the anaerobic metabolism. In total more than 30 batches at 3–l scale were run at 60 °C under non-sterile conditions. The process developed produced 37 g L^{-1} optically pure (98%) L-lactic acid in 20 h from 50 g L^{-1} raw potato starch. As co-metabolites smaller amounts (<7% w/v) of acetate, formate and ethanol were formed. Yields of lactic acid increased from 66% to 81% when potato residues from food processing were used as a starchy substrate in place of raw potato starch.

CONCLUSIONS: Potato starch and residues were successfully converted to lactic acid by *G. stearothermophilus*. The process described in this study provides major benefits in industrial applications and for the valorization of starch-rich waste streams.

Keywords: *Geobacillus stearothermophilus*; thermophiles; lactic acid; fermentation; starch; amylolytic bacteria

INTRODUCTION

Lactic acid (LA) has been widely used for decades in several industrial sectors such as food, pharmaceuticals, chemicals and cosmetics.[1] During the past 15 years LA gained additional importance as one of the top platform chemicals for the production of green solvents, fuel precursors and poly(lactic acid) (PLA), a biodegradable plastic.[2] As a result, by 2020 the global market of LA is expected to exceed 10^9 kg^1.

Around 90% of the entire production of LA derives from fermentation, while chemical synthesis is no longer of significant importance.[3] As claimed by Gao *et al.*,[4] the two bottlenecks for the fermentative production of LA at industrial scale are the cost of the substrate (carbon source) and the operating costs, especially with regard to sterilization (sterilization equipment, energy consumption and labor cost). In order to reduce costs for the raw materials, starch represents among others (molasses, lignocelluloses, etc.) a cheap alternative to refined sugars.[5] Starch from various origins can be used for LA production, including corn, cassava, potato and barley.[6] Corn starch is a standard substrate commonly used for the production of LA at an industrial level.[7] In these processes, however, the starch is not used directly but requires enzymatic hydrolysis to glucose prior to fermentation, since most of the LA bacteria cannot convert starch directly.[6] Therefore, the utilization of amylolytic microorganisms which can grow directly on starch and convert it to LA represents an interesting option.[8]

The second issue raised by Gao *et al.* can be overcome by the application of thermophilic microorganisms like *Bacillus coagulans*, *Geobacillus stearothermophilus* (formerly known as *Bacillus stearothermophilus*) and *Bacillus licheniformis*[9] which allows non-sterile fermentations operated at temperatures above 50 °C. The risk of contamination, which is of great concern especially at industrial scale, is reasonably low at such temperatures. It has of course to be argued that even if sterilization costs can be

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saved working at 60 °C, at the beginning of the process the fermentation medium has to be heated up. The desired temperature has also to be maintained during the lag phase. However, once growth starts most of the heating requirement is supplied by the metabolic heat released during the conversion of glucose to lactic acid.[10] Other advantages of operating at high temperature (up to 60 °C) include: reduction in the energy input due to the cooling requirements during LA fermentation at lower temperatures and also the reduced solubility of oxygen at 60 °C in comparison with 37 °C, which facilitates the preservation of anaerobic conditions in the fermentation broth.[10–12]

As can be expected, the combination of these desired qualities in one LA-producing strain is challenging. One good candidate is *Geobacillus stearothermophilus,* a facultatively anaerobic gram-positive bacterium of the phylum Firmicutes, with an optimal growth temperature between 50 and 60 °C and pH of 6.2–7.5. The species is well known as a producer of thermostable α-amylase[13] and as a spore-forming microorganism[14] used as a biological indicator for the deactivation of pathogens.[15] Members of the species can ferment hexose and pentose sugars, glycerol and starch to produce lactate, acetate, formate and ethanol.[14,16,17]

The thermophilic production of LA by *G. stearothermophilus* has previously been described by Danner *et al.,*[18] using sucrose as a substrate. Due to the well-known amylase activity of the species, we hypothesized that a similar process could be established even from raw starch. This would be also promising for biorefinery applications for the valorization of starch-rich waste streams, such as residues from agriculture and food processing, e.g. potato residues.[19]

The primary objective of this study was the characterization and optimization of a non-sterile process for the thermophilic production of LA by *G. stearothermophilus* directly from raw potato starch and its application also to potato residues from food processing. This work provides new insights into the potential application of this species for LA fermentation also at an industrial level.

MATERIALS AND METHODS

Bacterial strains

Ten *Geobacillus stearothermophilus* strains were obtained from the Leibnitz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. They are referred to by their DSM code number. In addition, two putative *G. stearothermophilus* strains were isolated from compost at the Institute for Environmental Biotechnology.

For conservation cultures were grown in shake flasks at 60 °C in a medium containing 8 g L^{-1} nutrient broth (NB), 4 g L^{-1} yeast extract (YE), 10 g L^{-1} starch (or glucose) and Na_2HPO_4/KH_2PO_4 buffer (0.07 mol L^{-1}) at pH 7. Aliquots of 1 mL of these cultures in the late exponential growth phase (pH higher than 5.5) were prepared for long term storage at −80 °C with the addition of glycerol 10% v/v.

Media and carbon sources

Corn steep liquor (CSL), MRS broth (de Man, Rogosa, Sharpe) and nutrient broth were purchased from Sigma-Aldrich (Austria). Other medium components include: yeast extract type CMRT from Ohly GmbH (Germany) and soy peptone E110 (SP) from Organotechnie S.A.S. (France). Potato starch of technical grade and glucose were purchased from AGRANA (Austria). Potato residues were obtained from Trasa (Spain) and were collected from food-processing industries in the region of Navarra (Spain).

Potato residues: physical pretreatment and characterization

Potato residues consisted of a viscous mash of puree and peels. 100 g of fresh weight are composed of 86.7 ± 0.3 g moisture, 6.3 ± 0.1 g starch, 1.2 ± 0.0 g proteins and 0.5 ± 0.0 g total LA. 62 ± 1.1% of the LA in the residues was present as D-LA. In order to preserve the product from degradation the water content was reduced from 86.7 ± 1.4% to 13 ± 1.4% in an industrial drum dryer. The particle size was then reduced from *c.* 150 mm to *c.* 4 mm in diameter with a hammer mill. The total starch in the dry biomass as well as the amylose/amylopectin ratio were determined with enzymatic assay kits (K-TSTA 07/11 and K-AMYL 07/11, respectively, Megazyme, Ireland). The crude protein content was estimated from the total Kjeldahl nitrogen using a conversion factor of 6.25.

Fermentations

Preliminary tests were performed in 100 mL screw cap, non-baffled flasks (Schott Duran®, Schott, Germany) with a working volume of 50 mL. After sterilization these were inoculated with 0.5 mL of a cryoculture and incubated at 60 °C and 90 rpm in a rotary shaker (Infors HT Multitron, Switzerland). Culture conditions were not strictly anaerobic, but it is assumed that oxygen levels were close to zero during the growth phase. pH values were measured externally. Unless otherwise specified, the medium used was NB 8 g L^{-1} and YE 4 g L^{-1}. All experiments were performed at least in duplicate.

Batch fermentations of raw potato starch were conducted in a Biostat ED 5-L fermenter (B. Braun Biotech International GmbH, Germany) with an initial working volume of *c.* 3 L. Two flat-bladed-disc-turbine impellers (six blades, outer diameter 70 mm) were mounted on the stirrer shaft and the agitation speed was set to 150 rpm. The initial starch concentration was *c.* 50 g L^{-1}. All experiments were performed at least in duplicate. Batch fermentations of potato residues were run in DASGIP parallel bioreactors of 1 L volume (DASGIP Information and Process Technology GmbH, Germany). The fermentation medium was prepared adding boiling water to the required amounts of SP, YE and dry potato residues for an initial working volume of 0.5 L. The experiments were performed in triplicate. The equivalent initial starch concentration was *c.* 56 g L^{-1}.

The fermentation medium (water, substrate and nutrients) was not aseptic (i.e. no sterilization applied) and the whole process was run under non-sterile conditions; this implies that feeding of the reactor and sampling took place from an upper port of the vessel by simply opening it to a non-sterile atmosphere. pH in the fermentation broth was regulated by controlled addition of NaOH 10 mol L^{-1}; the high concentration of the base was necessary to minimize dilution of the fermentation broth. Dissolved oxygen values in the liquid broth were monitored; however, no measures (such as sparging the broth with N_2) were taken to maintain

Table 1. Definition of factors and levels in the experimental design for medium optimization in shake flask cultures. Levels are defined as low, medium and high. Concentrations are given in g L^{-1}

	Levels (g L^{-1})		
Factors	low	medium	high
Yeast extract	2	4	6
Soy peptone	2	6	10

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strict anaerobic conditions throughout the process. Nevertheless, dissolved oxygen levels remained close to zero from the early exponential phase due to the microbial activity. Yields of LA on starch were calculated as the difference between the final and the initial amount (g) of LA in the fermentation broth, divided by the total amount (g) of starch added at the beginning of the process.

Multilevel factorial design for medium optimization

In order to identify the optimal medium composition, a multilevel full factorial design (3^2) was applied. Nine complex media were defined based on two factors (YE, SP) and three levels (low, medium, high) as shown in Table 1. These were compared with the standard medium used for the cultivation of *G. stearothermophilus* based on NB and YE as already described. Significant differences between means were evaluated with a multiple range test according to Fisher´s least significant difference (LSD) at the 95% confidence level.

HPLC analysis of sugars and organic acids

Sugars and organic acids were determined via HPLC in a Hewlett Packard series 1100 system, equipped with an ion exclusion column ION 300 (Transgenomic®, USA) heated at 45 °C and a refractive index detector (Agilent 1100, Agilent technologies, USA). The mobile phase consisted of a 0.005 mol L^{-1} H_2SO_4 solution with a flow rate of 0.325 mL min^{-1}.

Other analytical methods

In order to distinguish between the two optical isomers of LA an enzymatic assay kit (K-DLATE 06/08, Megazyme, Ireland) was used. Starch consumption was measured as the disappearance of blue color intensity given by the starch–iodine complex.[20]

Cell density was determined in a spectrophotometer by measuring the optical density (OD) at 600 nm. Cell counts were performed in a counting chamber (Thoma) after appropriate dilution. All measurements were performed in duplicate.

Enzyme assays

The extracellular activity of the bacterial amylase was determined from the cell-free supernatant of cultures grown aerobically on 0.1% w/v potato starch. After appropriate dilution (5–10-fold in bi-distilled water) 0.5 mL of the crude enzyme solution were incubated at 60 °C with 0.5 mL 1% w/v potato starch. After 20 min the reaction was stopped by the addition of 1 mL HCl 1 mol L^{-1}. Samples were transferred to 50 mL volumetric flasks and brought to volume with bi-distilled water and 2 mL of iodine (0.05% w/v I_2, 0.5% w/v KI) to develop the blue color. The OD was read at 620 nm after 20 min. The method was adapted from the original version of Fuwa.[20] One dextrinizing unit (U) is defined as the amount of enzyme that catalyzes the hydrolysis of 1 mg of iodine binding starch per min in the assay conditions. The measured values are expressed as follows:

$$U\ mL^{-1} = \frac{OD_{620\ BLANK} - OD_{620\ SAMPLE}}{OD_{620\ mg\ STARCH} * 20\ min\ * 0.5\ ml}$$

The effect of pH on the amylase activity was investigated preparing starch solutions in different acetate or phosphate buffers (0.1 mol L^{-1}) to cover pH values in the range 5–8. All assays were performed in duplicate.

RESULTS AND DISCUSSION

Strain selection

A total of 12 strains were screened for amylolytic activity and for the ability to form LA as the major product of anaerobic metabolism. Results are presented in Table 2. For ten of these strains by the end of the batch test no residual starch was measured, which indicates high enzyme activity. The substrate was converted mainly to LA and to variable minor amounts of formic acid (FA), acetic acid (AA) and ethanol (EtOH). A few strains released higher fractions of by-products (e.g. IFA 102, DSM 485 and 304) and were therefore considered unsuitable for the purpose of this work. In the other cases, the concentration of LA reached within 48 h ranged from 3.42 to 4.26 g L^{-1} from an initial amount of starch of 10 g L^{-1} (Table 2). Despite complete liquefaction of the starch the observed yields were generally low. This can be attributed to the fact that the medium was not buffered and acid production resulted in a decrease of pH. Based on the total LA production and the relative amounts of by-products, the strain *G. stearothermophilus* DSM 494 was selected for further studies.

Lactic acid batch fermentation from raw potato starch

Geobacillus stearothermophilus DSM 494 was tested in 3–l batch fermentations in a bioreactor at 60 °C in MRS medium. pH

Table 2. Overview on the consumption of starch and the formation of products for the listed strains of *Geobacillus stearothermophilus*

N	Strain	Residual starch	Lactic acid (g L^{-1})	Formic acid (g L^{-1})	Acetic acid (g L^{-1})	Ethanol (g L^{-1})	Final pH
1	DSM 297	no	3.86 ± 0.07	0.39 ± 0.08	0.37 ± 0.10	0.17 ± 0.04	5.32 ± 0.04
2	DSM 1550	no	3.48 ± 0.02	0.17 ± 0.03	0.45 ± 0.01	0.11 ± 0.03	5.31 ± 0.00
3	DSM 485	yes	0.35 ± 0.04	0.00 ± 0.00	0.24 ± 0.02	0.00 ± 0.00	6.79 ± 0.01
4	DSM 5934	no	3.42 ± 0.38	0.17 ± 0.09	0.35 ± 0.02	0.07 ± 0.03	5.37 ± 0.04
5	DSM 2313	no	3.66 ± 0.10	0.12 ± 0.08	0.40 ± 0.01	0.13 ± 0.02	5.43 ± 0.02
6	DSM 6790	no	3.73 ± 0.20	0.20 ± 0.10	0.48 ± 0.12	0.11 ± 0.03	5.33 ± 0.01
7	DSM 2357	no	3.95 ± 0.05	0.30 ± 0.01	0.28 ± 0.02	0.16 ± 0.04	5.31 ± 0.06
8	DSM 304	yes	0.24 ± 0.00	0.00 ± 0.00	0.42 ± 0.01	0.00 ± 0.00	6.79 ± 0.01
9	IFA 102	no	3.02 ± 1.06	0.18 ± 0.35	0.86 ± 0.26	0.18 ± 0.05	5.86 ± 0.58
10	IFA 9.1	no	4.41 ± 0.04	0.27 ± 0.07	0.20 ± 0.01	0.26 ± 0.01	5.31 ± 0.01
11	DSM 494	no	4.26 ± 0.02	0.20 ± 0.01	0.25 ± 0.00	0.21 ± 0.00	5.37 ± 0.07
12	DSM 22	no	3.83 ± 0.03	0.32 ± 0.12	0.38 ± 0.03	0.09 ± 0.00	5.35 ± 0.00

Cultures grown in shake flasks on NB 8 g L^{-1} - YE 4 g L^{-1} medium with 10 g L^{-1} raw potato starch. Errors are given as standard deviations (n = 2).

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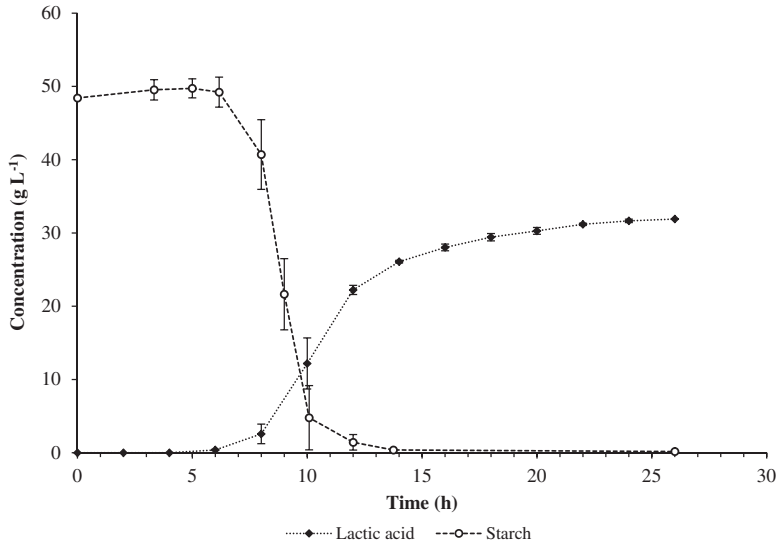

Figure 1. Starch consumption and lactic acid production trends during two batch fermentations at 60 °C and pH 7. Medium: MRS (de Man-Rogosa-Sharpe); C-source: raw potato starch. Error bars indicate the standard deviation (n = 2).

regulation and agitation enabled proper control of the conditions throughout the process, thus avoiding inhibition due to acidic conditions in the broth and prolonging the production of acid. In particular, it was possible to apply higher initial starch concentrations (50 g L^{-1}). With an inoculum of 1 mL volume the lag phase lasted *c.* 5–6 h.

During the exponential growth phase the starch was completely degraded, while the production of LA started until it stopped after approximately 24 h (Fig. 1). LA was produced up to a concentration of 31.9 g L^{-1}. The measured enantiomeric purity of the L-LA by the end of the fermentation was 98%. Considering the impurities (D-LA present in complex substrate compounds, e.g. yeast extract) the optical purity of L-LA produced by the strain was 99.5%. The optical purity of the product exceeds the required standards for technical grade L-LA (usually > 95% or > 97%). However, it does not fulfill the strict specifications for polymer-grade LA, where a purity higher than 99% is required to guarantee good mechanical properties for PLA.[21] It has to be addressed, however, that the reported values of optical purity of LA represent merely the ratio of the amount of L-LA over the total amount produced. These values are not indicative of the optical purity of the commercial product, since LA has not been purified from the fermentation broth.

The time course profile of LA production in this work using starch (Fig. 1) is comparable with the results reported by Danner *et al.*[18] using glucose as a substrate with the same strain. A detailed description of the levels of sugars (from the hydrolysis of starch) and of the metabolites produced during two parallel fermentations can be found in Table 3. It is evident that throughout the fermentation both glucose and maltose did not accumulate but were metabolized as soon as they were released. The minor products of the mixed acid fermentation (i.e. AA, EtOH and FA) were formed constantly during the fermentation and were not related to a specific growth phase. The maximum yield of LA was 47.9% ± 0.3%, which is partially due to the formation of co-metabolites. By the end of the fermentation process neither glucose nor starch was found in the broth, but residual maltodextrins could be observed in the chromatograms. This may be caused by a nutrient limitation or inhibitory effects exerted by the products. Therefore, a further medium optimization was conducted.

Medium optimization

Yeast extract, corn steep liquor and soy peptone as sources of organic nitrogen and growth factors were chosen as suitable

Table 3. Time course profile of the glucose and products of mixed acid fermentation released in two parallel batch fermentations at 60 °C on MRS (de Man-Rogosa-Sharpe) medium with 50 g L^{-1} raw potato starch as a sole carbon source at pH 7

Time (h)	0	3	6	8	10	12	14	25
Maltose (g L^{-1})	0.79 ± 0.01	0.79 ± 0.01	0.79 ± 0.01	0.70 ± 0.09	0.67 ± 0.03	0.41 ± 0.03	0.35 ± 0.03	0.29 ± 0.03
Glucose (g L^{-1})	0.06 ± 0.02	0.06 ± 0.01	< d.l.	< d.l.	0.44 ± 0.03	0.08 ± 0.00	0.04 ± 0.02	0.03 ± 0.01
Lactic acid (g L^{-1})	0.32 ± 0.01	0.31 ± 0.03	0.47 ± 0.00	1.75 ± 0.47	13.69 ± 2.46	23.78 ± 1.68	25.62 ± 0.18	31.91 ± 0.01
Formic acid (g L^{-1})	0.76 ± 0.07	0.70 ± 0.02	0.80 ± 0.01	0.75 ± 0.04	1.76 ± 0.04	2.81 ± 0.02	2.77 ± 0.02	2.90 ± 0.04
Acetic acid (g L^{-1})	3.70 ± 0.13	3.71 ± 0.04	3.61 ± 0.07	3.24 ± 0.41	3.73 ± 0.01	4.06 ± 0.06	3.91 ± 0.06	3.80 ± 0.04
Ethanol (g L^{-1})	0.90 ± 0.02	0.89 ± 0.02	0.91 ± 0.01	0.92 ± 0.06	1.89 ± 0.08	2.94 ± 0.20	3.11 ± 0.01	3.71 ± 0.05

Concentrations (g L^{-1}) are corrected according to final volumes for the dilution effect of the NaOH 10 mol L^{-1} added.
<d.l. 'stands for' below detection limits'. Errors are given as standard deviations (n = 2).

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candidates to fulfill the amino acid and vitamin requirements of *G. stearothermophilus*.[22,23] Preliminary results obtained from shake flasks and from batch fermentations showed that CSL could not enhance the production of LA. In fact, CSL performed worse than MRS and NB (as positive controls). In combination with YE, CSL led to even lower yields (results not shown). Also the effect of further addition of $MnSO_4$ – described as essential to some key enzymes for the metabolism of *G. stearothermophilus*[22] – was investigated, but appeared to be negligible (results not shown).

On the other hand, YE and SP showed a positive effect on LA production. The effects and the interactions of the two compounds were evaluated by a factorial experimental design.

The combination of SP and YE as nutrients for the production of LA provided highly satisfactory results. The statistical evaluation of the results obtained for cultures grown in shake flasks confirmed that there were significant differences in LA production among the 10 conditions tested (*P*-value of the F-test < 0.05). Moreover, as shown by the multiple range test at the 95% confidence level, almost all of the treatments performed better than the medium based on NB-YE used for conservation (Table 4). However, it appeared that too high concentrations of SP (10 g L^{-1}) had an inhibitory effect.

Some of the findings (e.g. the high yield of the SP 2 g L^{-1}-YE 2 g L^{-1} test) appear to be inconsistent, which may be explained by the limitations of experiments in shake flasks. Therefore, the most interesting treatments (SP 2 g L^{-1} - YE 2 g L^{-1}; SP 6 g L^{-1} - YE 2 g L^{-1}; SP 6 g L^{-1} - YE 6 g L^{-1}; SP 10 g L^{-1} - YE 6 g L^{-1}) were tested in 3 L batch fermentations with pH control. The general trends observed in shake flasks were confirmed in this new set of experiments: with increasing amounts of SP and YE higher titers of LA and higher productivities were obtained (Fig. 2). In particular, on MRS medium as well as on NB-YE the final titer of LA was 33.0 g L^{-1} (results not shown), similar to that obtained with SP 6 g L^{-1} - YE 2 g L^{-1}. The production of LA rose to 36.6 g L^{-1} with the medium SP 6 g L^{-1}- YE 6 g L^{-1}, with an increase in yield up to 66.2% ± 2.0%.

Table 4. Multiple range test (Fisher´s LSD, 95% confidence level) with soy peptone (SP) and yeast extract (YE) as factors affecting the production of lactic acid (LA) on raw potato starch

SP (g L^{-1})	YE (g L^{-1})	LA (g L^{-1})	Homogeneous groups
2	**2**	11.41 ± 0.24	def
2	**4**	9.57 ± 0.06	bcd
2	**6**	9.72 ± 0.15	cd
6	**2**	12.53 ± 0.82	ef
6	**4**	10.77 ± 1.76	de
6	**6**	13.12 ± 0.70	f
10	**2**	6.42 ± 0.15	a
10	**4**	7.28 ± 0.70	a
10	**6**	8.04 ± 0.47	abc
control		7.47 ± 0.02	ab

Comparison with the medium used for preservation (control): NB 8 g L^{-1} - YE 4 g L^{-1}. Errors are given as standard deviations (n = 2). Treatments with different letters in the homogeneous groups column have significantly different means ($P > 0.05$).

As expected, increasing the dose of SP to 10 g L^{-1} did not improve the titer and the yield, indicating that the vitamin and amino acid requirements were already fulfilled by the lower concentrated medium (SP 6 g L^{-1} - YE 6 g L^{-1}). This is supported by the data illustrated in Table 5, which compares the amino acid content of the different media with the optimum composition, as described by Rowe *et al.*[22] In extensive experiments the latter defined the amino acid composition of a medium that properly supported the aerobic growth of *G. stearothermophilus* NCA 1503. San Martin *et al.*[23] focused on the requirements for growth of *G. stearothermophilus* LLD-15 under anaerobic conditions and concluded that methionine, isoleucine, serine and glutamate were

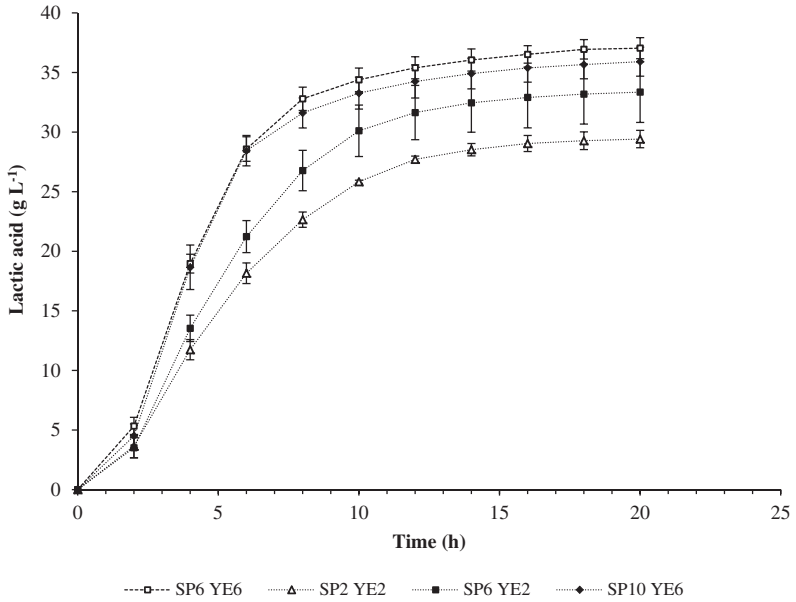

Figure 2. Effect of soy peptone (SP) and yeast extract (YE) on the production of lactic acid. Temperature: 60 °C; pH: 7; C-source: raw potato starch. SP6 - YE6 refers to a medium based on: SP 6 g L^{-1} - YE 6 g L^{-1}. Error bars indicate the standard deviation (n = 2).

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Table 5. Free amino acid content in the four media based on soy peptone (SP) and yeast extract (YE) tested in batch fermentations

Amino acid	ALA	ARG	ASN	ASP	CYS	GLU	GLN	GLY	HIS	ILE	LEU	LYS	MET	PHE	PRO	SER	THR	TRP	TYR	VAL
Reference (mg L^{-1})	84	64	50	130	50	400	50	50	42	100	164	140	52	86	100	140	84	30	56	126
SP 2 - YE 2 (%)	114	88	-	35	8	56	-	64	48	60	65	37	38	74	26	43	55	60	46	56
SP 2 - YE 6 (%)	162	169	-	51	16	100	-	88	86	92	106	60	62	126	34	74	83	100	82	87
SP 6 - YE 6 (%)	343	263	-	106	24	168	-	192	143	180	194	111	115	223	78	129	164	180	139	167
SP 6 - YE 10 (%)	390	344	-	122	32	212	-	216	181	212	235	134	138	274	86	160	193	220	175	198

Values according to the specifications in the technical data sheet of the respective products from Organotechnie. Reference as published by Rowe *et al.*[22] and values expressed as mg L^{-1}; all other values given as a percentage of the reference. SP6 - YE6 refers to a medium based on: SP 6 g L^{-1} - YE 6 g L^{-1}.

essential for the anaerobic metabolism of *G. stearothermophilus*. Table 5 shows that SP 6 g L^{-1} and YE 6 g L^{-1} provided the minimal dose required, especially with regard to those amino acids characterized as essential.

A higher viscosity in the YE/SP medium after the addition of starch caused difficulties in the control of T and pH. Increasing the inoculum volume to 10% v/v resulted in a good initial amylolytic activity and a shortened lag phase (Fig. 2).

pH dependence of the alpha amylase activity

In agreement with the observations of Pfueller and Elliott[24] and Wind *et al.*,[25] alpha amylase activity was found to be highest at pH 5.7 and reasonably good (above 90% of the maximum) in the range 5.3–6.7 (Fig. 3). At pH 7 the residual activity was 62.1%, but it decreased fast towards higher pH values. Activity values at a pH lower than 5 were considered irrelevant for these investigations, since all metabolic activities of DSM 494 are inhibited below this threshold value. For industrial production LA fermentations at lower pH levels are preferable because this reduces costs for the downstream processing.[6,26] Therefore, the observed optimum around pH 6 was considered very suitable. However, whether LA production was also optimal at pH 6 still had to be proven.

Influence of pH on LA fermentation

In a new set of experiments the impact of pH on LA production was investigated. As reported in Table 6, the production of LA (as well as co-metabolites) at pH 7 was higher than at pH 6. Final LA concentrations were 35% lower at pH 6 and 6.4 g L^{-1} of unconverted glucose were found by the end of the fermentation. It is well known from the literature[12,27,28] that the toxicity of LA is more pronounced at lower pH levels due to the effect of the undissociated form of the acid. This is the obvious cause for the observed limitation of product formation at pH 6.

The molar ratios of the metabolites at pH 7 and 6 were considerably different, being 7.6 LA : 1.0 FA : 0.4 AA : 1.1 EtOH versus 18.3 LA : 1.0 FA : 0.0 AA : 3.4 EtOH, respectively. A similar behavior was described by Hartley and Shama[29] and San Martin *et al.*,[30] who studied the anaerobic energy pathways of *G. stearothermophilus*, even though they focused on the fermentative production of EtOH. As suggested by these authors, the production and secretion of acids may be more difficult at lower pH since it goes against the pH gradient established by the cells between the extracellular and the intracellular environment to maintain the pH homeostasis. Moreover, acetic acid is a weaker acid than lactic acid and shows a stronger toxic effect at pH 6 due to the higher ratio of the undissociated form present already at high pH values.

At pH 8 the poor activity of the α-amylase resulted in high viscosity and the fermentation had to be aborted. For pH values above 7 the α-amylase activity of *G. stearothermophilus* drops quickly below 50% of the maximum (Fig. 3), therefore pH values higher than 7 should not be taken into consideration for this process.

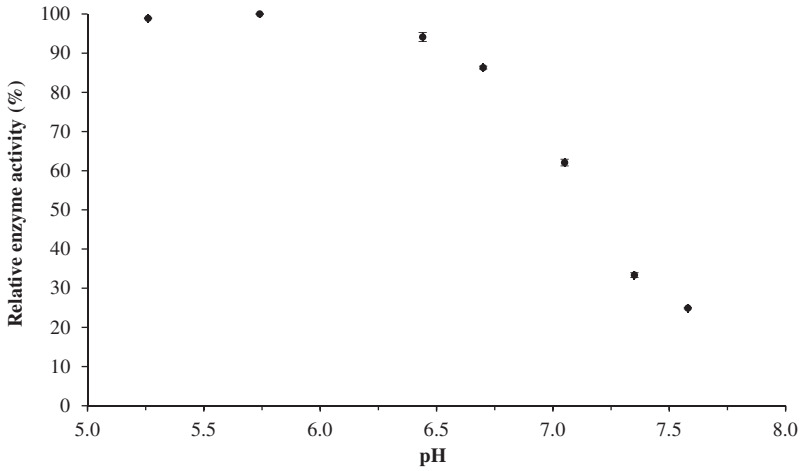

Figure 3. pH dependence of the extracellular alpha amylase from *G. stearothermophilus* DSM 494 at 60 °C. Values expressed as relative enzyme activity: 100% corresponds to 41.34 U mL^{-1}. Error bars indicate the standard deviation (n = 2).

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Table 6. Product formation and residual glucose (g L^{-1}) by the end of batch fermentations in the optimized medium with raw potato starch or potato residues as a C-source

	pH	Lactic acid (g L^{-1})	Formic acid (g L^{-1})	Acetic acid (g L^{-1})	Ethanol (g L^{-1})	Residual glucose (g L^{-1})
Raw potato starch	6	23.84 ± 0.54	0.67 ± 0.01	0.00 ± 0.08	2.27 ± 0.03	6.43 ± 0.05
Raw potato starch	7	36.62 ± 0.05	2.47 ± 0.30	1.17 ± 0.68	2.72 ± 0.07	0.47 ± 0.13
Potato residues	7	59.64 ± 0.28	0.52 ± 0.00	3.76 ± 0.48	1.92 ± 0.01	0.99 ± 0.34

Errors are given as standard deviations (n = 2 or n = 3).

Evaluation of process performances with raw potato starch as a C-source

The inhibitory effect of high concentrations of LA and of the co-metabolites was considered responsible for limiting the maximum LA concentration achievable at pH 7 to 37 g L^{-1}. In fact, a similar value, around 40 g L^{-1}, was also the maximum LA concentration reported by Danner *et al.*[18] with the same microorganism. As a result, the maximum yield of LA obtained in the optimized medium at pH 7 was 66.2% ± 2.0% and the total conversion of starch into LA and other metabolites was 77.8% ± 2.6%. Despite the undeniable limitation in yield, the process displays characteristics that are of particular interest concerning productivity and autoselectivity, as discussed below.

Among the examples of direct use of starch for LA production available in the literature and described in Table 7, Zhang and Cheryan[31] reached a concentration of LA of 93 g L^{-1}, with a productivity of 2.4 g L^{-1} h^{-1}, which is the highest reported so far. However, the production strain *Lactobacillus amylovorus* yielded a mixture of the L- and D-LA. Son and Kwon[32] also reported relatively high L-LA concentrations up to 71 g L^{-1} obtained from soluble starch by another *Lactobacillus* strain, namely *L. manihotivorans*, but in this case the productivity was very low: 0.6 g L^{-1} h^{-1}. Fossi *et al.*[33] used *Lactobacillus fermentum* achieving 53 g L^{-1} LA and a productivity of 1.1 g L^{-1} h^{-1}; no claims were made concerning the optical purity of the product. A few examples about the use of fungi for the direct conversion of starch to LA are also listed in Table 7. Among them, the best results were obtained by Yuwa-amornpitak and Chookietwattana[34] with Rhizopus *microsporus* reaching a concentration of 56 g L^{-1} pure L-LA and a productivity of 1.2 g L^{-1} h^{-1}. In comparison, the process described in this study for the direct production of LA with *G. stearothermophilus* has a productivity of 1.8 g L^{-1} h^{-1}, which is the second best only after Zhang and Cheryan[31] and has the economic and technical advantage of requiring no sterilization and of being run under non-sterile conditions throughout the whole process. Within this study more than 30 runs at the given temperature of 60 °C and of 1–2 days duration were conducted and the process proved to be robust and not susceptible of contaminations. This was confirmed on the one hand by microscopic inspections, but more importantly by the fact that no change in the production pattern of the metabolites was observed between the replicates. This aspect is of great advantage at an industrial scale, where keeping aseptic conditions is difficult and linked to high costs, whereas non-sterile handling makes the process easier and flexible. Two examples of the production of LA in non-sterile conditions were recently reported by Ouyang *et al.*[9] and Ma *et al.*[35] The former achieved a concentration of 73.03 g L^{-1} of LA with a productivity of 1.04 g L^{-1} h^{-1} with a *Bacillus* strain from a lignocellulosic hydrolyzate. The latter produced 97 g L^{-1} L-LA (>99% optically pure) with *Bacillus coagulans* at 50 °C from an enzymatic hydrolysate of tapioca starch and excess sludge hydrolysate as a N-source; the reported productivity was 2.8–3.1 g L^{-1} h^{-1}.

Even though a final concentration of 40 g L^{-1} of lactic acid is surely not attractive for industrial applications, the current process can be substantially improved by applying the following strategies. First, as already done in other studies focusing on ethanol production,[10] it is possible to knock out the genes responsible for the production of the co-metabolites. In this way, lactic acid yields can be increased. Moreover, the volumetric productivity of the process may be increased in continuous fermentations with membrane bioreactors. The fact that the process does not require strict sterile conditions in order to run stable would be of further advantage in this context. Finally, by integrating the purification step

Table 7. Examples of direct conversion of starch to lactic acid (LA) available in the literature

Microorganism	LA(g L^{-1})	Yield (%)	Productivity(g L^{-1} h^{-1})	L-LA purity	T (°C)	Sterile	Reference
*Lactococcus lactis**	19	94	0.8	high (–%)	30	yes	Bhanwar *et al.*[37]
Lactobacillus fermentum	53	-	1.1	-	45	yes	Fossi *et al.*[33]
Lactobacillus paracasei	37	93	0.8	92.5%	45	yes	Petrova and Petrov[38]
Enterococcus faecium	17	93	1.1	98.6%	30	yes	Shibata *et al.*[39]
Lactobacillus manihotivorans	71	85	0.6	100.0%	30	yes	Son and Kwon[32]
Lactobacillus amylophilus	33	85	0.3	high (–%)	37	yes	Vishnu *et al.*[40]
Rhizopus oryzae	26	87	0.4	100.0%	28	yes	Xiao *et al.*[41]
Rhizopus microsporus	56	-	1.2	100.0%	40	yes	Yuwa-amornpitak and Chookietwattana[34]
Lactobacillus amylovorus	93	77	2.4	low (–%)	40	yes	Zhang and Cheryan[31]
Geobacillus stearothermophilus	37	66	1.8	98.0%	60	no	This study

*in a dialysis bioreactor.

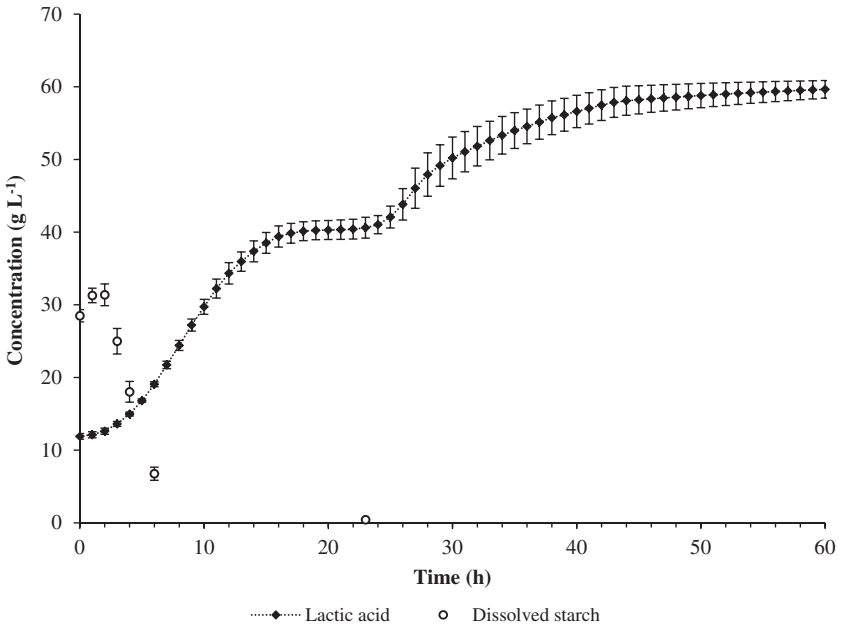

Figure 4. Time-course profile of starch consumption and lactic acid production during batch fermentations of potato residues as a C-source in soy peptone-yeast extract medium at 60 °C and pH 7. Error bars indicate the standard deviation (n = 3).

with the fermentative process (e.g. by ion exchange, electrodialysis or extraction) it would also be possible to reduce the amount of NaOH necessary to neutralize the product formed, since this would be continuously removed from the fermentation broth.[36]

The present work is the first example of LA fermentation boasting the combined features of the direct conversion of starch and non-sterile operational conditions.

Fermentation of potato residues as a C-source

When raw starch in the optimized medium was substituted with untreated potato residues as a C-source, *G. stearothermophilus* succeeded in the hydrolysis of the starchy substrate and its conversion to LA, as shown in Fig. 4 and Table 6. The given value for the starch concentration at the beginning of the process is lower than the actual total starch concentration in the medium because only the dissolved fraction could be measured. Beside 6.3% starch the potato residues also contained 0.5% LA. Therefore, the initial LA concentration in the fermentation medium was 11.9 g L^{-1} ± 0.35 g L^{-1} (46.5% L-LA). Consequently, although L-LA was produced during the course of the fermentation with an optical purity of 99.5%, the overall amount of L-LA by the end of the process was 89.5%.

The time-course profile of the first 20 h of fermentation resembled the observations at similar conditions (T = 60 °C, pH 7) on raw potato starch, with the only difference being the initial LA concentration. As evident in Fig. 4, after 18 h the production of LA stopped once an LA concentration of 40 g L^{-1} was achieved. This behavior was observed in previous experiments (data not shown) independently from the carbon source and also at higher substrate concentrations. In contrast to the experiments with raw starch, in this case a lag phase of 5 h occurred, during which glucose accumulated. After this period the production of LA restarted with a similar pattern of metabolites, until a concentration of 59 g L^{-1} LA was reached after 48 h. The process was run for another 12 h, with little increase in the production: 60 g L^{-1} LA was reached after 60 h. The total productivity during the first 18 h was 1.6 g L^{-1} h^{-1} and it decreased to 1.0 g L^{-1} h^{-1} considering a production period of 48 h.

Apparently the lag-phase served as a period of adaptation for the strain to high concentrations of LA, once the threshold value of 40 g L^{-1} was reached. This behavior was never observed with raw potato starch as a substrate even after 48 h but may depend on the presence of additional nutrients provided by the potato residues.

A higher fraction of the substrate may have been more easily available to the strain due to structural differences in the starch. Measurements showed that the percentage of amylose in the potato residues was 47% compared with 26% in the potato starch. Therefore, the starch in the potato residues is degraded to a higher extent and more fermentable sugar is released by the bacterial amylases. This explains the release of additional glucose during the lag phase after 18 h and the higher overall LA concentration in this case. Consequently the yield of LA at 48 h was 81.2%, which is considerably higher than the results obtained for raw starch (66.2%).

CONCLUSIONS

An effective process for the direct conversion of starch to lactic acid with *G. stearothermophilus* DSM 494 at 60 °C was developed. 37 g L^{-1} optically pure (98%) L-lactic acid were obtained in 20 h from 50 g L^{-1} raw potato starch. As co-metabolites smaller amounts of acetic acid, formic acid and ethanol were formed. In comparison with most literature data on similar direct fermentation processes high productivity (1.8 g L^{-1} h^{-1}) was obtained from raw potato starch. Moreover, the high growth temperature of the selected species provided distinct techno-economic advantages, e.g. no sterilization of the medium required, non-sterile operational conditions and a lower cooling demand throughout the

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fermentation. Finally the suitability of the process was demonstrated using an actual 'zero value' waste product, potato residues from food processing. With this substrate even higher lactic acid concentrations (59 g L^{-1}) were achieved with a yield of 81.2%.

ACKNOWLEDGEMENTS

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The authors especially thank Mr Marcus Pruckner for his valuable technical support.

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6. Conclusions and outlook

This work contributes to demonstrate the feasibility of a more sustainable production of lactic acid, based on the utilization of agro-industrial residues rather than fossil resources or first generation biomass feedstock. Different fermentation processes were set up on a laboratory-scale (up to 3-L working volume) using residues from the potato processing or wheat bran extracts from second-stage hydrolysis as feedstock. These substrates were chosen among others after a preliminary characterization.

Potato residues were investigated within the European project “CARBIO”. They can be considered as a suitable substrate for large-scale lactic acid production for a number of reasons, namely that they are generated in large amounts, provide starch as a carbon source for fermentation and also additional nutrients for microbial growth.

The production of lactic acid from potato residues has been pursued with two different approaches. Firstly, a conventional process with enzymatical hydrolysis of the starch-rich substrate followed by fermentation with *L. casei* was established. As feedstock, only the liquid fraction of the hydrolysate was used. Within this process, high product yields (92%) and titers (102 g/L) that meet current industrial requirements were obtained. Thus, the potential of starchy residues for the development of an easy and effective process for the production of lactic acid was demonstrated. Alternatively, *G. stearothermophilus* was used to develop an innovative process that combines the direct conversion of starch with thermophilic lactic acid fermentation. Differently from the case presented above, minor amounts of acetic acid, formic acid and ethanol were formed as co-metabolites, thus limiting product yield to 81% on potato residues. Productivities obtained in this study (1.0 g/L h) were satisfyingly better than most literature data on similar direct fermentation processes, even if they do not yet suffice for commercial applications. The most interesting feature of this strategy is represented by the distinct techno-economic advantages offered by the high growth temperature of the selected species. It was demonstrated that a stable process could be conducted in non-sterile conditions and without medium sterilization. Moreover, it is also expected that working at 60 ℃ reduces the cooling demand throughout the exothermic formation of lactic acid.

The information collected in these two studies offered new insights into the valorization of starchy residues as feedstock for lactic acid production. Results pointed out, however, that the fast-degrading nature of the substrate exclude applications of the lactic acid obtained from this source as a monomer for polymerization to poly (lactic acid). Uncontrolled fermentation by indigenous microorganism during transport and storage led to the formation of both D- and L- lactic acid that impaired final chiral purity.

Besides this, the fate of the solid fraction separated after starch hydrolysis (in the first study) or after fermentation (direct conversion by *G. stearothermophilus*) should be critically evaluated. A possible use is for instance in anaerobic digestion (FABbiogas, 2016). Furthermore, in a holistic perspective, the local availability of this raw material must also be addressed. The figures from the published studies show that potato residues cannot be the only raw material in use in this biorefinery. In fact, they would not support a commercially reasonable yearly lactic acid throughput. Thus, a process that can flexibly use a variety of locally available residues is required.

Alternatively to starchy feedstock, other opportunities were pursued by testing lignocellulosic raw materials. For this purpose, defatted and de-starched wheat bran hydrolysate was used as a substrate for lactic acid fermentation with *L. pentosus* . The substrate was provided by the Department of Food Science and Technology (BOKU, Vienna). Differently from the cases described above, the processes were run without media supplementation. It has to be noticed, however, that also sugar concentrations and thus expected lactic acid formation were significantly lower in this series of experiments. With *L. pentosus* a high consumption of glucose was observed, but a slower and not full consumption of xylose and arabinose, which also limited productivities (0.3 g/L h). The results presented in this work suggest targets for the screening and engineering of microbial strains. The latter should homofermentatively convert and efficiently use not only hexose but also pentose sugars, since both are released from the hydrolysis of lignocellulosic raw materials. A limiting aspect related to this substrate is the relatively low concentration of sugars (26 g/L). Attempts to increase the sugar concentration (58 g/L) resulted however in higher inhibition, as discussed in the related paper.

Overall, it can be concluded that starchy feedstock can be easier implemented and perform better than lignocellulosic ones for lactic acid production. Of course, this does not exclude that the gap can be minimized through a better pretreatment technology and more convenient microbial strains.

Generally, the published works underline a series of difficulties arising when residues from the food processing – rather than refined sugars – are used as raw materials for lactic acid fermentation. The main issues faced within these studies were caused by the chemical and physical nature of the residues and included: lack of homogeneity, high water content, logistics of conservation as well as the presence of compounds in the substrate that are inhibitive for the intended microbial strains. In particular, the following inhibitors were addressed: phenolic compounds present in the potato peels, furfural and acetic acid developed during the pre-treatment of wheat bran. Possible strategies to mitigate the inhibitive effects of phenolic compounds have also been discussed and data about the

observed degradation of furfural throughout fermentation have been presented. The findings and considerations presented here are seldom found in literature, despite their crucial importance in the evaluation of biorefinery processes.

Results also show how microbial strains play a crucial role in the effectiveness of a bio-based process. In fact, besides the standard requirements for an efficient lactic acid production (i.e. high yields, productivities, and chiral purity of the product) additional capabilities of the microbial strains are desirable when using residues as feedstock: efficient use of pentose sugars, direct conversion of starch, performance under thermophilic conditions and in presence of inhibitors. These qualities are strategic when it is intended to utilize residues from multiple sources as a substrate, as typical for a biorefinery.

From the data collected, it clearly emerged that process yields and economics must be further improved in order to establish sustainably-produced bio-based lactic acid as a competitive product on the market. Moreover, like in a classical petrochemical refinery, economical benefits must be pursued through the implementation of several production segments to offer a broad product range. This means first of all, that feedstock for the fermentative production of lactic acid must be differentiated, maximizing the use of local resources. For instance, residues from the food processing may be used together with other agro-industrial residues or municipal wastes. Secondly, production processes must be integrated into a wider context, expanding the product portfolio (i.e. deliver of other products besides lactic acid) and valorizing all side streams. Moreover, different forms of energy can be co-produced. Finally, in this way it is possible to take advantage of economies of scale and synergistic effects.

With regard to the abovementioned points, the concept promoted by Cellulac (see 2.2.1) appears to address this issue adequately. Of course, whether the developed technology is already mature for commercial deployment must still be proven. Nevertheless, there is evidence of growing awareness also within the industrial community that pushes the developement and integration of new technologies for more sustainable and profitable biorefineries.

List of Publications

Original Research Articles in SCI Journals

Smerilli, M., Neureiter, M., Haas, C., Frühauf, S., Fuchs, W. 2016. Valorization of Potato-processing Residues for the Production of Lactic Acid. *Chemical and Biochemical Engineering Quarterly*, **30**(2), 255-263.

Haas, C., El-Najjar, T., Virgolini, N., Smerilli, M., Neureiter, M. 2016. High cell-density P3HB production in a membrane bioreactor. *New Biotechnology*, **33**, Supplement, S7-S8.

Smerilli, M., Neureiter, M., Wurz, S., Haas, C., Fruehauf, S., Fuchs, W. 2015. Direct fermentation of potato starch and potato residues to lactic acid by *Geobacillus stearothermophilus* under non-sterile conditions. *Journal of Chemical Technology and Biotechnology*, **90**(4), 648-657.

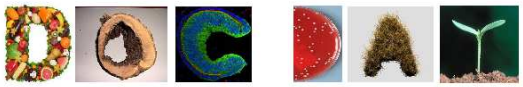
Tirpanalan, Ö., Reisinger, M., Smerilli, M., Huber, F., Neureiter, M., Kneifel, W., Novalin, S. 2015. Wheat bran biorefinery – An insight into the process chain for the production of lactic acid. *Bioresource Technology*, **180**, 242-249.

Haas, C., Steinwandter, V., De Apodaca, E.D., Madurga, B.M., Smerilli, M., Dietrich, T., Neureiter, M. 2015. Production of PHB from Chicory Roots - Comparison of Three Cupriavidus necator Strains. *Chemical and Biochemical Engineering Quarterly*, **29**(2), 99-112.

Haas, C., El-Najjar, T., Virgolini, N., Smerilli, M., Neureiter, M. 2017. High cell-density production of poly(3-hydroxybutyrate) in a membrane bioreactor. *New Biotechnology*, **37**, 117-122.

Oral presentations

Smerilli M., Haas C., Frühauf S., Pruckner M., Neureiter M. 2014. Production of lactic acid from potato residues from food processing, 2nd DocDay, Tulln an der Donau, Austria



Abstract

In the region of Navarra (Spain) $2.0 \cdot 10^6$ kg of waste potato peels and puree accumulate as food processing residues every year. They are collected from the local food industries between July and September and currently represent only a disposal burden.

Potato residues are rich in starch (46% in dry matter), which constitutes a valuable carbon source for many microbial products. Among them lactic acid (LA) is particularly promising, since the worldwide production capacities are continuously increasing since two decades and producers are interested in origins not in competition with food and feed. Furthermore, variable amounts (5 ± 2% in dry matter) of LA are found already in the potato by-products.

The potato mash was treated with commercial amylases at 60-70 ℃ for one day in order to release glucose (167 g/L) from starch. The hydrolysate was used as the substrate in 3 L batch fermentations with *Lactobacillus casei* at 37 ℃ and pH 5.5. After 66 hours all the glucose was used up and the final LA concentration was 102 g/L, with a yield of 92%. The optical purity of the product (L-LA) was only 92%, mainly due to the amounts of D-LA already present in the potato by-product. The produced LA is not suitable for the PLA industry, but it meets the current standards for technical grade LA.

Poster presentations

Smerilli M., Wurz S., Haas C., Frühauf S., Pruckner M., Neureiter M. 2014. Microbial production of lactic acid from starch and starch-rich residues: a comparison of two strategies. ProcessNet-Jahrestagung und 31. DECHEMA-Jahrestagung der Biotechnologen, Aachen, Germany

Abstract

Lactic acid is since long widely applied in several industrial sectors, but more recently the growing interest in poly-lactic acid (PLA) and environmental-friendly solvents (ethyl lactate) determined its considerable market expansion. Nowadays lactic acid is almost exclusively produced via microbial fermentation from refined sugars like glucose or sucrose. However, the use of starch as a substrate is also a possibility and more research could be focused on the use of agro- and food-residues. These are cheap and abundant resources, but require special pretreatments.

The valorization of food-processing residues was actually the aim of the European LEAD-ERA project CARBIO. In particular, in the region of Navarra (Spain) $2.0 \cdot 10^6$ kg of puree and peel wastes per year are collected from the local potato-processing industries. Within this project a comparison of two strategies for the valorization of starch-rich products was conducted with the aim of producing lactic acid of suitable grade for the emergent PLA industry. The first option is the conventional enzymatic hydrolysis of the starch fraction contained in the waste products (49% w/w dry weight) in order to release free glucose in a process similar to that well established for the hydrolysis of refined starch. The glucose-rich syrup (170-180 g/L) obtained after this process was supplemented with additional nutrients (yeast extract and corn steep liquor) and used in a fermentation process with *Lactobacillus casei* to produce L-lactic acid (92%) up to a concentration of 100 g/L.

An alternative is represented by the use of an amylolytic and thermophilic strain, namely *Geobacillus stearothermophilus,* that directly converts starch to lactic acid at optimum temperatures between 55 and 60 ℃, which allows to operate the process also at non-sterile conditions. The chiral purity of the L-lactic acid was 98%, but minor amounts of other organic acids were produced as well, thus limiting product yields to ca. 70%. Best results were obtained from 50 g/L starch, which could be converted to 37 g/L lactic acid in 20 hours.

While the first process meets already the current standards in industrial production of lactic acid, further improvements regarding yields and final product concentrations are instead required in the second one. Still the latter attracts a certain interest for being independent of costly enzyme formulations and sterile handling.

Smerilli M., Haas C., Frühauf S., Neureiter M. 2014. Production of lactic acid from waste potato residues from food processing. European Biorefinery Training School, Budapest, Hungary.

3rd European Biorefinery Training School

Abstract

In the region of Navarra (Spain) 2000 ton of waste potato peels and puree are accumulated as food processing residues every year. These are collected from the local food industries between July and September and currntly represent only a disposal issue, since they find no utilization. The waste biomass is rich in starch (46 g /100 g dry solids), which constitutes a valuable C-source for several microbial products, especially organic acids. Lactic acid (LA) is a favored product of this biorefinery, since epending on the storage conditions variable amounts (6 ± 2 g/ 100 g dry solids) of LA are found already in the potato residues. The potato mash was treated with commercial amyases at 60–70 ℃ for 27 hours in 2.5 L batches in order to release glucose from starch, which is otherwise not readily used as a substrate by most LA bacteria. The glucose concentration obtained was ca. 176 g/L. Afterwards the mash was centrifuged to separate the liquid fraction, which is more easily fed in a bioreactor. The liquid hydrolysate was supplemented with N- and vitamin B-rich yeast extract and corn steep liquor, in order to fulfill the metabolic requirements of *Lactobacillus casei*, the strain selected for the fermentation process. The latter took place at 37 ℃ in 3 L batches; pH was controlled to 5.5 with NaOH 10 M. After 66 hours all the glucose (102.3 g/L initially) was used up and the final LA concentration measured was 99.8 g/L, for a yield in LA of 92%. Despite the fact that the strain used is stereoselective for L-LA, the optical purity of the product was limited by the random amounts of D-LA present in the potato residues. Therefore, the product is not suitable for the PLA industry, where the optical purity is an important requisite for the mechanical properties of the polymer. However, the applications of LA are multiple and diverse, and the process described here meets the current standards for the industrial production of LA of technical or food grade.

Smerilli M., Wurz S., Haas C., Frühauf S., Neureiter M. 2014. Continuous production of lactic acid from starch by Geobacillus stearothermophilus: process design and reactor set-up. RRB-10 – 10th International Conference on Renewable Resources and Biorefineries, Valladolid, Spain

Abstract

Geobacillus stearothermophilus DSM 494 is a suitable strain for the production of lactic acid from several carbon sources including starch and xylose, which are abundant in biomass residues. It is a fast growing thermophilic bacterium with growth optimum between 55 and 60 ℃ and in batch processes the lag phase is usually extremely short. In presence of starch the strain produces amylolytic enzymes. The corresponding liquefaction and saccharification results in the formation of sugars which are subsequently fermented to lactic acid. A high chiral purity (98% L-lactic acid) makes the product interesting for polymer applications (PLA). In previous experiments 37 g/L lactic acid could be produced from 50 g/L starch within 20 hours and high productivities (7 g/L h) were observed during the exponential growth phase.

The aim of this work was to establish a continuous process for the direct production of lactic acid from starch, which is operated at non-sterile conditions at 60 ℃ and pH 7. The process is run in a 20 L-bioreactor (working volume: 12 L). In order to increase the volumetric productivity the system is equipped with a ceramic microfiltration module for the recycle of cells and unconverted sugars and/or starch. Dissolved fresh starch and a separated nutrient solution are continuously fed into the reactor and permeate is withdrawn at the outlet of the membrane module at the corresponding flow rate to guarantee the required dilution rate. In addition, pH control with NaOH and a cell bleed are implemented.

The developed set-up turned out to work satisfactorily for studying the effects of the major parameters on the production of lactic acid.

Smerilli M., Frühauf S., Neureiter M. 2013. Direct conversion of starch to lactic acid by *Geobacillus stearothermophilus*. Ghent Biobased Economy Summer School, Ghent, Belgium

Abstract

Lactic acid (LA) is a commodity chemical whose market has significantly expanded thanks to the most recent applications in green solvents (mainly ethyl lactate) and for the production of compostable plastics, namely polylactic acid (PLA). With regard to biorefineries, the development of a LA fermentation process from starch-rich vegetable residues looks attractive. Starch is particularly suitable as feedstock for the production of LA and it is already widely used for this purpose, even though enzymatic pretreatments for liquefaction and saccharification are required.

After the screening of fifty LA-producing bacteria, the thermophile *Geobacillus stearothermophilus* DSM 494 was selected for its capability of growing directly on potato starch as a sole carbon source thanks to its amylolytic activity, and of converting it to LA at temperatures around 60 °C. Another characteristic of this strain is the high selectivity (98%) for the L- optical isomer.

In 3 L lab-scale batch fermentations *G. stearothermophilus* DSM 494 produced up to 37 g/L LA. However, the production of other metabolites including ethanol, formic acid and acetic acid limited the yields to ca. 66%.

This strain is of great interest not only because it allows saving the costs for the purchase of amylolytic enzymes, but also it reduces process time and complexity thanks to the fact that no sterile handling is necessary to operate the process at 60 °C. Research on this topic is still in progress. The next steps include the scale-up to pilot scale and the establishment of a continuous process.

Smerilli M., Frühauf S., Neureiter M. 2013. Residues from vegetable processing for the production of bioplastics. RRB-9 – 9th International Conference on Renewable Resources and Biorefineries, Antwerp, Belgium

Abstract

Environmental issues, increasing oil prices and the introduction of environmental policies demand increasing efforts in order to develop sustainable and cost effective production processes for eco-friendly plastics.

The European LEAD-ERA project “CARBIO” aims to convert low-valued waste into valuable compounds, namely polylactic acid (PLA), polyhydroxybutyrate (PHB) and cellulose nanoparticles. The final purpose is to employ films of the above mentioned bio-based and compostable polymers for food packaging applications. The optimal use of the substrates and the development of effective and innovative production processes to keep prices competitive on the market are among the challenges.

Within this project, residues from vegetable processing are evaluated for their potential to produce a suitable substrate for fermentation processes. In particular, potato peels, sweet corn and chicory roots, collected from diverse food-industries in the region of Navarra (Spain), have been investigated. Thermochemical and enzymatic hydrolysis steps are developed in order to convert these biomass residues into valuable substrates for the fermentative production of lactic acid and PHB.

For the production of lactic acid an enzymatic hydrolysis process has been established to convert the starch contained in the potato peels (44% w/w based on dry weight) into concentrated glucose syrup (200 g/L), which is subsequently recovered as liquid fraction. Yields of this conversion process are around 65%. After sterilisation the hydrolysate can be directly applied for the production of lactic acid. In order to meet the demanding nitrogen and vitamin requirements of *Lactobacillus* species, the potato hydrolysate is supplemented with corn steep liquor and yeast extract. A factorial experimental design was used to optimize the medium composition. It turned out that the nutrient composition has a relevant impact on the process time. An optimized medium for a 3 L batch fermentation was prepared from hydrolysate of ca. 11 kg of fresh potato wastes and supplements and during the fermentation process a lactic acid concentration of around 100 g/L could be obtained in 60 h.

Smerilli M., Frühauf S., Neureiter M. 2013. Direct conversion of starch to lactic acid by *Geobacillus stearothermophilus*. Symposium on Biotechnology for Fuels and Chemicals, Portland, OR, USA

Abstract

Microorganisms capable of direct conversion of starch to lactic acid (LA) open new horizons in the scenario of alternative production processes. The major advantages are: saving costs for the purchase of amylolytic enzymes, reduction of process time and process complexity.

After the screening of fifty LA-producing bacteria, a *G. stearothermophilus* strain was selected for its capability of growing on potato starch as sole carbon source, and of converting it to LA with high selectivity for the L-optical isomer. Another characteristic of this strain is the optimum of growth and activity at temperatures between 55 and 60 ℃.

In this study a thermophilic process for direct use of starch with *G. stearothermophilus* is compared to a traditional LA process based on glucose operated at 37 ℃ with *L. casei*. While in the latter case glucose is converted with yields around 90%, in the thermophilic process yields on total starch are never higher than 60%, but productivities are 4-fold increased and no sterile handling is required. In 3 L lab-scale batch fermentations with pH control *L. casei* IFA 32 produced 108 g/L LA in 66 hours in MRS medium enriched of glucose with a yield of 88%. *G. stearothermophilus* IFA 301 produced instead 32 g/L LA in 24 hours (MRS medium without glucose) and 46 g/L LA in 13 hours (NB/YE medium) with yields of 58% and 50% respectively.

Research on this topic is still in progress. The next steps include the scale-up to pilot scale and the establishment of a continuous process

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List of Abbreviations

ASTM	American Society for Testing and Materials
DDGS	Dried distillers grains with solubles
EMP	Embden-Meyerhof-Parnas
PE	Polyethylene
PHB	Polyhydroxybutyrate
PLA	Poly (lactic acid)
PP	Polypropylene
SCI	Science Citation Index

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