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Master Thesis

Production and Purification of L-Lactic Acid from Food Waste of the Municipality of Heraklion

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‘History in general is therefore the development of Spirit in Time, as Nature is the development of the idea in Space’

Georg Wilhelm Friedrich Hegel

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Abbreviation

LA	Lactic Acid
PLA	Polylactic Acid
BuLA	Butyl Lactate
FW	Food Waste
MFW	Municipal Food Waste
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PBS	Polybutylene Succinate
PVA	Polyvinyl Alcohol
LAB	Lactic Acid Bacteria
ATP	Adenosine Triphosphate
NADH	Nicotinamide Adenine Dinucleotide (reduced)
PG	Phosphogluconate
PK	Phosphoketolase
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
TLC	Thin Layer Chromatography
NMR	Nuclear Magnetic Resonance
OD	Optical Density
RI	Refractive Index

Abstract

Societies around the world are facing serious challenges due to the thoughtless use of plastics derived from petrochemical derived synthetic polymers. In addition to the extensive use of oil sources, all these plastics end up in the ecosystem as industrial waste, resulting in a large environmental problem. According to the Food and Agriculture Organization (FAO), almost one-third of the total food produced annually is getting lost or wasted, in the food supply chain. Any solutions to these issues are required in order to reduce this tremendous environmental impact.

In previous decades many authors described Lactic Acid (LA) production from several waste sources, including molasses, bakery waste, water waste, and sugar cane. Lactic acid, 2-hydroxypropanic acid, is an organic compound which is widely used in food, pharmaceutical and chemical industries. In addition, lactic acid can be polymerized to form the biodegradable and compostable polylactic acid (PLA), which is considered a potential substitute for oil-produced plastics.

The present study particularly focuses on the optimization of lactic acid production from Municipal Food Waste (MFW), for the development of a scalable synthetic process. More specifically, a sequence consisting of subsequent fermentations to convert endogenous D, L-lactic acid from minced foods to optically pure L-lactic acid, followed by purification of L-lactic acid by ammonium lactate (NH_4LA) esterification and hydrolysis of the purified ester. These steps were performed in order to achieve optimization of bioplastic production for the municipal food waste of Heraklion and to obtain useful information for the design of a pilot unit. The yields and limitations of this process will be discussed.

Key words: Food waste, L-lactic acid, fermentation, bioplastic

Περίληψη

Οι κοινωνίες αντιμετωπίζουν σοβαρά προβλήματα λόγω της αλόγιστης χρήσης πλαστικών που προέρχονται από πετροχημικά συνθετικά πολυμερή. Εκτός από την εκτεταμένη χρήση πηγών πετρελαίου, όλα αυτά τα πλαστικά καταλήγουν στο οικοσύστημα ως βιομηχανικά απόβλητα, με αποτέλεσμα να δημιουργείται ένα τεράστιο περιβαλλοντικό πρόβλημα. Σύμφωνα με τον Οργανισμό Τροφίμων και Γεωργίας, σχεδόν το ένα τρίτο του συνόλου των τροφίμων που παράγονται ετησίως χάνεται ή σπαταλάται στην αλυσίδα εφοδιασμού τροφίμων. Απαιτούνται λύσεις σε αυτά τα ζητήματα προκειμένου να μειωθεί αυτή η τεράστια περιβαλλοντική επίπτωση.

Τις προηγούμενες δεκαετίες πολλοί συγγραφείς περιέγραψαν την παραγωγή γαλακτικού οξέος (LA) από διάφορες πηγές αποβλήτων, όπως μελάσα, απορρίμματα αρτοποιίας, απόβλητα νερού και ζαχαροκάλαμο. Το γαλακτικό οξύ, 2-υδροξυπροπανικό οξύ, είναι μια οργανική ένωση που χρησιμοποιείται ευρέως στις βιομηχανίες τροφίμων, φαρμάκων και χημικών. Επιπλέον, το LA μπορεί να πολυμεριστεί για να σχηματίσει το βιοαποικοδομήσιμο και λιπασματοποιήσιμο πολυγαλακτικό οξύ (PLA), το οποίο θεωρείται πιθανό υποκατάστατο των πλαστικών που παράγονται από πετρέλαιο.

Η παρούσα μελέτη εστιάζει ιδιαίτερα στη βελτιστοποίηση της παραγωγής του γαλακτικού οξέος από τα αστικά υπολείμματα τροφίμων, για την ανάπτυξη μιας επεκτάσιμης συνθετικής διαδικασίας. Πιο συγκεκριμένα, χρησιμοποιήθηκε μια ακολουθία επεξεργασίας τροφικών αποβλήτων που αποτελείται από ζυμώσεις για τη μετατροπή του ενδογενούς D, L-γαλακτικού οξέος από πολτοποιημένα τρόφιμα σε οπτικά καθαρό L-γαλακτικό οξύ, ακολουθούμενη από καθαρισμό του L-γαλακτικού οξέος με εστεροποίηση του γαλακτικού αμμωνίου και υδρόλυση του απομονωθέντος εστέρα. Αυτά η αλληλουχία διεργασιών πραγματοποιήθηκε ώστε να να επιτευχθεί βελτιστοποίηση της παραγωγής βιοπλαστικών και να εξαχθούν χρήσιμες πληροφορίες για το σχεδιασμό μίας πιλοτικής μονάδας. Θα συζητηθούν οι αποδόσεις καθώς και οι περιορισμοί αυτής της διαδικασίας.

Λέξεις κλειδιά: Υπολείμματα τροφών, L-γαλακτικό οξύ, ζύμωση, βιοπλαστικά

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Chapter 1: Introduction

1.1 Food Waste

Food is undoubtedly a valuable commodity and its production can be rich in nutrient resources. The survival and existence of life around the world directly depends on the food supply. However, the ever-changing lifestyle and rapid urbanisation of the worldwide population have led to increased production of food waste from various sources (agricultural, household, industrial).[1] The term food waste (FW), refers to any leftover or precooked food which generates biodegradable organic waste.[1] According to the definition given by the Food and Agriculture Organization of the United Nations (FAO), food waste concerns "*food losses of quality and quantity through the process of the supply chain taking place in production, post-harvest, and processing stages*".[1] Food waste can be produced at any phase of the supply chain, from the agricultural area to the processing plant and eventually to the retail market.[2] A major concern arises when this indispensable commodity *i.e.* food, is misused and mismanaged at any stage of the food life cycle resulting in serious social, economic, and environmental consequences.[1] Waste management should practically follow certain policies that support the concept of 3R's, *i.e.* reduce, reuse, and recycle.[3] According to the FAO, almost one-third of the total food produced annually is lost or wasted in the food supply chain. This situation has raised serious concern as not only there is a loss of vital resources but their disposal within the environment causes important problems. Food waste comprises a heterogeneous mixture of carbohydrates (starch, cellulose, hemicellulose or lignin), proteins, lipids, organic acids, and smaller amounts of inorganic matter.[2] Nowadays, most food waste recycled, mainly as feed and fertiliser but traditional approaches of landfilling and incineration could cause severe environmental and human health hazards by generating toxic gases.[1] Significant emissions of methane (CH_4), which is 23 times more potent than CO_2 (greenhouse gas emissions), are produced by these approaches and contribute significantly to global climate change.[4] However, utilisation of waste components could create many possibilities for producing valuable chemicals, fuels and products.[3] Production of biofuels (biodiesel, ethanol, hydrogen), electric power generation through conventional methods like incineration, compost, and landfill, as well as bioconversion of food waste to value-added products like biosurfactants and bioplastics are some great examples of the sustainable waste management system.

Plastics are traditionally synthesized from petrochemicals through mostly irreversible processes.[5] Approximately 140 million tonnes of petrochemical derived synthetic polymers are produced worldwide with a large proportion of them ending up in the ecosystems as industrial waste products.[6] The last decades more and more countries apply banning of plastic bags responsible for the widely known ''white pollution" around the world and replace them with biodegradable bags. Petroleum-derived polymers are difficult to degrade by microorganisms, and their high persistence poses serious environmental concerns.[1] An alternative to these synthetic plastics is bioplastics derived from renewable sources, *e.g.* by utilising various waste products, microorganisms can synthesize these bioplastics.

In the recent years, there is a tendency to use bioplastics over common petrochemical-based plastics, to reduce environmental burden.[1] Replacement of petrochemical plastics with

bioplastics contributes to global warming reduction, as the energy requirement for petroleum-based synthetic plastic production (77 MJ/kg) is higher when compared to that for bioplastics (57 MJ/kg).[7] On the other hand, landfilling leads to undesirable consequences such as groundwater contamination and augmenting of greenhouse gas emissions, and food waste has major contribution in this aspect. Bioconversion of food waste to generate bioplastics is considered as an profitable strategy for waste disposal.[1]

The bioconversion of food waste to biodegradable plastics requires pre-treatment to enhance its biological and physico-chemical properties.[1] Pre-treatment strategies include physical, chemical, biological and enzymatic degradation.[1] Physical treatment converts food waste into fermentable organic compounds using thermal and mechanical processes, including heating, milling, ultrasound, and microwaves.[5] Fermentable sugars can be formed using chemical treatment, which includes acid or alkali treatment, or biological treatment, that includes several microorganism strains, and can be used as fermentable substrates.[1] Polyhydroxyalkanoate (PHA), polyhydroxybutyrate (PHB), polybutylene succinate (PBS), starch blends, polyvinyl alcohol (PVA) and polylactic acid (PLA) are among the major synthetic biodegradable polymers.[1]

It is widely known, that fermentation processes account for about 80 – 90 % of the global lactic acid production.[9] Lactic acid is a highly versatile platform chemical with a wide variety of applications that cover the food, pharmaceutical, cosmetic, detergent and dairy industries. More recently, the market for lactic acid has been expanded in the production of polylactic acid (PLA). This biodegradable polymer further fuelled interest in the production of lactic acid and, with a continuously growing market, it is estimated that global demand for lactic acid would rise from 1220 kt in 2016 to 1960 kt by 2025.[9] Despite the positive outlook, production costs still need to be reduced. The production of PLA in particular, which has to compete with conventional petrochemical-based plastics, is currently limited by the cost of lactic acid. It has been estimated that the cost of lactic acid production must be reduced by at least 50% for PLA to be competitive on the market.[10,11]

1.2 Polylactic acid

Due to environmental problems and ecological issues associated with petroleum-based polymers, biodegradable and renewable polymers have attracted a great deal of interest. PLA is a biodegradable thermoplastic polyester, widely studied over the last decades.[12] PLA is a compostable polymer derived from renewable resources. It can be synthesized via a polycondensation of lactic acid or a ring-opening polymerisation (ROP) of cyclic lactides.[13] The most popular polymerisation process is the ROP of lactide (*i.e.* the cyclic dimer of lactic acid, Figure 1).[14] Initially, low molecular weight PLA (1 - 5 kDa) is formed and then depolymerized by internal transesterification to cyclic dimers, which can have three distinct isoforms:[14] poly(L-lactide) (L-PLA), poly(D-lactide) (D-PLA), and poly(DL-lactide) (DL-PLA) (Figure 1). Catalytic ring opening polymerization is then performed to synthesize a high molecular weight PLA.[14]

PLA is a hydrophobic polymer with a moderately high melting point (170 ^{o}C).[14] It is a fully biodegradable biopolymer with high tensile strength (70 MPa) that can be recycled from 7 to 10 times.[15] L-PLA and D-PLA are semi-crystalline and display high tensile strength and low elongation.[16] DL-PLA is amorphous and has random distribution of the two enantiomers of lactic acid.[16] However, the degree of DL-PLA crystallinity can be regulated through the proportion between the D and L enantiomer.[14]

PLA is a hydro-biodegradable aliphatic polyester since it requires high-temperature for its chemical hydrolysis.[14] Moisture breaks the macromolecules into smaller compounds many of which are consumed by bacteria and turned into carbon dioxide and water.[14] It can also be degraded by depolymerisation to the cyclic lactide under alkaline conditions.[14]

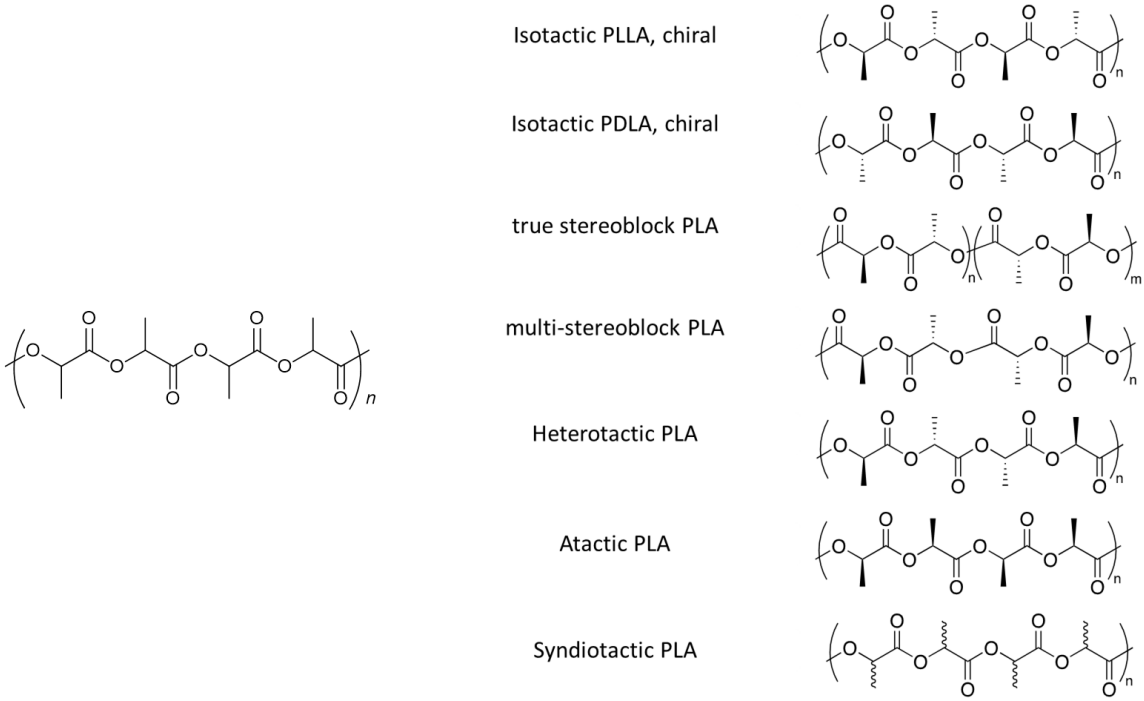


Figure 1. Structure of PLA.

For many years the primary uses of PLA were restricted to medical applications, such as for the construction of implants, tissue scaffolds and internal sutures, due to its high cost, poor availability and low molecular weight.[12] Recently, new synthetic approaches have allowed economical production of high molecular weight PLA and broadened its uses. Since PLA is compostable and can be obtained from sustainable sources, it is regarded as a promising material to reduce the waste disposal problem.[18] Its low toxicity, along with its eco-friendly features, has made PLA the preferred material for food packaging and other consumer products.[12]

There are many advantages considering PLA. First, it is an eco-friendly biopolymer. Apart from being derived from renewable resources, PLA is biodegradable, recyclable, and compostable. Its production also consumes carbon dioxide. These sustainable and eco-friendly characteristics made PLA an attractive biopolymer. The most attractive aspect of PLA,

especially regarding biomedical applications, is its biocompatibility. A biocompatible material should not produce toxic or carcinogenic effects in local tissues. Also, degradation products should not interfere with tissue healing. PLA hydrolyses to its constituent α-hydroxy acid, *i.e.* lactic acid, when implanted in living organisms, including the human body. It is then incorporated into the tricarboxylic acid cycle and excreted. As mentioned earlier, PLA degradation products are nontoxic, making it a natural choice for biomedical applications.[12] The Food and Drug Administration (FDA) in USA has also approved PLA for direct contacting with biological fluids. PLA has better thermal processability compared to other biopolymers such as polyhydroxyalkanoates (PHAs), polyethylene glycol (PEG) and, poly-ε-caprolactone (PCL) and is therefore suitable for both *in vitro* and *in vivo* applications where long-term mechanical stability is crucial.[19] However, PLA is fragile and the application of PLA relies not only on mechanical integrity and processability but also on regulated surface properties. For these reasons PLA has chemically been improved to boost its strength, degradation rate, hydrophilicity and chemical stability.[19–21] PLA can be processed by injection molding, film extrusion, blow molding, thermoforming, fiber spinning, and film forming.[12] Finally, PLA requires 25% - 55% less energy than petroleum-based polymers to manufacture, and this percentage can be further decreased in the near future to make its production cost-effective.

1.3 Lactic acid

Lactic acid, 2-hydroxypropanoic acid also known as milk acid, is perhaps the most abundant carboxylic acid in nature (Figure 1). It was first isolated in 1780 by the Swedish chemist Carl Wilhelm Scheele, but its commercially production started by Charles E. Avery at Littleton, Massachusetts in the USA in 1881.[22] Lactic acid is an essential chemical in many biological systems and in chemical and biochemical industry. As mentioned above, lactic acid is also a valuable monomer for the synthesis of biodegradable polymers like PLA.

1.3.1 Properties

Lactic acid is a three-carbon chiral organic acid consisting of two enantiomers, the L-(+)-lactic acid or (S)-lactic acid and its mirror image, D-(−)-lactic acid or (R)-lactic acid (Figure 2). Lactic acid isomers share the same physical and chemical properties but behave differently in living tissues. In living species L-lactic acid is found more frequently than D-lactic acid.[23] In the human body for example, only the L enantiomer is formed during muscle contraction.[23] Since the metabolic conversion of L-lactic acid is more rapid in the body, this enantiomer is also preferred for dietary and medical applications.[23, 24, 25] The optical purity of lactic acid is fundamental to the physical properties of PLA as mentioned earlier and its suitability for industrial use.[10] Conventional chemical synthesis can only produce a racemic mixture of lactic acid, whereas microbial fermentation has the benefit of producing optically pure enantiomers.[26] Lactic acid is miscible with water or ethanol, and features low volatility.

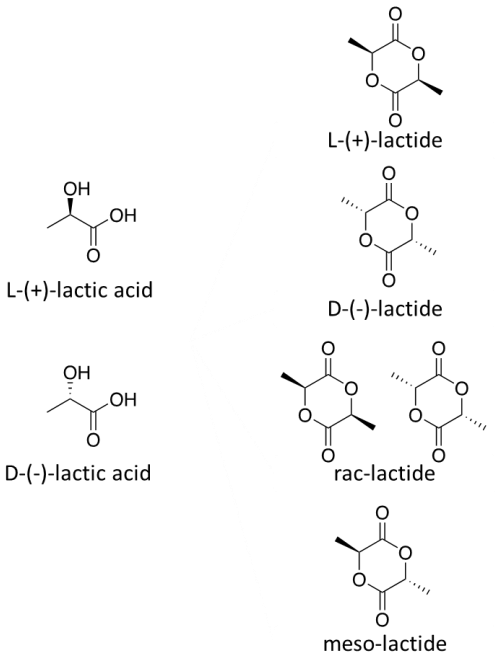


Figure 2. Structure of lactic acid and lactide.

Analytical techniques such as gas chromatography and liquid chromatography can be used to analyse and distinguish the optical isomers quantitatively.[22] Lactate dehydrogenase based NAD^+-based assays can also be used to quantify lactic acid.[22] It has a slight acidic flavour compared to other organic acids used in food industries. It is non-volatile and odourless. Although commercially accessible long ago, modern applications have resulted in a substantial rise in demand mostly in recent years (Figure 3). The global demand for lactic acid was estimated at 2.64 billion USD in 2018 and is projected to rise at an average annual growth rate of 18.7% from 2019 to 2025.[27]

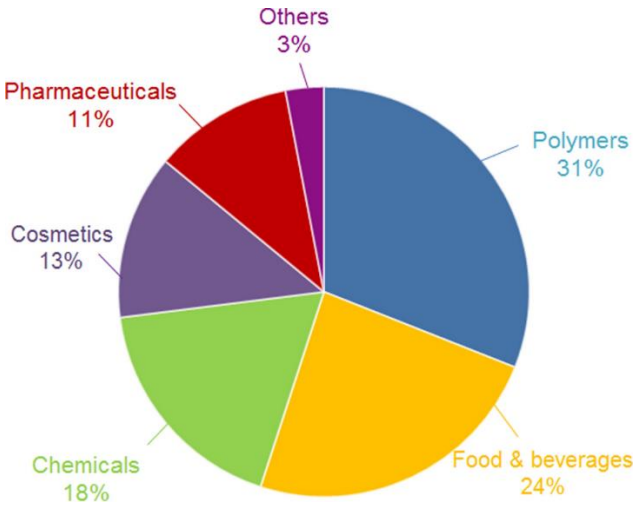


Figure 3. Demand for lactic acid in various sectors according to global market revenue of 2018.[28] Springer Rawoof, S. A. A.; Kumar, P. S.; Vo, D.-V. N.; Devaraj, K.; Mani, Y.; Devaraj, T.; Subramanian, S. Production of optically pure lactic acid by microbial fermentation: a review. *Environ. Chem. Lett.* **2020**. https://doi.org/10.1007/s10311-020-01083-w reproduced with permission of Springer.

Lactic acid is used as an acidulant (e.g. in vegetable and leather industry), flavouring, pH buffering or bacterial spoilage inhibitor in a wide variety of processed foods.[22] It has a wide variety of uses in chemical, pharmaceutical and food industry, and is a precursor to several products. LA is being also used in many small-scale applications such as pH adjustment for kinds cellophane (in food packaging) and textile printing developers, adhesive formulations, electroplating and electropolishing baths, etc.[22] It is an essential ingredient in fermented foods as well, such as yogurt, butter, and canned vegetables.[23] Moreover, lactic acid has numerous medicinal and cosmetic applications and formulas. In the pharmaceutical industry, lactic acid has a wide range of uses in implants, pills, dialysis, surgical sutures, and controlled drug release systems.[23] Due to its moisturising, antimicrobial and reinvigorating effects on the skin, lactic acid is also used in the cosmetic industry for the manufacture of hygiene, oral hygiene and aesthetic products.[23,29] Another very interesting use of lactic acid is in the synthesis of environmentally safe, *green*, solvents (lactate esters) which could substitute conventional solvents produced from petrochemical feedstock.[30] These are high boiling, non-toxic and degradable components.[30] The biodegradable polymer PLA derived from lactic acid has a variety of medical applications such as sutures, orthopaedic implants. As mentioned earlier, PLA is a biodegradable thermoplastic. It can be synthesized to be translucent and its degradation rate can be controlled by modifying its structure and molecular weight.[22] Low molecular weight poly-L-Lactic acid can be used for controlled release or degradable mulch films for large-scale agricultural applications.[31]

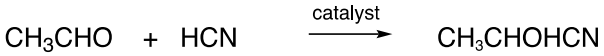
1.3.2 Lactic acid production

Lactic acid and its derivatives are naturally occurring compounds in plants, microorganisms, and animals, and can be formed via fermentation of carbohydrates or through chemical synthesis via coal, petroleum, and natural gas derived starting materials.[23] Industrially, chemical synthesis or enzymatic fermentation are the two principal methods by which lactic acid can be manufactured.

Chemical synthesis

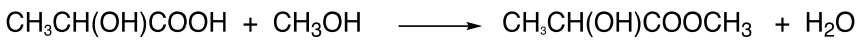
The commercial chemical synthetic method for lactic production is based on the lactonitrile route (Figure 4).[22] In the presence of catalyst, hydrogen cyanide is added to the acetaldehyde to yield lactonitrile.[32] This reaction occurs under high pressures in the liquid phase.[22] Crude lactonitrile is obtained and purified by distillation.[23] It is then hydrolysed with water, in presence of H_2SO_4 or HCl to obtain lactic acid and ammonium sulphate.[22] Lactic acid is in turn esterified with methanol to create methyl lactate which can be isolated from the aqueous phase, purified by distillation, and hydrolysed using an acidic catalyst to produce racemic lactic acid.[22] The reactions commonly involved in the chemical synthesis of lactic acid are summarized in Figure 3.

(a) Addition of hydrogen cyanide (HCN):



(b) Hydrolysis by sulphuric acid:

(c) Esterification:



(d) Hydrolysis:

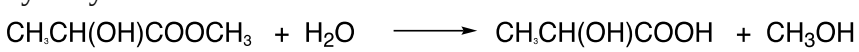


Figure 4. Chemical synthesis of lactic acid via the lactonitrile route.

With the exception of the lactonitrile route,[23] several other potential routes have been reported for lactic acid production through chemical synthesis which are unfortunately economically undrealistic.[33] Chemical production of lactic acid is costly and relies on derivatives from other industries originating from fossil fuels.[34] In addition, it leads to a racemic mixture of lactic acid, which is not considered to be useful for specific applications.[23]

Carbohydrate fermentation

Worldwide lactic acid production from microbial fermentation accounts for approximately 90% of the total production,[35, 36,37] and has drawn interest due to various advantages over the chemical synthesis, such as production of pure isomers and use of natural materials as fermentation substrates (Figure 4).[23] Industrial biotechnology focuses in utilizing inexpensive and renewable feedstocks as well as all metabolites of industrial interest generated by these procedures.[24] More and more industries have started new technological eco-friendly systems as environmental pollution has taken vast dimensions. Production of lactic acid via carbohydrate fermentation is a biotechnological method, which has drawn much interest from the research community the last decades.[10]

Lactic acid fermentation involves a biochemical mechanism by which carbohydrates such as glucose, fructose, and sucrose are transformed into cellular energy and the acidic metabolic product.[22] It is the anaerobic form of respiration that occurs in the absence of oxygen in many bacterial strains and animal cells.

Fermentation is mainly accomplished in batch mode as the closed structure of batch processing avoids contact of undesirable bacteria with the fermentation mixture and produces a higher concentration of lactic acid as compared to other fermentation processes.[38] A common problem often encountered in lactic acid cultures is inhibition of cell growth due to the low pH caused by the production of the acid itself. Since the microbial cells spend a vast amount of energy to maintain the pH at physiological values, pH decrease causes lactic acid production inhibition.[38] This common problem is addressed by constant pH monitoring and addition of neutralizing agents such as sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$) or ammonium hydroxide (NH_4OH).[39] The lactate anion ($C_3H_5O_3^-$) formed during pH neutralization is safer for the growth of microorganisms. Several batch fermentation protocols such as separate hydrolysis and fermentation and simultaneous saccharification and fermentation have been developed (Figure 5).[38, 40]

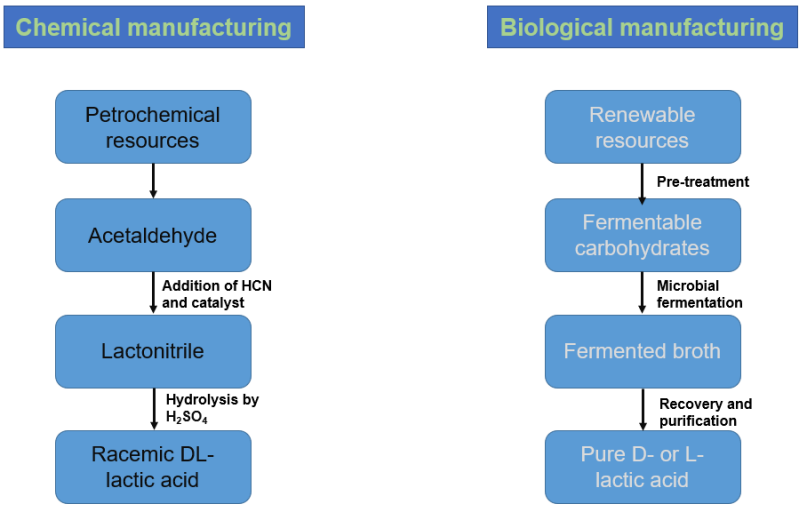


Figure 5. Schematic illustration of two models commonly followed for the production and purification of lactic acid.[35]

1.3.3 Raw materials

One of the most important factors in lactic acid's industrial manufacturing is the expense of raw materials.[23] Since there is a demand for vast amounts of lactic acid at relatively low price, inexpensive raw materials are required for its biotechnological manufacturing to be feasible.[10] High yields of lactic acid output, low cost, fast fermentation rate, the lowest amount of pollutants, and minimal to no by-product generation, are some essential characteristics of a raw materials that are appropriate for industrial lactic acid production.[35] Cheap raw materials, such as starch and cellulose, whey and molasses, have been used for manufacturing lactic acid over the years.[26] Among those, starch and cellulose materials are attracting a great deal of attention because they are inexpensive and sustainable.[41–43] The starchy materials used for lactic acid production include sweet sorghum, wheat, corn, cassava, potato, rice, rye, and barley.[42,44,45] According to *Wang et al.*, about 90% of the commercially available lactic acid was produced from submerged corn fermentation.[46] Before fermentation, these materials must be hydrolyzed into fermentable sugars, since they consist mainly of α-(1,4) and α-(1,6) glucose derivatives.[10]

Biomass lignocellulose is also a potential source of lactic acid production since it is the world's most plentiful source of biomass.[23] While the costs of lignocellulose biomass are low, the pre-treatment stages make the whole procedure cost-ineffective because of the increased cost of enzymes and chemicals, the inhibitory effects on the microorganisms used in fermentation (generation of organic acids during chemical hydrolysis) and the formation of mixed sugars such as pentoses, which cannot be fermented.[23]

Factory refuse products, such as whey and molasses, are also used as a regular substrate for lactic acid production. Whey is a significant by-product of the dairy industry which includes lactose, protein, fat, mineral salts and other essential nutrients for microbial growth.[10] In the past for manufacturing whey lactic acid through batch cultivation of *L. Casei* was also studied.[47–49] Molasses, are by-products of the sugar processing procedure that typically

contains a significant volume of sucrose.[26] Two well-known bacterial strains have been used in the past for the production of lactic acid from sugar molasses: *L. delbrueckii* and *E. faecalis*.[10]

Using microalgae is also a feasible alternative for reducing the cost of substrates. Microalgae have some benefits over lignocellulosic biomass. They have the ability to grow almost everywhere with an exceptionally short harvesting time of roughly 1 to 10 days and have high fermentable sugar content.[50]

Another ideal raw material for manufacturing lactic acid is food waste, since it is normally high in nutrients (carbohydrate content). Moreover, it is advantageous as an attractive solution for environmental waste treatment.[51–53] Sakai et al., has proposed a total recycle system for the production of L-lactic acid with a high optical purity, using municipal food waste as a substrate.[51] More recently, an improved method for manufacturing optically pure L- lactic acid from food waste was established via controlling key enzyme activities by combining supplementation of sewage sludge and discontinuous alkaline fermentation.[53]

1.3.4 Choosing the suitable microorganism

While several microorganisms are able to produce lactic acid, *Lactobacillus* species is by far the most widely used bacteria globally.[54] Some essential characteristics of *Lactobacillus* species are their high tolerance to acidic pH environments and the fact that they can undergo genetic modification to allow selective production of lactic acid isomers.[55] These microorganisms have long history of industrial use in many fields with no harmful impacts reported, both for employees and consumers.[56]

1.3.5 Lactic acid bacteria

Lactic acid bacteria (LAB) are among the best studied microorganisms.[57] These microorganisms produce lactic acid from sugars through fermentation. They are extremely adaptable, and have wide metabolic diversity.[9] The *Bacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* genera are remarkable members of this group.[22] The taxonomy of LAB was based on the gram reaction and the synthesis of lactic acid from different fermentable carbohydrates.[22]

Most LAB are facultative anaerobic, catalase negative, nonmotile, and non-spore forming.[23] Typically, they have a high acid tolerance with an optimum growth pH at 5.5-5.8 and complex nutritional requirements.[22] LAB usually have specific dietary needs, at the cost of their restricted efficiency to synthesize their own bioactive molecules for growth, such as B vitamins and amino acids.[10] They require elements, such as carbon and nitrogen, in the form of sugars, amino acids, vitamins and minerals for their growth.[58,59] The carbon to nitrogen (C:N) ratio sources is one of the key factors influencing the conversion of sugars to lactic acid.[60] Other essential factors that affect the growth of LAB and the production of lactic acid are temperature and pH.[26] Overall, the attractive features for industrial LAB are the ability to easily and thoroughly convert inexpensive raw materials to lactic acid with minimum nutritional requirements and to produce high yields of the desired stereoisomer without the generation of by-products.

LAB can be categorized into two main groups: homofermentative and heterofermentative.[10] Homofermentative LAB transforms glucose almost completely into lactic acid while, heterofermentative LAB catabolizes glucose into ethanol and CO_2 as well as lactic acid.[10] The homofermentative bacteria use the glycolytic pathway known as *Embden-Meyerhof-Parnas* pathway. The end product from this metabolic pathway is almost exclusively lactic acid and two moles of ATP (Figure 6).[61] More practically, fermentation is often considered to be homolactic when over 90% of hexoses are converted to lactic acid and 2 mol of ATP are produced per hexose molecule.[62] On the contrary, heterofermentative microorganisms use the 6-phosphogluconate/ phosphoketolase pathway (6-PG/PK), which results in other by-products in addition to lactic acid, such as ethanol, acetate and CO_2 (Figure 6).[63] The main distinction between these metabolic pathways is based on the essential enzymes used for each route:[63]

- Fructose 1,6-diphosphate (FDP) aldolase to glycolysis pathway
- Phosphoketolase to 6-PG/PK pathway

Homofermentative microorganisms generate lactic acid from glucose by using the glycolytic pathway and therefore cannot metabolise pentose sugars and associated compounds. The heterofermentative bacteria group produces lactic acid and other compounds using the 6-PG/PK hexose and pentose sugar pathways.[64]

Homolactic acid fermentation:

$$C_6H_{12}O_6 \; + \; 2\,ADP \; \rightarrow \; 2\,CH_3CH(OH)COOH \; + \; 2\,ATR$$

Heterolactic acid fermentation:

$$C_6H_{12}O_6 \; + \; ADP \; \rightarrow \; 2\,CH_3CH(OH)COOH \; + \; CH_3CH_2OH \; + \; CO_2$$

Figure 6. Homolactic and heterolactic acid fermentation.

The predominant fermentation microorganisms used recently are the homofermentative bacteria of the genus *Lactobacillus*, *Streptococcus* and *Pediococcus*.[65]

1.4 Production of lactic acid from food waste

In last decades many research groups have been involved in the production of lactic acid from food waste.[5,5 1, 66] Sakai and collaborators proposed in 2003, an innovative system for the production and isolation of lactic acid from urban food waste for the synthesis of polylactic acid (Figure 8).[51] The proposed recycling system combines fermentation and chemical processes to produce high-quality polylactic acid.

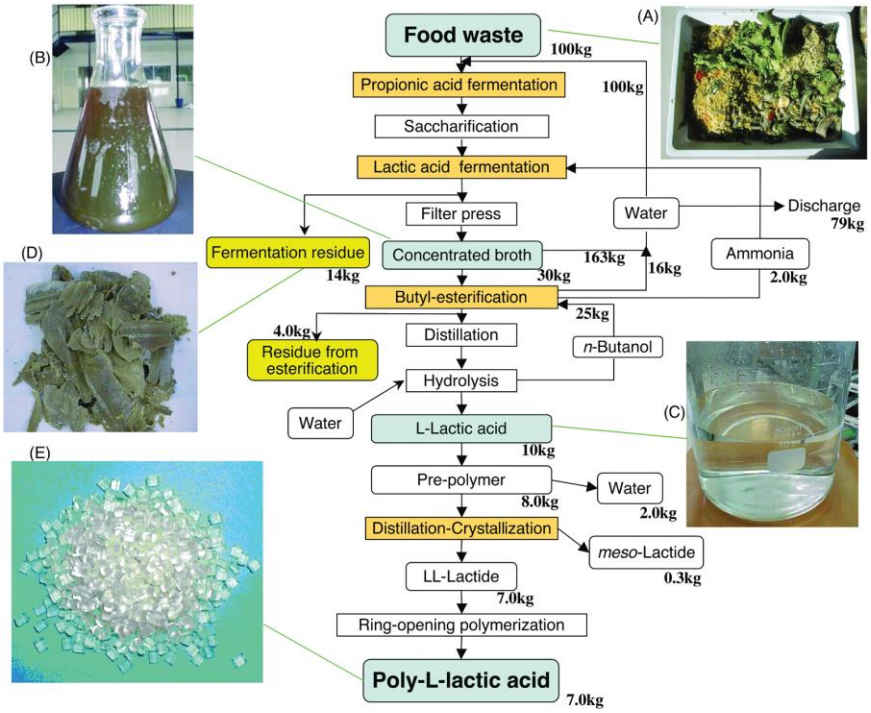


Figure 7. PLLA production from food waste as proposed by Sakai and collaborators.[51] Reprinted from Sakai, K., Taniguchi, M., Miura, S., Ohara, H., Matsumoto, T. and Shirai, Y. (2003), Making Plastics from Garbage. Journal of Industrial Ecology, 7: 63-74. https://doi.org/10.1162/108819803323059406 with permission from Wiley. Copyright 2008 Wiley

1.4.1 Propionic acid fermentation

The first step for the production of L-lactic acid by carbohydrate fermentation is the removal of naturally produced D- and L-lactic acid enantiomers from the food waste. It has been found that *Propionibacterium freudenreichii* preferentially consumes D- and L-lactic acids before sugars, as a carbon source under acidic conditions.[67] The ability of this strain is crucial in order for the production of enantiomerically pure lactic acid to be accomplished.

Generally, there are two classes defined in the *Propionibacterium* genus: classical or dairy and cutaneous *Propionibacteria*.[68] The *P. freudenreichii* belongs to the dairy community and is classified into two subspecies based on 2 biochemical processes: lactose fermentation and nitrate reductase function.[68] It is known that dairy *Propionibacteria* are not found in the human microbiome but can be isolated from other environments such as dairy products and other fermenting foods.[68] During the production of Swiss Emmental cheese, the *P. freudenreichii strain* ferments lactate and forms propionate ion, acetate ion salt, and carbon dioxide. On an industrial scale, *Propionibacterium* has various applications such as the synthesis of vitamin B_{12} as well as other biological molecules such as propionic acid.[69]

1.4.2 Saccharification

The next step for lactic acid production is the pre-treatment of the substrate. Biological pre-treatment, a cost effective technique aiming to improve the hydrolysis of the substrate during fermentation.[70] Without the pre-treatment step, fermentation efficacy can suffer by substrate inhibition.[70] In order to hydrolyze starch and other oligosaccharides or polysaccharides into simpler sugars, enzymatic hydrolysis of glycosidic bonds, commonly referred to as saccharification, is usually performed by hydrolytic enzymes such as glucoamylase, α- and β- amylase.[27, 70]

Glucoamylase (maltase) more specifically, is a commonly used enzyme which carries out saccharification of various substrates such as food waste resources, providing fermentable carbohydrates for lactic acid producing microorganisms.[27] Glucoamylase hydrolyzes the disaccharide maltose, derived from the hydrolysis of starch, into two molecules of glucose.

1.4.3 Lactic acid fermentation

As previously mentioned, the production of the L- or D- enantiomer of lactic acid is carried out by specific strains of lactic acid bacteria. At this stage, fermented sugars are used by microorganisms as a carbon source, by catabolizing glucose and other oligosaccharides, to produce the final product, lactic acid.

Several studies indicate that the optimum pH value for bacterial growth and lactic acid production is approximately 6.5 while the temperature should range between 30°C - 43°C.[71] Maintaining the pH at 6.5 and ensuring the optimum temperature for the microorganism, significantly improves the yield and production of lactic acid.[71]

Lactobacillus rhamnosus KY-3 is a homofermentative bacterium that was first isolated from a food mixture and it is commonly used for the industrial processing of L-lactic acid.[72] Recycling systems for municipal food waste have commonly this bacterial strain to produce high purity L-lactic acid.[73,51]

1.5 Downstream processing

The lactic acid solution derived from the fermentation processes cannot be used immediately for other applications. Usually the fermented broth is a liquid solution containing a quantity of lactic acid and its salts, various minerals, proteins, sugars and other by-products of the fermentation process. It is almost impossible to isolate crystalline lactic acid, even though the boiling points difference between water and lactic acid is significant. This occurs because lactic acid has a fairly high affinity to water, usually leading to dimers when in high concentrations.[23, 24] For chemical and associated industries, such separation procedures are vital since they account for about 40-70% of the operational and capital costs.[23] The design of an efficient method for lactic acid recovery from fermentation broths is therefore crucial in manufacturing process. The drawbacks may include high equipment costs, solvent recovery, and high energy consumption.[74]

Depending on the application, commercial LA is categorised in four distinct types: food grade lactic acid (25-90% content), medicinal and cosmetic grades (90% content), commercial grade

(88%-90% content), and specialty grades lactic acid (80%, 90% and 98% content).[24] Several steps in downstream lactic acid processing (Figure 7), are needed to obtain this degree of purity.

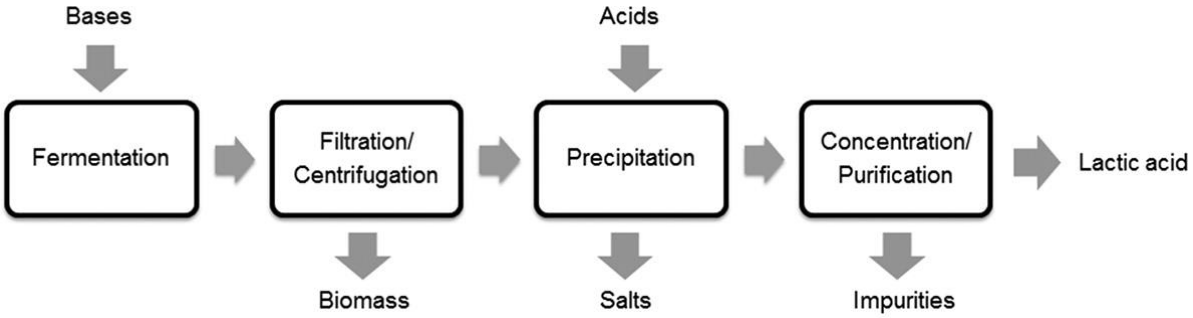


Figure 8. General process of lactic acid isolation procedure.[24]

1.5.1 Purification of lactic acid from food waste by esterification and hydrolysis method

Conversion of lactic acid or ammonium lactate (NH_4LA) into its corresponding esters, followed by hydrolysis of the purified ester to isolate the acid is widely accepted because highly pure lactic acid can be isolated.[22,51,75]

Initially, any solid residues have to be removed by filtration or centrifugation and the filtrate broth is then concentrated. Subsequently, the ammonium lactate produced by fermentation (if ammonium hydroxide has been added to adjust the pH) is esterified by the addition of alcohol (ethanol, methanol or butanol). In many cases, a catalyst is used to increase the esterification reaction yield. The lactic ester is then separated from other organic impurities via distillation and hydrolysed by adding water to obtain the final product, i.e. lactic acid.[76] By this means, high purity of lactic acid can be obtained.[22,51] Esterification is the only downstream method that distinguishes other organic acids from lactic acid.[77] Nevertheless, the combination of esterification and hydrolysis for the isolation of pure lactic acid requires considerable amounts of energy consumption.[38]

The most commonly used alcohol for esterification reaction is methanol.[78–81] However many studies and experiments have also been carried out with alcohols such as ethanol [82] and butanol.[96-98] The physical properties of the ester and the nature of the treatment process are affected by the type of alcohol used for the esterification process.[75] The greater the length of the carbon chain, the more the water solubility of the alcohol decreases, which makes this alcohol immiscible.[85] Since butanol is not miscible in water, an appropriate organic-aqueous volume ratio needs to be used.[86] Su and his collaborators examined the esterification processes of C1-C4 alcohols, and made an economic assessment.[87] Methanol and butanol are considered to be more cost effective in contrast to ethanol or isopropyl alcohol.[38] For short payback periods, the butanol approach is favoured while the approach of methanol is optimal for long payback periods.[38]

There are several factors beyond the choice of an alcohol which play an important role in the purification and recovery of lactic acid through extraction. Such factors are pH, mixing time, initial concentration of lactic acid, and volume ratio between the organic and the aqueous

phase.[86] When the pH of the aqueous solution decreases, the rate at which lactic acid is extracted and its distribution coefficient increase.[35]

1.5.2 Other purification approaches of lactic acid

So far, many of those bibliographical references have been suggested for the separation and isolation of lactic acid from fermented broth.[77] Conventional methods for purifying lactic acid include the use of calcium hydroxide ($Ca(OH)_2$) for precipitation. Excess sulfuric acid (H_2SO_4) is applied to recover lactic acid. In this way, however, large percentages of calcium sulphate ($CaSO_4$) are created as a by-product and in combination with the low purity of the final product and the extended use of chemicals, it is considered as a process anything but environmentally friendly.[9] The dissolved lactic acid is then sequentially purified using activated carbon, filtration, evaporation and crystallisation methods.[9] It has been reported that for every ton of lactic acid produced, approximately one more ton of calcium sulphate is created in parallel as a solid waste,[31] and this expensive procedure can hit up to 50% of the overall manufacturing cost of lactic acid.[88–90]

Studies are now focused on different approaches to extract lactic acid from intricate fermentation broths. The most promising approaches, although usually high-cost to date are ultra-and nano-filtration, electrodialysis, ion-exchange/adsorption, rapid distillation and short-haul hybrid evaporation.[9,38] Several of these approaches have only been validated in lactic acid model solutions or in well-defined media.[91]

Membrane separation processes are promising technologies for lactic acid extraction.[38,74] Such methods are based on the transfer of solutes through a semi-permeable physical barrier that separates two phases, restricting the transport of components from one phase to another.[74] In contrast to the traditional precipitation processes of lactic acid, new advances in separation technologies do not generate salt residues.[34] Microfiltration, ultrafiltration and electrodialysis are examples of these technologies.[38,74] The nanofiltration membrane with a pore size of 0.55 nm could retain approximately 90% of sugar, and allow more than 70% of lactic acid to permeate in single step operation.[38] Ultrafiltration is commonly used as a pre-treatment process for the removal of proteins and cell biomass from the fermentation broth. It was also used for the clarification of the fermentation broth.[38] Electro-dialysis on the other hand, is one of the most promising membrane techniques for the purification of lactic acid.[38] Electrodialysis is a separation method employing selectively permeable cationic and anionic exchange membranes.[74] This technique is applied to remove salts from any kind of solutions or to concentrate ionic substances.[74]

Recently, several experiments have been carried out to overcome the problems of conventional separation processes by means of non-traditional distillation separations.[24] Distillation is one of the oldest methods of separating mixtures in the chemical industry worldwide,[92] accounting for 90-95% of the total separations.[93] The role of distillation is based on the different boiling temperatures of the mixture components. As heat is supplied to a liquid mixture partial vaporization occurs, producing two phases (liquid and vapor) with different compositions due to the variation in volatility of the main components of the original liquid mixture. With the use of conventional distillation, at normal temperatures and pressure it is quite difficult to

obtain lactic acid from fermentation mixtures. Instead, it should first be converted into an ester (through the esterification reaction) and then hydrolysed using a reactive distillation column. It is a unique method that can be used to recover high efficiently and pure lactic acid.[81] This technology offers more advantages in comparison with the sequential approach of reaction and separation.[94] Molecular distillation is another method which can be used for the recovery of pure lactic acid. Molecular distillation is an diffusion mass transfer process used for the isolation of homogeneous liquid mixtures with low volatility, high molecular mass and thermal sensitivity,[24] and has been successfully applied in medicine, food, chemical and cosmetic industries.[95]

1.6 Aim of the thesis

Aim of this thesis was the production and purification of L-lactic acid (2-hydroxy propionic acid) from Municipal Food Waste following the approach proposed by Sakai and collaborators and to optimize the process at laboratory scale.[51] Four different food waste batches were used to carry out the experiments (food waste from the Hellenic Mediterranean University (HMU) restaurant, food waste recovered from Hotels, food waste enriched with 20% starch and 100% starch). Our goal was to isolate L-lactic acid from these four different food batches based on literature reports and to -ideally- optimize the process. To achieve this goal, we used two microorganism strains for the production of L-lactic acid into the refuse paste during the fermentation process. The first microorganism, *Propionibacterium freudenreichii*, was responsible for the consumption of naturally produced D- and L-lactic acid while the second one, *Lactobacillus rhamnosus KY-3*, was seeded into the refuse paste for the consumption and conversion immediately after, of the total sugars, to produce L-LA. Alpha-glucoamylase was added before the final fermentation to increase the total fermentable sugar content. All fermentations were performed under sterile conditions keeping all other conditions same as those reported in literature.[51]

To optimise the process, the esterification and hydrolysis reactions were studied in detail. We determined sugar, L-lactic acid and the acetic acid content by Nuclear Magnetic Resonance (NMR) spectroscopy. Chromatographic methods used for the analysis and optimisation of products throughout the entire process were High-Performance Liquid Chromatography (HPLC), and Thin Layer Chromatography (TLC). A polarimeter was used to determine the optical purity of the product. No catalyst was used in esterification and hydrolysis, thus reducing the overall cost of consumables while we achieved to recover L-lactic acid with reduced energy consumption, in an effort to make the entire process more environmentally friendly.

The two consecutive reactions were involved in the isolation of L-lactic acid namely, esterification of ammonium lactate (NH_4LA) to butyl lactate (BuLA) via butanol addition and hydrolysis of the produced ester to recover L-lactic acid (Scheme 1).

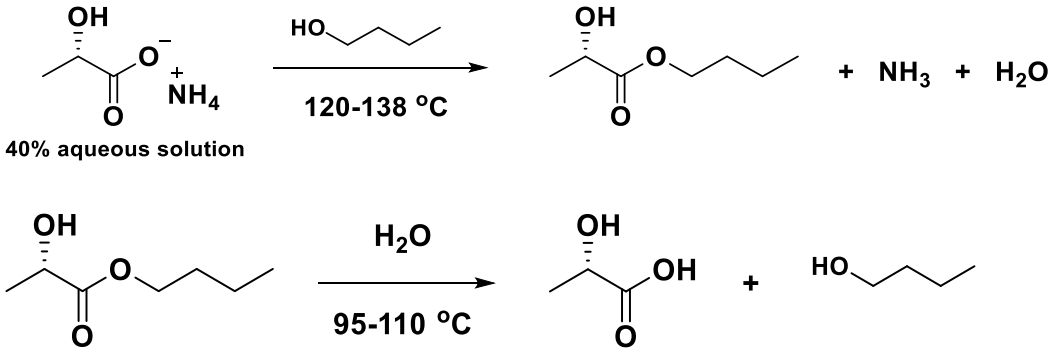


Figure 9. Isolation of L-lactic acid through esterification and hydrolysis.

Chapter 2: Results and Discussion

2.1 Food Waste batches

Four different food waste batches were used to carry out the experiments, the batches included in this study are:

- Food waste from the Hellenic Mediterranean University (HMU) restaurant,
- Food waste recovered from Hotels,
- Food waste enriched with 20% starch, and
- 100% starch.

Our aim was to reproduce L-lactic acid isolation and purification for food waste obtained in the municipality of Heraklion based on the approach reported by Sakai and collaborators.[51] We also aimed to optimize the processes in terms of energy consumption and yields in order to assist in the design of a pilot unit. Since the overall yield could in principle be affected by food waste quality (*e.g.* sugar and/or starch content), we included in this study a starch and a 20% enriched in starch food waste sample.

High-Performance Liquid Chromatography (HPLC) with refractive index (RI) detection was used to characterize the untreated food waste samples and the fermentation broths. A rapid HPLC method with refractive index detection (RI) was developed (LC-NH_2 chromatography column, mobile phase H_2O/CH_3CN 1/3, flow rate 0.8 mL/min, 32^oC) for the simultaneous analysis of glucose, fructose and sucrose (Figure 10). It should be noted that simultaneous detection of lactic acid and acetic acid was not possible using this or any other columns available in the laboratory.

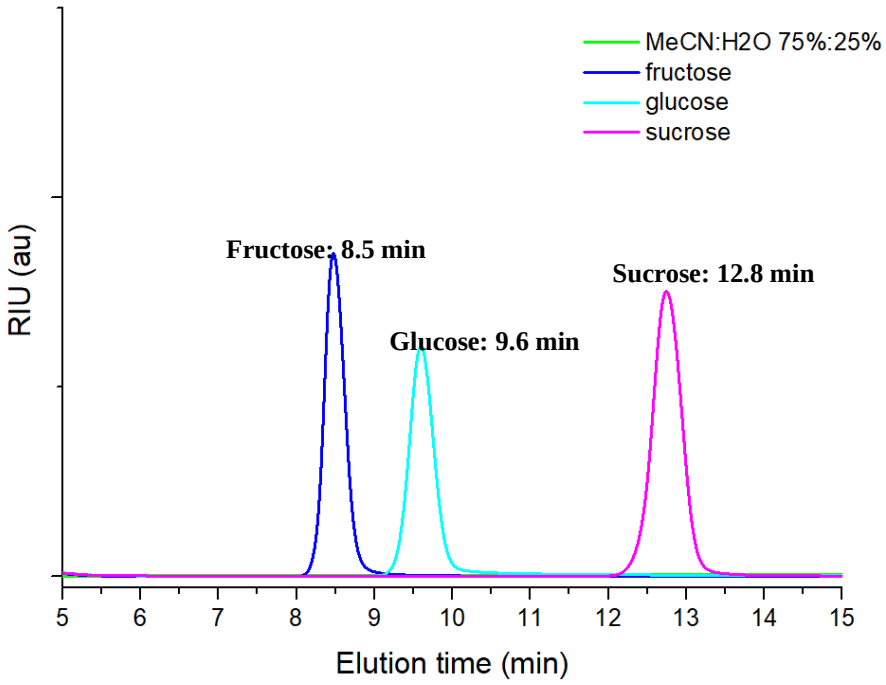


Figure 10. HPLC chromatogram of glucose, fructose and sucrose.

Minute (100 μL) food waste samples (prior and after fermentations) were extracted with water or mixtures of acetonitrile and water and were centrifuged (15 minutes, 10000 rpm) to remove solid waste before analysing with HPLC.

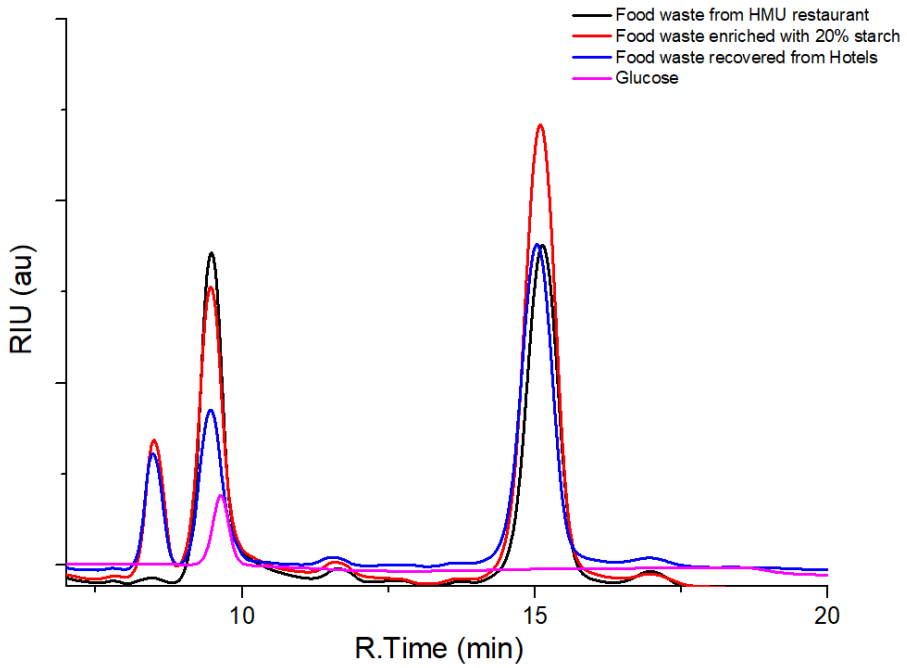


Figure 11. HPLC chromatogram of different food waste samples namely food waste recovered from the Hellenic Mediterranean University restaurant, food waste recovered from Hotels, food waste enriched with 20% starch, and 100% starch before fermentation.

HPLC analyses of the different food waste samples before fermentation (Figure 11) clearly demonstrated the presence of glucose in comparable levels in all samples. To accurately compare concentrations, measurements should be repeated in the future using an internal standard. All samples were found to contain one unknown major component (eluted at 15.1 min) -identification of this component needs to be performed in the future, possibly by using NMR and mass spectroscopy (MS) analysis. One clear difference observed in samples studied before fermentation was that the food waste obtained from the Hellenic Mediterranean University (HMU) restaurant did not contain fructose.

2.2 Propionic acid fermentation

The *Propionibacterium freudenreichii* strain (DSM 20271T) was used to carry out the fermentation of propionic acid,[51] aiming to consume the naturally occurring D- and L-lactic acid in food waste. In order to obtain a constant supply of the commercially available strain, the Laboratory of Synthetic Biomaterials in collaboration with Minotech (FORTH/IMBB) developed a sterile solid culture protocol at 37 °C. 20 % glycerol stocks were created and stored at -80 °C until needed. Immediately prior to food waste fermentations, a sample of the *Propionibacterium freudenreichii* glycerol stock was grown anaerobically at 37 °C in Brain Heart Infusion (BHI) medium. Optical Density at 600 nm (OD_{600}) was used to estimate bacteria

growth and the volume of liquid cell culture to be added in food waste fermentations according to literature (Table 1).

All food waste batches were minced with an equal amount of water, and their pH (acidic at approximately 3.5) was adjusted to 5.7 using a 5 N sodium hydroxide (NaOH) solution. The minced food waste was autoclaved (121 °C for 20 minutes) and then inoculated with the appropriate volume of a liquid *Propionibacterium freudenreichii* preculture (Table 1). The broths were gently shaken at 37 °C for 16 hours. pH values of food waste, OD_{600} and volumes of liquid precultures used during the propionic fermentation of food waste are summarized in Table 1.

Table 1. pH, OD_{600} and volumes of liquid preculture used during propionic fermentation with *Propionibacterium freudenreichii* aimed to consume the naturally occurring D- and L- lactic acid enantiomers.

Batches	**Initial pH of food waste samples**	**pH before fermentation**	**OD_{600} (AU)**	***Propionibacterium freudenreichii liquid* culture volume (mL)**
100% starch (700 mL)	3.78	5.67	1.636	17.5
20% starch in FW (1800 mL)	3.75	5.70	1.624	45
Food waste from Hotels (1600 mL)	3.77	5.95	1.592	40
Food waste from HMU restaurant (2000 mL)	3.97	5.40	1.656	50

2.3 Saccharification

Glucoamylase from *A. niger* in a maltodextrin and corn starch carrier (Saccazyme, The Alchemists Pantry) was used for the saccharification step aimed to degrade starch to simpler sugars such as glucose, maltose, and to some extent, dextrins. A sterile glucoamylase aqueous solution (5 g/100 mL of distilled water) was added to the fermented suspension and saccharification was allowed to proceed for four hours at 50 °C.

2.4 Lactic acid fermentation

Lactobacillus rhamnosus was used for the production of L-lactic acid, since *L. rhamnosus KY-3* is an L-forming homo-fermentative strain bacterium that is used widely for the industrial production of L-lactic acid.[51] Before inoculation, *Lactobacillus rhamnosus* (Hansen) was

cultivated at 37 °C in BHI medium under shaking (200 rpm). OD_{600} values of the precultures were used to determine bacteria growth and the volume of liquid cell culture needed to be added to food waste samples according to literature (Table 2).[51] It should be noted, that during lactic acid fermentation, sterile conditions are not important as the *Lactobacillus* is considered to be an aerotolerant anaerobe microorganism able to survive and carry out lactic acid production either in the absence or presence of oxygen.

Since the optimal pH for *Lactobacillus rhamnosus* fermentations is 6.5,[51] the pH of the treated refuse paste was adjusted accordingly by adding a 28 % – 30 % w/v aqueous solution of NH_4OH, 5 N (Table 2).

Table 2. Summary of pH after saccharification and before fermentation with *Lactobacillus rhamnosus*, OD_{600} values of with *Lactobacillus rhamnosus* precultures and volumes added.

Batches	**pH after saccharification**	**pH before LA fermentation**	**OD_{600} (AU)**	***Volume of Lactobacillus rhamnosus liquid culture (mL)***
100% starch	4.40	6.60	1.5	17.5
20% starch in FW	4.90	6.70	1.5	45
Food waste from Hotels	4.68	6.60	1.5	40
Food waste from HMU restaurant	4.80	6.60	1.5	50

HPLC analyses of the 100% starch broth sample after the fermentations revealed the presence of glucose after the fermentations (Figure 12). No significant difference was observed in glucose concentration upon pre-treatment of the sample for analysis (*i.e.* via extraction with water or water/acetonitrile mixtures prior to centrifugation).

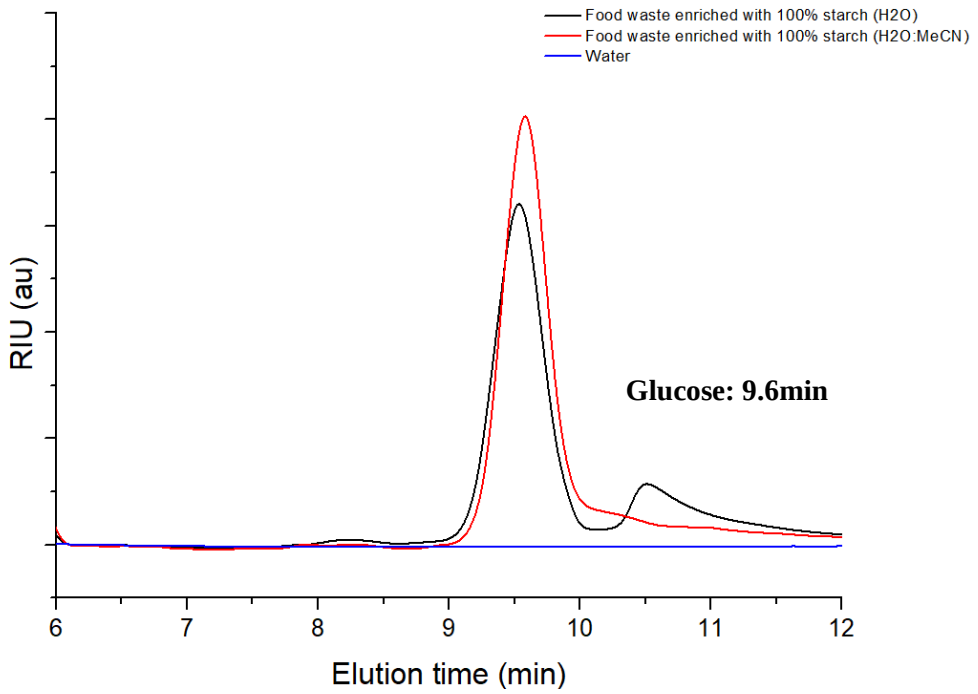


Figure 12. HPLC chromatogram of 100% starch broth after fermentations – a sample of the broth was treated with water or acetonitrile/water and centrifuged prior to analysis.

The detection of glucose after the fermentations, led to the conclusion that broth pH during the lactic acid fermentation is a very important parameter to control. We propose measuring and adjusting pH throughout this fermentation step in the pilot unit. HPLC analysis of all samples before, during and after the fermentations should be introduced as a standard protocol for future experiments.

All broths were stored at -20 °C until further use.

2.5 Purification of L-Lactic acid

For the purification of L-lactic acid experiments were based on the literature,[51] with the aim to verify the processes for the municipal food waste of Heraklion in laboratory scale, identify an improved procedure in the laboratory scale and assist our collaborators at the Hellenic Mediterranean University to design and implement a L-lactic acid/PLLA producing pilot unit. The processes involved in L-lactic acid purification were repeated two times, the optimized results are reported in this section.

2.5.1 Broth treatment

Centrifugation and filtration were performed prior to any of the isolation steps, in order to remove unwanted solid residues (cellular biomass, proteins, residual sugars, etc.) and enhance lactic acid isolation yields. In two samples (100 % starch and 20 % starch in food waste) centrifugation followed by conventional paper filtration was applied, while in the two other samples (food waste from Hotels and food waste from HMU restaurant) vacuum assisted filtration was carried out without prior centrifugation. Both approaches were found to be

suitable for the removal of the solid residues from the fermented broths, although the first one was judged to be more efficient and less time-consuming. We propose the combination of centrifugation and filter press for the pilot unit.

Condensation of the filtered broth aimed to remove the desired amount of water in order to produce a 40% w/v solution of ammonium lactate (NH_4LA, theoretical percentage proposed by Sakai and collaborators).[51] Condensation was performed both conventionally (*i.e.* use of temperatures higher than the boiling point of water (100 °C -120 °C), either under reduced pressure (*i.e.* lower temperatures, 50 °C – 70 °C, which can be translated in lower energy consumption at the pilot unit).

During the first cycle of experiments (not optimized), the distillation extend was based on calculations assuming a theoretical content of 40% w/v in NH_4LA in the broths. In the second cycle of experiments, distillation under reduced pressure was used in all batches. Importantly, having proved that the final NH_4LA concentration in the condensate is crucial to achieve esterification in good yields, ^{1}H NMR spectroscopy was used to quantify NH_4LA.

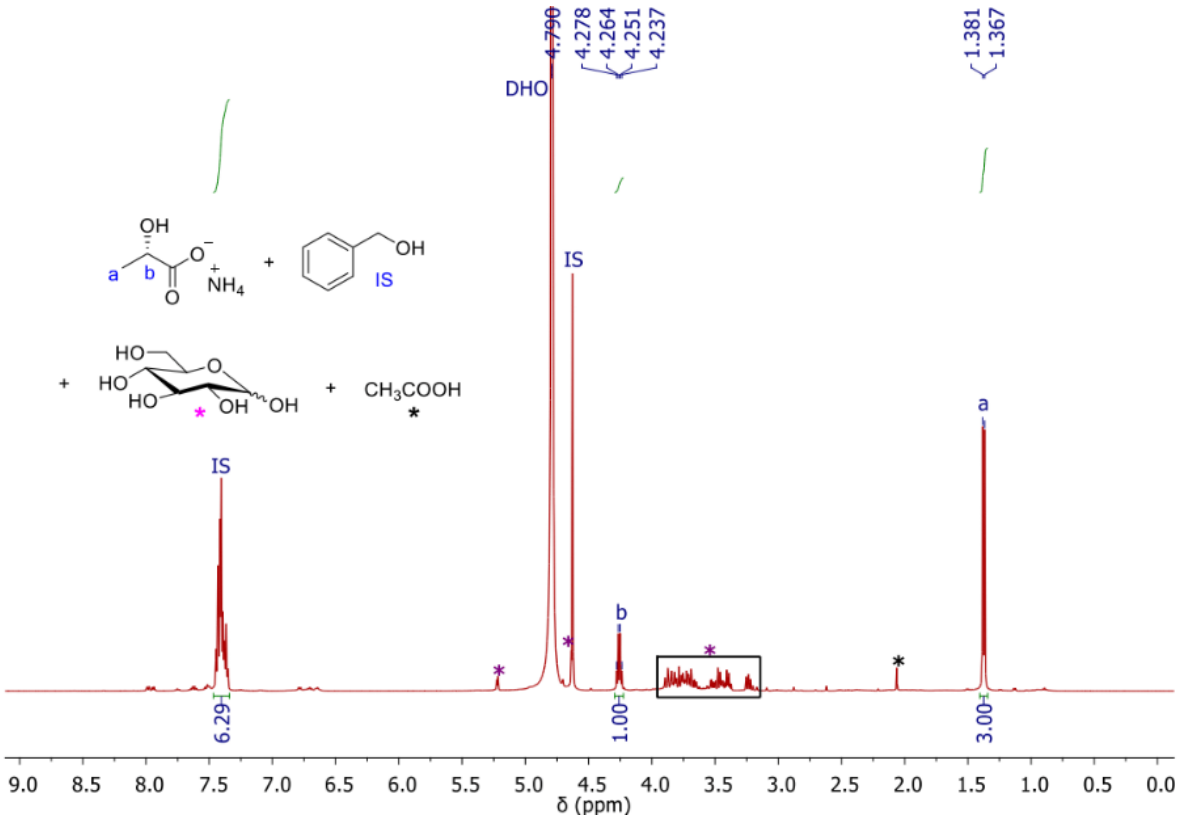


Figure 13. ^{1}H NMR spectrum of concentrated HMU restaurant food waste fermentation broth, using benzyl alcohol as Internal Standard (IS).

^{1}H NMR spectroscopy proved to be a powerful tool for the characterization of raw food waste samples. Spectra were recorded after simply mixing a minute volume of the fermented FW sample (100 µL) with deuterated water (500 µL) containing a fixed amount of an internal standard (IS, benzyl alcohol, Figure 13). As shown in a representative ^{1}H NMR spectrum presented in Figure 13, ammonium lactate was identified through the characteristic peaks of

the methyl group (three protons, 1.37 ppm) and the CH bearing the hydroxyl group (one proton, 4.25 ppm). The spectrum was surprisingly simple (taking into account the complexity of the broth) and allowed identifying other major components of the mixture such as glucose (hydroxyl methylene protons of glucose at between 3.2 ppm and 3.8 ppm) and acetic acid (2.082 ppm).[51,75] Since HPLC (with the columns available in the laboratory) could not simultaneously identify lactic acid and sugars, ^{1}H NMR spectroscopy was used throughout optimization experiments to calculate NH_4LA concentrations and sugar content before and after condensation.

The concentrations of NH_4LA calculated via ^{1}H NMR spectroscopy as compared to the theoretically predicted concentrations are summarized in Table 3. Taking into account that concentrations are crucial in the esterification step, and that the theoretically predicted w/v percentage concentrations (Table 3) were found larger than the experimental, we propose to use ^{1}H NMR spectroscopy prior to esterification. Since this cannot be easily implemented in a pilot unit, titration could also be considered as a simple alternative and should probably be studied in future experiments.

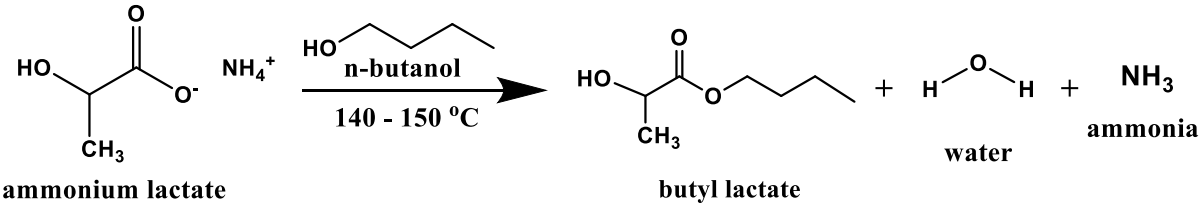
Table 3. Percentage (w/v) of ammonium lactate in food waste concentrate.

Concentrate	**100% starch**	**20% starch in FW**	**FW from University**	**FW from Hotels**
% NH_4LA/first round of experiments	72.5% [a]	40.0% [a]	43.9% [a]	40.0% [a]
% NH_4LA/second round of experiments	43.7% [b]		28% [b]	

[a] theoretical w/v percentage concentration, according to literature.[51]
[b] experimental w/v percentage concentration, according to ^{1}H NMR spectroscopy.

2.5.2 Esterification

A chemical trick was used to isolate lactic acid from the concentrated broth mixture containing sugars, acetic acid and -according to literature- propionic acid.[51] More specifically, an esterification reaction in *n*-butanol combined with azeotropic distillation was used to form butyl lactate in the organic phase where, water soluble proteins and salts precipitate (Figure 14). As mentioned above, the amount of *n*-butanol added to each sample, was based in either a theoretical or an experimental determination of NH_4LA concentration. According to literature a 3/1 mol ratio of *n*-butanol to NH_4LA is required for the coupled esterification and azeotropic distillation.[51] Taking advantage of the low solubility of *n*-butanol in water, the condensed organic phase separated into a light butanol phase and the heavy water phase. Esters, such as those of acetic acid and propionic acid, were separated during azeotropic distillation while butanol could be recovered and reused. This process was repeated several times, in an effort to maximise the efficiency of the esterification reaction within 8 hours, which was considered acceptable at the pilot unit scale. The ammonia released during distillation was trapped using a dilute aqueous solution (11% w/v) of hydrogen chloride (HCl).



ammonium lactate **butyl lactate**

Figure 14. Synthesis of butyl lactate ester from ammonium lactate salt.

The reaction was followed by Thin-layer Chromatography (TLC), using a silica plate and a 4/1 petroleum ether/ethyl acetate as mobile phase to separate butyl lactate from other reaction components or ethyl acetate to detect remaining lactic acid. TLC was also used to confirm the stability of butyl lactate under the harsh conditions (increased temperatures) used for esterification. Upon consumption of the lactic acid (8 to 10 hours), the dark product solution was filtrated when judged necessary to remove solid residues and the ester was distilled. For the later step, reduced pressure distillation was found to be more efficient. To identify the enantiomer formed by the production of lactic acid (during the fermentation and isolation process), we measured the enantiomeric excess of the formed ester in a polarimeter. The commercially available racemic solution of butyl lactate was used as a reference point. The enantiomeric excess of the purified ester was calculated to be 78% e.e..

The produced butyl lactate was isolated through distillation (170 °C - 200 °C) or distillation under reduced pressure (55 °C – 70 °C to recover residual butanol, 80 °C – 110 °C to recover the produced ester). The product was characterized by ^{1}H-NMR spectroscopy (Figure 15).

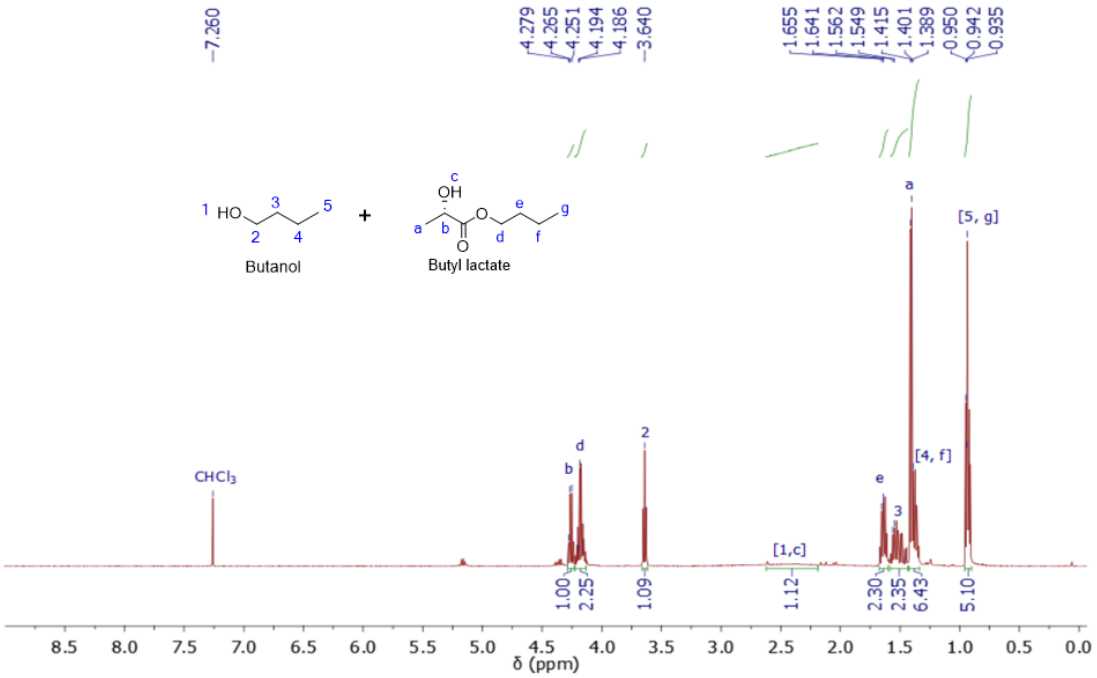


Figure 15. ^{1}H NMR Spectrum of butanol - butyl lactate mixture, after distillation of the ester (100 % starch content batch).

A characteristic ^{1}H-NMR spectrum of the distillate from the 100% starch broth is presented in Figure 15, revealing a mixture of 64.7 % butyl lactate and 35.3 % butanol. The molar ratio of

the products in the mixture was derived from the integrals of the signals assigned to an equal number of protons on the ^{1}H-NMR spectrum. More specifically, in the ^{1}H-NMR spectrum of the mixture obtained in deuterated chloroform, most protons exhibited well-separated resonances. Butyl lactate was identified through the characteristic signals assigned for protons of the methyl group (a, three protons, 1.39 ppm), the CH bearing the hydroxyl group (b, one proton, 4.26 ppm), the ester methylene protons (d, two protons, 4.18 ppm), the protons of the central methylene group (e, two protons, 1.65 ppm) and the alkyl methyl protons (g, two protons, 0.94 ppm). Butanol, was quantified through the characteristic signals for the protons of the carbon attached to the hydroxyl group (2, two protons, 3.64 ppm). All relevant calculations are presented in Table 4, and all relevant spectra are included in the Appendix.

Table 4. Esterification yields and product recovered during butyl lactate formation and isolation.

Batches	**100% starch**	**20% starch in FW**	**FW from University**	**FW from Hotels**
Initial Experiments				
% mass of *n*-BuLA	64.7	75.5 [b]	17.3 [b]	45.0 [b]
mass of *n*-BuLA (g)	3.092	1.220	0.259	0.094
Esterification yield %	8.9	6.3	0.8	0.25
Optimization experiments				
% mass of *n*-BuLA	14.0 [a]	27 [b]	36.7 [a]	52.9 [b]
Esterification yield %	5.9	1.1	13.8	2.3

[a] experimental percentage, according to NMR, [b] theoretical percentage, according to literature.

The results presented in Table 4, show that esterification yields are rather low in all experiments. We attribute this to the inherent nature of the reaction and the presence of residual water and propose that this reaction is optimized in the future either by drying or by adding an appropriate catalyst (*e.g.* a lipase).

In order to ascertain the optical purity of the butyl lactate formed, its optical rotation was measured using a polarimeter and found $[\alpha]_D^{19}$= -10 (1 M butyl lactate in MeOH at 19 °C). Comparing to bibliographic data ($[\alpha]_D^{20}$= -13± 1).[96]

2.5.3 Hydrolysis of the purified ester

Purification of the ester, and subsequent hydrolysis of the purified ester to lactic acid (Figure 16) was used as means of isolating lactic acid from the fermentation broth without generating calcium sulphate as a by-product.[94,97] The reaction of hydrolysis is commonly catalysed by a homogeneous catalyst such as sulfuric acid, anhydrous hydrogen chloride and many other mineral acids. Using the protocol proposed by Sakai and collaborators,[51] the hydrolysis of butyl lactate was performed in the absence of additional catalyst.

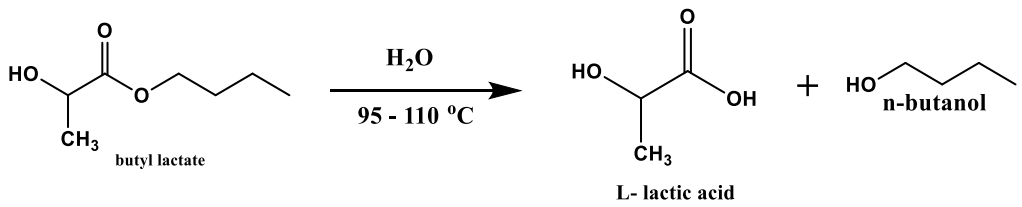


Figure 16. Hydrolysis of the purified ester for the synthesis of L-lactic acid.

More specifically, all butanol/butyl lactate mixtures produced by all samples of the first -not optimized- cycle of experiments were combined with thrice the volume of water, while the temperature was set at 110 °C. The reaction progress was monitored by TLC. The total mass of isolated lactic acid was calculated 1.9 g (21.2 mmol) and the overall hydrolysis yield was 66.3%.

During optimization, the hydrolysis was performed using a 20/1 molar ratio of water/butyl lactate under the same conditions. The biphasic mixture formed in this case delayed the course of the reaction (20 hours as compared to 7 hours determined for the homogeneous solution). The hydrolysis yield was found to be 55%, lower that in the previous case, a fact that can be attributed to the biphasic mixture formation. To avoid formation of a biphasic mixture, additional water or methanol, can be added to homogenize and carry out the hydrolysis reaction in significantly less time.

The product was characterized by ^{1}H and ^{13}C NMR spectroscopy (Figure 17 and Figure 18) The ^{1}H-NMR spectrum revealed all the characteristic lactic acid protons as mentioned above. The ^{13}C-NMR spectrum presented in Figure 18 was obtained in deuterated water and bears all resonances expected for lactic acid, *i.e.* the resonance at 19.2 ppm corresponding to the methyl group of lactic acid, the resonance at 66.4 ppm corresponding to the carbon bearing the hydroxyl group and the carboxylic carbon resonance at 178.5 ppm.

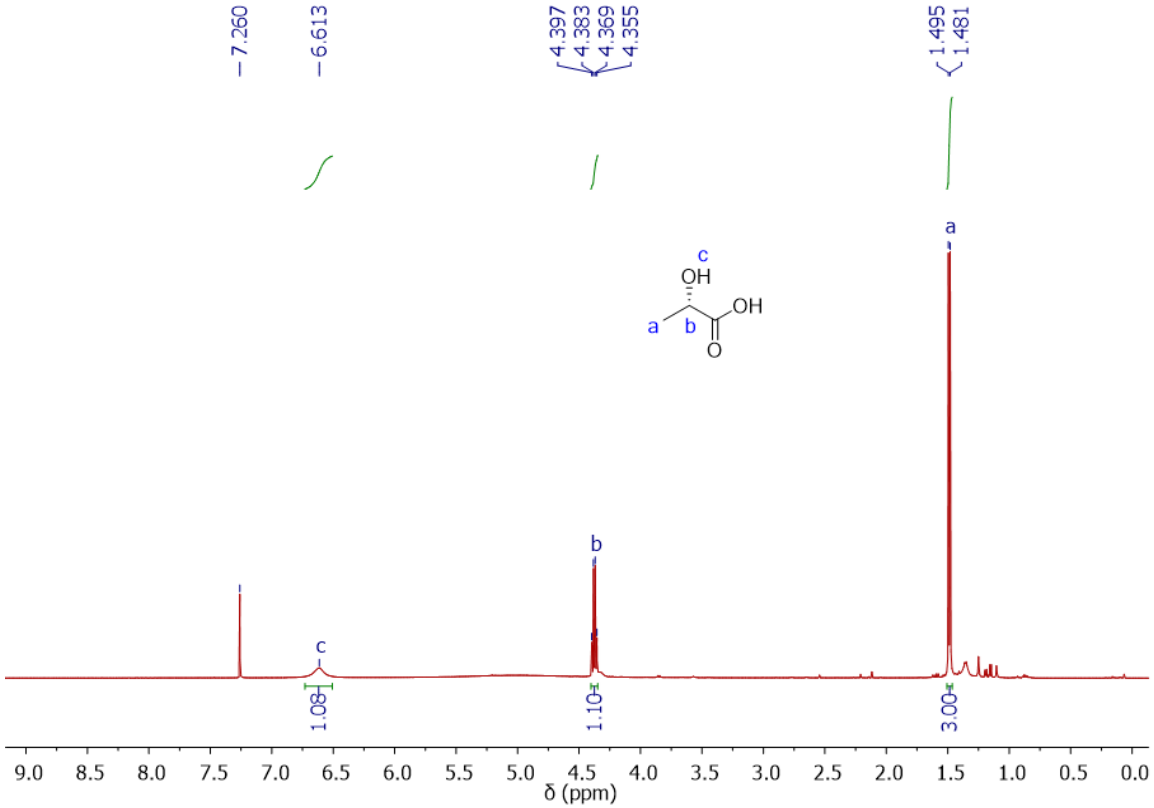


Figure 17. ^{1}H spectra of lactic acid in $CDCl_3$ produced after hydrolysis of butyl lactate derived from food waste.

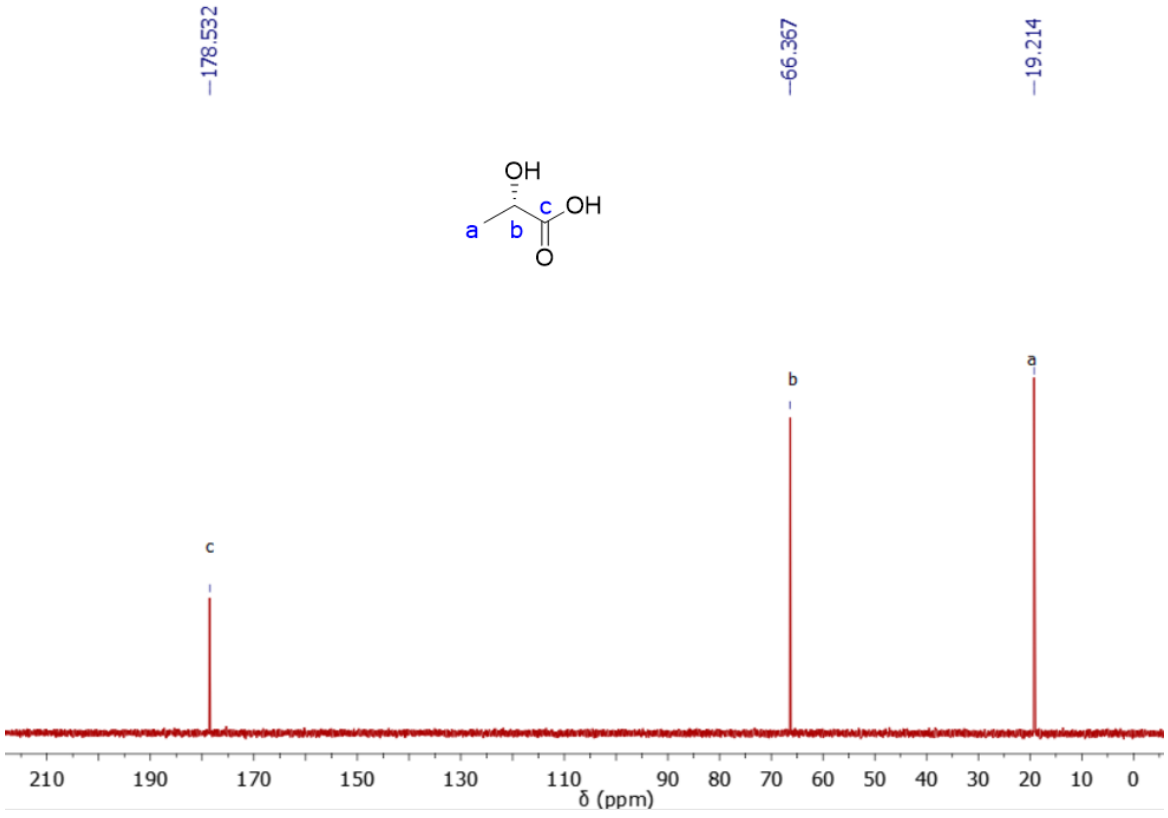


Figure 18. ^{13}C NMR spectra of lactic acid in D_2O produced after hydrolysis of the purified ester.

Conclusions

Overall, we succeeded in reproducing the procedure proposed by Sakai et al.[51] in different samples of food waste collected from the municipality of Heraklion and optimized the protocol in terms of reaction temperatures (bearing a significant effect on energy consumption in a pilot plant) and characterization (bearing a significant effect in the yield). The overall yield in lactic acid was roughly 100 times lower that the one reported by Sakai and collaborators (*i.e.* 1 g lactic acid/L of food waste. Since our experiments were performed on laboratory scale, a lower yield was expected.

Several important conclusions were drawn from this study.

A. Fermentations:
    i. Sterile conditions (not mentioned in the Sakai paper) necessary for the fermentations.
    ii. ^{1}H NMR spectroscopy, HPLC chromatography or -in the last fermentation- titration should be performed in samples of the broth to ensure fermentation efficiency. pH should be monitored and adjusted throughout fermentations.
    iii. High glucose content in the fermented broth renders handling of the broth difficult in the subsequent chemical steps.
    iv. No conclusions were drawn as to the effect of enriching food waste with starch. Further experiments need to be performed.
    v.
B. Lactic acid isolation
    i. Solid residue separation: best approach found to be centrifugation followed by filtration.
    ii. Esterification reaction: The initial concentration of ammonium lactate (40% w/v) and the butanol to ammonium lactate 3/1 molar ratio have crucial role on esterification. The reaction optimally proceeds at 130 °C - 140 °C.
    iii. Hydrolysis reaction: a water/butyl lactate molar ratio of 20/1 ensures the formation of a homogenous reaction mixture which was found to have an important effect on the yield. The reaction optimally proceeds at 100 °C - 110 °C.

Chapter 3: Experimental part

3.1 Materials

Food Waste Samples

Food waste samples were collected from Hotels and the restaurant at the Hellenic Mediterranean University. Four different food waste batches were used in all of our experiments:

- Food waste from the Hellenic Mediterranean University (HMU) restaurant,
- Food waste recovered from Hotels,
- Food waste enriched with 20% starch, and
- 100% starch.

The components of the municipal food waste used for fermentation were a mix of vegetables, fruits, meat, rice, spaghetti and others, in various percentages.

Culture and media

The stock culture of *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271T (DSMZ, Germany) was cultivated anaerobically at 30°C in BHI medium (Sigma Aldrich) containing (per litre) 5 g/L beef heart, 12.5 g/L calf brains, 2.5 g/L disodium hydrogen phosphate, 2 g/L D (+)-glucose, 10 g/L peptone and 5 g/L sodium chloride in 50 mL falcon tubes.

Lactobacillus Rhamnosus KY-3 was grown under stirring (200 rpm) at 37 °C overnight (18 h) in BHI medium.

Reagents

All materials used during the experiments were purchased from Sigma-Aldrich, Fischer Scientific or Carlo Erba and used without further purification. Deuterated solvents were obtained from Cambridge Isotope Laboratories. Glucoamylase from *A. niger* in a maltodextrin and corn starch carrier (Saccazyme) was purchased from The Alchemists Pantry (GB).

3.2 Analytical Methods

Optical Density

Cell growth was monitored by measuring the optical density (OD) at 600 nm in a 1.5 mL cuvette using a Jenway 6305spectrophotometer. Broth samples with suspended cells were diluted to an OD reading of less than 0.8 with distilled water.

Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H and ^{13}C spectra were recorded on a Bruker AMX-500 MHz spectrometer. Proton nuclear magnetic resonance spectra are reported in parts per million (ppm) on the δ scale and are referenced from the residual protium in the NMR solvent (chloroform-d_1: δ 7.26, water-d_2: 4.79). Data is reported as follows: chemical shift [multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constants (*J*) in Hertz, integration]. Carbon-13 nuclear magnetic resonance spectra are reported in parts per million (ppm) on the δ scale and are referenced from the carbon resonance of the solvent (chloroform-d_1: δ 77.00).

High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography was using a Shimadzu modular system comprising a DGU-14A solvent degasser, an LC-10AD pump, a CTO-10A column oven, a SIL-10AD auto-injector, a RID-10A refractive index detector and a SPD10A Shimadzu UV-Vis. spectrometer. The system was equipped either with a SUPELCOSIL^{TM} LC-NH_2 (5 μm, 25 cm × 4.6 mm) or with a Perfectsil^{TM} C8 (5 μm, 25 cm × 4.6 mm) column. The detection wavelengths, injection volume and flow rate were 254 nm, 10 μL and 0.5 - 0.8 mL min^{-1}, respectively. A 10-25% nanopure water solution in MeCN was used as a mobile phase at room temperature.

Optical Rotation

Optical rotation was measured on a P3000 automatic polarimeter (A. Krüss Optronic). $[\alpha]_D^T$ values are quoted in g/100 mL concentration using a 50 mm polarimeter tube, where D refers to the D-line of sodium (589 nm) and temperatures (T) are given in degrees Celsius (°C).

3.3 Experimental Section

3.3.1 Production of L-lactic acid

For the L- lactic acid production in the food waste, consumption of naturally occurring lactides followed by saccharification and fermentation were performed on laboratory scale.

Prior to fermentations, food waste samples were mince and homogenized with equal amount of deionized water.

Propionic acid and lactic acid fermentation

The minced and homogenized food waste batches were sterilized in an autoclave for 30 min at 120 °C. The pH of the sterile food waste was adjusted at 5.5 (using NaOH, 5 N) and then each food waste batch was inoculated with Propionibacterium *freudenreichii* culture. The suspension was rotated at 60 rpm for 16 hours at 37 °C to consume naturally occurring D-, L-lactic acid enantiomers.

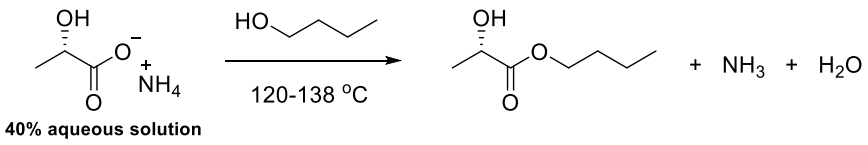
After the first fermentation, the temperature was increased to 50 °C and α-*glucoamylase* (20.000 units/g) was added to the food residues to hydrolyse starch into simpler sugars such as glucose. Saccharification proceeded for 4 hours.

The pH was then adjusted to 6.5 (using NH_4OH, 5 N) and *Lactobacillus rhamnosus* KY-3 was seeded at an initial cell density of ca. 1.2×10^8 cells/mL. The culture was incubated at 37 °C for 3-4 days under gentle stirring (60 rpm). To optimize L- lactic acid production, several samples were studied after periodical pH adjustment to 6.5 (with NH_4OH, 5 N).

Two cycles of experiments were carried out using the four different food waste batches for the purification of L- lactic acid.

3.3.2 Purification of L-lactic acid

Synthesis of butyl (S)-2-hydroxypropanoate from fermented broth



40% aqueous solution

The fermentation broth was centrifuged at 5,000 rpm for 1 hour at 4 °C to separate cell biomass. The supernatant was filtered under vacuum to separate solid residues from the fermented liquid. The filtrate was characterized by ^{1}H-NMR after which it was concentrated under reduced pressure to yield an average of 40% w/w ammonium lactate. Butanol (3 mol/mol of ammonium lactate) was added to the concentrate ammonium lactate solution and the resulting mixture was refluxed (107 °C initial internal temperature) for 7 hours, under a distillation apparatus. The azeotropic vapour of butanol and water were collected in the distillation flask in a two-phase condensate. The upper layer of the distillation flask was periodically returned to the reaction flask while the lower water layer was withdrawn. A 11% w/v hydrochloric acid solution equipped with a safety trap was used to absorb the produced ammonia. The temperature of the reaction mixture ranged between 107 °C and 122 °C within the 7-hour reaction (external

temperature range between 120 °C and 138 °C) after which, the removal of water was practically ceased. Finally, the remaining butanol was removed by distillation in vacuum (internal temperature range 55 °C -65 °C) and a solution of butyl lactate in butanol was collected via vacuum distillation (internal temperature range 70 °C -120 °C) as a yellowish oil.

^{1}H NMR of ammonium lactate (500 MHz, D_2O): 4.17 (q, *J* = 7.0 Hz, 1H, C*H*CH_3), 1.20 (d, *J* = 7.0 Hz, 3H, CHC*H*$_3$).
^{1}H NMR of butyl lactate (500 MHz, $CDCl_3$): δ 4.26 (q, *J* = 6.9 Hz, 1H, C*H*CH_3), 4.23-4.13 (m, 2H, OC*H*$_2$$CH_2$), 2.44 (br. s, 1H, O*H*), 1.68-1.61 (m, 2H, OCH_2C*H*$_2$), 1.41 (d, *J* = 6.8 Hz, 3H, CHC*H*$_3$), 1.42-1.34 (m, 2H, C*H*$_2$$CH_3$), 0.94 (t, *J* = 7.4 Hz, 3H, CH_2C*H*$_3$). ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.84 (*C*=O), 66.73 (*C*HCH_3), 65.53 (O*C*H$_2$$CH_2$), 30.54 ($OCH_2$*C*H$_2$), 20.43 (CH*C*H$_3$), 18.99 (*C*H$_2$$CH_3$), 13.61 ($CH_2$*C*H$_3$). $[\alpha]_D^{19}$= -10 (*c* 1.0, MeOH). R_f (20% ethyl acetate in petroleum ether): 0.60.

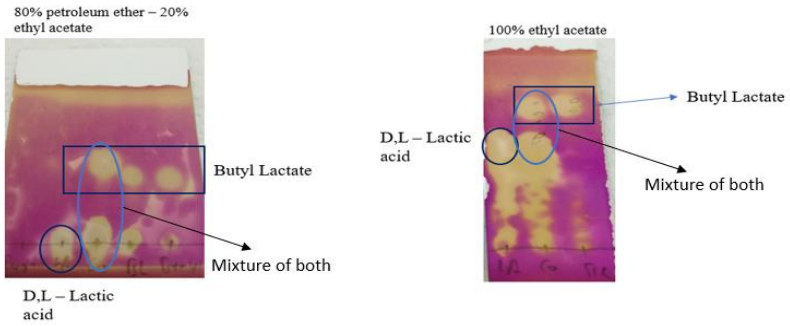
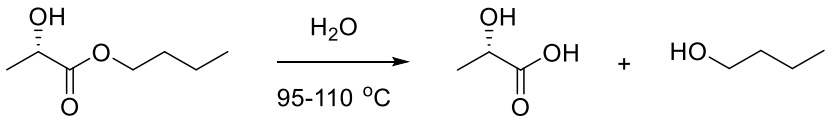


Figure 20. Representative TLC plates used during esterification for the sample of food waste enriched with 20% starch. Left: mobile phase 80% petroleum ether: 20% ethyl acetate , Right: mobile phase 100% ethyl acetate.

Synthesis of (S)-2-hydroxypropanoic acid (L-lactic acid)



^{1}H-NMR was used to calculate the relative butyl lactate/butanol concentrations. 24.8 mol H_2O/ester mol were added and the mixture was refluxed at 95-110 °C for 7 hours. Butanol and water were subsequently removed under vacuum distillation to furnish lactic acid as a yellowish oil.

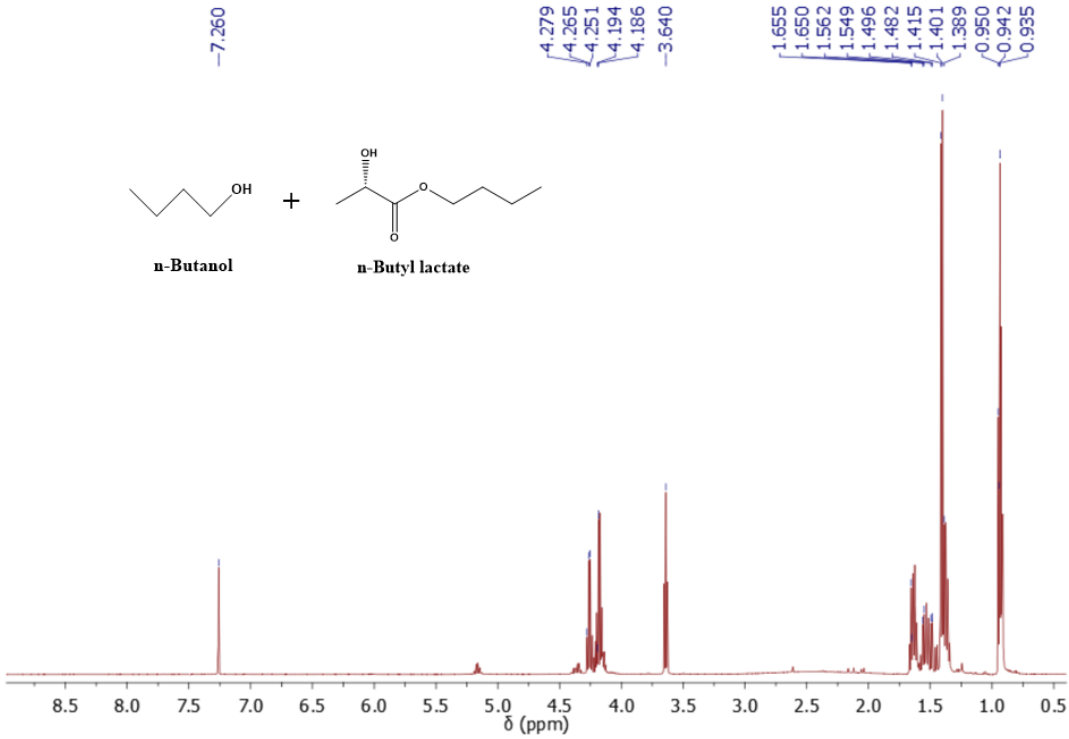
It is worth mentioned that in case of a low butyl lactate percentage in n-butanol, a biphasic mixture was observed leading to incomplete hydrolysis, unless methanol was used to homogenize the mixture.

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.61 (br. s, 1H, CHO*H*), 4.38 (q, *J* = 7.0 Hz, 1H, C*H*CH_3), 1.49 (d, *J* = 7.0 Hz, 3H, CHC*H*$_3$). ^{13}C NMR (125 MHz, $CDCl_3$): δ 179.89 (*C*=O), 66.54 (*C*HCH_3), 20.15 (CH*C*H_3). R_f (100% ethyl acetate): 0.47.

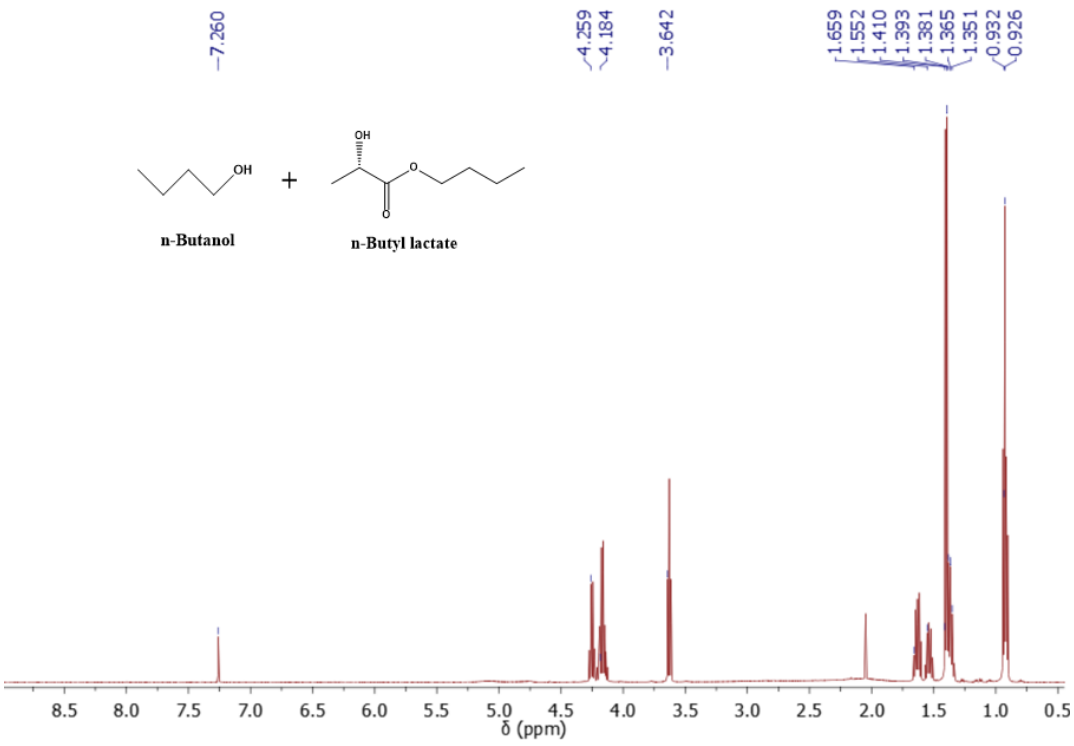
Appendix

1st cycle

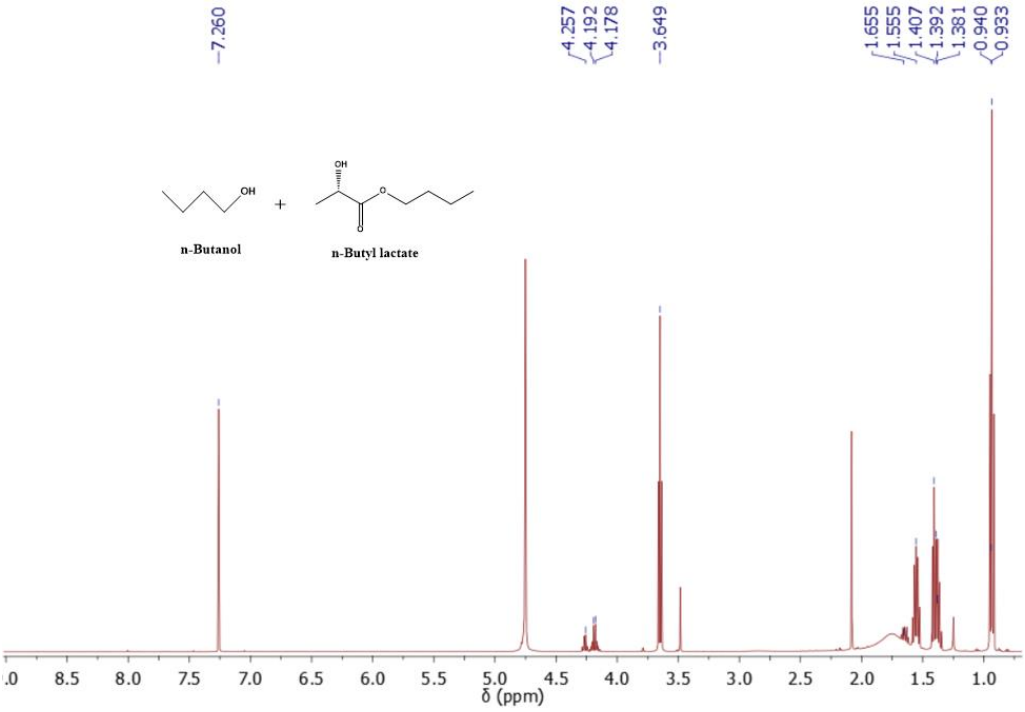
^{1}H NMR spectrum of butanol–butyl lactate mixture in $CDCl_3$ **(100% starch)**



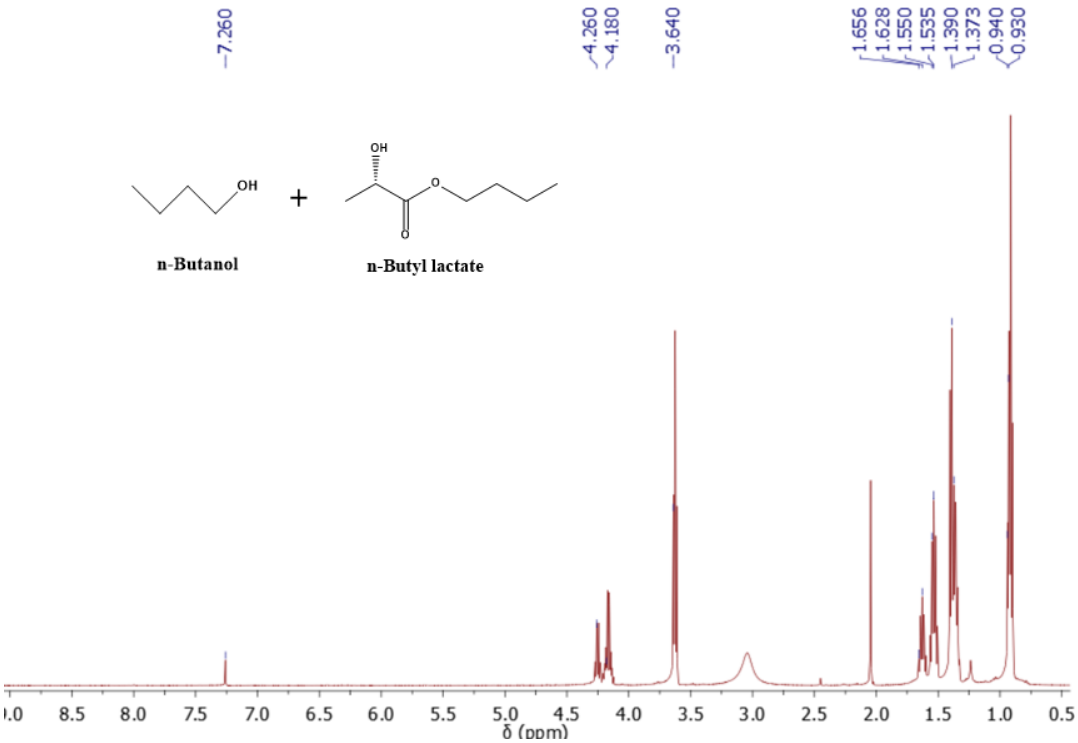
^{1}H NMR spectrum of butanol–butyl lactate mixture in $CDCl_3$ **(Food waste enriched with 20% starch)**



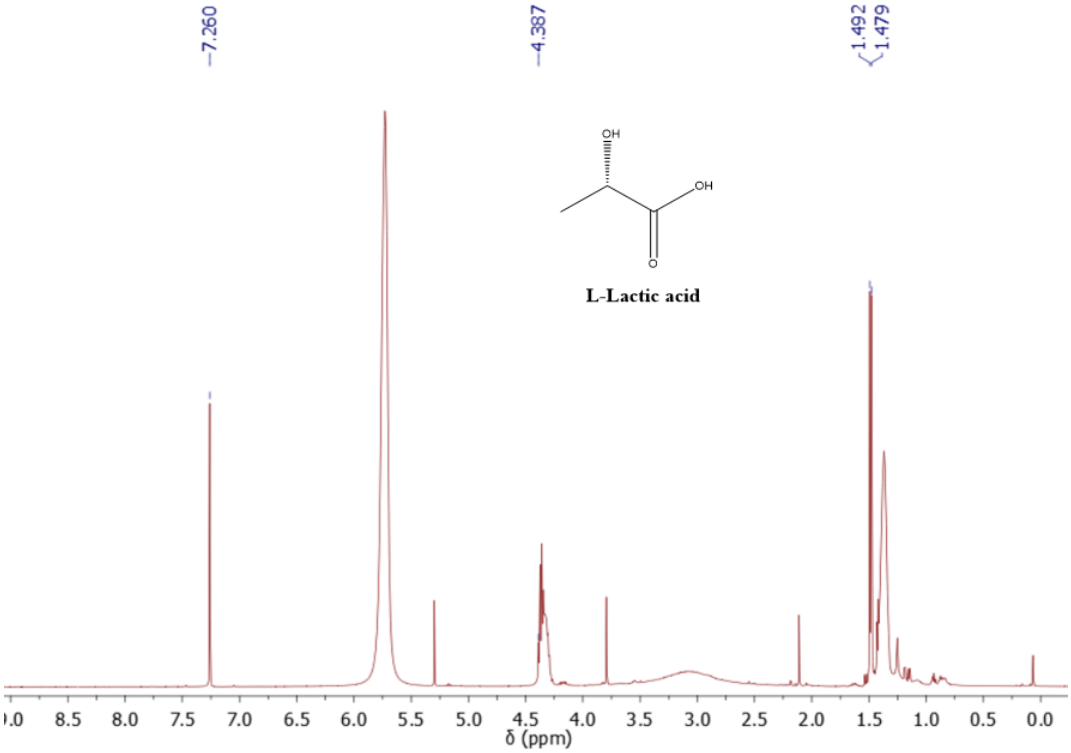
- ^{1}H NMR spectrum of bButanol–butyl lactate mixture in $CDCl_3$ (**Food waste from the Hellenic Mediterranean University (HMU) restaurant**)



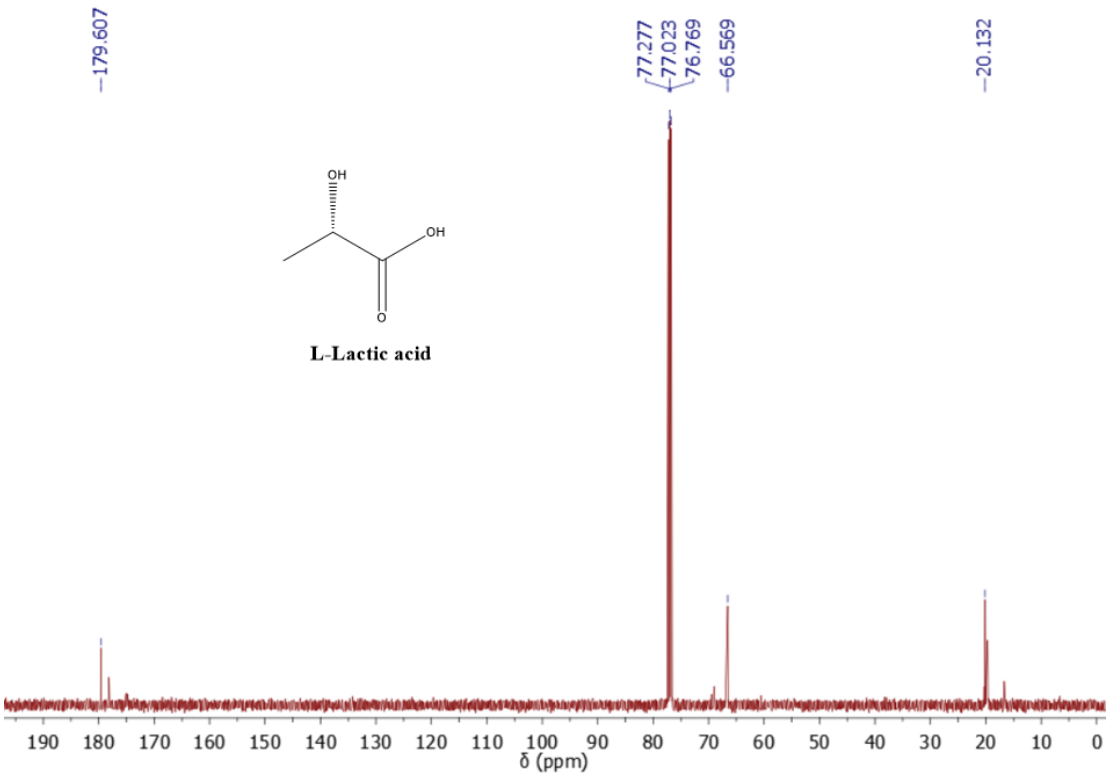
^{1}H NMR spectrum of bButanol–butyl lactate mixture in $CDCl_3$ **(Food waste recovered from Hotels)**



^{1}H NMR Spectrum of L-Lactic Acid in $CDCl_3$

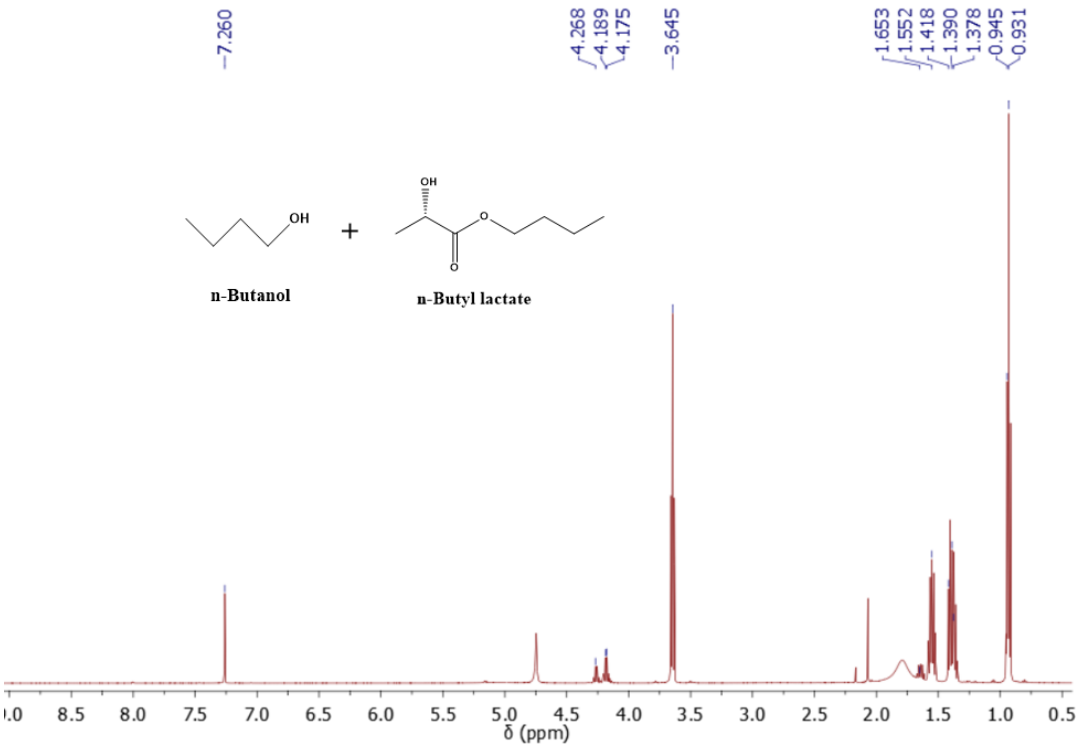


^{13}C NMR Spectrum of L-Lactic Acid in $CDCl_3$

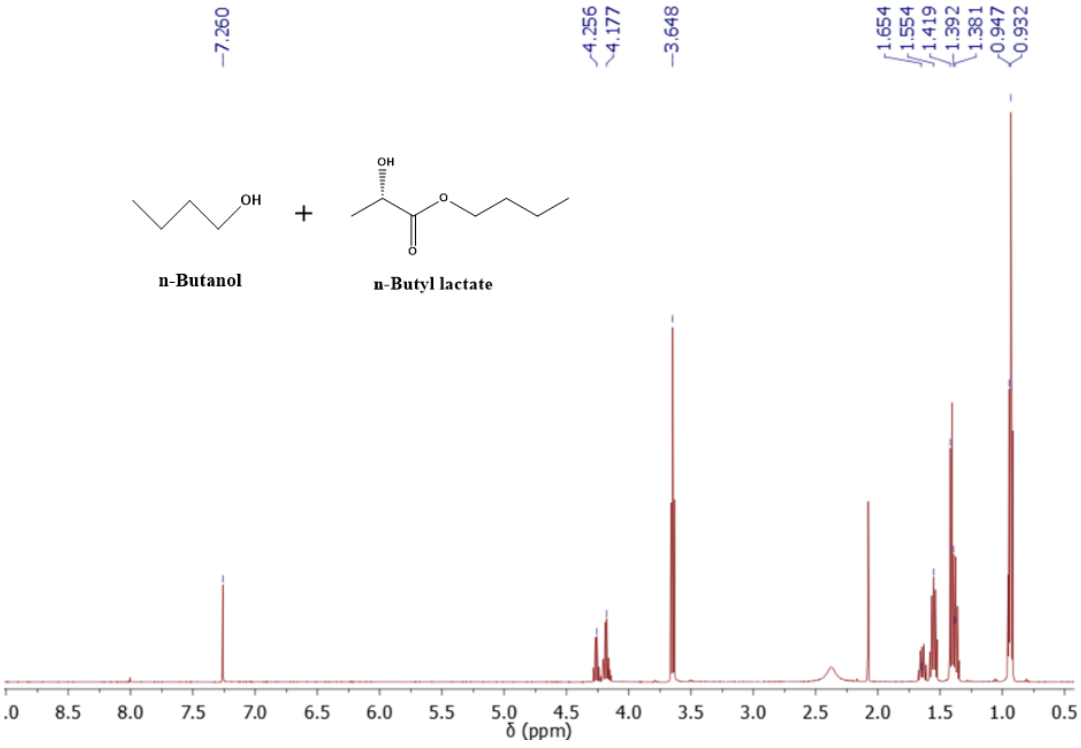


2nd cycle

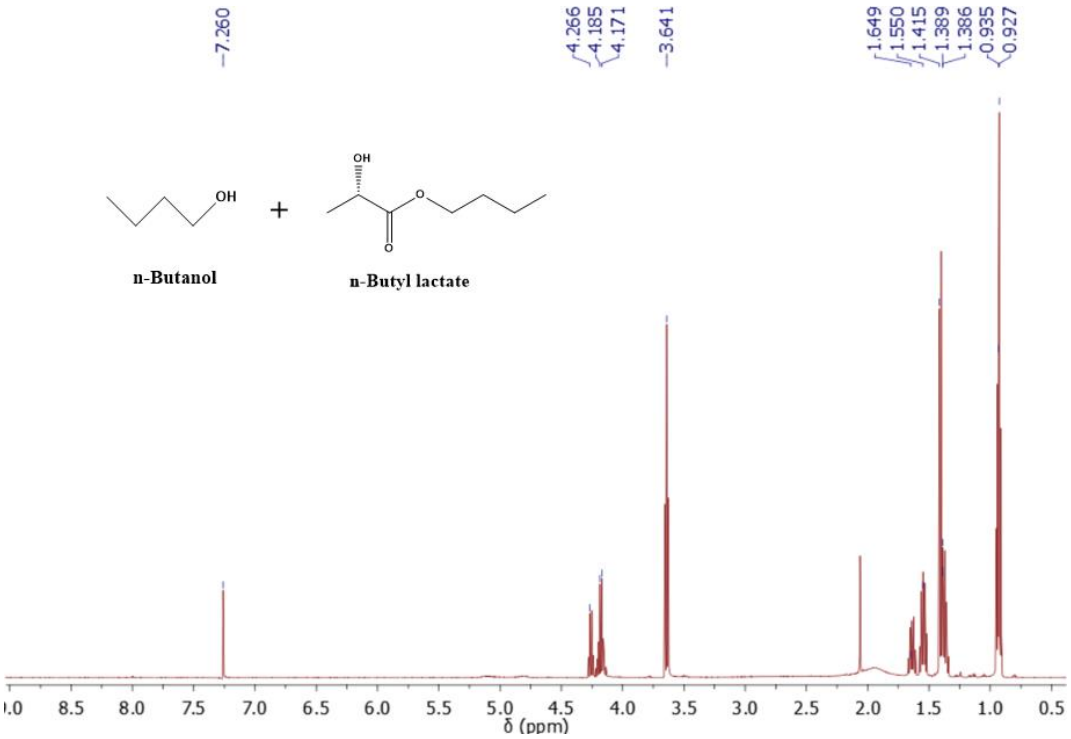
^{1}H NMR spectrum of butanol–butyl lactate mixture in $CDCl_3$ **(100% starch)**



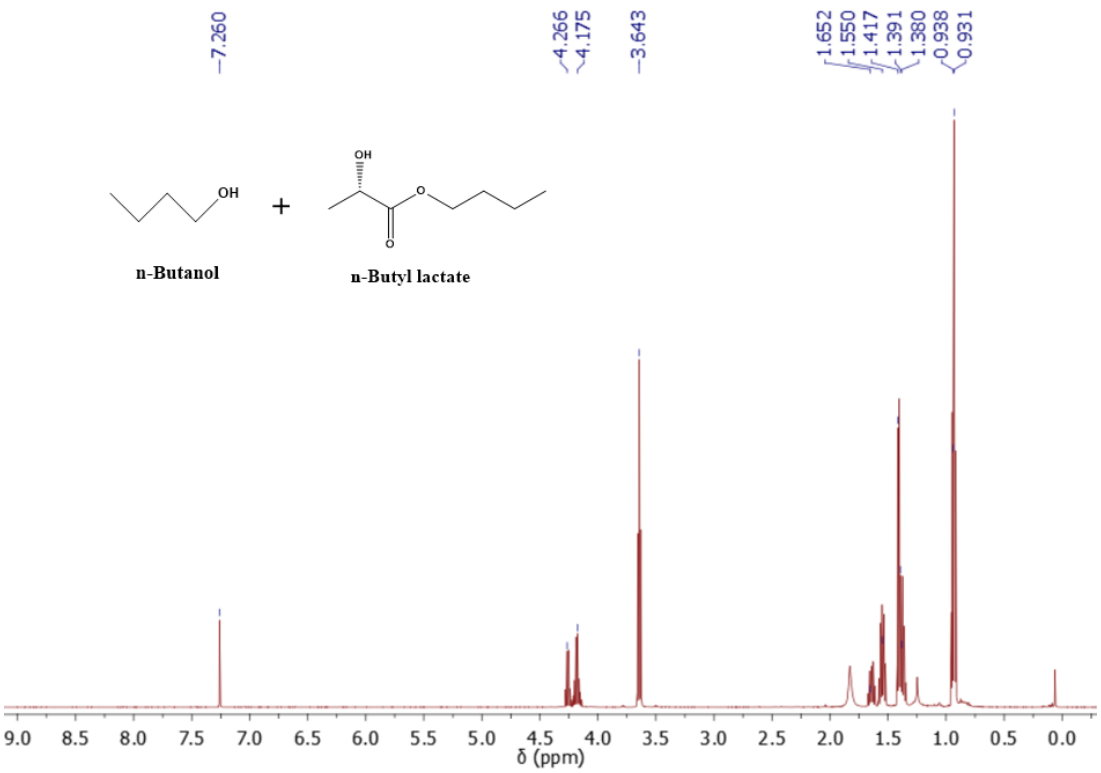
^{1}H NMR spectrum of butanol–butyl lactate mixture in $CDCl_3$ **(Food waste enriched with 20% starch)**



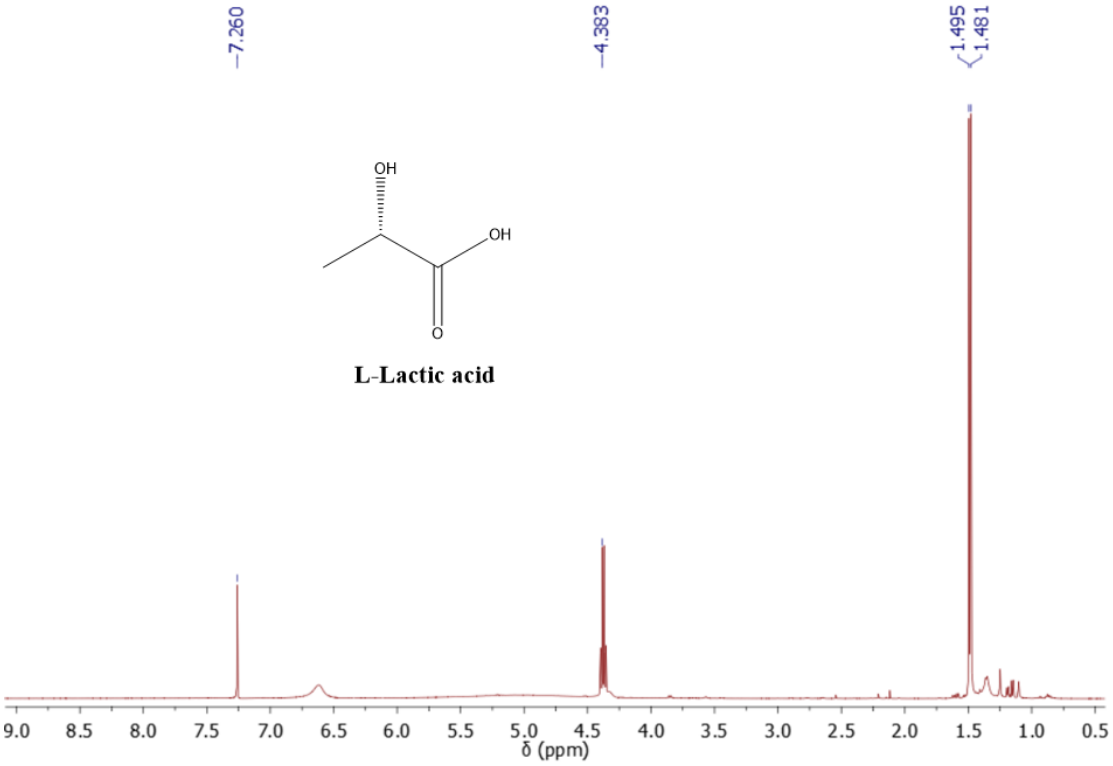
^{1}H NMR Spectrum of butanol–butyl lactate mixture in $CDCl_3$ **(Food waste from the Hellenic Mediterranean University (HMU) restaurant)**



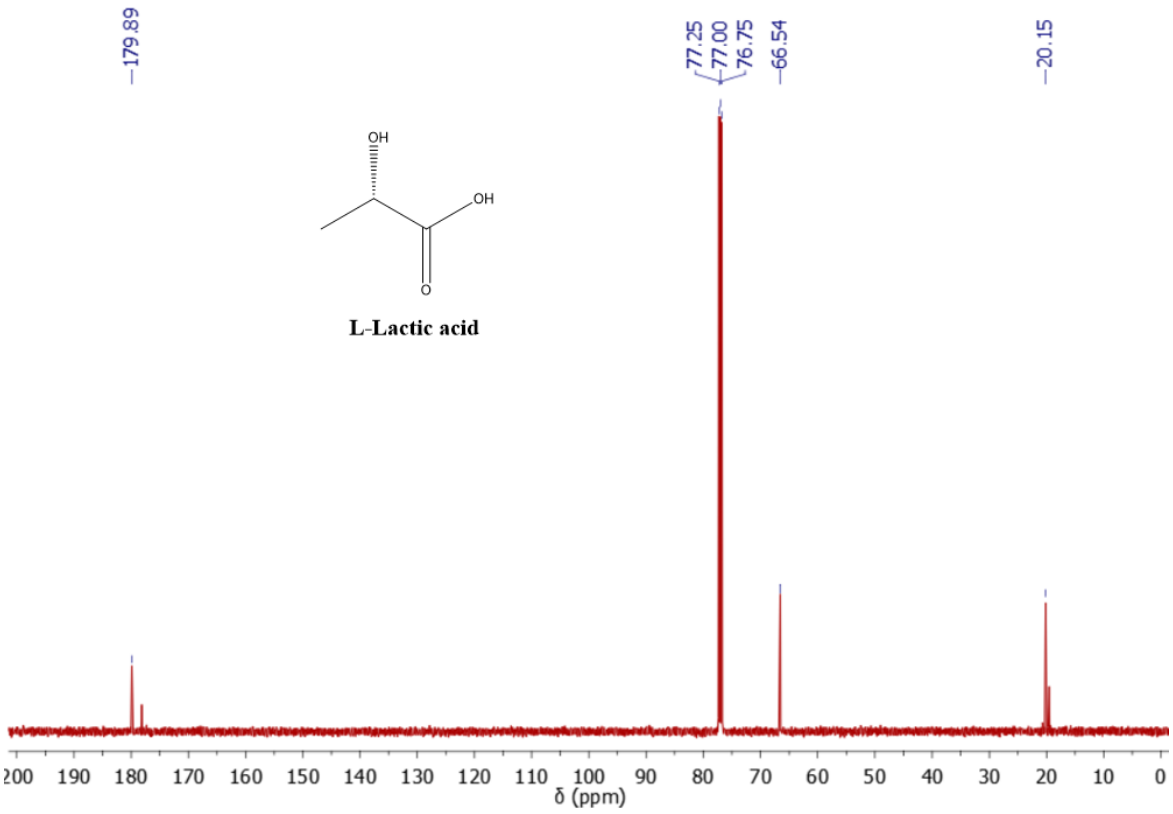
^{1}H NMR Spectrum of butanol–butyl lactate mixture in $CDCl_3$ **(Batch: Hotels)**



^{1}H NMR Spectrum of L-lactic acid in $CDCl_3$



^{13}C NMR Spectrum of L-lactic acid in $CDCl_3$



Summary tables

Table 1. Total initial volumes of food waste and butanol used throughout the procedure

Batches	**100% starch**	**Food waste enriched with 20% starch**	**Food waste from the Hellenic Mediterranean University (HMU) restaurant**	**Food waste recovered from Hotels**	**SUM**
Total volume after fermentation (mL)	700	1800	2000	1600	6100
FW used 1st round (mL)	335	322	500 (-422.4 mL)	500	1657
FW used 2nd round (mL)	340	600	700	700	2340
Remaining	25	878	378	400	1681
Total BuOH used in the first and second cycle (mL)	(70 + 15) = 85	(37+53) = 89	(56+79) = 135	(70+87) = 157	(233+234) = 467

1st cycle of experiments

Table 2. Summary table with all the results which arose during the first cycle of experiments

Batches	**100% starch**	**Food waste enriched with 20% starch**	**Food waste from the Hellenic Mediterranean University (HMU) restaurant**	**Food waste recovered from Hotels**
Fermented broth (mL)	335	322	500	500
Filtrate (mL)	148 [a]	203	339	387
Concentrate[b] (mL)	35 (72.5% NH_4LA)	36 (40.0% NH_4LA)	55 (43.9% NH_4LA)	69 (40.0% NH_4LA)
BuLA conc. in BuOH (%), mass of the ester (g)	77.8 **(3.014 g)**	75.5 **(1.220 g)**	17.3 **(0.259 g)**	45.0 **(0.094 g)**
Esterification yield (%)	15.9 n_{BL}= 20.61 mmol	6.3 n_{BL}= 8.35 mmol	0.8 n_{BL}= 1.77 mmol	0.25 n_{BL}= 0.64 mmol
Hydrolysis yield for 90% Lactic acid	**66.3%** **m_{LA}= 1.906 g** **n_{LA}= 21.16 mmol**			

[a] solid residue was further washed with H_2O, final filtrate volume: 356 mL.
[b] theoretical percentage, according to literature.

2nd cycle of experiments (optimization)

Table 3. Summary table with all the results which arose during the second cycle of experiments

Batches	**100% starch**	**Food waste enriched with 20% starch**	**Food waste from the Hellenic Mediterranean University (HMU) restaurant**	**Food waste recovered from Hotels**
Fermented broth (mL)	340	600	700	700
Filtrate (mL)	165 (3.6% NH_4LA)[a]	294	434	479
Concentrate (mL)	15 (43.7% NH_4LA)[a]	52 (40% NH_4LA)[b]	77 (28% NH_4LA)[a]	85 (40% NH_4LA)[b]
BuLA conc. in BuOH (%), mass of the ester (g)	14.0% **(0.532 g)**	27 % **(0.315 g)**	36.7 % **(4.080 g)**	52.9% **(1.060 g)**
Esterification yield (%)	5.9 n_{BL}= 3.63 mmol	1.1 n_{BL}= 2.15 mmol	13.8 n_{BL}= 27.91 mmol	2.3 n_{BL}= 7.25 mmol
Hydrolysis yield for 90% lactic acid	**56.6%** **m_{LA}= 2.04 g** **n_{LA}= 22.65 mmol**			

[a] experimental percentage, according to ^{1}H-NMR.
[b] theoretical percentage, according to literature.

Bibliography

(1) Sharma, P.; Gaur, V. K.; Kim, S.-H.; Pandey, A. Microbial strategies for bio-transforming food waste into resources. *Bioresource Technology* **2020**, *299*, 122580.

(2) Mamma, D. Food wastes: feedstock for value-added products. *Fermentation* **2020**, *6* (2), 47.

(3) Nayak, A.; Bhushan, B. An overview of the recent trends on the waste valorization techniques for food wastes. *Journal of Environmental Management* **2019**, *233*, 352–370.

(4) Lin, C. S. K.; Pfaltzgraff, L. A.; Herrero-Davila, L.; Mubofu, E. B.; Abderrahim, S.; Clark, J. H.; Koutinas, A. A.; Kopsahelis, N.; Stamatelatou, K.; Dickson, F.; Thankappan, S.; Mohamed, Z.; Brocklesby, R.; Luque, R. Food waste as a valuable resource for the production of chemicals, materials and fuels. current situation and global perspective. *Energy Environ. Sci.* **2013**, *6* (2), 426–464.

(5) Tsang, Y. F.; Kumar, V.; Samadar, P.; Yang, Y.; Lee, J.; Ok, Y. S.; Song, H.; Kim, K.-H.; Kwon, E. E.; Jeon, Y. J. Production of bioplastic through food waste valorisation. *Environment International* **2019**, *127*, 625–644.

(6) Shah, A. A.; Hasan, F.; Hameed, A.; Ahmed, S. Biological degradation of plastics: a comprehensive review. *Biotechnology Advances* **2008**, *26* (3), 246–265.

(7) Gironi, F.; Piemonte, V. Bioplastics and petroleum-based plastics: strengths and weaknesses. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects* **2011**, *33* (21), 1949–1959.

(8) López-Gómez, J. P.; Alexandri, M.; Schneider, R.; Venus, J. A review on the current developments in continuous lactic acid fermentations and case studies utilising inexpensive raw materials. *Process Biochemistry* **2019**, *79*, 1–10.

(9) Alves de Oliveira, R.; Komesu, A.; Vaz Rossell, C. E.; Maciel Filho, R. Challenges and opportunities in lactic acid bioprocess design—from economic to production aspects. *Biochemical Engineering Journal* **2018**, *133*, 219–239.

(10) Wee, Y.-J.; Kim, J.-S.; Ryu, H.-W. Biotechnological production of lactic acid and its recent applications; **2006**.

(11) Okano, K.; Tanaka, T.; Ogino, C.; Fukuda, H.; Kondo, A. Biotechnological production of enantiomeric pure lactic acid from renewable resources: recent achievements, perspectives, and limits. *Appl. Microbiol. Biotechnol.* **2010**, *85* (3), 413–423.

(12) Ren, J. *Biodegradable polylactic acid: synthesis, modification, processing and applications*; Berlin, Heidelberg, 2010; pp 1–3.

(13) Platel, R. H.; Hodgson, L. M.; Williams, C. K. Biocompatible initiators for lactide polymerization. *Polymer Reviews* **2008**, *48* (1), 11–63.

(14) Sanchez-Vazquez, S. A.; Hailes, H. C.; Evans, J. R. G. Hydrophobic polymers from food waste: resources and synthesis. *Polymer Reviews* **2013**, *53* (4), 627–694.

(15) Tokiwa, Y.; Calabia, B. P. Biodegradability and biodegradation of polylactide. *Appl. Microbiol. Biotechnol.* **2006**, *72* (2), 244–251.

(16) Żenkiewicz, M.; Richert, J.; Rytlewski, P.; Moraczewski, K.; Stepczyńska, M.; Karasiewicz, T. Characterisation of multi-extruded polylactic acid. *Polymer Testing* **2009**, *28* (4), 412–418.

(17) Song, R.; Murphy, M.; Li, C.; Ting, K.; Soo, C.; Zheng, Z. Current development of biodegradable polymeric materials for biomedical applications. *Drug Design, Development and Therapy* **2018**, *Volume 12*, 3117–3145.

(18) Ho, M.; Lau, K.; Wang, H.; Hui, D. Improvement on the properties of polylactic acid (PLA) using bamboo charcoal particles. *Composites Part B: Engineering* **2015**, *81*, 14–25.

(19) Liu, S.; Qin, S.; He, M.; Zhou, D.; Qin, Q.; Wang, H. Current applications of polylactic acid composites in tissue engineering and drug delivery. *Composites Part B: Engineering* **2020**, *199*, 108238.

(20) Ozdil, D.; Aydin, H. M. Polymers for medical and tissue engineering applications. *Journal of Chemical Technology & Biotechnology* **2014**, *89* (12), 1793–1810.

(21) Asghari, F.; Samiei, M.; Adibkia, K.; Akbarzadeh, A.; Davaran, S. Biodegradable and biocompatible polymers for tissue engineering application: a review. *Artificial Cells, Nanomedicine, and Biotechnology* **2017**, *45* (2), 185–192.

(22) Ren, J. *Biodegradable polylactic acid: synthesis, modification, processing and applications*; Ren, J., Ed.; Springer: Berlin, Heidelberg, 2010; pp 4–14.

(23) Komesu, A.; Oliveira, J. A. R. de; Martins, L.; Maciel, M.; Filho, R. M. Lactic acid production to purification: a review. **2017**.

(24) Komesu, A.; Maciel, M. R. W.; Oliveira, J. A. R. de; Martins, L. H. da S.; Filho, R. M. Purification of lactic acid produced by fermentation: focus on non-traditional distillation processes. *Separation & Purification Reviews* **2017**, *46* (3), 241–254.

(25) Sakai, K.; Poudel, P.; Shirai, Y. Total recycle system of food waste for poly-L-lactic acid output. *Advances in Applied Biotechnology* **2012**.

(26) Hofvendahl, Hahn-Hägerdal. Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme Microb. Technol.* **2000**, *26* (2–4), 87–107.

(27) Djukić-Vuković, A.; Mladenović, D.; Ivanović, J.; Pejin, J.; Mojović, L. Towards sustainability of lactic acid and poly-lactic acid polymers production. *Renewable and Sustainable Energy Reviews* **2019**, *108*, 238–252.

(28) Rawoof, S. A. A.; Kumar, P. S.; Vo, D.-V. N.; Devaraj, K.; Mani, Y.; Devaraj, T.; Subramanian, S. Production of optically pure lactic acid by microbial fermentation: a review. *Environ Chem Lett* **2020**. https://doi.org/10.1007/s10311-020-01083-w

(29) Castillo Martinez, F. A.; Balciunas, E. M.; Salgado, J. M.; Domínguez González, J. M.; Converti, A.; Oliveira, R. P. de S. Lactic acid properties, applications and production: a review. *Trends in Food Science & Technology* **2013**, *30* (1), 70–83.

(30) Doble, M.; Kruthiventi, A. K. Alternate solvents. *Green chemistry and engineering*; Chapter 5 Academic Press: Burlington, **2007**, 93–104.

(31) Datta, R.; Tsai, S.-P.; Bonsignore, P.; Moon, S.-H.; Frank, J. R. Technological and economic potential of polylactic acid and lactic acid derivatives. *FEMS Microbiol Rev* **1995**, *16* (2–3), 221–231.

(32) Pal, P.; Sikder, J.; Roy, S.; Giorno, L. Process intensification in lactic acid production: a review of membrane-based processes. *Chemical Engineering and Processing: Process Intensification* **2009**, *48* (11), 1549–1559.

(33) Gao, C.; Ma, C.; Xu, P. Biotechnological routes based on lactic acid production from biomass. *Biotechnology Advances* **2011**, *29* (6), 930–939.

(34) Datta, R.; Henry, M. Lactic acid: recent advances in products, processes and technologies-a review. *Journal of Chemical Technology & Biotechnology* **2006**, *81* (7), 1119–1129.

(35) Ghaffar, T.; Irshad, M.; Anwar, Z.; Aqil, T.; Zulifqar, Z.; Tariq, A.; Kamran, M.; Ehsan, N.; Mehmood, S. Recent trends in lactic acid biotechnology: a brief review on production to purification. *Journal of Radiation Research and Applied Sciences* **2014**, *7* (2), 222–229.

(36) Hofvendahl, Hahn-Hägerdal. Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme Microb. Technol.* **2000**, *26* (2–4), 87–107.

(37) Dumbrepatil, A.; Adsul, M.; Chaudhari, S.; Khire, J.; Gokhale, D. Utilization of molasses sugar for lactic acid production by lactobacillus delbrueckii subsp. delbrueckii mutant uc-3 in batch fermentation. *Appl. Environ. Microbiol.* **2008**, *74* (1), 333–335.

(38) Jantasee, S.; Kienberger, M.; Mungma, N.; Siebenhofer, M. Potential and assessment of lactic acid production and isolation – a review. *Journal of Chemical Technology & Biotechnology* **2017**, *92* (12), 2885–2893.

(39) Nakano, S.; Ugwu, C. U.; Tokiwa, Y. Efficient production of D-(−)-lactic acid from broken rice by lactobacillus delbrueckii using Ca $(OH)_2$ as a neutralizing agent. *Bioresource Technology* **2012**, *104*, 791–794.

(40) Hetényi, K.; Németh, Á.; Sevella, B. Investigation and modeling of lactic acid fermentation on wheat starch via SSF, CHF and SHF technology. **2011**.

(41) Åkerberg, C.; Zacchi, G. An economic evaluation of the fermentative production of lactic acid from wheat flour. *Bioresource Technology* **2000**, *75* (2), 119–126.

(42) Richter, K.; Berthold, C. Biotechnological conversion of sugar and starchy crops into lactic acid. *Journal of Agricultural Engineering Research* **1998**, *71* (2), 181–191.

(43) Venkatesh, K. V. Simultaneous Saccharification and fermentation of cellulose to lactic acid. *Bioresource Technology* **1997**, *62* (3), 91–98.

(44) Oh, H.; Wee, Y.-J.; Yun, J.-S.; Ho Han, S.; Jung, S.; Ryu, H.-W. Lactic acid production from agricultural resources as cheap raw materials. *Bioresour. Technol.* **2005**, *96* (13), 1492–1498.

(45) Xiaodong, W.; Xuan, G.; Rakshit, S. K. Direct fermentative production of lactic acid on cassava and other starch substrates. *Biotechnology Letters* **1997**, *19* (9), 841–843.

(46) Wang, Y.; Wang, M.; Cai, D.; Wang, B.; Wang, Z.; Qin, P.; Tan, T. Efficient L-lactic acid production from sweet sorghum bagasse by open simultaneous saccharification and fermentation. *RSC Adv.* **2016**, *6* (42), 35771–35777.

(47) Pauli, T.; Fitzpatrick, J. J. Malt combing nuts as a nutrient supplement to whey permeate for producing lactic by fermentation with *Lactobacillus casei*. *Process Biochemistry* **2002**, *38* (1), 1–6.

(48) Fitzpatrick, J. J.; Ahrens, M.; Smith, S. Effect of manganese on *Lactobacillus casei* fermentation to produce lactic acid from whey permeate. *Process Biochemistry* **2001**, *36* (7), 671–675.

(49) Büyükkileci, A. O.; Harsa, S. Batch production of L (+) lactic acid from whey by *Lactobacillus casei* (NRRL B-441). *Journal of Chemical Technology & Biotechnology* **2004**, *79* (9), 1036–1040.

(50) Nguyen, C. M.; Kim, J.-S.; Hwang, H. J.; Park, M. S.; Choi, G. J.; Choi, Y. H.; Jang, K. S.; Kim, J.-C. Production of L-lactic acid from a green microalga, hydrodictyon reticulum, by *Lactobacillus paracasei* LA104 Isolated from the traditional Korean food, makgeolli. *Bioresour. Technol.* **2012**, *110*, 552–559.

(51) Sakai, K.; Taniguchi, M.; Miura, S.; Ohara, H.; Matsumoto, T.; Shirai, Y. Making plastics from garbage. *Journal of Industrial Ecology* **2003**, *7* (3–4), 63–74.

(52) Abdel-Rahman, M. A.; Tashiro, Y.; Sonomoto, K. Recent advances in lactic acid production by microbial fermentation processes. *Biotechnology Advances* **2013**, *31* (6), 877–902.

(53) Abdel-Rahman, M. A.; Sonomoto, K. Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. *Journal of Biotechnology* **2016**, *236*, 176–192.

(54) Zhao, K.; Qiao, Q.; Chu, D.; Gu, H.; Dao, T. H.; Zhang, J.; Bao, J. Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium *Pediococcus acidilactici DQ2*. *Bioresour. Technol.* **2013**, *135*, 481–489.

(55) Kylä-Nikkilä, K.; Hujanen, M.; Leisola, M.; Palva, A. Metabolic engineering of *Lactobacillus helveticus CNRZ32* for production of pure L-(+)-lactic acid. *Appl Environ Microbiol* **2000**, *66* (9), 3835–3841.

(56) Lu, Z.; He, F.; Shi, Y.; Lu, M.; Yu, L. Fermentative production of L (+)-lactic Acid using hydrolyzed acorn starch, persimmon juice and wheat bran hydrolysate as nutrients. *Bioresource Technology* **2010**, *101* (10), 3642–3648.

(57) Pasolli, E.; De Filippis, F.; Mauriello, I. E.; Cumbo, F.; Walsh, A. M.; Leech, J.; Cotter, P. D.; Segata, N.; Ercolini, D. Large-scale genome-wide analysis links lactic acid bacteria from food with the gut microbiome. *Nature Communications* **2020**, *11* (1), 2610.

(58) Axelsson, L. T.; Salminen, S. Lactic acid bacteria: classification and physiology. **1993**.

(59) Amrane, A. Evaluation of lactic acid bacteria autolysate for the supplementation of lactic acid bacteria fermentation. *World Journal of Microbiology and Biotechnology* **2000**, *16* (2), 207–209.

(60) Wang, Y.; Tashiro, Y.; Sonomoto, K. Fermentative production of lactic acid from renewable materials: recent achievements, prospects, and limits. *Journal of Bioscience and Bioengineering* **2015**, *119* (1), 10–18.

(61) Kamm, B., Ed. Microorganisms in biorefineries; Microbiology Monographs; S 2015; Vol. 26.

(62) Kylä-Nikkilä, K. Genetic engineering of lactic acid bacteria to produce optically pure lactic acid and to develop a novel cell immobilization method suitable for industrial fermentations. **2016**.

(63) Salminen, S.; Wright, A. von; Ouwehand, A. *Lactic acid bacteria: microbiological and functional aspects*; New York, 2004.

(64) Endo, A.; Dicks, L. M. T. Physiology of the LAB. 2014; pp 13–30.

(65) Biddy, M. J.; Scarlata, C.; Kinchin, C. *Chemicals from biomass: a market assessment of bioproducts with near-term potential*; National Renewable Energy Lab. (NREL), Golden, CO (United States), 2016.

(66) Kim, K. I.; Kim, W. K.; Seo, D. K.; Yoo, I. S.; Kim, E. K.; Yoon, H. H. Production of lactic acid from food wastes. *Biotechnology for Fuels and Chemicals: The Twenty-Fourth Symposium*; Davison, B. H., Lee, J. W., Finkelstein, M., McMillan, J. D., Eds.; Applied Biochemistry and Biotechnology; Humana Press: Totowa, NJ, 2003; pp 637–647.

(67) Sakai, K.; Murata, Y.; Yamazumi, H.; Tau, Y.; Mori, M.; Moriguchi, M.; Shirai, Y. Selective Proliferation of Lactic acid bacteria and accumulation of lactic acid during open fermentation of kitchen refuse with intermittent pH adjustment. *Food Science and Technology Research* **2000**, *6* (2), 140–145.

(68) Koskinen, P.; Deptula, P.; Smolander, O.-P.; Tamene, F.; Kammonen, J.; Savijoki, K.; Paulin, L.; Piironen, V.; Auvinen, P.; Varmanen, P. Complete genome sequence of Propionibacterium *freudenreichii* DSM 20271T. *Stand Genomic Sci* **2015**, *10*.

(69) Xie, C.; Coda, R.; Chamlagain, B.; Varmanen, P.; Piironen, V.; Katina, K. Co-fermentation of *Propionibacterium freudenreichii* and *Lactobacillus brevis* in wheat bran for in situ production of vitamin B12. *Front. Microbiol.* **2019**, *10*.

(70) Rawoof, S. A. A.; Kumar, P. S.; Vo, D.-V. N.; Devaraj, K.; Mani, Y.; Devaraj, T.; Subramanian, S. Production of optically pure lactic acid by microbial fermentation: a review. *Environ Chem Lett* **2020**.

(71) Abdel-Rahman, M. A.; Tashiro, Y.; Sonomoto, K. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. *Journal of Biotechnology* **2011**, *156* (4), 286–301.

(72) Umeki, M.; Oue, K.; Mochizuki, S.; Shirai, Y.; Sakai, K. Effect of *Lactobacillus rhamnosus KY-3* and cellobiose as synbiotics on lipid metabolism in rats. *Journal of Nutritional Science and Vitaminology* **2004**, *50* (5), 330–334.

(73) Sakai, K.; Murata, Y.; Yamazumi, H.; Tau, Y.; Mori, M.; Moriguchi, M.; Shirai, Y. Selective Proliferation of Lactic acid bacteria and accumulation of lactic acid during open fermentation of kitchen refuse with intermittent pH adjustment. *Food Science and Technology Research* **2000**, *6* (2), 140–145.

(74) Komesu, A., Wolf Maciel, M. R., Maciel Filho, R. Separation and purification technologies for lactic acid – A brief review. Bio Resources **2017**, 12(3).

(75) Sun, X.; Wang, Q.; Zhao, W.; Ma, H.; Sakata, K. Extraction and purification of lactic acid from fermentation broth by esterification and hydrolysis method. *Separation and Purification Technology* **2006**, *49* (1), 43–48.

(76) Kumar, H.; Alén, R. Recovery of aliphatic low-molecular-mass carboxylic acids from hardwood kraft black liquor. *Separation and Purification Technology* **2015**, *142*, 293–298.

(77) Joglekar, H. G.; Rahman, I.; Babu, S.; Kulkarni, B. D.; Joshi, A. Comparative assessment of downstream processing options for lactic acid. *Separation and Purification Technology* **2006**, *52* (1), 1–17.

(78) Edreder, E. A.; Mujtaba, I. M.; Emtir, M. Optimal operation of different types of batch reactive distillation columns used for hydrolysis of methyl lactate to lactic acid. *Chemical Engineering Journal* **2011**, *172* (1), 467–475.

(79) Kamble, S. P.; Barve, P. P.; Joshi, J. B.; Rahman, I.; Kulkarni, B. D. Purification of lactic acid via esterification of lactic acid using a packed column, followed by hydrolysis of methyl lactate using three continuously stirred tank reactors (CSTRs) in series: a continuous pilot plant study. *Ind. Eng. Chem. Res.* **2012**, *51* (4), 1506–1514.

(80) Aqar, D. Y.; Rahmanian, N.; Mujtaba, I. M. Methyl lactate synthesis using batch reactive distillation: operational challenges and strategy for enhanced performance. *Separation and Purification Technology* **2016**, *158*, 193–203.

(81) Mo, L.; Shao-Tong, J.; Li-Jun, P.; Zhi, Z.; Shui-Zhong, L. Design and control of reactive distillation for hydrolysis of methyl lactate. *Chemical Engineering Research and Design* **2011**, *89* (11), 2199–2206.

(82) Komesu, A.; Martinez, P. F. M.; Lunelli, B. H.; Filho, R. M.; Maciel, M. R. W. Lactic acid purification by reactive distillation system using design of experiments. *Chemical Engineering and Processing: Process Intensification* **2015**, *95*, 26–30.

(83) Kumar, R.; Nanavati, H.; Noronha, S. B.; Mahajani, S. M. A continuous process for the recovery of lactic acid by reactive distillation. *Journal of Chemical Technology & Biotechnology* **2006**, *81* (11), 1767–1777.

(84) Sakai, K. Making plastics from garbage. *Journal of Industrial Ecology - Wiley Online Library*. **2003**

(85) Ingólfsson, H. I.; Andersen, O. S. Alcohols effects on lipid bilayer properties. *Biophys J* **2011**, *101* (4), 847–855.

(86) Chawong, K.; Rattanaphanee, P. N-butanol as an extractant for lactic acid recovery. *International Journal of Chemical, Materials and Biomolecular Sciences* **2011**, *4.0* (8).

(87) Su, C.-Y.; Yu, C.-C.; Chien, I.-L.; Ward, J. D. Plant-wide economic comparison of lactic acid recovery processes by reactive distillation with different alcohols. *Ind. Eng. Chem. Res.* **2013**, *52* (32), 11070–11083.

(88) Wasewar, K. L.; Heesink, A. B. M.; Versteeg, G. F.; Pangarkar, V. G. Reactive extraction of lactic acid using alamine 336 in MIBK: equilibria and kinetics. *J. Biotechnol.* **2002**, *97* (1), 59–68.

(89) Wasewar, K. L.; Pangarkar, V. G.; Heesink, A. B. M.; Versteeg, G. F. Intensification of enzymatic conversion of glucose to lactic acid by reactive extraction. *Chemical Engineering Science* **2003**, *58* (15), 3385–3393.

(90) Yankov, D.; Molinier, J.; Albet, J.; Malmary, G.; Kyuchoukov, G. Lactic acid extraction from aqueous solutions with tri-n-octylamine dissolved in decanol and dodecane. *Biochemical Engineering Journal* **2004**, *21* (1), 63–71.

(91) Olszewska-Widdrat, A.; Alexandri, M.; López-Gómez, J. P.; Schneider, R.; Mandl, M.; Venus, J. Production and purification of L-lactic acid in lab and pilot scales using sweet sorghum juice. *Fermentation* **2019**, *5* (2), 36.

(92) Petlyuk, F. B. Distillation theory and its application to optimal design of separation units; *Cambridge University Press*, 2004.

(93) Sirkar, K. K. Separation of molecules, macromolecules and particles: principles, phenomena and processes; *Cambridge University Press*, 2014.

(94) Sanz, M. T.; Murga, R.; Beltrán, S.; Cabezas, J. L.; Coca, J. Kinetic study for the reactive system of lactic acid esterification with methanol: methyl lactate hydrolysis reaction. *Ind. Eng. Chem. Res.* **2004**, *43* (9), 2049–2053.

(95) Song-lin, XU.; Tao, Z.; Shi-min, XU. The study of refining L-lactic acid by molecular distillation *Journal of Chemical Engineering of Chinese Universities*. **2004**.

(96) From, M.; Adlercreutz, P.; Mattiasson, B. Lipase catalyzed esterification of lactic acid. *Biotechnology Letters* **1997**, *19* (4), 315–318.

(97) Choi, J.-I.; Hong, W. H. Recovery of Lactic Acid by Batch Distillation with Chemical Reactions Using Ion Exchange Resin. *Journal of Chemical Engineering of Japan* **1999**, *32* (2), 184–189.