Cyprus University of Technology

Faculty of Geotechnical Sciences and Environmental Management

Doctoral Dissertation

Development of a citrus peel waste biorefinery for the production of high added-value commodities and biofuels

Maria Patsalou

Limassol, December 2019

CYPRUS UNIVERSITY OF TECHNOLOGY

FACULTY OF GEOTECHNICAL SCIENCES AND ENVIRONMENTAL MANAGEMENT

DEPARTMENT OF CHEMICAL ENGINEERING

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Approval Form

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DEVELOPMENT OF A CITRUS PEEL WASTE BIOREFINERY FOR THE PRODUCTION OF HIGH ADDED-VALUE COMMODITIES AND BIOFUELS

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ABSTRACT

Citrus fruits constitute one of the most highly utilized food products worldwide. The production of citrus fruits reaches over 124×10^6 t per year, while citrus juice manufacturing generates 25×10^6 t. During the processing of the fruit, half of its mass is converted into citrus peel waste (CPW) consisting of peels, seeds and segment membranes. Current management practices include the use of CPW as animal feed or disposal in landfills. However, CPW is composed of pectin, cellulose, hemicellulose, soluble sugars and essential oils, components that constitute CPW as a promising feedstock for extraction and production of added-value products and biofuels through the biorefinery platform.

The proposed biorefinery of this work combines physicochemical and biological treatments for extraction of essential oils and pectin as well as for production of succinic acid (platform chemical), ethanol and methane (biofuels) and a fertilizer. The first step employed distillation for extraction and collection of essential oils where the yield reached 0.43% and 0.24% (v/w) for "Mandora" and household citrus waste respectively. The next step of the proposed biorefinery included acid hydrolysis, where the optimized conditions comprised 116 ºC for 10 min using 5% (w/v) of dry raw material for both materials. Afterwards, the extraction of pectin, which reached 30.5% (w/w), was separated from the hydrolyzate generated through addition of ethanol. Subsequently, following ethanol removal, the hydrolyzate was microbially fermented to succinic acid or ethanol. Succinic acid production was enhanced with the addition of corn steep liquor in fermentations, while the addition of vitamins increased the production rate. A fed-batch experiment was also conducted and resulted in slight increase of both the final concentration of succinic acid as well as the product yield. Moreover, ethanol production was studied using *P. kudriavzevii* KVMP10, a newly thermotolerant yeast which was compared against two major industrial yeasts (*S. cerevisiae and K. marxianus*) and found to be a more efficient ethanol producer through use of CPW hydrolyzates. Finally, solid biorefinery residues were tested in anaerobic digestion for the production of biomethane and in agricultural applications as fertilizer targeting the development of a zero-waste process.

Keywords: Biorefinery, Succinic acid, Bioethanol, Biomethane, Fertilizer

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1 INTRODUCTION

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LIST OF ABBREVIATIONS AND MICROORGANISMS

Abbreviations

AA:	Acetic acid
ABE:	Acetone, n-butanol and ethanol
ADF:	Acid detergent fibre
ADL:	Acid detergent lignin
Aer:	Aerobic conditions
AFP:	Air-filled porosity
An:	Anaerobic conditions
AWHC:	Available water holding capacity
BD:	Bulk density
BH:	Bread hydrolysate
BTX:	Benzene, toluene and xylene
BR:	Biorefinery residues
C/N:	Carbon/nitrogen
CPW:	Citrus peel waste
CSL:	Corn steep liquor
DE:	Degree of esterification
DCPW:	Dried citrus peel waste
DDGS:	Dried distillers' grains with solubles
DM:	Dry matter
drm:	Dry raw material
EIA:	Energy information administration
e.o.:	Essential oils
eth:	Ethanol

FA:	Formic acid
FCI:	Fixed capital investment
FE:	Fungal extracts
FSC:	Final sugars concentration
FW:	Food waste
Gly:	Glycerol
HMF:	Hydroxymethylfurfural
HS:	Hestrin and Schramm
ISC:	Initial sugars concentration
ITS:	Internal transcribed spacer
LA:	Lactic acid
MA:	Malic acid
MDA:	Malonialdehyde
nd:	No data
nk:	Not known
NDF:	Neutral detergent fibre
OP:	Orange peel
OPW:	Orange peel waste
PA:	Propionic acid
Pcs:	Pretreated corn stover
Pep:	Peptone
PHA:	Polyhydroxyalkanoates
PHB:	Polyhydroxybutyrate
Pyr:	Pyruvic acid
QC:	Quality control

rm:	Raw material
SF:	Submerged fermentation
SSF:	Simultaneous sacharification and fermentation
SCP:	Single cell protein
SA:	Succinic acid
SFE:	Supercritical fluid extraction
TPS:	Total pore space
trs:	Total reducing sugars
Tryp:	Tryptone
TS:	Total solids
TSB:	Tryptic soy broth
tsc:	Total sugar consumed
Vit:	Vitamins
VS:	Volatile solids
WAIS:	West antarctic ice sheet
WM:	Wet mass
WB:	Wheat bran
WH:	Wheat hydrolysate
WS:	Wheat straw
YE:	Yeast extract

Microorganisms

Actinobacillus succinogenes	*A. succinogenes*
Aspergillus awamori	*A. awamori*
Aspergillus foetidus	*A. foetidus*

Aspergilus niger	*A. niger*
Aspergilus oryzae	*A. oryzae*
Bacillus subtilis	*B. subtilis*
Basfia succiniciproducens	*B. succiniciproducens*
Candida guilliermondii	*C. guilliermondii*
Candida shehatae	*C. shehatae*
Clostridium acetobutylicum	*C. acetobutylicum*
Clostridium beijerinckii	*C. beijerinckii*
Clostridium thermocellum	*C. thermocellum*
Clostridium tyrobutyricum	*C. tyrobutyricum*
Corynebacterium glutamicum	*C. glutamicum*
Cupriavidus necator	*C. necator*
Escherichia coli	*E. coli*
Fusarium oxysporum	*F. oxysporum*
Geotrichum candidum	*G. candidum*
Gluconacetobacter hansenii	*G. hansenii*
Gluconacetobacter xylinus	*G. xylinus*
Halomonas boliviensis	*H. boliviensis*
Issatcchenkia orientalis	*I. orientalis*
Kluyveromyces fragilis	*K. fragilis*
Kluyveromyces marxianus	*K. marxianus*
Komagataeibacter hansenii	*K. hansenii*
Komagataeibacter sucrofermentans	*K. sucrofermentans*
Lactobacillus amylophilus	*L. amylophilus*
Lactobacillus casei	*L. casei*

Lactobacillus delbrueckii	*L. delbrueckii*
Lactobacilluus pentosus	*L. pentosus*
Mannheimia succiniciproducens	*M. succiniciproducens*
Mucor indicus	*M. indicus*
Neurospora crassa	*N. crassa*
Pachysolen tannophilus	*P. tannophilus*
Penicillium charlessi	*P. charlessi*
Penicillium decumbens	*P. decumbens*
Phanerochaete sordida	*P. sordida*
Pichia kudriavzevii	*P. kudriavzevii*
Pichia stiplis	*P. stiplis*
Pycnoporus cinnabarinus	*P. cinnabarinus*
Saccharomyces cerevisiae	*S. cerevisiae*
Talaromyces flavus	*T. flavus*
Thermoanaerobacter ethanolicus	*T. ethanolicus,*
Thermoanaerobacterium sacchaarolyticum	*T. sacchaarolyticum*
Thermobacillus xylanilyticus	*T. xylanilyticus*
Tubercularia vulgaris	*T. vulgaris*
Zymomonas mobilis	*Z. mobilis*

1 INTRODUCTION

1.1 Food waste

Waste constitutes a major problem for societies worldwide, becoming increasingly important in developing countries, such as China and India, as well as in Europe (Lin et al., 2013). One third of the food produced gets lost or wasted (approximately 1.3 billion t annually). This estimate is based on the mass quantity of food produced for human consumption, which is lost and/or wasted from production to consumption stages. Food losses and waste amounts to about $ 680 billion in industrialized countries and $ 310 billion in developing countries, which correspond to approximately the same quantities of food (670 and 630 million t, respectively) (FAO, 2011). In reach developed country, the total per capita food production constitutes approximately 900 kg annually, while about 460 kg are produced in poorest areas. The foremost responsible sector for food losses or waste generation consists of the pre-consumption stage in both developed and developing regions (Figure 1.1.1). Thus, in developing countries 40% of losses occur at post-harvest and processing levels, while in industrialized countries more than 40% of losses take place at retail and consumer levels (FAO, 2011)

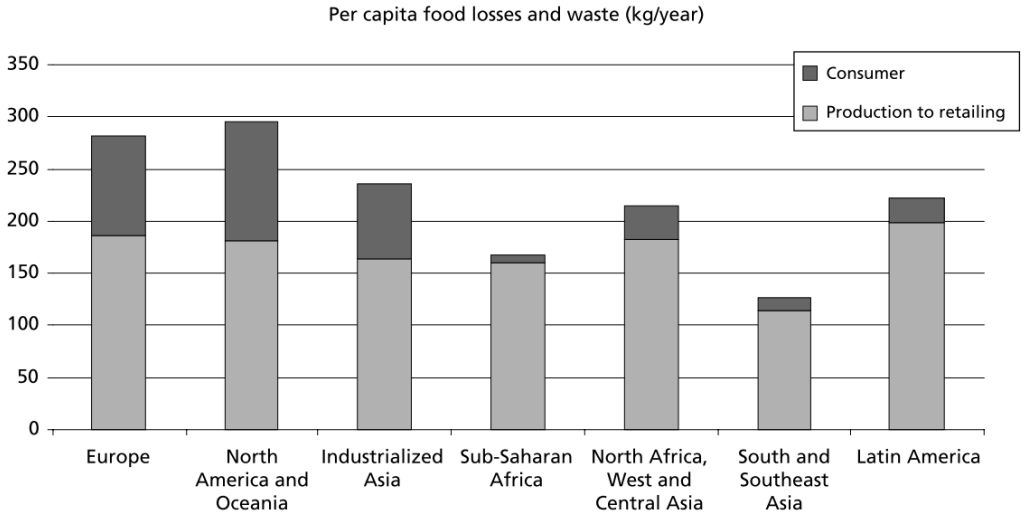

Figure 1.1.1: Per capita food losses and waste, at consumption and pre-consumptions stages, in different regions (adapted from FAO, 2011).

The food sector comprises over 50% of the total waste produced globally, where 60% consists of organic biodegradable matter generated in the different stages of the food supply chain. Food waste (FW) constitutes a global environmental, economic and societal problem, that should be addressed by a combination of prevention and valorization approaches (Turon et al., 2014). It has been estimated that 50% of the food produced is lost or wasted prior to consumption (Lin et al., 2013), while the waste streams generated can be classified in three main categories: (1) food/drink manufacturing waste, (2) grocery retail/catering business waste and (3) consumer/household waste (Kosseva, 2013). The food manufacturing sector is generating 38% of the 90 million t of FW produced by the European Union (Pfaltzgraff et al., 2013), while vegetables and fruits usually constitute the most-utilized items, followed by other commodities including grains/bakery, meat, fish and dairy products (Kosseva, 2013).

The management of food losses and waste require input of significant amounts of resources including water, land, energy, labor and capital. Moreover, the treatment of these waste produces greenhouse gas emissions, contributing to global warming and climate change (FAO, 2016). Current management practices of FW include first generation recycling methods, such as animal feed, composting, disposal in landfills and anaerobic digestion (Kiran et al., 2014). However, the opportunity to extract valuable components and to utilize the remaining biomass for regeneration of carbon-rich feedstocks is lost through these traditional methods (Turon et al., 2014). Alternatively, FW can be valorized through biotechnological approaches utilizing the high content of sugars, oligosaccharides and polysaccharides (e.g. cellulose, pectin) as well as other high-value molecules (lipids, amino acids, poly-phenols, phytochemicals, resins, starch) as a valuable resource (Pfaltzgraff et al., 2013).

1.2 Biorefineries: Multiproduct processes for biomass valorization

The need to replace the use of petroleum with new renewable resources for the production of fuels and chemicals and to identify novel practices for the reduction of biodegradable waste has led to the application of FW as a feedstock for

biorefineries (Lin et al., 2013). The term “biorefinery” describes a series of sustainable and low environmental impact technologies employed to convert biomass into fuels, chemicals, polymers, materials, food, feed and added-value ingredients (Koutinas et al., 2014a). Through the biorefinery concept, the first step for FW valorization includes determining biowaste constituents and extracting those of high value, while subsequent processing incorporates utilization of the remaining organic content for generation of case-specific fermentation media (Lin et al., 2013). Thus, the bioconversion of FW through biorefining aims in maximizing the production of energy, chemicals or materials from a renewable resource in a similar context to that of petroleum refinery.

Various FW-based biorefinery strategies have been previously developed for FW valorization, employing wheat, corn, bread, sugarcane and citrus derived waste for the production of biofuels and other added-value compounds. Apart from molasses, processing of the above raw materials generates residues considered as second-generation fermentation feedstocks, owing to their lignocellulosic nature, and require acid and/or enzymatic hydrolysis for the release of fermentable sugars (Koutinas et al., 2007; Liu et al., 2008a; Pourbafrani et al., 2010; Leung et al., 2012; Koutinas et al., 2014a). Nevertheless, a variety of other industrial food processing waste streams, including beer fermentation broth, winery waste, cheese whey and coffee waste, have been converted into different bioproducts through fermentation of their high content in soluble sugars (Schell et al., 2004; Neves et al., 2006; Devesa-Rey et al., 2011; Carvalho et al., 2013; Khattak et al., 2013).

1.2.1 Biorefineries proposed for the management of FW

1.2.1.1 Cereal crops

The cultivation of cereals covers a global area of 700 million ha ensuring a world production of approximately 2.5 billion t of grain per year (FAO, 2016). Cereals constitute essential resources for multiple applications that lead to the structuring of various industries, where food, pet food, starch and biofuels comprise some typical examples. The global production of the main cereals, including wheat, corn and rice, has increased over the last decades owing to the increase in human demand (cereals present 50% of the global utilization of food). Grains are processed through

various steps, such as milling, baking and malting, in order to form the final product. These industrial processes waste cellulose- and hemicellulose-rich by-products, which are environmental friendly and present sustainability advantages (Abecassis et al., 2014). Thus, cereal crops and industrial cereal processing waste comprise promising raw materials for future bioconversion (Koutinas et al., 2004).

Wheat waste

Wheat straw (WS), a crop waste product of the agricultural industry, constitutes a lignocellulosic material with global production of approximately 650 million t per year (Ruiz et al., 2013), while consisting the second largest biomass feedstock worldwide (Guo et al., 2019). WS presents a valuable raw material for application in biobased industries composed of 30% cellulose, 50% hemicellulose and 15% lignin. However, although WS is traditionally applied for swine and bovine breeding, environmental issues and modern animal breeding practices recommend its limited use. Moreover, the current practices associated with the management of agricultural crop waste, such as landfilling and cleaning of wheat fields by burning straw following harvesting/threshing of soils cause negative effects as a result of the high C/N ratio of the waste (Curreli et al., 2002).

Various alternative practices for the management of WS have been tested in the literature over the three last decades, including the development of WS-based biorefineries. WS constitutes a promising source of fermentable sugars owing to the fast growth of wheat as well as the relatively low lignin content (Curreli et al., 2002). Both physicochemical and biochemical pretreatment processes have been investigated for the conversion of cellulose and hemicellulose into glucose. Wet oxidation and alkaline hydrolysis of WS was tested by Bjerre et al. (1996) demonstrating a high conversion yield (85% w/w) of cellulose to glucose. Hatakka (1983) studied the bioconversion of cellulose to glucose obtained through pretreatment with *P. sordida* 37 and *P. cinnabarinus* 115, while the effect of thermal pretreatment of WS has been also studied on the biochemical methane potential of the residue (Ferreira et al., 2013). Additionally, the hemicellulose content of WS can be valorized via a number of biotechnological methods through the biorefinery platform. To this end, current hemicellulose extraction and isolation

methods include alkaline extraction through autohydrolysis or hydrothermal processing, which constitute viable and eco-friendly options (Ruiz et al., 2013).

A WS-biorefinery has been developed for separation of hemicellulose, cellulose and lignin based on acetic and formic acid organosolv fractionation technology (Figure 1.2.1). Following application of the organosolv technology, raw cellulose pulp was separated from the liquor (hemicellulose and lignin) through filtration. The raw cellulose pulp was delignified using hydrogen peroxide, while the rich in hemicellulose and lignin liquor was water processed for separation of the two fractions. The cellulose pulp, hemicellulose and lignin obtained in this biorefinery can be further exploited for the production of second generation biofuels, polymers, biodegradable packaging or other useful chemicals (Snelders et al., 2014). An alternative biorefinery concept using the organosolv technology combined with alkaline pretreatment was proposed by Yuan et al. (2018) (Figure 1.2.2). Following organosolv pre-extraction and filtration, lignin was separated from the liquid fraction and the solid fraction was alkaline pretreated. Cellulosic solids were produced as solid fraction of alkaline pretreatment, while silica and lignin were separated from the liquid fraction. Overliming detoxification was performed on the silica and lignin-free liquor, the liquid fraction was mixed with cellulosic solids for further treatment and ethanol production, while the solid fraction was used for energy production (Yuan et al., 2018).

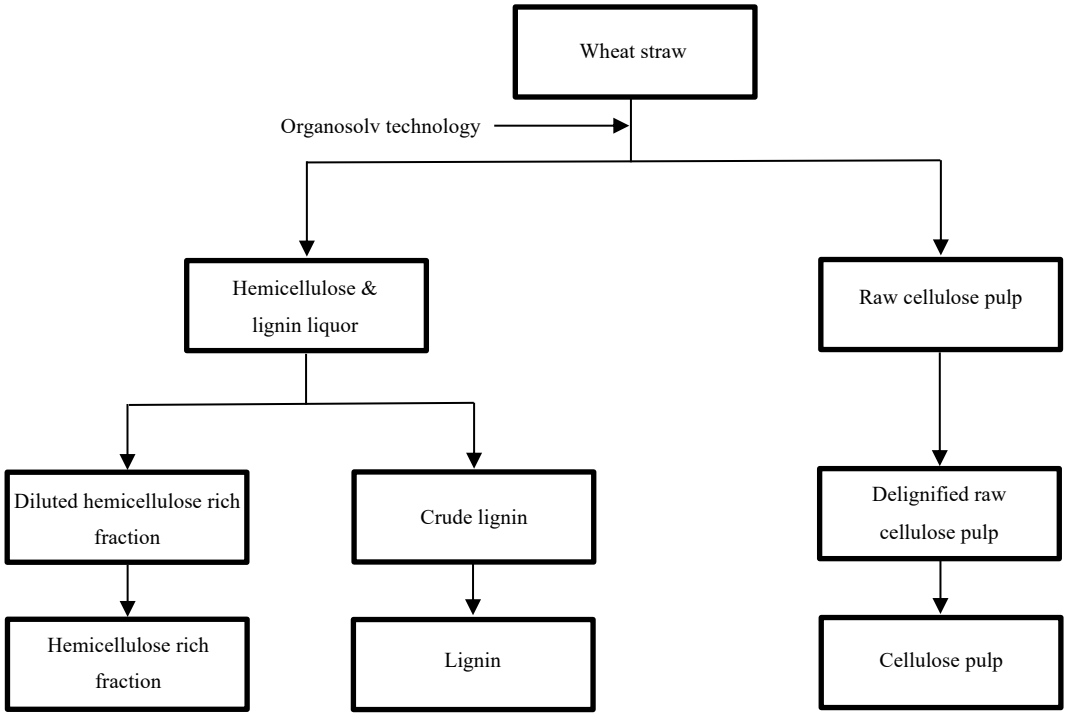

Figure 1.2.1: Process flow sheet of a wheat-based biorefinery applied for the production of cellulose, hemicellulose and lignin (adapted from Snelders et al., 2014).

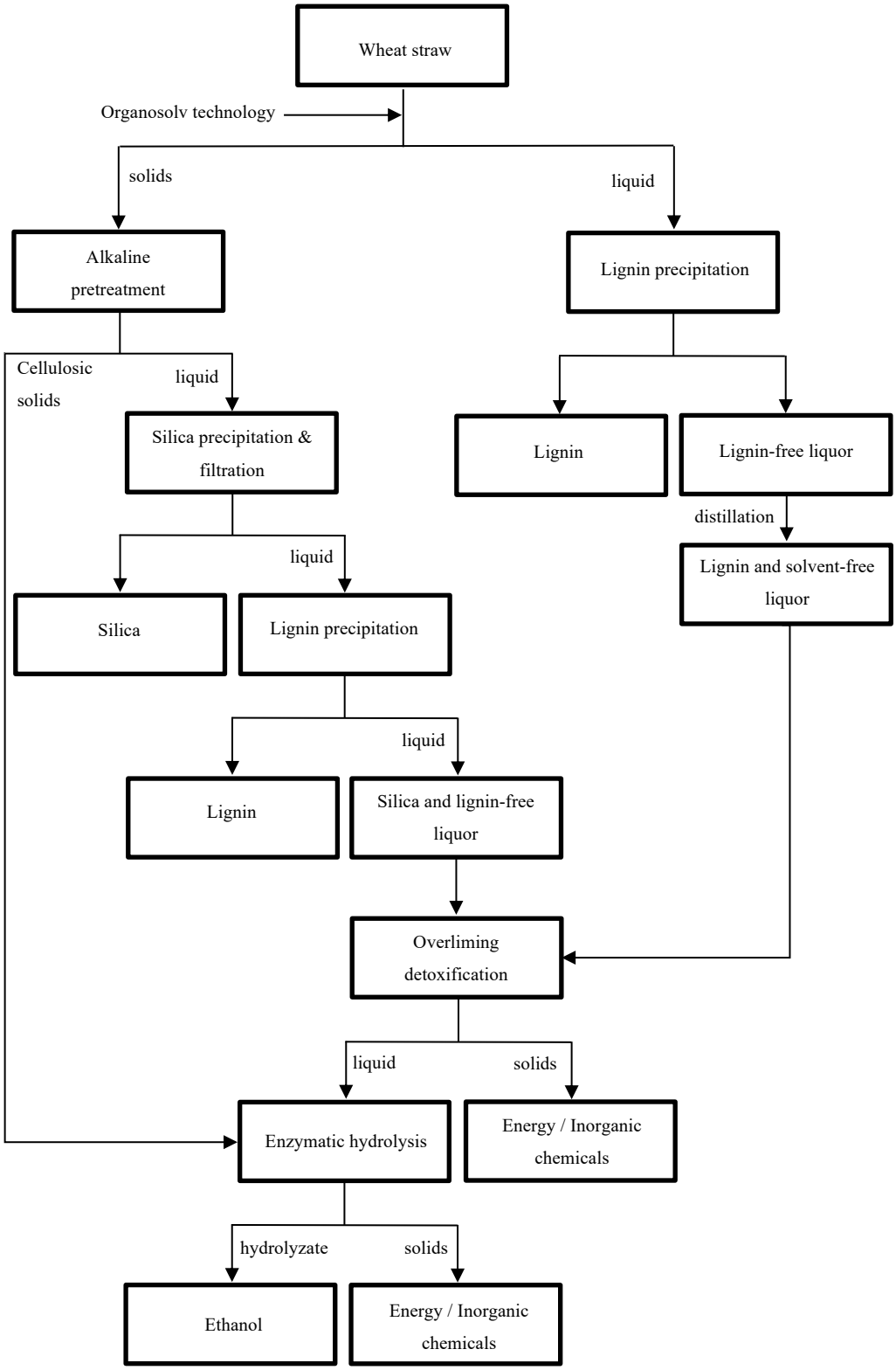

Figure 1.2.2: Process flow sheet of a wheat-based biorefinery applied for the production of lignin, ethanol and energy (adapted from Yuan et al., 2018).

WS-biorefinery concepts were also investigated for the production of high-added value products. A WS-biorefinery was investigated aiming to establish the

optimal conditions for the production of sugars and cellulose fibers using dilute-acid hydrolysis after drying and milling of WS (Jiang et al., 2018). Following dilute-acid hydrolysis, the liquid fraction was consisting of sugars, xylose, xylo-oligosacharide and glucose while the solid fraction was washed and drying for further treatment using soda-AQ delignification in order to obtain cellulose fibers (solid fraction) and lignin (liquid fraction) (Figure 1.2.3). Steam explosion was also used in a simple WS-biorefinery as a pretreatment of milled WS (Tomas-Pejo et al., 2017). Following steam explosion, a prehydrolyzate (liquid fraction) was generated for the production of high value bioproducts (biopolymers and bioplastics), while the solid fraction which contains water-insoluble solids was further used by simultaneous saccharification and fermentation (SSF) for bioethanol production. The residual solids from SSF comprising mainly lignin, were pyrolyzed in order to generate a bio-oil, which could be employed for the production of biochemicals and/or biofuels (Figure 1.2.4).

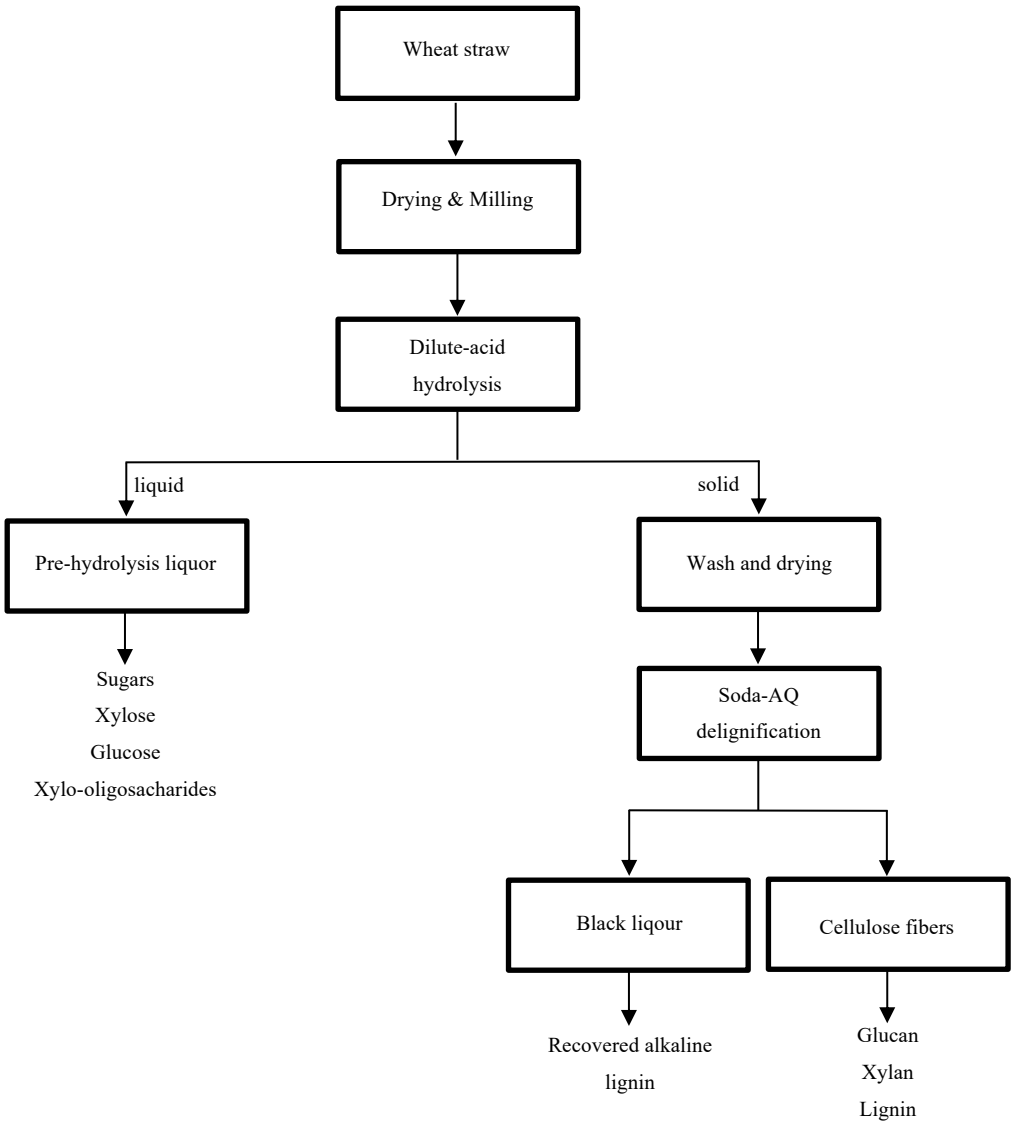

Figure 1.2.3: Process flow sheet of a wheat-based biorefinery applied for the production of sugars, lignin and cellulose fibers (adapted from Jiang et al., 2018).

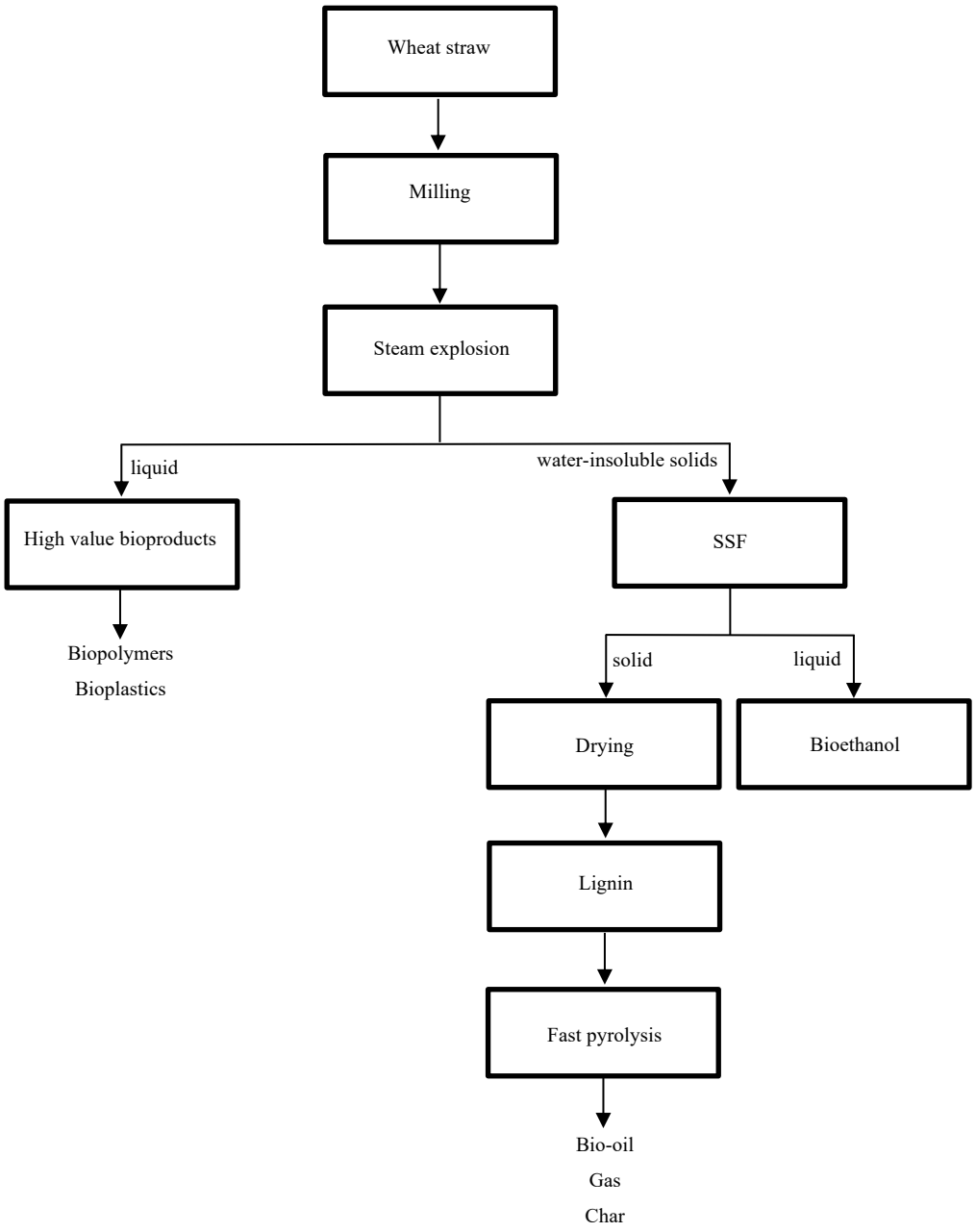

Figure 1.2.4: Process flow sheet of a wheat-based biorefinery applied for the production of high value bioproducts, bioethanol and bio-oil (adapted from Tomas-Pejo et al., 2017).

A pilot-scale process based on the biorefinery concept has been previously developed, for the production of biogas, ethanol, and energy from the waste (Figure 1.2.5). WS was air-dried followed by alkaline and mechanical dewatering pretreatment for preparation of a nutrient-rich feedstock applied in an SSF process.

The SSF process was performed using commercial hydrolytic enzymes and baker's yeast, while distillation of the fermentation broth was performed for ethanol extraction. Following separation of distillation residues, the liquid fraction was anaerobically digested to produce biogas, while the solid residue was used for the production of thermal energy (Maas et al., 2008).

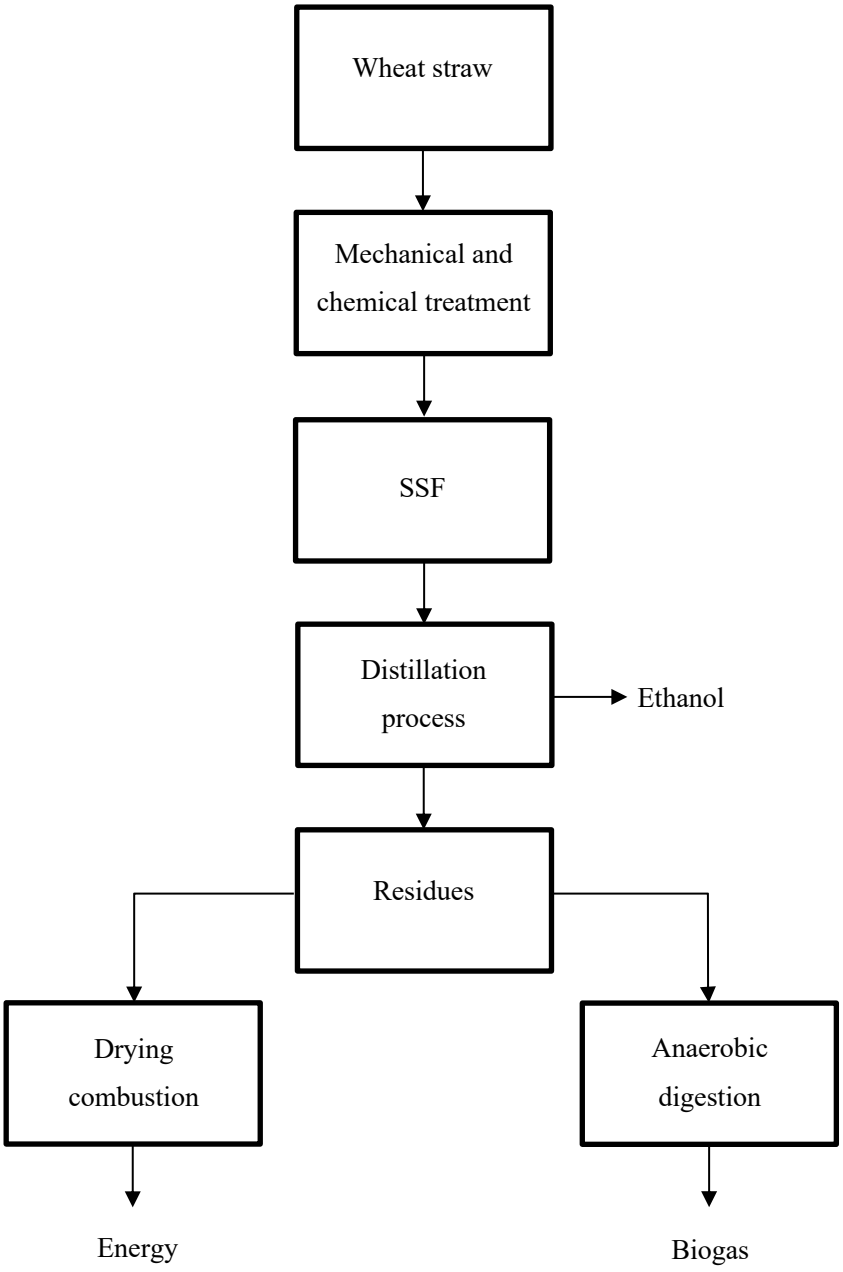

Figure 1.2.5: Process flow sheet of a wheat-based biorefinery applied for the production of biogas, ethanol, and energy (adapted from Maas et al., 2008).

Recently, Lopes et al. (2019) proposed a WS-biorefinery and performed a technoeconomic evaluation using WS for the production of xylo-oligosacharide and isobutene. Figure 1.2.6 presents a hydrothermal pretreatment of dried and milled wheat straw. The liquid fraction from hydrothermal pretreatment was used for xylo-oligosacharides recovery, while the solid fraction was enzymatically hydrolyzed in order to generate a glucose rich stream for isobutene production. The solids from enzymatic hydrolysis, which were rich in lignin, were used for electricity production in a combined heat and power plant (Lopes et al., 2019).

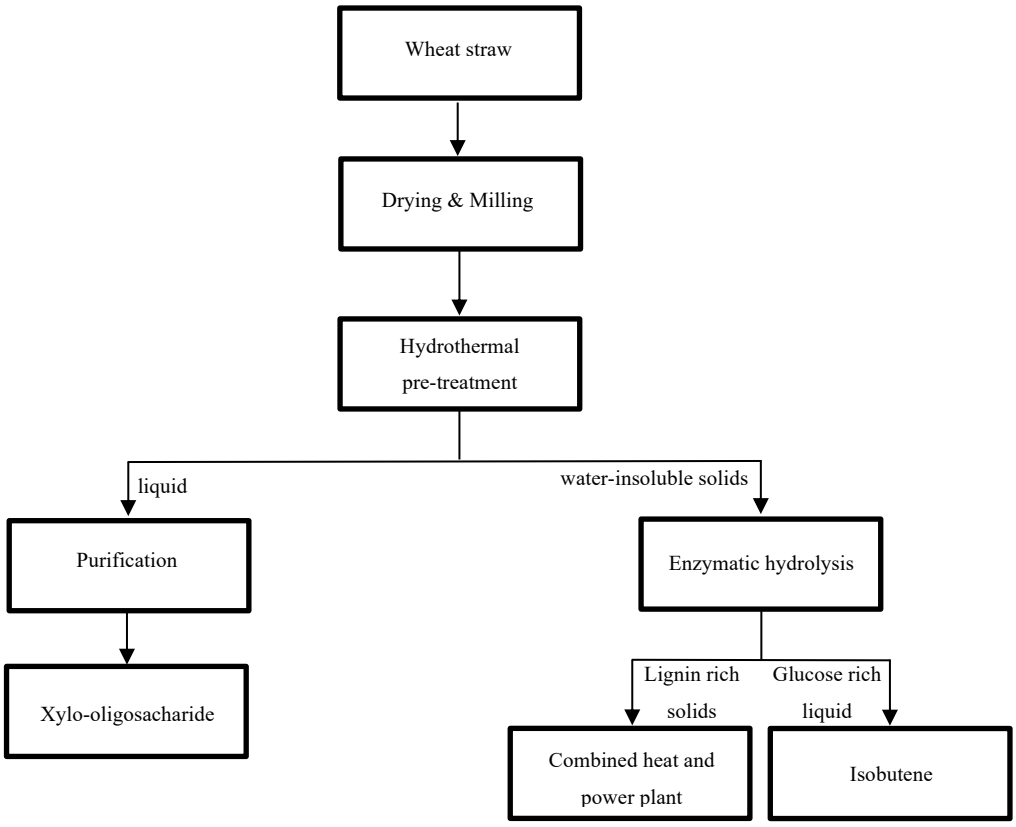

Figure 1.2.6: Process flow sheet of a wheat-based biorefinery applied for the production of xylo-oligosacharide, isobutene and electricity (adapted from Lopes et al., 2019).

Wheat bran (WB) constitutes a by-product of the wheat milling industry emitted during flour production that comprises approximately 150 million t on an annual basis. Depending on the milling process the flour production rate ranges between 73-77%, while the remaining 23-27% corresponds to by-products, consisting WB

and other components (e.g. wheat germ). The bran constitutes the main and most important fraction of milling by-products accounting for 25% of the grain's weight (Pruckler et al., 2014). WB is a hemicellulosic (arabinoxylan) material consisting mainly of starch (10-20%), protein (15-20%) and non-starch polysaccharides (41-60%), which could be used as the sole source of nitrogen in microbial fermentations (Palmarola-Adrados et al., 2005; Brijwani et al., 2010; Liu et al., 2010). The material represents a valuable source of phenolic compounds, such as hydroxybenzoic acids and hydroxycinnamic acids, known for their antioxidant activity. Among the phenolic compounds contained ferulic acid has been the most investigated, due to important physiological characteristics acting as antioxidant, anti-inflammatory, antimicrobial, anticancer and antithrombotic agent, while the ferulic acid bound to arabinoxylans from WB has been shown to be more bioavailable (Ou et al., 2007).

Although a common management practice for WB constitutes its application as animal feed, WB may serve as an important feedstock for bioconversion. Hydrolyzed WB, which consists of 21.3 g L^{-1} of glucose, 17.4 g L^{-1} of xylose and 10.6 g L^{-1} of arabinose, has been employed for acetone, butanol and ethanol production by *C. beijerinckii* ATCC 55025 (Liu et al., 2010). Furthermore, WB has been used as raw material for the production of lactic acid through solid-state fermentation using *L. amylophilus* GV6, as well as via co-culture of *L. casei* and *L. delbrueckii* (Naveena et al., 2005; John et al., 2006). WB has been utilized, for ethanol production using *S. cerevisiae* following pretreatment by acid and enzymatic hydrolysis (Palmarola-Adrados et al., 2005), while combined heat and enzymatic hydrolysis pretreatment of WB has been performed prior to ethanol production with the use of *S. cerevisiae* S1 (Favaro et al., 2012). Moreover, WB following enzymatic hydrolysis using a hemicellulasic cocktail obtained by *T. xylanilyticus*, was used for the production of ferulic acid, monomeric arabinose and xylose (Dupoiron et al., 2017).

The studies mentioned above highlight the use of WB as a promising raw material for the development of biorefineries. A WB-based biorefinery has been previously developed (Figure 1.2.7) including as major processing steps pre-extraction of water-soluble components, which included the recovery of 32% of the

feedstock, pretreatment with the use of hydrothermal/enzymatic hydrolysis (Reisinger et al., 2013) as well as lactic acid fermentation by *L. pentosus* (Tirpanalan et al., 2015). Furthermore, Wood et al. (2016) applied sequential amylase, protease and xylanase treatment followed by hydrothermal (lab scale) or steam explosion (pilot scale) pretreatment, developing a biorefinery for the valorization of WB into ethanol. However, a biorefinery approach for WB valorization was recently developed by Bedo et al. (2019) employing a two step dilute-acid hydrolysis to gain xylose-rich and arabinose-enriched hydrolyzates (Figure 1.2.8).

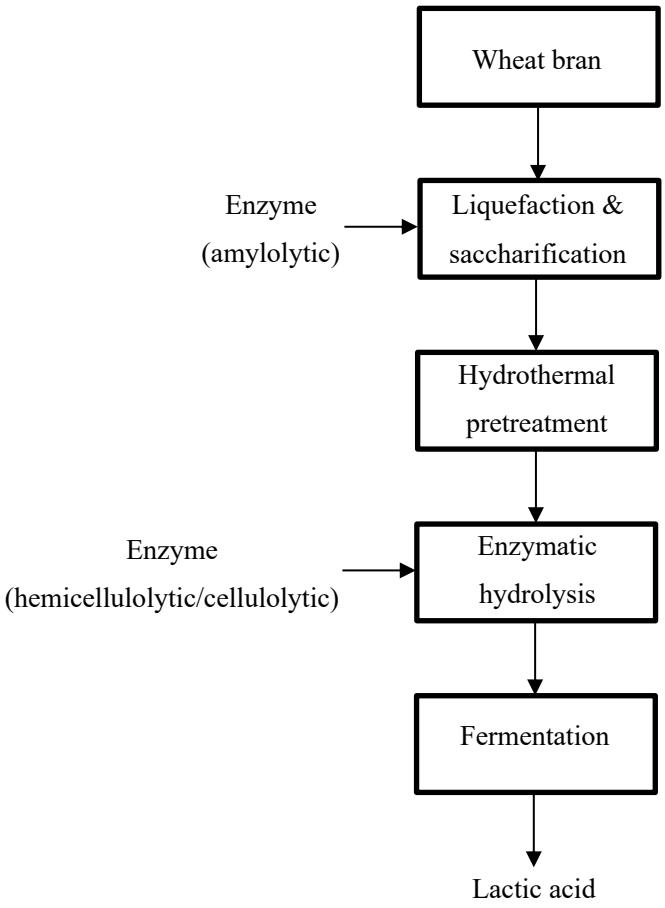

Figure 1.2.7: Process flow sheet of a wheat-based biorefinery applied for the production of lactic acid (adapted from Tirpanalan et al., 2015).

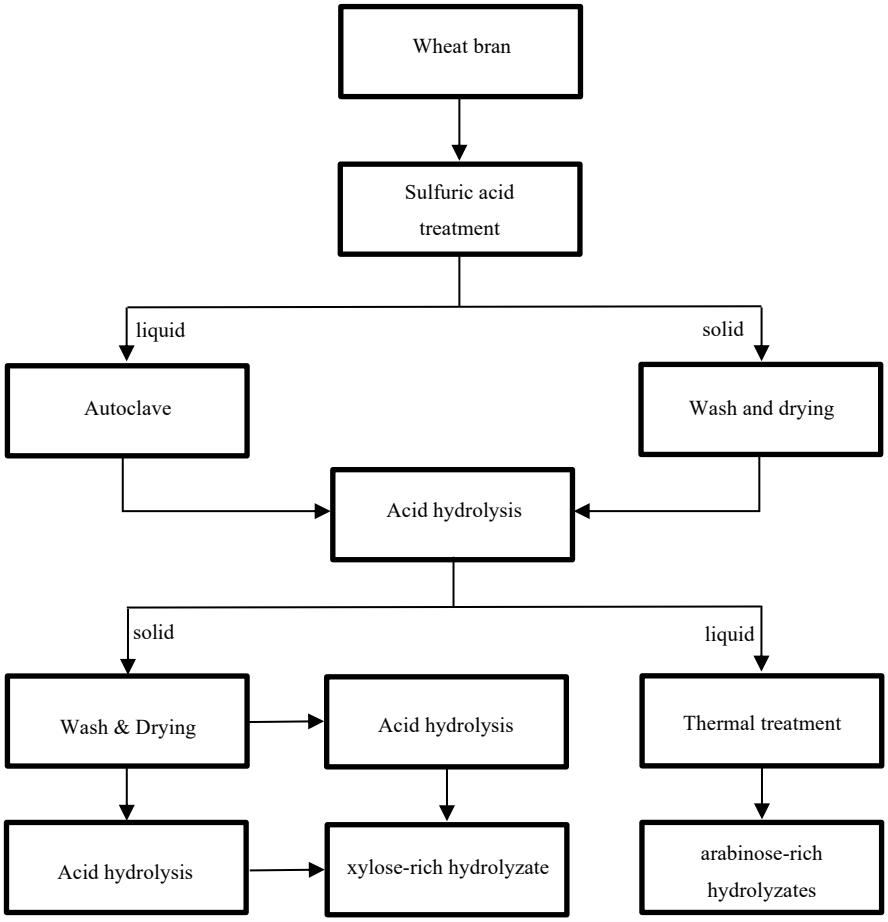

Figure 1.2.8: Process flow sheet of a wheat-based biorefinery applied for the production of xylose-rich and arabinose-enriched hydrolyzates (adapted from Bedo et al., 2019).

Apart from the materials generated during harvesting and processing of the crop, wheat constitutes a renewable and environmental friendly raw material, which can be also used as fermentation feedstock for industrial production of chemicals and biofuels (Koutinas et al., 2004). Therefore, first generation approaches have been also employed for the production of biofuels, biodegradable plastics and platform chemicals through the development of a complex whole-crop wheat biorefinery (Koutinas et al., 2006). Wheat grains have been used as a raw material for the production of polyhydroxybutyrate (PHB), where preprocessing steps of the biorefinery included mainly the production of a glucose-rich stream (WH-wheat hydrolyzate) and a complex nutrient source (FE-fungal extracts). *C. necator* was used for PHB production employing various mixtures of WH and FE as a substrate

(Koutinas et al., 2007; Xu et al., 2010). The same process flow diagram of the biorefinery employed for PHB production has been also adopted through application of different WH and FE ratios for bioethanol production (Arifeen et al., 2007). Furthermore, four different strategies have been developed for wheat pretreatment targeting the production of succinic acid. The first strategy involved fermentation of the fungal filtrate produced following fermentation of the whole-wheat flour by *A. awamori* 2B.361 U2/1. The second strategy employed the milled wheat for flour hydrolysis as well as in fungal fermentation. The fungal filtrate was used for flour hydrolysis of the wheat flour, while the flour hydrolyzate and the solids following fungal fermentation and autolysis were utilized for the production of succinic acid by *A. succinogenes* (Du et al., 2007). The third strategy comprised separating the wheat following milling into wheat flour and WB. Thus, following gluten extraction, the gluten free flour and WB was used as a substrate in solid-state fermentations by *A. awamori* for glucoamylace production. Moreover, WB was used by *A. oryzae* for protease production in a solid-state fermentation process. The glucoamylase solution was used for hydrolysis of the gluten-free flour and the protease solution was used for gluten hydrolysis. The resulting flour and gluten hydrolyzates were mixed and fermented by *A. succinogenes* for succinic acid production (Du et al., 2008). WB has been also used after milling of wheat for glucoamylace and protease production by *A. awamori* and *A. oryzae* respectively. The mixture of middlings fractions and milled bran was hydrolyzed by glucoamylace and protease for hydrolysis of starch and proteins forming carbon- and nitrogen-rich solutions, which was also employed as feedstock for the production of succinic acid by *A. succinogenes* (Dorado et al., 2009).

1.2.1.2 Bread & bakery waste

The global production of bread has been estimated at 100 million t per year (Melikoglu and Webb, 2013). However, approximately 2.5% of the total production from the baking industry constitutes waste generated as a result of production problems or stale bread returns, comprising one of the most heavily wasted food products in the world (Gelinas et al., 1999; Fazeli et al., 2004).

Waste from the baking industry are traditionally recycled as an animal feedstock (Gelinas et al., 1999) or digested anaerobically for biomethane production in

landfills (Melikoglu et al., 2013). However, bread comprises an excellent source of carbohydrates and nutrients consisting of 50% carbohydrates, 37% water and 8% protein (Demirci et al., 2016). Bread waste may serve as an ideal substrate for the production of added-value chemicals. The macromolecules contained (such as starch and protein) could be converted into glucose and free amino acids using amylolytic and proteolytic enzymes, which can be subsequently valorized through microbial bioconversion (Melikoglu et al., 2015). Specifically, approximately 60% of the bread waste's dry mass can be converted into glucose using significant activity of glucoamylases (Leung et al., 2012).

A biorefinery approach for bread waste valorization has been previously investigated (Figure 1.2.9), where the biowaste was employed as a feedstock in solid-state fermentations for the production of amylolytic and proteolytic enzymes by *A. awamori* and *A. oryzae* respectively. Following the production of hydrolytic enzymes the crude enzyme solution was added to the bread suspension for generation of a hydrolyzate composed of glucose and free amino-nitrogen, which was subsequently fermented for the production of succinic acid by *A. succinogenes* (Leung et al., 2012). However, Melikoglu et al. (2013) suggested a different approach for bread waste hydrolysis in the biorefinery developed. A multi-enzyme solution (which was rich in glucoamylase and protease generated by *A. awamori*) was initially produced through solid-state fermentation followed by hydrolysis of the blended waste bread. An alternative biorefinery approach using organic solvent and microwave technology followed by isomerization and dehydration (Figure 1.2. 10) was developed for the production of hydroxymethylofurfural (HMF) (Yu et al, 2017).

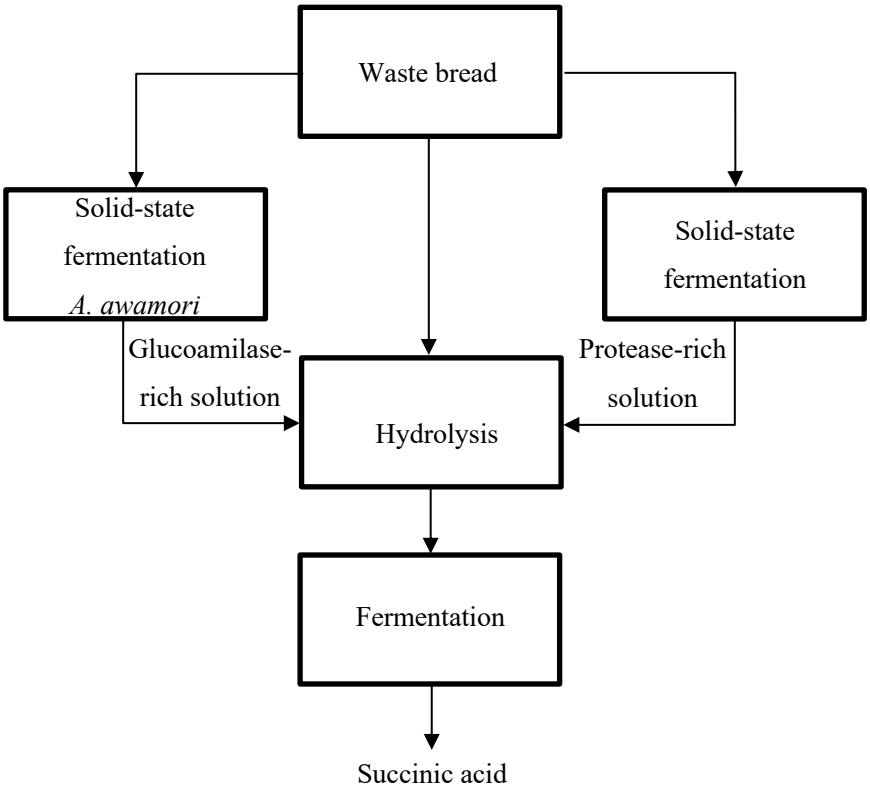

Figure 1.2.9: Process flow sheet of the bread waste-based biorefinery applied for the production of succinic acid (adapted from Leung et al., 2012).

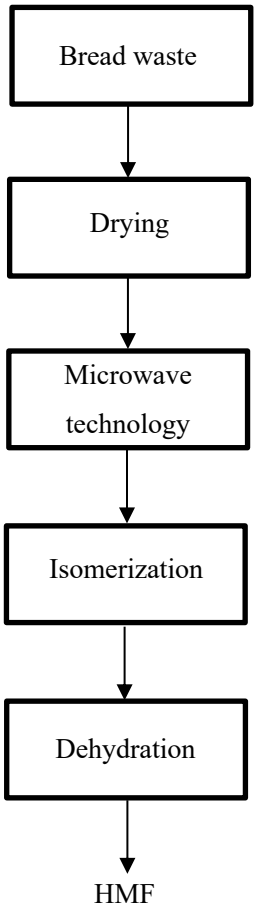

Figure 1.2.10: Process flow sheet of the bread waste-based biorefinery applied for the production of HMF (adapted from Yu et al., 2017).

Bakery waste including cakes and pastries has been also tested for its capacity to serve as a feedstock for biorefinery development in Hong Kong. Bakery waste constitutes a valuable nutrient source owning to its rich composition in carbon and nitrogen. Hydrolyzed pastries can generate a solution rich in soluble sugars due to the high content in starch (45%), carbohydrates (32%) and protein (7%). Although cakes involve a relatively low content of starch (13%), they comprise a significant amount of carbohydrates (62%) and protein (17%) (Zhang et al., 2013). Both types of bakery waste constitute a potential source for the generation of fermentable sugars, which could be bioconverted into added-value products (Arancon et al., 2013).

The valorization of bakery waste through the biorefinery concept has been also evaluated using a similar approach to the bread waste biorefineries presented above. Amylolytic and proteolytic enzymes were initially produced using bakery waste as

substrate by *A. awamori* and *A. oryzae* respectively. The resulting mixed-enzyme solution was added to bakery waste and a fermentation feedstock rich in glucose and free amino acids was obtained through enzymatic hydrolysis. Subsequently, *A. succinogenes* and *H. boliviensis* were employed for the production of succinic acid and PHB, respectively (Zhang et al., 2013).

1.2.1.3 Corn waste

Corn and corn stover are generally considered as primary feedstocks for the production of biofuels. Corn stover constitutes a highly sustainable resource for the production of added-value molecules, mainly consisting of cellulose (27%), xylan (18%) and water (20%), while also containing lower amounts of extractives (6.60%), galactan (1.08%), arabinan (3.26%), mannan (0.46%), lignin (8.52%), ash (4.74%), acetate (4.35%), protein (1.79%) and soluble solids (4.66%) (Ou et al., 2014).

Various corn stover pretreatment conditions followed by alkaline ethanol post-treatment have been tested for optimization of lignin and cellulose extraction based on the biorefinery concept (Figure 1.2.11). An alkaline ethanol system was investigated with different NaOH concentrations for the improvement of lignin removal from acid-steam-exploded corn stover. Subsequently, it was demonstrated that the modification of cellulose's crystalline structure using enzymatic hydrolysis formed a rich in fermentable sugars hydrolyzate, employed for biofuel production (Yang et al., 2016).

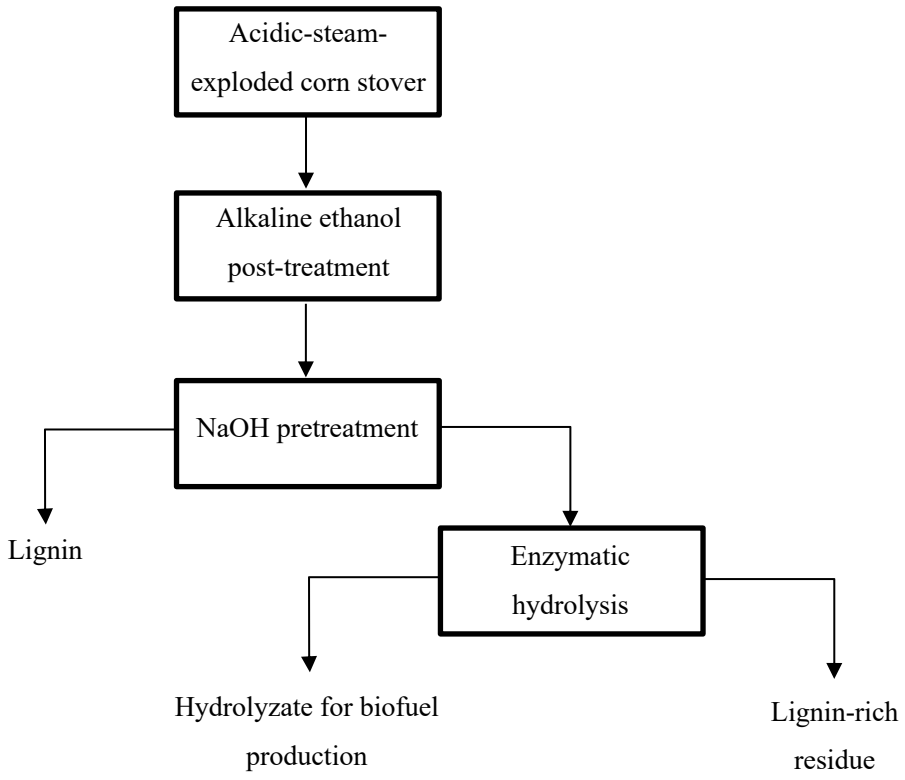

Figure 1.2.11: Process flow sheet of the acidic-steam-exploded corn stover-based biorefinery applied for the production of hydrolyzate employed for biofuel production and extraction of lignin, (adapted from Yang et al., 2016).

Corn constitutes starch (60%), water (15%), non-starch polysaccharides (7%), other solids (7%), soluble protein (2.5%) and oil (3.5%) (Ou et al., 2007). These components enabled biofuel production through a flow sheet that included processes for corn pretreatment, saccharification of polysaccharides, yeast fermentation of soluble sugars as well as ethanol distillation and dehydration (Wang et al., 2015). Following the distillation process, the solid content of the residues was dried to obtain dried distillers' grains with solubles (DDGS) as a protein source, which was used as animal feed (Bothast and Schlicher, 2005).

Overall, it is possible to produce ethanol from corn through two basic routs: the wet mill (33%) and the dry-grind (67%) methods. The wet mill includes processes for the pretreatment of corn generating high-value by-products, such as germ and corn oil from de-germ processing, fiber from de-fibered processing, and gluten. The dry mill includes grinding, cooking, as well as liquefaction of starch as corn pretreatment processes (Huang et al., 2008). The corn wet milling industry

generates 4 million t per year of corn fiber as by-product (Doner and Hicks, 1997). Corn fiber is generated through processing of maize pap and it could be considered as a potential raw material for valorization because of its rich composition and availability. Pericarp constitutes the major component of corn fiber consisting mainly of cellulose (18%), hemicellulose (35%) and starch (protein, fiber oil, lignin-20%). Although the traditional method for the disposal of corn fiber includes its use as animal feed (Gaspar et al., 2007), the low cost and high carbohydrate content of corn fiber makes it an attractive feedstock for microbial conversion to ethanol, serving as a potential raw material for future biorefineries.

Corn fiber has been evaluated as a raw material for bioethanol production by *S. cerevisiae*. Prior to fermentation, the raw material was pretreated through different alkaline solutions to dissolve hemicellulose, while the residue, which was mainly composed of cellulose, was hydrolyzed using cellulolytic enzymes (Gaspar et al., 2007). Mosier et al. (2005) explored the production of ethanol from pretreated corn fiber using *S. cerevisiae* 424A (LNH-ST) and *E. coli* achieving a yield that reached higher than 90%. Furthermore, corn fiber has been employed as a feedstock using different pretreatment methods for the production of butanol, acetone and ethanol through *C. acetobutylicum* (Qureshi et al., 2006; Qureshi et al., 2008), while the operation of a bioethanol pilot plant was also investigated applying corn fiber as a low cost feedstock (Schell et al., 2004).

Based on the traditional dry mill process, dry-grind processes have been previously developed employing the biorefinery concept for recovery of non-fermentable corn components, such as germ or both germ and fiber, prior to ethanol fermentation (Huang et al., 2008). Specifically, the “Quick Germ” ethanol process (Figure 1.2.12) has been demonstrated as a combination of a dry-grind and a wet-milling ethanol process to increase the coproduct’s (germ) value (Singh and Eckoff, 1997). The valorization of DDGS has been also evaluated through a biorefinery that involved catalytic process separation (Figure 1.2.13). DDGS treatment with catalytic pyrolysis generated a mixture of condensable aromatics and non-condensable gases, water and nitrogen products. An electrostatic precipitator was used for the collection of bio-oil including both the aqueous and oil phases. The aqueous phase, consisting of dissolved ammonia, water and hydrogen cyanide was

separated and treated in a waste-water operation. The oil phase was further separated in two fractions, the light fraction which included benzene, toluene, and p-xylene (BTX) as well as the heavy fraction that contained other aromatics. Benzene, toluene and p-xylene were purified from the light fraction and could be used as renewable petrochemicals. The heavy fraction was valorized through a hydroprocessing unit to obtain hydrocarbons with potential application as blendstock for gasoline and diesel production. Non-condensable gases were separated in a cryogenic separation unit for isolation of olefin, which could be used in the petrochemical industry (Wang et al., 2015).

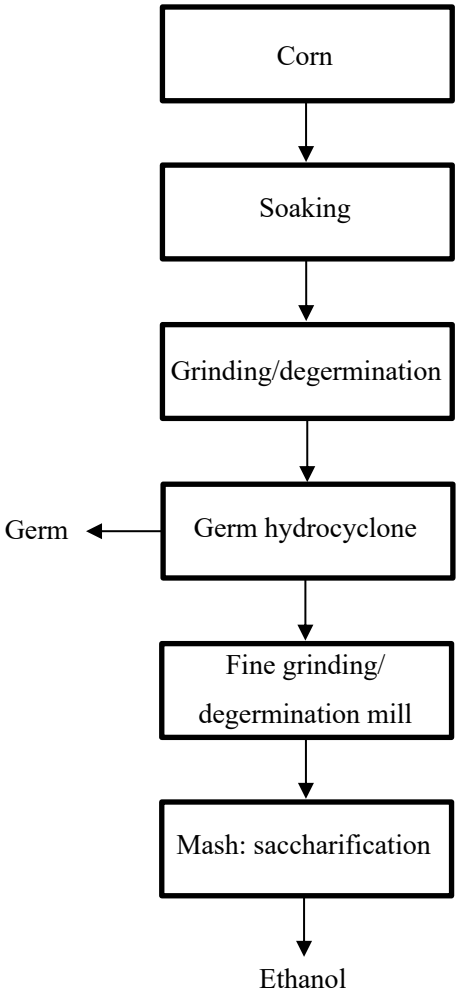

Figure 1.2.12: Process flow sheet of a corn-based biorefinery applied for the production of ethanol (adapted from Huang et al., 2008).

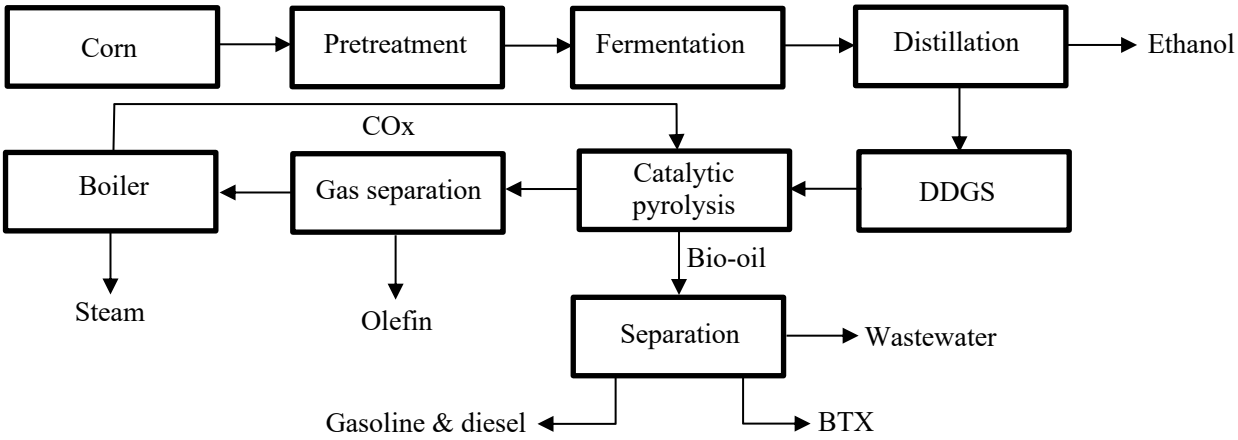

Figure 1.2.13: Process flow sheet of a corn-based biorefinery applied for the production of ethanol, gasoline, diesel and BTX as well as for the isolation of olefin (adapted from Wang et al., 2015).

1.2.1.4 Sugarcane waste

Sugarcane constitutes one of the most important energy crops, which is widely grown in tropical and subtropical countries. Brazil is the main producer of sugarcane providing approximately 39% of the global sugarcane production, followed by India (19%), China (7%), Thailand (5%) and Pakistan (4%) (Silalertruksa et al., 2016). In the Brazilian sugarcane industry, 70% of sugarcane processing units comprise integrated sugar mills with distillery plants where typically half of the sugarcane juice and molasses is employed for bioethanol production, while the other half is used for the production of sugar (Cavalett et al., 2012). The sugar and ethanol industry generates a large amount (1200 t per day) of lignocellulosic materials (sugarcane bagasse and trash) as residues (Ojeda et al., 2011). Approximately 1 t of sugarcane generates 280 kg of bagasse (Rabelo et al., 2011), which is currently used following sucrose extraction for the supply of electricity to sugar mills (Silalertruksa et al., 2016). Nevertheless, sugarcane trash (tops, dry and green leaves or straw), which was previously burnt, currently serves mainly for agricultural or industrial purposes (Dias et al., 2012).

Sugarcane straw can be used in the agriculture as well as a raw material for valorization through thermochemical or biochemical conversion processes and it has been applied as a biorefinery feedstock for the production of biofuels and high

added-value chemicals. Specifically, the valorization of sugarcane straw has been evaluated for biotechnological production of xylitol using *C. guilliermondii* FTI 20037 (Hernandez-Pérez et al., 2016a; Hernandez-Pérez et al., 2016b).

Sugarcane bagasse comprises the main residue from the sugar and ethanol industry and it can be sustainably applied for the production of first and second generation biofuels owing to its content in cellulose (37.65%), hemicellulose (29.44%) and lignin (32.91%) (Moncada et al., 2013). This composition enables application of sugarcane bagasse as a raw material supporting the development of a series of biorefinery-based valorization strategies (Ojeda et al., 2011). The production of ethanol, PHB and electricity has been studied in a process (Figure 1.2.14) that involved fractionation of hemicellulose into pentose through pretreatment with dilute acid, while the solid fraction (cellulose and lignin) was hydrolyzed and separated into glucose (liquid fraction) and lignin (solid fraction). The lignin generated was used for electricity production by combined cycle biomass gasification, glucose was applied for the production of ethanol and PHB by *S. cerevisiae* and *C. necator* respectively, while xylose was employed for ethanol production by recombinant *Z. mobilis* (Moncada et al., 2013). Further treatment of the hemicellulosic hydrolyzate formed during acid hydrolysis was examined to remove phenolic compounds, HMF and heavy metals through detoxification. Detoxification of the hydrolyzate was carried out using tannin-based biopolymers enhancing the formation of fermentation products (ethanol and xylitol) by *C. guilliermondii* (Silva-Fernandes et al., 2017).

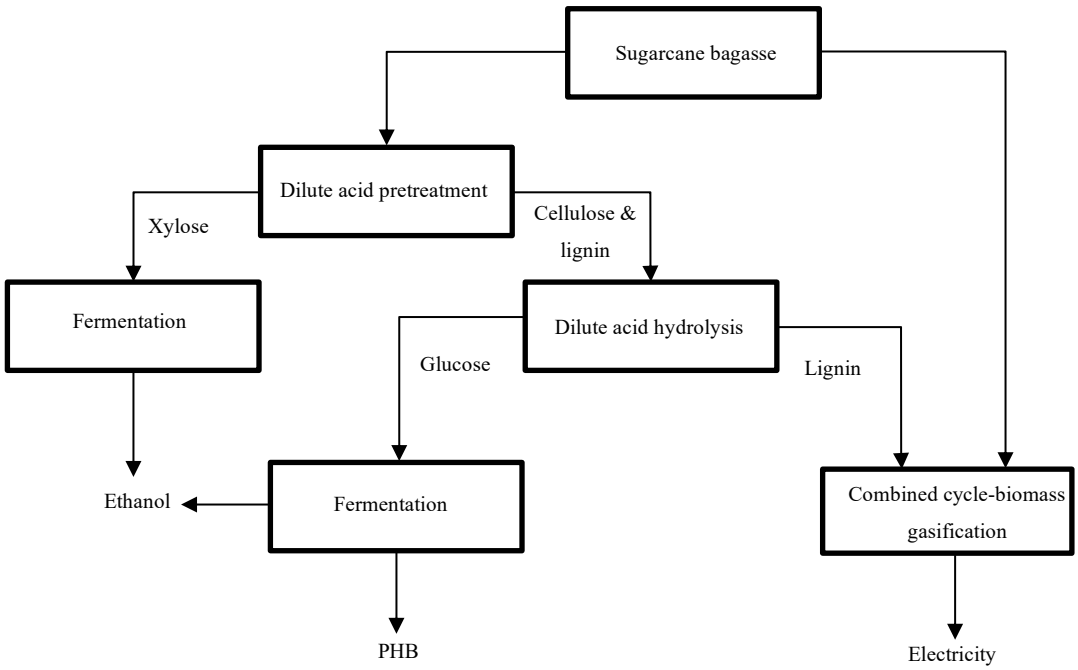

Figure 1.2.14: Process flow sheet of sugarcane bagasse-based biorefineries applied for the production of ethanol, PHB and electricity (adapted from Moncada et al., 2013).

Two pretreatment methods for sugarcane bagasse were tested by Rabelo et al. (2011) followed by ethanol and biomethane production (Figure 1.2.15). Alkaline hydrogen peroxide and lime pretreatments were performed under mild conditions for the recovery of lignin, while the resulting mixture was separated into solid and liquid fractions. The solid was enzymatically hydrolyzed and the hydrolyzate generated was fermented for bioethanol production. The solid residue from enzymatic hydrolysis was mixed with the residue from lignin extraction of the liquid fraction and applied in anaerobic digestion for biomethane production (Rabelo et al., 2011).

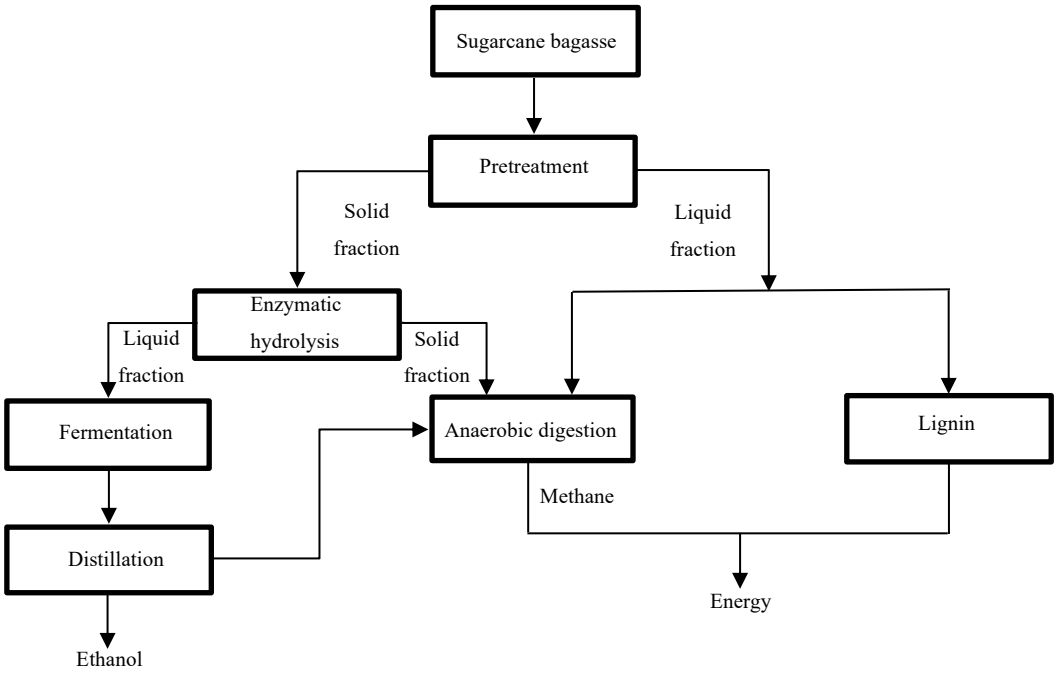

Figure 1.2.15: Process flow sheet of sugarcane bagasse-based biorefineries applied for the production of ethanol, methane and energy (adapted from Rabelo et al., 2011).

Application of sugarcane bagasse for production of biofuels has been also evaluated through application of life cycle analysis that compared different biorefinery strategies and proposed various process schemes based on the composition of bagasse. Specifically, liquid hot water, diluted acid, acid catalyzed steam explosion and organosolv processes were tested for bagasse pretreatment, while techno-economic analysis was primarily used for the selection of pretreatment methods. Following pretreatment, enzymatic hydrolysis technologies including simultaneous saccharification and co-fermentation, simultaneous saccharification and fermentation, consolidated bioprocessing and separate hydrolysis and fermentation were also tested. The combination of organosolv for pretreatment of sugarcane bagasse and simultaneous saccharification and co-fermentation generated the highest ethanol yield due to the high efficiency of hemicellulose hydrolysis achieved that resulted in increased xylose yields and lignin solubilization (Ojeda et al., 2011). Ethanol production combined to nanocellulose production was also examined by Albarelli et al. (2016). SO_2-catalized steam explosion, supercritical CO_2 explosion, organosolv fractionation

and supercritical CO_2 organosolv fractionation were tested for biorefinery development demonstating that supercritical CO_2 explosion incorporates promising economical parameters as a pretreatment method for ethanol and nanocellulose production (Albarelli et al., 2016).

Cane molasses constitutes the main by-product from the sugar industry consisting 35% (w/w) sucrose, 25% (w/w) water, 10% (w/w) soluble sugars such as glucose and fructose, 9.6% (w/w) ash, 8.9% (w/w) metal ions (including K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cl^-, SO_4^{2-}), 4.6% (w/w) salt, 4.3% (w/w) crude protein, 2.5% (w/w) other carbohydrates and 0.06% (w/w) crude fat (Jiang et al., 2009; Dai et al., 2015). The specific feedstock constitutes a low-cost raw material demonstrating a series of industrial applications and it has been used industrially for the production of ethanol, lactic acid, citric acid and polysaccharides (Liu et al., 2008a; Jung et al., 2013). Furthermore, various methods have been tested for cane molasses pretreatment indicating that acid hydrolysis is the most efficient for the release of sugars and resulting product yields (Jiang et al., 2009). Several studies have investigated the application of cane molasses as a raw material for the production of high added-value products. The production of succinic acid by *A. succinogenes* has been tested from cane molasses following pretreatment with acid hydrolysis (Liu et al., 2008a), while butyrate fermentation was also studied using *C. tyrobutyricum* (Jiang et al., 2009). Moreover, cane molasses has been used to generate reactor operation strategies for the development of a three-stage process to produce polyhydroxyalkanoates (PHAs). The process consisted of (1) molasses fermentation by acidogenic bacteria, (2) selection of PHA-accumulating cultures and (3) accumulation of PHA from fermented molasses (Albuquerque et al., 2007). Additionally, molasses has been demonstrated as a promising feedstock for the production of 2,3-butanediol (Jung et al., 2013).

Sugarcane may serve as a feedstock for the production of first-generation ethanol, electricity and high added-value products (Silalertruksa et al., 2016; Hernandez-Perez et al., 2016b). The development of sugarcane-based biorefineries constitutes a promising valorization strategy due to the rich composition of its biomass (Silalertruksa et al., 2016), which includes a content of 15.3% (w/w) of total reducing sugars in wet basis, while consisting of 13% sugarcane fibers,

43.38% sugarcane bagasse/trash cellulose, 25.63% sugarcane bagasse/trash hemicellulose and 23.24% sugarcane bagasse/trash lignin on a dry basis (Dias et al., 2012). Sugarcane bagasse/trash mixture was evaluated for valorization into ethanol, methanol and lactic acid or co-production of ethanol and lactic acid following different pretreatment methods by Mandegari et al. (2018) (Figure 1.2.16). Furthermore, the production of second-generation ethanol from pentoses has been suggested to increase the yield of first generation ethanol in sugarcane biorefineries. Thus, co-fermentation of pentoses (derived from bagasse and trash) with juice and molasses to produce bioethanol with the use of a yeast engineered to ferment xylose could serve as a viable direction for the sugarcane ethanol industry (Losordo et al., 2016).

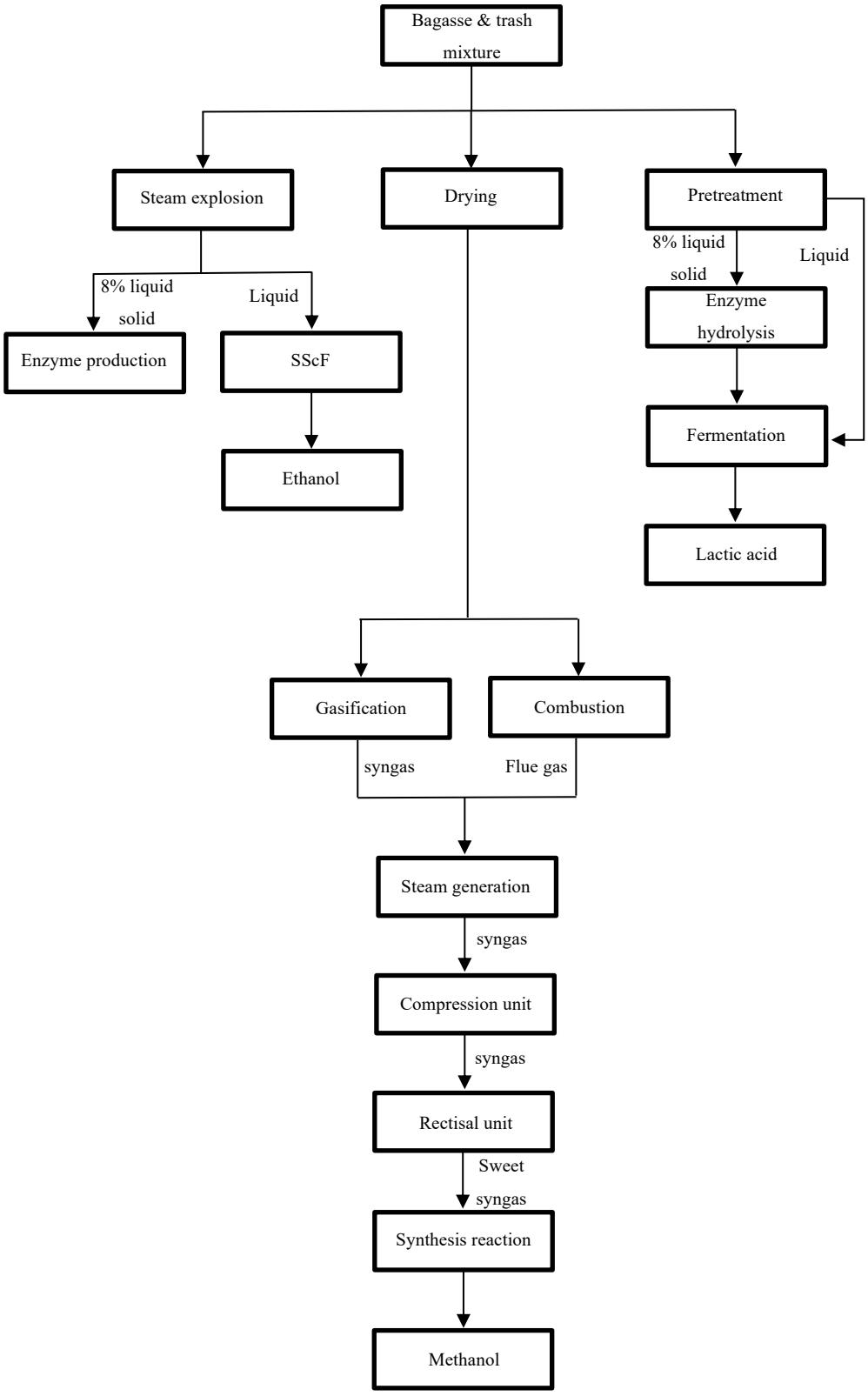

Figure 1.2.16: Process flow sheet of sugarcane bagasse-based biorefineries applied for the production of ethanol, methanol and lactic acid (adapted from Mandegari et al., 2018).

Sugarcane has been applied for acetone, n-butanol and ethanol (ABE) production in a biorefinery that involved integrated first and second generation processes as well as power co-generation. The production of butanol was conducted either using ABE fermentation from C5 sugars or through catalysis of ethanol (Figure 1.2.17). Following sugarcane juice extraction, straw and bagasse were used for electricity co-generation. However, bagasse was also pretreated and hydrolyzed, while the hydrolyzate was mixed with juice serving as a feedstock for the production of ethanol. The hydrolysis residues contributed to co-generation for electricity production, and the pentoses generated from bagasse pretreatment were fermented into ethanol, butanol and acetone. Following distillation and rectification of the fermentation broth as well as ethanol dehydration, ethanol was catalytically converted to n-butanol and co-products (Pereira et al., 2015).

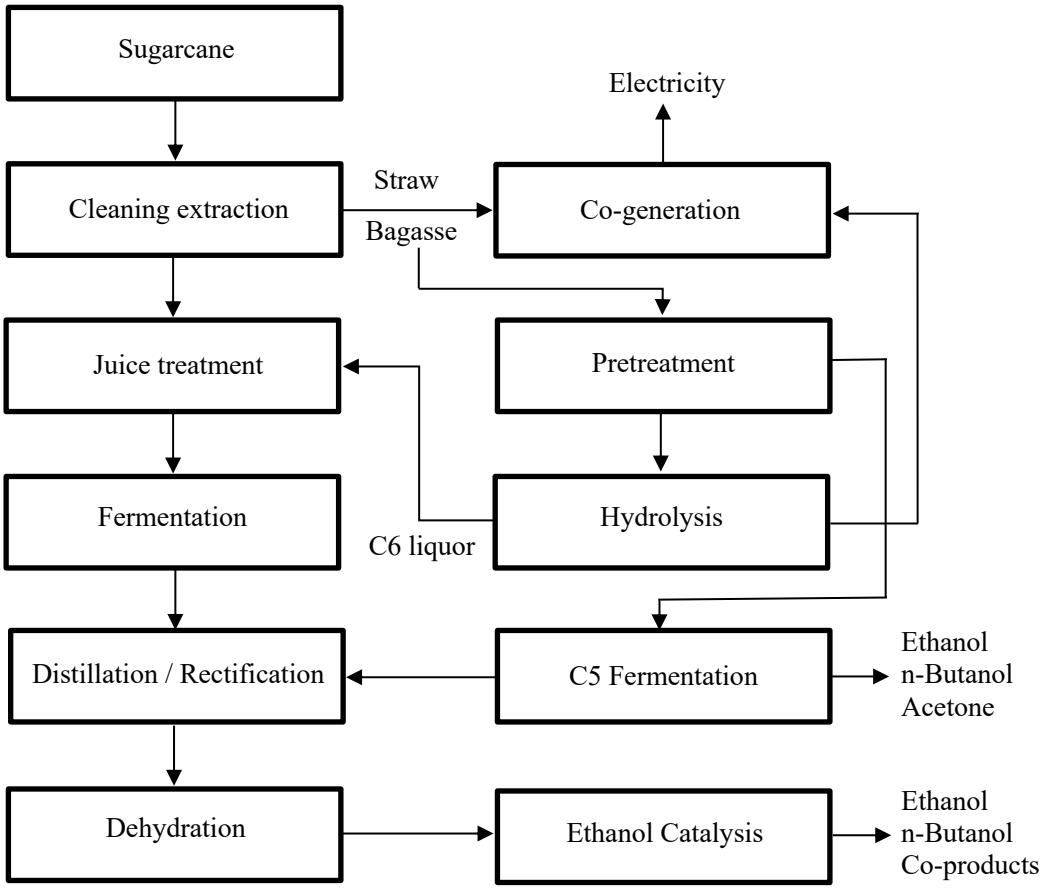

Figure 1.2.17: Process flow sheet of a sugarcane bagasse-based biorefinery applied for the production of ethanol, n-butanol, acetone and electricity (adapted from Pereira et al. 2015).

A similar sugarcane biorefinery strategy was adopted for the production of butanol, acetone and ethanol through the common processing option, which requires application of sugarcane for the production of ethanol and sugar. Therefore, the residual solution from sugar crystallization (molasses) was introduced as a co-product in the biorefinery and also applied for ethanol production (Mariano et al., 2013). Furthermore, Dias et al. (2012) used sugarcane for the production of anhydrous ethanol, while the main difference with previous approaches was the application of pentoses, which were both fermented into ethanol and anaerobically processed for biogas generation and energy production in a co-generation system. However, Albarelli et al. (2018) investigated a third generation biorefinery targeting biofuel production with the use of microalgae (Figure 1.2.18).

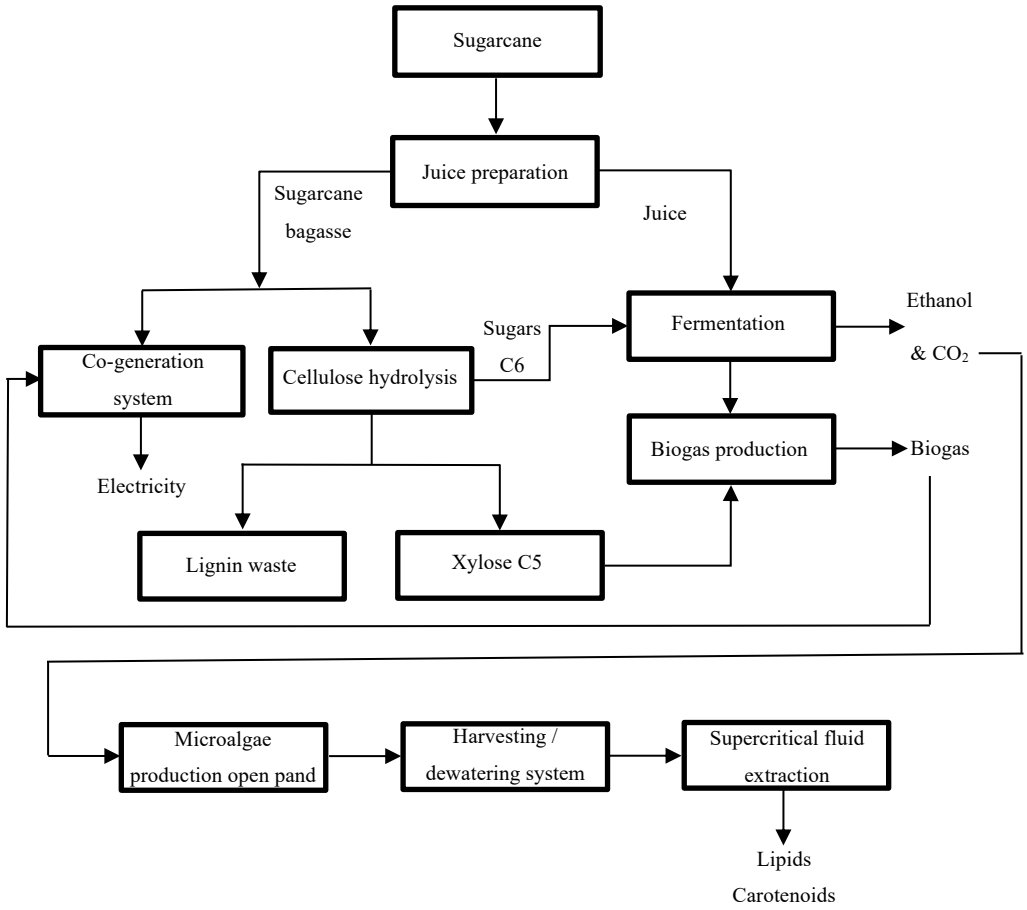

Figure 1.2.18: Process flow sheet of sugarcane-based biorefineries applied for the production of ethanol, methane, electricity, lipids and carotenoids (adapted from Albarelli et al., 2018).

1.2.1.5 Citrus peel waste

The valorization of citrus peel waste (CPW) has been also previously explored. A biorefinery developed for the management of CPW was first evaluated by Pourbafrani et al. (2010). CPW was hydrolyzed by dilute-acid hydrolysis at 150 ^{o}C for 6 min, while hydrolysis conditions were optimized through application of central composite rotatable experimental design achieving a maximum sugar yield of 0.41 g g^{-1} of total dry CW. Subsequently, pectin was extracted from the residue obtaining 77.6% (w/w) of the total pectin content. An expansion tank was employed for essential oils recovery from the citrus peel hydrolyzate, which was eventually employed for bioethanol production with the use of baker's yeast. The yield of ethanol reached 0.43 g g^{-1} of fermentable sugars, while the solid residues as well as the stillage were anaerobically digested for biomethane production (Pourbafrani et al., 2010).

An alternative approach for OPW valorization through the biorefinery concept was proposed by Pfaltzgraff et al. (2013). D-limonene, sugars, pectin and a form of mesoporous cellulose were produced by the biorefinery suggested using a hydrothermal low temperature microwave process that did not require any additional pretreatment. The D-limonene yield reached 1.52% (v/w), while the use of microwave heating (dynamic mode: 1800 W) at 120 ^{o}C for 15 min resulted in 10.80% (w/w) pectin yield obtained at a scale of 3 L (Pfaltzgraff et al., 2013, Clark et al., 2016) (Figure 1.2.19). However, microwave hydrodiffusion and gravity were used for OPW pretreatment targeting the production of essential oils, polyphenols and pectin through the biorefinery presented in Figure 1.2.20. The specific technology included physicochemical treatment including ultrasound for polyphenols' extraction and microwave for pectin isolation. The microwave technology reduced the time for essential oils extraction as compared to distillation, while the polyphenols yield was increased by 30%. The maximum pectin yield obtained was 24.2% using microwave power of 500 W for 3 min (Boukroufa et al., 2015).

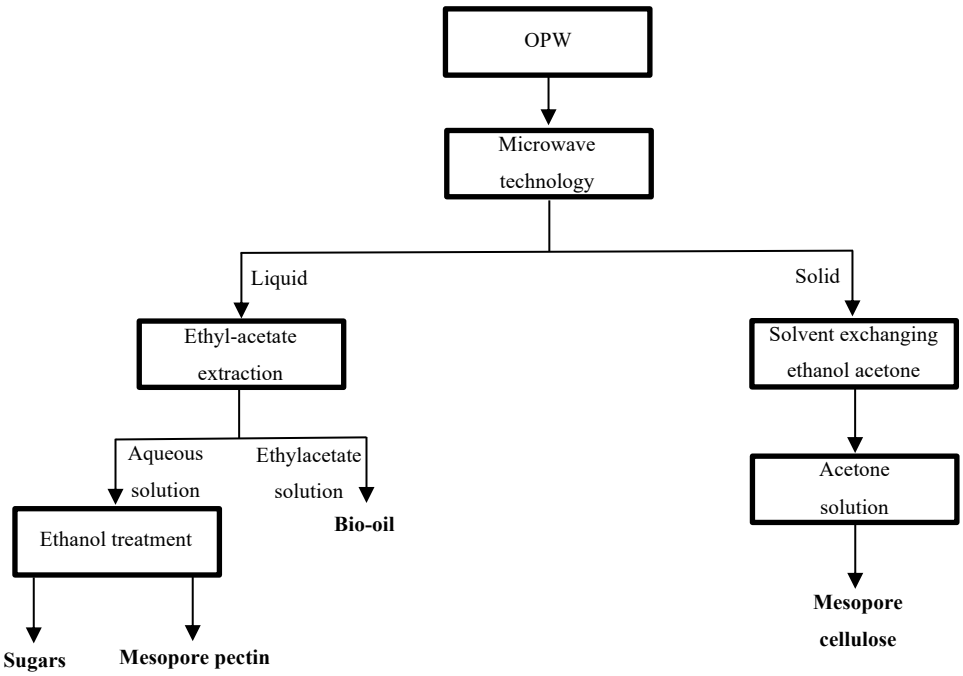

Figure 1.2.19: Process flow sheet of OPW-based biorefineries applied for the production of sugars, pectin, bio-oil and mesopore cellulose (adapted from Clark et al., 2016).

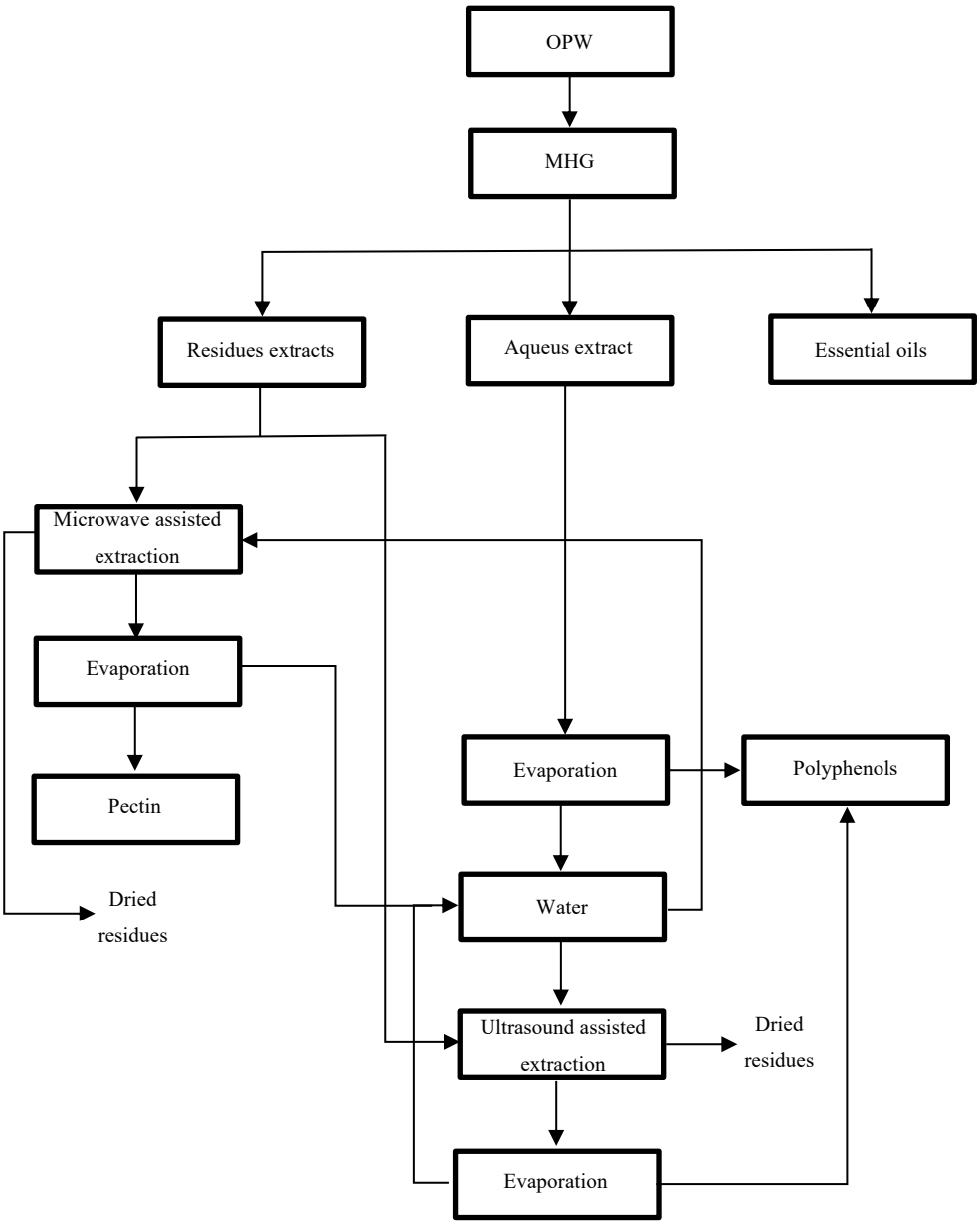

Figure 1.2.20: Process flow sheet of OPW-based biorefineries applied for the production of essential oils, pectin and polyphenols (adapted from Boukroufa et al., 2015).

A valorization approach for simultaneous pectin extraction and pretreatment of CPW was proposed by Satari et al. (2017). Citrus waste, following dilute-acid treatment with nitric acid, was subject to ethanol-acid treatment in order to enhance pectin extraction obtaining a yield of 45.5%. Moreover, solar hydrodistillation was employed in a simpler OPW biorefinery (Figure 1.2.21), which was used for extraction of essential oils, polyphenols, flavonoids and pectin. The polyphenol and

flavonoid (narirutin and hesperidin) content extracted was higher as compared to the conventional procedure (Hilali et al., 2019).

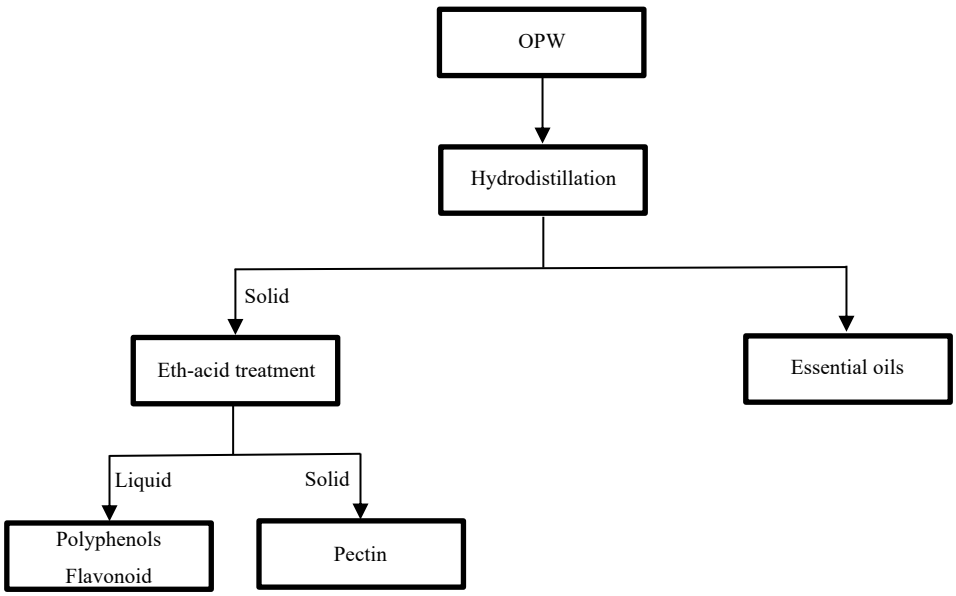

Figure 1.2.21: Process flow sheet of CPW-based biorefineries applied for the production of essential oils, polyphenols, flavonoids and pectin (adapted from Hilali et al., 2019).

1.3 Citrus processing: Residues generated and valorization approaches

1.3.1 Characteristics of citrus fruits

The global citrus production constituted over 124×10^6 t for 2016 according to the Food and Agriculture Organization of the United Nations. The fruits included in the annual compilation of statistics covered comprise oranges, tangerines, lemons, limes and grapefruit. Oranges and tangerines incorporated the highest word production that reached 67×10^6 t and 33×10^6 t, respectively. The production of lemons and limes was 16×10^6 t while the production of grapefruit was constituted 8×10^6 t (FAO, 2017).

Citrus fruits belong to one of the most important fruit crops in the world given that they are globally well-accepted by consumers due to their aroma, attractive colors and pleasant flavors (Zou et al., 2016). Although consumption of fresh citrus fruits is popular all over the world, about 20% of citrus fruits are industrially processed to obtain a wide range of products (FAO, 2017). The citrus industry constitutes one of the most important fruit-processing industries followed by wine manufacturing (Izquierdo and Sendra, 1993). Hence, the worldwide industrial generation of CPW exceeds 25×10^6 t per year (FAO, 2016).

Citrus fruits are well-known for a series of favorable effects to biological functions of the human health such as antioxidant, antimutagenicity, antiinflammation, anticarcinogenicity and anti-aging, which can be attributed to the phytochemical compounds that exist in the fruit. These phytochemicals include vitamins A, C and E, pectin, flavonoids, limonoids, carotenoids, mineral elements as well as other useful compounds (Zou et al., 2016). Thus, citrus industries process value-added molecules that incorporate quality, nutritional characteristics and purity which could be useful to various sectors. Since these three aspects are closely related to the composition of the fruit, analysis of citrus constituents is commonly applied to identify novel processes and application (Izquierdo and Sendra, 1993).

1.3.2 Citrus peel waste

1.3.2.1 Composition of CPW

The CPW formed during the processing of citrus for juice extraction consist of peels, seeds and segment membranes accounting for 50% (w/w) of the whole fruit (Marin et al., 2007; Wilkins, 2009). Thus, in order to propose a potential valorization technology for CPW, it is essential to determine the chemical composition of this feedstock. According to Rivas et al. (2008), orange peel consists of 42.5% (w/w) pectin, 16.9% (w/w) soluble sugars, 10.5% (w/w) hemicellulose, 9.21% (w/w) cellulose, 6.50% (w/w) protein, 3.75% (w/w) starch, 3.50% (w/w) ash, 1.95% (w/w) fat, 0.84% (w/w) lignin as well as a small amount of 4.35% (w/w) which includes organic acids such as citric acid, malic acid, malonic acid, oxalic acid and ascorbic acid (Vitamin C). The composition of orange peels was also investigated by Bampidis and Robinson (2006) demonstrating that the dry matter (dm) content of orange peel is mainly organic (975 g kg_{dm}^{-1}), containing 58 g kg_{dm}^{-1} proteins, 200 g kg_{dm}^{-1} neutral detergent fibre, 129 g kg_{dm}^{-1} acid detergent fiber, organic acids (23 g kg_{dm}^{-1} lactic acid, 20 g kg_{dm}^{-1} acetic acid, 0.3 g kg_{dm} propionic acid, 0.6 g kg_{dm}^{-1} isobutyric acid as well as 7.3 g kg_{dm}^{-1} calcium and 1.7 g kg_{dm}^{-1} phosphate. Furthermore, orange peel waste (OPW) include 0.5% (g g_{wm}^{-1}) of essential oils, consisting 90% (v/v) of D-limonene (Li et al., 2010a) that could be employed for the production of food, medicines, flavorings and antimicrobial agents (Martin et al., 2010). Moreover, the low pH-value (3.64) of the peel requires neutralization for some applications. However, the low pH-values as well as the chemical composition of CPW are dependent on a number of factors the include citrus cultivation conditions, maturity, rootstock, varieties and climate (Kale and Adsule, 1995)

1.3.2.2 Current management practices

Direct utilization of CPW without any pretreatment is commonly applied, incorporating traditional management practices. The management options used include application as animal feed and organic fertilizer as well as disposal in landfills.

As with most plant substrates, CPW constitutes an attractive nutrient source as animal feed. Plant materials could be often easily assimilated by animals due to the adaptation capabilities of microbial communities that inhabit in their rumen (Bampidis and Robinson, 2006). During the industrial processing of the fruit in a juice factory, a semi-solid residue is obtained as waste product in the form of juice centrifugation pulp as well as the solid residues consisting mainly of peels. Tripodo et al. (2004) demonstrated that treating the semi-solid residue enzymatically and/or through an alkaline method resulted in a product with excellent digestibility which could be favorably compared against a wide range of other agroindustrial waste products currently used as components of animal feed. Nevertheless, the quantity of CPW that could be added to the animal feed includes a maximum level given that high concentrations of the material could cause rumen parakeratosis. In addition, the presence of citrus by-products can lead to the development of mycotoxins that could be harmful to ruminants (Bampidis and Robinson, 2006). Thus, a drying step prior to the addition of CPW in the feed would be necessary in order to avoid generation of toxins. However, the drying process could increase the price of the final product, while poor safekeeping may lead to uncontrolled production of methane. Thus, the traditional management of CPW through use as animal feed consist a risk (Lopez et al., 2010) stimulating research towards the development of new alternative CPW applications for the production of high added-value products and biofuels.

The application of CPW as organic fertilizer constitutes another traditional management practice for the specific residue. Specifically, CPW could be converted into a fertilizer via composting. This can be achieved by modifying the waste through adjustment of important parameters such as pH-value, moisture, and C/N ratio, while the process could be completed within 3 months under appropriate conditions (van Heerden et al., 2002). Although mature compost produced from CPW demonstrated acceptable levels of phytotoxicity, while air-filled porosity and water holding capacity stimulated plant growth, the relatively high conductivity of the compost may contribute to increased salinity (Lopez et al., 2010). However, field experiments demonstrated that the addition of compost in soil improved the growth of citrus trees by 25% as compared to control that comprised trees planted

in non-modified soil. Guerrero et al. (1995) studied the potential of OPW for application as organic fertilizer for lettuce growth. In this study, both fresh and dry matter of either orange pulp or peel was applied as organic fertilizer into a poor of nutrients soil following drying and grinding, in order to increase its nitrogen content. The results indicated that lettuce growth increase between 30% and 130% while no phytotoxicity was observed.

Overall, current management options for CPW include burying in landfills as well as application as animal feed and organic fertilizer after drying. However, the thermal dehydration process of CPW to produce animal feeds is energy consuming and thus not always cost-effective (Negro et al., 2018), while the final product consists a rather poor animal feed due to low protein content and high quantity of sugars (Mamma and Christakopoulos, 2014). Moreover, the waste includes elevated organic matter (~95% of total solids) and water content (~80%-90%) as well as low pH-value (3-4) constituting CPW inappropriate for landfilling based on the latest EU Waste Framework Directive 2008/98/EC (Ruiz and Flotats, 2014). Although the disposal of CPW is opposed to EU regulations, the waste could serve as a valuable feedstock for the manufacture of biofuels and other commodities.

1.3.2.3 CPW valorization approaches

Nowadays, the need to replace the use of petroleum with new renewable resources for the production of fuels and chemicals and to identify novel practices for the reduction of biodegradable waste has led to the application of FW as a feedstock for advanced waste management technologies (Lin et al., 2013). The valuable composition of the peel renders CPW a promising feedstock for the biotechnological production of biofuels (ethanol and methane) and added-value commodities (succinic acid, industrial enzymes, single cell protein and bacterial cellulose) enabling the extraction of useful compounds (essential oils, pectin) included in the waste. Several studies have previously investigated the valorization of CPW through bioprocessing (Wilkins, 2009; Martin et al., 2010; Pourbafrani et al., 2010; Koutinas et al., 2016a; Ruiz and Flotats, 2016). However, CPW cannot be always directly applied and pretreatment of the material prior to the bioprocess usually requires the removal of essential oils due to the antimicrobial properties of D-limonene that may cause inhibition of the biosystem. Provided that the removal

of D-limonene constitutes CPW a relatively expensive raw material, the alternative use of microorganisms less susceptible to D-limonene could reduce the fermentation cost.

Hydrolyzates obtained from CPW following acid or enzymatic hydrolysis comprise a high content of soluble sugars and monosaccharides that could serve as a nutrient rich fermentation feedstock for the production of biofuels or other products. Different methods of CPW pretreatment have been employed for generation of hydrolyzates used in microbial fermentations. Steam explosion, hydrothermal sterilization, dilute acid hydrolysis and enzymatic hydrolysis through application of pectinase, cellulase and β-glucosidase have been used for the release of sugars from the cellulose, hemicellulose and pectin content of CPW (Grohmann and Baldwin, 1992; Grohmann et al., 1995; Wilkins et al., 2007a).

Bioethanol production from CPW hydrolyzate was investigated in several studies using *S. cerevisiae*, *M. indicus* and *P. kudriavzevii* (Wilkins et al., 2007b; Pourbafrani et al., 2010; Boluda-Aguilar et al., 2010; Oberoi et al., 2011). Moreover, acid pretreated OPW have been tested for the production of single cell protein (SCP) that contained 35-40% of crude protein including high *in vitro* digestibility using *G. candidum* (Vaccarino et al., 1989; Lo Curto et al., 1992). (73-88%), while following acidic pretreatment of OPW hesperidin, a high-value by-product was obtained from the solid residue, reaching yields that ranged between 3.7% and 4.5% (w/w_{dm}) (Lo Curto et al., 1992).

In recent years, research on CPW fermentation for the production of bacterial cellulose has received increasing attention. A hydrolyzate generated from acid treatment of citrus peels was tested for the production of bacterial cellulose by *K. hansenii* GA2016 demonstrating that the biopolymer generated from the particular feedstock included high water holding capacity, thin fiber diameter, elevated thermal stability and high crystallinity (Guzel and Akpinar, 2017). Kuo et al. (2019) performed enzyme hydrolysis of OPW using cellulases and pectinases produced by *G. xylinus*, demonstrating that the OPW media formed could not inhibit the fermentation performed for bacterial cellulose production, although the high concentration of reducing sugars. The addition of nitrogen source resulted in bacterial cellulose production 4.2-6.3 times higher as compared to the use of

traditional HS (Hestrin and Schramm) medium (Kuo et al., 2019). Furthermore, the production of bacterial cellulose by *G. xylinus* and *G. hansenii* was tested with the use of citrus pulp water obtaining similar results to the industrial production (Cao et al., 2018). Citrus juices as well as aqueous extracts from citrus peel processing waste were also used for the production of bacterial cellulose by *K. sucrofermentans* DSM15973 (Andritsou et al., 2018). The bacterial cellulose formed from the orange peel aqueous extract presented improved water-holding capacity, degree of polymerization and crystallinity index as compared to the use of the cellulosic fraction isolated from depectinated orange peel.

CPW has been also tested as a substrate in anaerobic digestion for methane production under thermophilic and mesophilic conditions (Koppar and Pullammanappallil, 2013; Sanjaya et al., 2016). Calabro et al. (2016) demonstrated the inhibitory effect of increasing concentration of essential oils on the biomethanization of OPW. A similar trend was also reported by Lotito et al. (2018), during anaerobic digestion of fresh and stored CPW for biomethane production. Some researchers used a pretreatment method for CPW prior its application on anaerobic digestion in order to remove essential oils (Gunaseelan, 2004; Martin et al., 2010) reventing bioprocess inhibition (Negro et al., 2018).

CPW have been also valorized for the production of industrial enzymes, activated citrus peel extract and paper pulp supplemented as well as in applications as carriers for biocatalyst development and as pollutant adsorbent. A high percentage of CPW mass consists of pectin, which can induce the synthesis of pectic enzymes (Lopez et al., 2010). Pectic enzymes contribute useful functions for the fruit juice industry facilitated by extraction and clarification of juices (Rombouts and Pilnik, 1986). Garzon and Hours (1992) employed CPW for the production of pectic enzyme by *A. foetidus* in solid-state cultures obtaining a yield of pectinases which was 25% greater as compared to the yield achieved with apple pomace using the same microorganism. *T. flavus*, *P. charlessi* and *T. vulgaris* were tested for the production of pectic enzymes using citrus pulp waste in solid-state cultures and the most activated pectinase was observed in *P. charlessi* and *T. flavus* cultures (Siessere and Said, 1989). Furthermore, the production of multienzyme preparations containing pectinases, cellulases as well as xylanases by *A. niger* BTL,

F. oxysporum F3, *N. crassa* DSM 1129 and *P. decumbens* using dry orange peel under solid-state fermentation demonstrated that the regulation of initial pH-value and moisture were beneficial for the production of the enzyme. *A. niger* BTL was grown on orange peel under optimal conditions producing the greatest yield of polygalacturonase, pectate lyase, xylanase, β-xylosidase and invertase, while *N. crassa* (DSM 1129) resulted in the highest yield of endoglucanase (Mamma et al., 2008). Continuous cultures of *B. subtilis* 11089 fed with orange peel as carbon source were used for the production of extracellular hydrolytic enzymes, obtaining improved yields as compared to the use of glucose potentially due to the nutrient-rich nature of orange peel (Mahmood et al., 1998).

OPW was also used as a carrier for the development of biocatalysts in alcoholic fermentations. Thus, following sterilization the material was used as a support for *S. cerevisiae* cells immobilization. The results demonstrated that OPW could be employed for the development of immobilized biocatalysts demonstrating higher fermentation efficiency as compared to free cells (Plessas et al., 2007). CPW has been also tested as an adsorbing agent for contaminants present in wastewater. The material demonstrated enhanced adsorptive removal of methylene blue (redox indicator) and Direct Red 23 and 80 (textile dyes) (Arami et al., 2005; Namasivayam et al., 2003). Moreover, CPW could effectively adsorb heavy metal ions such as Cd^{2+}, Zn^{2+} and Pb^{2+} as well as binary mixtures Cd^{2+}-Zn^{2+}, Cd^{2+}-Pb^{2+}, and Zn^{2+}-Pb^{2+} (Ajmal et al., 2000; Perez-Marin et al., 2008).

The use of CPW to produce an activated citrus peel extract was investigated from Medvedev and Kat (2004). The composition of the activated citrus peel extract consisted of flavonoid glycosides, triglycerides, fatty acids, short peptides as well as oligosaccharides and it was effective for use as a dermatological treatment and for preserving food, beverages and cosmetics. The composition of CPW render them suitable for use as a paper pulp supplement because of high concentration of cellulose and hemicellulose combined with low concentrations of lignin and ash (Ververis et al., 2007). Various advantages have been demonstrated for the use of CPW as a paper pulp supplement that did not affect the breaking length, while decreasing tearing resistance and increasing bursting strength. Furthermore, the

cost of orange peel was about 45% lower as compared to conventional pulp, resulting in 0.9-4.5% reduction of the final paper price.

1.4 Production of useful commodities from CPW

1.4.1 Essential oils

All plants hold the ability to produce volatile compounds, but quite often only in traces. “Essential oil plants” include plant species producing an essential oil of commercial interest, constituting biological, cultural, and technological resources. They have been used since the furthest antiquities as spices and remedies for the treatment of diseases and in religious ceremonies because of healing properties and pleasant odors (Bakkali et al., 2008). Flowers such as rose, jasmine, carnation, clove, mimosa, rosemary and lavender, leaves such as mint, lemongrass, *Ocimum* spp. and jamrosa, leaves and stems such as cinnamom, petitgrain, patchouli, verbena and geranium, bark such as canella, cassia and cinnamon, wood like cedar, sandal and pine, roots such as angelica, sassafras, vetiver, valerian and saussurea, seeds like fennel, coriander, dill, nutmeg and caraway, fruits like orange, lemon, grapefruit and juniper, rhizomes such as curcuma, calamus, ginger and orris as well as gums or oleoresin exudations like balsam of Peru, Myroxylon balsamum, storax, myrrh and benzoin constitute the natural sources of essential oils (Talati, 2017).

The global need for pure natural ingredients in various industrial fields increase the demand for essential oils (Dhifi et al., 2016). Thus, the variety of available essential oils enable the improvement as well as formulation of old and new natural flavorings. Moreover, although natural essential oil ingredients include superior properties as compared to synthetic ones. They often vary in composition which affects processing characteristics and final product performance. The worldwide supply of essential oils is strongly dependent on the companies producing flavorings and fragrances, where Givaudan, Firmenich and IFF constitute the main producers holding 46% of total sales (Barbieri and Borsotto, 2018).

Essential oils constitute one of the most important products of the agriculture-based industry, employed as flavoring as well as antimicrobial agents in food and drink products, pharmaceuticals and cosmetics (Do et al., 2015). Approximately 3000 essential oils have been produced by using at least 2000 different plant species, out of which 300 are important from commercial point of view (Rault and Karuppayl, 2014). The estimation of the global essential oils production in 2017

was more than 150 000 t (tripling in volume since 1990), which corresponds to a market of approximately $ 6 billion. Several economic analyses foresee that by 2020 the production of essential oils is expected to reach 370 000 t annually reaching a value of more than $ 10 billion. Among the crops used for extraction of essential oils, the most important options include orange, mint and lemon, the production reaches about 100 000 t (two-thirds of the total production of essential oils) (Barbieri and Borsotto, 2018). However, the price of essential oils varies significantly mainly due to the scarcity of raw material, harvesting issues, climate dependence or extraction yield (Do et al., 2015).

A significant amount of fruits from the genus *Citrus* include the most popular natural essential oils, accounting for the largest proportion of commercial natural flavors and fragrances (Sawamura, 2010). The genus *Citrus* belongs to the Rutaceae or Rue family and includes about 140 genera and 1300 species. *Citrus sinensis* (orange), *Citrus paradisi* (grapefruit), *Citrus limon* (lemon), *Citrus reticulata* (tangerine), *Citrus grandis* (shaddock), *Citrus aurantium* (sour orange), *Citrus medica* (citron), and *Citrus aurantifolia* (lime) comprise some well-known fruits of specific genus (Kamal et al., 2011). Citrus essential oils are obtained as by-product of citrus processing, with a wide range of application in the food and pharmaceutical industries as well as in perfumes and cosmetics (Cristani et al., 2007). In the food/drink industry essential oils are employed as aroma flavor in a multitude of food products, including alcoholic and non-alcoholic beverages, candy and gelatins. Pharmaceutical industries employ essential oils as flavoring agents to cover unpleasant tastes of drugs, while the perfumery and cosmetic industries apply their product in various preparations (Bousbia et al., 2009).

Citrus essential oils contain 85-99% volatile (mixture of monoterpenes) and 1-15% non-volatile components (Acar et al., 2015). The peel of grapefruit contains almost only limonene and some myrcene, but lime peel oil demonstrates a mixed composition of β-pinene, γ-terpinene and limonene (Gancel et al., 2003). Orange peel typically contains more than 0.5% (w/w) essential oils and approximately 90% of its content constitutes D-limonene, a hydrocarbon classified as a cyclic terpene that is a colorless liquid at room temperature with an orange aroma (Lopez et al., 2010).

Traditional extraction of essential oils includes cold pressing, where the oil glands within the peels and cuticle are mechanically crushed to release their content. A watery emulsion of the oil is generated, which is subsequently centrifuged to separate the essential oils. Water or steam distillation can be also applied to remove the essential oils through evaporation. As steam and essential oil vapors they are condensed, collected and separated in a vessel known as the "Florentine flask" (Bousbia et al., 2009). Although, the process of steam distillation is the most widely accepted method for the production of essential oils at large scale, several different methods can be also used for essential oils' extraction from the peel. The four major processes currently used include the pelatrice and sfumatrice methods, which are mainly used in Italy, as well as the Brown peel shaver and the FMC extractor applied in North and South America. Isolation of essential oils can be also achieved through solvent extraction using different organic solvents, with the application of liquid carbon dioxide or by supercritical fluid extraction (SFE) (Talati, 2017).

1.4.2 Pectin

Pectin constitutes a structural heteropolysaccharide of higher plants that exists mainly in the middle lamella and the primary cell walls of dicotyledonous plants. This natural biopolymer plays a crucial role in cell growth, mechanical strength and defense mechanisms of the plant (Kaya et al., 2014). The structure and composition of pectin have still not been fully investigated even though pectin was explored over 200 years ago. Pectin holds the ability to change during isolation from plants, storage and processing of plant material and thus, the structure of this molecule is very difficult to determine (Novosel'skaya et al., 2000). At present, pectin is thought to consist mainly of D-galacturonic acid (GalA) units linked through α-(1-4) glycosidic bonds. These uronic acids include carboxyl groups, some of which are naturally present as methyl esters. Apart from the standard structural feature of the galacturonyl polymer, several side chains containing sugars, such as xylose, arabinose, glucose, fucose, mannose or galactose have been found to be linked to the main backbone structure. (Muller-Maatsch et al., 2016)

Although pectin is present in most of the plants, the commercial manufacture of pectin is very limited and depends on the ability of pectins to form gel. The molecular size and the degree of esterification (DE) comprise two main characteristics affecting the gelling ability of pectin (Sriamornsak, 1998). At present, commercial pectins are mainly produced from citrus peel or apple pomace. Apple pomace contains 10-15% of pectin, while citrus peel contains 20-30% of the biopolymer on a dry matter basis. Thus, CPW can be considered as an important source of this high value commodity (Lopez et al., 2010).

Pectin is widely used in food and pharmaceutical industries as thickening and gelling agent. The food industry applies pectin in fillings, sweets and as a stabilizer in fruit juices and milk drinks (Lopez et al., 2010). Pectin is mainly used for the production of jams, jellies, frozen foods and low-calorie foods as a fat and/or sugar replacer (Thakur et al., 1997). Furthermore, the heteropolysaccharide has medical uses which include antidiarrhea, detoxification as well as blood glucose and cholesterol lowering (Sotanaphun et al., 2011; Thakur et al., 1997), while it can be potentially employed in pharmaceutical preparation and drug formulation as a carrier for a wide range of biologically active agents (Sriamornsak, 1998). Other applications of pectin include use in paper substitutes, foams, edible films, and plasticizers (Thakur et al., 1997).

A well-established method for production of the heteropolysaccharide includes extraction and recovery of pectin from CPW. Briefly, CPW is first dried and applied to acid hydrolysis. The hydrolyzate is subsequently removed and pectin extracted using precipitation with ethanol, while the gel formed is separated and dried at 50 °C (Faravash and Ashtiani., 2007). Moreover, Donaghy and McKay (1994) tested the extraction of pectin using polygalacturonase generated by inoculating whey with the yeast *K. fragilis.*

1.4.3 Succinic acid

Succinic acid ($C_4H_6O_4$) is nowadays established as a key platform chemical for the bio-economy era according to several reports and increasing industrial interest for commercialization (Bozell and Petersen, 2010; Jansen and van Gulik, 2014). Its significance as a platform intermediate is based on the reactivity of the two

functional carboxylic groups leading to versatile end-products, high fermentation efficiency, utilization of numerous carbon sources and inexpensive renewable resources as feedstocks, as well as the cost-competitiveness of biotechnological production over petrochemical synthesis.

Various chemical technologies have been developed for the production of succinic acid (SA), including paraffin oxidation (Polly, 1950) and catalytic hydrogenation or electrolytic reduction of either maleic acid or maleic anhydride (Cok et al., 2014; Muzumdar et al., 2004). The paraffin oxidation technology employs a calcium or manganese catalyst to obtain a mixture of dicarboxylic acids. Subsequently, distillation, crystallization and drying are used to purify succinic acid, which is produced in relatively low yield and purity through this process. The catalytic hydrogenation technology is a mature industrial process that could be carried out in homogeneous or heterogeneous catalyst systems. Although succinic acid can be obtained in high yield and purity, the operation of the hydrogenation technology is expensive and may cause serious environmental problems.

Conventional industrial applications for succinic acid include the production of polybutylene succinate and polybutylene succinate-terephthalate (9%), polyester polyols (6.2%), the food industry as acidulant, flavorant and sweetener (12.6%), the pharmaceutical industry (15.1%), and the production of resins, coatings and pigments (19.3%) (Bioconcept, 2014). In the bio-economy era, succinic acid is expected to evolve into a platform intermediate, as a replacement for maleic anhydride, for the production of various bulk/intermediate chemicals such as 1,4-butanediol, γ-butyrolactone, tetrahydrofuran, N-methyl-2-pyrrolidone, 2-pyrrolidone, succinimide, succinic esters, maleic acid/maleic anhydride among others (Song and Lee, 2006). Succinate and its derivatives (e.g. adipic acid and 1,4-butanediol) could be applied for the manufacture of biodegradable polymers (e.g. polyamides and polyesters). For instance, its market is expected to grow in the production of polybutylene succinate and polyurethanes such as polyethylene succinate.

The utilization of succinic acid as platform chemical necessitates its production at a cost around $1 per kg succinic acid as it is required for the production of commodity products by the chemical industry. Microbial bioconversion could lead

to cost-competitive production of succinic acid due to certain advantages including high carbon source to succinic acid theoretical conversion yield, significant reduction of greenhouse gas emissions and non-renewable energy consumption (Cok et al., 2014; Hermann et al., 2007) and high potential for CO_2 sequestration due to CO_2 fixation involved in the reductive TCA cycle leading to succinic acid production. The market potential and the advantages provided by bioprocessing have led to investment by several companies (Table 1.4.1) in the construction of industrial facilities for fermentative production of succinic acid with varying capacities (Jansen and van Gulik, 2014; Carus, 2012; Taylor et al., 2015). The current industrial activity for succinic acid production is currently positioned at a Technology Readiness Level of 8 with manufacturing facilities constructed in Europe and North America (Taylor et al., 2015). The market price of bio-based succinic acid is around $ 2.94 per kg, while the respective market price of both bio- and fossil-based succinic acid is around $ 2.5 per kg (Taylor et al., 2015). The annual production capacity of bio-based succinic acid in the period 2013-2014 was around 38,000 t that constitutes 49% of the total market (Taylor et al., 2015). The bio-based succinic acid market is expected to reach 600,000 t by 2020 with a projected market size of $ 539×10^6, but this is regarded as an optimistic scenario because a production cost of $ 1 per kg has been considered and the current production cost is much higher (Taylor et al., 2015).

Table 1.4.1: Industrial facilities for bio-based succinic acid production.

Company	Capacity	Start-up	Raw material	Fermentation/ Microorganism	Dowenstream recovery critical stage	Investment made in	Reference
BioAmber (DNP/ard)	3,000 t y^{-1} demo plant	2010	Wheat glucose	*E. coli*	Electrodialysis	Europe, Pomacle, France	Carus (2012), Taylor et al. (2015)
BioAmber, Mitsui	30,000-50,000 t y^{-1}	Under construction	Corn glucose	Low pH-value culture is targeted using *Candida krusei*	Direct succinic acid separation when low pH-value conditions are used	Sarnia, Ontario, Canada	Jansen and van Gulik (2014), Taylor et al. (2015)
BioAmber, Mitsui	70,000-200,000 t y^{-1}	Two plants to be contructed 2012	-	-	-	North America	Taylor et al. (2015)
Reverdia (joint venture between Roquette & DSM)	10, 000 t y^{-1}		Starch / sugars	Low pH-value culture is targeted by *S. cerevisiae*	Direct separation of the succinic acid	Cassano, Spinola, Italy	Jansen and van Gulik (2014), Taylor et al. (2015)
Myriant, ThyssenKrupp	1,000 t y^{-1}	2013	Glucose	*E. coli*	Ammonia precipitation	Leuna, Germany	Taylor et al. (2015)
Myriant	14,000 t y^{-1}	2013	Corn glucose	*E. coli*	Ammonia precipitation	Lake Providence, Louisiana, USA	Carus (2012), Jansen and van Gulik (2014), Taylor et al. (2015)
Succinity (joint venture between BASF & Corbion-Purac)	10,000 t y^{-1}	2013	Glycerol/ sugars	*B. succiniciproducens*	Magnesium hydroxide as neautralizer followed by recycling	Montmelo, Spain	Jansen and van Gulik (2014), Taylor et al. (2015)

Bioprocessing costs and environmental impact are highly dependent on the selection of the raw material, the upstream pre-treatment stages required to produce a nutrient-complete fermentation medium, the fermentation stage and the downstream separation and purification of succinic acid (especially in the case of high purity grade required for biopolymer formulation). All these stages are highly dependent on the microorganism and the fermentation conditions employed. Producing bulk bio-based platform chemicals will require the construction of industrial plants including several bioreactors with capacities in the scale of hundrents of cubic meters per bioreactor. Therefore, the use of facultative anaerobic microorganisms, as in the case of *A. succinogenes*, will reduce bioreactor costs due to the absence of aeration that increases significantly capital and operating costs. Therefore, the selection of the microorganism depends on both bioprocessing and physiological aspects (e.g. oxygen and specific nutrient requirements, flexibility in raw material utilization, optimum fermentation parameters).

The carbon sources used in current industrial fermentations constitute mainly purified sugars or glucose syrups from corn. The utilization of agricultural residues and industrial side streams is necessary in order to create a sustainable bio-based succinic acid production. Although succinic acid producing bacteria can directly assimilate some industrial by-product streams (e.g. cheese whey and cane molasses), other raw materials (e.g. lignocellulosic residues and starch-rich waste streams) cannot be readily consumed. In the latter case, optimum pre-treatment schemes should be developed increasing also the complexity of downstream separation stages due to the remaining nutrients in the fermentation broth. Therefore, the utilization of crude renewable resources will eventually lead to sustainable production of bio-based succinic acid only through refining of the original resource in an analogous manner that refining has been applied to corn and petroleum (Koutinas et al., 2014a). In this way, the production of value-added co-products will provide the profitability margin required for the development of sustainable bioprocesses.

1.4.3.1 Succinic acid producers

Table 1.4.2 presents information regarding fermentation efficiency and conditions reported for various succinic acid producing strains. Fermentative succinic acid production has been accomplished by both wild-type and genetically engineered strains. *A. succinogenes*, *B. succiniciproducens* and *M. succiniciproducens* are the most promising wild-type bacterial strains as they are capable of consuming numerous carbon sources, constituting facultative anaerobes achieving high fermentation efficiency and classified as biosafety level 1 microorganisms by DSMZ and ATCC. These strains have been isolated from the rumen. The highest succinate concentration (105.8 g L^{-1}) has been produced by *A. succinogenes* FZ53 mutant using glucose with a yield and productivity of 0.82 g_{SA} $g_{glucose}^{-1}$ and 1.36 g L^{-1} h^{-1}, respectively (Guettler et al., 1996). Contrary to other bacterial strains, *B. succiniciproducens* has not been studied to a great extent. Its metabolic fluxes have been investigated and two mutant strains have been developed (Becker et al., 2013).

Highly efficient genetically engineered *E. coli* strains have been constructed for succinic acid production, such as *E. coli* strain AFP111/pTrc99A–pyc that produced 99.2 g L^{-1} of succinic acid concentration in dual phase fermentations with the highest reported yield of 1.1 g_{SA} $g_{glucose}^{-1}$ and a productivity of 1.3 g L^{-1} h^{-1} (Vemuri et al., 2002). *C. glutamicum* ΔldhA-pCRA717 could be a promising microorganism for succinic acid production due to the high productivity (3.17 g L^{-1} h^{-1}), final concentration (146 g L h^{-1}) and yield (0.92 g_{SA} $g_{glucose}^{-1}$) achieved (Okino et al., 2008). Recent research focuses on the development of genetically engineered yeast strains that can produce succinic acid at low pH-value in order to reduce the unit operations in downstream separation and purification of succinic acid (Jansen and van Gulik, 2014; Van De Craaf et al., 2012).

The main advantages of *A. succinogenes* exploitation for succinic acid production include the utilization of numerous carbon sources, adequate tolerance to inhibitors and sufficient fermentation efficiency even with crude renewable resources. The main disadvantages lie on the fastidious nature of *A. succinogenes* for nitrogen sources (e.g. yeast extract) and vitamins (e.g. biotin), the near neutral optimum pH-value required and the limited genetic engineering tools for its genetic

manipulation. The implementation of succinic acid production by *A. succinogenes* in integrated biorefineries using complex renewable resources would provide nutrient-complete fermentation media at lower cost than commercial nutrient sources. The use of neutralizers and the neutral pH-value of the fermentation broth resulting from *A. succinogenes* cultures affect both fermentation and downstream separation costs. Lower pH-values of the fermentation broth reduce the downstream separation cost as the pH-value affects the dissociation level of succinic acid (pKa_1 = 4.16 and pKa_2 = 5.6) (Jansen and van Gulik, 2014).

Table 1.4.2: Fermentation efficiency and conditions for different succinic acid producing strains.

Fermentation parameters	*A. succinogenes* FZ53	*M. succiniciproducens* LPK7	*B. succiniciproducens* JF4016	*E. coli* AFP 111/pTrc99A-pyc	*E. coli* KJ060	*C. glutamicum* ΔldhA-pCRA717	*S. cerevisiae* SUC-297	*Y. lipolytica* Y-3314
Carbon source	Glucose	Glucose	Glucose	Glucose	Glucose	Glucose	Glucose	Glycerol
Nutrient sources (g L^{-1})	Yeast extract (5-15), corn steep liquor (10-15), vitamins	Yeast extract (5)	Yeast extract (5), peptone (5), vitamins	Yeast extract (10), Tryptone (20), biotin, thiamine	$(NH_4)_2HPO_4$, $NH_4H_2PO_4$, thiamine, betaine	Urea (2), yeast extract (2), casamino acid (7), $(NH_4)_2SO_4$ (7), biotin, thiamine	$(NH_4)_2SO_4$, vitamins	Yeast extract (10), Peptone (10), uracil, leucine
Gas supply	CO_2 (0.05-0.1 vvm)	CO_2 (0.25 vvm)	CO_2 atmosphere	Dual-phase cultures	Anaerobic environment	Oxygen deprivation	Air and CO_2	Aerobic shake flasks
pH-value	7.2-6	6.5	nk[a]	7	7	nk[a]	5	6.8-5.8
Neutralisers	$MgCO_3$, $Mg(OH)_2$	Ammonia solution	$MgCO_3$	NaOH, HCl	$NaHCO_3$, KOH, K_2CO_3	NaOH, bicarbonate	KOH	$CaCO_3$
Yield (g $g_{glucose}^{-1}$)	0.82	0.76	0.49	1.1	0.92	0.92	nk	0.36
Productivity (g L^{-1} h^{-1})	1.36	1.8	0.53	1.3	0.9	3.17	0.45	0.27
SA (g L^{-1})	105.8	52.4	20	99.2	86.6	146	43	45.5
By-products[b]	AA: PA: Pyr	MA: Pyr	FA: AA: LA: Eth	AA: Eth	MA: AA: LA	AA: LA: Mal: Pyr	Eth: Gly: MA	nk[a]
Reference	Guettler et al. (1996)	Lee et al. (2006)	Becker et al. (2013)	Vemuri et al. (2002)	Jantama et al. (2008)	Okino et al. (2008)	Van De Graaf et al. (2012)	Yuzbashev et al. (2010)

[a] nk: not known.

[b] AA: acetic acid, FA: formic acid, Pyr: pyruvic acid, PA: propionic acid, LA: lactic acid, MA: malic acid, Eth: ethanol, Cly: glycerol.

1.4.3.2 Succinic acid production by A. succinogenes

Actinobacillus succinogenes is a Gram-negative, facultative anaerobic, non-motile, non-spore forming, capnophilic, pleomorphic rod, which was isolated from bovine rumen (Guettler et al., 1999) and taxonomically was placed in the *Pasteurellaceae* family, based on 16S rRNA amplification. Its taxonomical order has been formed as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; *Pasteurellaceae*; *Actinobacillus succinogenes*. *A. succinogenes* is mesophilic and grows well at 37-39 ^{o}C in chemically defined media. The microorganism is capable of consuming a wide range of C5 and C6 sugars as well as various disaccharides and other carbon sources, such as glucose, xylose, arabinose, mannose, galactose, fructose, sucrose, lactose, cellobiose, mannitol, maltose and glycerol (Carvalho et al., 2014; Wan et al., 2008; Zheng et al., 2009; Li et al., 2010b; Jiang et al., 2013; Jiang et al., 2014). The use of more reduced carbon sources than glucose, such as sorbitol, glycerol and mannitol, results in higher succinic acid yields. However, the utilization of C5 sugars, such as xylose and arabinose, results in lower succinate yields (Li et al., 2010b).

Various industrial waste and by-product streams (e.g. sugar cane molasses, cheese whey, crude glycerol from biodiesel production, wheat milling by-products, sake lees) and agricultural residues (e.g. corn fiber and corncob, sugarcane bagasse, bio-waste cotton) have been evaluated for the production of succinic acid mainly by *A. succinogenes* (Table 1.4.3). Production of succinic acid requires significant quantities of complex nitrogen sources such as yeast extract. The reduction of succinic acid production cost necessitates the utilization of low-cost nitrogen sources supplied either by separate renewable resources, such as corn steep liquor (CSL), or by the same renewable resource that also provides the carbon source (e.g. wheat milling by-products, waste bread). For instance, the use of whey as carbon source achieved succinic acid yield of 0.72 g_{SA} $g_{lactose}^{-1}$ in the presence of yeast extract, which was only slightly reduced (0.71 g_{SA} $g_{lactose}^{-1}$) in the presence of CSL (Lee et al., 2003). The utilization of agroindustrial waste and by-product streams may also supply other nutrients, such as minerals and vitamins.

Sugar cane molasses has been employed, after pretreatment with sulfuric acid, in fed-batch cultures for succinic acid production by *A. succinogenes* CGMCC1593 leading to the production of 55.2 g L^{-1} at a productivity of 1.15 g L^{-1} h^{-1} (Liu et al., 2008a). Shen et al. (2014) identified the optimum concentrations of total sugars of cane molasses (85 g L^{-1}), yeast extract (8.8 g L^{-1}), and $MgCO_3$ (63.1 g L^{-1}) that led to the production of 64.3 g L^{-1} of succinic acid concentration at 60 h fed-batch fermentation (Shen et al., 2014). As a comparison, the succinic acid concentration (37.3 g L^{-1} and 55.8 g L^{-1}) and productivity (1.04 g L^{-1} h^{-1} and 0.77 g L^{-1} h^{-1}) achieved by genetically engineered *E. coli* strains were approximately in the same range (Ma et al., 2014; Chan et al., 2012b).

Crude glycerol is a highly promising industrial by-product stream for succinic acid production because glycerol is a more reduced carbon source than C5 and C6 sugars. Vlysidis et al. (2011) reported the production of 26.7 g L^{-1} of succinate concentration at a yield and productivity of 0.96 g g^{-1} and 0.23 g L^{-1} h^{-1}, respectively. Limited glycerol consumption during cell growth by *A. succinogenes* could be improved by the supplementation of external electron acceptors such as dimethylsulfoxide that led to the production of 49.6 g L^{-1} of succinic acid concentration with a productivity of 0.96 g L^{-1} h^{-1} and a yield of 0.64 g_{SA} $g_{glycerol}^{-1}$ in fed-batch cultivation (Cavalho et al., 2014).

Wheat milling by-products have been utilised for the production of succinic acid employing a two-stage bioprocess (Dorado et al., 2009). Initially, amylolytic and proteolytic enzymes were produced via solid state fermentations on bran-rich wheat milling streams using the fungal strains *A. awamori* and *A. oryzae*. Crude fermented solids were subsequently used to hydrolyse the starch and protein contained in wheat milling by-products. The hydrolyzates were used as the sole fermentation feedstock for the production of 50.6 g L^{-1} succinic acid using the strain *A. succinogenes.* The utilization of 20% (v/v) inoculum at the beginning of fermentation led to the production of 62.1 g L^{-1} succinic acid.

Enzymatic hydrolyzates of sake lees (pretreated with 0.5% sulfuric acid) supplemented with 2.5 g L^{-1} of yeast extract and 0.2 mg L^{-1} biotin led to the production of 36.3 g L^{-1} succinic acid with a productivity of 1.21 g L^{-1} h^{-1} and a yield of 0.59 g_{SA} $g_{glucose}^{-1}$ (Chen et al., 2012).

Lignocellulosic biomass has also been used for succinic acid production. Pretreatment of lignocellulosic biomass should be carried out via combined thermo-chemical and enzymatic treatment in order to produce C5 and C6 sugars. Chen et al. (2010) utilized corn fiber (containing 31.6% hemicellulose, 21.7% cellulose and 15.4% starch) hydrolyzates produced via sulfuric acid pretreatment followed by $CaCO_3$ neutralization and activated carbon adsorption (targeting the removal of furfural) for the production of 35.4 g L^{-1} succinic acid with a yield of 0.72 g g_{sugars}^{-1} and a productivity of 0.98 g L^{-1} h^{-1} using the strain *A. succinogenes* NJ113 (Chen et al., 2010). Hydrolyzates from waste corn-cob produced via dilute acid pretreatment were used as xylose and arabinose rich media (constituting around 90% of total sugars) supplemented with yeast extract (11 g L^{-1}) and $MgCO_3$ (38 g L^{-1}) as neutralization agent for the production of 23.6 g L^{-1} succinic acid with a yield of 0.58 g_{SA} per g consumed sugars and a productivity of 0.49 g L^{-1} h^{-1} (Yu et al., 2010).

Cereal straws are abundant renewable resources with 35-45% cellulose, 20-30% hemicelluloses and 8-15% lignin (Zheng et al., 2009). Corn straw hydrolyzate has been demonstrated to be more efficient for the production of succinic acid compared to hydrolyzates derived from rice and wheat straw (Zheng et al., 2009). Fed-batch fermentation with the strain *A. succinogenes* CGMCC1593 cultivated on corn straw hydrolyzates, rich mainly in glucose and xylose, produced by combined alkali pretreatment followed by enzymatic hydrolysis led to the production of 53.2 g L^{-1} of succinic acid concentration with a yield of 0.82 g g^{-1} and a productivity of 1.21 g L^{-1} h^{-1} (Zheng et al., 2009). Cotton stalks pretreated by steam explosion followed by $NaOH/H_2O_2$ treatment were employed in simultaneous saccharification and fermentation at 40 ^oC for the production of succinic acid (63 g L^{-1}) by *A. succinogenes* 130Z with a productivity of 1.17 g L^{-1} h^{-1} and a conversion yield of 0.64 g g^{-1} (Li et al., 2013).

Sugarcane bagasse hydrolyzates containing glucose (8 g L^{-1}), arabinose (5 g L^{-1}), xylose (4 g L^{-1}) and cellobiose (25.7 g L^{-1}) led to complete consumption of all sugars and production of 20 g L^{-1} of succinic acid concentration with a yield of 0.65 g g^{-1} and a productivity of 0.61 g L^{-1} h^{-1} using the strain *A. succinogenes* NJ113 (Jiang et al., 2013). Ultrasonic pretreatment of sugarcane bagasse followed by

hydrolysis with dilute acid led to the production of 23.7 g L^{-1} of succinic acid concentration with a yield and productivity of 0.79 g g^{-1} and 0.99 g L^{-1} h^{-1}, respectively (Chen et al., 2012).

Chen et al. (2011) demonstrated that rapeseed meal could be employed as a renewable resource for succinic acid production providing both carbon and nitrogen sources. The rapeseed meal hydrolyzate was produced via pretreatment with dilute sulfuric acid followed by hydrolysis using a commercial pectinase preparation. Fed-batch fermentations with *A. succinogenes* ATCC 55618 were carried out with simultaneous saccharification using a pectinase formulation leading to the production of a succinic acid concentration of 23.4 g L^{-1} with a yield of 0.115 g_{SA} g_{dm}^{-1} and a productivity of 0.33 g L^{-1} h^{-1} (Chen et al., 2011).

The pulp and paper industry produce significant quantities of spent liquors that contain high concentrations of sugars derived mainly from hemicellulose degradation. Alexandri et al. (2016) reported the production of succinic acid by *A. succinogenes* and *B. succiniciproducens* in batch cultures using crude and pretreated spent sulphite liquor produced by the sulphite pulping process. The spent sulphite liquor contains predominantly xylose with lower quantities of galactose, glucose, mannose and arabinose. Besides *A. succinogenes*, *E. coli* AFP 184 can also consume xylose (Donnelly et al., 2003). When this strain was cultivated in diluted spent sulphite liquor in dual phase fermentations, where aeration was required for cell growth in the first phase and CO_2 supply was required for the production of succinic acid in the second phase, a succinic acid concentration of 5.2 g L^{-1} was produced from 13.9 g L^{-1} total sugars (Pateraki et al., 2016).

Waste and by-product streams from the food-industry could be employed for the production of succinic acid. Around 47.3 g L^{-1} of succinic acid with productivity of 1.12 g L^{-1} h^{-1} were produced by *A. succinogenes* cultivated on waste bread hydrolyzates produced via hydrolysis of starch and protein contained in waste bread by crude enzymes produced via solid state fermentation (Leung et al., 2012). Spent yeast from breweries and wineries could be employed for nitrogen and other nutrient supplementation after autolysis or enzymatic hydrolysis in order to release the intracellular nutrients. Jiang et al. (2010) reported that spent brewer's yeast hydrolyzate supplemented with vitamins could successfully replace the addition of

15 g L^{-1} of yeast extract, resulting in the production of 46.8 g L^{-1} of succinic acid concentration with a yield of 0.69 g_{SA} $g_{glucose}^{-1}$. Besides spent yeast, the corn steep liquor derived from corn refining could be employed as nutrient-rich supplement (Xi et al., 2013).

The high carbohydrate content of macroalgae (up to 60% dry matter) could be used for succinic acid production. Fermentation of algal hydrolyzates (Morales et al., 2015), containing around 45 g L^{-1} and 7.5 g L^{-1} of glucose and mannitol respectively, with *A. succinogenes* 130Z resulted in 33.78 g L^{-1} of succinic acid with a yield of 0.63 g_{SA} g_{tsc}^{-1} and a productivity of 1.5 g L^{-1} h^{-1}.

Table 1.4.3: Bio-based succinic acid production in fermentations utilizing different raw materials and microbial strains.

Carbon source	Strain	Nitrogen-nutrient source (g L^{-1})	Type of fermentation, working volume	SA Concen. (g L^{-1})	SA productivity (g L^{-1} h^{-1})	Yield (g_{SA} g_{tsc}^{-1})	SA: LA: FA: AA (mol mol^{-1})*	Reference
Representative succinic acid production from pure carbon sources by *A. succinogenes*								
Glucose	*A. succinogenes* 130Z	YE (6)/CSL (10)	CO_2 sparging, continuous, 0.158 L	48.5	nd	0.84	1:0:0:0.38	Bradfield and Nicol (2014)
Glucose	*A. succinogenes* CGMCC 1593	YE (10)/CSL (6)/Vit	CO_2 sparging, fed-batch, bioreactor 3L	60.2	1.3	0.75	1:0: 0.13:0.31	Liu et al. (2008b)
Glycerol	*A. succinogenes* 130Z	YE (5-10)/Vit	CO_2 sparging, batch, bottle reactors, 0.07 L	26.7	0.23	0.96	1:0:0.15:0.14	Vlysidis et al. (2011)
Glycerol	*A. succinogenes* 130Z	YE (10)	CO_2 sparging, fed-batch, bioreactor, 1.5 L	49.6	0.62	0.92	1:0:0.39:0.16	Carvalho et al. (2014)
Sucrose	*A. succinogenes* NJ113	YE (10)/CSL (5)	CO_2 sparging, fed-batch, bioreactor, 1.5 L	60.4	2.16	0.72	1:0:0.55:0.29	Jiang et al., 2014
Cellobiose	*A. succinogenes* NJ113	YE (10)/CSL (5)	CO_2 sparging, batch, bottles 0.03 L	38.9	1.08	0.66	1:0:0:0.69	Jiang et al., 2013
Representative succinic acid production from crude renewable resources by *A. succinogenes*								
Corn fiber	*A. succinogenes* FZ6 (mutant)	YE (10)/Biotin (µg)	CO_2 sparging, batch, vials 0.01 L	70.6	0.70	0.88	1:0:0.01:0.08:f	Guettler et al. (1996)
	A. succinogenes NJ113	YE (10)/CSL (5)	CO_2 sparging, batch, bioreactor, 4.5 L	35.4	0.98	0.72	nd	Chen et al. (2010)
Corncob	*A. succinogenes* CICC 11014	YE (11)	CO_2 sparging, batch anaerobic bottles, 0.025 mL	23.6	0.49	0.58	nd	Yu et al. (2010)
Corn stover	*A. succinogenes* CGMCC 1593	CSL (20)	CO_2 sparging, batch SSF[a], bioreactor, 2 L	47.4	0.99	0.72[c]	1:0.06:0.06:0.44	Zheng et al. (2010)
Corn straw	*A. succinogenes* CGMCC 1593	YE (15)	CO_2 sparging, batch, bioreactor, n	53.2	1.21	0.82	1:0:0:0.22	Zheng et al. (2009)
Corn stalk	*A. succinogenes* CGMCC 2650 or BE-1	YE (30)/Urea (2)	CO_2 sparging, batch, nd	17.8	0.56	0.66	nd	Li et al. (2010c)
Wheat milling by-products	*A. succinogenes* 130Z	YE (2.5)	CO_2 sparging, batch, bioreactor, 0.5 L	62.1	0.91	1.02	nd	Dorado et al. (2009)
Waste Bread	*A. succinogenes* 130Z	Bread hydrolyzate (200 mg L^{-1} free amino nitrogen)	CO_2 sparging, batch, bioreactor, nd	47.3	1.12	nd	nd	Leung et al. (2012)
Cotton stalk	*A. succinogenes* 130Z	YE (30)/Urea (2)	CO_2 sparging, batch SSF[b], flasks, nd	63	1.17	0.64	nd	Li et al. (2013)
Cane molasses	*A. succinogenes* CGMCC 1593	YE (10)	CO_2 sparging, fed-batch, bioreactor, nd	55.2	1.15	nd	1:0:0.16:0.32	Liu et al. (2008b)
Cane molasses	*A. succinogenes* GXAS137	YE (8.8)	CO_2 sparging, fed-batch, bioreactor, 0,8 L	64.3	1.07	0.76	1:0:0:0.39	Shen et al. (2014)

Sugarcane bagasse cellulose	*A. succinogenes* NJ113	YE (10)/CSL (5)	CO_2 sparging, batch, bioreactor, 1.5 L	20	0.61	0.65	1:0:0:1.28	Jiang et al. (2013)
Sugar cane bagasse	*A. succinogenes* NJ113	YE (10)/CSL (5)	CO_2 sparging, batch, bioreactor, 1.5 L	23.7	0.99	0.79	1:0:0:0.37	Xi et al. (2013)
Sugarcane bagasse	*A. succinogenes* CIP 106512	YE (2)	CO_2 sparging, batch, bioreactor, 1.5 L	22.5	1.01	0.43	nd	Borges and Pereira Jr. (2011)
Macroalgal hydrolyzate	*A. succinogenes* 130Z	YE (16.7)	CO_2 sparging, batch, bioreactor, 1.5 L	33.0	1.27	0.75	1:0.18:0.28:0.54:g	Morales et al. (2008)
Rapeseed meal	*A. succinogenes* 130 Z	YE (15)	CO_2 sparging, fed-batch SSF[b], bioreactor, 1.2 L	23.4	0.33	0.115[d]	1:0:0:0.71	Chen et al. (2011)
Whey	*A. succinogenes* 130Z	YE (5)/Pep (10)	CO_2 sparging, batch, bioreactor, 1.2L	21.3	0.43[e]	0.44	1:0.02:0.68:0.78:h	Wan et al. (2008)
Sake lees hydrolyzate	*A. succinogenes* 130Z	SLH/YE/biot	CO_2 sparging, batch, bioreactor, 1.5L	52.3	1.74	0.85	1:0:0:0.30	Chen et al. (2012)
Representative succinic acid production from pure carbon sources by various strains								
Glucose	*A. succiniciproducens* ATCC 53488	YE (5)/Pep (10)/$(NH_4)_2SO_4$ (5)	CO_2 sparging, batch, bioreactor, nd	32.2	1.19	0.90	1:0:0:0.52	Nghiem et al. (1997)
Galactose	*A. succiniciproducens* ATCC 29305	YE (2.5)/Pep (2.5)/$(NH_4)_2SO_4$ (5)	CO_2 sparging, batch, bioreactor, 1 L	15.3	1.46	0.90	1:0:0:0.60	Lee et al. (2008)
Glucose				45.4	2.84	0.92	1:0:0:0.24	
Xylose	*E. coli* AFP184	CSL (33)/$(NH_4)_2SO_4$ (3)	Dual phase, batch, bioreactor, 8 L	29.2	1.79	0.69	1:0:0:0.45	Berglund et al. (2007)
Fructose				27.7	1.54	0.46	1:0:0:0.34	
Glucose	*E. coli* AFP111	$(NH_4)2HPO_4$ (8)/NH_4Cl (0.2)/$(NH_4)_2SO_4$ (0.8)/Vit	Dual phase, fed-batch, bioreactor, 3 L	101	1.18	0.78	1:0:0:0.07	Ma et al. (2011)
Representative succinic acid production from crude renewable resources by various strains								
Corn stalk	*E. coli* SD121	YE (10)/Tryp (20)/ $(NH_4)_2SO_4{\cdot}7H_2O$(3)	Dual phase, batch, bioreactor, 1L	57.8	0.96	0.87	1:0:0:0.29:i	Wang et al. (2011)
	A. succiniciproducens ATCC 29305	CSL (20)/Tryptophane (0.02)	CO_2 sparging, batch, bioreactor, nd	34.7	1.02	0.91	nd	Samuelov et al. (1999)
	A. succiniciproducens ATCC 29305	CSL (20)/Tryptophane (0.02)	CO_2 sparging, batch, bioreactor, nd	19.8	3	0.64	nd	
Whey	*M. succiniciproducens* MBEL55E	CSL (7.5)	CO_2 sparging, batch, bioreactor, 1L	13.4	1.18	0.71	1:0.06:1.10:0.73	
	M. succiniciproducens MBEL55E	YE (2.5)	CO_2 sparging, batch, bioreactor, 1L	13.5	1.21	0.72	1:0.05:1.11:0.74	Lee et al. (2003)
	M. succiniciproducens MBEL55E	CSL (5)	CO_2 sparging, batch, bioreactor, 0.5L	10[e]	3.9[e]	0.69[e]	1:0:0.80:0.79	
Cane molasses	*E. coli* AFP111/pTrcC-cscA	$(NH_4)2HPO_4$ (8)/NH_4Cl (0.2)/$(NH_4)_2SO_4$ (0.7)/Vit	Dual phase, fed-batch, bioreactor, 1.5 L	37.3	1.04	0.79	1:0:0:0.17:j	Ma et al. (2014)
Cane molasses	*E. coli* KJ122-pKJSUC-24T	$(NH_4)_2HPO_4$ (19.9)/$NH_4H_2PO_4$ (7.5)/Vit	CO_2 sparging, batch, bioreactor, 7.5 L	55.8	0.77	0.96	1:0:0:0.18	Chan et al. (2012b)

Softwood hydrolyzate	*E. coli* AFP184	YE (15)/CSL (15) / $(NH_4)_2SO_4$ (3.3)	Dual phase, batch, bioreactor, 0.7 L	42.2	1.00	0.72	nd	Hodge et al. (2009)
Pre-treated wood hydrolyzate	*M. succiniciproducens* MBEL55E	YE (5)	CO_2 sparging, batch, bioreactor, 1 L	11.73	1.17	0.56	1:0.23:0.45:0.59	Kim et al. (2004)
Pre-treated wood hydrolyzate	*M. succiniciproducens* MBEL55E	YE (5)	CO_2 sparging, batch, bioreactor, 0.5 L	7.98	3.19	0.55	nd	

Nitrogen Source: YE: Yeast extract, CSL: Corn steep liquor, Tryp: Tryptone, Pep: Peptone, Vit: Vitamin supplementation.
nd: No data.

[a] mol mol^{-1} ratio of fermentation by-products SA: Succinic acid, LA: Lactic acid, FA: Formic acid, AA: Acetic acid.

[b] Simultaneous saccharification and fermentation.

[c] Yield: $g_{SA}\ g_{substrate}^{-1}$.

[d] Yield: $g_{SA}\ g_{dm}^{-1}$.

[e] Maximum value observed during continuous fermentation at different dilution rates.

[f] Also propionic acid (3 g L^{-1}).

[g] Also ethanol: (2.5 g L^{-1}).

[h] Also ethanol (3 g L^{-1}).

[i] Also ethanol (1.62 g L^{-1}).

[j] Also pyruvic acid (1.2 g L^{-1}).

1.5 Biofuels

The global climate change associated to the extensile release of greenhouse gases, has raised concerns about the application of fossilized hydrocarbons as the main energy source (Liao et al., 2016). If the current upward trend continues, oil requirements are expected to increase from 60% to 75% by 2030 (Abo et al., 2019), while CO_2 emissions could double by 2050. This situation would lead to a global warming of approximately 2 ^oC above the level that existed in 1900 (Liao et al., 2016). A range of negative effects could be caused if this extent of temperature increase persisted such as the disintegration of the West Antarctic Ice Sheet (WAIS) (O'Neill and Oppenheimer, 2002). In line with the above, Abo et al. (2019) highlighted that *"The European Commission has set an example by setting a target of 20% renewable energy in total energy consumption by 2020 and expects a significant contribution of biofuels to the achievement of this objective"*. Therefore, in recent years, exploitation of new renewable resources for production of biofuels as a replacement to the use of the nonrenewable source of petroleum has demonstrated manifold global research interest (Jiang et al., 2018). The ability of microorganisms to use renewable resources for biofuel synthesis is exploitered by the current industry manufacturing biofuels (e.g. bioethanol, biomethane, biobutanol) mainly from sugarcane, corn and wheat (Mohanty and Swain, 2019).

1.5.1 Bioethanol production from bioresources

The total fuel ethanol production in major countries and regions in 2018 reached 28.7 billion gallons, out of which 16.1 billion gallons were produced safely in the United States (Statista, 2018). The industrial production of ethanol is currently performed by catalytic hydration of ethylene (chemical method) (Mohsenzadeh et al., 2017) and by fermenting agricultural feedstocks (biochemical method) (Mohanty and Swain, 2019). Ethanol can serve as a green energy source, which is mainly produced using starch, sugar and carbohydrates, such as corn, potato, molasses, sugarcane and lignocellulosic biomass (Lin and Tanaka, 2006). Sugars can be directly converted to ethanol, while starchy and cellulosic materials should be first pretreated mainly using enzymes or chemicals to hydrolyze the polymers

into sugars (Taghizadeh-Alisaraei et al., 2017). Thus, ethanol production can proceed in three distinct ways. First generation ethanol includes the production of biofuel from raw materials considered as food crops such as sugarcane, corn and wheat, second generation incorporates ethanol production from non-food biomass (plant or animal waste) and third generation refers to the production of the biofuel from algal biomass (Baeyens et al., 2015).

Ethanol obtained from a fermentation process requires further separation and purification. Fractional distillation is employed to separated ethanol from fermentation broth based on the volatilities of the different components entailed. Most large-scale industries and biorefineries use a continuous distillation column system (Limayem and Ricke, 2012). During the industrial fermentation process various stress conditions for the yeast can be present, such as high temperature, high ethanol concentration, osmotic stress as well as bacterial contamination (Vohra et al., 2014). Thus, the main challenges of ethanol production constitute the selection of a strain adapted to the stress conditions mentioned above.

A few microorganisms demonstrated resistance to various stresses present during ethanol processing. *S. cerevisiae* and *Z. mobilis* could be commonly used to convert C6 sugars to the biofuel with high ethanol tolerance. However, both strains were unable to ferment C5 sugars. Although *C. shehatae* and *P. stiplis* offered the potential to ferment C5 sugars into ethanol, the strains achieved low product yield and ethanol tolerance, while *P. tannophilus* and *E. coli* were capable of converting both C5 and C6 sugars. *K. marxianus* constitutes a thermophilic yeast which is capable of fermenting a broad spectrum of sugars. However, high concentration of sugars affects the product yield, while low ethanol tolerance and yield on xylose fermentation significantly reduce the performance of the strain. As far as the production of ethanol using ethanologenic bacteria is concerned, thermophilic strains (e.g. *T. sacchaarolyticum*, *T. ethanolicus*, *C. thermocellum*) constitute extreme microorganisms, which can be resistant to extremely high temperatures (e.g. 70 ^{o}C) fermenting a variety of sugars. However, these bacteria are known to include low tolerance to ethanol (Baeyens et al., 2015).

In recent years, research on ethanol production from biowaste has become very popular. CPW constitutes a cellulosic material that requires physicochemical and/or

biochemical pretreatment for ethanol fermentation. Pretreatment of CPW prior to the bioprocess usually requires the removal of essential oils due to the antimicrobial properties of D-limonene that may cause inhibition of the biosystem. Although the removal of D-limonene constitutes CPW a relatively expensive raw material, the alternative use of a microorganism less susceptible to D-limonene could reduce the fermentation cost. Thus, the effect of orange peel oil on alcoholic fermentations of hydrolyzates derived from CPW has been investigated with the use of *K. marxianus* (Wilkins et al., 2007c), *Z. mobilis* (Wilkins, 2009), *Rhizopus sp.*, *M. indicus* (Lennartsson et al., 2012) as well as using the newly isolated thermotolerant yeast *P. kudriavzevvi* KVMP10 (Koutinas et al., 2016a). In most cases, increasing concentrations of D-limonene caused reduction in ethanol concentration and increase in the duration of the lag phase. However, *M. indicus* was the strain less susceptible to inhibition and it was capable of producing 20 g L^{-1} of ethanol in the presence of 1-2% (v/v) D-limonene.

Previous research has demonstrated a variety of approaches for the pretreatment of CPW followed by fermentation for ethanol production by several microorganisms. Ethanol production was investigated in a citrus peel derived hydrolyzate following steam explosion using *S. cerevisiae,* which yielded a final ethanol concentration of 39 g L^{-1} (Wilkins et al., 2007b). The hydrolyzate generated following dilute-acid hydrolysis and pectin recovery was used in fermentations of the same strain where the production of ethanol reached 40 L $t_{\text{raw material(rm)}}^{-1}$ (Pourbafrani et al., 2010). Furthermore, a hydrolyzate generated from mandarin waste was tested for ethanol production by *S. cerevisiae* following hydrothermal sterilization and the concentration of ethanol reached 42 g L^{-1} (Oberoi et al., 2011). However, when enzymatic hydrolysis was employed using a mixture of cellulase, β-glucosidase and pectinase, the production of 34 g L^{-1} of ethanol was achieved in *P. kudriavzevii* fermentations (Sandhu et al., 2012). Mandarin and lemon waste have been pretreated through steam explosion, while the hydrolyzate formed produced 60 L t_{rm}^{-1} and 68 L t_{rm}^{-1} of ethanol in *S. cerevisiae* cultures respectively (Boluda-Aguilar et al., 2010; Boluda-Aguilar and Lopez-Gomez, 2013). Popping and enzymatic hydrolysis has been investigated for mandarin waste pretreatment producing 46 g L^{-1} of ethanol in *S. cerevisiae* fermentations (Choi et al., 2013).

Furthermore, the production of ethanol has been evaluated using *M. indicus* in an enzymatically derived hydrolyzate of OPW where ethanol concentration reached 15 g L^{-1} (Lennartsson et al., 2012). The capacity of *P. kudriavzevii* to ferment the hydrolyzates generated at 40-42 ^{o}C as compared to *S. cerevisiae* and *M. indicus*, which were used for ethanol production at a range of temperatures between 30-37 ^{o}C constitutes *P. kudriavzevii* a promising strain for ethanol production from the specific waste. Moreover, it is evident that the combination of different pretreatments employed for hydrolyzate generation constitutes a crucial step for the release of fermentable substrates affecting the productivity of the bioprocess.

1.5.1.1 *P. kudriavzevii* KVMP10 as a yeast for ethanol production

Pichia kudriavzevii KVMP10 is a thermotolerant yeast, which was isolated from soil beneath an apple tree (Koutinas et al., 2016a). Based on the phylogenetic and physiological characteristics, the strain was designated as *P. kudriavzevii* KVMP10 and the internal transcribed spacer (ITS) sequence was deposited in GenBank (accession number KP690977). Thus, a phylogenetic tree based on ITS was constructed (Figure 1.5.1) with the use of the multiple alignment software CLUSTALW (MEGA 5.05) (Thompson et al., 1994) aiming to correlate *P. kudriavzevii* KVMP10, with other *P. kudriavzevii*, *K. marxianus* and *Saccharomyces* sp. that shared ITS gene sequence similarities. *K. marxianus* NBRC 1777 and *Saccharomyces* sp. KCH were used as outgroup. As shown on the phylogenetic tree the isolated strain exhibited close similarity to *P. kudriavzevii* d89a (accession number KP674621) and *P. kudriavzevii* B-WHX-12-12 (accession number KC756946.1). Similarly to strain KVMP10, *P. kudriavzevii* B-WHX-12-12 was isolated from apple orchards in China and demonstrated osmotolerant characteristics (unpublished work, NCBI). Furthermore, *P. kudriavzevii* TY11 (KC905770.1), which only slightly varied from the isolated KVMP10 (Figure 1.5.1), has also demonstrated significant thermotolerance and ethanol productivity (unpublished work, NCBI). *P. kudriavzevii* was previously named as *I. orientalis* and it has been characterized as robust and multistress-tolerant yeast, resisting low pH-values, elevated temperatures and salt contents (Toivari et al., 2013).

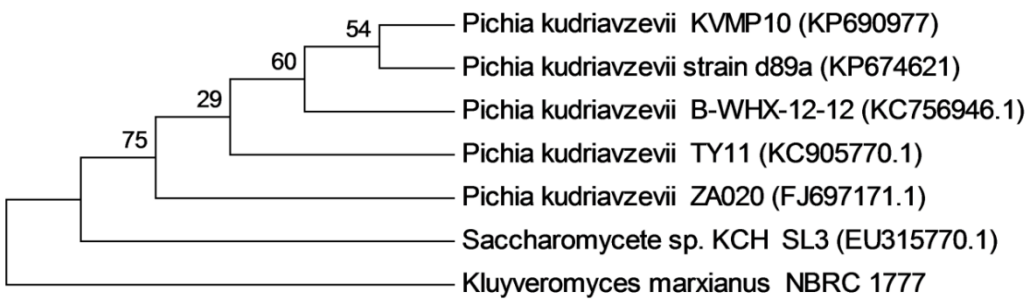

Figure 1.5.1: Phylogenetic tree of *P. kudriavzevii* KVMP10 obtained by neighbor-joining analysis of ITS sequences, showing the position among different *P. kudriavzevii*, *K. marxianus* and *Saccharomycete sp*. strains. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points.

P. kudriavzevii strains have been previously tested for ethanol production using different lignocellulosic materials. Ethanol production from alkali- and ozone-treated cotton stalks was investigated with the use of *P. kudriavzevii* HOP-01 obtaining biofuel concentrations of 19.82 g L^{-1} and 10.96 g L^{-1} respectively (Kaur et al., 2012). Alkali-treated rice straw was also evaluated for ethanol production using the same strain and ethanol formation reached 24.25 g L^{-1} (Oberoi et al., 2012). A cassava starch hydrolyzate was tested using *P. kudriavzevii* DMKU 3-ET15, which was capable of generating ethanol at high temperatures that reached 45 ^{o}C (Yuangsaard et al., 2013). Furthermore, *P. kudriavzevii* constitutes an osmotolerant yeast as confirmed by the capacity of *P. kudriavzevii* ITV-S42 to ferment up to 200 g L^{-1} of initial glucose concentration (Diaz-Nava et al., 2017).

1.5.2 Methane production from waste and renewable resources

Methane comprises the main component of biogas, which is produced from biomass through the process of anaerobic decomposition in municipal solid waste landfills or under controlled conditions via anaerobic digestion processes. The composition of biogas varies between 40-60% methane and 60-40% carbon dioxide, containing small amounts of hydrogen sulfide (Kapdi et al., 2005). The Clean Air Act regulation of the United States demands a specific size of municipal solid waste landfills to be equipped with a control system for landfill gas collection. Various methods exist for the management of landfill gas. Some landfills reduce emissions through capturing and burning of the gas, which generates mainly CO_2.

Another practice that various landfills follow includes collection of the gas followed by treatment for the removal of CO_2, hydrogen sulfide and water vapor, selling the methane produced to sites that use the biofuel for electricity production. The U.S. Energy Information Administration (EIA) estimated that in 2017 about 8 billion m^3 of landfill gas was collected at 370 U.S. landfills. The gas was burnt to generate about 11.5 billion kWh of electricity, which corresponds to 0.3% of the total U.S. utility-scale electricity generation in 2017 (EIA, 2019). Many industries such as paper mills as well as food processors use anaerobic digesters as part of their waste treatment processes. These industries use the biogas produced to heat the digesters enhancing the anaerobic process or generate electricity, which is either commercialized or applied at the facility. A total of 1.2 billion kWh of electricity was estimated by EIA to be produced by 107 of the aforementioned waste treatment facilities in the U.S. during 2017. Furthermore, approximately 116 million kWh of electricity was produced from biogas in nine large dairy industries in the U.S., where the methane produced could be applied as a fuel in generators for farming needs or burnt to provide heating water for buildings (EIA, 2019).

A wide variety of materials has been used for methane production. Lignocellulosic waste, municipal solid waste, food waste, livestock manure as well as waste activated sludge were applied for the generation of methane under thermophilic and mesophilic conditions (Mao et al., 2015). Lignocellulosic residues mainly include crop waste, which cannot be easily digested due to the presence of recalcitrant components such as lignin and cellulose. Thus, in most cases a pretreatment method should be applied prior to the use of anaerobic digestion for the treatment of the specific materials.

CPW consist a lignocellulosic material with low percentage of lignin (Lopez et al., 2010). However, the effect of D-limonene was evaluated during anaerobic digestion of the waste indicating a reversible inhibitory response of the system (Ruiz and Flotats, 2016). Biomethanization of CPW has been tested following D-limonene extraction under thermophilic and mesophilic conditions demonstrating that pre-treatment of the waste prior to anaerobic digestion enhances the availability of nitrogen and phosphorus constituting the supplementation of nutrients unnecessary, hence reducing the cost of the process (Martin et al., 2010).

Mesophilic conditions were tested using dried CPW and the methane production achieved ranged between 455-732 mL g_{VS}^{-1} (Gunaseelan, 2004). Steam distillation was also evaluated as CPW pretreatment prior to the application of anaerobic digestion and methane formation reached 230 mL g_{VS}^{-1} under mesophilic conditions (Martin et al., 2010). CPW were applied using acid hydrolysis as well as steam distillation as pretreatments for thermophilic anaerobic digestion, while the methane production achieved was 363 mL g_{VS}^{-1} and 537 mL g_{VS}^{-1} respectively (Pourbafrani et al., 2010; Forgacs et al., 2011).

1.5.3 Production of other biofuels from waste

Apart from the molecules mentioned above, biodiesel and biobutanol constitute widely known biofuels that could be produced from waste. Specifically, biodiesel could be derived from waste cooking and vegetable oils (Kirubakaran and Arul Mozhi Selvan, 2018), while biobutanol could be produced from food crops (sugarcane, maize, cereal grains), lignocellulosic materials (agricultural residues such as corn, rice, wheat), algae as well as photobiological solar fuels and electrofuels (Huzir et al., 2018). The production of a range of other biofuels such as biomethanol (Iaquaniello et al., 2017), bioethers (Gonzalez Prieto et al., 2019), dimethyl-ether (Inayat et al., 2017), biohydrogen (Argun and Dao, 2017) and syngas (Nanda et al., 2018) from wastes (municipal solid waste, biomass, palm waste, waste peach pulp, cooking oil) has been also evaluated in previous studies. Moreover, new generation biofuels have been proposed comprising esters of low molecular weight organic acids manufactured through an integrated technology using waste such as whey, vinasse and lignocellulosic biomass as raw materials (Koutinas et al., 2016b). Thus, delignified wheat straw was tested for organic acids production, which were subsequently enzymatically esterified using 1-butanol as a solvent resulting in high yields (90%) (Dima et al., 2017). Furthermore, industrial bioethanol distilleries waste was also tested for organic acids production. The organic acids were esterified with selected alcohols using immobilized Candida Antarctica Lipase-B that provided higher ester yield (97%), thus demonstrating an alternative method for the production of biofuels (Foukis et al., 2017). Moreover, the lipase catalyzed esterification reaction mechanism was further studied in anhydrous *n*-hexane (Foukis et al., 2018) and in anhydrous solvent-free system for

ethyl butyrate synthesis (Foukis et al., 2019) demonstrating that the synthesis follows an entirely different mechanism in the two cases.

1.6 Aim of current work

The studies presented above indicate that CPW comprise an abundant residue, which entails a rich composition in valuable components and nutrients for fermentation. Thus, a holistic approach should be followed for valorization of the specific bioresource. The current study proposed the development of a zero-waste CPW biorefinery (Figure 1.6.1), which employed steam distillation for isolation of essential oils and acid/enzyme hydrolysis for extraction of pectin and generation of a liquid hydrolyzate. The latter comprised high content of soluble sugars that was used as a nutrient rich fermentation feedstock for the production of succinic acid or ethanol. Moreover, the remaining solid biorefinery residues were tested in agricultural applications as fertilizer and in anaerobic digestion for the production of methane closing the loop in the management of CPW through development of a zero-waste process.

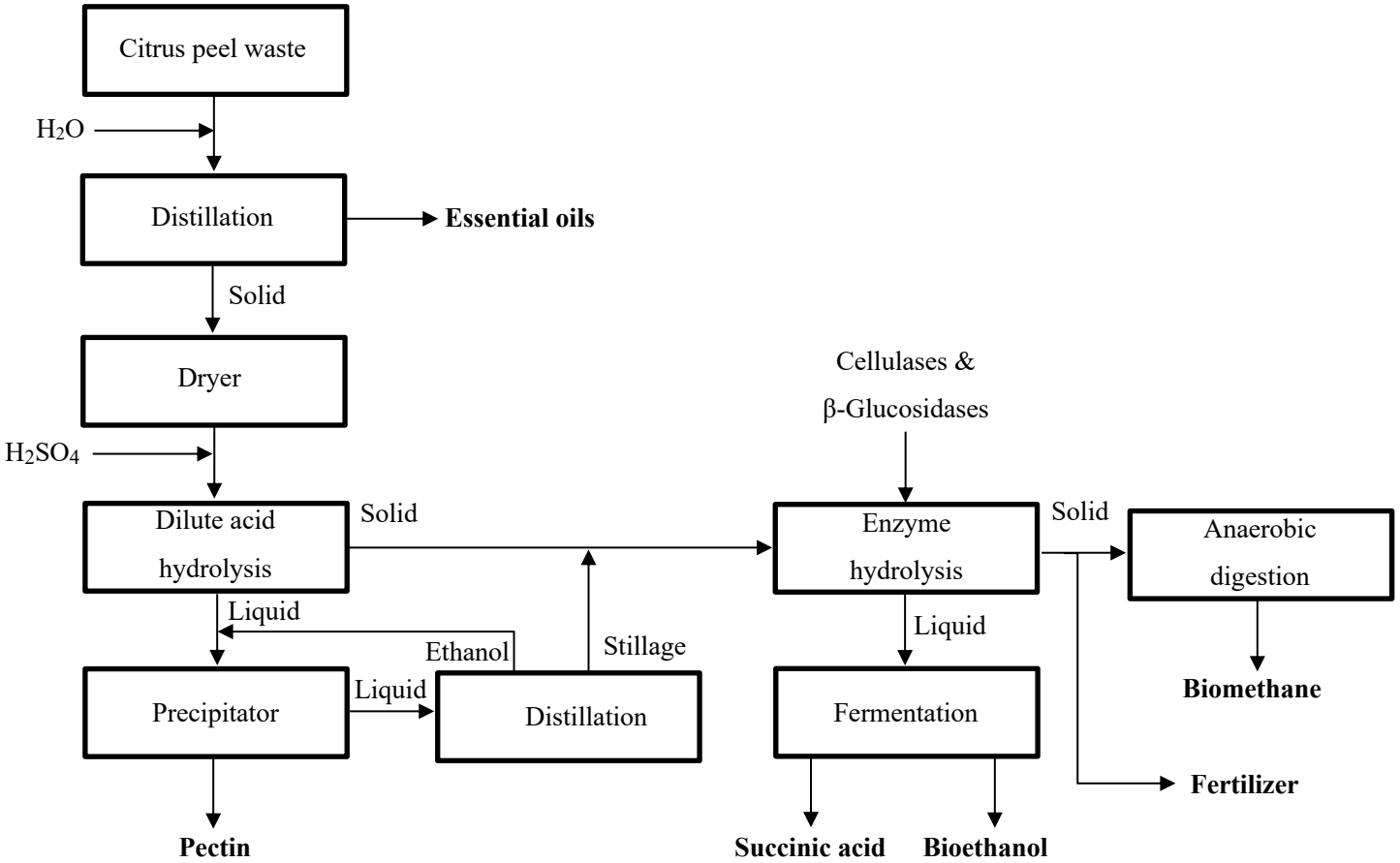

Figure 1.6.1: Process flow sheet of the biorefinery used for CPW valorization.

1.6.1 Objectives

The development of the CPW-based biorefinery of the current study requires the characterization of the feedstock in different constituents as well as to conduct fermentations for the production of the commodities envisaged. Furthermore, subsequent optimization of succinic acid and ethanol fermentations is essential through evaluation of a range of parameters, while potential valorization of the solid biorefinery residues generated should be explored to construct a zero-waste process. The main objectives of the study are as follows:

- Characterization of the raw material to identify the content in cellulose, hemicellulose and lignin before and after pretreatment. Moreover, the quantity and composition of essential oils and pectin isolated from the waste as added-value products should be assessed to determine their marketability.

- The newly isolated *P. kudriavzevii* KVMP10 yeast, which is capable for elevated ethanol production from CPW at increased temperatures, should be evaluated for production of the biofuel from the waste under a range of parameters.

- Suitable dilute acid and enzyme hydrolysis conditions for enhancing the release of fermentable sugars from CPW in the hydrolyzate generated should be obtained. The hydrolyzates formed were applied as fermentation feedstocks for the production of succinic acid and ethanol, while the release of metal ions and fermentation inhibitors in the different hydrolysis approaches followed has been also evaluated.

- Succinic acid and ethanol fermentations were further investigated to maximize the concentration of the final product. The addition of nitrogen sources, vitamins as well as the use of batch and fed-batch fermentations were explored in fermentations.

- The solid residue generated as biorefinery side-stream was explored for use in agricultural applications as well as in anaerobic digestion aiming to generate a zero-waste process.

2 RESEARCH METHOLOGY

2.1 CPW: origin, storage and handling

The CPW used in the present work for the development of the biorefinery for succinic acid production constituted “Mandora” residues consisting of peels, seeds and segment membranes, which were obtained from a local juice factory (KEAN, Limassol, Cyprus) and stored at -20 ^{o}C until further use. “Mandora” fruit constitutes a Cypriot interspecific hybrid variety. Citrus household waste was used for the production of ethanol and consisted of OPW. Both residues were thawed and ground to particles less than 2 mm in diameter using a laboratory blender (Waring Commercial, Texas, USA) prior application to the experiments.

2.2 Isolation of essential oils and pectin

The first step of CPW pretreatment required extraction and collection of essential oils through the addition of water to the raw material at a ratio of 6:1 (w/w) and boiling for 1 h. Essential oils were collected through distillation (Li et al., 2010b) and the residue was dried at 70 ^{o}C for 24 h (Wilkins et al., 2007a).

An autoclave (SANYO MLS-3781L, Panasonic, Tottori, Japan) was used for dilute acid hydrolysis. A preliminary study was carried out in order to optimize the conditions of acid hydrolysis. Specifically, dry CPW was diluted with distilled water at 1:20 and 1:10 (w/v) ratios, while sulfuric acid was added to the mixture at a concentration of 0.5% (v/v) and hydrolysis proceeded at temperatures ranging between 109 ^{o}C and 116 ^{o}C for 10 and 20 min (Table 2.2.1) to produce the feedstock required for succinic acid fermentations. This procedure was slightly modified prior ethanol fermentations. Thus, dry CPW was diluted with 0.5% (v/v) H_2SO_4 at 1:20 (w/v) ratio and hydrolysis proceeded at temperatures ranging between 108 ^{o}C and 125 ^{o}C for 10 and 20 min. Experiments were performed in duplicate.

Centrifugation and filtration followed dilute acid hydrolysis in order to obtain the supernatant, which was mixed with an equal volume of ethanol (96% v/v) to precipitate pectin at room temperature for 4 h (Pourbafrani et al., 2010). Subsequently, the mixture was centrifuged at 3000 rpm for 30 min. The precipitate was washed five times with ethanol (45% v/v) followed by drying at 50 ^{o}C to obtain pectin (Faravash and Ashtiani, 2007).

Table 2.2.1: Conditions of acid hydrolysis used as pretreatment of CPW prior succinic acid fermentations.

Experiment	Temperature (ºC)	Reaction time (min)	% (w/v) dry raw material (drm)
1	116	10	5
2	116	20	5
3	109	10	5
4	109	20	5
5	116	10	10
6	116	20	10
7	109	10	10
8	109	20	10

drm: dry raw material.

2.3 Enzyme hydrolysis

Following pectin extraction and ethanol removal from the supernatant through distillation at 80 ^{o}C, the hydrolyzate was mixed with solid residues from acid hydrolysis and it was subject to enzymatic treatment. The pH-value of the mixture was adjusted to 4.8 with the use of 1 M NaOH to ensure that the conditions for the process were within the optimal pH-value range 4.5-5.0 for the enzymes employed. The enzymes applied for enzyme hydrolysis included cellulases and β-glucosidases.

2.3.1 Application of enzymes for CPW pretreatment

Different enzyme ratios were selected and tested to optimize the step of enzyme treatment. Enzyme hydrolysis was performed in duplicate experiments for 48 h at 50 ^{o}C in shake flasks stirred at 100 rpm in a waterbath. Cellulases (Chem Cruz, Texas, USA) and β-glucosidases/pectinases (Oenozym FW, Lamothe-Abiet, Canejan/Bordeaux, France) from *Aspergillus niger* were employed in different ratios during the process based on Zheng et al. (2010). The enzyme ratios used in each experiment are provided on Table 2.2.2, while as soon as hydrolysis was completed the samples were heated in an oven at 105 ^{o}C for 15 min to inactivate the enzymes (Wilkins et al., 2005).

Table 2.2.2: Load of enzyme units employed in hydrolysis experiments (enzymatic treatment was performed in hydrolyzates generated through the conditions of acid treatment specified in experiment 1 - Table 2.2.1).

Experiment	Cellulases (IU g_{drm}^{-1})	β-Glucosidases (BGL g_{drm}^{-1})
A	20	25
B	30	25
C	20	35
D	30	35
E	20	50
F	30	50

drm: dry raw material

2.4 Microorganisms and culture conditions

2.4.1 Succinic acid fermentations

A. succinogenes Z130 was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The strain was maintained at -80 ºC in glycerol stock cultures and prior to the experiments the inoculum was cultured in 30 g L^{-1} of tryptic soy broth (TSB). TSB was sterilized at 121 ºC for 20 min and *A. succinogenes* was incubated at 37 ºC for inoculum preparation in an orbital shaker stirred at 100 rpm for 14 h.

2.4.1.1 Batch experiments on shake flasks

Succinic acid fermentations were performed in batch mode using 130 mL Duran bottles with a working volume of 100 mL. The reducing sugars obtained from dilute acid hydrolysis or combined acid and enzyme hydrolysis of CPW were used as carbon source for the experiments. The fermentation feedstock was additionally supplemented with 30 g L^{-1} $MgCO_3$ and 5 g L^{-1} yeast extract, while continuous sparging of CO_2 was supplied with a flow rate of 0.5 vvm. Stirring was controlled at 100 rpm in a rotary shaking waterbath and temperature was maintained constant at 37 ºC.

The size of the inoculum that was added in fermentations was initially optimized. Different percentages of inoculum were tested (7%, 13% and 20% v/v) in a hydrolyzate generated using a combination of acid (5% (w/v) raw material, 105 ºC, 60 min) and enzyme hydrolysis (7 FPU mL^{-1}). The fermentation process was performed at 37 ºC with the addition of 40 g L^{-1} $MgCO_3$, but without the supply of CO_2. Moreover, simple sugars (glucose, fructose, galactose) as well as galacturonic acid with initial concentration of 10 g L^{-1} were tested as single substrates in *A. succinogenes* fermentations with the supplementation of 5 g L^{-1} yeast extract, 30 g L^{-1} $MgCO_3$ and continuous sparging of 0.5 vvm CO_2. The specific fermentations were performed at 37 ºC and 100 rpm.

Recycling of the stillage remaining following distillation for extraction of essential oil was also tested to increase the content of soluble sugars in the hydrolyzate. The effect of four different nitrogen sources (ammonium sulfate, yeast

extract and corn steep liquor) fed at a concentration of 5 g L^{-1} was evaluated in *A. succinogenes* fermentations using the hydrolyzate obtained from acid hydrolysis through the optimal conditions selected. Subsequently, the effect of vitamins addition (B12, 1 μg L^{-1}; biotin, 20 μg L^{-1}; folic acid, 20 μg L^{-1}; thiamine, 50 μg L^{-1}; riboflavin, 50 μg L^{-1}; niacin, 50 μg L^{-1}; pantothenate, 50 μg L^{-1}; p-aminobenzoate, 50 μg L^{-1}; lipoic acid, 50 μg L^{-1}; B6, 100 μg L^{-1}) was examined in fermentations. All shake flask experiments were performed in duplicate, while two samples were analyzed for each replicate constituting analyses of a total of 4 samples in each experiment.

2.4.1.2 Lab-scale bioreactor experiments

Different bioreactor operations namely batch, SSF and feb-batch, were tested in a bioreactor (Minifor, Labda, Brno, Czech Republic) with an initial working volume of 0.3 L (0.45 L total volume). The bioreactor was autoclaved at 121 ºC for 15 min and the pH-value was controlled at 6.8 in batch and fed-batch experiments, while SSF was run at pH-value of 6.4. 30 g L^{-1} $MgCO_3$ and 5 g L^{-1} CSL were supplemented in the hydrolyzate prior to fermentation, while continuous sparging of CO_2 was supplied with a flow rate of 0.5 vvm. The temperature of batch and fed-batch fermentations was 37 ºC, whereas that of the SSF process was set at 38 ºC. The agitation was regulated in the reactor at 100 rpm in all experiments.

The feedstock used in batch and fed-batch experiments was the rich in carbohydrates hydrolyzate generated from dilute acid hydrolysis of CPW performed at 116 ºC for 10 min applying 5% (w/v) dry raw material following the extraction of pectin. In the SSF process the same hydrolyzate was used with the addition of solid residues from acid hydrolysis. The most efficient combination of cellulases and β-glucosidases as determined in the experiments was added in SSF.

2.4.2 Ethanol fermentations

P. kudriavzevii KVMP10 was previously isolated as a thermotolerant ethanologenic yeast within our research group (Koutinas et al., 2016a), while *K. marxianus* was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Commercial pressed

baker's yeast was used as a source of *S. cerevisiae*. All strains were maintained at -80 ^{o}C in glycerol stock cultures.

P. kudriavzevii KVMP10 and *S. cerevisiae* were precultured in liquid media simulating a Valencia OPW hydrolyzate (Wilkins et al., 2009), while the inoculum of *K. marxianus* was prepared using universal media for yeast strains (containing (g L^{-1}): yeast extract 3, malt extract 3, peptone 5 and glucose 10) incubated at 30 ^{o}C in shake flasks stirred at 100 rpm.

2.4.2.1 Evaluation of P. kudriavzevii KVMP10 fermentation parameters

P. kudriavzevii KVMP10 was tested for bioethanol production under five different temperatures (30 ^{o}C, 37 ^{o}C, 40 ^{o}C, 42 ^{o}C and 45 ^{o}C) as well as three different pH-values (4, 4.8 and 7) to optimize the temperature and the pH-value during fermentation of the hydrolyzed Valencia OPW model solution. *P. kudriavzevii* KVMP10 was tested for assimilation of simple sugars (glucose, fructose, galactose, sucrose as well as xylose) with initial concentration of 10 g L^{-1}. The batch experiments were conducted in 100 mL flasks with a working volume of 60 mL in an incubator operated at a temperature according to the specification of each experiment and reciprocal shaking at 100 rpm. All chemicals were obtained from Sigma-Aldrich Company Ltd (Dorset, UK) and were of ANALAR grade. The effect of D-limonene on *P. kudriavzevii* KVMP10 fermentations was also tested. OP oil was procured from a local orange juice factory (Kean Soft Drinks Ltd., Limassol, Cyprus).

2.4.2.2 Ethanol fermentations on CPW hydrolyzates

The mixture of reducing sugars obtained from CPW pretreatment (dilute acid hydrolysis only or combined acid and enzyme hydrolysis) were used as carbon source for the experiments. Bioethanol fermentations were performed in batch experiments using 100 mL flasks with a working volume of 60 mL at 42 ^{o}C and 100 rpm. The feedstock was supplemented with 10 g L^{-1} of yeast extract as nitrogen source. Moreover, recycling of the liquid stillage remaining following essential oils extraction into the hydrolysis process was tested to enhance the soluble sugars' content in the hydrolyzate. All experiments were performed in duplicate, while

three samples were analyzed for each replicate constituting analyses of 6 samples in each time point.

2.5 Anaerobic digestion of different citrus-based waste

CPW, dried citrus peel waste (DCPW) and biorefinery residues (BR, remaining solids following acid hydrolysis of CPW), were used as feedstock in anaerobic digestion. Equal quantities of volatile solids (VS, 6 g L^{-1}) were employed to produce biogas under mesophilic conditions (37 ^{o}C), while anaerobic digestion was performed in batch experiments using 250 mL flasks with a working volume of 150 mL. The nutrient medium was prepared according to the composition used in Angelidaki et al. (2009). Each bottle was supplemented with 6 g of granular sludge withdrawn from a full-scale UASB reactor (Charalambides Christis Ltd, Limassol, Cyprus) used for the treatment of dairy wastewater at pH-value 6.8-7.3. Granular sludge was washed with distilled water and applied as active inoculum (4% w/v), while each bottle was flushed with 100% CO_2 gas to ensure anaerobic conditions. Experiments were performed in triplicate and biomethane accumulation was determined for 121 d. Following 35 d, a refeed of each material (6 g_{VS} L^{-1}) was applied in each digestion and a subsequent refeed was also conducted at 78 d. Furthermore, different initial VS contents (3, 6, 12 and 24 g L^{-1}) of CPW were applied in triplicate anaerobic digestion experiments for 92 d to evaluate the effect of essential oils in the process.

2.6 Application of BR as fertilizer

BR was initially dried at 40 ºC in an air circulating oven until constant weight was achieved and then milled. Different ratios of the dried residue in commercial peat substrate were used to evaluate lettuce seedling production (0%, 1%, 2.5%, 5% and 10% w/w).

Substrate mixtures were placed in plastic seedling trays and 3 seeds of lettuce (*Lactuca sativa* var. Paris Island) were placed per module (40 cm^3 module capacity). Nine modules per treatment were used as replications. Trays were placed in walk-in growth chambers (temperature of 25±1 ºC max, 20±1 ºC min; RH: 65-70%) and watered according to plant needs, while daily observations were recorded for seed emergence. Following 25 d of cultivation, seedling growth was assessed and six plants per treatment were harvested. Plant height (in terms of longest leaf), leaf number, fresh weight and dry upper plant matter were measured. Maximum Fv/Fm photochemical quantum yields of PSII were measured using OptiSci OS-30p Chlorophyll Fluorometer (Opti-Sciences, Hudson, USA). Chlorophyll content was measured as described by Chrysargyris et al. (2016) using DMSO.

2.7 Analyses

2.7.1 Cellulose, hemicellulose and lignin

CPW and hydrolyzed CPW were dried in an oven at 70 ºC for 24 h prior analysis for lignocellulosic composition. Specifically, a manual FibreBag system (Gerhardt Analytical Systems, Konigswinter, Germany) was employed for determination of cellulose, hemicellulose, lignin and N-compounds content in each material. Thus, the FibreBag system was used to determine crude fibre as well as acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL) fractions in each material following the protocols provided by Gerhardt.

ADF

- German Official Method: Methodenbuch des Vernades Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, Band III, Bestimmung der Saure-Detergentien-Fased (ADF)
- AOAC Official Method 973.18, Feber (Acid Detergent and Lignin (H_2SO_4) in Animal Feed, First Action 1973, Final Action 1977)

Principle: The part of components which are not eluted by an acid detergent solution is called acid detergent fibre and it indicates the portion of cellulose, lignin and lignin-N-compounds. The insoluble residue is dried, weighted, and then incinerated. The difference between the ash content and the insoluble residue is the ADF content.

Chemicals:

- ADF-solution: 20 g N-cetyl-N, N, N-trimethyl-ammoniumbromide are diluted in 1 L H_2SO_4 (0.5 mol L^{-1})
- Anti-foam agent: decahydronaphthalene

Procedure:

Preparation:

- The number of FibreBags needed is dried at 105 +/- 1 ºC for 1 h in the drying chamber. The weight of the FibreBags is value m_1 for the balance

protocol. When storing the FibreBags in a desiccator they only have to be dried once and then, can be weighed directly.

- The weight of the sample to be put into the FibreBag should be 1 g weighed (m_2).
- The blank value is determined in parallel to the regular analysis by blank determination (FibreBag without sample). It should be < 1 mg per FibreBag.
- The glass spacers are put into the FibreBags and both together are inserted in the carousel.

Digestion:

- 360 mL ADF solution are placed into a beaker. Boiling stones and, as an anti-foam agent, decahydronaphthalene are added. The handling tool is attached to the carousel and the carousel is gently lowered into the beaker containing the ADF solution. The carousel is rotated for about 1 min so that the FibreBags are completely filled with the solution.
- The extraction beaker is subsequently placed on the preheated hotplate and brought to boil by setting it full (takes about 3-5 min). As soon as the solution starts boiling the heating power is reduced.
- Simmer gently for 60 min, the samples should float freely in the FibreBags. This can be assisted by gently rotating the carousel with the handling tool or by softly swirling the beaker.
- Exactly 60 min following the beginning of boiling, remove the beaker from the hotplate, lift the carousel out of the beaker using the handling tool to drain the solution from the FibreBags.
- Discard the detergent solution with the solute whithin the beaker as the solution should be disposed of separately.
- The samples are washed 3-5 times with hot water (about 100 mL) till they are free of detergents.

Drying of FibreBags and Preparation of Crucibles:

- The FibreBags together with the glass spacers are taken out of the carousel. Subsequently, the spacers are removed from the FibreBags rinsing them carefully with water.
- The drained FibreBag is placed into a crucible, which has been pre-ashed at 600 °C and weighed (value m_6 for the balance protocol). Then, it is placed into a drying chamber at 105 °C for minimum 4 h or overnight and afterwards into the desiccator to cool down to ambient temperature.
- FibreBag and crucible after digestion and drying is value m_3.

Incineration of Samples:

- The sample residue together with the FibreBag is incinerated at 600 °C for at least 4 h or overnight.
- The resulting vapours are not hazardous. Following incineration, the crucible is left to cool in the drying chamber for 30 min and afterwards in the desiccator to cool down to ambient temperature.
- Then, it is weighed and value m_4 is obtained for the weighing protocol.

Calculation:

The ADF content is the non-soluble part remaining after boiling in the acid detergent solution reduced by the content of ash. Thus, it is calculated as follows:

$\%ADF = [((m_3 - m_1)-(m_4 - m_5))x100] / m_2$

Blank Value $m_5 = m_7 - m_6$

meaning:

m_1 = weight of FibreBag, g

m_2 = initial sample weight, g (has to be adjusted according to the dry content)

m_3 = weight of crucible with dried FibreBag and sample residue after digestion, g

m_4 = weight of crucible with ash, g

m_5 = blank value of empty FibreBag, g

m_6 = weight of crucible, g

m_7 = weight of crucible with ash of the empty FibreBag, g

NDF

- German Official Method: Methodenbuch des Verbandes Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, Band III, Bestimmung der Neutral-Detergentien-Faser (NDF), 1988

Principle: The part of components which are not eluted by a neutral detergent solution is called neutral detergent fibre (NDF). In vegetable feed, the content of NDF indicates the portion of cellulose, hemicellulose, lignin and lignin-N-compounds. The insoluble residue is dried, weighted, and then incinerated. The difference between the ash content and the insoluble residue is the ash corrected NDF. The process of dilution and filtration is simplified by use of the FibreBags.

Chemicals:

- NDF-Solution: While being heated gently, 93 g EDTA ethylenediamine tetra acetic acid-disodium salt and 34 g disodium tetra borate-decahydrate are dissolved in a beaker in 2 L water. After cooling off, 150 g dodecylsulphate-sodium salt and 50 mL triethylene glycol. In another beaker, 22.8 g sodium dihydrogenphosphate are dissolved in 2 L water being gently heated up and after cooling off, are added to the first batch. The solution is filled up with distilled water to make about 5 L; the pH-value has to be adjusted between 6.9 and 7.1 (if necessary, standardize with phosphoric acid, acetic acid or sodium hydroxide solution).
- Heat-stable α-amylase – 2 mL α-amylase per sample (no amylase to be added to blank value sample)
- Anti-foam agent (silicone-antifoam agent)

Procedure:

Preparation:

- The number of needed FibreBags is dried in the drying chamber at 105 +/- 1 °C for 1 h.

- The weight of the FibreBags is value m_1 for the balance protocol. When storing the FibreBags in a desiccator they only have to be dried once and then, can be weighed directly.
- The sample weight per FibreBag should be 1 g and should be weighed with 1 mg preciseness; this gives value m_2 for the weighing protocol.
- The glass spacers are put into the FibreBags and both together are inserted into the carousel.
- The blank value is determined in parallel to the regular analysis by blank determination (FibreBag without sample). It should be < 5 mg per FibreBag.
- The dry matter of the sample should be determined separately and is considered for the calculation of the content (result is related to the dry matter).

Digestion:

- 360 mL NDF-solution are put into a beaker. Boiling stones and anti-foam agent are added. The handling tool is attached to the carousel and the carousel is gently lowered into the beaker containing the NDF-solution. The carousel is rotated for about 1 min so that the FibreBags are completely filled with the solution.
- Then the extraction beaker is placed on the preheated hotplate and brought to boil by setting it full (takes about 3-5 min). As soon as the solution starts boiling the heating power is reduced and some amylase is added (higher starch contents may require another addition of amylase after 10 min).
- Simmer gently for 60 min, the samples should float freely in the FibreBags. This can be helped by gently rotating the carousel with the handling tool or by softly swirling the beaker.
- Exactly 60 min after boiling start, remove the beaker from the hotplate, lift the carousel out of the beaker using the handling tool and drain the solution from the FibreBags.
- Discard the detergent solution with the solute within the beaker - the solution has to be disposed of separately.

- The samples are washed 3-5 times with hot water (about 100 mL) till they are free of detergents.

Drying of FibreBags and Preparation of Crucibles:

- The FibreBags together with the glass spacers are taken out of the carousel. Then the spacers are removed from the FibreBags rinsing them carefully with water.
- The drained FibreBag is put into a crucible, which has been pre-ashed at 600 °C and weighed (value m_6 for the balance protocol). Then, it is dried in the drying chamber at 105 °C for minimum 4 h or overnight and afterwards it is placed into the desiccator to cool down to ambient temperature.
- FibreBag and crucible after digestion and drying is value m_3.

Incineration of Samples:

- The sample residue together with the FibreBag is incinerated at 600 °C for at least 4 h or overnight.
- After incineration, the crucible is left to cool in the drying chamber at 105 °C for 30 min and afterwards in the desiccator to cool down to ambient temperature.
- Then, it is weighed and value m_4 is obtained for the weighing protocol.

Calculation:

The NDF content constitutes the non-soluble part remaining after boiling in the neutral detergent solution minus the content of ash and it is calculated as follows:

$\%NDF = [((m_3 - m_1)-(m_4 - m_5))x100] / m_2$

Blank Value $m_5 = m_7 - m_6$

meaning:

m_1 = weight of FibreBag, g

m_2 = initial sample weight, g

m_3 = weight of crucible with dried FibreBag and sample residue after digestion, g

m_4 = weight of crucible with ash, g

m_5 = blank value of empty FibreBag, g

m_6 = weight of crucible, g

m_7 = weight of crucible with ash of the empty FibreBag, g

ADL

- German Official Method: Methodenbuch des Verbandes Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, Band III, Bestimmung der Säure-Detergentien-Faser (ADF) und Bestimmung des Säure-Detergentien-Lignins (Rohlignin)
- AOAC Official Method 973.18, Fiber (Acid Detergent) and Lignin (H2SO4) in Animal Feed, First Action 1973, Final Action 1977

Principle: For the acid-detergent lignin (ADL) procedure, the acid-detergent fiber (ADF) procedure is used as a preparatory step. The components cellulose and lignin are not eluted from the sample by the acid detergent solution. The cellulose is dissolved by treatment with 72% sulphuric acid.

2.7.2 Composition of essential oils

"Mandora" CPW which was obtained from a local factory, grounded "Mandora" and citrus household waste were used for determining the quantity as well as to identify the composition of essential oils. Three samples of each material were chopped and approximately 15-20 g of sample was subject to hydrodistillation for 3 h using Clevenger apparatus. The essential oil (dried over anhydrous sodium sulphate) yield was measured and calculated as µL of oil per g wet tissue. Oils were kept in amber glass bottles at -20 ◦C until GC-MS analysis.

Analytical gas chromatography was carried out on a Shimadzu GC2010 gas chromatograph interfaced Shimadzu GC-MS QP2010 plus mass spectrometer (Kyoto, Japan). An aliquot of 2 µL was injected in a split mode (split ratio 20:1) into the gas chromatograph fitted with a ZB-5 column (Zebron, Phenomenex, USA) coated with 5% pheny-95% dimethylpolysiloxane with film thickness of 0.25 µm, length of 30.0 m and a diameter of 0.25 mm. The flow of the carrier gas (Helium)

was 1.03 mL min^{-1}. The injector temperature was set at 230 ^{o}C. Electron impact mass spectra with ionization energy of 70 eV was recorded at the 35-400 m/z. The column temperature was programmed to rise from 60 ^{o}C to 240 ^{o}C at a rate of 5 ^{o}C min^{-1}, with a 5 min hold at 240 ^{o}C. The solution of standard alkanes mixtures (C8–C20) was also analyzed using the above conditions.

The identity of the oil components was assigned by comparison of their retention indices relative to (C8-C20) n-alkanes with those of literature or with those of authentic compounds available in our laboratory. Further identification was made by matching their recorded mass spectra with those stored in the NIST08 mass spectral library of the GC-MS data system and other published mass spectra (Adams, 2012). The percentage determination was based on peak area normalization without using correction factors.

2.7.3 Pectin

Pectin from "Mandora" and citrus households was isolated as descripted in section 2.2. Fourier Transform Infrared (FTIR) spectra were recorded in the range 450–4000 cm^{-1} with a nominal resolution of 1 cm^{-1} and averaging 5 spectra. All samples were prepared as KBr pellets and analyzed on a Perkin-Elmer Spectrum 100 spectrophotometer.

2.7.4 Organic acids

Determination of organic acids in A. succinogenes fermentations: The organic acids produced during succinic acid fermentations were identified and quantified through High Pressure Liquid Chromatography (HPLC). There included succinic acid, which was the main product formed as well as formic and acetic acids generated as fermentation by-products.

Culture samples were centrifuged at 13000 rpm for 5 min and filtered with 0.45 μm syringe filters. A Shimadzu LC-20AD liquid chromatograph (Shimadzu, Milton Keynes, UK) equipped with a Shimadzu SPD-20A UV/VIS detector, a Shimadzu SIL-20A HT auto sampler and a CTO-10AS VP column oven was used. The column was eluted isocratically at a rate of 0.6 mL min^{-1} from an organic analysis column (Rezex RHM-Monosaccharide H+ (8%) column, Phenomenex, USA) with

5 mM H_2SO_4 at 50 °C. The injection volume was 20 µL. Succinic acid as well acetic acid and formic acid concentration was calculated interpolating from a previously established calibration curve and the coefficient of variation for 3 samples was 0.5%, 0.2% and 0.17% respectively at a concentration level of 0.75 g L^{-1}.

Determination of volatile fatty acids (VFAs): The concentration of VFAs (acetate, formate, butyrate, propionate and valerate) formed during anaerobic digestion was measured through HPLC using the method described above following acidifications. This included column elution at a rate of 0.7 mL min^{-1} as well as the use of 1 µL as injection volume. Culture samples were centrifuged at 13000 rpm for 3 min and filtered using 0.22 µm syringe filters. Acetate, formate, butyrate, propionate and valerate concentration was calculated interpolating from a previously established calibration curve and the coefficients of variation for 3 samples were 2.2%, 1.4%, 1.3%, 1% and 1.8% at a concentration level of 2 g L^{-1} respectively.

2.7.5 Determination of ethanol concentration

Gas Chromatography using a flame ionization detector was employed for the determination of ethanol concentration. A Shimadzu GC-2014 (Shimadzu, Milton Keynes, UK) and a 30 m long Zebron ZB-5 capillary column (Phenomenex, Macclesfield, UK) with 0.25 mm internal diameter was used. The stationary phase of the column was 5%-phenyl and 95% dimethylpolysiloxane, while the mobile phase applied was nitrogen. Samples obtained during bioethanol fermentations were centrifuged for 3 min at 13000 rpm and the supernatant was filtered through 0.2 µm syringe filters. Ethanol was extracted by vortexing 1 mL of the filtered sample with 2 mL of hexane for 1 min. Approximately 1 µL of the extract was injected and the temperature of the column was kept constant at 40 °C for 3 min. Ethanol concentration was calculated interpolating from a previously established calibration curve and the coefficient of variation for 3 samples was 1.47% at a concentration level of 4 g L^{-1}.

2.7.6 Biogas composition

Biogas composition (H_2, O_2, N_2, CH_4 and CO_2) was analyzed using a gas chromatograph (Agilent Technologies, 7820OA, Santa Clara, USA) fitted with a ShinCarbon ST 50/80 (2 m length, 2.2 mm ID) mesh column (Restek Corporation, Bellefonte, USA) and thermal conductivity detector as described by Vardanyan et al. (2018).

2.7.7 Reducing sugars

Depending on the requirements of each experiment two methods (NMR and phenol-sulfuric acid) were applied for determination of carbohydrates concentration.

The content of reducing sugars in the CPW hydrolyzate obtained from the experiments that aimed in selecting the optimal conditions for dilute acid hydrolysis prior to succinic acid fermentations was measured through NMR analysis. Samples of each hydrolyzate (120 μL) were transferred to a 5 mm NMR tube and sodium acetate (50 μL, 5 mM) was added as internal quantification standard. Deuterium oxide was added up to a final volume of 500 μL, while all experiments were prepared twice. ^{1}H NMR experiments of freshly prepared solutions were recorded on a 300 MHz Bruker Avance spectrometer (Coventry, UK), using a pre-saturating pulse for suppressing the water absorption peak, relaxation delay 5 s, 2925 Hz spectral window, and 128 scans. Data analysis was performed using the MestReNova software (Mesterlab Research, Santiago de Compostela, Spain).

During succinic acid as well as ethanol fermentations reducing sugars were analyzed by the phenol-sulfuric acid method (Dubois et al., 1956) to reduce the time required for analyses. This method is based on the phenol-sulfuric acid reaction and it is useful for the determination of simple sugars, oligosaccharides, polysaccharides and their derivatives.

2.7.8 Elemental analysis

An inductively coupled plasma mass spectrometer (Thermo X-Series II, Germany) was used for elemental determination of hydrolyzates obtained from

dilute acid hydrolysis (conditions: 109 ^{o}C, 10 min, 5% of dry raw material) and a combination of acid/enzyme hydrolysis (109 ^{o}C, 10 min, 5% of dry solids/20 IU crude cellulase solution g_{drm}^{-1}) as well as of the crude cellulase solution. Calibration curves with at least 6 points in the range of 5-100 μg L^{-1} were prepared for 21 trace elements: As, Be, Ca, Cd, Co, Cu, Cr, Fe, Li, Mo, Mn, Mg, Ni, P, Pb, Sb, Se, Sr, Ti, Tl and V. The calibration curve with the highest correlation coefficient was used for each element, while for the preparation of working standards a multi-analyte calibration standard (Thermo Scientific, Germany) was applied. All samples (15 mL) were acidified with 2% HNO_3 and 30 μL of an internal standard mixture of Ga, Lu and In were added prior to analysis. Each sample was analyzed in duplicate and for each duplicate at least 30 mass scans were performed. The concentration of trace elements in the samples was based on monitoring the analyte and its corresponding internal standard. A quality control (QC) in which a recovery of 80-120% of spikes and standards was used.

2.7.9 Total and Volatile solids

The total solids (TS) and VS content of granular sludge, CPW, DCPW and BR were determined according to Standard Methods (APHA, 2012).

2.7.10 Fertilizer properties assessment

The main physicochemical properties of the mixtures were determined. Organic matter content was determined after ashing in oven at 550 ^{o}C and organic carbon was then calculated accordingly. Total pore space (TPS), air-filled porosity (AFP), available water holding capacity (AWHC) and bulk density (BD) were also measured along with EC and pH-value, using the 1:5 dilution method as described previously (Chrysargyris et al., 2018).

For the seed germination tests, 6 serial dilutions (up to 10^{-6}) of the dried material in water (extract was stirred for 24 h at ambient temperature) were used. Filter paper was placed in petri dishes and it was moistened daily (4 replicates/treatment, 15 seeds/replicate). Seeds were considered germinated upon radicle emergence. Mean shoot and root length was evaluated on the eighth day.

Following the hydrochloric digestion of plant ash, potassium (K) and sodium (Na) content was determined using microprocessor flame photometer (Lasany Model 1382) and phosphorus (P) content using the vanadate/molybdate (yellow method) as described by Gupta et al. (2008). Nitrogen (N) content was determined through Kjeldahl method. The above methods were used to determine K, Na, P and N content into the substrate mixtures as well.

Plant stress level and damage indices were determined in terms of malonialdehyde (MDA) and hydrogen peroxide (H_2O_2) content, as described by Chrysargyris et al. (2017), following homogenization of plant tissue with 0.1% trichloroacetic acid.

3 RESULTS AND DISCUSSION

3.1 Characterization of the feedstock's composition

3.1.1 Content of cellulose, hemicellulose and lignin in CPW and BR

CPW constitutes an industrial waste that may serve as a promising feedstock for valorization due to the presence of a valuable composition in added-value components and fermentation nutrients. Previous research has focused on characterizing the material in terms of its content in lignocellulosic components and other constituents. Marin et al. (2007), studied the composition of CPW obtained from various citrus processing industries demonstrating that CPW consist of (w/w) 2.56% ash, 9.57% sugar, 4% fat, 9.06% protein 4.50% flavonoids, 23.02% pectin, 7.52% lignin, 37.08% cellulose and 11.04% hemicellulose based on dry mass. However, Rivas et al. (2008) reported a slightly different composition that consisted of 3.5% ash, 16.9% soluble sugars, 1.95% fat, 6.5% protein, 42.5% pectin, 0.84% lignin, 9.21% cellulose and 10.5% hemicellulose.

Herein, the content of cellulose, hemicellulose and lignin entailed in the CPW used in the biorefinery was determined and compared to previous studies (Table 3.1.1). Cellulose, hemicellulose and lignin determined as percentages of dry CPW mass were measured at 22.45%, 8.05% and 0.66% respectively. The differences in the results of the current study and that of the relevant literature could be attributed to a wide range of parameters associated to crop cultivation. Moreover, the composition of the aforementioned macromolecules was determined in the solid material remaining as BR following the process of acid hydrolysis. Results indicate that the percentage of cellulose did not change. However, the percentage of hemicellulose was decreased significantly to 3.42% following the chemical pretreatment demonstrating that acid hydrolysis assists mainly in breaking down hemicellulose into simple sugars.

Table 3.1.1: Lignocellulosic content of CPW and BR. Values represent the percentage (w/w) of each constituent in the dry mass of each material.

Material	CPW	CPW	CPW	BR
Cellulose	37.08	9.21	22.45	22.32
Hemicellulose	11.04	10.5	8.05	3.42
Lignin	7.52	0.84	0.66	1.34
References	Marin et al. (2007)	Rivas et al. (2008)	Current study	

CPW: Citrus peel waste, BR: Biorefinery residues.

3.1.2 Pectin

Pectin constitutes one of the major CPW components consisting a complex of polysaccharides mainly composed of 1-4 linked α-D-galacturonic acid (Khan et al., 2015). The isolation of pectin is technologically important based on the wide application of the heteropolysaccharide in the food industry, as a jelling and thickening agent in jams and jellies, as well as in the pharmaceutical sector (Thakur et al., 1997).

Dried citrus peel included 30% of pectin according to Khan et al. (2015). However, Marin et al. (2007) and Rivas et al. (2008) reported different pectin contents that reached 23.02% and 42.5% respectively. Extraction of the biopolymer is known to require the presence of an acidic solution, while the yield of pectin achieved depends on extraction duration, temperature and pH-value (Khan et al., 2015). Nevertheless, Tiwari et al. (2017) demonstrated that pH-value was the most important parameter for pectin extraction isolating pectin contents ranging between 7.3-52.9%.

The isolation of pectin in the current study was carried out using the extraction method described in section 2.2. Following an optimization study of the conditions used in acid hydrolysis, the temperature and reaction time applied was 116 ^{o}C and 10 min respectively, employing 5% of dry raw material and 0.5% H_2SO_4. The results demonstrated that the pectin extracted reached 23.25% (w/w), while when

the stillage from essential oils extraction was used in acid hydrolysis instead of water, the content of pectin isolated was substantially increased to 30.54% (w/w). The FTIR spectra of pectin extracted from household and “Mandora” CPW as well as commercial pectin was investigated to determine the purity of the bioproduct (Figure 3.1.1). The results demonstrate that the FTIR spectra of isolated pectins were very similar to the spectrum of the commercial heteropolysaccharide.

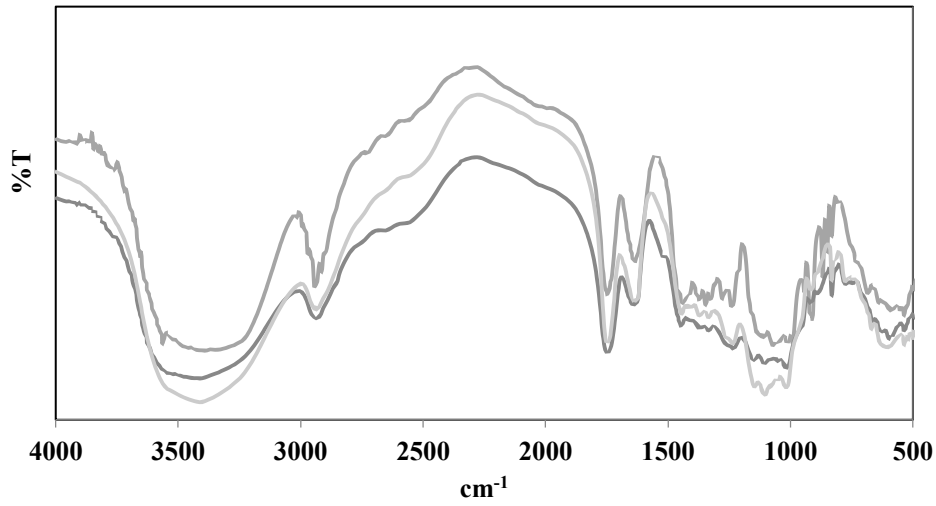

Figure 3.1.1: FTIR spectra of commercial pectin (——), pectin from “Mandora” (——) and pectin from CPW (——).

The following characteristic bands were identified in all samples. The broad band centered at 3408 cm^{-1} coresponds to a characteristic O–H stretching band (Monfregola et al., 2011; Kumar and Chauhan, 2010). The smaller intensity band at 2934 cm^{-1} is due to the C-H bond vibration (Monfregola et al., 2011; Kumar and Chauhan, 2010; Guolin et al., 2012). The peak at approximately 1747 cm^{-1} relates to the C=O bond vibration indicating the methylated carboxyl groups ($COCH_3$) in pectin (Monfregola et al., 2011; Kumar and Chauhan, 2010; Guolin et al., 2012). The characteristic peak at 1634 cm^{-1} is due to the –O– tensile vibration band (Guolin et al., 2012; Shi and Gunasekaran, 2008; Teresa Pacheco et al., 2019) and the three peaks at 1445 cm^{-1}, 1371 cm^{-1} and 1333 cm^{-1} represent the C–O–H in the bending vibration (Guolin et al., 2012). The peak at 1236 cm^{-1} is due to an asymmetric C–O–C tensile vibration and indicates the abundance of –O–CH3 (methoxyl) groups (Guolin et al., 2012). The peak at approximately 1050 cm^{-1} corresponds to the –

COC– stretching of the galactouronic acid (Kumar and Chauhan, 2010). The peak at around 1075 cm^{-1} is assigned to C=O or C=C double bond of pectin (Shi and Gunasekaran, 2008; Teresa Pacheco et al., 2019). The various bands at 1145, 1105, 1015 and 915 cm^{-1} correspond to skeletal C–O and C–C vibration bands of glycosidic bonds and pyranoid ring (Monfregola et al., 2011; Guolin et al., 2012; Kalapathi and Proctor, 2001). The results presented above indicate that the biopolymers extracted comprised true pectin compounds given that the FTIR spectra did not exhibit any structural differences in pectins derived from different sources.

3.1.3 Essential oils

It has been well documented that essential oils are considered as high added value compounds based on various biological properties and aroma. Research on different scientific fields such as chemistry, biology, pharmacology and food science are influenced by the use of these natural plant products (Kale and Adsule, 1995), while among the existing essential oils, citrus essential oils comprise the most widely used worldwide. Citrus essential oils can be obtained as by-products of citrus processing and they are widely used in the food industry as aroma flavor in candies, gelatins, alcoholic and non-alcoholic drinks, as well as in the perfumery and cosmetics industry in various preparations. Moreover, citrus essential oils can be applied in pharmaceutical industries as flavoring agents to mask unpleasant tastes of drugs (Bousbia et al., 2009).

Citrus essential oils contain 85-99% volatile and 1-15% non-volatile components, while the volatile constituents comprise a mixture of monoterpene and sesquiterpene, hydrocarbons and their oxygenated derivatives (Acar et al., 2015). The content and composition of essential oils from CPW has been previously determined in various studies. Kamaliroosta et al. (2016) applied a Clevenger distillation apparatus and obtained 1.48%, 0.90%, 0.54% and 0.46% (w/w) as essential oils average yield from orange, tangerine, sweet lemon and sour lemon respectively. The main component identified was D-limonene, which accounted for 64.87% and 28.10% of orange peel and targentine peel essential oils respectively.

In the present work, essential oils from two different types of CPW, “Mandora” and household citrus waste were analyzed in terms of carbohydrate composition (Table 3.1.2). Thus, hydrodistillation of wet citrus tissue resulted in essential oil yields that reached 0.43%, 0.19% and 0.24% (v/w) for grounded “Mandora” peel, non-grounded “Mandora” peel and household citrus waste. The analysis applied identified approximately 20 compounds in each sample tested, which is in agreement to previous studies. Among the molecules identified D-limonene included most of the oil’s content and reached higher percentages as compared to the literature (Espina et al., 2011; Nagy et al., 2018). Thus, 96.36%, 96.70% and 94.41% (v/v) of grounded “Mandora” peel, non-grounded “Mandora” peel and household citrus waste’s essential oils constituted D-limonene.

Table 3.1.2: Chemical composition (% v/v, average of 3 different runs ±SE) of essential oils following GC/MS analysis. RI is calculated relatively to C8-C20 alkanes at ZB-5 column.

Compound	RI	Mandarin	Orange	Ripe fruit peel oil	Grounded "Mandora" peel oil	Non-grounded "Mandora" peel oil	Citrus household waste
a-Pinene	**933**	0.27	0.30	0.70	**0.31±0.012**	**0.34±0.008**	**0.28±0.016**
Sabinene	**973**	0.42	0.43	1.21	0.10±0.006	**0.13±0.001**	**0.26±0.013**
β-Myrcene	**991**	0.73	0.92	2.19	1.35±0.022	**1.53±0.017**	**1.32±0.042**
Octanal	**1003**	0.22	0.27	2.82	0.48±0.013	**0.30±0.002**	**1.29±0.040**
Δ-3-Carene	**1013**			-	0.01±0.009		**0.06±0.007**
D-limonene	**1028**	74.38	85.50	91.68	96.36±0.085	**96.70±0.021**	**94.41±0.198**
Trans-cimene	**1046**			0.01	-	**0.06±0.002**	
Linalool	**1100**	0.54	0.47	1.01	0.53±0.014	**0.18±0.005**	**1.30±0.035**
Nonanal	**1104**		0.07	0.05	0.06±0.005	**-**	**0.09±0.009**
Citronellal	**1153**	0.08	0.08		-	**0.05±0.003**	**0.01±0.001**
Terpinen-4-ol	**1178**			0.08	-	**0.04±0.002**	**0.07±0.004**
a-Terpineol	**1191**			0.08	0.05±0.004	**0.02±0.002**	**0.16±0.005**
Decanal	**1206**	0.41	0.43	0.53	0.58±0.011	**0.16±0.007**	**0.39±0.010**
Nerol	**1242**			0.04	0.02±0.001	**0.02±0.001**	**0.08±0.005**
Geranial	**1271**		0.07		0.04±0.001	**0.05±0.002**	**0.17±0.006**
Perilla aldeyde	**1275**	0.24	0.08	0.03	-	**0.02±0.004**	**-**
Valencene	**1509**	0.09	0.34		0.07±0.003	**0.34±0.015**	**0.06±0.003**
Monoterpene hydrocarbons		76.08	87.51	96.36	98.13±0.047	**98.76±0.040**	**96.33±0.121**
Sesquiterpene hydrocarbons		0.26	0.70	0.07	0.07±0.003	**0.34±0.015**	**0.06±0.003**
Oxygenated monoterpenes		13.62	5.21	1.27	1.22±0.030	**0.54±0.017**	**2.17±0.065**
Oxygenated sesquiterpenes		0	0.18		0	0	0
Other					0.55±0.018	**0.30±0.002**	**1.38±0.049**
Total		95.99	92.65	99.59	99.97±0.002	**99.94±0.012**	**99.94±0.006**
		Espina et al. (2011)		Nagy et al. (2018)	Current study	Current study	Current study

3.2 Preliminary study for the development of a CPW-based biorefinery for the production of succinic acid

3.2.1 Assesment of suitable fermentation conditions

Selection of inoculum size: In order to understand the growth properties of the strain, the microbial production of succinic acid was first investigated in batch cultures containing different quantities of *A. succinogenes* Z130 inoculums. Thus, following essential oils extraction and drying, CPW was subject to dilute acid and enzyme hydrolysis, which was performed at 105 ^{o}C for 60 min while crude cellulase media (7 IU g_{drm}^{-1}) was used for enzyme hydrolysis. The initial inoculum content tested included 7%, 13% and 20% and their addition in succinic acid fermentations demonstrated that 13% was optimum choice based on the cell growth achieved.

Utilization of simple sugars: Glucose, fructose and galactose comprise sugars which are often present in biowaste-based fermentation feedstocks, while galacturonic acid constitutes the main carbohydrate generated from hydrolysis of the pectin content of CPW. Thus, the above mentioned carbon sources were applied as the sole substrates for succinic acid production by *A. succinogenes* aiming to identify if they could be consumed by the strain. As shown in Figure 3.2.1 glucose and fructose were completely assimilated by *A. succinogenes* while galactose and galacturonic acid were not consumed. The product yields reached during the fermentation were 0.59 g_{sa} $g_{glucose}^{-1}$ and 0.50 g_{sa} $g_{fructose}^{-1}$.

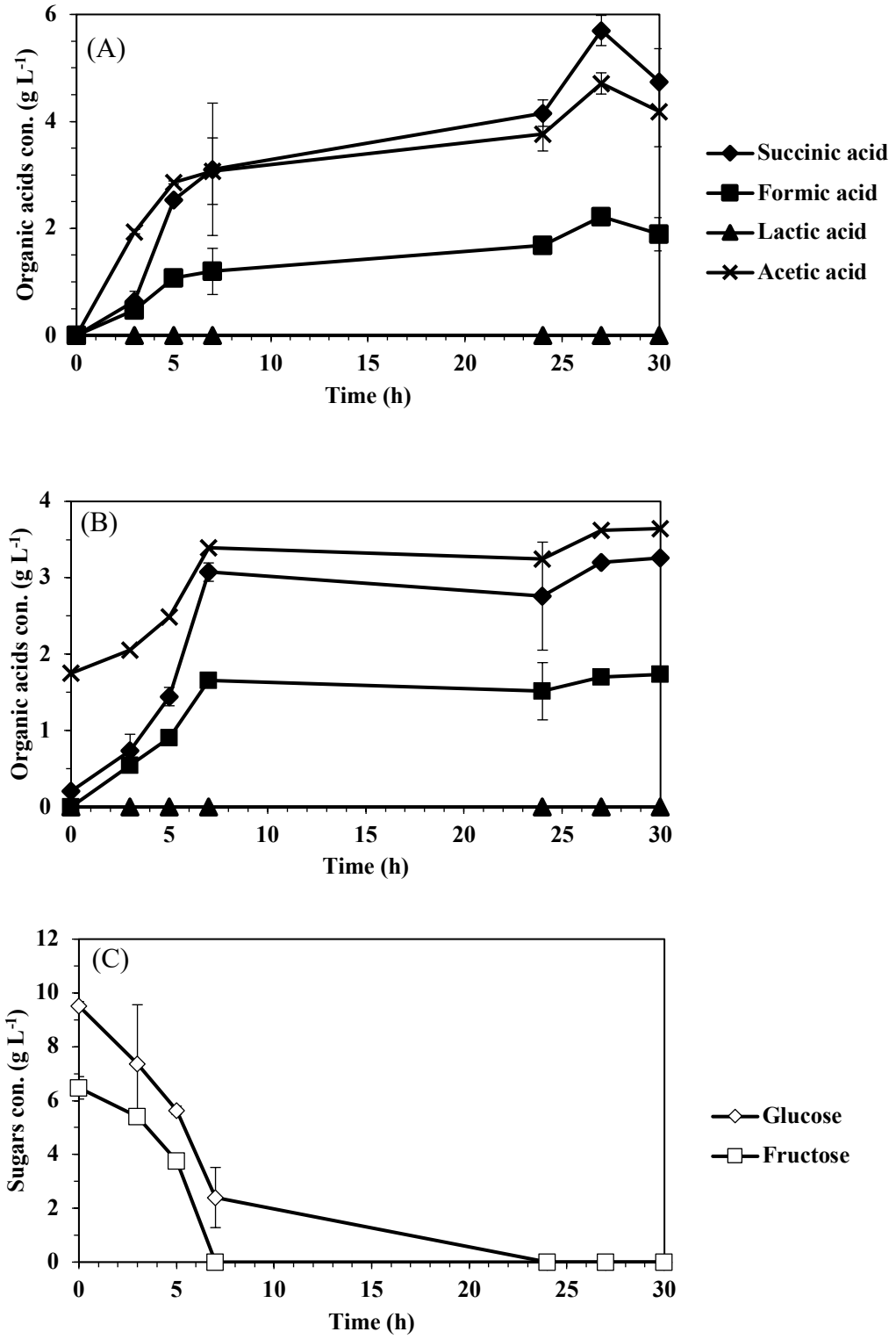

Figure 3.2.1: Succinic acid and by-products concentrations in *A. succinogenes* fermentations fed with (A) glucose and (B) fructose. (C) Utilization for sugars in each culture.

3.2.2 Succinic acid derived from CPW

CPW was treated through a series of physicochemical and biochemical processes constituting the biorefinery depicted on Figure 1.6.1. Essential oils (0.43% w/w) were first extracted from the waste while the solid residue was dried and applied to dilute acid hydrolysis. The choice of conditions for acid hydrolysis aimed at generating a hydrolyzate rich in carbohydrates assimilable by *A. succinogenes* based on existing literature as well as on the experiments presented below in section 3.2.2.2. Talebnia et al. (2008) demonstrated that the optimal conditions for dilute acid hydrolysis of CPW, based on the maximum total sugar yield and the minimum yield of HMF constitute the use of 0.5% (v/v) H_2SO_4 at 116 ^oC for 13 min. Thus, the presence of HMF, a common inhibitor that is often present in lignocellulosic hydrolyzates could inhibit *A. succinogenes* fermentations and its content should be carefully considered (Gunnarsson et al., 2015). Furthermore, the structures of arabinose and galactose are more stable at temperatures higher than 120 ^oC, while fructose is more stable between 100 ^oC and 120 ^oC as compared to higher temperatures (Grohmann et al., 1995). Thus, since *A. succinogenes* demonstrates lower succinate yields when fed with galactose and arabinose as compared to fructose (Pateraki et al., 2016), the range of selected temperatures for dilute acid hydrolysis was limited below 120 ^oC. Dilute acid hydrolysis of CPW was tested with the use of the conditions specified in section 2.2, which were screened based on measurement of the total sugars yield as well as the formation of the final fermentation product. Pectin (23.25% w/w) was extracted from the hydrolyzate through ethanol addition according to the protocol presented in Pourbafrani et al. (2010) and described in section 2.2, while distillation was applied at 80 ^oC for the removal of ethanol from the hydrolyzate. Subsequently, the hydrolyzate was mixed with the solid residues from dilute acid hydrolysis and it was enzymatically hydrolyzed with cellulases and β-glucosidases applied in different ratios aiming to enhance the release of fermentable sugars. All the hydrolyzates generated through dilute acid treatment or with a combination of acid/enzyme hydrolysis were tested as feedstocks for the production of succinic acid by *A. succinogenes*.

3.2.2.1 Elemental analysis

Hydrolyzates obtained through acid hydrolysis (conditions: 109 ^{o}C, 10 min, 5% of dry raw material) as well as a combination of sequential acid and enzyme hydrolysis (109 ^{o}C, 10 min, 5% of dry raw material/20 IU crude cellulase solution g_{drm}^{-1}) of CPW, were analyzed through ICP-MS to evaluate the release of metal ions by each process. Additionally, the analysis was also performed for the crude cellulase solution to determinate the ion content contributed through enzymes' addition. ICP-MS analysis demonstrated the release of Mg^{2+}, Ca^{2+} and Fe^{2+} in substantially higher concentrations as compared to other detected metal ions, such as Li^{+1}, Be^{+2}, Ti^{+4}, V^{+5}, Mn^{+2}, Co^{+2}, Ni^{+2}, Cu^{+2}, Sr^{+2}, Mo^{+6}, Sb^{+5} and Pb^{+2} (Figure 3.2.2) for both hydrolyzates obtained through acid and the combined acid/enzyme hydrolysis. Specifically, 45.03 mg L^{-1} Mg^{2+}, 20.75 mg L^{-1} Ca^{2+} and 2.51 mg L^{-1} Fe^{2+} were measured in hydrolyzates obtained with the use of acid hydrolysis, while the rest of the metal ions detected were present in trace concentrations which were lower than 0.2 mg L^{-1}. Furthermore, the hydrolyzate obtained via a combination of sequential acid and enzyme hydrolysis (Figure 3.2.2) comprised substantially higher concentrations of Mg^{2+} (139.46 mg L^{-1}), Ca^{2+} (65.65 mg L^{-1}), Ti^{+4} (1.14 mg L^{-1}) and Mn^{+2} (1.33 mg L^{-1}) as compared to the hydrolyzate generated through acid hydrolysis. The rest of the substances detected did not demonstrate notable concentration difference between the two treatments. Mg^{2+} was detected in relatively high concentrations in the crude cellulase solution applied, demonstrating that following subtraction of the enzymatic solution's content in ions the net liberation of Mg^{2+} during enzyme hydrolysis constituted approximately 22 mg L^{-1}. However, the rest of the ions tested in the cellulase media included concentrations below the detection limit and did not affect the ionic composition of hydrolyzates.

Metal ions can be important factors affecting the efficiency of microbial fermentations. The concentrations of Cu^{2+}, Pb^{2+} and Ni^{2+} released from CPW by both pretreatment approaches (Figure 3.2.2) were substantially lower as compared to the inhibitory levels previously identified for rumen micloflora (Forsberg, 1978). Specifically, 150 mg L^{-1} of Pb^{2+} and 200 mg L^{-1} of Ni^{2+} did not present any negative effect on bacterial growth, while 21 mg L^{-1} of Cu^{2+} was required to inhibit cells by 50%. Thus, based on the release of two orders of magnitude lower ion

concentrations, the hydrolyzates generated are not expected to include metal ions at inhibitory levels for *A. succinogenes*, which is a strain known to have been isolated from rumen microflora. Furthermore, Mg^{2+} and Ca^{2+} ions serve as important cofactors for *A. succinogenes* fermentations. Ca^{2+} are necessary for preserving the fluidity and permeability of the cell membrane, thus facilitating energy and transfer regulation (Norris et al., 1996; Li et al., 2011). Moreover, apart from the positive influence of $MgCO_3$ as a neutralizing agent for succinic acid production, which needs to be supplemented in *A. succinogenes* fermentations for optimal performance, Mg^{2+} ions do not interrupt the stability of the membrane and cell flocculation is not observed (Pateraki et al., 2016). The results presented on Figure 3.2.2 demonstrate that during acid/enzyme hydrolysis, Mg^{2+} and Ca^{2+} ions were liberated at substantially higher concentrations as compared to acid hydrolysis highlighting the favorable effect of combining the two hydrolysis methods for the release of ions that usually need to be supplemented to the fermentation medium.

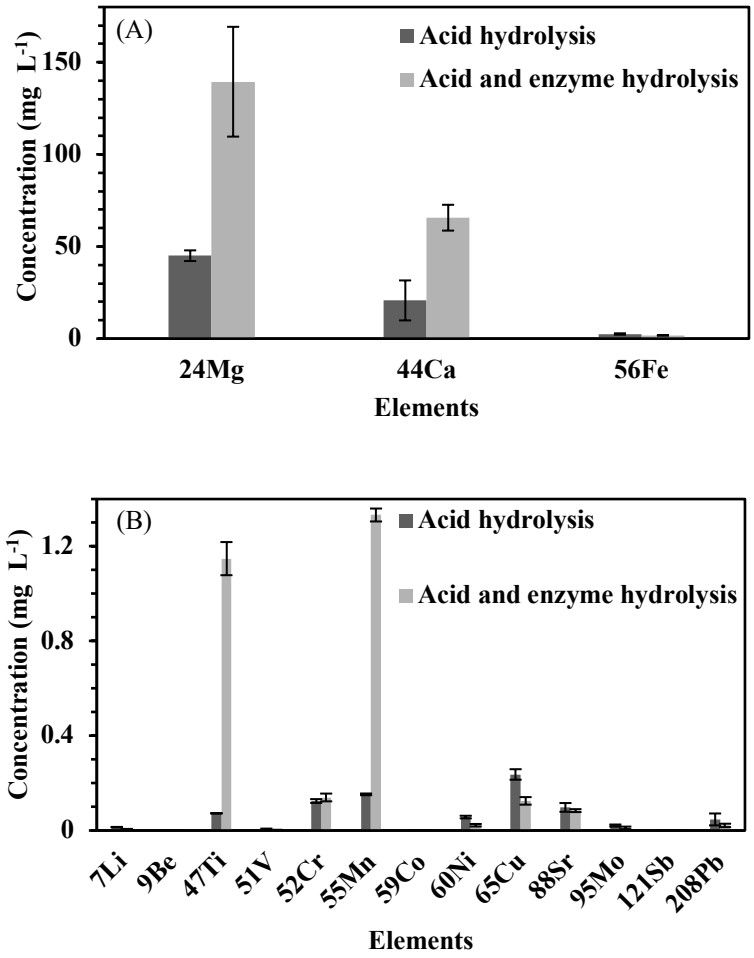

Figure 3.2.2: Concentration of metal ions generated using dilute acid hydrolysis and a combination of sequential acid and enzyme hydrolysis. (A) Metal ions with concentrations higher than 1.5 mg L^{-1}; (B) Metal ions with concentrations lower than 1.5 mg L^{-1}. Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

3.2.2.2 Succinic acid production using hydrolyzates of CPW obtained through acid treatment

Dilute acid hydrolysis was applied to break down cellulose and hemicellulose into simple sugars, constituting also a necessary processing step for extracting pectin from the waste (Kaya et al., 2014). Acid hydrolysis conditions were selected based on the relevant literature as described in section 3.2.2 and the conditions tested in the experiments are listed on Table 2.2.1. The yields of reducing sugars (Figure 3.2.3) released through the process were obtained using NMR. The highest

sugar yields were achieved using 5% (w/w) of dry CPW and ranged between 0.17-0.21 $g_{total\ reducing\ sugars(trs)}\ g_{drm}^{-1}$. Thus, the maximum yield of glucose equivalents liberated from the raw material was achieved at 116 ºC for 10 min reaction time with 5% (w/w) of dry CPW, through the addition of 0.5% (v/v) sulfuric acid. However, the use of 10% (w/w) of dry CPW substantially reduced the yields obtained to a level of 0.08-0.12 $g_{trs}\ g_{drm}^{-1}$ demonstrating that low material contents enhanced the effect of acid hydrolysis. This conclusion was clarified by the t-test ($p<0.05$) performed to identify statistically significant differences in the mean values obtained for the various acid hydrolysis conditions stated above. Pourbafrani et al. (2010) achieved a maximum sugar yield of 0.41 g g^{-1} of total dry citrus waste optimizing hydrolysis conditions through application of central composite rotatable experimental design that involved dilute acid hydrolysis of CPW at a substantially higher temperature (150 ºC) for 6 min as compared to the current work. However, although higher sugar yields were obtained the considerably elevated temperatures employed are expected to raise processing costs as well as to potentially increase the content of inhibitors in the feedstock.

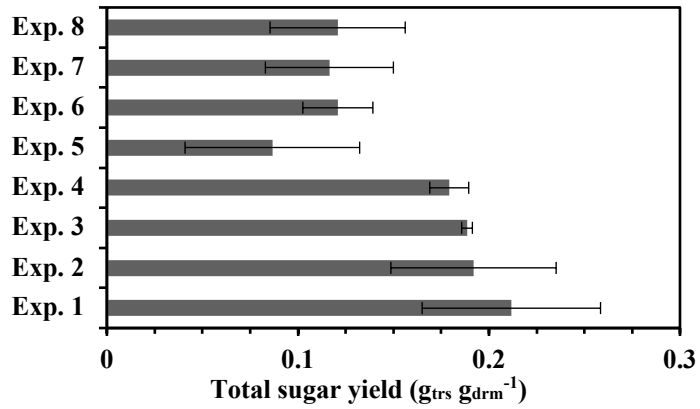

Figure 3.2.3: Yields of sugars obtained through acid hydrolysis of CPW. NMR was used for detection of monosacharides and disaccharides in the hydrolyzates generated, the concentration of which has been converted into glucose equivalents (yield: g_{ts} g_{drm}^{-1}, trs: total reducing sugars, drm: dry raw material). Exp. 1: 116 ^{o}C, 10 min, 5% (w/v) drm; Exp. 2: 116 ^{o}C, 20 min, 5% (w/v) drm; Exp. 3: 109 ^{o}C, 10 min, 5% (w/v) drm; Exp. 4: 109 ^{o}C, 20 min, 5% (w/v) drm; Exp. 5: 116 ^{o}C, 10 min, 10% (w/v) drm; Exp. 6: 116 ^{o}C, 20 min, 10% (w/v) drm; Exp. 7: 109 ^{o}C, 10 min, 10% (w/v) drm; Exp. 8: 109 ^{o}C, 20 min, 10% (w/v) drm. Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

The presence of HMF, a common product formed during acid hydrolysis of lignocellulose due to dehydration of hexoses, was also investigated by NMR aiming to assess the influence of process conditions on the formation of fermentation inhibitors in the hydrolyzate (Figure 3.2.4). Concentrations of HMF lower than 0.35 g L^{-1} have been previously demonstrated not to impose an inhibitory effect on *A. succinogenes* fermentations (Gunnarsson et al., 2015). HMF analysis performed in all the hydrolyzates generated involved concentrations lower than 0.038 g L^{-1}, highlighting that the content of the inhibitor in the feedstocks produced was an order of magnitude lower than the non-inhibitory concentrations reported in the literature. The influence of hydrolysis temperature, time and acid concentration on the generation of fermentation inhibitors (Palmqvist and Hahn-hagerdal, 2000) has been previously explored demonstrating that the concentration of HMF could continuously increase up to 120 h and 240 ^{o}C under dilute acid hydrolysis conditions (Asghari and Yoshida, 2006). Thus, it is expected that experiment 2 would exhibit the highest concentration of HMF since it was conducted at the

highest temperature tested (116 ºC) for 20 minutes (longest reaction time explored), while containing the lowest quantity of raw material employed (5% w/w). The t-test analysis performed demonstrated that the concentration of HMF in experiment 2 was the highest achieved exhibiting statistical difference as compared to the rest of the results including p-values that ranged between 0.02-0.04.

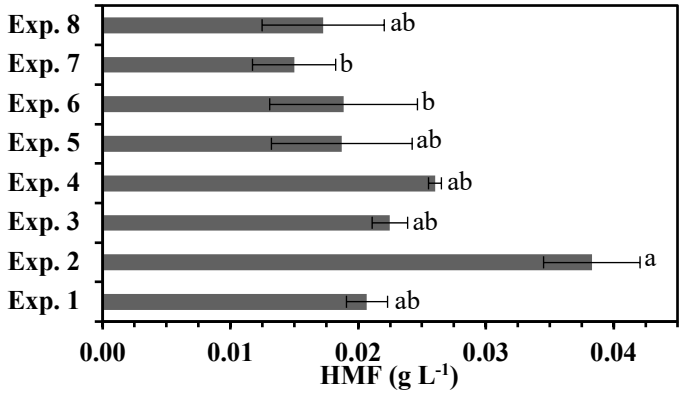

Figure 3.2.4: Concentration of HMF in dilute acid hydrolyzates. Exp. 1: 116 ºC, 10 min, 5% (w/v) drm; Exp. 2: 116 ºC, 20 min, 5% (w/v) drm; Exp. 3: 109 ºC, 10 min, 5% (w/v) drm; Exp. 4: 109 ºC, 20 min, 5% (w/v) drm; Exp. 5: 116 ºC, 10 min, 10% (w/v) drm; Exp. 6: 116 ºC, 20 min, 10% (w/v) drm; Exp. 7: 109 ºC, 10 min, 10% (w/v) drm; Exp. 8: 109 ºC, 20 min, 10% (w/v) drm. Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

The CPW hydrolyzates formed through the acid hydrolysis experiments described above were applied following pectin recovery for the production of succinic acid in *A. succinogenes* fermentations (Figure 3.2.5). Biomass production was maintained at the same levels during all fermentations, while the total consumption of sugars as well as acids' production are presented on Table 3.2.1. *A. succinogenes* could not completely metabolize the total carbohydrate content at the end of fermentation and apart from the formation of succinic acid, other organic acids such as formic, lactic and acetic acid were also generated in lower concentrations. The concentration of formic and lactic acid (reported as a combined concentration of the two metabolites) remained lower than 2.4 g L^{-1} in all experiments. However, the concentration of acetic acid was overall higher and reached 4.86 g L^{-1} in experiment 5. Although the concentration of succinic acid in the specific experiment was 9.11 g L^{-1}, the additional production of other organic

acids was 7.26 g L^{-1} highlighting that following future optimization of fermentation conditions, aiming to minimize the generation of by-products, succinic acid yields could be substantially improved.

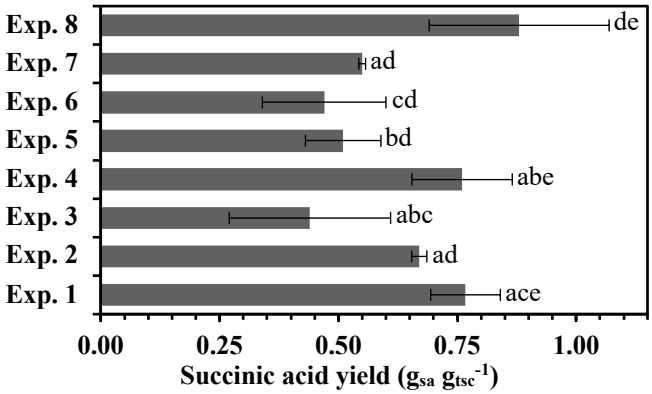

Figure 3.2.5: Succinic acid yields achieved in *A. succinogenes* fermentations of hydrolyzates obtained through dilute acid treatment of CPW (yield= g_{sa} g_{tsc}^{-1}, sa: succinic acid, tsc: total sugars consumed). Exp. 1: 116 ºC, 10 min, 5% (w/v) drm; Exp. 2: 116 ºC, 20 min, 5% (w/v) drm; Exp. 3: 109 ºC, 10 min, 5% (w/v) drm; Exp. 4: 109 ºC, 20 min, 5% (w/v) drm; Exp. 5: 116 ºC, 10 min, 10% (w/v) drm; Exp. 6: 116 ºC, 20 min, 10% (w/v) drm; Exp. 7: 109 ºC, 10 min, 10% (w/v) drm; Exp. 8: 109 ºC, 20 min, 10% (w/v) drm. Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

Table 3.2.1: Total sugars' consumption and final titre of products following fermentation of acid hydrolyzates.

Experiment	Concentration of sugars (g L^{-1})			Final product titers (g L^{-1})		
	ISC**	FSC**	TSC**	SA*	FA & LA*	AA*
1	12.00	4.01	7.99	6.13	1.29	2.17
2	11.91	3.73	8.18	5.48	1.39	2.67
3	15.79	2.15	13.64	5.95	1.73	2.79
4	12.94	4.85	8.09	6.17	1.42	2.60
5	21.83	4.08	17.75	9.11	2.40	4.86
6	15.84	3.09	12.75	5.95	1.73	2.79
7	9.47	2.12	7.35	4.05	1.27	1.59
8	16.15	5.26	10.89	9.57	1.13	3.03

*SA: Succinic acid, FA & LA: Formic and lactic acid, AA: Acetic acid.
**ISC: Initial sugars concentration, FSC: Final sugars concentration, TSC: Total sugars consumed.

In line with the above, the succinic acid yields presented (Figure 3.2.5) were calculated as the final concentration of succinic acid generated over the total concentration of sugars consumed. The highest yield reached was 0.88 g_{sa} g_{tsc}^{-1} and it was obtained through fermentation of the hydrolyzate obtained at 109 ºC for 20 min using 10% of dried CPW. However, Figure 3.2.3 indicates that the highest yield of total sugars released was achieved in the dilute acid hydrolysis performed at 116 ºC for 10 min reaction time using 5% (w/w) of dry CPW (presenting a yield of 0.77 g_{sa} g_{tsc}^{-1}). Thus, although higher succinic acid yields would be expected for process conditions generating elevated sugar yields, the slightly improved generation of succinic acid at 109 ºC could be due to the release of higher contents of sugars assimilable by *A. succinogenes* at lower temperatures (Grohmann et al., 1995). However, the t-test analysis performed between the results of Figure 3.2.5 did not

present statistical difference between the mean values of experiments 1 and 8 (p=0.44) demonstrating that either of the two conditions could be used.

Similarly to the present work, different methods of CPW pretreatment have been employed for generation of hydrolyzates applied as feedstocks in microbial fermentations. Steam explosion, hydrothermal sterilization, dilute acid hydrolysis and enzymatic hydrolysis (through application of pectinases, cellulases and β-glucosidases) have been used for the release of sugars from the cellulose, hemicellulose and pectin content of the waste (Grohmann and Baldwin, 1992; Grohmann et al., 1995; Wilkins et al., 2007a). Thus, the production of bioethanol in *S. cerevisiae* fermentations has been investigated in a citrus peel derived hydrolyzate generated through steam explosion (Wilkins et al., 2007b) followed by dilute acid hydrolysis and pectin recovery (Pourbafrani et al., 2010), where the yields obtained reached 0.43 g_{eth} g_{tsc}^{-1} for both pretreatment approaches applied. Moreover, acid pretreated CPW have been tested for the production of SCP that contained 35-40% of crude protein including high in vitro digestibility (73-88%) with the use of *G. candidum* (Vaccarino et al., 1989).

3.2.2.3 Enzyme hydrolysis

A combination of sequential acid and enzyme hydrolysis was tested aiming to produce higher amounts of fermentable sugars as well as to enhance the formation of succinic acid. The dilute acid hydrolyzate obtained at 116 ^{o}C for 10 min treatment of 5% (w/w) dried CPW was chosen for further enzymatic processing, given that the yield of total carbohydrates liberated under the specific conditions was high. Moreover, the lower initial raw material content applied would be expected to enhance the enzymatic conversion of polysaccharide molecules into fermentable sugars. Thus, enzyme hydrolysis was performed as described in section 2.3.1 and the enzyme load applied in each experiment is given on Table 2.2.2. The hydrolyzate employed for enzyme treatment contained an initial concentration of total carbohydrates of 16.6 g L^{-1} generated through the previous step of dilute acid hydrolysis (Figure 3.2.6). The net release of total sugars through enzyme hydrolysis in all experiments conducted ranged between 10-15 g L^{-1}. The highest release of total sugars was obtained in experiments B and F were following 40 h of incubation the final total sugar concentration was 29 g L^{-1} for both experiments, while the total

sugar yield reached 0.58 g_{trs} g_{drm}^{-1}. The specific yield was substantially higher as compared to the yield achieved through acid hydrolysis applied as a single treatment (0.21 g_{trs} g_{drm}^{-1}). Thus, the highest yields were achieved with the use of 30 IU of cellulases as well as 25 BGL (experiment B) and 50 BGL (experiment F) of β-glucosidases, respectively. In an attempt to reduce the cost of the developed process a combination of 30 IU of cellulases and 25 BGL of β-glucosidases was chosen as the most suitable enzyme load combination between the enzyme hydrolysis conditions tested for application in the proposed biorefinery.

Various pretreatments of citrus waste have been previously explored for their capacity to release fermentable sugars (Table 3.2.2). Wilkins et al. (2005) and Lennartson et al. (2012) have evaluated enzyme hydrolysis as the sole pretreatment applied for orange peel waste hydrolysis through the use of a mixture of 1.4 mg cellulase protein g_{drm}^{-1} and 1.7 mg β-glucosidase protein g_{drm}^{-1} as well as 0.24 FPU g_{drm}^{-1} of cellulase, 3.9 IU g_{drm}^{-1} of β-glucosidase and 1163 IU g_{drm}^{-1} of pectinase respectively. Although similar conditions (50 ^{o}C and pH-value of 4.8) were applied as compared to the current study the total sugar yield reached a lower level in both studies that ranged between 0.25-0.32 g_{trs} g_{drm}^{-1}. However, various researchers have previously suggested application of pretreatment in two sequential steps as a more efficient approach for CPW valorization. Thus, two stage acid hydrolysis (Oberoi et al., 2010), dilute acid hydrolysis and pectin recovery (Pourbafrani et al., 2010) as well as popping and enzyme hydrolysis (Choi et al., 2013) have been explored for citrus waste pretreatment and the total sugar yields achieved were 0.23 g_{ts} g_{drm}^{-1}, 0.41 g_{trs} g_{drm}^{-1} and 0.63 g_{trs} g_{rm}^{-1} respectively. The present work demonstrates that the yield of sugars released from the raw material could approximately double when a combination of acid and enzyme treatment is applied in CPW for the production of a carbohydrate-rich fermentation supplement as ehibited by the high efficiency achieved as compared to the literature (Table 3.2.2).

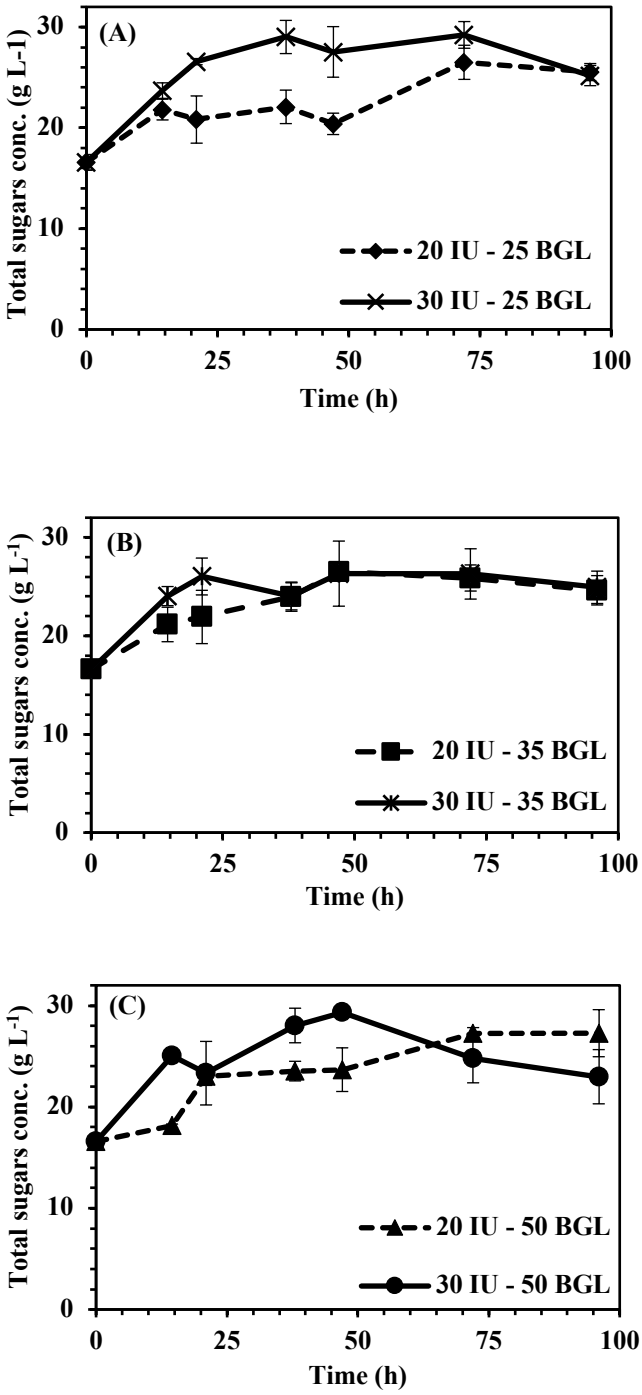

Figure 3.2.6: Concentrations of total liberated sugars during enzyme hydrolysis of CPW through addition of a combination of cellulases and β-glucosidases supplied at a content of 25 BGL (A), 35 BGL (B) and 50 BGL (C). Enzymatic hydrolyses was performed using hydrolyzates generated through the conditions of acid treatment specified in experiment 1 (Table 2.2.1). Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

Table 3.2.2 Yields of total reducing sugars released through citrus waste pretreatment.

Raw material	Pretreatment method	Yield of total reducing sugars	References
Orange peel waste	Enzyme hydrolysis	0.25-0.30 (g g_{drm}^{-1})	Wilkins et al. (2005)
Mandarin waste and banana peels	Steam depressurization	0.17 (g g_{drm}^{-1})	Sharma et al. (2007)
Orange peel hydrolyzate	Two stage acid hydrolysis	0.23 (g g_{drm}^{-1})	Oberoi et al. (2010)
Citrus waste	Dilute-acid hydrolysis and pectin recovery	0.41 (g g_{drm}^{-1})	Pourbafrani et al. (2010)
Orange peel waste	Enzyme hydrolysis	0.32 (g g_{drm}^{-1})	Lennartsson et al. (2012)
Mandarin peel waste	Popping and enzyme hydrolysis	0.63 (g g_{rm}^{-1})	Choi et al. (2013)
Citrus peel waste	Dilute acid hydrolysis and enzyme hydrolysis	0.58 (g g_{drm}^{-1})	Current study

3.2.2.4 Succinic acid production using hydrolyzates of CPW obtained through acid and enzyme treatment

The hydrolyzate generated through the two sequential steps of dilute acid (116 ^{o}C, 5% (w/w) for 10 min) and enzyme (30 IU of cellulases and 25 BGL of β-glucosidases) hydrolysis demonstrating the highest sugar yield was applied as feedstock for the production on succinic acid by *A. succinogenes* achieving a maximum succinic acid concentration of 8.25 g L^{-1} (0.70 g_{sa} g_{tsc}^{-1}). Based on the data generated, without optimization of fermentation conditions the product yield achieved reached 62.5% of the theoretical yield considering that the maximum glucose to succinic acid conversion yield for *A. succinogenes* corresponds to 1.12 g_{sa} $g_{glucose}^{-1}$ (Pateraki et al., 2016). *F. succinogenes* S85 has been previously employed for the production of succinic acid using pretreated CPW following D-limonene removal and drying (Li et al., 2010b). However, the succinic acid yield

obtained reached only 0.12 g_{sa} g^{-1} pre-treated citrus peel highlighting that the material should have been further hydrolyzed to generate a sugar-rich feedstock prior to fermentation. Previous studies demonstrated the release of reducing sugars through various technologies such as acid or enzyme hydrolysis as well as popping (Table 3.2.2). The results presented show that CPW treatment with relatively high reducing sugar yields entail higher energy demand. Acid hydrolysis experiments, including both two stage acid (Oberoi et al., 2010) and dilute-acid hydrolysis (Pourbafrani et al., 2010) as well as popping were performed at temperatures that ranged between 121-150 ^{o}C. Nevertheless, the current approach of CPW pretreatment integrates lower energy and environmental friendly technologies, due to the use of reduced acid hydrolysis temperatures (116 ^{o}C) combined with enzyme hydrolysis, while approximately reaching the maximum yield that exists in the literature (Table 3.2.2).

3.2.2.5 Economic analyses

The bio-based succinic acid production from renewable feedstocks holds the potential for sustainable replacement of its petroleum-based manufacturing (Jansen and van Gulik, 2014). The raw material (maleic anhydride) costs of the chemical process constitute 1.027 \$ kg^{-1} of succinic acid exhibiting an overall conversion yield of 95% w/w (Song and Lee, 2006). However, glucose costs 0.39 \$ kg^{-1} of succinic acid including an overall conversion of 91% w/w. Moreover, lignocellulosic biomass constitutes a low-cost alternative to the use of glucose as raw material for the production of succinic acid (Akhatar et al., 2014). Thus, the biochemical production of succinic acid from renewable resources could be a competitive alternative to petroleum-based processes saving fossil reserves and contributing environmental benefits as well as economic feasibility (Pinazo et al., 2015).

The economic feasibility of the biorefinery with the combined use of the enzymatic and the chemical treatment of CPW was assessed through comparison of the additional revenue contributed through enzyme hydrolysis and the costs incurred. The annual cost of the fixed capital investment (FCI) as well as the total production cost were estimated (based on Lam et al., 2014), considering treatment of 1 t CPW d^{-1} for 300 d per year (Table 3.2.3). The annual revenue of products was

estimated based on two scenarios. Apart from essential oils and pectin extraction, Scenario 1 included acid treatment as the sole hydrolysis process applied exhibiting production of 6.13 g L^{-1} (0.77 g_{sa} g_{tsc}^{-1}) of succinic acid. Scenario 2 involved combination of acid and enzyme hydrolysis where the concentration of succinic acid reached 8.30 g L^{-1} (0.70 g_{sa} g_{tsc}^{-1}). 4.3 kg of essential oils and 23.25 kg of pectin could be extracted from pretreatment of 1 t of CPW in both Scenarios, including annual revenues of 83,700 $ and 12,900 $ respectively (Table 3.2.3). 1 t of CPW could yield 12.26 kg and 16.6 kg of succinic acid in Scenarios 1 and 2 respectively, while the addition of enzymatic treatment increased the revenue by 11,718 $. However, the total production cost (excluding utilities) and the depreciation of the FCI calculated using the 20-years straight-line method constituted 17,886.50 $, indicating that the use of enzyme hydrolysis cannot be competitive given that the additional cost involved in enzyme hydrolysis is substantially higher compared to the revenue incurred by the increase in succinic acid production.

Table 3.2.3: Overall annual revenue generated from the succinic acid production facility for Scenarios 1 and 2, as well as the total fixed-capital investment and total production cost of enzyme hydrolysis.

Revenue			
		Scenario 1	**Scenario 2**
Succinic acid	9 \$ kg^{-1} (Lam et al., 2014)	**\$ 33,102**	**\$ 44,820**
Pectin	12 \$ kg^{-1} (Davila et al., 2015)	**\$ 83,700**	**\$ 83,700**
Essential oils	10 \$ kg^{-1} (Vlysidis et al., 2017)	**\$ 12,900**	**\$ 12,900**
Total fixed-capital investment for enzyme hydrolysis			
Type	**Component**	**Percentage of FCI* (%)**	**Cost (\$)**
Direct	Purchased equipment	30	22,307.50
	Purchased-equipment installation	10	7,435.83
	Instrumentation and controls (installed)	4	2,974.33
	Piping (installed)	6	4,461.50
	Electrical systems (installed)	4	2,974.33
	Building (including services)	10	7,435.83
	Yard improvements	2	1,487.16
	Service facilities (installed)	5	3,717.91
	Land	0	0
Indirect	Engineering and supervision	6	4,461.50
	Construction expenses	8	5,948.66
	Legal expenses	2	1,487.16
	Contractor's fee	3	2,230.75
	Contingency	10	7,435.83
		Total FCI	**74,358.33**
Total production and depreciation cost of enzyme hydrolysis			
Component	**Description**		**Cost (\$ $year^{-1}$)**
Raw materials	enzymes based on Olofsson et al., 2017		5,325
Utilities	Nd		Nd
Operating labor cost	0.25 x 30,000 \$ based on Vlysidis et al., 2017		7,500
Maintenance & repair	1% of FCI		743.58
Laboratory charges	8% of operating labor cost		600
Depreciation	5% of FCI		3,717.92
	Total production and depreciation cost		**17,886.50**

*The percentages were estimated based on Lam et al. (2014).

nd: No data.

3.3 Enhancement of succinic acid fermentation

3.3.1 Effect of nitrogen source and vitamins

The source of nitrogen constitutes a crucial parameter in microbial fermentations providing a nutrient that could be directly linked to cell proliferation and metabolite biosynthesis. Yeast extract, which is often applied as a source of nitrogen in fermentation, provides proteins, lipids, vitamins and other compounds constituting an important co-factor for cell growth in succinic acid fermentations. However, the significant cost contributed by the application of yeast extract in industrial processes (Borges and Pereira Jr., 2011), increases the need for investigating the use of alternative nitrogen resources. Various rich in carbohydrates hydrolyzates have been previously tested for the production of succinic acid by *A. succinogenes* through supplementation of CO_2, as well as nitrogen source (yeast extract) and vitamins. Specifically, the use of feedstocks derived from wheat, bread, cotton stalk and macroalgae in fermentations resulted in production of 62.1 g L^{-1} (1.02 g_{sa} g^{-1}_{tsc}), 47.3 g L^{-1}, 63 g L^{-1} (0.64 g_{sa} g^{-1}_{tsc}) and 33.8 g L^{-1} (0.63 g_{sa} g^{-1}_{tsc}) of succinic acid respectively (Vemuri et al., 2002; Jantama et al., 2008; Okino et al., 2008; Van De Graaf et al., 2012).

The current study tested the production of succinic acid in *A. succinogenes* fermentations, evaluating the effect of different nitrogen sources (ammonium sulfate, yeast extract and corn steep liquor) as well as the addition of vitamins in CPW hydrolyzates generated following acid hydrolysis and pectin recovery. The highest succinic acid yield was achieved with the use of corn steep liquor and yeast extract (0.49 and 0.48 g_{sa} g^{-1}_{tsc} respectively), while the highest succinic acid concentration reached 17.8 g L^{-1} at 36 h when corn steep liquor was applied (Figure 3.3.1A). The formation of acetic and formic acid was measured at concentration lower than 3 g L^{-1} in all succinic acid fermentations performed. The use of corn steep liquor and yeast extract was combined with the addition of vitamins in *A. succinogenes* fermentations obtaining 0.48 and 0.47 g_{sa} g^{-1}_{tsc} respectively. Moreover, the concentration of succinic acid reached 14.43 g L^{-1} and 10.28 g L^{-1} at 24 h with the addition of corn steep liquor and yeast extract respectively, reducing the duration of fermentation (Figure 3.3.1B). However, the concentration of by-

products was increased in both cultures. Specifically, formic and acetic acid were produced at 6.3 g L^{-1} and 7.5 g L^{-1} respectively, in fermentations supplemented with yeast extract, while 5.6 g L^{-1} of formic acid and 8.8 g L^{-1} of acetic acid were produced in succinic acid fermentations where corn steep liquor was added as the nitrogen source.

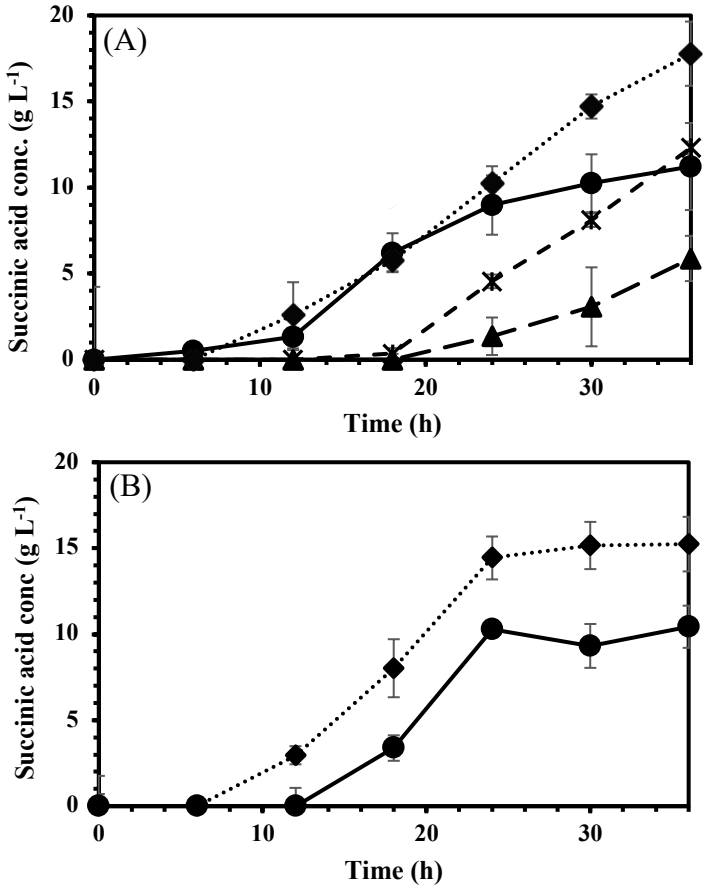

Figure 3.3.1: Concentration of succinic acid achieved in *A. succinogenes* fermentations of hydrolyzates obtained through dilute acid treatment of CPW with the addition of: ammonium sulfate (—▲—), yeast extract (—●—), corn steep liquor (···◆···), and without the addition of nitrogen source (– ✱ –). (A) Without vitamins addition and (B) with the supplementation of vitamins

The combined addition of corn steep liquor and vitamins in CPW hydrolyzates was selected in subsequent experiments based on the highest concentration of succinic acid formed under the specific conditions as compared to the rest of the experiments. Batch fermentation was performed using a 0.45 L bioreactor. The maximum succinic acid concentration and product yield observed following 27 h

were 18.5 g L^{-1} and 0.62 g_{sa} g_{tsc}^{-1} respectively. At the specific time point formic and acetic acid were also formed at a concentration of 5.5 g L^{-1} and 11.3 g L^{-1} respectively (Figure 3.3.2). The final concentration of succinic acid as well as the final product yield were increased as compared to the results obtained in shake flasks where the pH-value was not controlled. Figure 3.3.3 presents an overall mass balance of the CPW biorefinery proposed. Thus, following steam distillation of 1 t of raw material, 4.3 kg of essential oils can be obtained. Acid hydrolysis, which constitutes the subsequent processing step, was performed using the stillage that contained 72.9 g of total sugars and 130 kg of solids. The hydrolyzate generated contained 100.4 kg and 73.8 kg of BR and total sugars respectively. During pectin extraction 39.7 kg of the heteropolysacharide were obtained. The remaining hydrolyzate contained 27.8 kg of total sugars producing 10.3 kg of succinic acid and 9.4 kg by-products (acetic and formic acid). Nevertheless, the *A. succinogenes* fermentation showed that 11.1 kg of total sugars remained in the fermentation broth demonstrating the presence of carbohydrates that cannot be assimilated by the microorganism. Furthermore, the overall mass balance demonstrated that acid hydrolysis did not enhance the total sugar content, given that the total sugars before the process were 72.9 kg which was slightly increased to 73.8 kg following hydrolysis. According to the results obtained in section 3.3.1 hemicellulose decreased by 4.6%, while the utilization of C5 sugars by *A. succinogenes*, such as xylose and arabinose, is known to result in lower succinate yields (Li et al., 2010b) demonstrating that acid hydrolysis cannot significantly enhance succinic acid production. However, acid hydrolysis constitutes an important step for pectin extraction (Kaya et al., 2014), which is an important high added value product of the proposed biorefinery.

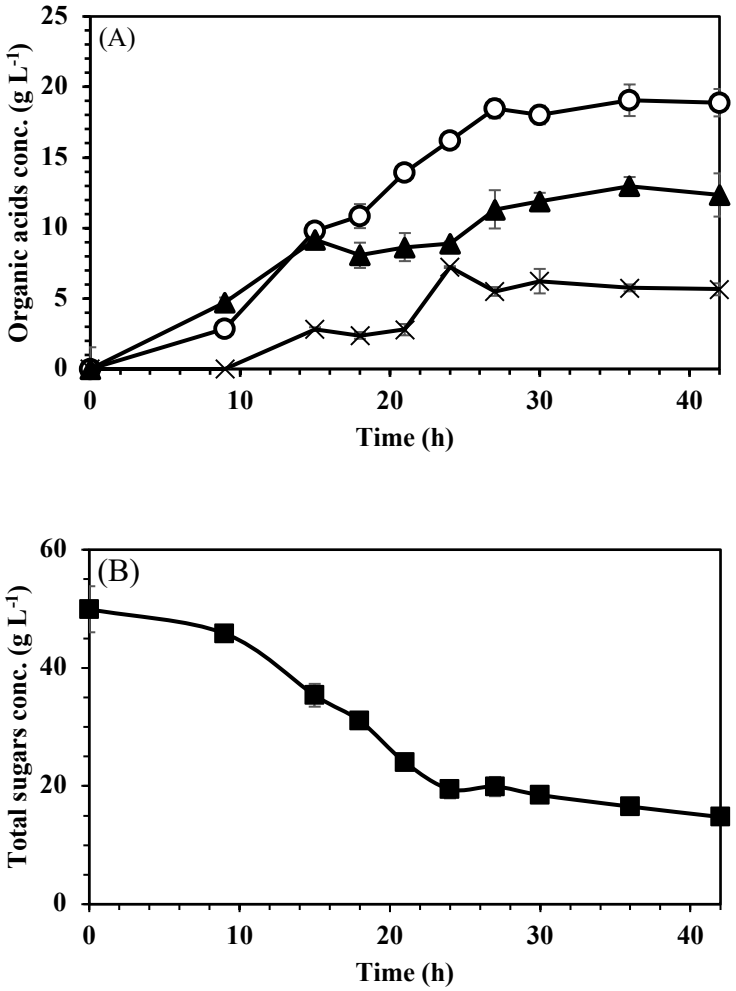

Figure 3.3.2: Succinic acid (—○—) and by-products (formic (—×—) and acetic (—▲—) acids) concentrations in *A. succinogenes* fermentations (A) and utilization of total sugars (B).

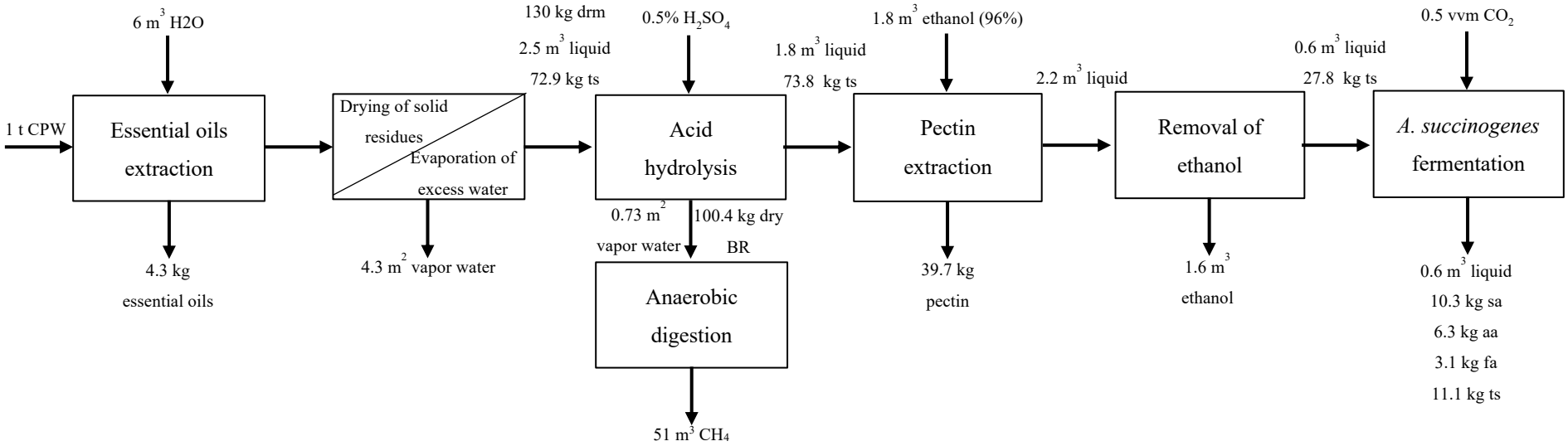

Figure 3.3.3: Overal mass balance of CPW treatment for succinic acid production.

3.3.2 Simultaneous saccharification and fermentation

SSF was applied in a hydrolyzate obtained following acid hydrolysis performed under the optimal conditions previously identified in an attempt to enhance succinic acid production. As mentioned above, enzyme hydrolysis was carried out at 50 ºC at pH-value that ranged between 4.5-5. However, the optimum pH-value and temperature for *A. succinogenes* fermentations are known to vary between 6-7.2 and 37-39 ºC respectively (Pateraki et al., 2016). Li et al. (2013) demonstrated that *A. succinogenes* could not grow at 50 ºC, while the microorganism's activity was reduced at temperatures higher than 40 ºC. Therefore, given that saccharification and fermentation require different temperatures and pH-values, their combined use should consider the conditions required in both processes. Thus, in the current work the pH-value and temperature needed were chosen based on Chen et al. (2011), which indicated that the SSF process could be performed at 38 ºC and pH-value of 6.4.

The experiments presented above demonstrate that succinic acid fermentations of CPW hydrolyzates were more efficient through supplementation of corn steep liquor as nitrogen source and vitamins' addition. The preliminary study conducted also indicated that the most suitable enzyme ratio included the use of 30 IU cellulases and 25 BGL β-glucosidases. Therefore, batch SSF was implemented in a 0.45 L suspended growth bioreactor under controlled pH-value, temperature and stirring. Following 63 h of cultivation the highest succinic acid concentration achieved was 11.9 g L^{-1}, while the product yield reached 0.39 g_{sa} g_{tsc}^{-1}. The by-products (formic and acetic acid) formed during the fermentation process were maintained to concentrations lower than 2.4 g L^{-1} (Figure 3.3.4A).

In the SSF process, total sugars' concentration was stable for the first 18 h of fermentation (Figure 3.3.4B), while the final concentration of succinic acid as well as the final product yield remained at lower levels as compared to the experiments of the previous section, which were conducted without the use of enzymes. Therefore, the lower production rate and final concentration of succinic acid achieved suggest that enzymatic hydrolysis was potentially the limiting step in SSF under the present conditions. A similar dilute acid hydrolysis process to the present

work has been applied as pretreatment achieving final succinic acid production that reached 16.4 g L^{-1} and substantially lower product yield 0.13 g_{SA} g^{-1}_{drm} (Chen et al., 2011). Previous SSF studies have been carried out using *A. succinogenes* for the production of succinic acid from lignocellulosic materials such as empty fruit bunces (Akhatar and Idris, 2017) , duckweed (*Landoltia punctate*) (Shen et al., 2018)), corn stover (Zheng et al., 2010) and rapeseed meal (Chen et al., 2011) employing a similar range of temperatures (37-38 ^{o}C) and pH-value (6.4-7) with the current study. However, the agitation used in fermentation as well as the pretreatment method followed in each case was different (Table 3.3.1).

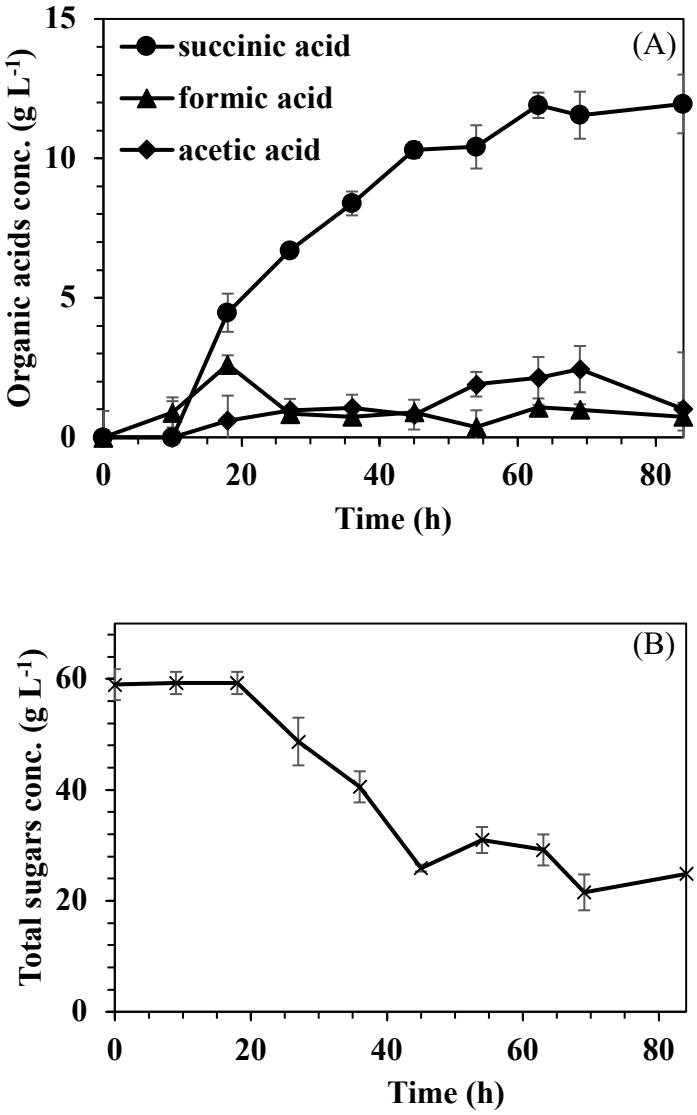

Figure 3.3.4: SSF of a CPW hydrolyzate for the production of succinic acid conducted at 38 °C and pH-value of 6.4. The enzymes employed included the use of 30 IU cellulases and 25 BGL β-glucosidases. (A) Concentration of organic acids and (B) concentration of total sugars during the experiment.

Table 3.3.1: Simultaneous saccharification and fermentations of different raw material by *A. succinogenes*.

Raw material	Pretreatment	T (°C)	pH-value	rpm	SA (g L^{-1})	Yield	Reference
Rapeseed meal	dilute acid	38	6.4	300	16.4	0.13 (g_{SA} g_{drm}^{-1})	Chen et al. (2011)
Corn stover	preheated dilute alkaline	38	6.5-7	nd	47.4	0.72 (g_{SA} g_{pcs}^{-1})	Zheng et al. (2010)
Empty fruit bunches	without treatment	38	6.5	210	16.1	0.23 (g_{SA} g_{rm}^{-1})	Akhatar and Idris (2017)
	autoclave alkali				20.9	0.29 (g_{SA} g_{rm}^{-1})	
Duckweed	Liquefaction	37	7	100	52.41	nd	Shen et al. (2018)
CPW	dilute acid	38	6.4	100	11.9	0.1 (g_{SA} g_{drm}^{-1})	Current study

SA: Succinic acid, drm: Dry raw material, pcs: Pretreated corn stover, rm: Raw material, nd: No data, CPW: Citrus peel waste.

3.3.3 Fed-batch fermentation

The inhibition of *A. succinogenes* growth due to high initial concentrations of glucose or sucrose has been previously evaluated (Liu et al., 2008b; Zheng et al., 2009; Jiang et al., 2014). The results obtained demonstrated that *A. succinogenes* could grow in a medium containing 100 g L^{-1} of total sugars and succinic acid was produced as the major product. However, the production of succinic acid was significantly increased at a range of initial sugar concentrations between 35-65 g L^{-1}, while the maximum production was obtained using an initial total sugar content of 65 g L^{-1} (Liu et al., 2008b; Zheng et al., 2009). According to Chen et al. (2011), *"the fed-batch procedure could provide a way of dealing with the initial mixing problems at high substrate loadings"*. Therefore, a fed-batch experiment was performed in the bioreactor aiming to improve succinic acid production through addition of the CPW hydrolyzate at regular time intervals during the cultivation. Succinic acid and by-products formation as well as the total sugars consumed are depicted in Figure 3.3.5.

The reactor was loaded with the CPW hydrolyzate that included initial total sugars concentration of 53.84 g L^{-1} derived from 70 g_{drm} and supplemented with 5 g L^{-1} of csl and vitamins. The production of succinic acid reached 12.9 g L^{-1} following 24 h cultivation, while the succinic acid yield obtained at the specific time point was 0.75 g_{sa} g_{tsc}^{-1}. Although the concentration of succinic acid was slightly lower as compared to that of the experiment conducted in section 3.3.1 which was performed under similar conditions (16.2 g L^{-1} at 24 h), the product yield was higher than the one measured before (0.53 g_{sa} g_{tsc}^{-1}).

As shown in Figure 3.3.5, following two supplementations of CPW hydrolyzate derived from processing of 35 g of drm at 24 and 48 h, the succinic acid concentration was increased to 22.4 g L^{-1}. Moreover, a yield of 0.73 g_{sa} g_{tsc}^{-1} was achieved while the productivity reached 0.45 g L^{-1} h^{-1}. However, significant amounts of formic and acetic acid were produced as by-products, the concentration of which was measured at 7.7 and 12.2 g L^{-1} respectively. Moreover, although the addition of the hydrolyzate at 24 h resulted in net succinic acid production of 12.9 g L^{-1}, the subsequent dose at the feedstock exhibited a lower effect fermenting net

production of 9.5 g L^{-1}. Jiang et al. (2014) reported that the production of formic acid by *A. succinogenes* is increased in the presence of sucrose. However, although the characterization of hydrolyzates composition in simple sugars was not possible in the current study due to the instrumentation available, Wilkins et al. (2005) demonstrated the presence of sucrose in OPW hydrolyzates. Furthermore, Chen et al. (2011) studied the fermentation of acid-pretreated rapeseed meal for succinic acid production using *A. succinogenes* through a fed-batch approach obtaining a similar performance as compared to the current study. The final succinic acid production reached 23.4 g L^{-1}, while the productivity was 0.33 g L^{-1} h^{-1}. However, high concentration of acetic acid was also accumulated highlighting that the low production of succinic acid formed could be due to acetic acid production when rapeseed meal was applied as substrate (Chen et al., 2011). Furthermore, Borges and Pereira Jr. (2011) investigated the production of succinic acid from sugarcane, reporting that nitrogen deficiency could trigger the formation of other metabolic by-products that include acetic and formic acid. Thus, the effect of nitrogen source concentration added in CPW hydrolyzate fermentations should be carefully considered. Table 3.3.2 presents the concentration, yield and productivity of succinic acid achieved in different batch and fed-batch fermentations of *A. succinogenes*. Similarly to the results obtained here the concentration of succinic acid and product yield were only slightly increased with the use of fed-batch condition in all studies performed using pretreated materials (e.g. corn straw, cane molasses).

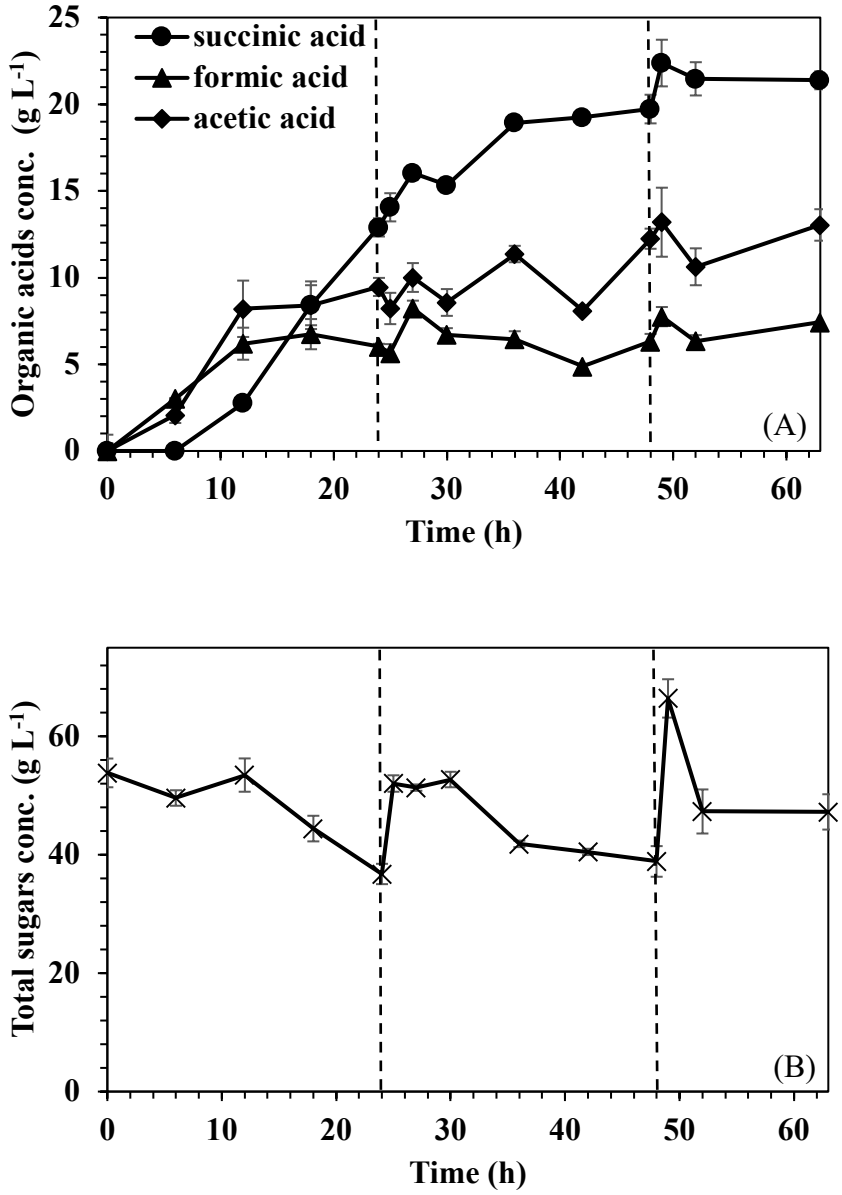

Figure 3.3.5: Fed-batch fermentation of CPW hydrolyzate applied for the production of succinic acid. The CPW hydrolyzate was added to the reactor following 24 and 48 h of cultivation, while each dose was produced through combination of acid and enzyme hydrolysis using 35 g of drm. (A) Concentration of organic acids, and (B) concentration of total sugars during the experiment.

Table 3.3.2: Succinic acid concentration and yield achieved in batch and fed-batch fermentations by *A. succinogenes*.

Carbon source	Batch			Fed Batch			Reference
	Succinic acid ($g\ L^{-1}$)	Yield ($g_{sa}\ g_{ts}^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	Succinic acid ($g\ L^{-1}$)	Yield ($g_{sa}\ g_{ts}^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	
Glucose	19	0.76	1.4	60.2	0.75	1.3	Liu et al. (2008a)
Glycerol	24.4	0.95	2.13	49.6	0.64	0.96	Carvalho et al. (2014)
Sucrose	57.1	0.72	Nd	60.5	0.83	2.16	Jiang et al. (2014)
Raw carob pods	9.4	0.54	1.32	18.97	0.94	1.43	Carvalho et al. (2016)
Corn straw	45.5	0.80	0.95	53.2	0.82	1.21	Zheng et al. (2009)
Cane molasses	46.4	0.79	0.97	55.2	0.80	1.15	Liu et al. (2008b)
Cane molasses	58.0	0.68	0.97	64.3	0.76	1.07	Shen et al. (2014)
Cassava root	93.34	0.77	1.87	151.44	1.51	3.22	Thuy et al. (2017)
CPW	18.5	0.62	0.69	22.4	0.73	0.45	Current study

CPW: Citrus peel waste, SA: Succinic acid, ts: total sugars.

3.4 CO_2 utilization in *A. succinogenes* fermentations

A. succinogenes is predicted to be an industrially important microorganism because of its high efficiency in succinic acid production, while the use of CO_2 in fermentations constitutes a very promising aspect contributing a series of environmental benefits. Wheat, bread, cotton stalk and macroalgal hydrolyzates, as well as glycerol, rapeseed meal and whey have been previously tested for the production of succinic acid by *A. succinogenes* through supplementation of CO_2, obtaining a range of yields between 0.115-1.02 g_{sa} g_{tsc}^{-1} (Table 3.4.1). Pateraki et al., (2016) underlined the importance of CO_2 on the environmental impact of the process emphasizing that anthropogenic energy-related CO_2 emissions could be employed for the production of succinic acid. Therefore, not only carbon assimilation and CO_2 recycling is targeted, but also the consumption of CO_2 during fermentation maintaining the reaction equilibrium. Furthermore, the use of CO_2 constitutes a crucial factor in microbial fermentations as it is inhibitory for the growth of a number of microorganisms (Dixon and Kell, 1989). Thus, the use of *A. succinogenes* contributes two major advantages including the high yield of succinic acid production as well as the consumption of CO_2, which make it competitive in succinic acid fermentations. The results of the fed-batch fermentation study demonstrate the ability of *A. succinogenes* to valorize CPW with high product yields (0.73 g_{sa} g_{tsc}^{-1}) demonstrating the potential for optimization of this novel CPW valorization route under environmental friendly conditions.

Table 3.4.1: Succinic acid production in fermentations utilizing different raw materials.

Raw material	Nitrogen source	Fermentation	Succinic acid ($g\ L^{-1}$)	Yield ($g_{sa}\ g_{tsc}^{-1}$)	Reference
Glycerol	YE (10 $g\ L^{-1}$)	Fed-batch, bioreactor	49.6	0.64	Carvalho et al. (2014)
Wheat hydrolyzate	YE (5 $g\ L^{-1}$) / Vit	Batch, bioreactor	62.1	1.02	Dorado et al. (2009)
Bread hydrolyzate	BH (200 $mg\ L^{-1}$ FAN)	Batch, bioreactor	47.3	nd	Leung et al. (2012)
Cotton stalk hydrolyzate	YE (30 $g\ L^{-1}$) / Urea (2 $g\ L^{-1}$)	Batch SSF*, shake flasks	63.0	0.64	Li et al., (2013)
Macroalgal hydrolyzate	YE (16.7 $g\ L^{-1}$)	Batch, bioreactor	33.8	0.63	Morales et al. (2015)
Rapeseed meal	YE (15 $g\ L^{-1}$)	Fed-batch SSF*, bioreactor	23.4	0.115	Chen et al. (2011)
Whey	YE (5 $g\ L^{-1}$) / Pep (10 $g\ L^{-1}$)	Batch, bioreactor	22.2	0.57	Wan et al. (2008)
CPW hydrolyzate	YE (5 $g\ L^{-1}$)	Fed-batch, bioreactor	22.4	0.73	Current study

YE: yeast extract, Vit: vitamins, BH: bread hydrolyzate, Pep: peptone, SA: succinic acid, tsc: total sugars consumed, nd: No data.

*Simultaneous saccharification and fermentation.

3.5 Ethanol production

The industrial production of ethanol is currently performed by catalytic hydration of ethylene (chemical method) (Mohsenzadeh et al., 2017) and by fermenting agricultural feedstocks (biochemical method) (Mohanty and Swain, 2019). Ethanol can serve as a green energy source, which is mainly produced using starch, sugar and carbohydrates, such as corn, potato, molasses, sugarcane and lignocellulosic biomass (Lin and Tanaka, 2006). Sugars can be directly converted to ethanol, while starchy and cellulosic materials should be first pretreated mainly using enzymes or chemicals to hydrolyze the polymers into sugars (Taghizadeh-Alisaraei et al., 2017). Herein, CPW has been applied as a cellulosic material, which includes an additional significant content of soluble sugars that could be applied for production of a hydrolyzate rich in carbon sources for ethanol fermentations. The thermotolerant strain *P. kudriavzevii* KVMP10 was tested and compared against two industrial yeasts (*K. marxianus* and *S. cerevisiae*) for ethanol production from CPW hydrolyzates under technologically favorable fermentation conditions. Thus, elevated bioprocess temperature (42 ^{o}C) was applied in an attempt to reduce operational costs associated to decreased energy use for cooling and lower contamination risk (Kyriakou et al., 2019; Tavares et al., 2019).

3.5.1 Evaluation of *P. kudriavzevii* KVMP10 fermentation parameters

3.5.1.1 The effect of D-limonene concentration on bioethanol production by P. kudriavzevii KVMP10

A newly isolated yeast (*P. kudriavzevii* KVMP10) was applied in alcoholic fermentations employing OPW. Prior to application in fermentation the inhibitory effect of D-limonene on *P. kudriavzevii* KVMP10 was first tested in solid media of the hydrolyzed OPW model solution supplemented with OP oil at concentrations that ranged between 0-1% (v/v) under aerobic conditions. Although increased concentrations of D-limonene resulted in longer lag-phases and substantial reduction in biomass formation, the microorganism was capable of growing on all OP oil concentrations tested and the colonies formed exhibited a cream color

(Appendix I). The duration of the lag-phase varied between 0-48 h, while maximum biomass formation was observed following 72-168 h of cultivation (Appendix I).

OP oil was also added in cultures conducted at 30 ºC (Figure 3.5.1). Increasing contents of the inhibitor resulted in prolonged lag-phases and slight reduction of ethanol formation. Thus, although without the inhibitor the maximum product concentration occurred within 24 h of cultivation, the addition of 0.01%, 0.05% and 0.10% (v/v) of OP oil resulted in maximum ethanol generation after 48, 54 and 73 h respectively. Moreover, OP oil slightly reduced ethanol production from 25 g L^{-1} in OP oil free media to 21-22 g L^{-1} with the use of the inhibitor. Various studies have shown the inhibitory effect of D-limonene highlighting the need for its removal prior to the bioprocess (Table 3.5.1). The lag-phases of *K. marxianus* and *S. cerevisiae* gradually increased for rising contents of OP oil between 0-0.2% (v/v), which also inhibited the formation of ethanol (Wilkins et al., 2007c). Prolonged lag-phases were not observed in simultaneous saccharification and fermentation of citrus peel supplemented with 0.08-0.043% (v/v) of D-limonene using *S. cerevisiae* (Wilkins et al. 2007b), while *Z. mobilis* and *M. indicus* have shown significant resistance to D-limonene (Wilkins, 2009; Lennartsson et al., 2012).

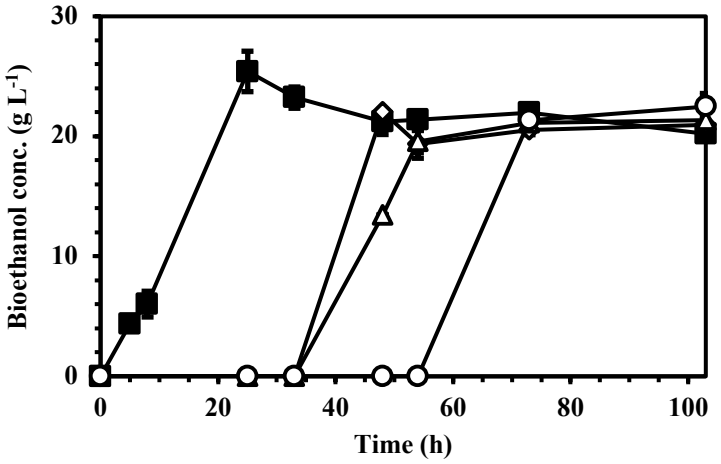

Figure 3.5.1: Bioethanol concentration in *P. kudriavzevii* KVMP10 fermentations conducted at 30 ºC for different OP oil contents. ■ : 0.00% (v/v) OP oil; ◇ : 0.01% (v/v) OP oil; △ : 0.05% (v/v) OP oil; ○ : 0.10% (v/v) OP oil.

Table 3.5.1: The inhibitory effect of D-limonene in ethanol bioprocesses.

Raw material	Microorganism	Process conditions	Range of initial D-limonene conc. (v/v)	Effect on lag-phase	Effect on ethanol conc.	Effect on product yield ($g_{ethanol\ (eth)}$ g_{sugars}^{-1})	Reference
Orange peel hydrolyzate	*K. marxianus*	SF, Aer, 37 ºC	0.00-0.20%	Gradual increase in lag-phase from 0 to 72 h	Gradual decrease from 37 to 13 g L^{-1}	Varying yields between 0.34-0.58	Wilkins et al. (2007c)
Orange peel hydrolyzate	S. cerevisiae	SF, Aer, 37 ºC	0.00-0.20%	Gradual increase in lag-phase from 0 to 72 h	Gradual decrease from 41 to 23 g L-1	No effect (0.43-0.45)	Wilkins et al. (2007c)
Citrus peel waste	*S. cerevisiae*	SSF, 37 ºC	0.08% - 0.43%	No effect (lag-phase duration > 24 h)	Gradual decrease from 39 to 7 g L^{-1}	No effect (0.43)	Wilkins et al. (2007b)
Orange peel hydrolyzate	*Z. mobilis*	SF, 30 ºC	0.00-0.20%	Gradual increase in lag-phase from 0 to 96 h	Substantial reduction for 0.20% (v/v) from 40-43 to 15 g L^{-1}	nd	Wilkins (2009)
Orange peel hydrolyzate	*Z. mobilis*	SF, 37 ºC	0.00-0.20%	Gradual increase in lag-phase from 0 to 72 h	Reduction for 0.20% (v/v) from a level of 41-43.5 to 36.5 g L^{-1}	nd	Wilkins (2009)
Orange peel hydrolyzate	*Rhizopus sp.*	SF, Aer, 32 ºC	0-2%	nd	nd	Varying yields between 0.28-0.37	Lennartsson et al. (2012)
Orange peel hydrolyzate	*M. indicus*	SF, Aer, 32 ºC	0-2%	No effect (26 h lag-phase duration)	nd	No effect (0.39-0.43)	Lennartsson et al. (2012)
Orange peel hydrolyzate	*Rhizopus sp.*	SF, An, 32 ºC	0-2%	nd	nd	Substantial reduction for 1-2% (v/v) from 0.31-0.39 to 0.20-0.25	Lennartsson et al. (2012)
Orange peel hydrolyzate	*M. indicus*	SF, An, 32 ºC	0-2%	No effect (34 h lag-phase duration)	nd	Varying yields between 0.36-0.43	Lennartsson et al. (2012)
Orange peel hydrolyzate	*M. indicus*	SF, Aer, 32 ºC	1% and 2%	Decrease from 55 to 28 h	No effect (19-20 g L^{-1})	Increase from 0.38 to 0.53	Lennartsson et al. (2012)
Orange peel hydrolyzate	*P. kudriavzevii* KVMP10	SF, 30 ºC	0.00-0.10%	Gradual increase in lag-phase from 0 to 54 h	Varying concentrations between 25-21 g L^{-1}	nd	Current study
Orange peel hydrolyzate	*P. kudriavzevii* KVMP10	SF, 42 ºC	0.00-0.05%	Gradual increase in lag-phase from 0 to 72 h	Gradual decrease from 54 to 21 g L^{-1}	nd	Current study

SF: Submerged fermentation, SSF: Simultaneous saccharification and fermentation, Aer: Aerobic conditions, An: Anaerobic conditions, nd: No data.

3.5.1.2 Bioethanol production at different temperatures

Temperature is considered as a major parameter of ethanol bioprocesses reducing the costs through more efficient product recovery and cooling of the bioreactor, higher growth and saccharification rates and reduction of microbial contamination (Banat et al., 1998). Thus, batch cultures of *P. kudriavzevii* KVMP10 were conducted at different temperatures aiming to determine the optimal conditions for ethanol production. Figure 3.5.2 presents the concentration of ethanol in experiments where the temperature ranged between 30-42 ^{o}C. The bioethanol concentration achieved was 54 g L^{-1} at 42 ^{o}C and it reached 39 and 40 g L^{-1} at 37 and 40 ^{o}C respectively. An experiment conducted at 45 ^{o}C resulted in 60 h of lag phase and substantial reduction in ethanol concentration to a maximum of 10 g L^{-1} indicating an inhibitory effect at higher temperatures.

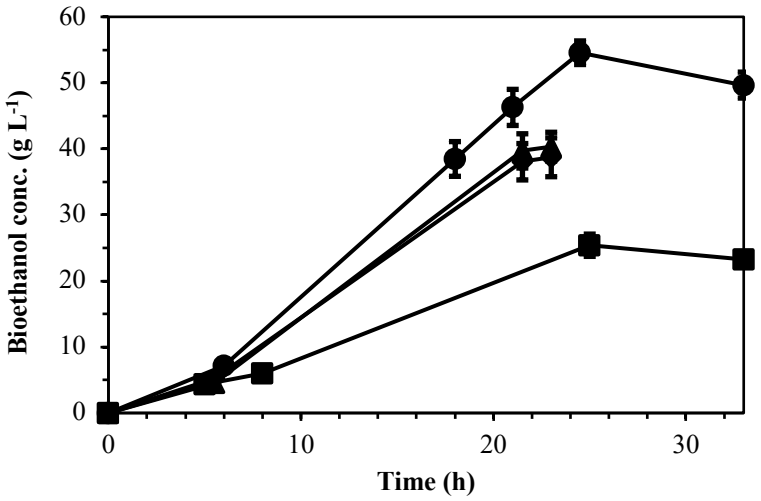

Figure 3.5.2: Bioethanol concentration in *P. kudriavzevii* KVMP10 fermentations conducted at different temperatures. Bioethanol produced at: ■ : 30 ^{o}C; ◆ : 37 ^{o}C; ▲ : 40 ^{o}C; ● : 42 ^{o}C.

A comparison of the data obtained here with past studies exemplifies that the isolated strain is a highly efficient bioethanol producer (Table 3.5.2). *P. kudriavzevii* KVMP10 could produce substantially higher ethanol concentrations to the 37, 41 and 43.5 g L^{-1} formed by the industrial strains *K. marxianus*, *S. sereviaciae* and *Z. mobilis* respectively, using the same model solution of OPW (Wilkins et al., 2007c; Wilkins, 2009). Moreover, the highest ethanol production

was obtained at 42 ºC, which is considerably higher to the 37 ºC used in the above studies. However, although *P. kudriavzevii* KVMP10 demonstrated elevated ethanol productivity (2.25 g L^{-1} h^{-1}), *S. cerevisiae* could generate ethanol at a productivity of 3.85 g L^{-1} h^{-1} in fermentations of mandarin peel waste (Choi et al., 2013). Although the capacity of *K. marxianus* to produce ethanol at elevated temperatures has received substantial interest (Banat et al., 1998; Koutinas et al., 2014b), several studies have recently studied the ethanologenic potential of the highly thermotolerant yeast *P. kudriavzevii*.

Although over the past decades *K. marxianus* has received substantial research interest due to a range of favorable characteristics and the capacity to produce ethanol at elevated temperatures (Banat et al. 1998; Koutinas et al. 2014b), several studies have recently investigated the ethanologenic potential of the highly thermotolerant yeast *P. kudriavzevii* under different conditions. Cassava starch hydrolyzates containing 180 g L^{-1} of total sugars have been employed in alcoholic fermentations of *P. kudriavzevii* PBB511-1 and DMKU 3-ET15 respectively (Yuangsaard et al., 2013; Kaewkrajay et al., 2014). The maximum ethanol concentrations monitored reached 37 g L^{-1} for PBB511-1 at 45 ºC and 78.6 g L^{-1} for DMKU 3-ET15 cultures performed at 40 ºC. Kaur et al. (2012) achieved an ethanol concentration of 19.82 g L^{-1} in *P. kudriavzevii* HOP-1 fermentations of a cotton stalk hydrolyzate that contained 42.29 g L^{-1} of glucose formed through alkali pretreatment. Furthermore, HOP-1 was used in SSF that generated 24.25 g L^{-1} of bioethanol from alkali treated rice straw, forming 35% and 200% more product as compared to *S. cerevisiae* at 40 and 45 ºC respectively (Oberoi et al., 2012). *P. kudriavzevii* D1C achieved maximum ethanol concentration and productivity of 74 g L^{-1} and 7.43 g L^{-1} h^{-1} respectively, in fermentations that utilized molasses containing 150 g L^{-1} of glucose at 40 ºC (Dandi et al., 2013). Enzymatically pretreated mandarin peel waste has been used in SSF of *P. kudriavzevii* at 40 ºC generating 33.87 g L^{-1} of ethanol with a productivity of 2.82 g L^{-1} h^{-1} (Sandhu et al., 2012), while the strain has also effectively utilised sugarcane juice with a total sugar content of 170 g L^{-1} exhibiting 71.9 g L^{-1} of ethanol concentration and a productivity of 4 g L^{-1} h^{-1} (Dhaliwal et al., 2011).

Table 3.5.2: Production of ethanol from citrus waste in microbial fermentations.

Raw material	Pretreatment method	Process conditions	Microorganism	Total initial sugar content	Ethanol concentration	Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	Yield ($g_{eth}\ g_{sugars}^{-1}$)	References
Orange peel hydrolyzate	nd	SF	*S. cerevisiae*	Nd	40-45 ($g\ L^{-1}$)	0.82-0.90	nd	Grohmann et al. (1994)
Orange peel hydrolyzate	nd	SF	*E. coli*	111 ($g\ L^{-1}$)	35-38 ($g\ L^{-1}$)	0.42-0.80	nd	Grohmann et al. (1995)
Orange peel hydrolyzate	nd	SF, 37 ºC	*K. marxianus*	90.6 ($g\ L^{-1}$)	37 ($g\ L^{-1}$)	0.51	0.44	Wilkins et al. (2007c)
Orange peel hydrolyzate	nd	SF, 37 ºC	*S. cerevisiae*	90.6 ($g\ L^{-1}$)	41 ($g\ L^{-1}$)	0.56	0.45	Wilkins et al. (2007c)
Citrus peel waste	Steam explosion	SSF, 37 ºC, 0.08% e.o.	*S. cerevisiae*	0.31 ($g\ g^{-1}_{drm}$)	39.03 ($g\ L^{-1}$)	1.62	0.43	Wilkins et al. (2007b)
Orange peel hydrolyzate	nd	SF, 37 ºC, 0.05% e.o.	*Z. mobilis*	90.6 ($g\ L^{-1}$)	43.5 ($g\ L^{-1}$)	0.60	0.48	Wilkins 2009
Mandarin waste and banana peels	Steam depressurization	SSF, 30 ºC	*S. cerevisiae* and *P. tannophilus*	0.17 ($g\ g^{-1}_{drm}$)	26.84 ($g\ L^{-1}$)	0.55	0.42	Sharma et al. (2007)
Orange peel hydrolyzate	Two stage acid hydrolysis	SF, 34 ºC	*S. cerevisiae*	27.54 ($g\ L^{-1}$)	30.33 ($g\ L^{-1}$)	3.37	0.46	Oberoi et al. (2010)
Mandarin waste	Hydrothermal sterilization	SSF, 37 ºC	*S. cerevisiae*	74 ($g\ L^{-1}$)	42 ($g\ L^{-1}$)	3.50	0.48	Oberoi et al. (2011)
Citrus waste	Dilute-acid hydrolysis and pectin recovery	SF, 30 ºC	*S. cerevisiae*	32.97 ($g\ L^{-1}$)	39.64 ($L\ tn_{wet\ rm}^{-1}$) 14.17 ($g\ L^{-1}$)	nd	0.43	Pourbafrani et al. (2010)
Mandarin waste	Enzyme hydrolysis	SSF, 40 ºC	*P. kudriavzevii*	64 ($g\ L^{-1}$)	33.87 ($g\ L^{-1}$)	2.82	0.67	Sandhu et al. (2012)
Mandarin waste	Steam explosion	SSF, 37 ºC	*S. cerevisiae*	Nd	60 ($L\ tn_{rm}^{-1}$)	nd	nd	Boluda-Aguilar et a.l (2010)
Lemon peel waste	Steam explosion	SSF, 37 ºC	*S. cerevisiae*	Nd	67.83 ($L\ tn_{rm}^{-1}$)	nd	nd	Boluda-Aguilar et al. (2013)
Orange peel waste	Enzyme hydrolysis	Aer, 32 ºC	*M. indicus*	39 ($g\ L^{-1}$)	15 ($g\ L^{-1}$)	0.62	0.39	Lennartsson et al. (2012)

Orange peel waste hydrolyzate	nd	SF, Aer, 32 ºC	*Rhizopus sp.*	50 (g L^{-1})	nd	nd	0.37	Lennartsson et al. (2012)
Orange peel waste hydrolyzate	nd	SF, Aer, 32 ºC	*M. indicus*	50 (g L^{-1})	nd	nd	0.41	Lennartsson et al. (2012)
Orange peel waste hydrolyzate	nd	SF, An, 32 ºC	*Rhizopus sp.*	50 (g L^{-1})	nd	nd	0.39	Lennartsson et al. (2012)
Orange peel waste hydrolyzate	nd	SF, An, 32 ºC	*M. indicus*	50 (g L^{-1})	nd	nd	0.43	Lennartsson et al. (2012)
Mandarin peel waste	Popping and enzyme hydrolysis	SF, 30 ºC	*S. cerevisiae*	0.63 (g g^{-1}_{rm})	46.2 (g L^{-1})	3.85	0.91	Choi et al. (2013)
Orange peel hydrolyzate	nd	SF, 30 ºC	*P. kudriavzevii* KVMP10	101 (g L^{-1})	25 (g L^{-1})	1.08	nd	Current study
Orange peel hydrolyzate	nd	SF, 42 ºC	*P. kudriavzevii* KVMP10	101 (g L^{-1})	54 (g L^{-1})	2.25	nd	Current study

SF: Submerged fermentation, SSF: Simultaneous saccharification and fermentation, nd: No data, Aer: Aerobic conditions, An: Anaerobic conditions, e.o.: Essential oils, eth: Ethanol, rm: Raw material.

3.5.1.3 The effect of D-limonene on P. kudriavzevii KVMP10 cultures under optimal conditions

In order to understand the capacity of *P. kudriavzevii* KVMP10 for application in OP valorization approaches, the inhibitory effect of D-limonene should be explored under conditions that maximize the production of ethanol. Thus, increasing OP oil contents were added in *P. kudriavzevii* KVMP10 cultures performed with a pH-value of 4.8 at 42 ºC (Figure 3.5.2). The use of 0.01% (v/v) peel oil reduced the maximum ethanol concentration formed from 54 g L^{-1} obtained in the absence of the inhibitor to 33 g L^{-1}. Moreover, the application of higher OP oil concentrations resulted in further reduction of the product concentration, which reached 23 and 21 g L^{-1} for OP oil contents of 0.025% and 0.05% (v/v) respectively. The bioethanol concentration obtained with the use of OP oil at 42 ºC was similar to that obtained at 30 ºC (Figure 3.5.1), demonstrating that the product yield was substantially reduced due to the inhibitor. *S. cerevisiae* is inhibited by D-limonene concentrations higher than 0.12% (v/v), while other strains have demonstrated microbial inhibition for D-limonene contents of 0.05-0.25% (v/v) (Wilkins et al., 2007b; Lennartsson et al., 2012).

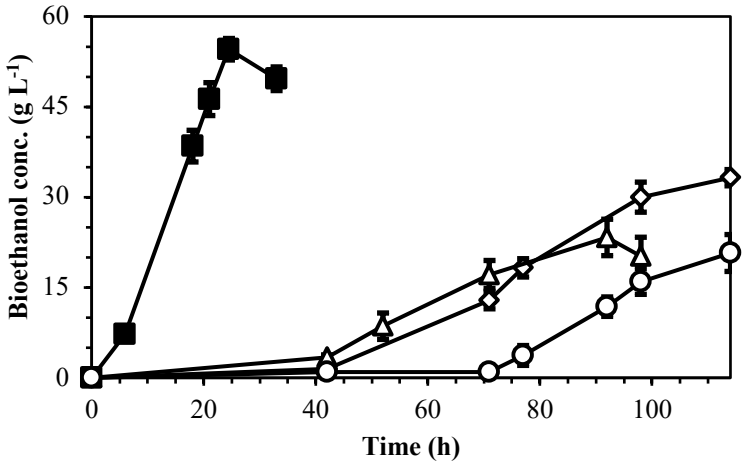

Figure 3.5.3: Bioethanol concentration in *P. kudriavzevii* KVMP10 fermentations conducted at 42 ºC for different OP oil contents. —■— : 0.00% (v/v) OP oil; —◇— : 0.01% (v/v) OP oil; —△— : 0.025% (v/v) OP oil; —○— : 0.05% (v/v) OP oil.

3.5.1.4 Ethanol production by individual sugars

The capacity of *P. kudriavzevii* KVMP10 to produce ethanol from individual sugars was tested in cultures supplemented with 10 g L^{-1} of each carbon source, which were maintained at 42 ºC with a pH-value of 4.8 (Figure 3.5.3). Glucose, sucrose and fructose generated the highest ethanol content that reached 4.5, 4.9 and 5.0 g L^{-1} (98, 106 and 108 mM) respectively, while when galactose was fed 3.5 g L^{-1} (76 mM) were formed. Moreover, a culture fed with 10 g L^{-1} of D-xylose generated 1.9 g L^{-1} (41 mM) of the product. One of the most important parameters for the development of cost effective bioethanol processes is the use of yeasts converting both hexoses and pentoses to the product at high rates, yields and final concentrations. Although *P. kudriavzevii* was reported as not capable of assimilating D-xylose (Oberoi et al., 2012; Sandhu et al., 2012; Dandi et al., 2013), similarly to this study *P. kudriavzevii* VVT-C-75010 was a xylose-fermenting strain (Toivari et al., 2013), while the catabolic genes of D-xylose have been identified in *P. kudriavzevii* M12 (Chan et al., 2012a).

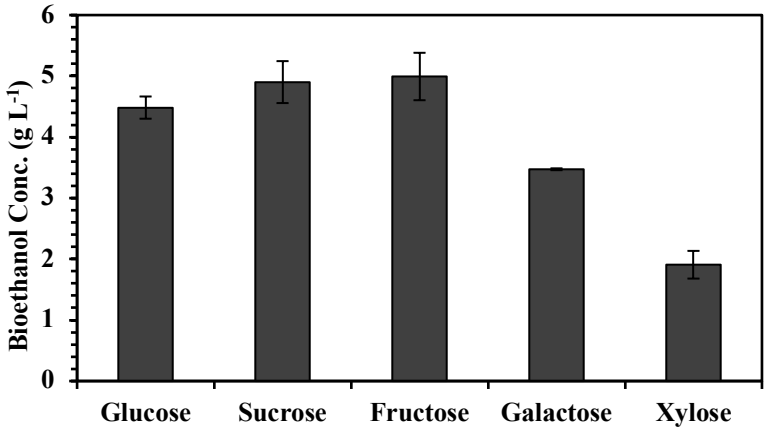

Figure 3.5.4: Bioethanol formed in single substrate experiments. Each bar corresponds to the concentration of bioethanol produced using an initial concentration of 10 g L^{-1} of the sugar indicated.

3.5.1.5 Critical aspects for the use of P. kudriavzevii KVMP10 in ethanol bioprocess

The energy demand of distillation could be up to 40% of the energy required for bioethanol manufacture (Nagy and Boldyryev, 2013). Moreover, when the bioprocess is conducted at high temperatures, evaporation of significant quantities of ethanol is enabled, which could be further liquefied using a heat exchanger. The cost effective production of fuel-grade ethanol in alcohol distilleries requires low energy demand emphasizing the importance of employing thermotolerant yeasts, such as *P. kudriavzevii* KVMP10. The yeast can ferment sugars at higher temperatures compared to other traditional strains (Yuangsaard et al., 2013) and it consumes glucose, sucrose, fructose, galactose and xylose suggesting its potential for application in the development of bioethanol processes based on cellulosic feedstocks. Moreover, *P. kudriavzevii* KVMP10 achieved a high product yield, generating higher ethanol concentrations compared to *S. cerevisiae*, *K. marxianus* and *Z. mobilis* using the same model solution of OPW (Table 3.5.2).

P. kudriavzevii cultures performed with 150 g L^{-1} of glucose have resulted in ethanol contents higher than 9% (v/v) (Dandi et al., 2013) confirming that the yeast may achieve substantially high alcohol concentrations. Thus, although other common strains were more resistant to D-limonene (Table 3.5.1), the capacity of *P. kudriavzevii* KVMP10 to ferment a variety of carbohydrates (including xylose) demonstrates that it is a superior yeast holding great potential for the development of OP based biorefinery concepts for the production of fuel-grade or potable alcohol. The use of the hydrolyzed OPW model solution in *P. kudriavzevii* KVMP10 fermentations produced 6.8% (v/v) of ethanol, which is a typical alcoholic degree achieved industrially, exemplifying its potential for application in potable and fuel-grade alcohol production from OP.

The valorization of food waste using biotechnological approaches could serve as a sustainable practice for generation of various commodities valuable to the society. It has been shown that *P. kudriavzevii* KVMP10 was not highly resistant to the presence of D-limonene. However, a range of favorable characteristics, such as strong ethanologenic capacity, high temperature fermentation and utilization of both hexose and pentose sugars demonstrate that *P. kudriavzevii* KVMP10 is a

versatile yeast holding great potential for application in ethanol processes from lignocellulosic biomass.

3.5.1.6 Ethanol production using CPW hydrolyzates obtained through acid hydrolysis

Dilute acid hydrolysis of CPW served the dual objective of breaking down polymers (cellulose, hemicellulose) into soluble sugars, while consisting an essential processing step for pectin isolation (Kaya et al., 2014). Thus, six CPW hydrolyzates were produced using three hydrolysis temperatures (108 ^{o}C, 116 ^{o}C, 125 ^{o}C) for 10 min and 20 min respectively. Optimal conditions for CPW saccharification through dilute acid hydrolysis were identified as 116 ^{o}C for 10 min based on the concentration of ethanol produced and the final product yield during fermentations (Figure 3.5.4). Thus, ethanol concentration and product yield reached 5.8 g L^{-1} and 0.48 g_{eth} g^{-1}_{tsc} respectively using *P. kudriavzevii* KVMP10, while *K. marxianus* and *S. cerevisiae* produced 4.6 g L^{-1} and 4.2 g L^{-1} of ethanol respectively. Although, the highest ethanol concentration (6.7 g L^{-1}) was obtained using the hydrolyzate generated at 125 ^{o}C for 20 min with application of *P. kudriavzevii* KVMP10, the product yield decreased significantly to 0.32 g_{eth} g^{-1}_{tsc}. A t-test ($p < 0.05$) was performed to statistically assess differences between the mean values of ethanol concentration (Table 3.5.3). Significant statistical difference was observed between ethanol titres obtained in *P. kudriavzevii* KVMP10 and *S. cerevisiae* fermentations using both hydrolyzates exhibiting the highest product formation (116 ^{o}C for 10 min and 125 ^{o}C for 20 min). Nevertheless, the titre of the biofuel obtained in *P. kudriavzevii* KVMP10 fermentations fed with the aforementioned hydrolyzates were not statistically different. Overall, nearly in all experiments performed using *K. marxianus* and *S. cerevisiae* the increase in hydrolysis duration reduced the final product titre and yield indicating the potential formation of inhibitors at elevated preprocessing duration. This is in line with previous studies demonstrating the inhibitory effect that may occur in ethanol fermentations of *K. marxianus* (Almeida et al., 2007) and *S. cerevisiae* (Palmqvist and Hahn-Hagerdal, 2000) fed with feedstocks pretreated employing increased acid hydrolysis temperatures and duration. Therefore, considering that the overall performance of *K. marxianus* and *S. cerevisiae* was decreased, the lower product yield of *P.*

kudrivzevii KVMP10 and the elevated energy demand expected with application of 125 ºC for 20 min in hydrolysis, the use of 116 ºC for 10 min was selected as suitable conditions for CPW hydrolysis. These results are in good agreement with other studies demonstrating that the optimal conditions for dilute acid hydrolysis of CPW comprise application of 116 ºC for 10-13 min with the use of 0.5% (v/v) H_2SO_4 and 5-6% (w/v) dry solids of the raw material (Patsalou et al., 2017; Talebnia et al., 2008).

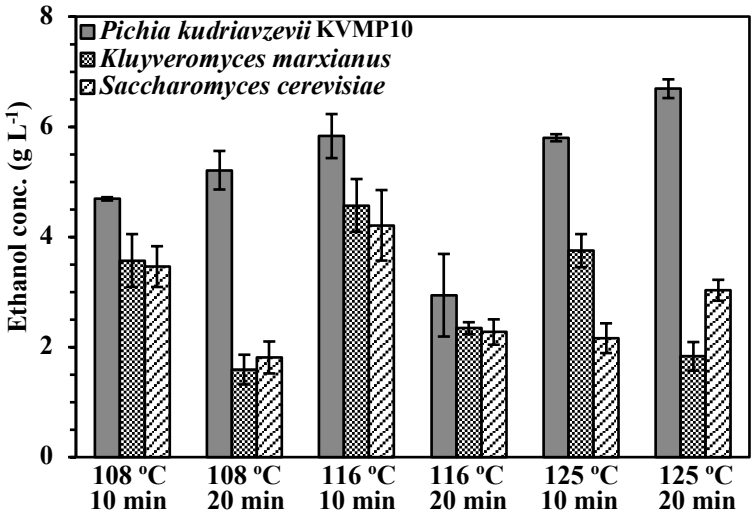

Figure 3.5.5: Ethanol titre achieved in *P. kudriavzevii* KVMP10, *S. cerevisiae* and *K. marxianus* fermentations of hydrolyzates obtained through dilute acid treatment of CPW.

Table 3.5.3: Statistically assessed differences between the mean values of ethanol concentration.

		108 °C, 10 min			**108 °C, 20 min**			**116 °C, 10 min**			**116 °C, 20 min**			**125 °C, 10 min**			**125 °C, 20 min**		
		P. kudriavzevii KVMP10	*K. marxianus*	*S. cerevisiae*	*P. kudriavzevii* KVMP10	*K. marxianus*	*S. cerevisiae*	*P. kudriavzevii* KVMP10	*K. marxianus*	*S. cerevisiae*	*P. kudriavzevii* KVMP10	*K. marxianus*	*S. cerevisiae*	*P. kudriavzevii* KVMP10	*K. marxianus*	*S. cerevisiae*	*P. kudriavzevii* KVMP10	*K. marxianus*	*S. cerevisiae*
108 °C, 10 min	*P. kudriavzevii* KVMP10		.0726	.0514	.1546	**.0407**	**.0227**	**.0335**	.1464	.0966	.2710	**.0159**	**.0253**	**.0070**	.4034	**.0278**	.1973	**.0307**	**.0248**
	K. marxianus			.1348	.0988	**.0135**	**.0033**	**.0098**	.5836	.1220	.6289	.0688	.0682	**.0264**	**.0291**	.0702	.1033	**.0084**	.0801
	S. cerevisiae				**.0185**	.5630	.1778	**.0409**	**.0066**	.4855	.3888	.1319	.0526	**.0354**	.0869	**.0366**	**.0225**	**.0105**	.1899
108 °C, 20 min	*P. kudriavzevii* KVMP10					.0609	**.0379**	.0622	**.0347**	.1079	.2424	**.0096**	**.0029**	.0880	.5719	**.0055**	.0571	**.0480**	**.0013**
	K. marxianus						**.0051**	**.0050**	.1426	.3674	.3115	.2537	.1532	**.0221**	**.0083**	.1516	.0611	**.0002**	.2587
	S. cerevisiae							**.0050**	.0694	.0645	.1295	.1319	.4363	**.0144**	**.0071**	.5458	**.0372**	**.0105**	.1735
116 °C, 10 min	*P. kudriavzevii* KVMP10								.0543	**.0132**	.0547	**.0259**	**.0295**	.0508	**.0025**	**.0307**	.0568	**.0044**	**.0297**
	K. marxianus									.2477	.6112	**.0383**	**.0128**	.0577	.2142	**.0085**	**.0469**	.0955	**.0237**
	S. cerevisiae										.2881	.2936	.1962	.0537	**.0368**	.1926	.1111	.1035	.2930
116 °C, 20 min	*P. kudriavzevii* KVMP10											.2911	.2264	.1350	.1710	.2229	.2573	.1802	.2909
	K. marxianus												.0663	**.0129**	.0520	.0736	**.0072**	.4721	.2876
	S. cerevisiae													**.0199**	.0545	.1293	**.0053**	.3896	**.0279**
125 °C, 10 min	*P. kudriavzevii* KVMP10														.0852	**.0217**	.0686	**.0185**	.0592
	K. marxianus															.0562	.8135	**.0064**	**.0189**
	S. cerevisiae																**.0080**	.3523	**.0412**
125 °C, 20 min	*P. kudriavzevii* KVMP10																	**.0476**	**.0014**
	K. marxianus																		.6689
	S. cerevisiae																		

P-value<0.05 is statistically significant

A variety of CPW pretreatment approaches have been previously evaluated for ethanol production by different yeasts. The production of the biofuel was investigated in *S. cerevisiae* fermentations, using a CPW-derived hydrolyzate obtained through application of steam explosion as well as dilute-acid hydrolysis and pectin recovery, achieving a yield of 0.43 g_{eth} g_{tsc}^{-1} (Pourbafrani et al., 2010; Wilkins et al., 2007b). *S. cerevisiae* was also applied for bioethanol production using CPW hydrolyzates obtained through hydrothermal sterilization, steam explosion as well as a combination of popping and enzyme hydrolysis demonstrating bioethanol production of 42 g L^{-1} (Oberoi et al., 2011), 60 L tn_{rm}^{-1} (Boluda-Aguilar et al., 2010) and 46.2 g L^{-1} respectively (Choi et al., 2013). OPW was pretreated by two stage acid and enzyme hydrolysis, while the hydrolyzate formed was used as fermentation feedstock for bioethanol production by *S. cerevisiae* and *M. indicus* achieving final product concentrations of 30.3 g L^{-1} and 15 g L^{-1} with a yield of 0.46 g_{eth} g_{tsc}^{-1} and 0.39 g_{eth} g_{tsc}^{-1} respectively (Oberoi et al., 2010; Lennartsson et al., 2012). Moreover, mandarin waste was subject to enzyme hydrolysis producing a fermentation feedstock that achieved 0.67 g_{eth} g_{tsc}^{-1} and 33.9 g L^{-1} of the biofuel with the use of *P. kudriavzevii* (Sandhu et al., 2012).

3.5.2 Enhancement of *P. kudriavzevii* KVMP10 ethanol fermentations

The final ethanol titre achieved using the hydrolyzates obtained were low compared to the relevant literature. Therefore, a number of parameters were evaluated to improve ethanol production through the biorefinery proposed.

3.5.2.1 Effect of nitrogen source

The effect of nitrogen source supplementation was initially tested to increase biofuel production. However, although nitrogenous compounds can substantially enhance the fermentation rate of ethanol (Harun et al., 2010; Torija et al., 2003), the use of yeast extract as nitrogen source did not demonstrate any noticeable effect on ethanol formation in all yeast fermentations conducted.

3.5.2.2 Effect of the addition of enzyme hydrolysis

Acid and enzyme hydrolysis were combined as sequential pretreatments to enhance the production of ethanol due to potential increased release of fermentable sugars. The addition of the enzymatic pretreatment step in the biorefinery substantially enhanced the titre of ethanol to 9.2 g L^{-1} in *P. kudriavzevii* KVMP10 fermentations resulting in a product yield of 0.42 g_{eth} g^{-1}_{tsc}. Pretreatment processes that involve two steps can be more efficient in lignocellulosic biomass saccharification releasing increased concentrations of simple sugars that enhance ethanol generation (Choi et al., 2013; Oberoi et al., 2010). Thus, sequentially applied acid and enzyme hydrolysis of CPW is capable of producing substantially elevated sugar yields that reached 0.58 g_{trs} g_{drm}^{-1} (Patsalou et al., 2017).

3.5.2.3 Effect of recycling the liquid stillage

Recycling of the liquid stillage remaining following essential oils extraction into the hydrolysis process was applied in an attempt to further increase the concentration of monosaccharides in the hydrolyzate and ethanol formation, as well as to reuse the process water generated for plant application. Stillage water recycling contributed to three-fold increase of bioethanol concentration that reached 30.7 g L^{-1} in *P. kudriavzevii* KVMP10 fermentations conducted at 42 ^{o}C, while biofuel production was also enhanced in *K. marxianus* cultures reaching 26.3 g L^{-1}. Nevertheless, ethanol production from *S. cerevisiae* remained at low levels with the reuse of the stillage potentially due to the elevated process temperature employed.

The present findings demonstrate that *P. kudriavzevii* KVMP10 constitutes a more efficient bioethanol producer with application of CPW hydrolyzates as compared against the industrial yeasts tested (*K. marxianus* and *S. cerevisiae*). Moreover, the increased ethanol production was achieved at elevated fermentation temperature, which is in agreement with other studies highlighting that *P. kudriavzevii* strains are robust ethanol producers exhibiting multiple tolerance at high temperatures and acidic environments (Seong et al., 2017). Key novelties of the results obtained comprise that high ethanol production was achieved from CPW under mild pretreatment and harsh fermentation conditions, while saving of

valuable resources was achieved through process water recycling. Thus, in accordance to the water reuse achieved, the remaining solid residue from CPW hydrolysis was applied in anaerobic digestion to produce biogas targeting the operation of a zero-waste biorefinery as described below.

3.6 Valorization of biorefinery residues

3.6.1 Anaerobic digestion

The biomethanization of CPW has been mainly studied following essential oils extraction, which serves the dual purpose of generating an added-value product and reducing the anaerobic digestion inhibition caused by the antimicrobial properties of the oil (Negro et al., 2018). However, although essential oils were extracted from CPW in several studies prior use in methane production (Martin et al., 2010; Pourbafrani et al., 2010), untreated CPW have been also used in anaerobic digestion of fresh or dried citrus fragments (peel, pulp and seeds) under thermophilic and mesophilic conditions (Table 3.6.1). Herein, methane production was tested using solid BR remaining as side stream from acid hydrolysis of CPW. Moreover, the capacity of BR to produce the biofuel was compared against the application of untreated CPW as well as DCPW under mesophilic conditions.

Table 3.6.1: Methane production from CPW under mesophilic and thermophilic conditions.

Material	Working / Total Volume (mL/mL)	Conditions	Inoculum of sludge	Medium	Substrate content	Biomethane (mL g_{VS}^{-1})	Reference
Mesophilic conditions							
***Citrus sinensis* (Tight skinned sweet orange)-dried peels**	75 / 135	35 °C / N_2:CO_2 (70:30)	20% (v/v) from mesophilic CSTR	Basic nutrient medium	0.7% (w/v)	455	Gunaseelan (2004)
***Citrus sinensis* (Tight skinned sweet orange)-dried pressings**						502	
***Citrus reticulata* (Loose skinned mandarin)-dried peels**						486	
***Citrus reticulata* (Loose skinned mandarin)-dried pressings**						433	
***Citrus reticulata* (Loose skinned mandarin)-dried seeds**						732	
Orange peel waste-steam distilled	nd / 3500	37 °C	12 g_{VSS} L^{-1} granular sludge	Nutrient medium and trace elements	2 g_{VS} L^{-1}	230	Martin et al. (2010)
Fresh ripe orange-seeds	30 / 120	35 °C	2% (w/v) from biogas digester	Distilled water	0.5% (w/v)	581	Sanjaya et al. (2016)
Fresh ripe orange-pulp						288	
Fresh ripe orange-peel						72	
Rotten orange-seeds						658	
Rotten orange-pulp						312	
Rotten orange-peel						48	
DCPW	150 / 250	37 °C / CO_2	4% (w/v) from a full-scale UASB reactor	Nutrient medium	0.6% (w/v)	356	Current study
BR						342	
CPW					0.3% (w/v)	366	
CPW					0.6% (w/v)	294-339	
CPW					1.2% (w/v)	332	

CPW					2.4% (w/v)	281	
Thermophilic conditions							
Fermentation stillage and solids from dilute acid hydrolysis-neutrilized	nd / 2000	55 ºC / N_2:CO_2 (80:20)	400 g_{VS} from a municipal waste digester	nd	6 g_{VS} in each bottle	363	Pourbafrani et al. (2010)
CPW-steam distilled	nd / 3500	67 ºC	12 g_{VSS} L^{-1} granular sludge	Nutrient and trace elements solution	2 g_{VS} L^{-1}	332	Martin et al. (2010)
Citrus waste-steam explosion	600 / 1200	55 ºC / N_2:CO_2 (80:20)	400 mL from large scale thermophilic digester	Distilled water	0.75%	537	Forgacs et al. (2011)
Citrus waste-untreated						102	
CPW	4000 / 5000	55 ºC	2000 mL	nd	0.45 kg	644	Koppar and Pullammanappallil (2013)

DCPW: Dried citrus peel waste, BR: Biorefinery residues, CPW: Citrus peel waste, nd: No data, VS: Volatile solids.

3.6.1.1 Methane production using BR, CPW and DCPW

Biogas and methane production from BR as well as CPW and DCPW was evaluated with the addition of 6 g L^{-1} as initial content of volatile solids in anaerobic digestion (Figure 3.6.1). During the first batch, DCPW exhibited substantially higher production of methane at 35 d, as compared to the rest of the materials, generating 303 mL. The use of BR demonstrated prolonged biogas inhibition for the first 15 d of the process accumulating high levels of acetate that reached 9.7 g L^{-1} (Figure 3.6.2), while CPW also caused inhibition of methane formation. However, the concentration of acetate was subsequently reduced in the experiment fed with BR resulting in methane production that reached 264 mL at 35 d, similarly to biofuel production from CPW. Following 36 d a subsequent batch was conducted through refeeding of the same content of each material in fermentations. The inhibitory effect was substantially reduced in the second batch, regarding the kinetics of methane production, indicating potential adaptation of the sludge to each material. Specifically, the production rate of methane was 11.3 mL d^{-1}, 15.9 mL d^{-1} and 7.5 mL d^{-1} during the first batch experiment, while at the second batch the rate increased to 14.9 mL d^{-1}, 25.6 mL d^{-1} and 13.6 mL d^{-1} for CPW, DCPW and BR respectively. Following the second refeed of each material, the methane production rate was further increased only in the digestion of DCPW reaching 27.2 mL d^{-1}. Moreover, cumulative methane formation was significantly increased with the use of BR reaching 314 mL. Following 78 d a second refeed of each material was applied to evaluate whether methane production was further increased. The production of methane was only slightly enhanced in the third batch reaching 305-320 mL, for all materials tested.

The data obtained demonstrate the capacity of BR to generate significant amounts of methane at a similar level to the use of dried or untreated CPW. Moreover, although methane production is consistent with other studies that reached similar biofuel formation from CPW (Table 3.6.1), the production of the biofuel achieved here under mesophilic conditions was comparable to that of studies employing thermophilic anaerobic digestion temperatures. Therefore, the approach followed not only enables the development of a zero-waste biorefinery, but also provides significant energy gains due to the substantially reduced temperature used in anaerobic digestion.

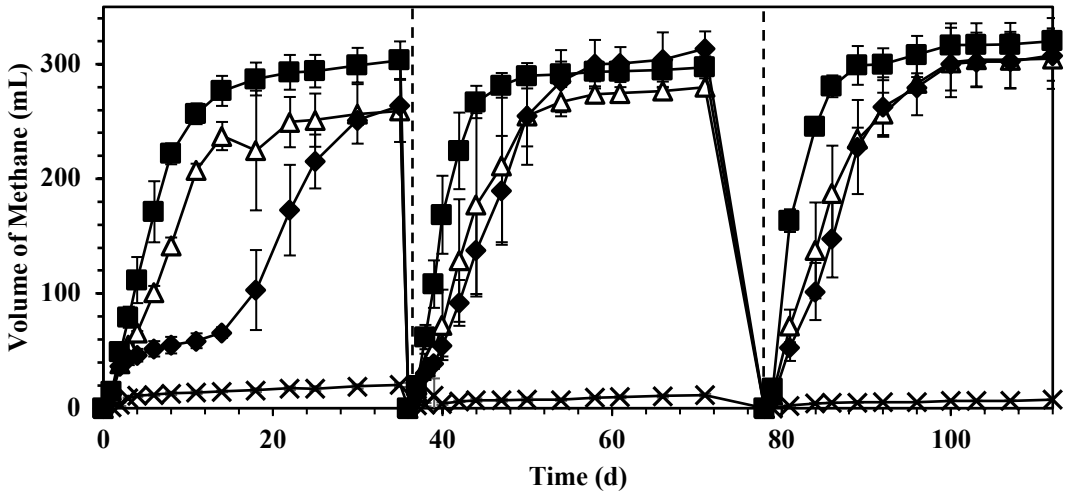

Figure 3.6.1: Cumulative methane production using 6 g_{VS} L^{-1} of: i) untreated CPW (—△—), ii) DCPW (—■—), and iii) solid BR (—◆—). A control fermentation was conducted without the addition of CPW (—✕—), while all experiments were conducted at 37 ^{o}C. Dashed lines represent the time where substrate refeed was applied.

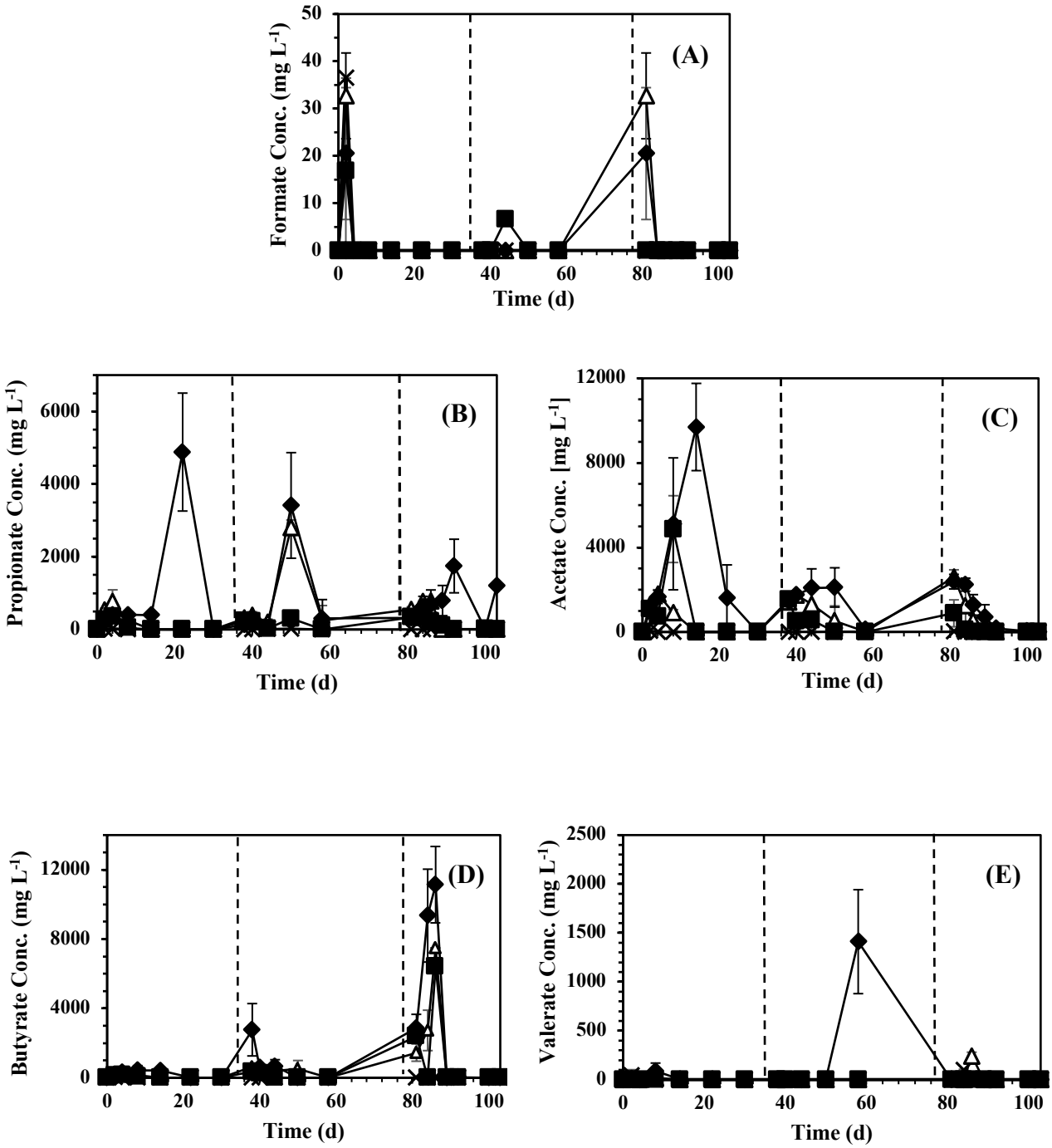

Figure 3.6.2: Concentration of volatile fatty acids (A: formate; B: propionate; C: acetate, D: butyrate; E: valerate) formed during anaerobic digestion of: i) untreated CPW (—△—), ii) DCPW (—■—), iii) solid BR (—◆—), and in the control experiment (—✕—).

Although the use of DCPW in anaerobic digestion resulted in the highest production of methane in the first batch as compared to the application of CPW and BR, calculation of bioprocess yield exhibited that employing CPW in the system enhanced the overall waste-to-energy conversion (Figure 3.6.3). The methane yield, defined as mL of methane produced per g of raw material (rm) used, was substantially higher with application of untreated CPW ranging between 72-84 $mL_{methane}$ g_{rm}^{-1} respectively. However, employing DCPW and BR resulted in similar and lower process yield that reached 42-51 $mL_{methane}$ g_{rm}^{-1} for both materials. The enhanced methane yield performed with the use of CPW was expected given the higher organic content remaining in the specific material for treatment in anaerobic digestion as compared to DCPW and BR, where volatile compounds and/or hydrolyzed carbohydrates have been removed. This conclusion was clarified by the t-test ($p < 0.05$) performed to identify statistically significant differences in the mean values obtained for the final methane production (mL) per g of rm with each feedstock in the first two batches. Furthermore, a statistically significant increase ($p < 0.05$) was observed in the volume of biogas produced per g of rm between 35 d and 112 d with the use of BR, demonstrating potential adaptation of the culture to the specific material following the first batch. Direct comparison of the three different citrus waste fractions demonstrated that even BR could produce high contents of methane, highlighting the applicability of biorefinery side streams for valorization through anaerobic digestion.

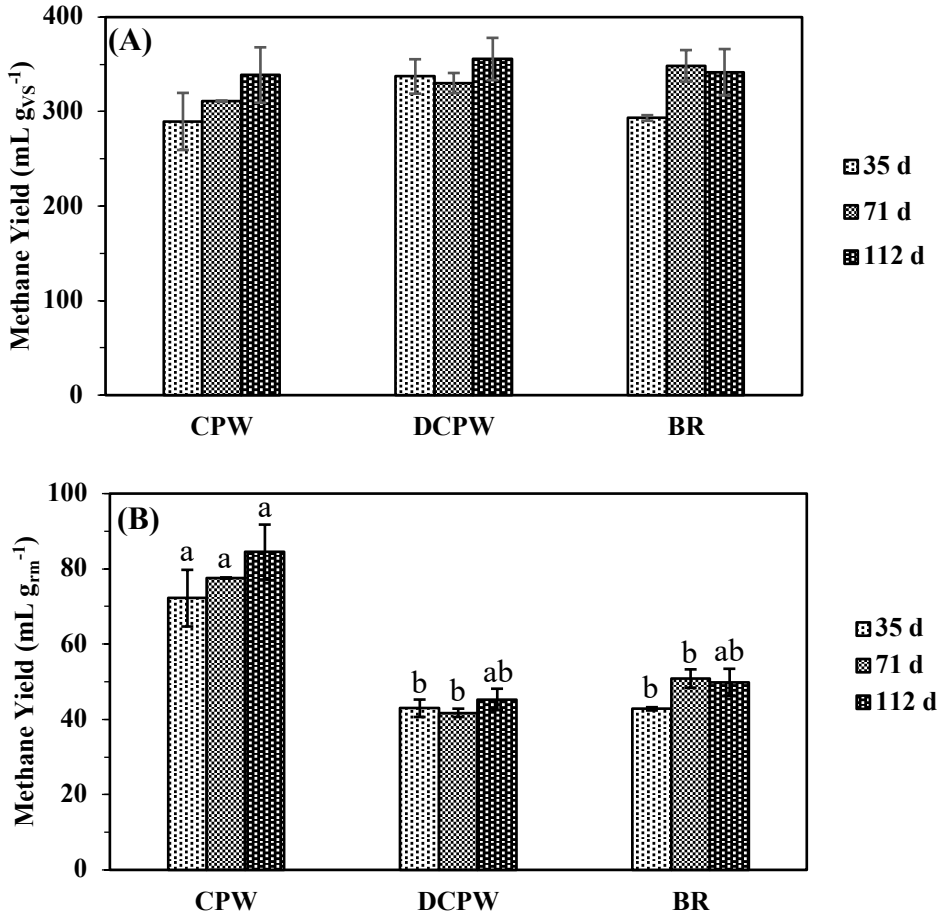

Figure 3.6.3: Yield of methane as (A) mL of product per g of initial VS and (B) mL of product per g of raw material (rm) generated from anaerobic digestion of CPW, DCPW and BR following completion of each batch.

3.6.1.2 Biomethane production using different initial quantities of CPW

Biomethanization of untreated CPW was previously evaluated by various studies (Table 3.6.1). Forgacs et al. (2011) achieved significantly low production of methane from untreated citrus waste that reached 102 mL g_{VS}^{-1}, while Koppar and Pullammanappallil, (2013) generated 644 mL g_{VS}^{-1} from untreated CPW under thermophilic conditions. The use of peel, seeds and pulp of fresh ripe orange as distinct feedstocks in anaerobic digestion under mesophilic conditions resulted in methane production that reached 72 mL g_{VS}^{-1}, 581 mL g_{VS}^{-1} and 288 mL g_{VS}^{-1} respectively (Sanjaya et al., 2016). Herein, different initial quantities of untreated CPW volatile solids (ranging between 3-24 g L^{-1}) were applied in anaerobic digestion to evaluate the effect of increasing contents of organic material as well as essential oils in the bioprocess (Figure

3.6.4). The maximum cumulative production of methane in each experiment was monitored as 165 mL, 264 mL, 598 mL and 1011 mL employing 3 g_{VS} L^{-1}, 6 g_{VS} L^{-1}, 12 g_{VS} L^{-1} and 24 g_{VS} L^{-1} respectively. Methane production per g of VS applied was not significantly different in each experiment ranging between 294-366 mL g_{VS}^{-1} (Table 3.6.1). However, the increase in feedstock concentration resulted in prolonged lag phase (Figure 3.6.4B).

It has been previously shown that the increase in essential oils' concentration can negatively impact biomethanization of orange peel waste inhibiting the methanogenesis (Calabro et al., 2016). However, the results obtained here demonstrate that anaerobic digestion of CPW under mesophilic conditions can generate high methane yields comparable to those obtained under thermophilic conditions. Thus, the maximum yield of methane achieved in the present study ranged between 294-366 mL g_{VS}^{-1}, which was three-fold higher than the yield achieved by Forgacs et al. (2011) that reached 102 mL g_{VS}^{-1} under thermophilic conditions. A similar effect was reported by Lotito et al. (2018), where the use of fresh and stored citrus peel waste achieved methane production that ranged between 333-471 mL g_{VS}^{-1} under mesophilic conditions. Nevertheless, significant quantities of α-terpineol and p-cymene were detected as final degradation products towards the end of digestion, while D-limonene was no longer present (Calabro et al. 2016; Lotito et al., 2018).

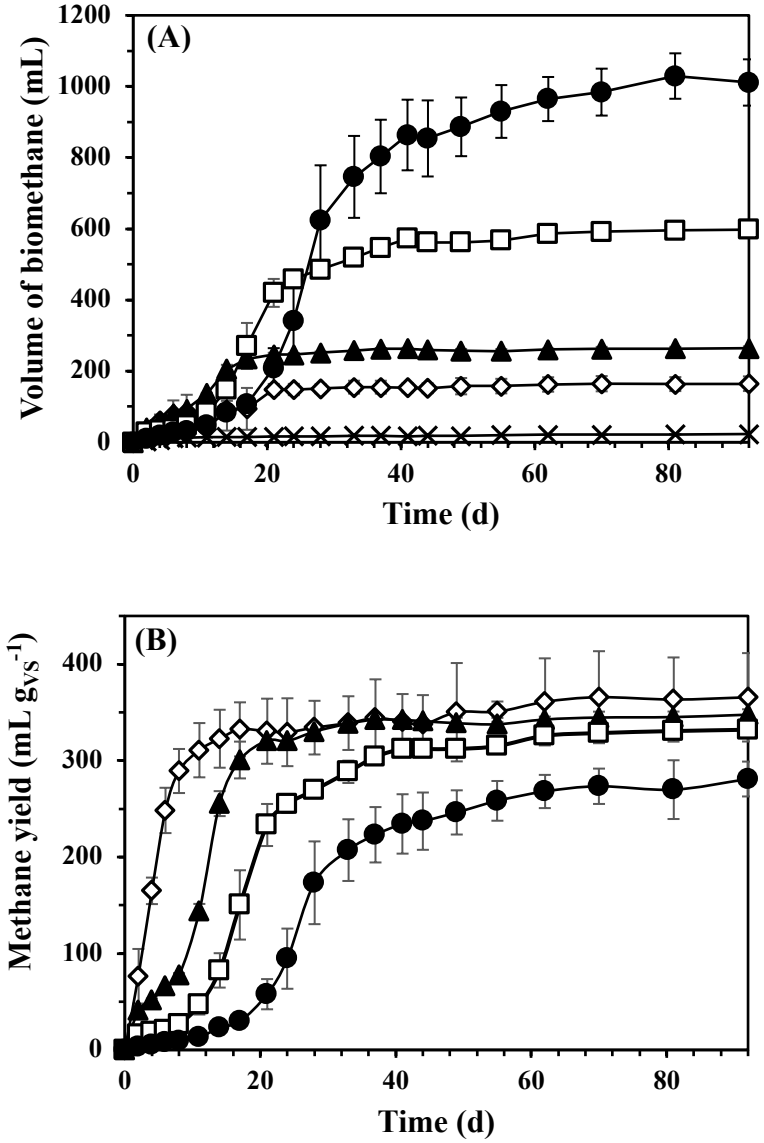

Figure 3.6.4: Cumulative methane production (A) and methane content per g of initial VS (B) using: i) 3 g L^{-1} (—◇—), ii) 6 g L^{-1} (—▲—), iii) 12 g L^{-1} (—□—), and iv) 24 g L^{-1} (—●—) initial volatile solids of CPW. A control fermentation was performed without the addition of CPW (—✕—), while all experiments were conducted at 37 ^{o}C.

3.6.2 Assessing the applicability of BR as fertilizer

Solid BR remaining as side stream from the application of the biorefinery were tested in an agricultural application as a fertilizer. Different ratios of the dried residue in commercial peat substrate (0%, 1%, 2.5%, 5% and 10% w/w) were used to evaluate lettuce seedling production *in vivo*. Furthermore, seed germination tests, 6 serial dilutions (up to 10^{-6}) of the dried material in water, were used as *in vitro* experiments to assess the effect of BR use for plant cultivation.

The effects of the solid residue on the substrate's properties are presented on Table 3.6.2. Addition of the material in peat gradually increased the conductivity and reduced the pH-value of the substrate mixture. This response is due to the low pH-value and the high EC of BR, which were measured at 1.52 and 14.77 mS cm^{-1} respectively. Potassium, phosphorus and nitrogen increased in content in the mixture, as the partition of the residue in the substrate increased, as a result of the higher content of these elements in the organic residue as compared to the control (100% peat). Total and air-filled porosity included higher values using the substrates that employed 5% and 10% of BR, because of the texture of the solid fine grinded residue.

Table 3.6.2: Physicochemical properties of substrate mixtures. The different rations of BR in the peat mixture resulted in 5 different substrates.

	0%	1%	2.5%	5%	10%
pH-value	5.59 ± 0.0318a[Y]	5.10 ± 0.0289b	4.85 ± 0.0289c	4.03 ± 0.0231d	3.31 ± 0.0203e
EC (µS cm^{-1})	1215 ± 7.10e	1374.1 ± 8.03d	2085.9 ± 12.15c	2778.9 ±16.19b	3970.9 ± 23.15a
Organic matter %	97.87 ± 0.372ab	98.57 ± 0.248a	98.18 ± 0.003ab	97.84 ± 0.003b	97.99 ± 0.121ab
Organic C%	56.77 ± 0.217ab	57.18 ± 0.147a	56.95 ± 0.000ab	56.75 ± 0.000b	56.84 ±0.069ab
Total N (g kg^{-1})	4.60 ± 0.087d	6.02 ±0.318b	5.13 ± 0.044cd	5.55 ± 0.193ab	6.65 ± 0.017a
K (g kg^{-1})	0.62 ± 0.003e	0.69 ± 0.015d	0.76 ± 0.002c	0.83 ± 0.025b	1.10 ± 0.003a
P (g kg^{-1})	0.402 ± 0.0364b	0.357 ±0.0165b	0.396 ± 0.0069b	0.414 ± 0.0147ab	0.473 ± 0.0040a
Na (g kg^{-1})	0.172 ± 0.0012b	0.182 ± 0.0038b	0.179 ± 0.0009b	0.180 ± 0.0121b	0.215 ± 0.0090a
Total porosity % (v/v)	85.4 ± 0.84bc	82.3 ± 1.06c	82.7 ± 0.10c	85.9 ± 1.59ab	88.8 ± 0.31a
Air filled porosity % (v/v)	11.8 ±1.01bc	9.3 ± 0.21d	10.6 ± 0.20cd	12.7 ±0.61ab	14.2 ± 0.70a
Container capacity % (v/v)	73.6 ± 0.17ab	73.0 ±0.85ab	72.1 ±0.29b	73.3 ± 0.97ab	74.6 ± 0.39a
Bulk density (g cm^{-3})	25.0 ± 0.33a	24.5 ± 0.16a	23.6 ± 0.26b	24.5 ±0.24bc	25.2 ± 0.28a

[Y] values (n=3) in rows followed by the same letter are not significantly different, $P \leq 0.05$.

Seed germination decreased at 10^{-1} dilution for the first 4 d, exhibiting zero germination for the pure solid residue (SR) extract (Figure 3.6.5). Similar effects were observed for the shoot and root length in vitro for seeds in Petri dishes (Appendix II). *In vivo*, while increasing the percentage of SR in the mixture, plants appeared shorter and developed a lower number of leaves, resulting to lower fresh and dry weight (Table 3.6.3, Appendix II). This effect was apparent even at low SR concentrations of 1%. Plants appeared stressed following addition of the residue in the substrate. The chlorophyll content gradually declined, while stress indicators (as the overproduction of H_2O_2 and MDA levels) increased, supporting the high abiotic stress that SR triggered to the plants. Seed emergence has been delayed as well, adding 3.5 d to seeds developed at 10%.

Results indicated that increasing concentrations of SR in the substrate elevated the stress imposed on plant growth (Appendix II). Furthermore, according to the data of Table 3.6.2, the addition of the material in peat gradually reduced the pH-value of the substrate mixture. Therefore, while the pH-value was reduced, plant growth was inhibited.

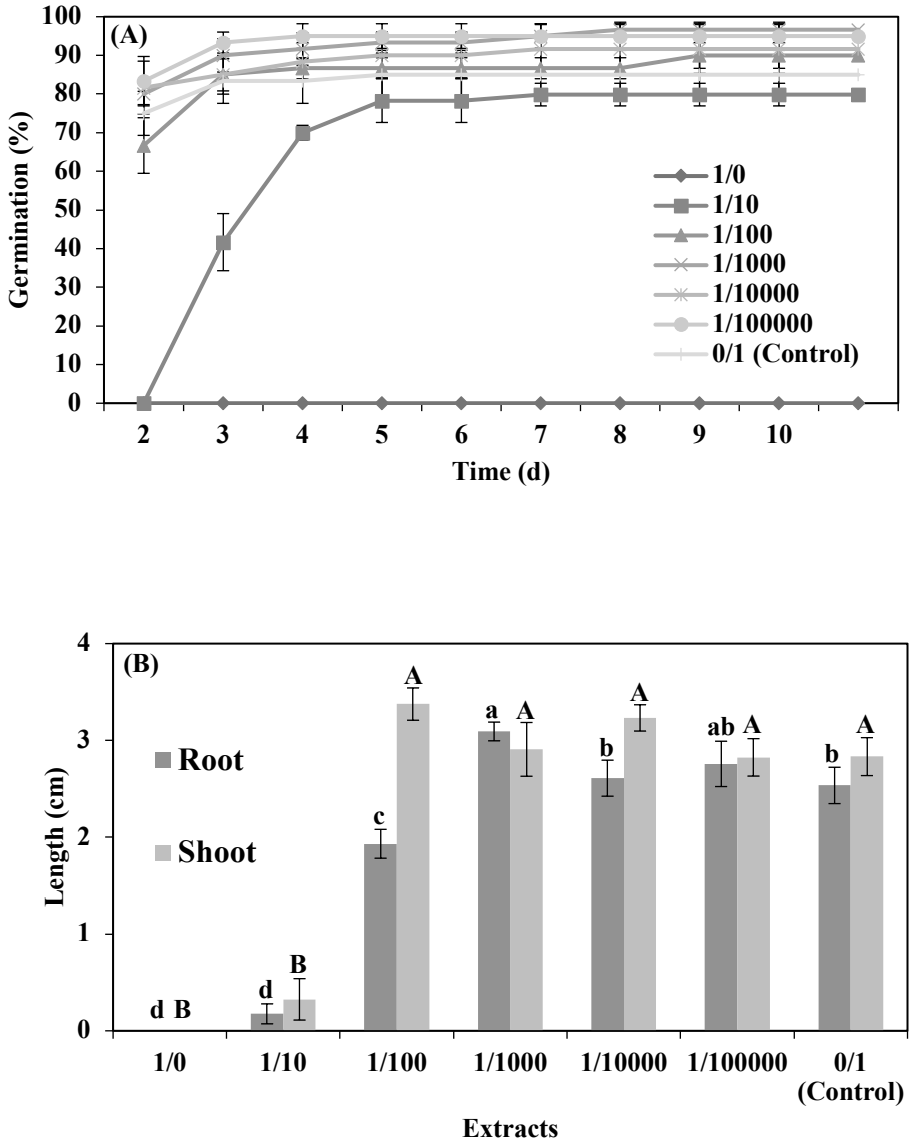

Figure 3.6.5: Effects of SR extracts at concentrations (10^{0}–10^{-6}) on (A) cumulative seed germination and (B) on shoot and root length of lettuce *in vitro*. Values represent mean (±SE) of measurements made on four Petri dishes (15 seeds and 5 radicles/dish) per treatment. Mean values followed by the same letter do not differ significantly at P=0.05 according to Duncan's MRT.

Table 3.6.3: Effect of the different mixtures tested on plant growth, mineral analysis and plant stress condition on lettuce seedlings, following 24 d of cultivation.

	0%	1%	2.5%	5%	10%
Plant height (cm)	8.16 ± 0.120a,[Y]	6.03 ± 0.070b	4.38 ± 0.177c	1.96 ± 0.095d	1.00 ± 0.047e
Leaf number	7.00 ± 0.365a	5.66± 0.333b	5.00 ± 0.000b	2.16 ±0.166c	2.01 ± 0.009c
Fresh weight (g)	2.48 ± 0.556a	1.09 ± 0.049b	0.78 ± 0.133bc	0.03 ± 0.002c	0.01 ± 0.000c
Dry weight (g)	0.27 ± 0.061a	0.11 ± 0.009b	0.08 ±0.026b	nd	nd
Total N (g kg^{-1})	18.30 ±0.262a	15.37 ±0.235b	15.89 ± 0.511b	nd	nd
P (g kg^{-1})	4.21 ± 0.019a	4.04 ± 0.059b	4.30 ± 0.017a	nd	nd
K (g kg^{-1})	27.63 ± 1.166b	30.68 ±0.321a	31.73 ± 0.924a	nd	nd
Na (g kg^{-1})	11.61 ± 0.0447a	11.09 ± 0.479a	11.00 ± 0.038a	nd	nd
Chlorophyll Fluorescence	0.821 ± 0.002a	0.809 ± 0.006a	0.0810 ± 0.003a	nd	nd
Chrorophyll a (mg g^{-1} FW)	1.070 ±0.160a	0.910 ± 0.076ab	0.673 ± 0.059b	0.237 ±0.003c	nd
Chrorophyll b (mg g^{-1} FW)	0.243 ±0.035a	0.230 ± 0.020ab	0.160 ± 0.015b	0.06 ±0.000c	nd
Total Chlorophyll (mg g^{-1} FW)	1.350 ±0.200a	1.143 ± 0.096ab	0.833 ± 0.074b	0.293 ±0.003c	nd
MDA (nmol g^{-1} FW)	17.11 ± 0.665c	27.54 ±0.506b	21.52 ±0.201c	60.27 ± 0.375a	nd
H_2O_2 (µmol g^{-1} FW)	0.17 ± 0.005d	0.46 ±0.006b	0.39 ± 0.000c	1.42 ±0.009a	nd
Mean germination time (d)	4.39 ± 0.104c	4.43 ±0.108c	4.93 ± 0.338c	5.98 ±0.513b	7.36 ±0.322a

[Y] values (n=6 for plant growth and n=3 for minerals/plant physiology) in rows followed by the same letter are not significantly different, $P \leq 0.05$. (nd: no detected).

4 PROJECT REVIEW

The research objectives of this project (presented in section 1.6) have been achieved. The main conclusions derived from the results of the present study are presented below.

4.1 Conclusions

4.1.1 Characterization of raw material

The first objective of the current thesis required characterizing the raw material to identify the quantity as well as the quality of essential oils and pectin isolated. The essential oil production yields reached 0.43% (v/w) and 0.24 (v/w) for "Mandora" and household citrus waste respectively, while analysis of the product's composition identified the presence of approximately 20 compounds. Among the molecules detected D-limonene included the highest content, which was measured at 96.36% and 94.41% for "Mandora" and household citrus waste respectively. Furthermore, the lignocellulosic content of "Mandora" was determined and included 22.45%, 8.05% and 0.66% of cellulose, hemicellulose as well as lignin respectively. The quantity of pectin extracted from CPW in the current biorefinery reached 30.5% and the FTIR analysis applied clarified that there were no structural differences between commercialy available pectin and the product isolated.

4.1.2 Preliminary study for the development of a CPW-based biorefinery for the production of succinic acid

4.1.2.1 Assessment of suitable fermentation conditions

A preliminary study of *A. succinogenes* fermentation was carried out to desing the process required for succinic acid production through the biorefinery. The content of inoculum that should be added in fermentations was investigated demonstrating that 13% of preculture should be used. The preliminary study also included assessing the capacity of the strain to consume glucose, fructose, galactose as well as galacturonic acid. *A. succinogenes* was capable of valorizing glucose and fructose for succinic acid production, while the product was not formed in fermentations of galactose and galacturonic acid.

4.1.2.2 Succinic acid derived from CPW

A preliminary study for the development of a CPW biorefinery has been proposed and applied for valorization of the waste with the use of *A. succinogenes*. Following extraction of essential oils (0.43% v/w) and pectin (30.54% w/w), the residue was hydrolyzed to fermentable sugars using a combination of dilute acid and enzyme hydrolysis. The release of metal ions was enhanced through applying a combination of sequential acid and enzyme hydrolysis demonstrating that the concentrations of ions released were substantially lower to the inhibitory levels identified for rumen microflora, while Mg^{2+} and Ca^{2+} ions were formed at substantially higher concentrations in the combined treatment potentially reducing the requirement for their addition to the fermentation medium. The most suitable conditions for the release of fermentable sugars through dilute acid and enzyme hydrolysis included 116 ^{o}C for 10 min and 5% (w/v) of dry CPW followed by the addition of 30 IU of cellulases and 25 BGL of b-glucosidases respectively, resulting in a substantially high total yield of 0.58 g_{ts} g_{drm}^{-1}. The hydrolyzate generated based on the combined pretreatment was fermented by *A. succinogenes* for the production of succinic acid at high yields. Nevertheless, economic analysis confirmed that the combined CPW preprocessing approach could not be competitive due to the elevated cost of enzymatic treatment.

4.1.3 Bioreactor level succinic acid fermentations

The process was up-scaled using a laboratory bioreactor to enhance the production of succinic acid by *A. succinogenes*. The effect of varying nitrogen sources as well as the addition of vitamins were evaluated demonstrating that corn steep liquor could enhance the production of succinic acid, while the addition of vitamins increased the production rate. The SSF experiment performed exhibited that the enzymatic hydrolysis conducted during the fermentation could not enhance succinic acid production. However, comparing the results obtained in batch and fed-batch fermentations conducted in the bioreactor, demonstrated that the fed-batch experiment could increase both the final concentration of succinic acid as well as the product yield.

4.1.4 Ethanol production

4.1.4.1 Evaluation of *P. kudriavzevii* KVMP10 fermentation parameters

A preliminary study of applying *P. kudrivazevii* KVMP10 in alcoholic fermentations was performed in order to evaluate the strain's applicability for ethanol production in the CPW biorefinery. The efficiency of *P. kudrivazevii* KVMP10 was tested in different temperatures and pH-value levels to test the strain's tolerance in varying D-limonene contents as well as the capacity to assimilate simple sugars. The results demonstrated that the optimum conditions for *P. kudrivazevii* KVMP10 fermentation comprised 4.8 and 42 ^{o}C of pH-value and temperature respectively, while the strain could successfully assimilate glucose, sucrose, fructose, galactose and xylose. *P. kudriavzevii* KVMP10 was highly thermotolerant and utilized both hexoses and pentoses for ethanol production, which was achieved at elevated rates, highlighting its great potential for application in ethanol production processes from citrus peel.

4.1.4.2 Ethanol production using CPW hydrolyzates obtained through acid hydrolysis

The above mentioned yeast was applied for the development of the CPW-biorefinery targeting ethanol production through fermentation. Optimal conditions for CPW saccharification through dilute acid hydrolysis were identified as 116 ^{o}C for 10 min based on the concentration of ethanol produced and the final product yield obtained during fermentations. Moreover, the thermotolerant *P. kudriavzevii* KVMP10 was a more efficient ethanol producer at elevated fermentation temperatures as compared to major industrial yeasts (*S. cerevisiae* and *K. marxianus*).

4.1.4.3 Bioethanol fermentations

Aiming to enhance the alcoholic fermentation process, the addition of nitrogen source, the combination of acid and enzyme hydrolysis as well as recycling of the stillage remaining from essential oils extraction was tested. The results indicate that the application of enzyme hydrolysis did not demonstrate any noticeable effect, while the combination of acid and enzyme hydrolysis enhanced the production of ethanol due to potential increased release of fermentable sugars. The recycling of stillage contributed a three-fold increase in ethanol concentration. The present findings demonstrate that *P.*

kudriavzevii KVMP10 constitutes a more efficient ethanol producer through use of CPW hydrolyzates as compared to the industrial yeasts tested (*K. marxianus* and *S. cerevisiae*).

4.1.5 Biorefinery residues

4.1.5.1 Anaerobic digestion

The use of the biorefinery's side stream in anaerobic digestion demonstrated the capacity of BR to generate significant amounts of methane at a similar level to the use of dried or untreated CPW. The production of the biofuel achieved here under mesophilic conditions was similar to that of studies employing thermophilic anaerobic digestion temperatures. Therefore, the approach followed not only enables the development of a zero-waste biorefinery, but also provides significant energy gains due to the substantially reduced temperature used in anaerobic digestion.

4.1.5.2 Assessing the applicability of BR as fertilizer

Solid BR was tested in an agricultural application as a fertilizer. Addition of the material in peat gradually increased the conductivity and reduced the pH-value of the substrate mixture. This response was caused potentially due to the low pH-value and the high EC of BR, while the total and air filled porosity increased at the substrates that employed 5% and 10% of BR. Plants appeared stressed following addition of the residue in the substrate. The chlorophyll content gradually declined, while stress indicators (as the overproduction of H_2O_2 and MDA levels) increased, supporting the high abiotic stress that SR triggered to the plants.

4.2 Future work

Following completion of the present thesis, the topics presented below could be proposed as future work relevant to the CPW biorefinery.

4.2.1 CPW pretreatment technologies

The current work proposed dilute acid hydrolysis followed by enzyme hydrolysis as pretreatment steps for CPW processing, following extraction of essential oils, in order to enhance the concentration of soluble sugars in the fermentation broth. However, the use of ultrasounds has been previously proposed for extraction of essential oils, polyphenols and pectin (Boukroufa et al., 2015) or for the reduction of polysaccharides contained in lignocellulosic materials (Rehman et al., 2014; Villa-Velez et al., 2015). Thus, the use of ultrasounds in the biorefinery proposed could enhance the formation of soluble sugars prior to fermentation as well as to assist the extraction of essential oils and pectin. Furthermore, the use of ultrasounds prior application of enzyme hydrolysis could increase the yield of sugars released during the enzymatic process.

4.2.2 Succinic acid fermentations

The scale up succinic acid fermentations to laboratory bioreactor demonstrated a slight increase in by-products formation in *A. succinogenes* fermentations. As mentioned in section 3.2.2.5 not only carbon assimilation is targeted during succinic acid production, but also the consumption of CO_2 maintaining the reaction equilibrium during fermentation. The supplementation of CO_2 in *A. succinogenes* cultivation is known to minimize the formation of by-product and to enhance the final succinic acid concentration and yield. Thus, although CO2 was supplied in the fermentation conducted in the present study, the volumetric flow rate applied should be optimized to reduce the generation of by-products. Moreover, the use of nitrogen source plays a crucial role in *A. succinogenes* fermentations as mentioned in section 3.3.1.3. Therefore, it would be important to evaluate the effect of different nitrogen source concentrations during batch and fed-batch fermentations.

4.2.3 Ethanol fermentations

P. kudriavzevii KVMP10 constitutes a thermotolerant yeast which is capable of producing high concentrations of ethanol at the elevated temperature of 42 ºC and pH-value of 4.8. Thus, given that enzyme hydrolysis could be optimally performed at 50 ºC and pH-value of 5.5 a simultaneous saccharification and fermentation process could be developed. However, optimizing the temperature of the process would be required to achieve high product yield.

4.2.4 BR management

BR was tested, as mentioned in section 3.6.2, in an agricultural application demonstrating that the addition of the residue in peat reduced the pH-value of the mixture, while increasing the stress improsed on plant growth. Given that BR was used in the specific application without prior adjustment of the pH-value, it would be essential to test the use of BR in agricultural applications as fertilizer following neutralization of the pH-value to avoid the stress triggered. Furthermore, OPW has been previously applied as support for the development of immobilized biocatalyst following sterilization generating promising results (Plessas et al., 2007). Thus, given that one of the most commonly used polysaccharides for cell immobilization is cellulose (Kourkoutas et al., 2004) and BR consists mainly of cellulose, the side stream generated could be also used as a carrier for cell immobilization to increase the formation of the final product.

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APPENDIX I

P. kudriavzevii KVMP10 grown in solid media supplemented with increasing concentrations of orange peel oil

Table AP1: Duration of lag-phase and time required for maximum biomass formation of *P. kudriavzevii* KVMP10 grown in solid media supplemented with increasing concentrations of orange peel oil.

Orange peel oil content % (v/v)	Observed duration of lag-phase (h)	Observation time of maximum biomass formation (h)
0.0	0-12	72
0.1	0-12	168
0.2	0-12	168
0.3	0-24	168
0.4	0-12	168
0.5	0-24	144
0.6	0-24	144
0.7	0-48	120
0.8	0-24	120
0.9	0-24	120
1.0	0-48	168

Figure AP1: Cultivation of *P. kudriavzevii* KVMP10 in solid media supplemented with various orange peel oil concentrations. 50 µL of pregrown cultures were inoculated in each petri dish and the whole surface of the plate was streaked in parallel lines. The cultures were incubated at 30 ºC. Shown are pictures taken at the time points specified for cultures containing a peel oil content (v/v) of: A) 0.0%; B) 0.1%; C) 0.2%; D) 0.3%; E) 0.4%; F) 0.5%; G) 0.6%; H) 0.7%; I) 0.8%; J) 0.9%; K) 1.0%.

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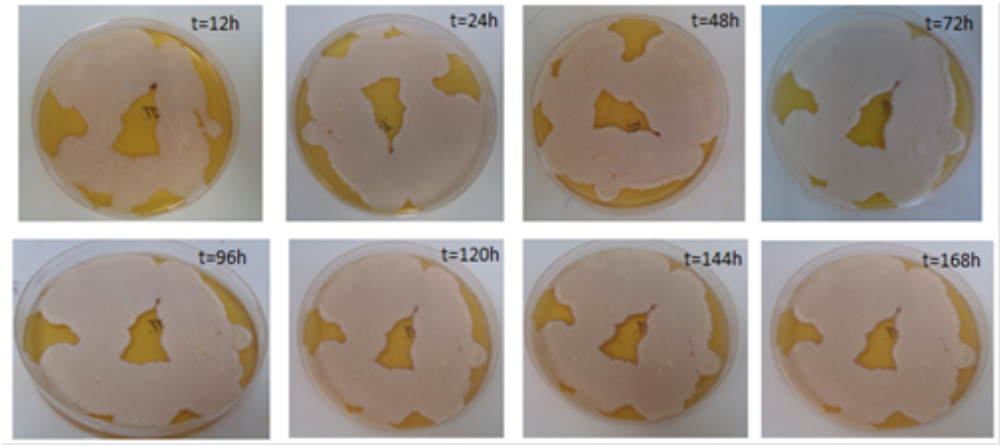

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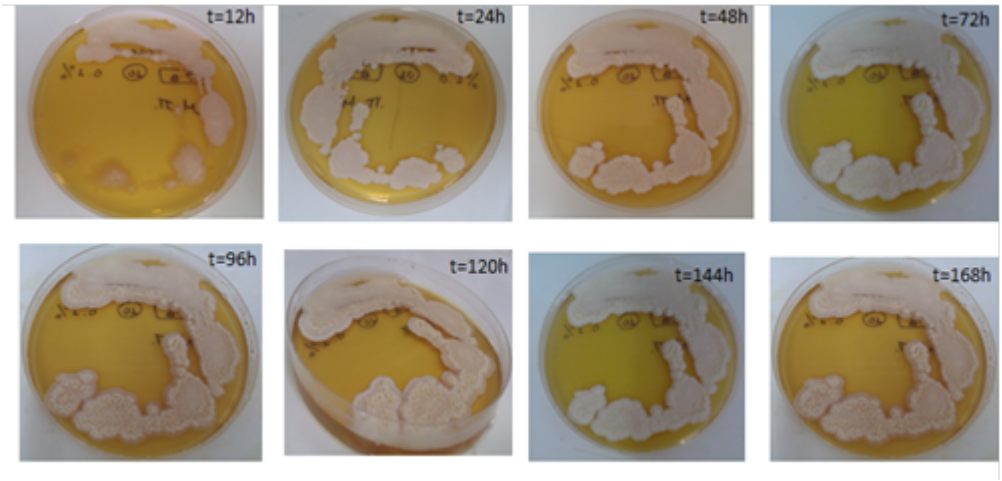

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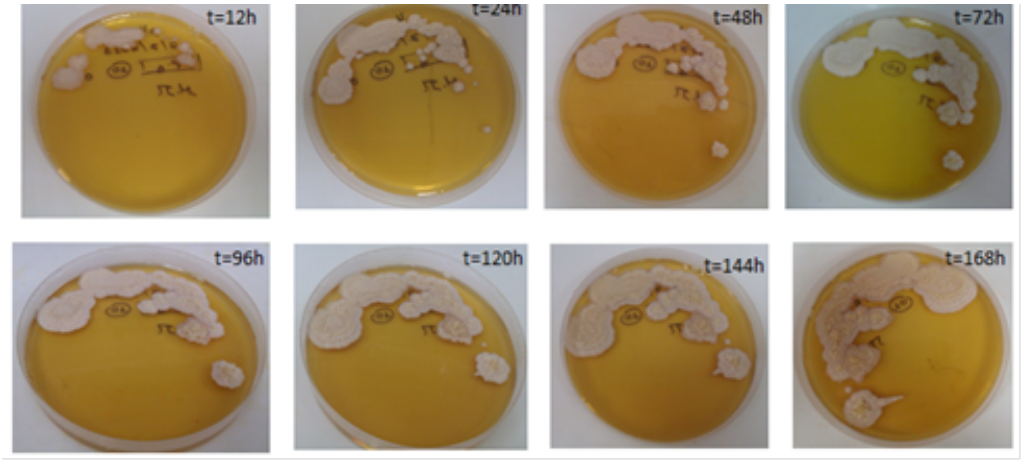
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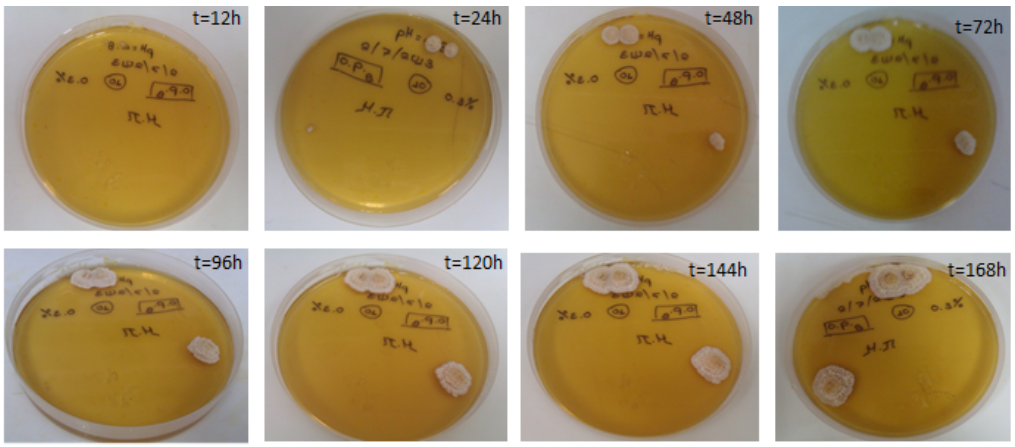
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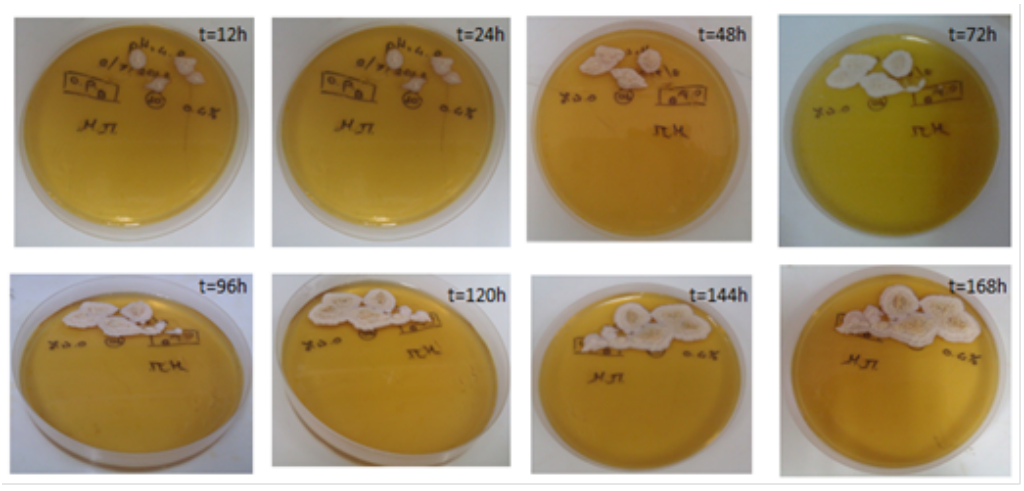
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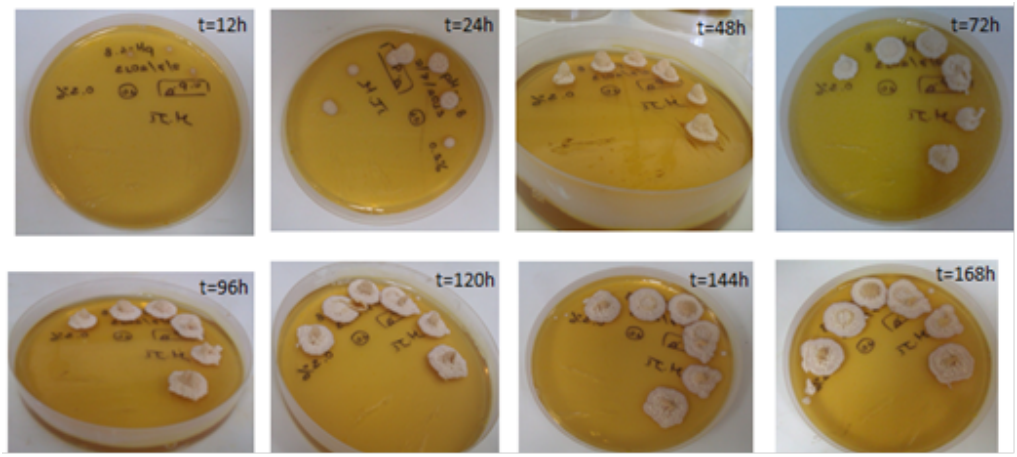
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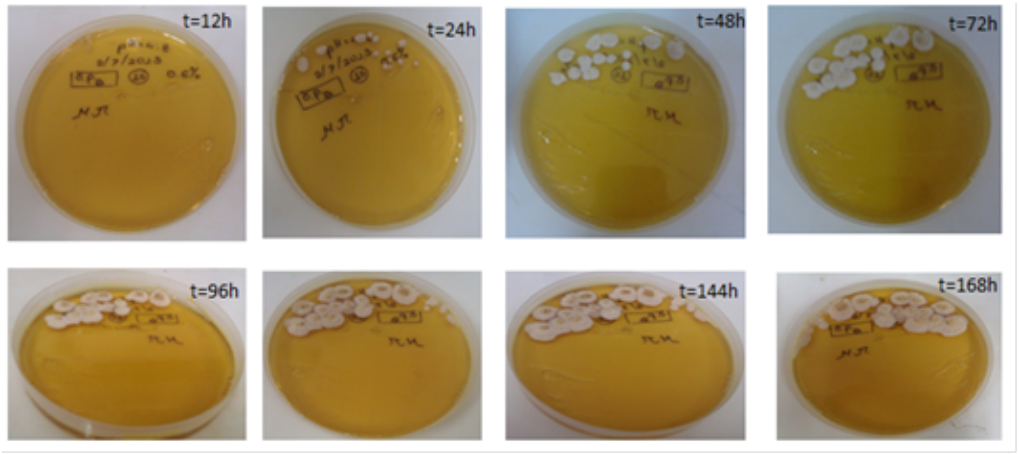
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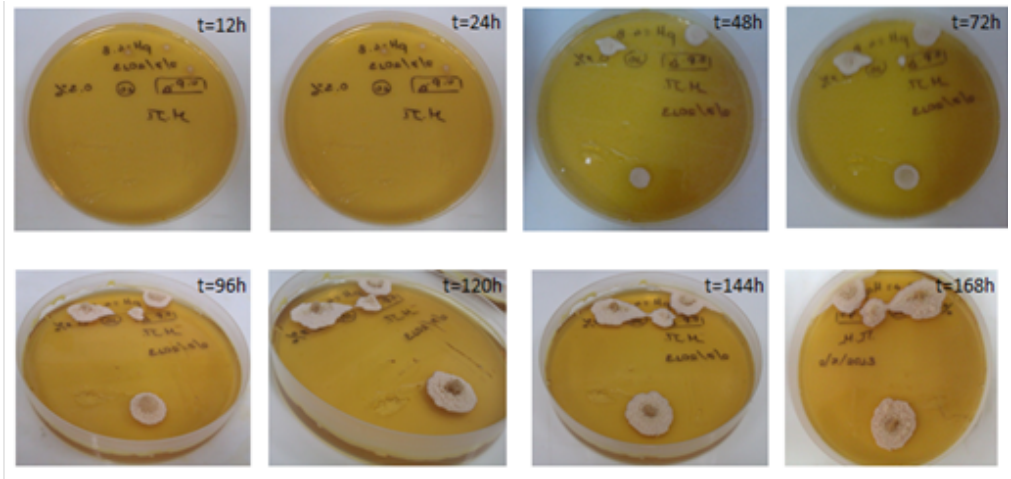
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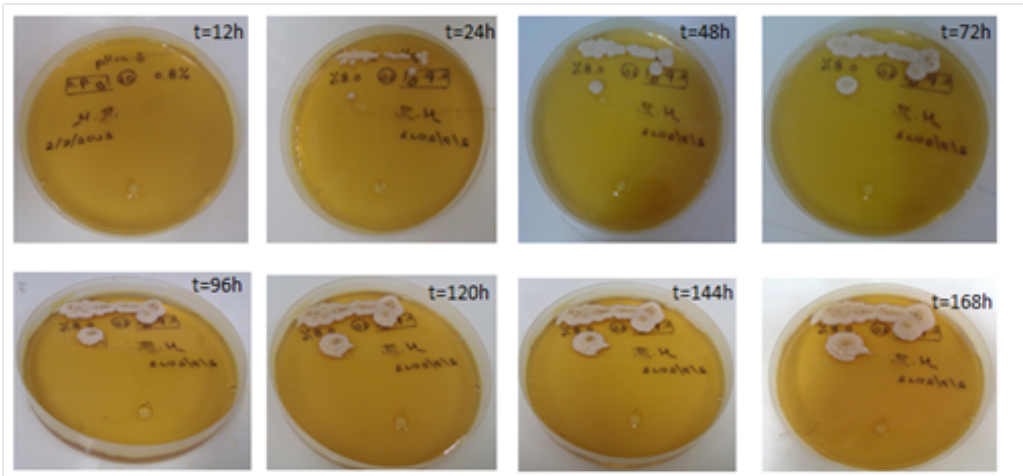
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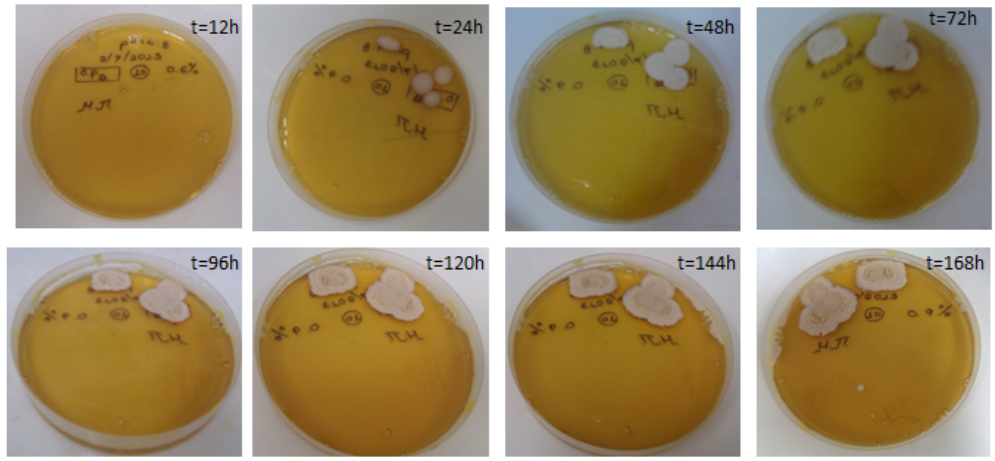
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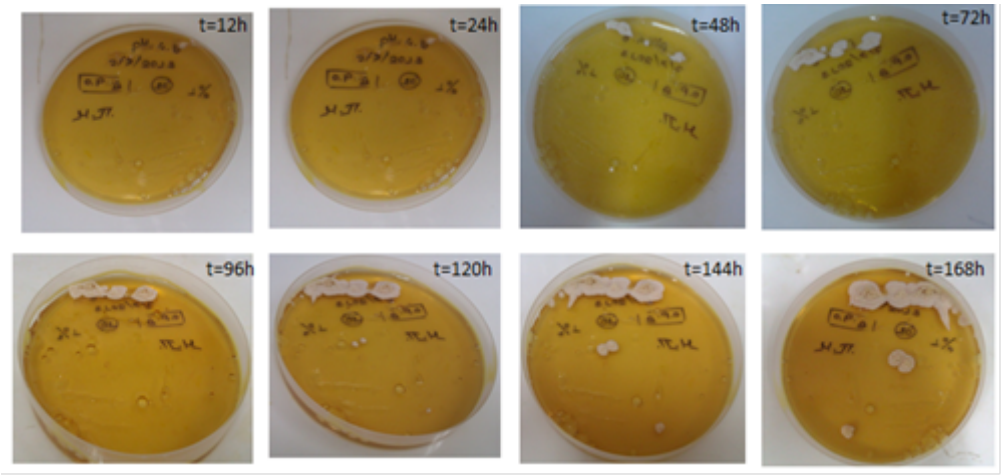
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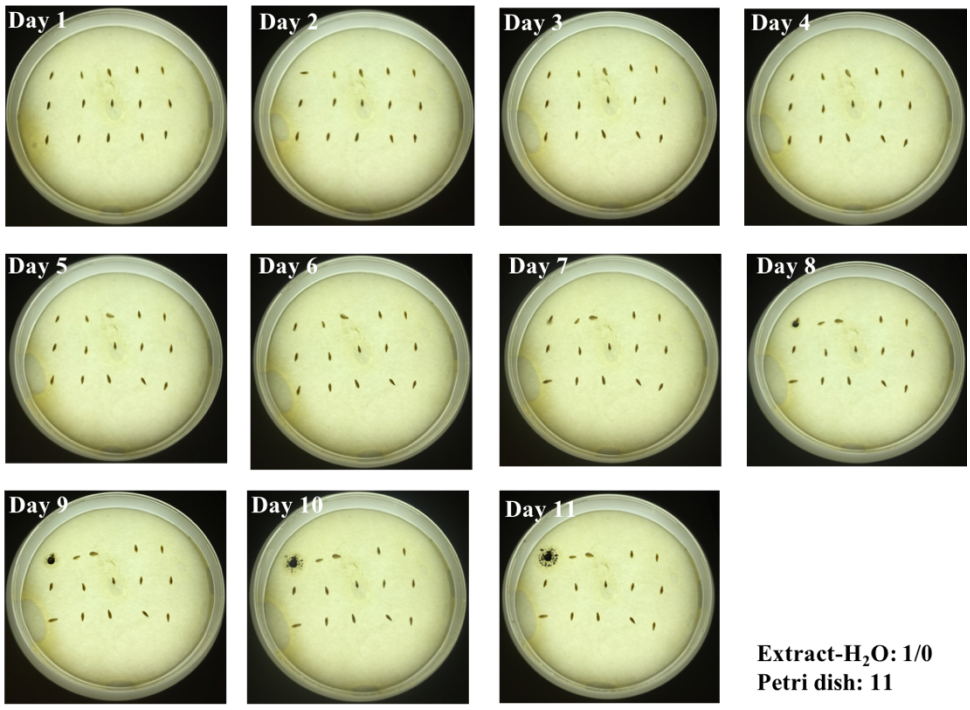
APPENDIX II

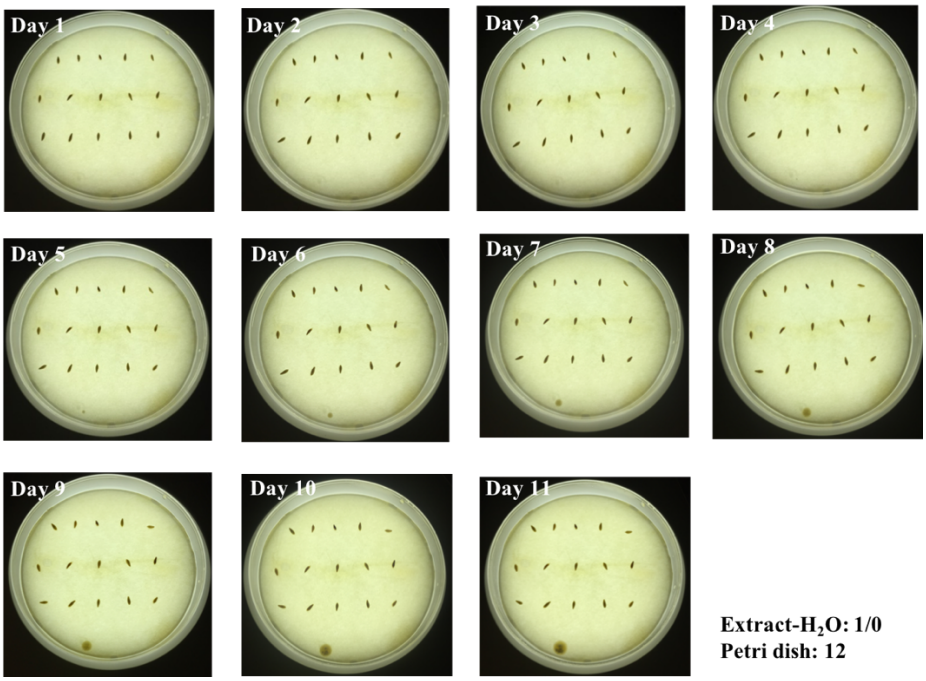
Seed germination test for shoot and root length of lettuce under the presence of BR extract

Figure AP2A: Seed germination tests, 6 serial dilutions (up to 10^{-6}) of the dried material in water (extract was stirred for 24 h at ambient temperature), were used. Filter paper was placed in Petri dishes and it was moistened daily (4 replicates/treatment, 15 seeds/replicate). Seeds were considered germinated upon radicle emergence.

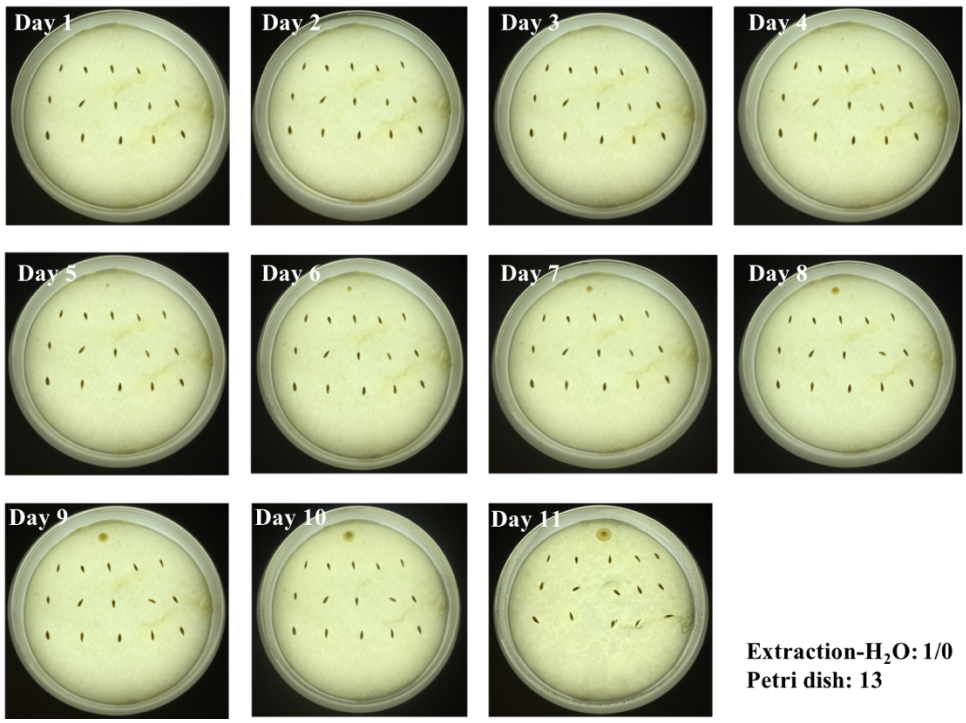
1.



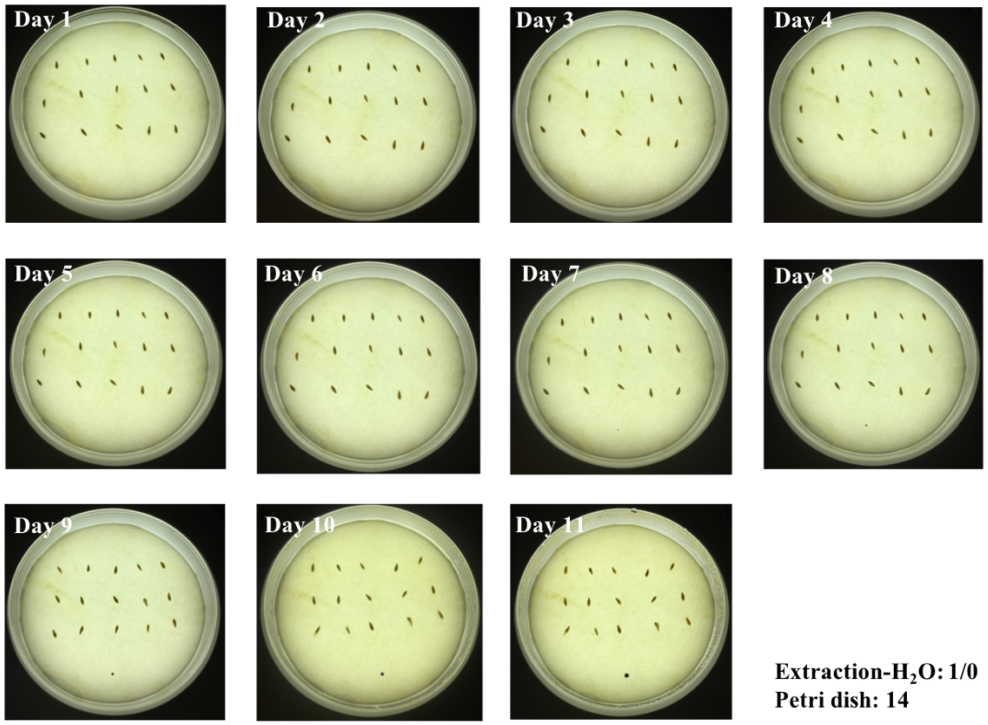
2.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extract-H_2O: 1/0
Petri dish: 12

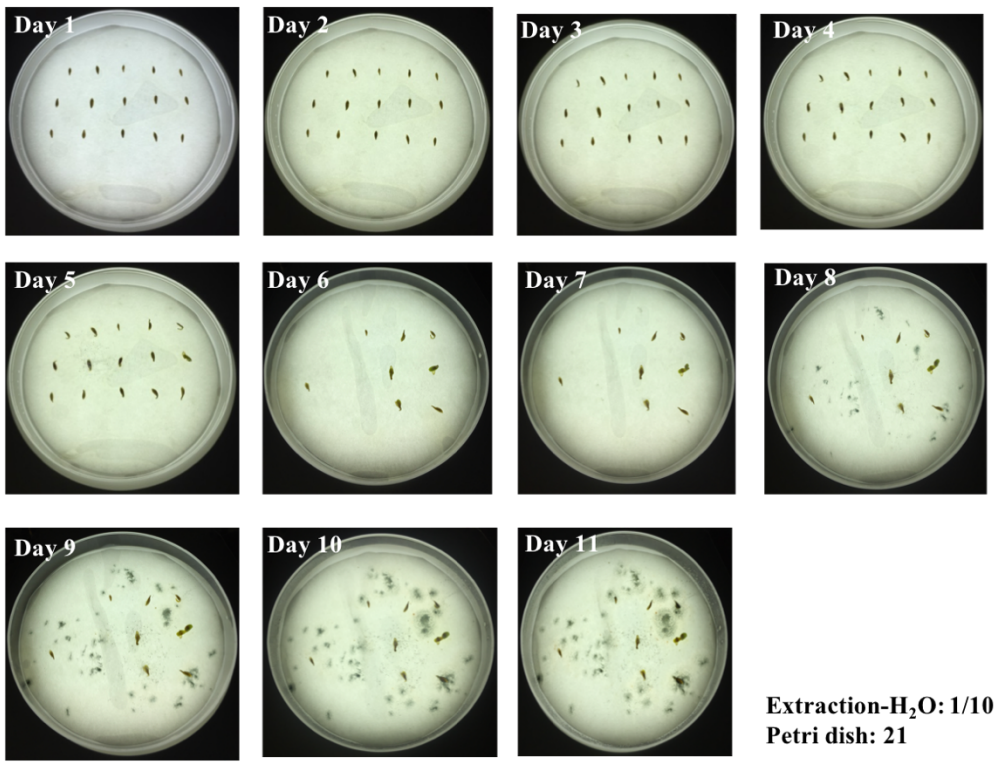
3.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/0
Petri dish: 13

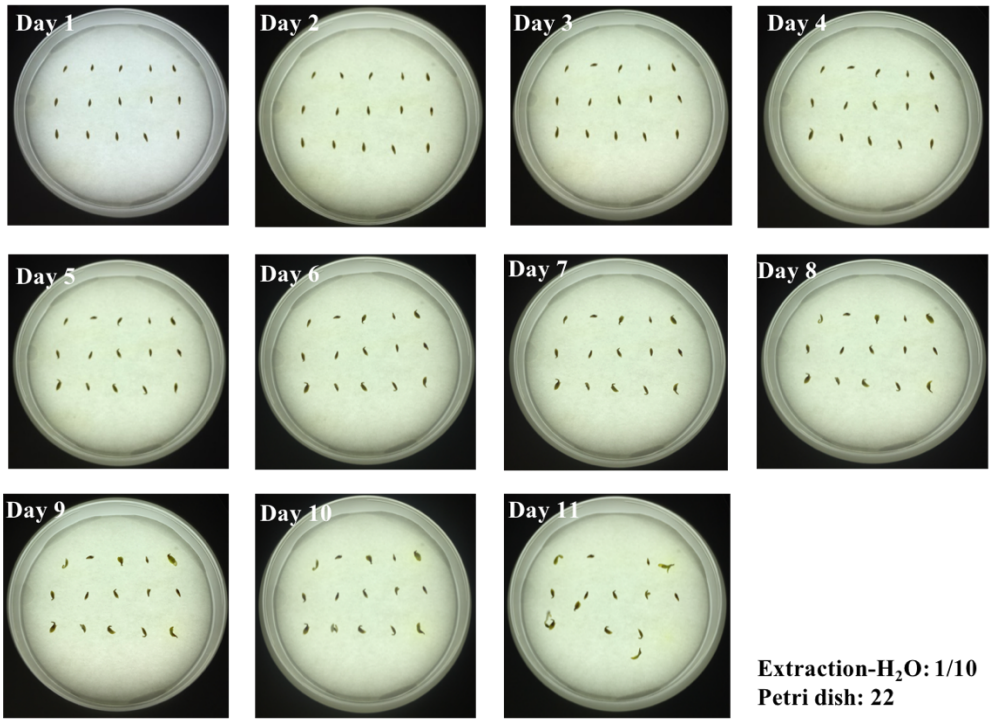
4.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/0
Petri dish: 14

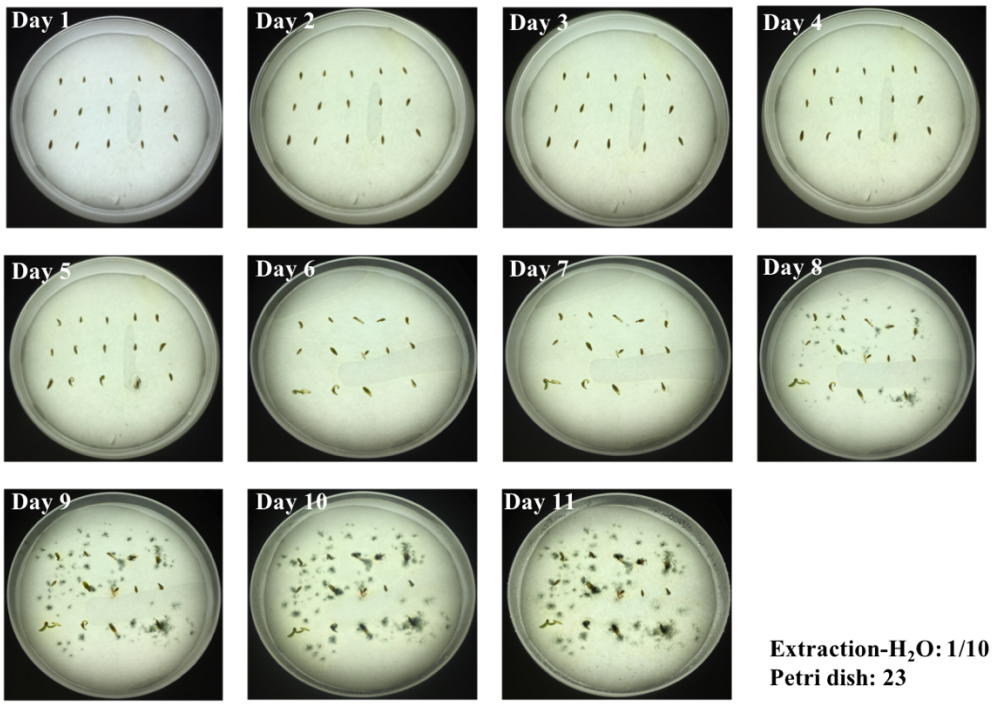
5.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/10
Petri dish: 21

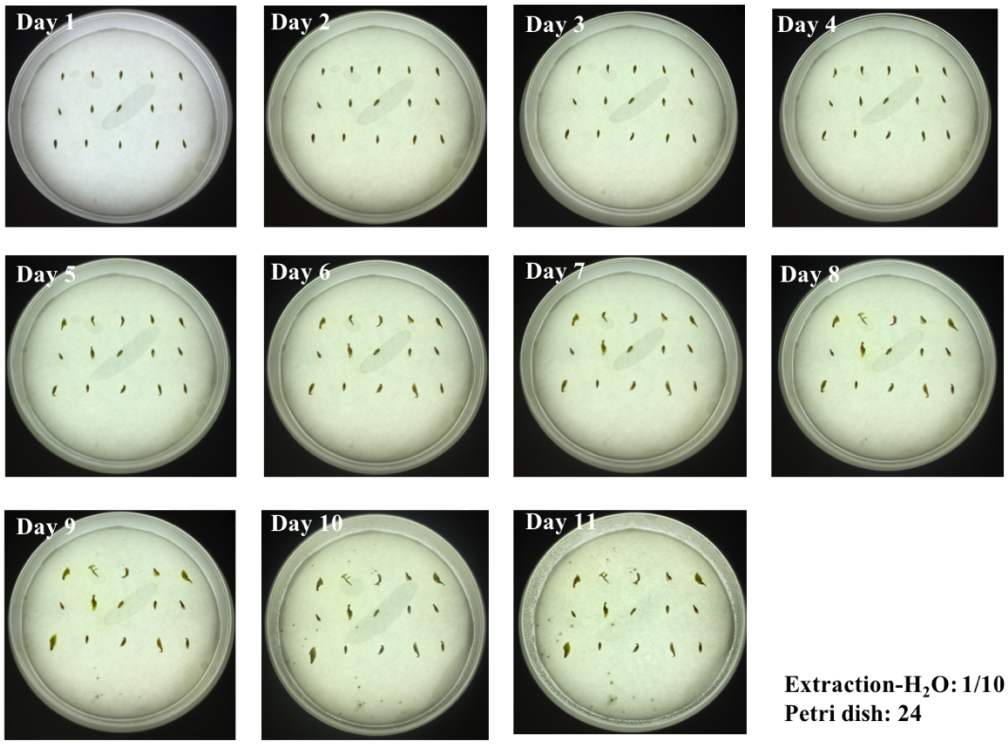
6.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/10
Petri dish: 22

7.

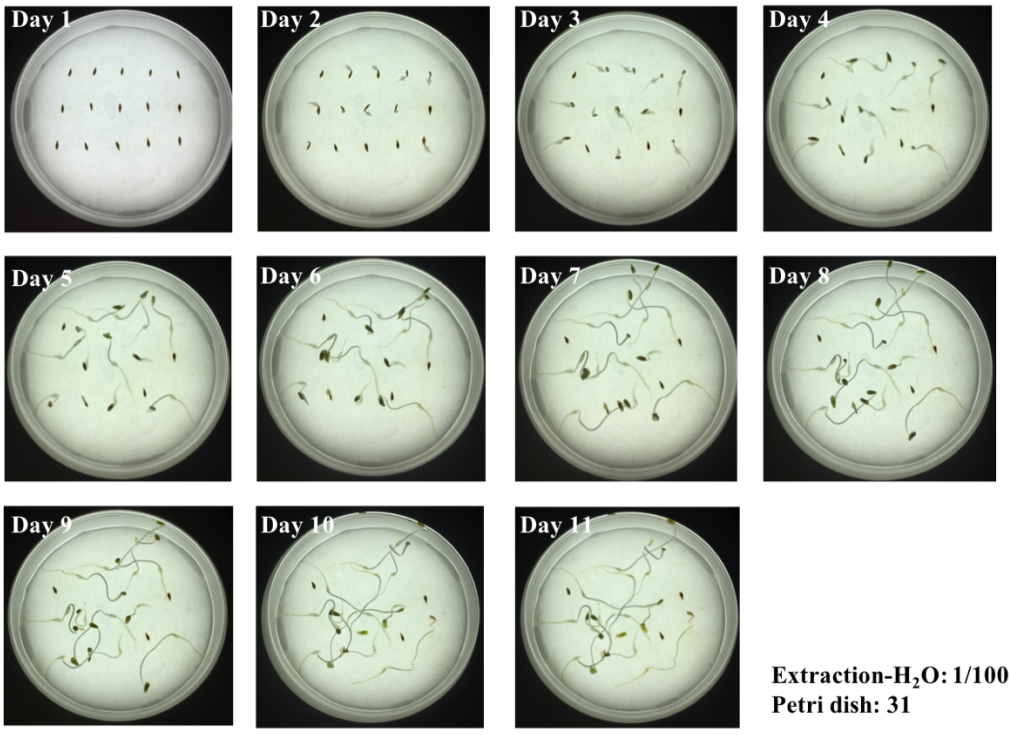

Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/10
Petri dish: 23

8.



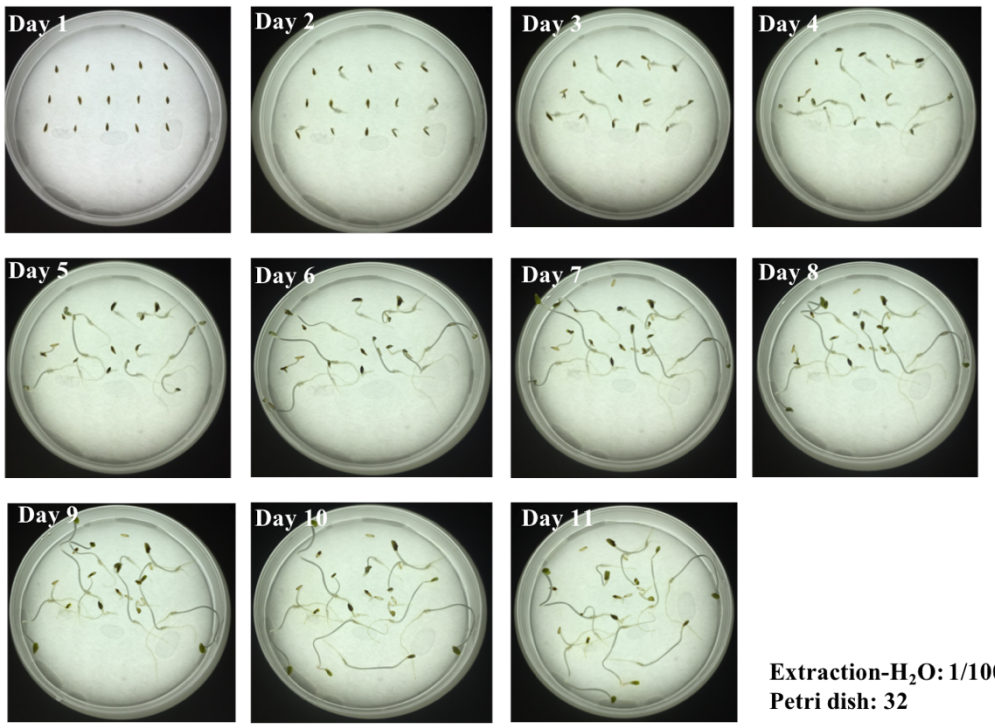
Extraction-H_2O: 1/10
Petri dish: 24

9.



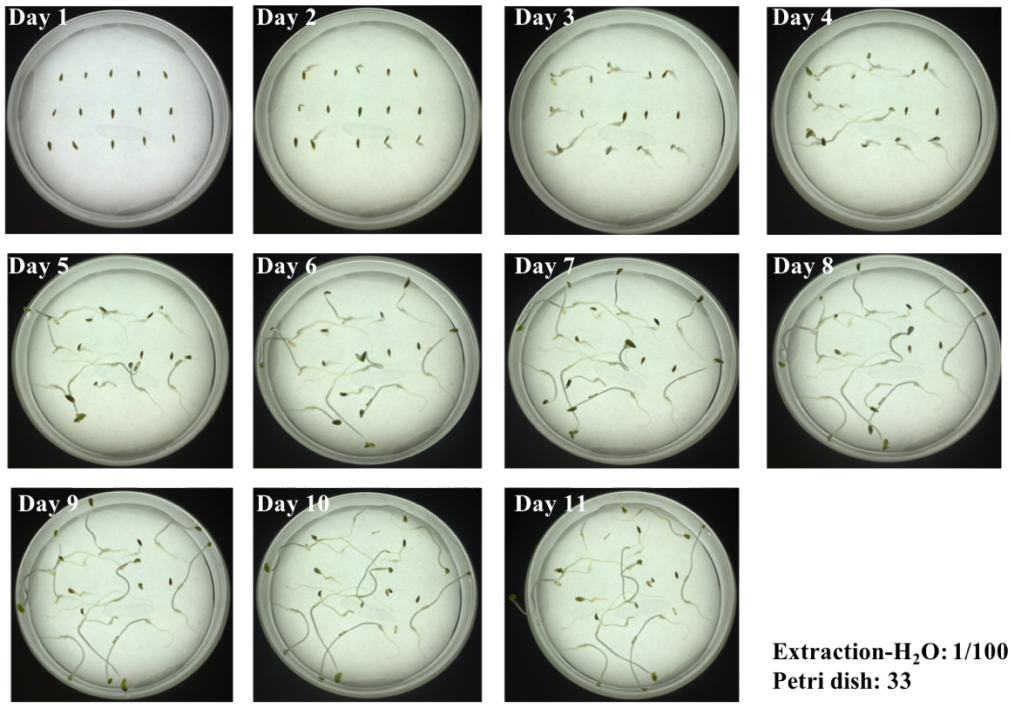
Extraction-H_2O: 1/100
Petri dish: 31

10.



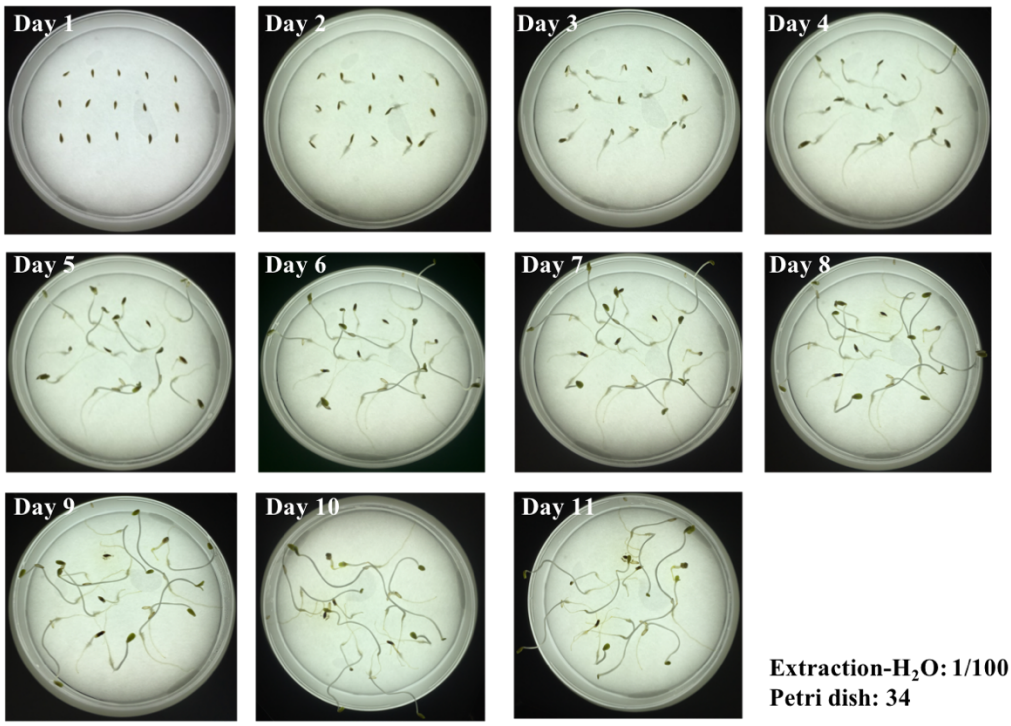
Extraction-H_2O: 1/100
Petri dish: 32

11.



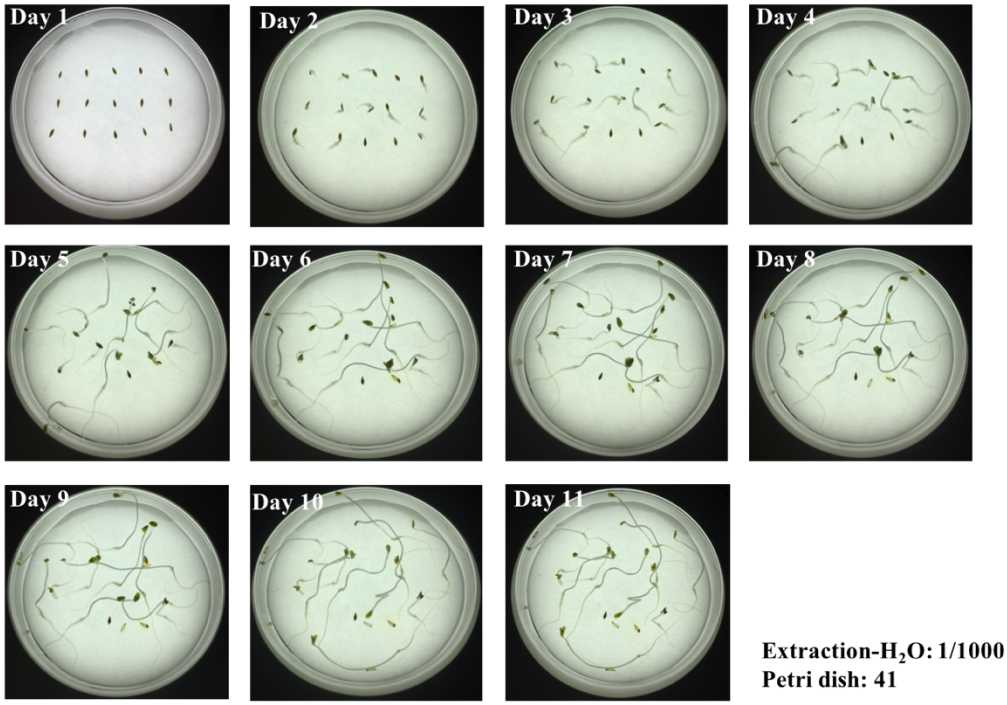
Extraction-H_2O: 1/100
Petri dish: 33

12.



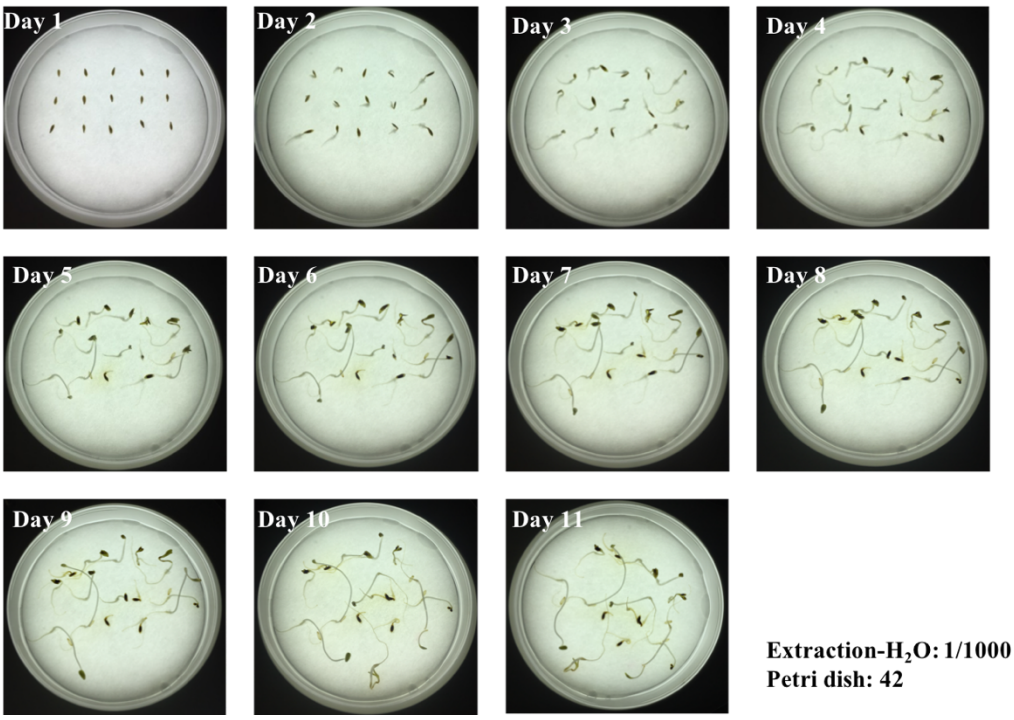
Extraction-H_2O: 1/100
Petri dish: 34

13.

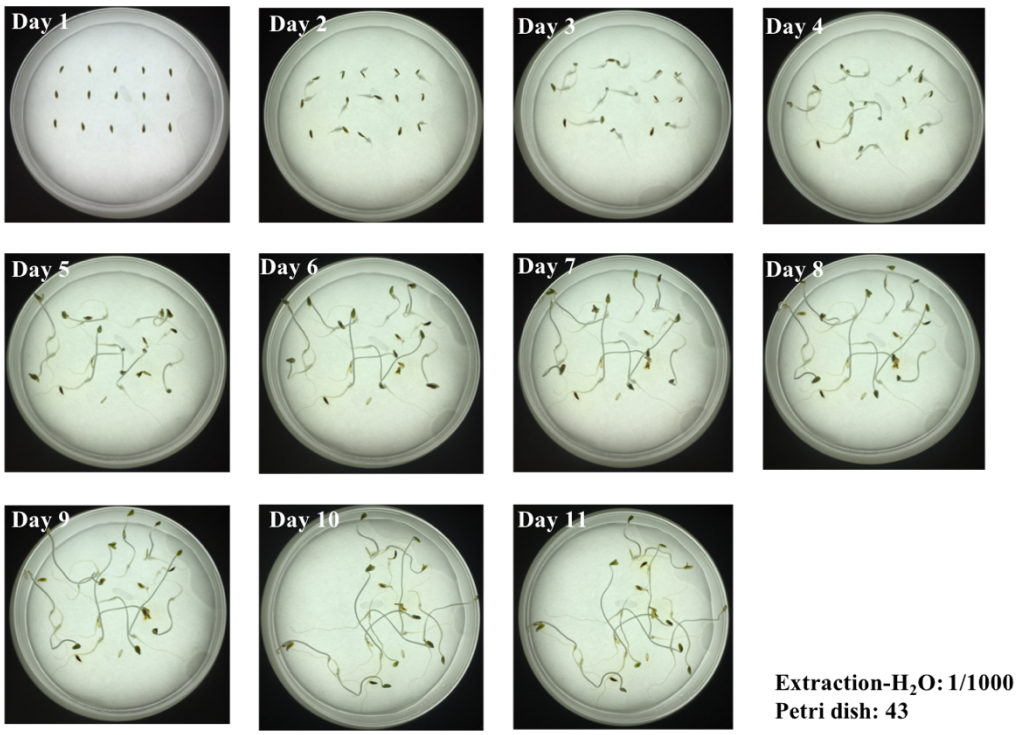


Extraction-H_2O: 1/1000
Petri dish: 41

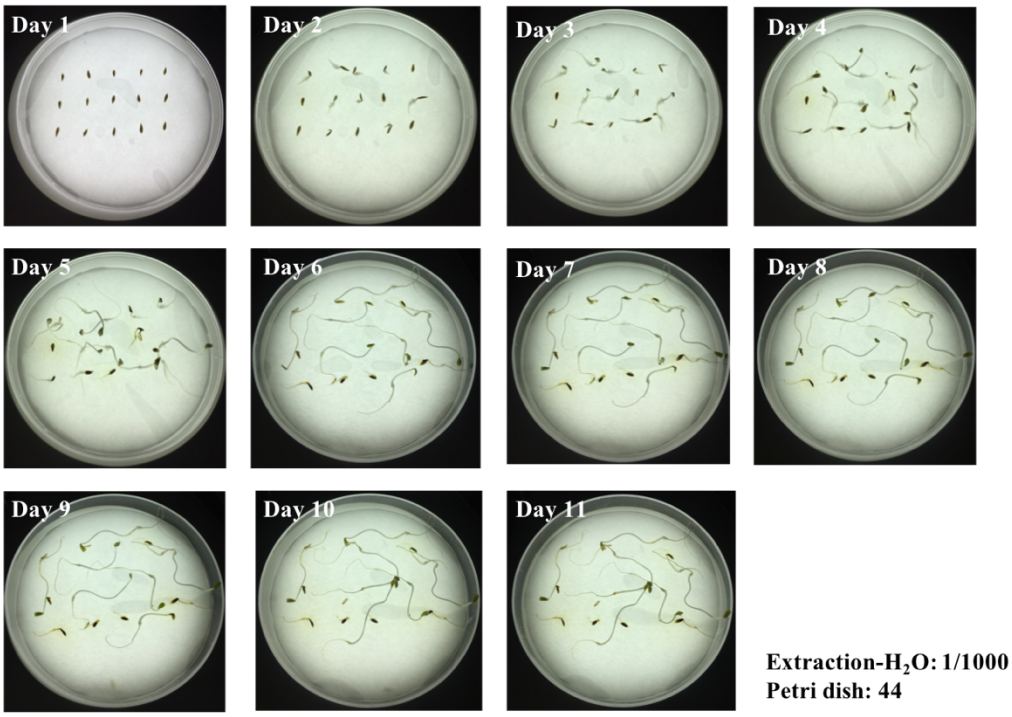
14.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/1000
Petri dish: 42

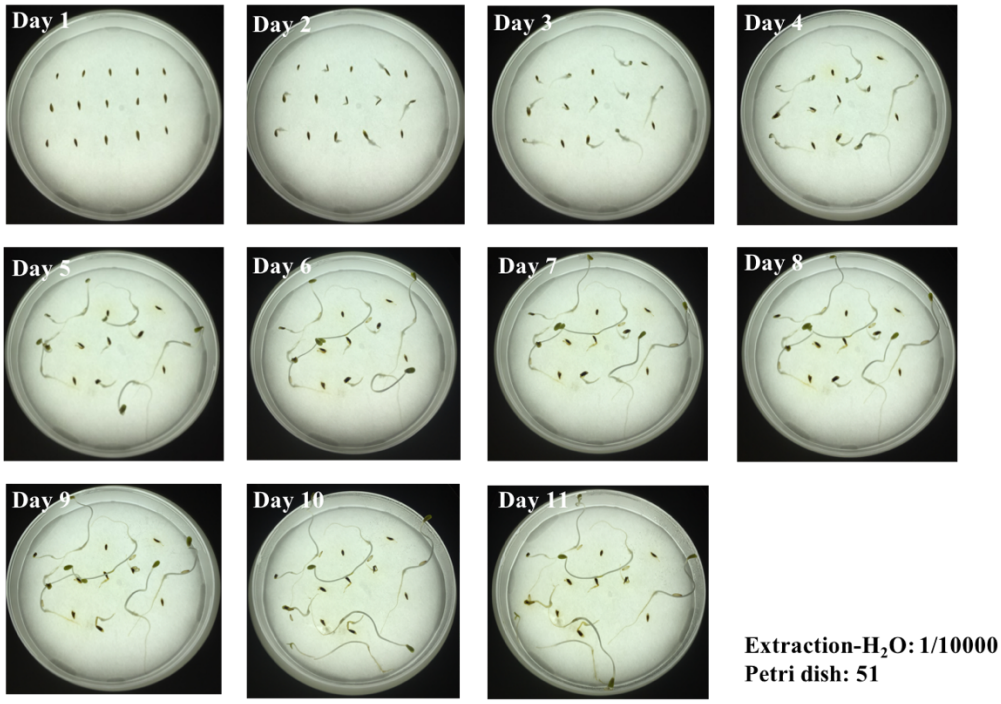
15.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/1000
Petri dish: 43

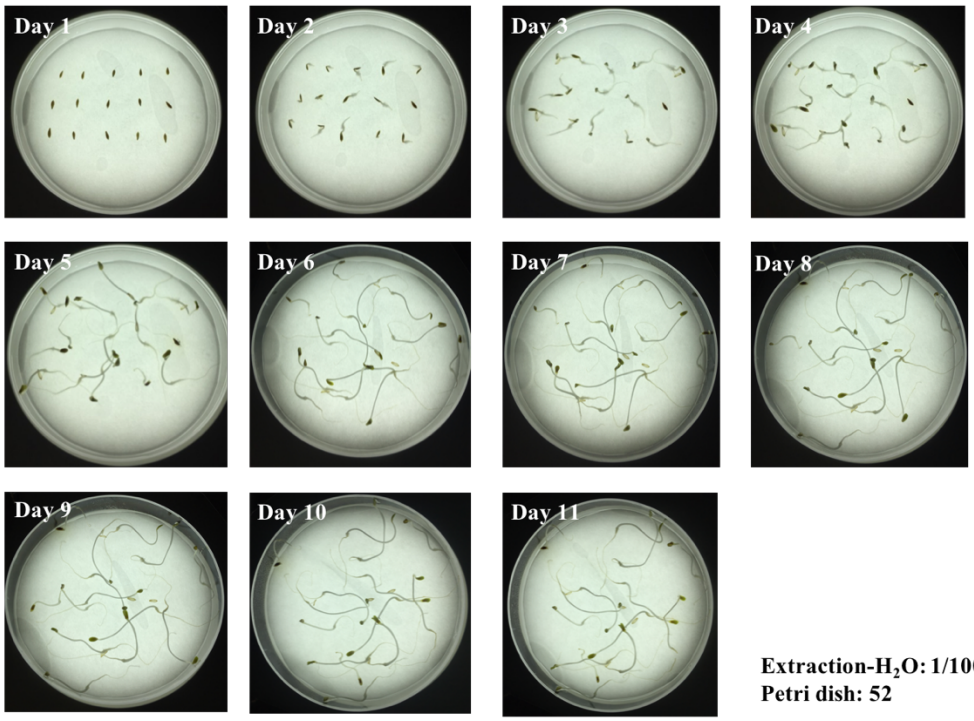
16.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/1000
Petri dish: 44

17.

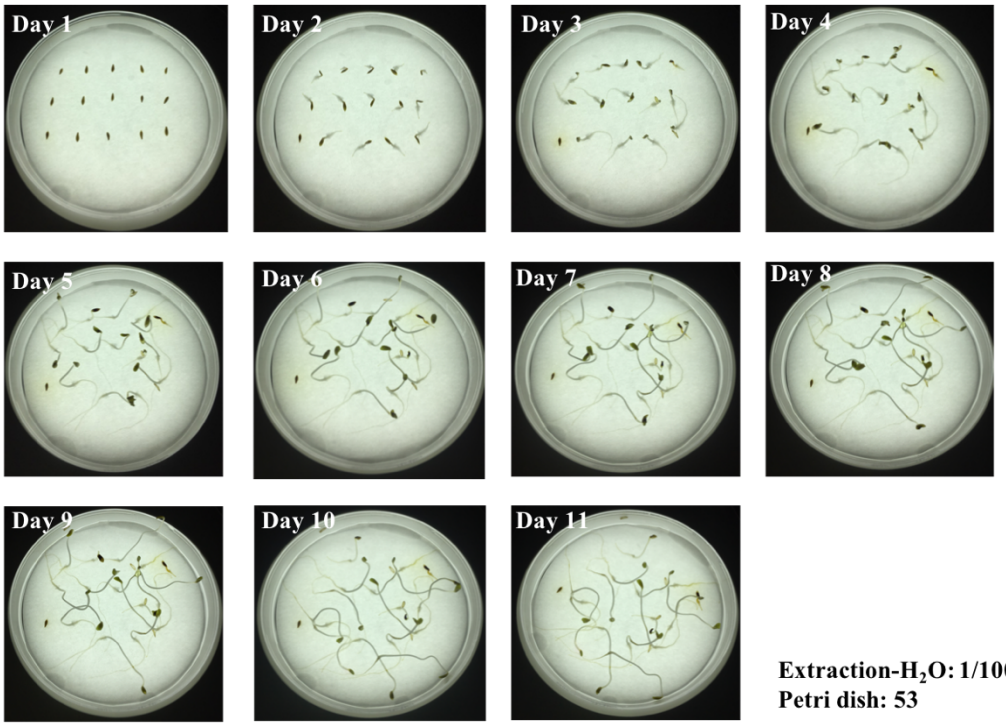

Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
Extraction-H_2O: 1/10000
Petri dish: 51

18.



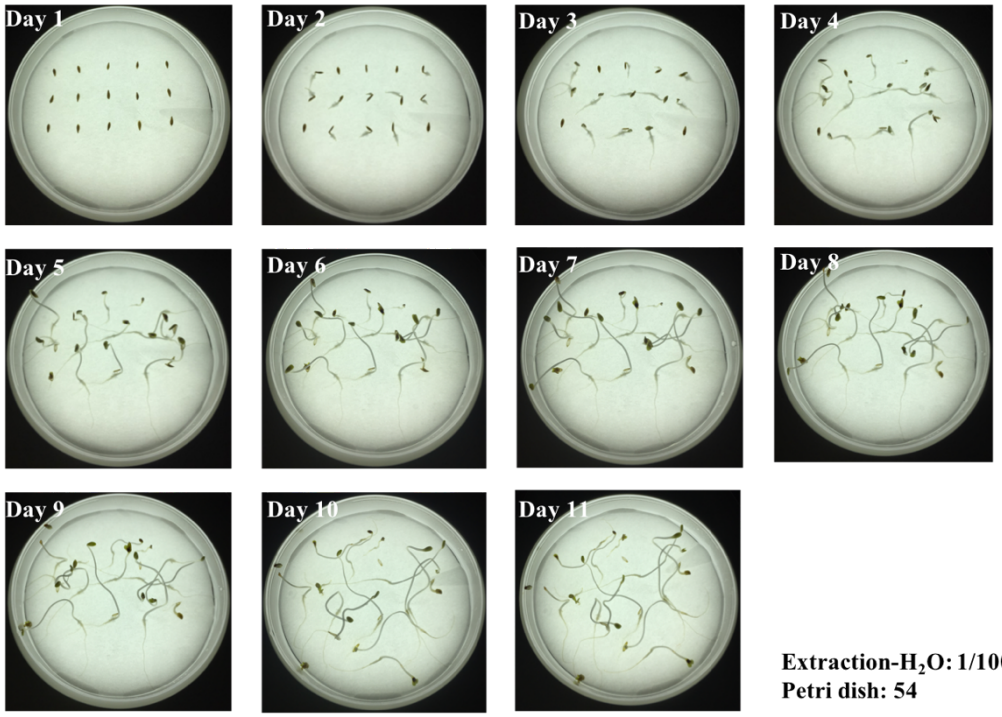
Extraction-H_2O: 1/10000
Petri dish: 52

19.



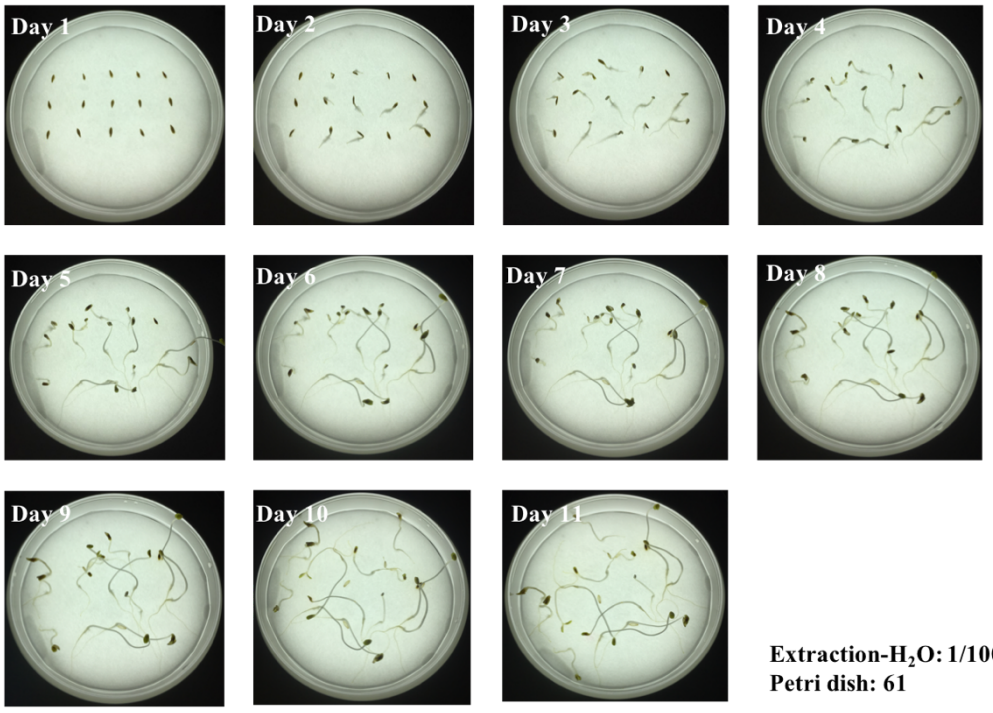
Extraction-H_2O: 1/10000
Petri dish: 53

20.



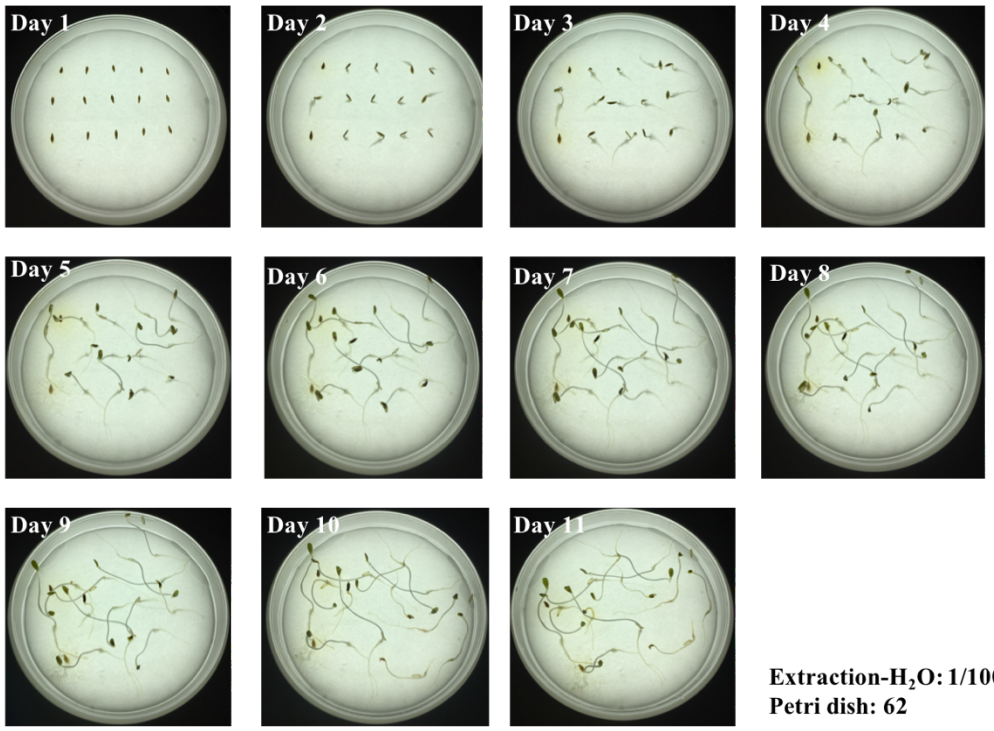
Extraction-H_2O: 1/10000
Petri dish: 54

21.



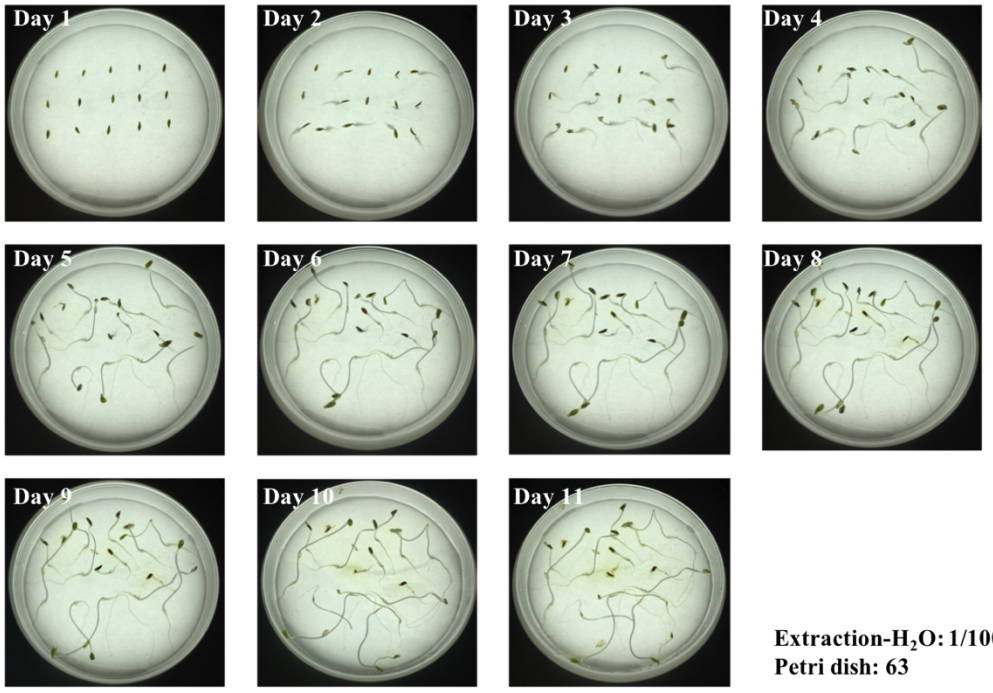
Extraction-H_2O: 1/100000
Petri dish: 61

22.



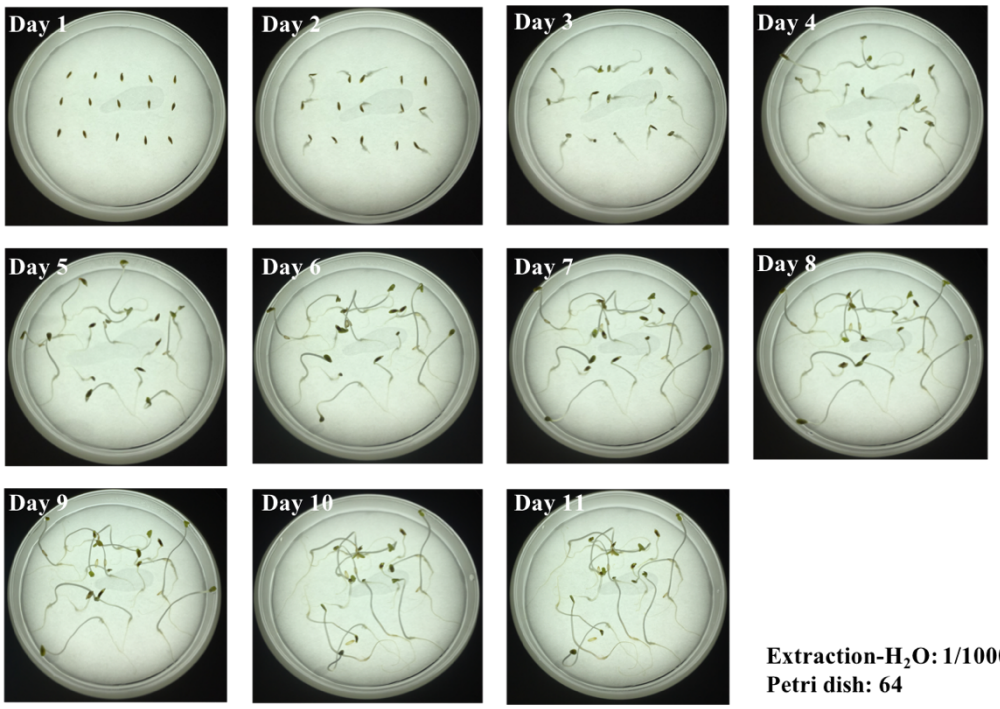
Extraction-H_2O: 1/100000
Petri dish: 62

23



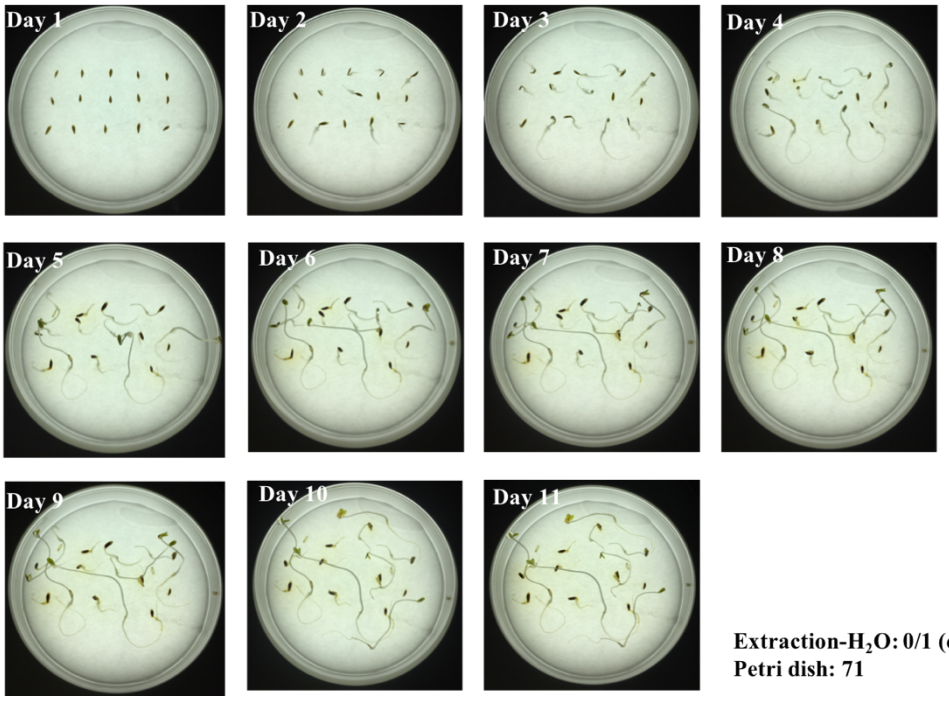
Extraction-H_2O: 1/100000
Petri dish: 63

24.



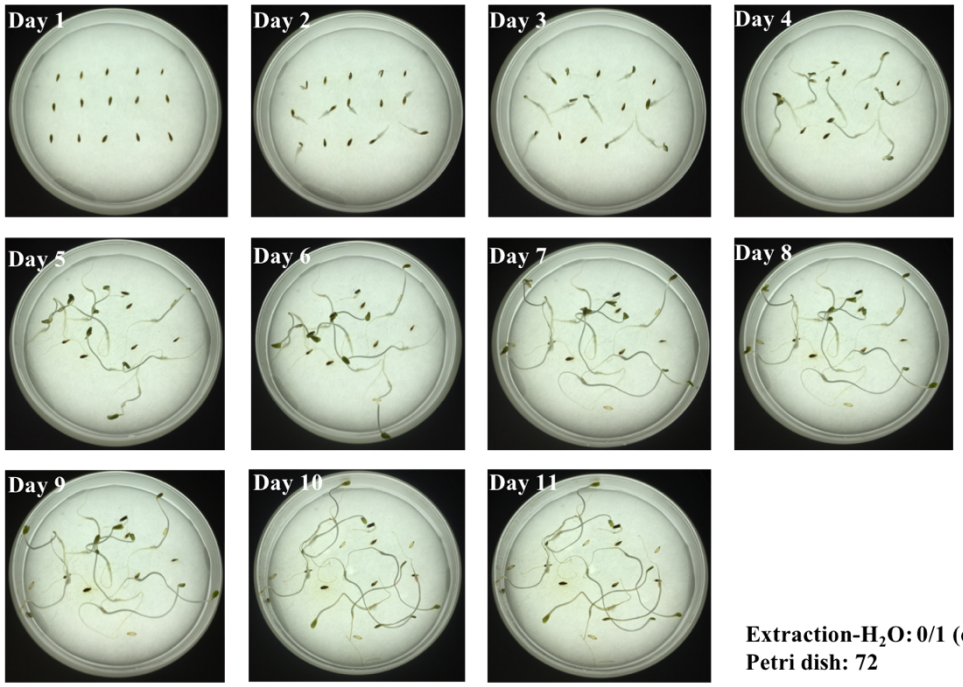
Extraction-H_2O: 1/100000
Petri dish: 64

25.



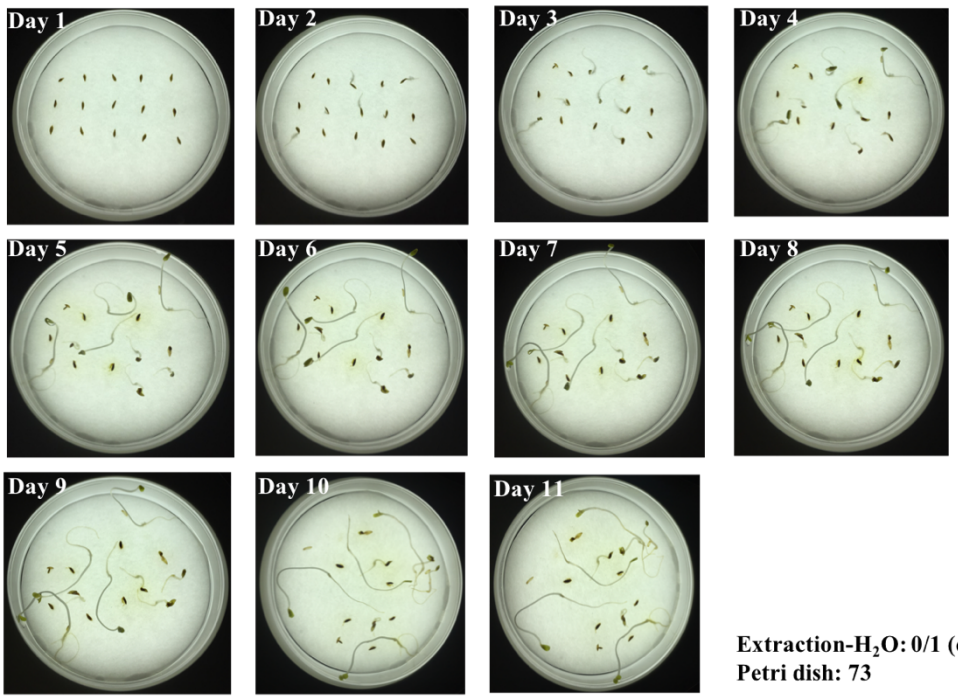
Extraction-H_2O: 0/1 (control)
Petri dish: 71

26.



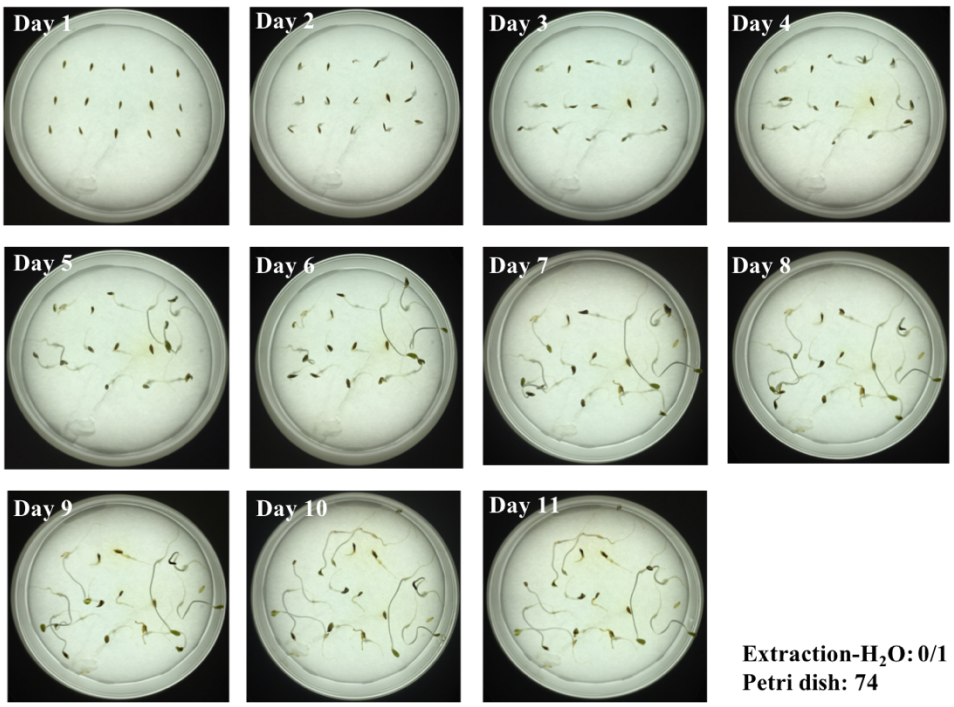
Extraction-H_2O: 0/1 (control)
Petri dish: 72

27.



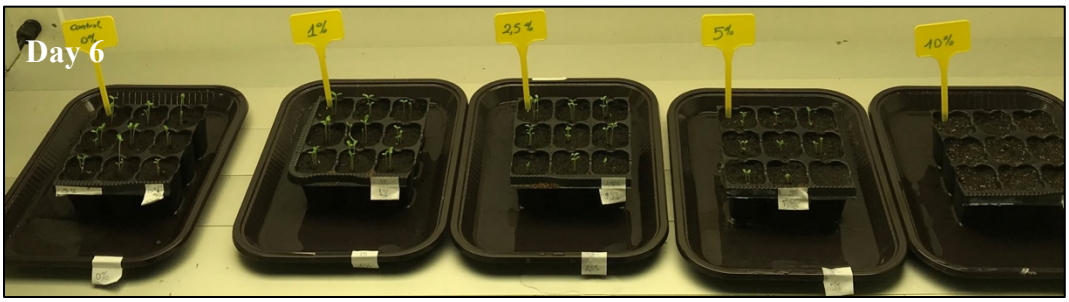
Extraction-H_2O: 0/1 (control)
Petri dish: 73

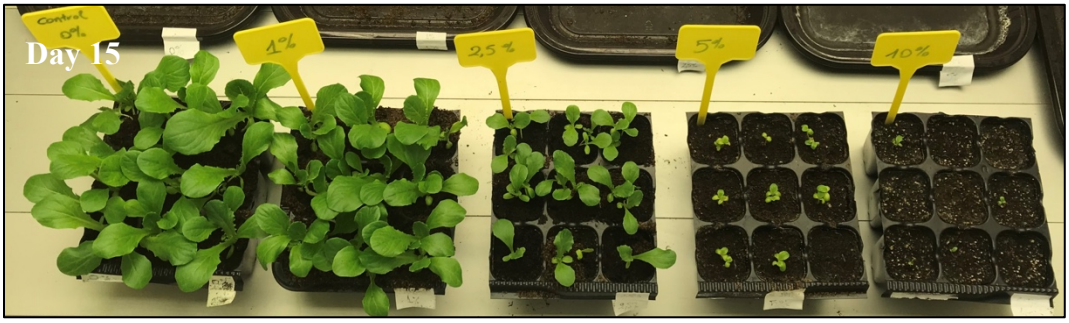
28.

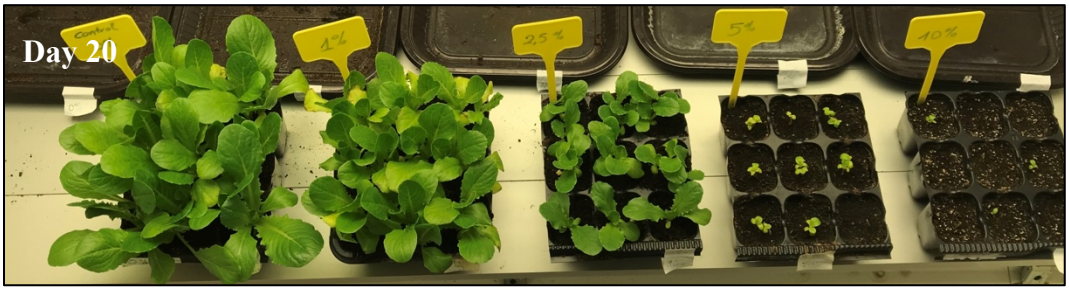


Extraction-H_2O: 0/1 (control)
Petri dish: 74

Figure AP2B: Different ratios of the dried BR in commercial peat substrate (0%, 1%, 2.5%, 5% and 10% w/w) were used to evaluate lettuce seedling production *in vivo*.




Day 15
Control 0%
1%
2,5 %
5%
10%


Day 20
Control
1%
2,5 %
5%
10%