Evaluation of Second-Generation Biofuels Production from Halophytes by Focusing on Wet-Fractionation of Native Halophyte *Salicornia sinus-persica*

By

Ayah M. S. Alassali

A Thesis Presented to the

Masdar Institute of Science and Technology

in Partial Fulfillment of the Requirements for the Degree of

Master of Science in

Chemical Engineering

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May 2014

AUTHOR'S DECLARATION

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Abstract

Abu Dhabi exemplifies a coastal desert that is surrounded by seawater from three sides, allowing for cultivation of salt-tolerant crops (halophytes). In this study, the halophytes *Salicornia bigelovii* (dry) and *Salicornia sinus-persica* (fresh) were evaluated for their potentials to be used as feedstocks for bioethanol production.

The main focus of the study was to utilize the native halophyte *Salicornia sinus-perica* harvested fresh (green). *Salicornia sinus-perica* contains about 70% of water. For such green biomass direct fractionation and fermentation is advantageous, since it allows water preservation in the system.

Chemical characterization and ethanol production potential of the fractionated *Salicornia sinus-perica* were examined. The fresh untreated pulp fraction showed sugar content that is comparable to conventional crops; particularly 16.27 ± 1.47 g/100 g dry mass (DM) glucose, 11.57 ± 1.00 g/100 g DM of fructose and xylose combined, 1.79 ± 0.03 g/100g DM galactose and 11.04 ± 1.19 g/100g DM arabinose.

In order to optimize sugar recovery and fermentability of the pulp fraction, a pre-treatment study was conducted, showing that ethanol yields obtained are directly proportional to the applied pre-treatment temperature (for temperatures ranging between 120 ˚C and 170 ˚C). Ethanol yields increased by 10 folds for pulp pre-treated at 170 ˚C compared to the pulp treated at 120 ˚C when the residence time was fixed at 10 minutes.

Juice fermentation obtained high ethanol yields (~100%), however due to its low sugar content (1.0-1.5 %), production of other value-added chemicals was considered. Juice fermentation showed noticeable production of organic acids, which is probably achieved due to contamination with bacteria. Juice sterilization and acid hydrolysis

did not enhance the sugar convertibility of the juice, hence fresh juice utilization is recommended.

For future work, *Salicornia sinus-persica* needs to be evaluated for production of added-value chemicals (proteins and nutraceuticals), which can improve the economy of salicornia-based biorefinery.

This research was supported by the Government of Abu Dhabi to help fulfill the vision of the late President Sheikh Zayed Bin Sultan Al Nahyan for sustainable development and empowerment of the UAE and humankind.

Acknowledgments

Foremost, I would like to express my sincere gratitude to my advisor Dr. Mette H. Thomsen for the continuous support of my Master work and research, for her patience, motivation, guidance and immense knowledge. I also would like to thank my co-advisor Dr. Farrukh Ahmad for his guidance in my thesis writing. I would like to thank my thesis committee: Dr. Farrukh Ahmad and Dr. Alejandro Ríos Galvan, for their time, help and insightful comments. I also would like to express my appreciation to Dr. Jonathan Jed Brown for his help is samples collection and identification. Furthermore, I would like to thank every member and colleague in the Chemical Engineering department at Masdar Institute; everyone has added a precious value to this two-year journey.

I would like express my sincere gratitude to Carlo A. Amadei and Fabian Bonk for the valuable comments and recommendations for my thesis work.

To all friends at Masdar Institute, thank you for helping me and standing by me, I could never do it without having such friends around me. Nahid, Celia, Lutfiye and Vandana, you were a great company in the lab, I appreciate it. To all Masdarian weirdos, psychos, creepy, funny or those who think they are, it was fun to have you around.

My deepest appreciations and thanks to my family for their continuous support throughout this journey. My deepest gratitude to Mohammad Said, who always encouraged me and believed in me and Sawsan who never forgot me from her prayers, love you baba and mama. My sisters and brother, I love you! I would also like to thank Khaled for his patience and support throughout this journey, all my appreciation and love. Many thanks to every person who made it possible to reach this stage of my life.

Last but not least, Masdar taught me to strive being a value rather than a success.

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CHAPTER 1

1. Introduction

1.1. Background

Commercial aviation, with a universal capacity of about 15,750 aircraft, is strongly dependent on a fuel source that is obtained from fossil fuels, contributing in about 2–3% of global carbon emissions in 2009 [1]. Emissions generated from aviation operations lead to alterations in the chemical and particle microphysical characteristics of the upper atmosphere, resulting in fluctuations in radiative energy in the climate's system. This can potentially lead to climate change influences and eventually cause destruction and welfare/ecosystem loss [1].

Furthermore, the excessive usage of fossil energy to run the regular aircraft operations makes it strongly impacted by both, oil price instability and climate change regulations. The relative rate of development of the sector together with concerns about the environmental impact and the future security of fuels resources have obliged the sector to speedily inspect the potential use of alternative fuels in aviation gas turbines. The aviation sector is aiming at and committed to reduce GHG emissions and to meet growing transportation demand while being challenged to manage fuel price volatility [2]. Moreover, future guidelines are expected to impose clean energy

sources to counterbalance emissions. For instance, the International Air Transport Association (IATA) put the objective for its members is to use 10 percent alternative fuels by 2017 [3]. In order to achieve these advancements in this field, drop-in fuels are needed. Those fuels must be reliably and safely incorporated, while being completely interchangeable with the existing jet fuel product to evade the logistic problems of airports handling multiple fuels of different qualities and the commercial restrictions this could impose. Furthermore, the sustainability of a commercial jet refers to any candidate fuel that is suitable for use in existing engine technology. For these motives, the main research drive has been around the development of "drop-in" fuels which can be used in the existing units [2]. Converting alcohols to jet from alcohol fermentation of sugary substrates is one route mmong the different examined routes used to produce bio jet fuel, [4], [5].

The Sustainable Bioenergy Research Consortium (SBRC), founded by Boeing, Etihad, UOP Honeywell and Masdar, is focused on the research and development of sustainable aviation biofuels. Principally, SBRC has started a research utilizing halophytic (saltwater tolerant) plants [6]. Bioenergy research on oil-rich halophytes emphasizes on biodiesel/kerosene fuel from the oil-rich seeds [7], [8], [9], while looking into ethanol and biogas production from the lignocellulosic residue [10], [11], [12].

Biofuels

Increasing the demand on the limited fossil fuels has led to increasing fuel price, associated with the threat of fossil fuels scantiness. In addition, environmental problems such as greenhouse gas emissions (GHG) and pollution have also generated and have urged the industry to find renewable fossil-fuels substitutes that are secure

and can be produced at a reasonable price. Different renewable energy options have been proposed, such as wind, solar, hydropower, geothermal, and biomass [13], [14].

According to the Energy Information Administration (EIA), 2011 [15], biorenewable energy is one of the most rapidly developing industries in the world. Guo, Fang [16] discussed the predicted high potential of biofuel production from cellulosic material, which is globally growing by 6.7% per year and predicted to reach 2.7 X 10^6 barrels of oil equivalents per day in 2030, hence, the interest in liquid biofuels is increasing [14].

Biofuel is referred to liquid or gaseous fuels generated from almost any biomass, including starch and sugar-rich crops, lignocellulosic material, woody and herbaceous plants, as well as vegetal oils [17]. Biofuels are renewable, locally available, and easily accessible while being a carbon neutral source of energy [18]. Different technologies are applied to produce targeted fuels from various biomass, such as ethanol and diesel, in addition to butanol, hydrogen and methane [19, 20], [21]. Liquid biofuels (i.e. bioethanol, biodiesel, and biobutanol) have great potentials to renewably replace petroleum [15].

Biofuels production route is energy intensive and its implementation can be relatively expensive. Nevertheless, high-value byproducts can be potentially produced alongside the biofuels chain in a biorefinery, making this route viable for application [22]. Examples of such biochemicals are acetic acid, malic acid, acetone, lactic acid, which are all currently derived from petroleum [16].

1.2. Motivation

Biomass shows advantages by being a renewable and maintainable energy source; it also has positive environmental properties, since it is able to offset the negative

effects of fuels emissions by the fact that the CO_2 generated through combustion of the fuel will be taken up by the next generation of plants. Plants are the most common source of biomass [17]. Food resources (i.e. sugar and starch-based crops) – also called 1st generation crops– should be avoided in sustainable biofuels systems (to avoid competing with food production) [23], instead the focus in biofuels production should be on so-called 2nd generation feedstocks that are waste products from food production, agricultural byproducts, or plant cultivated on marginal land [24].

In lignocellulosic biomass, about 90% of plants' dry weight is composed of cellulose, hemicellulose, lignin, and pectin [13]. Lignocellulosic biomass has shown advantages over first generation biomass; it produce much higher yields per hectare ((135 GJ/ha) compared to corn kernel (85 GJ/ha) and soy (18 GJ/ha)) [25], alongside exhibiting significant greater energy return and improved carbon sequestration compared to the first generation biofuel crops [25]. For the 2nd generation biomass utilization, high pressure and high temperature pretreatment methods are recommended in order to disrupt the recalcitrant lignicellulosic structure and to improve the enzymes accessibility to the cellulosic carbohydrates [26], [11]. These pre-treatment methods have shown their efficiency and low negative-impacts on the environment [27], as they mainly depend on pressure and temperature optimization rather than chemicals application.

Arid coastal land is an example of land that can be used for sustainable biofuels production. However, the salinity of seawater exceeds the limits of the tolerability of conventional crop plants [28]. Hence, searching or developing salt-tolerant cultivars can help many developing countries to meet future food and energy needs [29]. Salt tolerance is often governed by the anatomical and physiological intricacy of the plant.

Consequently, it is difficult to burgeon salt tolerance in terrestrial species on a wide scale [30].

Brackish plants and halophytes crops allow for direct salty water irrigation [31], [32]. Among the 13 mineral nutrients needed by plants, 11 are found in seawater in adequate concentrations. Accordingly, the disadvantages addressed for low-quality soil and brackish water for conventional crops could be turned into positive attributes for halophyte agriculture [29].

Furthermore, total global costal deserts provide 1.3×10^8 ha of cropland, which could be brought into exploitation [33]. Hence, halophytes cultivation in such areas should be techno-economically evaluated in terms of large-scale production and ability to produce biofuels to substitute depleting fossil fuels.

1.3. Relevance to Abu Dhabi

Abu Dhabi exemplifies a coastal desert. The absence of fresh water and fertile soil creates great restrictions for crop cultivation. However, seawater could be utilized for cultivating high-salt-concentrations and swamping accommodating species [29]. Thus, vegetation occurring in salty environments (such as algae, sea weeds, mangroves and halophytes) should be sought and studied for their chemical composition and convertibility potentials to biofuels. Other studies associated with the economic feasibility of its germination besides the ability of broad applications of such biomass to be also considered.

Research taking place at Masdar Institute has evaluated an integrated seawater agriculture system, where aquaculture, halo-agriculture of an oilseed crop (*Salicornia bigelovii*), and mangrove siviculture (*Avicennia marina*) were the core of the studied system [34]. An Integrated Seawater Energy Agriculture System, abbreviated ISEAS,

is a biofuel, food (fish) and feed production system, intended to take advantage of the interactions existing between several integrated desert ecosystems [35]. The novelty lies in using seawater to irrigate the natively available non-arable plants on coastal desert land.

Significant amounts of lignocellulosic biomass are produced in the integrated seawater agriculture system in the form of straw and trimmings. To confirm the high yields of the produced straw, a field trial was conducted by Glenn et. al, 1999 on *Salicornia bigelovii* over a period of 6 years, where straw accounted for approximately 90 percent of the harvestable mass [36] with yields ranging between 12.70 to 24.60 t/ha of lignocellulosic (dry matter) biomass versus 1.39 to 2.45 t/ha of oilseeds over a 200-days [37]. Comparable results were obtained by studying the commercial development of *Salicornia bigelovii* in Mexico through a 5-years project, where salicornia produced 14.0 to 22.0 t/ha of forage (dry matter) versus 0.50 – 2.50 t/ha of oil seeds [38]. Also, the aboveground biomass of mangroves was periodically thinned, producing lignocellulosic biomass.

Establishing an optimized, economic feasible process will permit for implementing sustainable and renewable bioenergy systems. Consequently, an efficient diversification from petrochemical industry could be managed.

The suggested process is very important for desert-like regions with extended coast line. This research is remarkably relevant to **Abu Dhabi**, due to its geographic location with approachable and direct **exposure to seawater**. Moreover, studying the application of a green biorefinery has a great significance in **addressing the Food - Water - Energy nexus,** especially that the water could be reserved in the system and, also, added-value chemicals and proteins could be extracted from the fresh biomass.

1.4. Previous Work

Laboratory experiments were conducted to evaluate efficient pretreatment methods for extraction of mineral salts and hemicellulose sugars from the dry lignocellulosic fraction of *S. bigellovii* (post-oilseeds removal). Characterizing the raw material of the seedless *S. bigelovii* revealed extremely high ash content (43.08 g/100 g DM). The material shows typical lignocellulosic crop characteristics. Results of the compositional analysis were as follows: 9.1 ± 1.5 g/100 g DM glucan, 7.7 ± 0.4 g/100 g DM xylan, 5.5 ± 2.1 g/100 g DM arabinan, 6.8 ± 1.4 g/100 g DM Klason lignin, 6.8 ± 0.1 g/100 g DM structural ash, and 53.7 ± 3.6 g/100 g DM total extractives (including extractable ash).

Water extractive showed that a substantial part of the plant, containing ‘‘extractable’’ ash which is mostly represented by salty deposit on the plant tissues. This indicates that most of ash could be removed by washing by water [39], [40], as explained later in section 2.4.1.

S. bigelovii was pretreated hydrothermally at 190, 200 and 210°C without adding catalyst and also at 200°C with adding a catalyst (0.5% H_2SO_4). Processing time was maintained at 10 min, with 6% dry matter loading. Pretreatment temperatures higher than 200 ˚C resulted in increased production of furfural, which can be inhibitory to fermentative microorganisms. The highest glucose recovery (86%) was attained at 210°C pre-treatment, where xylose recovery in the filtrate was significantly ($p<0.05$) lower than other pre-treatments. Maximum recoveries of xylose and arabinose were 50% and 38%, respectively, at pre-treatment temperature of 190°C [40].

Generally, all the applied pretreatment conditions lead to a major enhancement in the initial glucan-to-glucose enzymatic convertibility of *S. bigelovii.* Improved enzymatic hydrolysis is obtained when lignocellulosic biomass is pre-treated, achieving high

glucan convertibility (ranging from 87% to 92%). The different pre-treatment conditions showed no significant difference, suggesting maintaining the lowest applied temperature (190 ˚C) and examining lower serverity trreatment conditions. Due to the salicornia's high-salinity, excess water is needed for washing, resulting in increasing the pre-treatment cost in comparison to terrestrial biomasses [39]. Hence, optimizing water input is very important in Salicornia pretreatment.

For future work, local halophytes species need to be identified and characterized in terms of biofuels production and other applications.

1.5. Aims and Objectives

The main objective of this study is to **evaluate and chemically-characterize** the biomass fraction of halophyte salicornia with goal of bioethanol production with a focus on the **fresh green salicornia biomass of a locally available species**. This is driven by the fact that using fresh salicornia biomass instead of dry lignocellulosic biomass would enable decreased processing severity (hence cost) and most importantly preserve water in the process.

This thesis focuses mainly on experimental work carried out via the following specific studies:

I. Evaluating dry *Salicornia bigelovii* biomass:

Chemical characterization and fermentability studies were conducted on the raw material of *Salicornia bigelovii*, which is being cultivated under different conditions (three fertilizer levels: 1 gN/m^2, 1.5 g N/m^2 and 2 g N/m^2 and two salinity levels: 10 ppt and 50 ppt salt)) in the International Centre of Biosaline Agriculture (ICBA).

II. Evaluating of fresh, green *Salicornia sinus-persica*:

A wet-fractionation study was conducted on a local, fresh lignocellulosic halophyte *Salicornia sinus-persica*, for which two fractions were obtained:

Fiber fraction (pulp) characterized by conducting the following:

- Sugar analysis and fermentability studies on fresh/untreated pulp.
- A low-severity pre-treatment study, where parameters were optimized in order to enhance biofuels production from fibers (pulp fraction).

Juice fraction determined by conducting the following:

- Sugar analysis and a fermentability study.
- Evaluation of other valuable byproducts production from juice fermentation.
- Added-value chemicals analysis by GC-MS.
- Juice fermentation by salt-tolerant yeast (*Debaryomyces hansenii)* in order to evaluate its performance on the saline substrate in comparison to standard Baker's Yeast (*Saccharomyces cerevisiae*).

III. Evaluation of *Salicornia sinus-persica*-based green biorefinery.

After evaluating the sugar composition of fresh salicornia, in addition to studying the valuable products produced by fractionated-biomass fermentation, a green biorefinery was accordingly suggested. Different processes were proposed demonstrating the best way to exploit the biomass in its fresh form. Nevertheless, further studies to be conducted in the future, to assess the implementation of a salicornia-based green biorefinery.

CHAPTER 2

2. Literature Review

2.1. Lignocellulosic Biomass

2.1.1. Plant cell wall

Plant cell walls are advanced active, systematic fibrous networks, which achieve a wide variety of biotic roles of the plant [41]. Three main divisions of the cell wall are recognized. These are: the primary cell wall, which is created during cell division, increases its surface area quickly throughout plant growth. The second part is the middle lamella, creating an interface between the primary walls of neighboring cells. Thirdly is the secondary cell wall, a complex structure exceptionally appropriate for the cell functions [42]. The main constituents of plant's cell wall include different polymers, such as polysaccharides, aromatic substances, proteins, in addition to other ions and water. Secondary walls are quite rich in polysaccharides (cellulose and xylans), lignin, specific structural proteins, and negligible amounts of pectin [43] (Figure 2-1 and Figure 2-2).

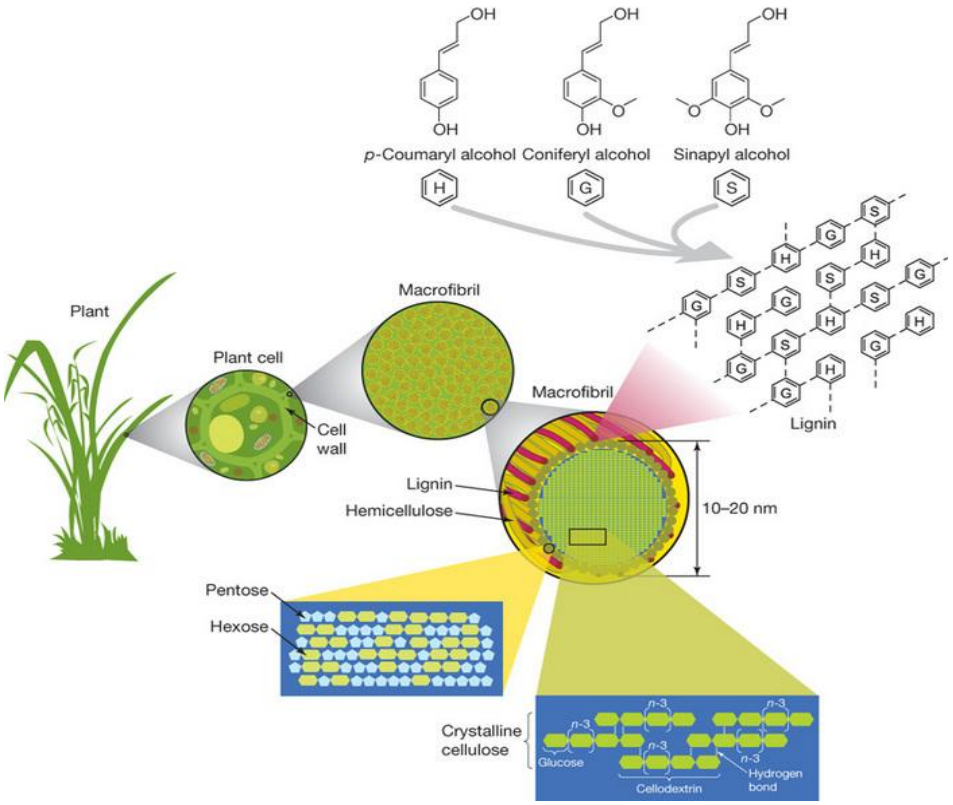


Figure 2-1. Structural organization of the plant cell wall. The secondary cell wall composed of microfibrils, showing a lignocellulosic building blocks, where hemicelluloses and lignin prevent cellulose degradation [44]

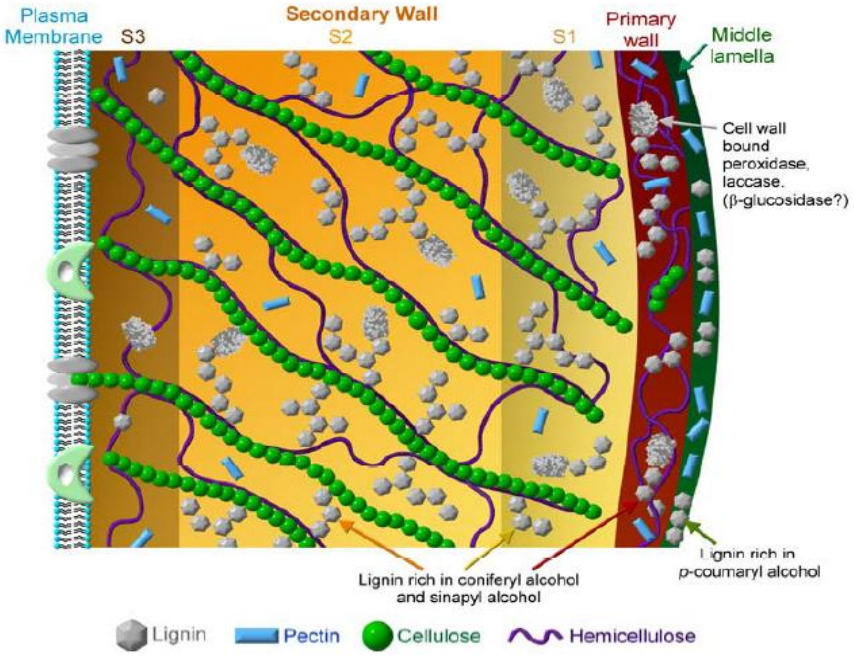


Figure 2-2. A close image for the secondary cell wall, showing the main constituents; cellulose, hemicellulose and lignin [45].

2.1.2. Lignocellulosic material

The cell wall of lignocellulosic biomass is a complex arrangement of crystalline cellulose fibrils bound by non-crystalline hemicellulose, where a matrix of hemicellulose and lignin is surrounding the cellulose-hemicellulose structure [46], as shown in Figure 2-1. Hemicellulose, cellulose and lignin represent respectively 20 – 40 wt.%, 40 – 60 wt.%, and 10 – 25 wt.% of the lignocellulosic biomass [47, 48]. Low molecular-weight components (extractives and minerals) are minor components and differ from one species to another.

The macromolecular substances of lingocellulosic structure, formed in the fibrous part of trees, plants, grasses or agricultural residues [49], can be separated into carbohydrate-derived and lignin-derived products. Carbohydrate-derived material consists mostly of polysaccharides, oligosaccharides, monosaccharides, and their degraded products, while lignin-derived products are composed of aromatic monomers and oligomers [50]. All recent commercial applications of lignin take advantage of the polymer and polyelectrolyte properties of this component, in addition to combustion and synthesis of dimethylsulfoxide and vanillin. Nevertheless there are difficulties allied to the structure complexity and the changes occurring while biomass treatment, resulting in difficulties in extracting it [51].

a. Cellulose

Cellulose is dispersed in all types of plants, representing about 50% of both hard- and softwoods. The chemical and physical properties of cellulose allow it to represent the main structural component of the plant cell wall [52].

Cellulose is a linear homopolysaccharide that is formed from glucose (D-glucopyranose) units joined together by β-(1-4) glycosidic bonds [46]. Pyranose has a

chemical structure that includes a six-membered ring involving of five carbon atoms and one oxygen atom. The linear cellulose backbone fluctuates in width and length besides containing various degree of polymerization and degree of order [53], [46].

Cellulose structure can be assessed at either global or ultra-structural levels. At the global level, spectroscopy and crystallography are the main information source [54, 55]. Inter-and intramolecular bonds in cellulose situate in planes, forming an arrangement of hydrogen-bonded sheets, as shown in Figure 2-3, forming lattice forces that are responsible for maintaining the crystalline structure [55]. Most cellulose crystalline models exhibit no hydrogen bonds between chains standing in different crystal layers, having the aliphatic hydrogen atoms in axial sites and the polar hydroxyl groups in equatorial sites. The uppermost and lowermost parts of the cellulose chains are in fact entirely hydrophobic, whereas the sides of the chains are hydrophilic and allow for hydrogen bonding. This structure is significant in packing the chains to form crystal-like product. In anticipated cellulose crystal packing patterns, the chains are laden with hydrophobic regions, resulting in an insolubility of cellulose under normal conditions. Furthermore, the nonpolar sites are linked to hydrophobic surfaces of the binding proteins, such as tryptophan or phenylalanine side chains [56]. A key fact is that systematic chain folding can be ruled out to have cellulose as extended chain polymer made of single crystals [55].

At the ultra-structural level, individual cellulose chains could be directly visualized using advances in transmission electron microscopy (TEM) [57]. The complete width of cellulose microfibril is made from monocrystalline domains (inhabiting the core) [57, 58], where the amorphous phase of cellulose represents the chains found on the outer surface of microfibril [59] as illustrated in Figure 2-4. Cellulose microfibrils comprise the core of the plant cell wall as shown in Figure 2-1 [42].

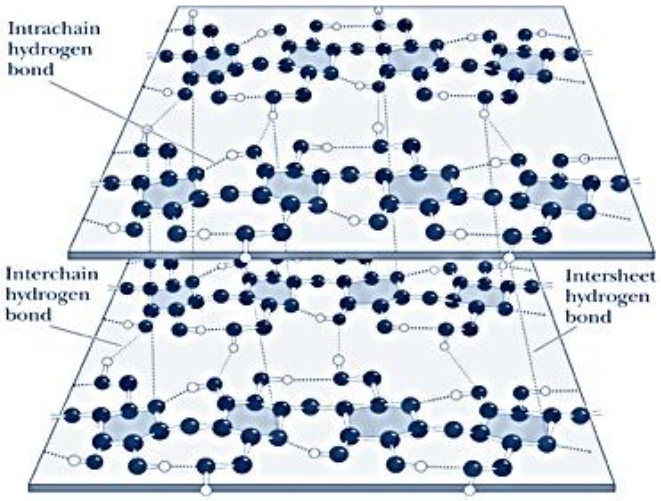


Figure 2-3. Intra-chain, inter-chain and inter-sheet hydrogen bonding in the cellulose fibrils resulting in a strong structure [60].

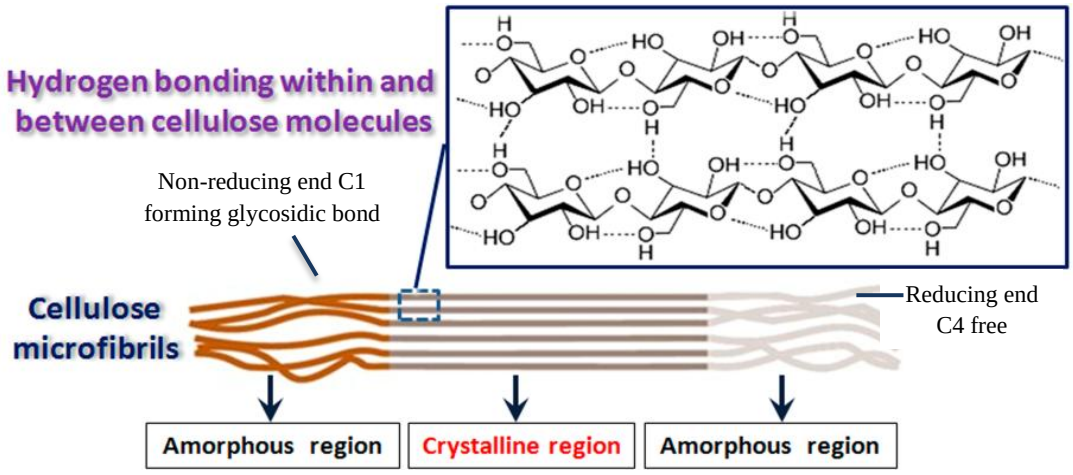


Figure 2-4. Cellulose structure, where the crystalline core is centering the amorphous structure in the cellulose microfibril [61].

- *Two amorphous ends are illustrated, one reducing carbon free and other non-reducing carbon forming glycosidic bond.*

Typically, degree of polymerization (DPs) differs based on the source. For instance, in primary plant the degree of polymerization falls in the range between 5000 and 7500 glucose monomer units, however DP in wood happens at around 10,000 and around 15,000 for cellulose from cotton [56].

b. Hemicellulose

Hemicelluloses represent the second most abundant polysaccharide in nature, following cellulose [62] and similar to cellulose, its monomer units can be fermented to ethanol [63]. Hemicellulose with a molecular weight of <30,000 [48], principally functions as a stabilizer to the cell wall, yet it is water-soluble at normal conditions due to the branched and heterogeneous structure [56], as shown in Figure 2-5. The configuration of hemicellulose shows an assortment of polysaccharides, composed essentially of hexoses (glucose, mannose, and galactose), pentoses (xylose and arabinose) in addition to uronic acids (methylglucuronic and galaturonic acids) [48]. Other sugars such as a-L-rhamnose and a-L-fucose might also be present in insignificant quantities. The hydroxyl groups of sugars can to some extent be replaced with acetyl groups. However, the most relevant abundant hemicelluloses are xylans in hard wood and glucomannans in soft wood [64]. The side groups of xylan is characterized to have an important role in the bonding of lignin to hemicellulose [56]. Sugars are linked together by ß-1,4- and occasionally by ß-1,3-glycosidic bonds.

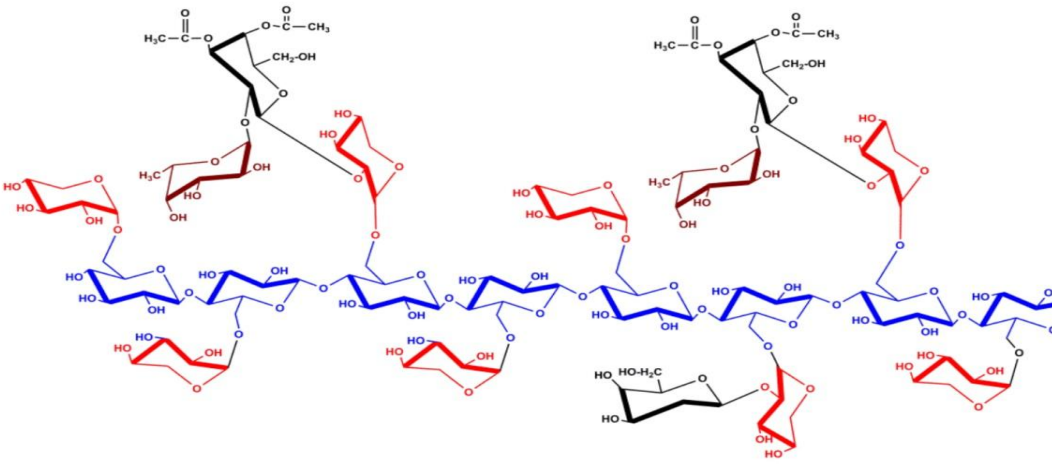


Figure 2-5. Macromolecular structure of hemicellulose. Principally composed of xyloglucan. In blue backbone β-D-glucans; in red α-D-xylose; in black α-D-galactose and in brown α-L-fucose residues [42].

Hemicellulose exhibits high degrees of polymerization; it is also linked to substituents on the main chain or its branches. The branching, substituents and linkages vary, creating a complex structure of hemicellulose and that forms various conformations and functions [63]. Wyman et al. [56] anticipated that there are no chemical bonds between cellulose and hemicellulose, nonetheless mutual adhesion is obtained by hydrogen bonds and van der Waals forces. This is explained due to the hemicellulose heterogeneous structure of short branched polysaccharides [65], which bind firmly to the surface of cellulose micro-fibril [48]. According to the fundamental pattern, hemicelluloses fill the voids around cellulose fibrils and afford connection to the lignins [66].

The main difference between hemicellulose and cellulose is that hemicellulose consists mainly of xylose and other five-carbon monosaccharides. Hemicellulose is amorphous in structure and easily hydrolyzable [65].

c. Lignin

Lignin is an aromatic polymer formed from phenylpropanoid precursors [45, 48]. Lignin exists in the cellular cell wall, connected to cellulose and hemicellulose allowing for structural backing, protection, impermeability and microbial and oxidative stress resistance [65]. It has been described as natural glues that bind tightly to polysaccharides [50]. However this polymer hinders hydrolytic enzymes from accessing the sugar due to the complex connections it forms with cellulose and hemicellulose [45]. This component of biomass is normally burned as boiler fuel, as its extraction is very complex, which restricts its fermentation applications [67].

Lignin displays diverse structures in plants, which in contrast to what is seen in cellulose [68]. Lignin is composed of three main monolignols (phenylpropane) units,

called hydroxycinnamyl alcohols. The main difference for these units is their degree of methoxylation as explained in Figure 2-6.

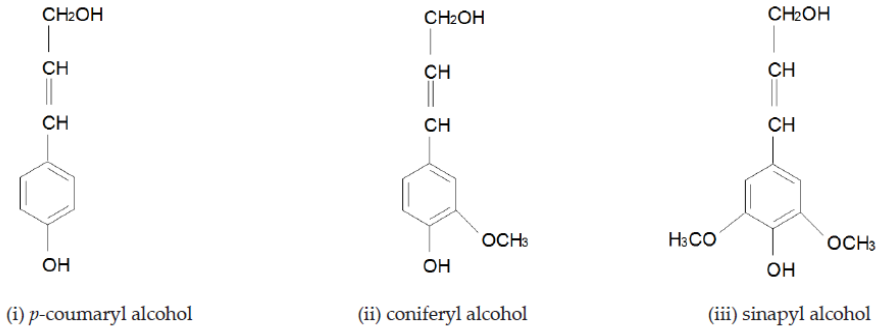


Figure 2-6. The phenolic precursors, from which lignin is formed [23].

The polymer synthesis is originated by free radicals, generated from peroxidase-mediated dehydrogenation of three phenyl propionic alcohols: coniferyl alcohol, coumaryl alcohol, and sinapyl alcohol. The ultimate structure is heterogeneous, where units are connected by C-C and aryl-ether linkages, to have a predominant structure of aryl-glycerol as in Figure 2-7 [65]. The structure of lignin is an amorphous heteropolymer, which doesn't dissolve in water under standard conditions [65]. Moreover, lignins are optically inactive due to their racemic nature [68].

Generally, the molecular mass of lignin ranges from tens of thousands of Daltons to much larger (infinite) [69].

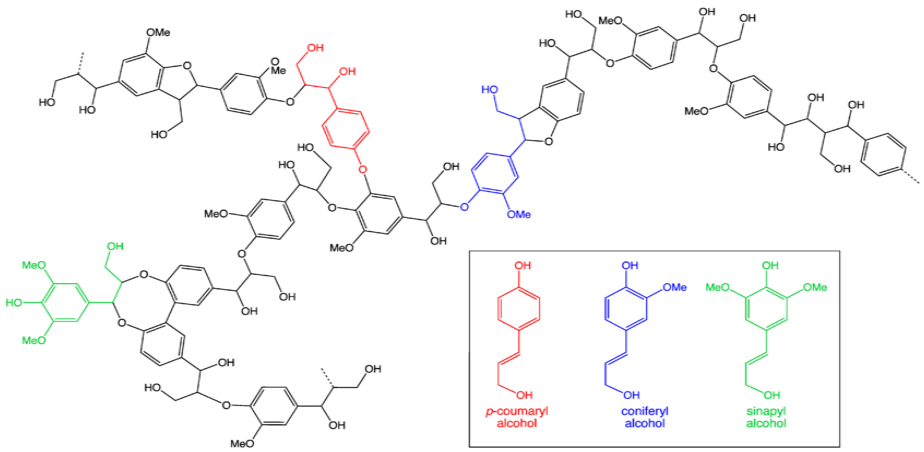


Figure 2-7. The heterogeneous structure of lignin resulted from the three basic phynelpropane units [70].

2.1. Halophytes

Several studies has been conducted to evaluate halophytes for their potential use [71, 72]. Halophytes are plants that can grow and complete their life cycle in saline habitats. Generally, they are capable of tolerating NaCl concentrations higher than 0.5% [73]. Most halophytes employ the controlled buildup of ions such as sodium (Na^+), chloride (Cl^-), magnesium (Mg^{2+}), and sulfate (SO_4^{2-}), which results in increasing the osmotic potential in their tissues that pushes moisture to move from soil into tissues [74].

Halophytes are reported as one of the richest sources in terms of lignocellulosic biomass [75]. The use of halophytes as biofuel crop has good potentials, since they do not compete with conventional crops for high quality soil and freshwater [76], [11]. Calculations have shown that halophytes have heat content comparable to what lignite coal has. Environmental Research Laboratory (ERL) at the University of Arizona suggested that using halophyte in 500 MW power plant (ratio of 1:2 halophyte to coal) would produce about 10% less heat per unit mass when compared to using coal alone; however, 25% reduction in carbon emission could be achieved [77]. Moreover, the ERL proposed using refined halophyte oil to blend with diesel [78]. However, annual lignocellulosic materials generally contain substantial amounts of alkali metals such as sodium and potassium, in addition to alkaline earth elements e.g., calcium and magnesium [79], which makes its challenging to apply high-temperature processes such as combustion on halophytes. Alkali metals are swiftly released in form of gases during combustion and interact with other elements, resulting in problems with fouling, slagging, and corrosion [80].

Other applications of this biomass, such in livestock feeding, could result in adverse effects on animal production; hence its application regulation should be considered [81]. In conclusion, the high-ash content of halophytes is not well suited for traditional utilization (direct animal feed or combustion) and alternative utilization routes need to be developed.

2.2. Salicornia

Salicornia is an annual, small, vascular, salt-tolerant plant (halophyte), which is ordinarily found in coastal estuaries and salt swamps [77]. Salicornia is composed of a central stem, from which short opposite cylindrical branches are sprouted [82]. The leafless stems are photosynthetically active, with green color, but their foliage turn red in autumn [77]. World-wide there are about 13 different Salicornia species, which are diverse in many aspects [83]. Different types of distinctions suggest that the different species are mainly resultants of differences of location and native environments, resulting in different inbreeding populations. Generally, elevated salinity trauma due to dry hot conditions during summer periods may induce prompt changes in the density and diversity of species in halophyte groups [84]. There are other names in the literature for salicornia; it is called pickleweed, sea asparagus, samphire, dwarf saltwort, pousee-pied, sea beans, or glasswort [85], [86], [87], [88].

Chemical characterization of salicornia

Compatible organic solutes are generated and accumulated in cytoplasm in the cells of salt-tolerant spices. Those solutes intend to balance the osmotic potential created by the accumulation of Na^+ and Cl^+ ions [89]. There are various chemicals to achieve this function in halophytes, with different levels of existence, such as sugars (e.g., sucrose), sugar alcohols (e.g., sorbitol), betaines (e.g., glycinebetaine), amino acids

(e.g., proline), methylated proline-related compounds (e.g., methyl-proline) and methylated sulphonio compounds (e.g., dimethylsulphonioproprionate) [90], [75]. This indicates the possibility of finding some of those chemicals in *Salicornia*. It was reported by Greenway and Munns [91] that saltwort contains 29 mg of choline (quaternary ammonium salt) and 888 mg of betaine (any neutral chemical compound with a positively charged cationic functional group) per 100 g, moreover large quantities of salts and minerals such as K, Mg, Ca, and Fe can be found in this plant [92].

Chemical characterization of the lignocellulosic composition, extractives and ash content of *Salicornia bigelovii* straw, in relation to different irrigation salinities (10-50 ppt) and fertilizer grades (use varied between 1 and 2 gN/m^2), was conducted at Masdar Institute by our research group. These results will be described in the results section. Main findings showed that the composition of the *Salicornia bigelovii* was comparable to traditional lignocellulosic biomasses, containing glucan (up to 27 g/100 gTS), xylan (up to 23 g/100 gTS) and lignin (24 g/100 gTS). High amount of ash (up to 53 g/100 gTS) and water-ethanol soluble extractives (up to 25 g/100 gTS) were also detected [40], [93].

As mentioned earlier, *Salicornia bigelovii Torr* produces oil seeds. Oil extraction from the seeds produces salicornia meal (SM) as a by-product of this process. The composition of seeds as described by Glenn et al., 1999 [36] showed about 31% protein, 28% oil, and 10% of equal amounts of fiber and ash. The oil showed high concentrations of polyunsaturated fatty acids, particularly linoleic acid (74% of total). The chemical characterization of salicornia meal showed that it contains 34 (w/w)% of crude protein, 6.45 (w/w)% of ether extract and 3.60 (w/w)% of crude fiber [94]. In another study, the chemical composition of *Salicornia herbacea* powder

found to contain dietary fiber (60.66%), total sugar (15.2%), in addition to uronic acid (2.6%) [92].

Salicornia could be considered as a raw material in different industries, especially in nutraceuticals production [95]. For instance, phytochemicals (provided that they could be efficiently recovered) have been acknowledged in different halophytes. For instance immunomodulatory polysaccharides were found in *S. herbacea* [96]. Antibacterial properties similar to typical antibiotics were identified in the ethanol extracts of *S. maritime,* [97]. Also, a polysaccharide exhibiting activity against HIV was found in its leaves [98]. Triterpenoids [97, 98], flavonoid sulfates, alkaloids, tannins and steroids were detected in *S. maritima* extracts [99, 100]. Another species, *S. vermiculata,* an edible halophyte, has been recognized for its hypoglycemic and hypolipidemic properties and has showed to hold activity against cancer [101]. According to Benwahhoud et al., 2001 [102], water extracts of *S. vermiculata* exhibited hypoglycemic and hypolipidemic effects when applied to rats, which is thought to be generated because of the existence of flavonoids [101]. Moreover, *S. europaea* was found to contain alkaloids, saponins, flavonoids in addition to other phenolic compounds [103]. Oleanolic acid glucoside extracted from *S. europaea* has been categorized with antidiabetogenic properties on rats [104], while containing an anti-obesity agent when extracting chikusetsusaponin methyl ester [105]. Additionally, flavonoid compounds extracted from *S. europaea* were assumed to be in charge for the antimicrobial effect of the ethanol extracts [106]. Generally, phytochemicals generated in halophyte species that are locally available/grown in the UAE have also been reported to be exist in halophyte species in other areas of the world, which includes antioxidants (e.g. hydroxycinnamic acids and their derivatives) found in *Salicornia spp.* growing in Europe or Korea [103, 107].

In conclusion, information provided by literature confirms the great potential for coastal halophytes of the United Arab Emirates to be used as raw material in biorefinery processes where biofuels production from the plants carbohydrates could be combined with production of value added nutraceutical products.

2.2.1. Salicornia bigelovii

Salicornia bigelovii which was used in the dry lignocellulosic pretreatment study (described in section 1.4) is a member of the flowering plant family Chenopodiaceae [108], which contains about 20% of all halophyte species [109]. It is a C_3, dicotyledonous annual halophyte (salt-tolerant plant) [110] that is native to North America and the Caribbean [111]. On the upper one-third of the plant with many small (weighing approximately 0.6 to 0.9 mg) oilseeds [111] and can grow up to 50 cm tall as shown in Figure 2-8.

Family: *Amaranthaceaen.* current name: *Salicornia bigelovii Torr.* Location: USA. Florida. Description: Tall stems, bright red in fall. Collector: J.M. Carlton s.n. Date: 24 May 1973.

Family: *Amaranthaceaen.* current name: *Salicornia bigelovii* Torr. Location: USA. Florida. Volusia Co.: Upper St. Johns River, Puzzle Lake #3, Transect E.bk. Collector: D. Auth 97. Date: 15 Sep 1971.

Figure 2-8. Leafless, succulent *Salicornia bigelovii* [112]

Salicornia is considered one of the most salt tolerant vascular plant [108], showing ability to yield as much biomass and seed as conventional crops even with soil solution exceeding 70 ppt TDS (about twice the salinity of seawater) [37], [113]. Nevertheless, irrigation rates should be optimized, since soil-moisture salinity exceeding 75 ppt TDS in the top 15 cm (in the root zone) will result in decreasing the growth rates of salicornia [113].

The oilseed halophyte, *S. bigelovii* yields 2 t/ha of seed containing 28% oil and 31% protein, similar to soybean yield and seed quality. *S. bigelovii* has sufficient genetic diversity among wild accessions and supports cultivators to promote a crop improvement program [114]. The high NaCl content possessed by *S.bigelovii* can be reduced by washing and then pressing the straw [115]. In a study carried out by Weeks et al., 1986 [116], the plants grown in the 45 ppt salinities had common final dry weights and seed yields of about 60 g and 11 g, respectively, while the 1 ppt plants had 28 g dry weight and nearly 5 g seed yield. This relationship between water salinity, growth and seed production of the plant makes it suitable for cultivation in the UAE. *S. bigelovii*, which is rich in oil, is a potential feedstock for biodiesel or kerosene fuel production [117]. The lignocellulosic part of the plant (stems and seed spikes) has a similar composition to other halophytic shrubs (10-30% cellulose, 10-30% hemicellulose and 2-10% lignin) [11]. This makes the biomass leftover after the seed separation an attractive lignocellulosic feedstock for production of ethanol, biogas and other value-added by-products.

2.2.2. Salicornia sinus-persica

Salicornia sinus-persica is a C_3 halophyte species that was found in Iran, specifically in the central provinces [118] and also in areas surrounding the Arabian Gulf. It is an

annual plant, smooth, dark green in color, becoming orange-reddish on the lower part of the stems, as shown in Figure 2-9.

Alkhani et. al., 2003 [118] described *Salicornia sinus-persica* growing in Iran. Its height ranges between 25 and 60 cm, with a canopy diameter reaches 80 cm. Stems ascend in dense form, getting salt accumulations on the lower older shoots forming tubercles. An upper central flower in each segment takes the shape of reversed pentagonal to sub-rhombic, stretch, truncate, 2.0–2.8 x 1.3–1.8 mm; however, side flowers are triangular, 1.2–1.6 mm wide at base. Seeds are elliptic, being larger in size for the central flowers (1.7–1.8 x 0.8–1.0 mm) compared to the side flowers 1.3–l.6 x 0.8–0.9 mm. With increasing salinity, water potential in *S. persica* became more negative; indicating that *S. persica* osmotically adjusts in response to salinity intensification [30].

Figure 2-9. Salicornia sinus-persica collected from shores of United Arab Emirates

Studying the impacts of different concentrations of NaCl (in irrigation water) on *S. persica* in terms of growth, ions accumulation, content of soluble sugar and water, in addition to the significance of proline and glycine betaine increase (due to salt stress) was done by Ahmad et. al., 2012 [30] under controlled experimental conditions. In this study, the highest growth was observed at concentration of 11.69 ppt of NaCl and

significantly decreased at higher concentrations, however all plants survived at salinities as high as 40.91 ppt NaCl. In terms of water content, the highest was found at NaCl concentrations of 11.69 ppt and 17.53 ppt in shoots and roots, respectively.

Under salt stress, plants accumulate numerous compatible solutes, like proline (α-amino acid), soluble sugar, soluble protein and quaternary amino acids. These solutes adjust the osmosis and protect the structure of cell proteins, however they do not have negative effects on plant metabolism even with high concentrations [119]. An increase in total solute concentration was observed in particular at salt stress caused by inorganic ions [30].

2.3. Biofuels from Lignocellulosic Biomass

Many countries look into liquid fuels production from renewable resources. The goal is to replace fossil fuels or to decrease its usage as much as possible, in order to rely on a sustainable and secure energy supply. To date, biodiesel and bioalcohol are considered the most applied biofuels in the EU [120].

Cellulosic biorefineries could provide industrial ethanol and other valuable chemicals [121]. Bioethanol can be easily produced from various raw materials [32], such as cereal crops, sugar beets [120] and lignocellulosic material such as agricultural and forest residues, some dedicated crops (salix, switch grass), halophytes, algae and sea grass/plants. Bioethanol has been introduced in large/production scales in Brazil, USA and some European countries [32].

To produce bioethanol from lignocellulosic material, the following should be applied: i) Physico-chemical pretreatment ii) hemicellulose hydrolysis; iii) cellulose hydrolysis; iv) fermentation of the pre-hydrolyzed material; v) separation of byproducts; and vi) ethanol recovery and concentration [122]. Figure 2-10 illustrates a simplified process flowchart for ethanol production from lignocellulosic material.

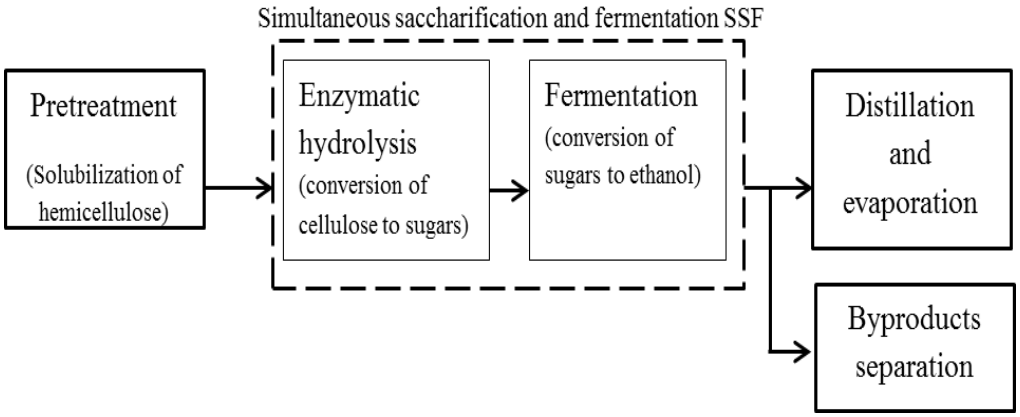


Figure 2-10. Simplified flowchart of ethanol production from lignocellulosic biomass (this figure is based on Galbe and Zacchi [32]).

Having a salt-tolerant biomass with high non-structural ash content requires washing before processing. Washing is necessary in order to protect process equipment from corrosion and rusting in addition to its positive effects in fermentation. For instance, Glenn, Hodges [123] suggested reducing the high NaCl content possessed by *S. bigelovii* by straw washing and pressing.

2.4. Lignocellulosic Biomass Processing

2.4.1. Washing of salt-containing biomass

In the characterization study of *Salicornia bigelovii* conducted by Chaturvedi [39], water extractives exemplified a substantial part of the plant, containing extractable ash (mostly represented by salty deposit on the plant tissues), as indicated in Table 1.

Table 1. Description of the non-volatile extractives obtained from the water-ethanol extraction of the mixed (stems + pods) *S.bigelovii* feedstock [39].

Extractives (non-volatile)			
	Content (g/100gTS)	**Ash content (%)**	**Ash-free content (g/100gTS)**
Water extractives	49.10	73.89	12.82
Ethanol extractives	8.94	20.48	7.11
Total extractives	**58.04**		**19.93**

Since freshwater is scares in UAE, it was important to evaluate biomass washing using salty-water. Accordingly, water with different salinity levels was used for the washing study, where a ratio of 10%w/v: biomass/water was applied. The different water salinities applied were: 0.03, 5, 10 and 30 ppt.

The results showed that fresh water is needed in order to remove significant amount of salt from the biomass as indicated in Figure 2-11. No significant amount of salt was removed from the biomass, when saline water was used, even when it contained low salinity (i.e. 5 ppt). Generally these results indicated that using fresh water can extract significant amounts of salt at low temperature in a timely manner (10 minutes). As a result of this study, feedstock washing was done at 25°C for 10 minutes. This washing showed that 15% of the non-structural ash remains in the solid fractions after washing and a subsequent filtration. Measuring the COD of the washed away extractives stream yielded a value of 7g/L COD [39].

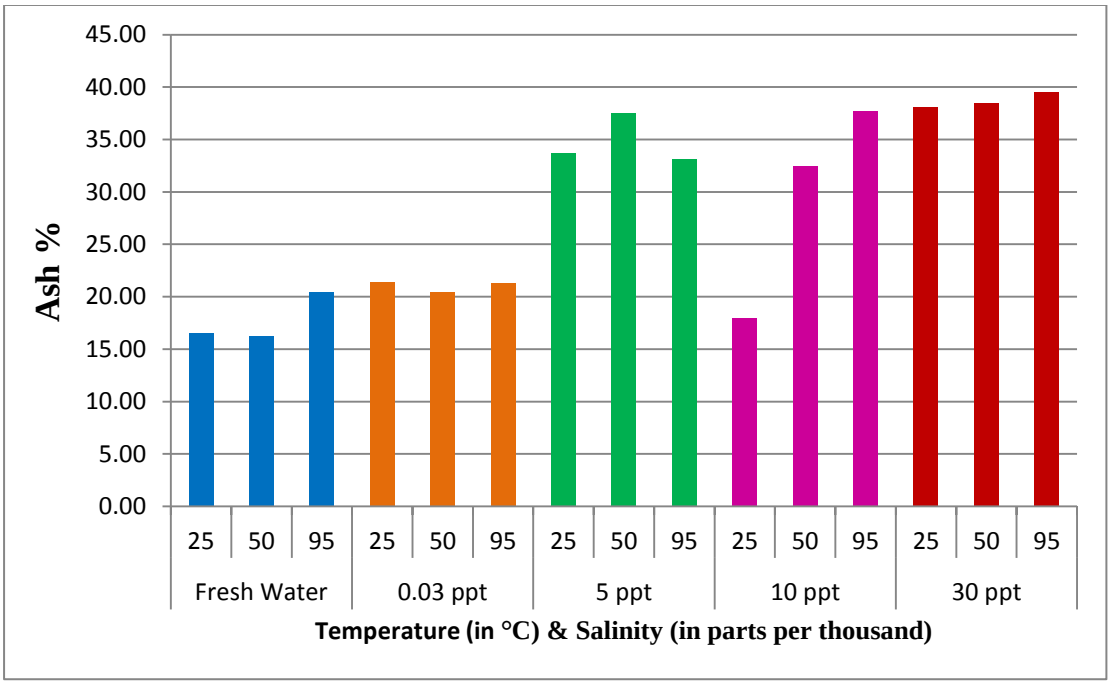


Figure 2-11. The ash % obtained at different temperatures and salinity of washing [39].

2.4.2. Pre-treatment

Pre-treatment is needed for the best utilization of lignocellulosic biomasses in biofuels production process. In order to obtain the monomeric sugars from lignocellulosic material, efficient pre-treatment is required, which can open up the lignocellulosic structure by partially hydrolyzing the amorphous hemicelluloses, thereby removing lignin [26, 124], possibly due to lignin dissolution (depolymerization and repolymerization impact) as hemicellulose is hydrolyzed [125]. Proper pre-treatment also helps in decreasing the crystallinity of cellulose [26, 124]. Pretreatment is used to alter the lignocellulosic structure physically and chemically for better hydrolysis rates [13].

The pre-treatment effectiveness, as discussed by Laser et al., 2002 [126], is dependent on the cellulose pore volume [127], cellulose crystallinity [128] and the lignin and hemicellulose removability [127]. Moreover, the differences in the microscopic structure presented by the middle lamella as well as by the primary and secondary cell walls result in heterogeneity in the lignocellulosic material [129]. Accordingly, it is needed to optimize pre-treatment conditions for each lignocellulosic biomass. Pre-treatment should achieve low operational and capital cost while still being effective. The effectiveness of a pre-treatment method is assessed by measuring the rate of material loading and fuel generating, in addition to the recovery portions of the lignocellulosic components. Through pre-treatment, low energy demand should be considered and inhibitors' generation should be avoided and/or minimized [130]. Pre-treatment optimization is done by adjusting process parameters, such as temperature, pressure, pH, residence time, biomass concentration and also catalyst usage [11]. Other pre-treatment parameters should be deliberated such as the safety and cost of

catalysts and the ability of recycling them. Moreover, generating high-value lignin co-product can be advantageous for increased process feasibility [131].

Incomplete pretreatment results in incomplete processing of the sugar content [129], however, applying severe pretreatment (by increasing temperature, catalyst concentration, or time of reaction) can result in sugar degradation and inhibitor formation [132]. To evaluate and compare the severity of different pretreatment methods, a parameter called the "severity factor" has been defined [32]. Severity factor approximately calculates the combined effect of acidity, temperature, and length of pre-treatment [130], as indicated in the following formula:

$$\log(R_o)=\log(t\ \exp(\frac{T-100}{14.75})) \qquad \text{Equation 1} \qquad [133]$$

- log(Ro): pre-treatment severity,
- t: pre-treatment residence time and
- T: pre-treatment temperature

To assess the best pretreatment method, the following parameters should be considered: Analyzing the sugar recovery and yield (in enzymatic hydrolysis), studying the fermentability of the pretreated biomass to indicate if there is production of inhibitory products, and studying the potential of production value-added components by the pretreatment method [130].

There are many pretreatment approaches, depending on the process parameters and catalysts/chemicals, such as chemical treatment (acid pre-treatment, alkaline pre-treatment, wet oxidation, organosolv pre-treatment), physical treatment (size reduction, pyrolysis, microwave and electron beam irradiation pre-treatment), physicochemical treatment (steam explosion or hydrothermal hydrolysis, hot water, CO_2 explosion and ammonia fiber explosion) and biological pre-treatment [13, 130,

134]. The impact of pre-treatment is shown in Figure 2-12. After pre-treatment, acids or enzymes are used to disrupt the cellulose into constituent sugars [13].

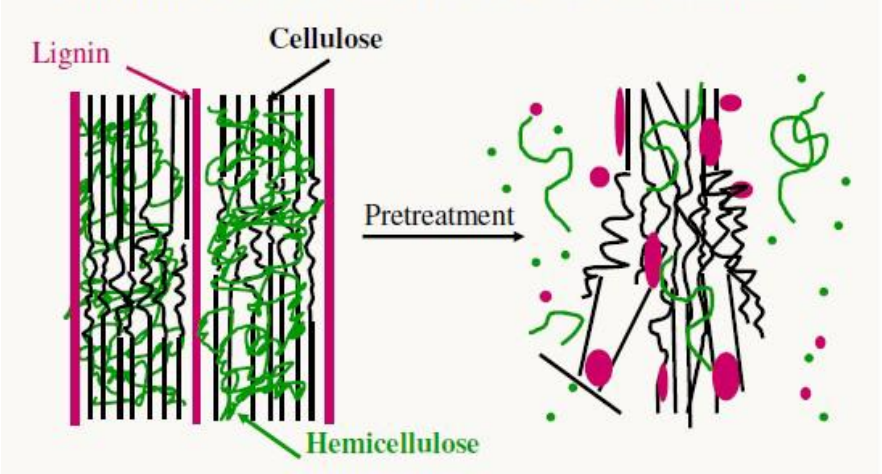


Figure 2-12. The effect of pretreatment on lignocellulosic material [131].

a. Mechanical pretreatment

Mechanical pre-treatment, also called physical pre-treatment, is the first step applied on the raw biomass. It is responsible for biomass cleaning, size reduction and cell structure breaking to increase the exposure for further chemical, biological or biochemical treatments. Mechanical pretreatment provide larger surface area offering better catalyst transfer and faster reaction times [135].

The energy consumed in particle-size reduction is high; it can take up to one third of the power needed for the entire process, based on the National Renewable Energy Laboratory (NREL) [136].

b. Lignin removal and hemicellulose hydrolysis

The first step after physical processing of the biomass is hemicellulose hydrolysis and lignin removal. This step is important to break the lignocellulosic structure into its primary components, where lignin and all/or part of the hemicellulose are solubilized, either chemically, physically, physico-chemically or even biologically. Subsequently, the free hemicellulose can be hydrolyzed into monomeric sugars by adding water or

steam. The liquid phase (water or steam) will be primarily dissolving xylose, mannose, arabinose, and galactose. Minor portions of cellulose may also be converted into dissolved glucose; however, the majority of the cellulose hydrolysis is achieved in a separate step [135].

Physico-chemical pretreatment (hydrothermal pretreatment)

Hydrothermal treatment involves biomass cooking in water at high temperatures and pressures. This pretreatment method results in great increase in the surface area of cellulose (by non-chemical swelling), which significantly improves enzyme access [124, 137]. By applying hydrothermal pre-treatment, only minor extents of lignin can be removed (the acid soluble part). Additionally, the lignin structure is changed by melting, coagulation and its following repolymerization on cellulose fibers. Hence, it is impossible to extract lignin in its functional form from the hydrothermally pretreated entities [138], [139].

In hydrothermal treatment, the reactor is slowly heated and then cooled after the process (can be referred to as hot-water treatment or liquid hot-water cooking) and no explosion is involved. Generally, applying high pre-treatment temperatures requires short residence times (as low as a few seconds), where low temperatures require long residence times (up to a few hours) [140].

Hydrothermal pre-treatment is gaining interest as a pre-treatment method for the ethanol industry, since it does not necessitate chemicals' application and also it is not demanding to operate. This method has been implemented in pilot scale at DONG Energy facility in Skærbek, Denmark, in a screw-conveying reactor [141], [142], [143].

Different studies were conducted by applying hydrothermal treatment on different types of lingocellulosic biomasses. Mok et al., 1992 [144] discussed that the treatment

efficiency depends mainly on the feedstock type rather than the reaction conditions. Compressed hot water percolation was realized to obtain 76-100% of hemicellulose removal (varies according to the material type) and up to 60 % of lignin removal at a severity parameter (log R_o shown in Equation 1) equal to 4.1 (at 230 °C for 2 min duration), nonetheless, fermentation of the pretreated samples was not evaluated [144]. Studying olive tree residues, the highest glucose yields obtained in enzymatic hydrolysis were found applying pretreatment temperatures between 200 ˚C and 210 ˚C, while the highest xylose recovery occurred at lower temperatures (~170 ˚C) [145]. 64% of hemicellulose solubility was achieved applying hydrothermal pretreatment with a severity factor of 3.96 (corresponding to a temperature of 215 ˚C) on wheat straw. Hemicellulose removal lead to concentrating the solids in cellulose (to reach up to 61%), where acceptable inhibitors formation was reported [146]. Weil et al., 1997 [147] studied hydrothermal pretreatments on yellow poplar sawdust, through which 90% yield of glucose was obtained during enzymatic hydrolysis at pre-treatment temperature of 240 °C. Nevertheless low ethanol yields were achieved (50%), due to the inhibitory compounds formed during the treatment [147].

Hydrothermal pre-treatment results in slurry production, which can either be followed by separating the solid fraction from the liquid fraction, or by feeding the slurry product into the next step as it is (i.e., without separating the two fractions) [148]. However, utilizing the slurry without separating the liquid and solid fractions could have inhibitory effects of the fermentation process, due to the high concentrations of sugar degradation products generated by the pre-treatments process. Degradation products/chemicals include furfural (from dehydration of pentoses) and 5-hydroxyl-methyl-furfural (from dehydration of hexoses). Degradation products and acetic acid, in addition to some minor concentrations of other organic acids (e.g., levulinic and

formic acid) formed during the treatment, result in yeast and other fermenting microorganisms inhibition, especially when present at high concentrations. Other by-products can include lignin degradation products [138, 140, 142, 149].

Inhibitors formation

Inhibitors are usually divided in three major groups based on their origin; which are weak acids, furan derivatives, and phenolic compounds following the main degradation pathways shown in Figure 2-13. By degrading hemicellulose, xylose, mannose, acetic acid, galactose, and glucose are degraded. Through applying harsh treatment conditions (high temperature and pressure), xylose is degraded to furfural [150]. Likewise, 5-hydroxymethyl furfural (HMF) is formed by hexose degradation. Further breaking and degradation of furfural and HMF generates formic acid, while levulinic acid is formed by HMF degradation [151]. Nevertheless, hexose degradation to by-products becomes more favorable at temperatures above 210-220 ˚C [140].

Phenolic compounds are generated from incomplete breakdown of lignin [152], and also through carbohydrate degradation [153].

Many other inhibitory compounds were detected in the hydrolysate, such as Hibbert's ketones found in in pine hydrolys ate [154], and spruce [155], vanillic acid and vanillin (generated by degrading the guaiacylpropane units of lignin), found in hydrolysates from willow, spruce, red oak, poplar, and pine [155]. Syringaldehyde and syringic acid, formed in the degradation of syringyl propane, have been discussed by Jönsson, Palmqvist [156], which was found in hardwood hydrolysates. Moreover, hydroquinone (1,4-di-hydroxybenzene), catechol (1,2-di-hydroxybenzene), 4-Hydroxybenzoic acid, substantial amount of the alcoholic hydroxyl groups and other extractives (i.e. caproic acid, caprylic acid, pelargonic acid, and palmitic acid)

formation has been reported in the literature [152], [156], [157] as summarized by [155].

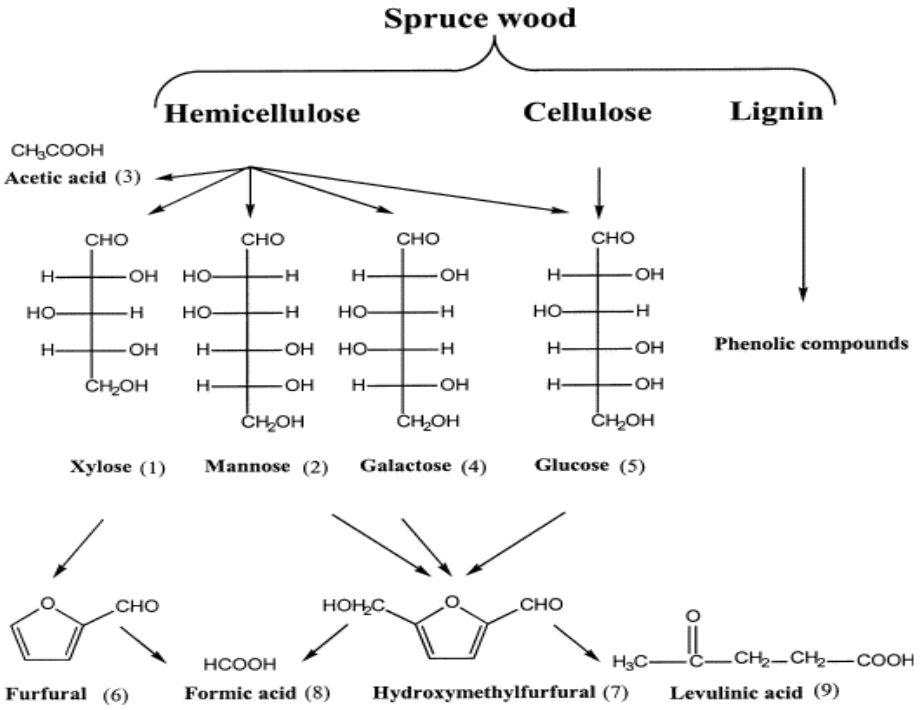


Figure 2-13. Reactions occurring during hydrolysis of lignocellulosic materials. The furan derivatives and phenolic compounds will react further to form some polymeric material.

Inhibitors formation results in a coincided decrease in the concentration of fermentable sugars. These compounds might to some extent limit the effective application of the hydrolysates for ethanol production by fermentation. Fermentation is accordingly inhibited and ethanol yield is decreased due to the presence of such compounds [155].

To obtain both high yields of fermentable sugars and high fermentability the pre-treatment process should be optimized by keeping the combined severity not higher than 3 [158]. Furthermore, specific detoxification methods can be developed for efficient removal of inhibitors prior to fermentation of strongly inhibiting hydrolysates [159]. A study done by Tengborg et al., 1998 [158], where hydrolysis using SO_2 or H_2SO_4 at the same combined severity has shown equal sugar yields,

whereas the fermentability was better when SO_2 was used, indicating that less inhibitors were formed in this case.

2.4.3. Enzymatic hydrolysis

Enzymes are specific substrates, function as biological catalysts; they have the ability to enhance reaction rate or reaction velocity without being changed in the overall process. Generally, enzymes lower the activation energy of a reaction, for instance by providing an alternative pathway and stabilizing the transition state as can be seen in Figure 2-14. Hence, the rates of the enzyme-catalyzed reactions are faster than those of uncatalyzed reactions by the factor of 10^6 - 10^{12} [160].

After opening the lignocellulosic structure by pretreatment and making the cellulose available in its crystalline structure, saccharification (enzymatic hydrolysis) applying enzymes is needed to produce fermentable sugars [161] as shown in Figure 2-15.

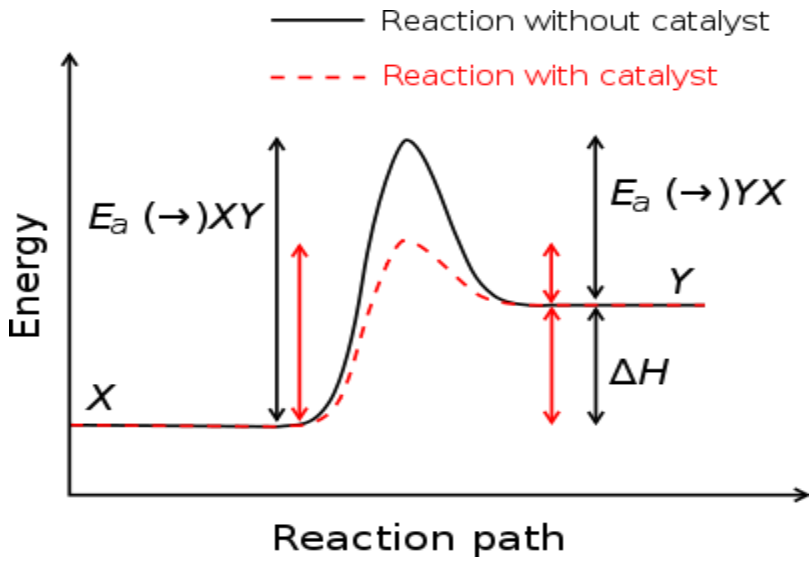


Figure 2-14. Comparing the activation energy for enzymatic catalyzed reactions with uncatalyzed reactions.

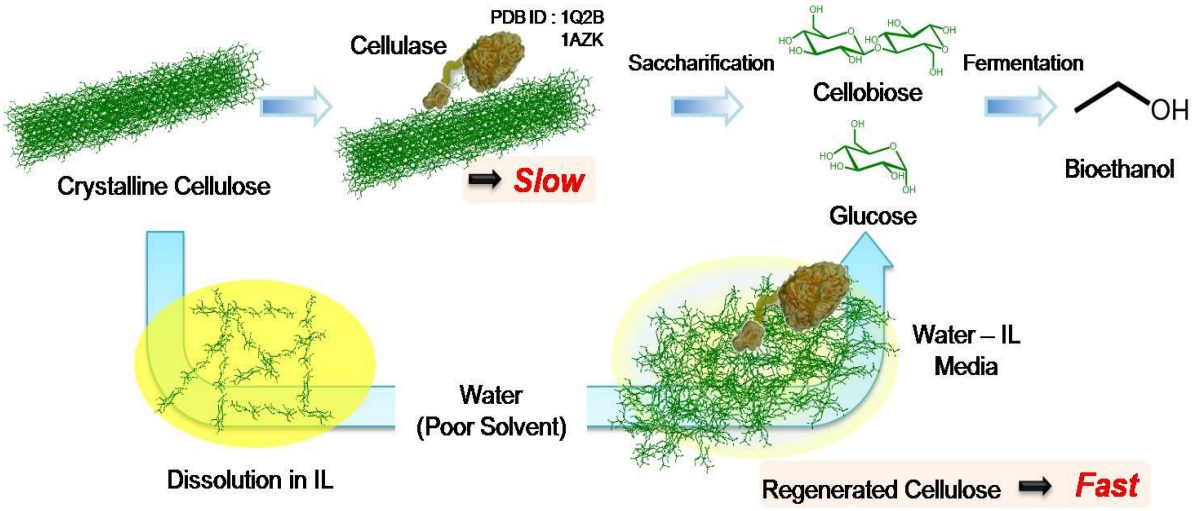


Figure 2-15. Enzymatic in situ saccharification process in aqueous-ionic liquid (IL) media [162].

Enzymatic hydrolysis of cellulose is implemented using cellulolytic enzymes system, which is a blend of endo-ß-1,4-glucanglucanhydrolases, exo- ß-1,4-glucancellobiohydrolases (cellulases) and ß-glucosidase [163]. Cellulase depolymerizes cellulose to give soluble sugars [164] by breaking cellulose down to cellobiose, which is subsequently cleaved to glucose by ß-glucosidase. It is generally recognized as the most often employed method to hydrolyze cellulose to glucose because of its mild reaction conditions and precise action in hydrolysis, in addition to its advantage in minimal production of glucose degradation products. Cellulose hydrolysis rate is determined by accessibility of cellulase to the limited adsorption sites on crystalline cellulose structure [165].

Enzyme-catalyzed conversion of cellulose to glucose is slow due to its stiff crystalline arrangement; hence pre-treatment is a prerequisite, which also helps in reaching high yields and making the process commercially viable [166]. Nevertheless, some pre-treated lignocelluloses show resistance to enzymatic hydrolysis by commercially-available cellulases. This behavior is triggered by the presence of lignin (despite its relocation during treatment), which represents a physical barrier reducing the

cellulose accessibility besides the possibility of adsorbing enzymes non-productively [167], [168].

The efficiency of the hydrolysis may decline due to the inhibitory effects of cellobiose and glucose on cellulases and β-glucosidase [169]. Comparing enzymatic hydrolysis to dilute-acid hydrolysis, higher yields of monosaccharides by the enzymatic hydrolysis were obtained because cellulase enzymes catalyze only hydrolysis reactions and not sugar degradation reactions [163]. Moreover, enzymes are naturally occurring compounds which are biodegradable and therefore environmental friendly [155].

Enzymes recovery would create completely new opportunities in large-scale enzymology applications [170], since this will help in process optimization and cost reduction [171].

2.4.4. Fermentation

Microbial conversion of plant biomass-derived sugars consists of multiple processes to produce different chemicals/products, such as ethanol, lactate, acetate, succinate, butanol, acetone, and other products. Fermentation processes that include conversion of sugars from lignocellulose offer great potential for expanding the renewable production of fuels and chemicals [172]. Natural microorganisms in the industrially relevant products ferment soluble sugars, such as species in the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, and others (producing lactic acid), *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis* (producing ethanol) and also *Clostridium acetobutylicum*, *Clostridium beijerinckii* (producing butanol).

Generally, fermentations classification is done according to the end product and the microorganism used, such as: (i) alcohol fermentation; (ii) vinegars (acetic acid); (iii) milks (lactic acids); and, (iv) amino acid fermentation [173].

a. Ethanol fermentation

Generally yeasts metabolize sugars (e.g. glucose) to produce ATP (energy for the cell). ATP hydrolysis produces adenosine-5′-diphosphate (ADP) and inorganic phosphate (Pi), the energy released is utilized by the cell for various reactions and transformations. Glucose metabolism occurs in a series of stages known as glycolysis, where two of three carbon compounds (glyceraldehyde-3-phosphate and dihydroxyacetonephosphate) are formed by glucose metabolism. Those compounds are transformed into two molecules of pyruvate. Two main fermentation conditions are known; aerobic and anaerobic. Aerobic is when oxygen is a terminal electron acceptor and when reoxidation of reduced pyridine nucleotide through cytochrome system occurs by electron transmission. In Aerobic fermentation, pyruvate enters Krebs cycle as shown in Figure 2-16. Under anaerobic conditions, reduced pyridine nucleotide oxidation is related to the reduction of an organic compound [174]. Usually, the organic compound is a product of the catabolic pathway, e.g., in fermentation with application of yeast, NADH is regenerated by the reduction of pyruvic acid to ethanol, since pyruvate is decarboxylated and reduced to yield two carbon dioxide and alcohol as shown in Figure 2-17 [175].

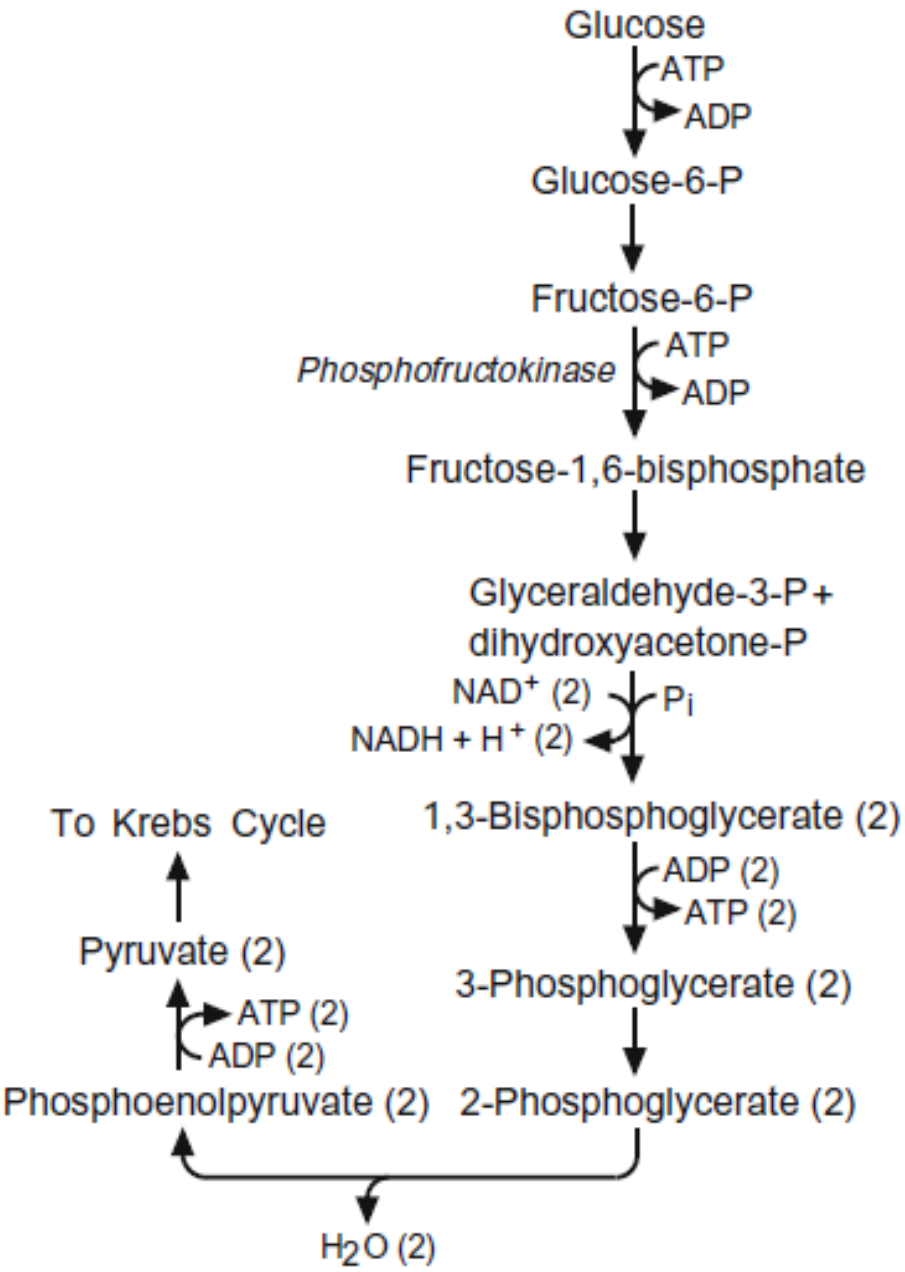


Figure 2-16. Utilization of glucose by *Saccharomyces* through Embden–Meyerhof–Parnas (EMP) pathway, phosphofructokinase pathway, entner doudoroff pathway or glycolysis pathway.

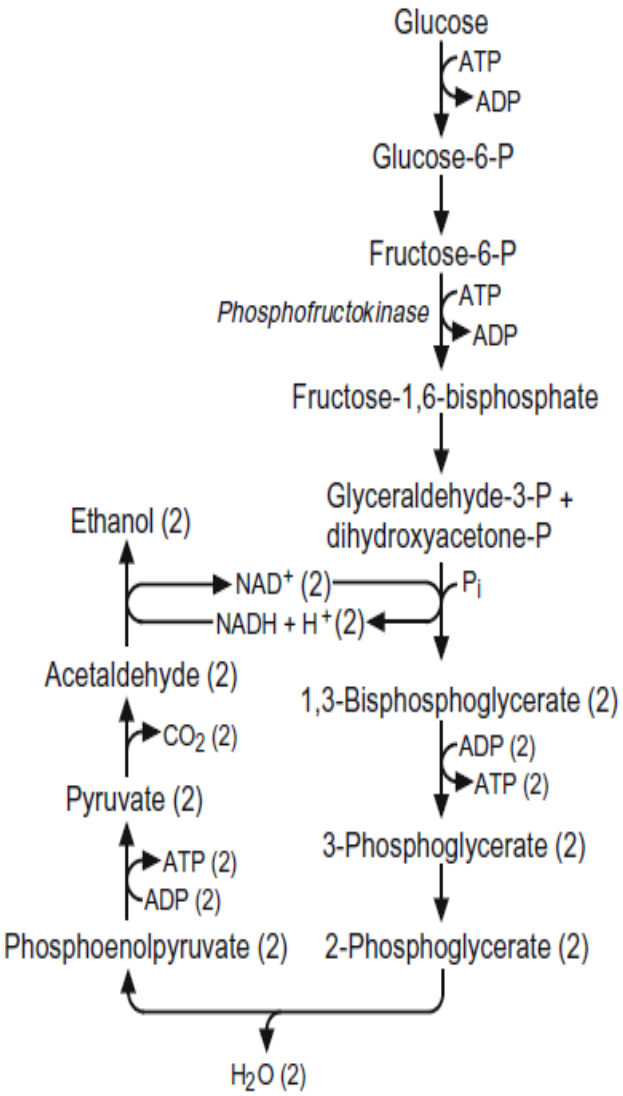


Figure 2-17. Utilization of glucose by *Saccharomyces* under anaerobic fermentation.

Yeast: Saccharomyces cerevisiae

A microorganism capable of efficient fermentation of the main sugars in hydrolysates of lignocellulosic biomass (glucose, xylose) would be very desirable in large-scale industrial processes. *Saccharomyces cerevisiae* is a GRAS (Generally Regarded as Safe) microorganism for this purpose [176] and a top candidate to be applied for biofuel production in terms of robustness and efficiency of glucose fermentation [177].

Colonies of *saccharomyces* are smooth, usually flat, and sometimes dense. *Saccharomyces* ferments glucose, sucrose, and raffinose and assimilate glucose, sucrose, maltose, raffinose, and ethanol but not nitrate [175]. Sugars fermentation by *Saccharomyces* produces alcohol and carbon dioxide, where 1 mole sugar yields 2 moles of each ethanol and carbon dioxide (Equation 2).

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2 \qquad \text{Equation 2}$$

The rate of ethanol produced by *Saccharomyces cerevisiae* can reach 8×10^7 to 9×10^7 molecules of ethanol per yeast cell per second depending on the applied conditions [178].

Saccharomyces cerevisiae strain is a widespread microorganism used in bioethanol production. This is because of the high efficiency of glucose fermentation and high ethanol tolerance of this strain. Furthermore, Baker's yeast are quite resistant to acetic acid and other inhibitors exist in lignocellulosic hydrolysates [67]. The biggest drawback of *S. cerevisiae* is its inability to ferment C5 sugars. It is deficient in a xylose-assimilation pathway and adequate levels of key pentose phosphate pathway enzymes. The inability of *S. cerevisiae* to grow on xylose has been attributed to inefficient xylose uptake. Hence, metabolic engineering has been widely used to overcome this drawback [176]. A fermentation study conducted by Eklund and Zacchi [179] showed that an increase in the cell concentration results in higher ethanol yields. In fermenting pretreated willow, a maximum ethanol yield of 85.6% was obtained when applying yeast concentration of 10 g/L. At a level of 3 g/L, the same ethanol yield was obtained. However at a level of only 1 g/L, a minor decrease in the obtained ethanol yield was observed (i.e., 80%).

Zymomonas mobilis has also been suggested for ethanol production. Hence, a comparison between *Saccharomyces cerevisiae* and *Zymomonas mobilis* was performed by Eklund and Zacchi [179]. The results show that yeast was superior concerning ethanol yield after 72 hours of simultaneous saccharification and fermentation (SSF); however, the variance was minor. Regarding by-product formation, *S. cerevisiae* showed higher concentrations after 72 hours SSF (the total by-product formation was about 2 to 2.5 g/L for the bacterium and about 3 to 4 g/L for the yeast). Moreover, succinic acid was not detected in *Z. mobilis* fermentation, where yeast was able to produce it.

Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) involves cellulose to glucose hydrolysis (applying enzymes) followed by the transformation of fermentable sugars. Both processes happen in the same container [180], as illustrated in Figure 2-18. This method saves cost (by almost 20% of the capital investment [181]) by decreasing the number of used vessels in comparison to separate hydrolysis and fermentation. Moreover, it results in increasing the hydrolysis rate due to decreased product inhibition, since cellulases are inhibited by glucose and cellobiose [179]. The avoidance of end-product inhibition is the most important reason for applying SSF. Gauss, Suzuki [180] could show that larger total ethanol yield was obtained when using SSF, in addition to other several potential advantages, such as the advantage that glucose does not need to be separated from the lignin fraction following a separate enzymatic hydrolysis step, thus avoiding/minimizing-loss potentials [180], [181].

Nevertheless, SSF encounters difficulties in optimizing the conditions applied; since the optimum conditions for the cellulases and micro-organisms vary. The micro-

organism (such as *Saccharomyces cerevisiae*) does not tolerate temperatures higher than 40 ˚C, which is lower than the optimum temperature at which cellulases have the maximum activity (50 ˚C) [179]. Additionally, in SSF the yeast cannot be reused due to the problems of splitting the yeast from the lignin after fermentation [181].

Eklund et al., 1995 [179] studied the differences between separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) on pretreated willow. A glucose yield of 95% was obtained after 72 hours of enzymatic hydrolysis for SHF. The fermentation of this hydrolysate produced ethanol at a yield of 85% after 48 hours (total reaction time of 120 for the SHF). The same ethanol yield was obtained applying SSF with a total reaction time of 72 hours, concluding that there is a gain of 48 hours using SSF in comparison to SHF. Another advantage was concluded by this study was the amount of by-products generated (3.2 g/L for SSF compared to 5.0 g/L for the SHF).

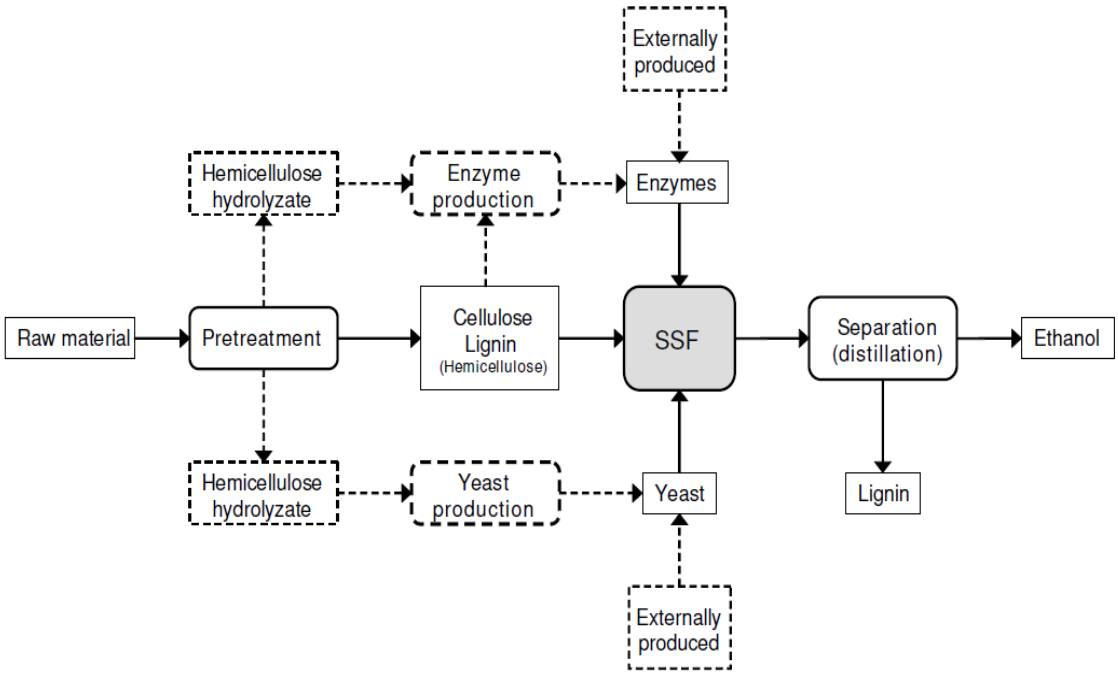


Figure 2-18. Schematic representation of an SSF process [181].

b. Acetic acid fermentation

Acetic acid is a significant feedstock for many chemicals including vinyl acetate polymer, terephthalic acid/dimethyl terephthalate, acetic acid esters, cellulose acetate, acetic anhydride, and calcium magnesium acetate. Currently, these products are obtained from petroleum-derived acetic acid [182]. The fermentation process is a renewable and sustainable alternative for producing acetic acid, which has been studied extensively since the late 1970s [183].

Acetic acid bacteria (AAB) are rod shaped, strictly aerobic, gram-negative microorganisms [184]. They belong to the Acetobacteraceae family and are classified into 12 genera and 59 species and the main source of these bacteria is food. AAB play a role as plant-associated bacteria (N_2 fixing), human pathogens and symbionts of insects [185]. Industrially, they have advantages (food production) as well as disadvantages (spoilage of fermented beverages). Some AAB are used as biocatalysts for the industrial production of a range of compounds. Certain AAB strains (i.e., *Gluconobacter oxydans*) produce enzymes involved in amino acids synthesis e.g., glutamic and aspartic acids [186].

Despite the negative effect of oxygen limitation on AAB growth, AAB do not necessarily die with a lack of oxygen. For example, Acetobacter may enter a "viable-but-not-culturable" phase and exhibit growth at pH values higher than 3.75. In low oxygen environments, AAB utilize electron acceptors other than oxygen [187], as shown in Figure 2-19 [188] illustrating that addition of activated charcoal used to remove phenolic compounds from a wine also affected growth of *Acetobacter pasteurianus*.

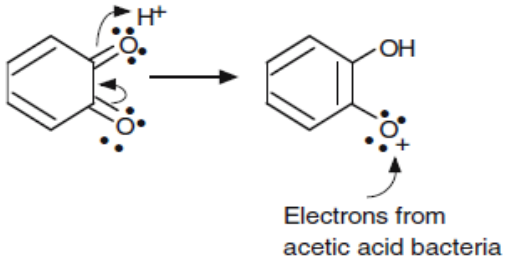


Figure 2-19. The behavior of phenolics as electron acceptors under low oxygen conditions [175].

Likewise, *Gluconobacter oxydans* can use p-hydroxyquinone as a terminal electron acceptor to grow on glycerol under anaerobic conditions [188].

Through oxidative fermentation of AAB, specific oxidation reactions occur, releasing electrons to molecular oxygen [185]. The metabolic potential of AAB is illustrated by the partial oxidation of carbohydrates in the media, releasing the consistent products (ketones, aldehydes, and organic acids) [189]. Acetic acid bacteria are characterised by a unique resistance to ethanol and acetic acid [184]. Growth of acetic acid bacteria in ethanol containing media results in oxidation of ethanol to acetic acid (acidification process). Besides formation of other odour- and flavor-active metabolites in addition to possible generation of polysaccharides including dextrans and levans [190], [191], [175]. Some AAB oxidise ethanol to acetic acid by two serial reactions catalysed by the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzymes [184].

Acetate can have either bacteriostatic or bactericidal effects therefore considered cytotoxic, even at low concentrations (i.e. 0.5% (v/v)) [192]. The toxicity of acetic acid forms from the high lipophilic nature of the undissociated acetate. Acetate is characterized as a lipid-permeable molecule, easily penetrating into the cytoplasm, leading to acetate anion accumulation and, consequently, decreasing the intracellular pH, creating disruption in the proton-motive force [193].

Dilution with citric acid and its effects on acetic acid ferementation

Throughout our experimental set, juice was fermented while being diluted with citric acid. Citric acid is extensively utilized by the food and pharmaceutical industries. It is characterized for its safety, high solubility in water, and for its chelating and buffering properties. Industrially it is used as acidifying agent, pH-adjuster and antioxidant. It also has the ability to minimize sucrose inversion and preventing its crystallization [194].

Citrate has a vital function in intermediary metabolism. Four enzymes are involved in the catalysis of citrate formation or dissolution through aldol- (or retroaldol-) type reaction involving equilibrium between citrate on one hand and oxaloacetate and an acetyl-moiety on the other, as shown in Equation 3. Citrate lyase occurs only in bacterial sources, mainly as a citrate-induced activity. ATP-citrate lyase, is a cytosolic enzyme, generally existing in eukaryotes. It requires ATP, CoA and a divalent metal ion such as Mg^{2+} to cleave citrate (Equation 4). Third enzyme is si-Citrate synthase, which has the effect to catalyze citrate formation, as shown in Equation 5. re-Citrate synthase found in some anaerobic bacteria. The enzyme shows an opposite stereochemistry to what si-citrate synthase shows [195].

$$\text{Citrate} \xleftrightarrow{Mg^{+2}} \text{Oxaloacetate + acetate} \qquad \text{Equation 3}$$

$$\text{Citrate + CoA + ATP} \xleftrightarrow{Mg^{+2}} \text{Acetyl-CoA + oxaloacetate + ADP + } P_i \qquad \text{Equation 4}$$

$$\text{Citrate + CoA + } H^- \xleftrightarrow{Mg^{+2}} \text{Acetyl-CoA + oxaloacetate + } H_2O \qquad \text{Equation 5}$$

Some species of Clostridium utilize citrate as carbon and energy source and also contain the enzyme citrate lyase [195]. In the citrate fermentation pathway, citrate lyase is a key enzyme in commencing the anaerobic utilization of citrate by some bacteria. The general reaction catalyzed by citrate lyase is towards citrate cleavage, producing oxaloacetate and acetate as initial products, as was studied in Aerobacter indologenes [196]. The reaction goes into completion with excess amount of enzymes [197]. Additional catabolism of oxaloacetate formed takes place either by decarboxylation or by reduction. In some microorganisms, the oxaloacetate is decarboxylated to pyruvate by the enzyme oxaloacetate decarboxylase (this enzyme is induced in these sources in the presence of citrate). The subsequent oxidative decarboxylation of pyruvate to acetyl-CoA, a source of ATP generation and also power reducion, is catalyzed by the pyruvate dehydrogenase complex [195].

The stereospecificity was studied by Walther et al., 1977 [198] on Clostridium sphenoids growing in citrate media, where C4 and C5 of citrate yield acetate and that Cl and C6 become the carboxyl groups of oxaloacetate. Oxaloacetate is further degraded to C2 compounds (i.e., ethanol and acetate) forming CO_2 originating from Cl and C6 of citrate [198].

Citrate lyase occurs constitutively in *Streptococcus diacetilactis* only. Citrate lyase or citrate lyase activity was found in some strains of *Leuconostoc citrovorum*, *Streptococcus liquefaciens* [195], *Rhodopseudomonas gelatinosa* and *Rhodopseudomonas palustris* [199]. Anaerobic growth activity of citrate lyase on citrate was reported in *Aerobacter cloacae*, which did not need sodium ions [200] and also in *Proteus rettgeri* [195].

Many species of clostridia show the ability to utilize citrate as carbon and energy source besides containing the enzyme citrate lyase, such as *C. sphenoides*, which

fermented citrate to acetate, ethanol, carbon dioxide, and hydrogen [198]. Anaerobic growth of *Aerobacter aerogenes* on citrate needed Na^+, having the growth rate directly related to Na^+ concentration. Nonetheless, Na^+ is not required for *Aerobacter aerogenes* growth on glucose. Na^+ cannot, however, be replace with K^+, NH^{4+}, Li^+, Cs^+, or Rb^+. The optimal growth happened at 0.10 M Na^+ with the existence of 0.15 mM K^+. Na^+ was needed to stimulate the oxaloacetate decarboxylase activity, which is specifically induced by the growth on citrate [200]. NaCl increased acid production in *Proteus vulgaris* utilizing glucose, which was mainly explained by the existence of Na^+ [201]. For maximal rate of oxidation of marine bacteria with citrate as a substrate, about 200 mM Na^+ is needed [202].

c. Fermentation challenges

Pentose sugar fermentation

Hemicellulose is one of the major components of renewable biomass, comprising 20 - 35% of plant material [203]. Hemicellulose yields a mixture of sugars, with D-xylose as the major product when hydrolyzed [204]. Recently, hemicellulose has shown useful applications in various agro-industrial processes, such as effective conversion of hemicellulosic biomass to fuels and chemicals, delignification of paper pulp, clarification of juices, digestibility improvement of animal feedstock, and improvement in the consistency of beer. The utilization of hemicellulosic sugars is crucial for efficient conversion of lignocellulosic materials to valuable fermentable products, with the focus on fuel ethanol [205].

One of the leading challenges faced in the fermentation process is the ability to ferment both C6 and C5 sugars to ethanol using a single organism while minimizing production of other byproducts [206]. The yeast *Saccharomyces cerevisiae* has proved its applicability and well-adaption to industrial use due to its close-to-theoretical

ethanol yields, its tolerance to a wide range of inhibitors and to its high osmotic pressure [207]. However, traditional *S. cerevisiae* cannot ferment xylose and arabinose, while glucose fermentation is achieved rapidly and efficiently. *Zymomonas mobilis* behaves the same way [205], [208]. *S. cerevisiae* contains all necessary enzymes for the conversion of xylose to ethanol, except xylose isomerase (XI). Efforts have been focusing on the utilization of xylose, where native strains capable of fermenting xylose have been identified among bacteria [209]. *S. cereviciae* is incapable to ferment pentoses directly [210]. However, xylose isomerization to xylulose with the enzyme glucose (xylose) isomerase (XI; EC 5.3.1.5) allows for a subsequent fermentation. Direct xylose fermentation was discussed by Hahn-HäGerdal et al., 1991 by using the strains: *Pachysolen tannophilus, Candida shehatae*, and *Pichia stipis* [211]. Xylose fermenting yeasts generally have limited ethanol tolerance. In direct fermentation, low and well-controlled oxygenation is necessary in addition to the continuous removal of inhibitors. On the other hand, xylose isomerization prior to fermentation with *S. cerevisiae* gives ethanol yields similar to those obtained in hexose fermentations without oxygenation and removal of inhibitors. Nonetheless, the enzyme is not very stable in a lignocellulose hydrolysate. Additionally, *S. cerevisiae* has a poorly established pentose phosphate shunt [211].

Different yeast strains (*Candida diddensii, Candida utilis, Rhodosporidium toruloides, Rhodotorula glutinis var. salivaria, Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces diastaticus, Saccharomycopsis fibuligera, Torula candida,* and *Trichosporon melibiosaceum*) were tested by Gong et al., 1981 [206] for ethanol production from D-glucose, D-xylulose and xylitol in the presence of D-xylose isomerizing enzyme. All of the yeast strains showed ability to utilize all the tested sugars as carbon and energy sources, except the respiratory-deficient mutant

of *Saccharomyces cerevisiae*, which is not capable of catabolizing D-xylose or xylitol aerobically. Generally, D-glucose and xylitol are better carbon sources for vegetative growth. Yet, ethanol was produced from both D-glucose and D-xylulose but not from xylitol. The amounts of ethanol produced varied between the species, with *Candida utilis* producing very small amounts of ethanol from D-xylose. In this study it was proved that the rate of fermenting D-glucose by *S. cerevisiae* is higher than the rate of fermenting D-xylulose. Nonetheless, the slow rate could be overcome by increasing the cell density [206], [212]. In another study done by Chiang et al., 1981 [212], *S. cerevisiae* was used to study the conversion of D-xylulose to ethanol in the presence of D-xylose. This study confirmed that increasing ethanol-production rates needs higher yeast cell densities. The optimum parameters were also identified for D-xylulose fermentation, with a temperature of 35°C and pH range between 4 and 6. Fermenting D-xylulose by yeast obtained ethanol as a major product; small amounts of xylitol and glycerol were also generated. The production of xylitol was subjective to pH as well as temperature. High pH values and low temperatures enhanced xylitol production. At ethanol concentrations higher than 4%, a decrease in D-xylulose fermentation rate occurred [212]. Saha [205] reported that the yeasts *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehate* have the capability to ferment xylose to ethanol after converting xylose using the enzyme xylose isomerase to xylulose, which can then be fermented to ethanol. Generally, commercial exploitation of xylose is restricted due to the low ethanol tolerance, slow rates of fermentation, difficulty in controlling the rate of oxygen supply at the optimal level, plus sensitivity to inhibitors generated during pretreatment and hydrolysis of lignocellulosic substrates [213].

In conclusion, developing a proper pretreatment method, minimizing the generation of inhibitory compounds, and tailoring a proper mix of hemicellulases (enzyme cocktail) for each biomass conversion will influence the utilization of hemicelluloses in fuels and chemicals production by fermentation [205].

Salt stress

Osmotic stress is a general phenomenon from bacteria and plants to humans. Osmotic stress tends to knock off the balance of the cellular homeostasis in the organism. Osmoadaptation takes place as a cellular response to restore the normality [214]. Normally, osmotically stressed yeasts accumulate osmoregulatory solutes such as glycerol and arabitol [215]. These neutral, low molecular mass solutes protect enzymes and structural proteins against inactivation, inhibition and denaturation [216]. NaCl concentrations of the medium affect the glycerol yields in *Saccharomyces rouxii*, salt-tolerant soy yeast, remarkably [215]. Identifying polyalcohols produced by aerobic fermentation in glucose and sodium chloride-concentrated media, about 70% of polyalcohols produced were glycerol, while in the concentrated glucose medium, a larger amount of D-arabitol was produced than glycerol [215].

Zygosaccharomyces rouxii and *Saccaromyces cerevisiae* are both well-known yeasts in food fermentation industry. However, *Z. rouxii* can tolerate higher salt concentrations in comparison to *S. cerevisiae* [217].

Osmoadaptation in *S. cerevisiae* is a highly complex with coordinated flow of proceedings, which can be distinguished as follows: (i) immediate cellular changes that occur directly due to the physico-mechanical forces operating under these conditions; (ii) main defense processes provoked in order to set protection, restoration and recovery in motion and (iii) continuous adaptive events that permit repair of cellular homeostasis under the new conditions [218]. In order to understand the

complex actions, keen insights at any point of time are required [214], which will help in understanding the yeast behavior at the molecular level. Osmotic stress tolerance has significant implications for commercial applications.

Parmar et al., 2011 [219] discussed the effects of the higher salinity conditions on yeast growth, which include growth rate, yield of biomass, lag phase of growth and cell composition. Minimized glucose uptake has been observed in hyperosmotic shock in *S. cerevisiae* with an associated escalation in the lag phase in a concentration-dependent manner [219]. Spencer et al., 1956 [220] found that osmophilic *Zygosaccharomyces,* isolated from some sources like honey fermentation, produces one or more of the following sugar alcohols: glycerol, erythritol, mannitol, D-arabitol, and two unidentified polyhydric alcohols under aerobic conditions.

2.4.5. Green Biorefiney

Recently, there is an increasing demand on bio-based chemicals, especially from renewable resources. Most of the reviews focus on converting lignocellulosic (mostly carbohydrate-based) materials into broad range of chemicals [221], which are normally oil refinery-based chemicals. However, lower yields and higher costs are associated [221]. In that term, the DOE/NREL report "Top Value Added Chemicals from Biomass", carbohydrate-based building blocks with a unique arrangement were described. Twelve sugar-based building blocks were identified, which are:1,4-diacids (succinic, malic and fumaric), 2,5-furan dicarboxylic acid, aspartic acid, 3-hydroxy propionic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, sorbitol, glycerol, and xylitol/arabinitol. Despite the abundance of chemicals in the biomass, technical barriers exist, preventing the widespread use exploitation of biomass for products and chemicals [222].

Namely, building blocks of various biomass resources are converted into value added products, biofuels, energy (biogas) and chemicals through biorefineries. Increased productivity and efficiency of a biorefinery can be achieved through decreasing the overall energy consumed by unit operations, exploiting the use of all possible feedstock components and also through utilizing all byproducts and waste streams [222].

Different types of biorefineries were discussed by Ecker et al., 2012 [223], which are: the lignocellulosic feedstock biorefinery [224] and the whole crop biorefinery; containing the sugar platform biorefinery and the Green Biorefinery [223]. Products of special interest like added-value extractives, proteins and nutraceutical chemicals (e.g., hormones and antibiotics) can substantially improve the economy of a biorefinery, making it more economically feasible and industrially attractive [225], hence there is a great potential in utilizing the green biomass in green biorefinery. This way excessive heat and mechanical pretreatment are avoided, the activity of the fresh biomass is maintained and inhibitors' formation is eliminated and/or minimized, in addition to decreasing the cost associated with excessive pretreatment and the use of enzymes [226], [227].

Green Biorefinery uses whole green crops, like different kinds of grass [223]. Traditionally, green biomass has been a waste product from the green crop drying industry in Europe, where the green crop-drying factories would heat and press fresh, green biomass before drying with the aim of producing green pellets (as animal feed) from the resultant pulp. The waste stream from this process is a juice rich in nutrients, called brown juice [228]. Green or brown juices generated from this process used to be spread on the fields as fertilizer. However, problems with pollution of the ground water with nitrate, especially in the late autumn have led to strict regulations for the

use of plant juice as fertilizer in many countries [229]. Moreover, the green pellet factories can efficiently be transformed into green biorefineries [230], where juice may be used for production of proteins, many different phyto-chemicals and fermentation products [229]. In the green biorefinery the green crops are wet-fractionated by applying mechanical force, such as crushing, shearing or grinding to produce two main fractions; juice and fibers [231]. Fibers are a result of concentrating the solid material in plant cells and contain particles and insoluble high molecular weight compounds. The liquid fraction, on the other hand, contains soluble compounds. Vitamins, enzymes, colors and other phytochemicals can be isolated directly from the juice or press cake [232], [233]. After extraction of the high value compounds from the juice, it can be used as a substrate for fermentation. The fermentation products can be any organic compounds such as enzymes, antibiotics, biodegradable plastics, organic acids, alcohols, and amino acids [228]. The press cake can be used either to produce forage for animal feed or for energy production, applying different methods, like combustion and fermentation to produce several liquid fuels or digestion to produce gaseous fuels [233]. Nevertheless, anaerobic fermentation eliminates the adverse effect of biomass burning on the environment [77].

There were also attempts for wet fractionation to extract and concentrate soluble leaf proteins to be used in food products, especially in countries that were scarce in important nutrients like proteins [234]. Pirie showed that pulping and pressing of different feedstocks results in 50-70% of crude protein yield of the extraction phase (depending on the feedstock) [235].

CHAPTER 3

3. Materials and methods

3.1. *Salicornia Bigelovii* under Different Cultivation Conditions [93]

3.1.1. Raw material

S. bigelovii seeds (wild collected seeds) were provided by Masdar Institute from the U.S. state of Texas to the International Center for Biosaline Agriculture (ICBA) for the ISEAS project [236]. Seed cultivation was carried out by applying different growth conditions to evaluate the growth and biomass (total and seed) production at different levels of salinity (10, 20, 30, 40 and 50 ppt of NaCl) and fertilizer (ammonium nitrate) treatments (1.0, 1.5 and 2.0 g N/m^2) [236], [93].

The produced biomass, composed of stems, seedless inflorescences, and branches, was harvested, seeds were separated from the stems and the remaining biomass was dried. Samples for chemical characterization were milled using a knife mill (IKA, 10 MF Basic) [93].

3.1.2. Chemical characterization

After drying the washed grounded biomass, *S. bigelovii* was subjected to complete biomass compositional analysis by studying its total solids, ash content and also sugar

and acid composition following standard analytical protocols developed by National Renewable Energy Laboratory (NREL) [237, 238].

a. Determination of water- and ethanol-soluble extractives

In order to determine the extractives content, the weight loss of the extracted solid was measured and then solids were analyzed in both, water extract and ethanol extract [238]. Dried and finely ground samples were subjected to water extraction followed by ethanol extraction using a Soxhlet apparatus. The biomass (5g) was loaded into a cellulose thimble and subjected to 7 hours of extraction with 200 g of water (with 3-4 siphon cycles per hour) and 7 hours extraction with 200 g of ethanol (5-6 siphon cycles per hour). After the extraction, the solid was removed from the thimble, dried in the drying oven (at 105 °C) overnight and weighed to determine the total extractives amount (including volatile and non-volatile extractives) (Equation 6) [93].

$$\text{Total extractives (TE)}\left(\frac{\text{g}}{100\text{gTS}}\right) = \frac{\text{TS} - \text{W}_{\text{dried extracted biomass}}}{\text{TS}} * 100 \qquad \text{Equation 6}$$

- TS – total solids of the original biomass [g].
- $W_{\text{dried extracted biomass}}$ – weight of the extractives-free biomass removed from the thimble and dried [g].

The non-volatile water- and ethanol-soluble extractives were measured by evaporating the extracts to dryness and thus determining the solids content gravimetrically (Equation 7).

$$\text{Nonvolatile extractives(NE)}\left(\frac{\text{g}}{100\text{gTS}}\right) = \frac{\text{W}_{\text{dried water or ethanol extract}}}{\text{TS}} * 100 \qquad \text{Equation 7}$$

$W_{\text{dried water or ethanol extract}}$ – weight of the extract (evaporated to dryness) [g].

Water extracts were additionally analyzed for free sugars (glucose, xylose and arabinose) and organic acids (acetic, lactic and formic acids) using High Performance Liquid Chromatography (HPLC) Agilent 1260 Infinity Bio-inert Binary LC). The Hi Plex-H column (Agilent) and refractive index detector (RID) were used to determine the concentrations of glucose, xylose, and arabinose at 65°C using 0.005 M H_2SO_4 as the mobile phase (eluent) with a flow rate of 0.6 ml/min. The content of sugars and organic acids was calculated per total solids of the original biomass sample to estimate the amount of these components that have been released during extraction (Equation 8) [93].

$$\text{Sugar/acid}_{\text{extract}}\left(\frac{\text{g}}{100\text{gTS}}\right) = \frac{C_{extract} * V_{extract} * \frac{1\text{g}}{1000\text{mg}}}{\text{TS}} * 100 \qquad \text{Equation 8}$$

- $C_{extract}$ – concentration of a measured component (sugar or organic acid) analyzed by the HPLC [mg/ml]
- $V_{extract}$ – volume of the extract [ml]

b. <u>Total solids & ash determination</u>

Total solids and ash determination was carried out by following the NREL protocol [239, 240]. There are two types of the inorganic material in biomass; structural and extractable. The amount of inorganic material can be measured as part of the total composition. Structural ash is bound in the physical structure of the biomass (salt deposits), while extractable ash is in the inorganic material that can be removed by washing or extracting the material with water [241]. For total solids and ash determination, ceramic crucibles were needed, since they endure extreme temperatures. Crucibles were ignited by heating them at 575°C. The biomass sample was placed in the crucibles and dried at 105°C overnight to determine the total solids content. The difference between the initial and final weight after drying was used for

calculating the total solids (Equation 9 and Equation 10). In order to measure the ash content, the crucibles were ignited at 575 °C in the muffle furnace. The total ash content was calculated using Equation 11.

$$Total\ Solids\ of\ Biomass\ (TS)\ \% = \frac{W_d}{W_i} * 100 \qquad \text{Equation 9}$$

$$Total\ Moisture\ Content\ \% = (1 - \frac{W_d}{W_i}) * 100 \qquad \text{Equation 10}$$

W_d = Weight of the biomass after drying at 105°C (g) to constant weight

W_i = Weight of the initial biomass (g)

$$Total\ Ash\ \% = \frac{W_r}{W_d} * 100 \qquad \text{Equation 11}$$

W_r = Weight of the residue after drying at 575°C (g) to constant weight

W_d = Weight of the biomass after drying at 105°C (g) to constant weight

c. <u>Chemical characterization by strong acid hydrolysis of dry biomass</u>

The dried material was hydrolyzed by strong acid [237]. Each sample was analyzed in duplicate. Dried samples (0.3 g) were treated with 72% (w/w) H_2SO_4 (3.0 ml) at 30 °C for 1 h with continual centrifugation (every 10 minutes). The resultant solutions were then diluted with 84 ml water (acid diluted to 4%) and autoclaved at 120 °C for 1 h. At the same time a standard was prepared (under the name spiked sample), in which 82 ml water and 2 ml of spike solution were added to dilute after treating with concentrated acid. The spike solution is composed of a solution of the three sugars (glucose, xylose and arabinose) at concentration of 30 g/L of each. The preparation of the spiked solution followed the following procedure: 3.00 g D-xylose, 3.00 g L-arabinose and 3.30 g D-glucose-monohydrate were weighed separately in a weighing

boat and transferred quantitatively to a 100 ml measuring flask with Millipore water. Water was filled to the mark and the solution was kept frozen until used.

The hydrolysate was filtered, and the Klason lignin content was determined as the difference between the weight of the insoluble residue and the ash content. The samples were analyzed for carbohydrates and other by-products (organic acids) using HPLC (Agilent 1260 Infinity Bio-inert Binary LC). The Hi Plex-H column (Agilent) and refractive index detector (RID) were used to determine the concentrations of glucose, xylose, and arabinose at a temperature of 65 ˚C by means of 0.005 M H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min. Accordingly, the content of sugars and organic acids was calculated per total liquid of the original sample as shown in Equation 12 [93].

$$\text{Sugar/acid}_{\text{extract}}\left(\frac{\text{g}}{100\text{gTS}}\right) = \frac{\text{C}_{\text{extract}} * \text{V}_{\text{extract}} * \frac{1\text{g}}{1000\text{mg}}}{\text{TS}} * 100 \qquad \text{Equation 12}$$

- $C_{extract}$: concentration of a measured component (sugar or organic acid) analyzed by the HPLC [mg/ml]
- $V_{extract}$: volume of the extract [ml]

The recovery factor (R_f) of the individual sugars was calculated according to Equation 13 in order to account for any sugar degradation arising during acid hydrolysis, which was achieved by evaluating the behavior of the spiked samples and comparing it to the samples. Afterwards, a recovery factor was obtained, which was used as a correction factor, as explained in Equation 14.

$$R_f = \frac{C_{h+s\,(measured)}}{C_{s\,(added)} + C_{h\,(measured)}} \qquad \text{Equation 13}$$

C_{h+s}: Sugar in acid hydrolysate with standard addition (g/100 g DM).

C_s: Sugar standard added (g/100 g DM).

C_h: Sugar in acid hydrolysate without standard addition (g/100 g DM).

$$C_{corr} = \frac{C_{h\,(measured)}}{R_f} \qquad \text{Equation 14}$$

Concentration of the polymeric sugars was calculated from the corresponding monomeric sugars concentration (e.g., glucose to glucan or xylose to xylan conversion), applying an anhydro correction of 0.88 (or 132/150) for C5 sugars (xylose and arabinose) and a correction of 0.90 (or162/180) for C6 sugars (glucose, galactose, and mannose).

$$\mathrm{C_{anhydro}} = \mathrm{C_{corr}} * \text{Anhydro correction} \qquad \text{Equation 15}$$

Acid insoluble lignin (Klason lignin) content in the "as received" sample was calculated using Equation 16.

$$\%\ \mathrm{AIL}_{received\ sample} = \frac{(\mathrm{W_b} - \mathrm{W_a})}{\mathrm{W_s}} * 100\% \qquad \text{Equation 16}$$

Where,

%AIL = acid insoluble lignin (%)

$W_{\boldsymbol{b}}$ = weight of residue ***before*** drying at 105^{o}C (g)

$W_{\boldsymbol{a}}$ = weight of residue ***after*** drying at 105^{o}C (g)

W_s = weight of a sample (g)

3.1.3. Chemical characterization of fresh untreated Salicornia bigelovii (a mixture of the differently-cultivated biomass)

A mixture of all cultivated samples (under different cultivation conditions; three fertilizer levels (F1: 1 g N/m^2, F2: 1.5 g N/m^2 and F3: 2 g N/m^2) and two salinity levels (S1: 10 ppt and S5: 50 ppt salt)) [93] was first grounded, after which its dry matter and ash composition (following section 3.1.2-b) and its sugar composition (following section 3.1.2-c) were determined.

3.1.4. Biomass washing

Before pre-treating the biomass, a washing step was conducted to decrease the salt content. Salt might interfere with the resultant products and could also result in instrument corrosion. The grounded material was suspended in a beaker and mixed with water in a ratio of 10% w/v (biomass/water) for half an hour according to the results obtained by [39]. The resultant suspension was filtered through a cloth filter to separate the biomass from water. Total solids and ash content of the obtained solid fractions were measured by following the procedure in section 3.1.2-b. The wet biomass was used in the hydrothermal pretreatment study, after measuring the material's dry matter content.

3.1.5. Hydrothermal pre-treatment

Hydrothermal pre-treatment was performed at 6% dry matter loading at a temperature of 190 ˚C without any catalyst application for a residence time of 10 minutes. Hydrothermal pre-treatment was tested for both washed and unwashed biomass, in order to understand the impact of biomass washing on sugar extraction and conversion. Generally, washing is required to avoid equipment corrosion, hence

decreasing unit cost. The pretreatment was conducted in a 1.0 L reactor, with a vertical setup, and continuous mixing as shown in Figure 3-1.

Figure 3-1. The reactor used for hydrothermal pre-treatment of salicornia biomass

A batch of 500 ml was prepared by weighing a 30 g-equivalent biomass and adding 500 ml-equivalent deionized (DI) water. Heating was controlled automatically, where it happens gradually until it reaches the desired temperature. However, cooling is done manually by dipping the reactor in cold water. The relaxation time mainly depends on the temperature of the cooling water as well as the number of times it is changed. The cooling process was done in a timely manner. The pre-treated slurry was filtered to separate the solid fraction (fibers) from the liquid fraction (pre-treatment liquid). Dry matter and ash content were measured for both fractions. Both fractions were preserved at -18 $^{\circ}$C until analysis and further processing. Further processing included: strong acid hydrolysis (with the measurement of the Klason

lignin) of the fiber fraction, analysis of the monomeric and oligomeric sugar composition as well as degradation by-products in the pre-treatment liquid, and fibers fermentability (with and without adding the pre-treatment liquid as fermentation medium).

a. Total solids and ash content of the pre-treated biomass

Total solids and ash content of the two obtained fractions (liquid and solid) were measured following the procedure described in section 3.1.2-b. Each fraction was handled separately.

b. Free sugars

Carbohydrate content was analyzed for the pretreated biomass (fibers and pre-treatment liquid). The analysis followed standard analytical protocols developed by NREL [237] and was performed by determining the lignocellulosic components; carbohydrates and lignin. Weak acid hydrolysis [242] and strong acid hydrolysis [237] were respectively conducted on the liquid fraction and the solid fraction generated from biomass pre-treatment, in order to break the cellulosic biomass into monomeric sugar.

Strong acid hydrolysis

The solid fraction of the pre-treated material was dried before conducting the strong acid hydrolysis study. Strong acid hydrolysis was achieved by following the description in section 3.1.2-c.

Weak acid hydrolysis

Weak acid hydrolysis of the juice was done by taking a sample of 10 mL and digesting it with 10 ml sulfuric acid (8 w/w%). The solution was autoclaved at 120 ˚C for 10 minutes. After cooling the solution to room temperature, it was analyzed for

carbohydrates and other by-products using HPLC. The Hi Plex-H column (Agilent) and RI detector were used to determine the concentrations of glucose, xylose, and arabinose at the same conditions mentioned for strong acid hydrolysis. Accordingly, the content of sugars and organic acids was calculated per total liquid of the original sample as explained in Equation 17. The equation is multiplied by 2 to eliminate the effect of dilution, where the pre-treatment liquid was diluted by a factor of 2.

$$\text{Sugar/acid}_{\text{extract}}\left(\frac{\text{g}}{\text{L liquid}}\right) = C_{\text{extract}} * \frac{1\text{g}}{1000\text{mg}} * \frac{1000\text{ mL}}{1\text{ L}} * 2 \qquad \text{Equation 17}$$

$C_{extract}$: concentration of a measured component (sugar or organic acid) analyzed by the HPLC [mg/mL].

c. <u>Klason lignin content</u>

The hydrolysate generated from strong acid hydrolysis of the fiber fraction was filtered through fritted ceramic funnels; the weight of the acid insoluble filtrate represented the Klason lignin content. Equation 16 is used to calculate the Klason lignin content in the original sample.

3.1.6. Glucan-to-glucose convertibility and fermentability

The glucan-to-glucose convertibility was conducted for the fibers (solid fraction of the pretreated biomass), which was done using 10% dry content loading. Fermentation samples were prepared by adding wet sample that is equivalent to 10 g dry biomass, deionized (DI) water was added to form a slurry containing 100 ml DI water.

Enzymatic hydrolysis was carried out at 50 ˚C for 24 hours, by adding Celluclast 1.5 and Novozyme 188 or Cellubrix, with enzymatic hydrolysis of 15 FPU/g DM and having Novozyme 188 to Celluclast ratio of approximately 1/9th. This amount of

enzyme is defined as one filter paper unit (FPU) [243]. Intensive shaking was applied (110 rpm). After enzymatic hydrolysis, the samples were cooled to room temperature by the aid of an ice bath. pH was measured and tuned (if needed) to a pH value of 4.8. 0.2 ml of sterile filtered urea was added to each fermentation flask, after which a second portion of enzymes was added (exactly the same amounts added before). Baker's yeast was then added to initiate fermentation at a concentration of 2.0 g/L. Fermentation was carried out in an incubator 32 ˚C while shaking at 70 rpm for 7 days.

3.2. Evaluation of Second Generation Biofuels Production from Wet-fractionated *Salicornia sinus-persica*

3.2.1. Raw material

A native strain of Salicornia (*Salicornia sinus-persica*) was collected from Umm Al Quwain shores in the United Arab Emirates; the site is shown in Figure 3-2.

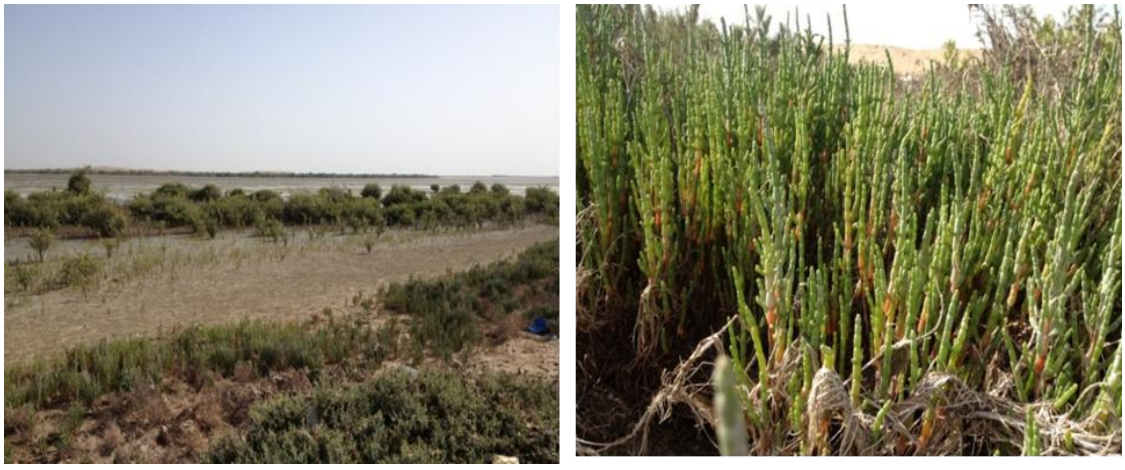


Figure 3-2. Native strain of *Salicornia sinus-persica* collecting from Umm Al Quwain shores.

The collected samples were divided into two batches; washed and unwashed. Washing was done using tap water, where the plant was soaked and rubbed inside a water pot to remove as much as possible of salt deposits accumulated on the plants

surface. The two batches underwent juicing, where two fractions were obtained; liquid fraction (juice) and solid fraction (pulp) as shown in the Figure 3-3. Both fractions were preserved at -18 ˚C until being further processed. Another portion of the pulp was dried at temperature of 105 ˚C before executing carbohydrate composition analysis.

Figure 3-3. The two obtained fractions from salicornia juicing (juice and fibers).

3.2.2. Processing the fresh biomass

Total solid (dry matter %) and ash content were measured for the fresh biomass as a whole and for the fresh juice and pulp generated by wet-fractionation of *Salicornia sinus-persica*, following the procedure and equations describes in section 3.1.2-b as per the NREL reports [239, 240].

Also protein content was measured for the fractionated biomass (pulp and juice) by measuring total nitrogen content applying Kjeldahl method. The dried and homogenized material was digested with sulfuric acid at 390 ± 5 ˚C for couple of hours. By adding sodium hydroxide to the digestion solution, all nitrogen-containing

components produce ammonium, which is evaporated by distillation as ammonia. This is condensed in a conical flask with boric acid solution [244].

3.2.3. Processing of the fresh pulp

The wet pulp was split into two portions, one was dried at 105 ˚C overnight to be analyzed for its sugar composition and the other was kept in the freezer (at -18 ˚C), wet for further processing (discussed in the following sections).

a. Sugar analysis of the dry pulp

Carbohydrate composition was analysed for the fresh pulp (without pre-treatment) after being dried, for washed and unwashed biomass. The analysis followed standard analytical protocols developed by NREL and was achieved by determining the lignocellulosic components; carbohydrates and lignin. Strong acid hydrolysis results in breaking the cellulosic biomass into monomeric fermentable sugars to be analysed, where the same procedure explained in section 3.1.2-c was followed. Acid insoluble lignin (Klason lignin) was determined as the residual, undissolved matter obtained after strong acid hydrolysis of the material.

b. Pulp fermentation after mild pre-treatment

Before conducting fermentation on the pulp fraction, pulp was mildly pretreated (at 120 ˚C for 30 minutes once and for 60 minutes another time). The mild pretreatment aimed at opening the lignocellulosic structure for better fermentation yields, yet without applying extensive energy. Each pre-treatment was conducted for both, washed and unwashed biomass, where each batch was composed of 500 ml total volume, containing 6% dry matter (DM). Applying different pretreatment duration was studied for its impact on the process's fermentability.

Dry matter and ash content of each batch were measured using the procedure described in section 3.1.2-b. After which fermentation was conducted by preparing 8 batches, for washed and unwashed biomass, pre-treated for 30 minutes or 60 minutes by adding either DI water or liquid produced from pulp pre-treatment as a fermentation medium, the description of the 8 batches is illustrated in Table 2. The experiment was conducted in duplicates; by preparing 10% DM in 25 ml total volume for each of the described batches. Fermentation was conducted following the procedure described in section 3.1.6.

Table 2. The different batches prepared for pulp fermentation, where different pre-treatment conditions and fermentation media were used

Batch no.	Feedstock characteristic	Pre-treatment temperature (˚C)	Pre-treatment duration (min)	Fermentation medium
1	Washed	120	30	Water
2	Washed	120	30	Pre-treatment liquid
3	Washed	120	60	Water
4	Washed	120	60	Pre-treatment liquid
5	Unwashed	120	30	Water
6	Unwashed	120	30	Pre-treatment liquid
7	Unwashed	120	60	Water
8	Unwashed	120	60	Pre-treatment liquid

c. Pulp hydrothermal pre-treatment

The pulp fraction produced from biomass juicing underwent hydrothermal pre-treatment, where 10% dry matter loading was applied. Three different pre-treatments were studied; where temperature was the parameter to be evaluated. The pre-treatment

temperature was assessed by studying the lignocellulosic structure besides inhibitors formation (which is evaluated by sugar composition and consecutive fermentability). Three pre-treatment temperatures were applied: 120 ˚C, 150 ˚C and 170 ˚C, while setting the pre-treatment residence time at 10 minutes. The reactor used for hydrothermal processing is shown in Figure 3-1, with the specifications described in section 3.1.5. After cooling the pre-treated biomass (slurry form), the fiber fraction was separated from the pre-treatment liquid by filtration through a cloth. Both fractions underwent sugar- and inhibitor-composition analysis as described below. The pre-treated biomass (both fractions) was preserved in a freezer at -18 ˚C until further processing.

d. Chemical characterization of the solid fraction of the pre-treated pulp

A portion of the solid fraction of the pre-treated pulp was dried overnight at 105 ˚C, after which dry matter [239], ash content [240] (described in section 3.1.2-b) and strong acid hydrolysis [237] (described in 3.1.2-c) were conducted. The analysis of the sugars and organic acid contents was done by HPLC (Agilent) as explained earlier.

e. Glucan-to-glucose convertibility and fermentability

The fermentability study was conducted on the solid fraction produced by pulp pre-treating (i.e., fibers) by preparing 5% of dry matter loading in a total volume of 100 ml. This experiment was done once by adding DI water and another by adding the pre-treatment liquid as fermentation medium, both were done in duplicates. The aim was to assess any inhibitory effects which could be encountered by adding the pre-treatment liquid as a fermentation medium. The inhibitory components are foreseen by-products of the pre-treatment process. The inhibitory assessment is achieved by weighing the ethanol yields for both fermentation media.

Enzymatic hydrolysis was done at 50 ˚C for 24 hours, by adding Celluclast 1.5 and Novozyme 188 or Cellubrix, with enzymatic hydrolysis of 15 FPU/g DM and having Novozyme 188 to Celluclast ratio of $1/9^{th}$. Intensive shaking was applied (150 rpm). After enzymatic hydrolysis, the samples were cooled to room temperature using a cold water bath. pH was measured and adjusted (if needed) to a pH value of 4.8. A second portion of enzymes was added (Celluclast 1.5 and Novozyme 188 or Cellubrix, with enzymatic hydrolysis of 15 FPU/g DM and having Novozyme 188 to Celluclast ratio of $1/9^{th}$). Baker's yeast was then added to initiate fermentation at a concentration of 2 g/L. Bottles were purged with N_2 before sealing them with glycerol-filled fermentation caps. Fermentation was carried out in an incubator at 32 ˚C while shaking at 70 rpm for 7 days. Ethanol, residual sugars and organic acids content analysis was achieved by HPLC using Hi Plex-H column (Agilent) and RI detector.

3.2.4. Processing of the fresh juice

a. Sugar content of fresh juice

The carbohydrate composition of fresh juice was analyzed directly. Fresh juice was filtered through 0.2 µm nylon filter before being introduced to the HPLC. Hi Plex-H column (Agilent) and RI detector were used to determine sugar, ethanol and organic acid concentrations. Same analysis conditions mentioned in section 3.1.2-c were applied.

b. Sugar content of fresh, pH-adjusted juice

pH value was adjusted for the juice to value of 3.0 by applying different acids; lactic acid, hydrochloric acid and sulfuric acid. The idea behind this experiment was to evaluate the effect of changing the pH value as well as the different behaviors encountered by adding different acids. A duplicate was prepared for each acid and

samples afterward were run on the HPLC using Hi Plex-H column (Agilent) and RI detector.

c. Fresh juice pre-processing

Before conducting juice fermentation, juice was pre-processed to study the best utilization of the juice. Four juice batches were prepared. The first batch was pre-hydrolysed by enzymes (Simultaneous saccharification and fermentation (SSF) by adding Celluclast 1.5 and Novozyme 188 or Cellubrix with enzymatic concentration of 15 FPU/g DM and having Novozyme 188 to Celluclast ratio of approximately $1/9^{th}$ for 2 hours pre-hydrolysis at 50 ˚C, under intensive shaking (140 rpm)). The second batch was pre-hydrolysed by acid (following the procedure of weak acid hydrolysis described in section 3.1.5-b), where the third batch was pre-processed by autoclaving (juice was cooked at 121 ˚C for 20 minutes). The fourth batch was the control batch where it represented fresh, untreated juice. The trials were conducted in duplicates.

d. Juice fermentation (fermentability study)

The pre-processed juices as well as the control samples (untreated) were subjected to fermentation after bringing their temperature to room temperature and adjusting their pH to 4.8. *S. cerevisiae* was added to each bottle (with concentration of 2 g/L), where bottles were sealed by glycerol-filled caps. Fermentation took place over 7 days at 32 ˚C, with continuous shaking at 70 rpm. The experiment was carried out in duplicates.

e. Large-scale fresh juice fermentation

The extent of glucan-to-glucose convertibility was studied in large scale for fresh juice (without juice pre-processing). pH was adjusted to 4.8 at a temperature of 32 ˚C in a fermenter (shown in Figure 3-4). *S. cerevisiae* was added in a concentration of 2 g/L, where continuous shaking at 200 rpm was applied. Having the fermentation done

in a bioreactor allowed for controlling the process parameters (pH and temperature) throughout the fermentation process.

Figure 3-4. Bioreactor (fermenter) used for large scale (1L) juice fermentation.

3.3. Acetic acid fermentation

3.3.1. The effect of adding citric acid buffer

In order to study potential of producing other valuable products from salicornia juice, since the sugar composition is fairly low (1.0-1.5%), juice samples were diluted by citric acid with a dilution factor of 2. Citric acid buffer was prepared in a concentration of 21 g/L (i. e., 0.109 M) and pH of the buffer was adjusted to 4.8. Fermentation was then conducted on diluted juice samples, with and without applying a pre-hydrolysis step. Enzymatic pre-hydrolysis was done for 2 hours at 50 ˚C with continuous shaking at 130 rpm. Fermentation was then conducted, using the yeast *S. serevisiae* (concentration of 2 g/L) following the procedure explained in section 3.2.4-d. Juice composition was analysed before and after conducting the fermentation step to study the different products generated.

CHAPTER 4

4. Results and Discussions

4.1. Characterization of the Chemical Composition of the Halophyte *Salicornia bigelovii*

4.1.1. Chemical characterization of the raw material under different cultivations

As explained in the materials and methods, chapter 3, section 3.1.1, six main samples of *Salicornia bigelovii* were cultivated as a part of ISEAS at three fertilizer levels (F1: 1 g N/m^2, F2: 1.5 g N/m^2 and F3: 2 g N/m^2) and two salinity levels (S1: 10 ppt and S5: 50 ppt salt). The samples were analyzed in terms of chemical composition and bioethanol potential. The six samples were fractionated into stem and seed spikes for chemical characterization as illustrated in (Table 3). The analysis showed that *S. bigelovii* contained total solids ranging between 27.85 and 66.37 g/100 g TS of total extractives (including extractable ash), 16.31–55.67 g/100 g TS of carbohydrates, 5.42–16.60 g/100 g TS of lignin and 2.18–9.68 g/100 g TS of ash incorporated in the plant matrix (structural ash). The composition varied according to the plant fraction and cultivation conditions. The results show clearly that *S. bigelovii* seed spikes have a considerably different composition than the stems ($P < 0.01$). Fertilizer level was found to have a significant influence ($P < 0.05$) on the carbohydrates, extractives and ash content, while salinity was a significant factor for ash and extractives content in

both fractions of the plants ($P < 0.05$). The results suggest that *S. bigelovii* stems cultivated at low salinity (10 ppt) and medium fertilizer grade (1.5 g N/m^2) contain the highest carbohydrate content and accordingly could be of high value for biofuels production, while seed spikes can be more suitable for extracting value-added active components (having a high extractives content) [245].

Table 3. Chemical composition of the different samples of S. bigelovii cultivated at three fertilizer levels (F1: 1 gN/m^2, F2: 1.5 gN/m^2 and F3: 2 gN/m^2) and two salinity levels (S1: 10 ppt and S5:50 ppt) [245]

S. bigelovii sample	Glucan (g/100 g DM)	Xylan (g/100 g DM)	Arabinan (g/100 g DM)	Total sugars (g/100 g DM)	Lignin (g/100 DM)	Structural ash (g/100 g DM)	Extractives (g/100 g DM)
Stem, S1, F1	16.55	11.18	3.34	31.07	16.60	6.27	37.22
Stem, S1, F2	27.12	22.63	5.93	55.67	14.08	2.34	27.85
Stem, S1, F3	22.31	17.80	4.48	44.59	15.58	2.18	29.10
Stem, S2, F1	20.11	18.04	2.31	40.47	12.19	2.35	37.32
Stems, S2, F2	20.54	18.91	4.62	44.07	13.56	3.42	36.65
Stems, S2. F3	18.08	15.38	3.82	37.27	10.49	5.04	42.95
Spike, S1, F1	9.02	7.39	6.61	23.02	5.42	7.99	57.54
Spike, S1, F2	7.79	7.34	5.26	20.39	7.29	5.64	59.25
Spike, S1, F3	8.92	7.72	6.39	23.03	9.37	7.04	54.13
Spike, S2, F1	7.01	5.39	5.69	18.09	7.97	6.86	66.37
Spike, S2, F2	7.72	7.34	4.85	19.91	7.76	8.31	63.10
Spike, S2, F3	6.58	5.54	4.20	16.31	7.44	9.68	65.03

4.1.2. Chemical characterization of a raw material mixture (combined from differently cultivated samples)

Carbohydrate content analysis of the *Salicornia bigelovii* raw material (mixed sample of differently-cultivated samples) was conducted prior to conducting pre-treatment trial in our lab. The raw material of dry *salicornia bigelovii* was ground and washed with tap water (as explained in section 3.1.4) and then dried before being analysed for sugar composition. With dry matter (DM) of 97.29%, *S. bigelovii* showed the following composition: 15.91% of the DM (structural ash), 10.67% of the DM (glucan), 8.14% of the DM (xylan) and 6.17% of the DM (arabinan). Sugar composition of the raw material is similar to the results obtained in the previous work (section 1.4); however higher ash content was obtained due to not applying water and ethanol extraction prior to the composition analysis.

4.1.3. Chemical characterization of pre-treated biomass

A pre-treatment trial was conducted at Masdar Institute by our group to evaluate the efficiency of the Parr hydrothermal pre-treatment reactor and compare its productivity to the loop-reactor, which was used in the previous work (section 1.4) [39]. A pre-treatment (under the conditions: 190 ˚C for 10 minutes residence time) was conducted, showing more than 90% of glucan recovery (compared to a recovery of about 80% from the previous study).

Studying the hydrothermal pre-treatment on both washed and unwashed biomasses was intended to understand the impact of salt removal on the pre-treatment outputs. Washing the biomass tends to concentrate the lignocellulosic content by extracting the non-structural ash (salt) and the easily extractable organic compounds. Hence, higher sugar concentrations were observed for washed biomass (in both liquid and solid

fractions). Sugar content (including only glucose, fructose and xylose) of the washed pre-treated biomass made up to 30% of the dry matter (DM), where the unwashed biomass showed only about 25% total sugar in the DM of the raw material. The ash content was about the double for the unwashed biomass compared to the washed (13.91 versus 8.24) as shown in Figure 4-1.

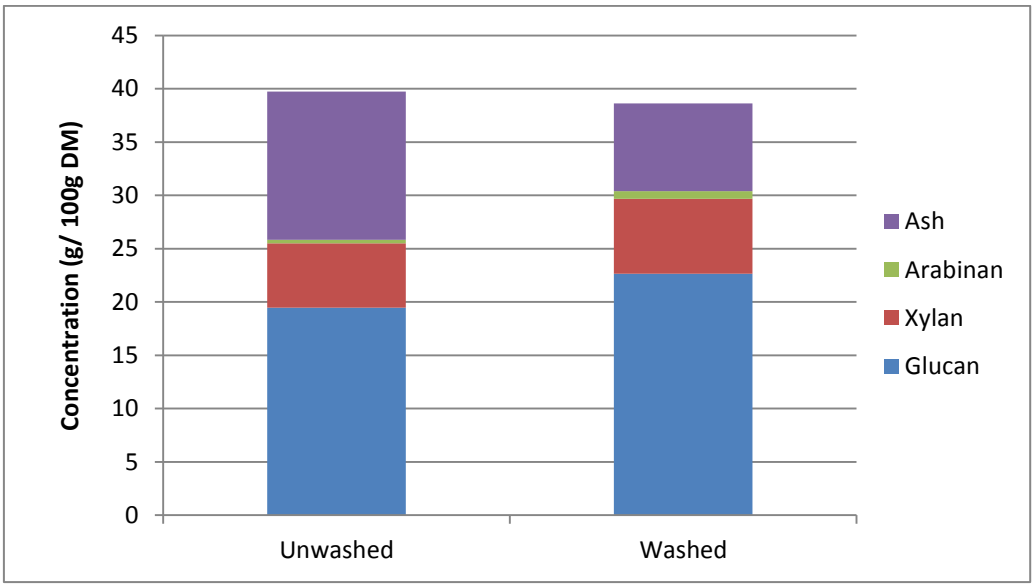


Figure 4-1. Total sugar composition of pre-treated *S. bigelovii* obtained from pulp fraction of the pre-treated material

Liquid generated from the pre-treatment process of the washed biomass also showed higher sugar concentrations than the pre-treatment liquid of the unwashed biomass, while indicating substantial concentrations of arabinose. The unwashed biomass, on the other hand, did not release arabinose sugar as indicated in Figure 4-2. Nevertheless, formic acid was only detected for the unwashed biomass.

By calculating the sugar recovery after biomass pre-treatment, negative effects were observed when the biomass was not washed beforehand. The sugar recovery in unwashed biomass was 2 folds and 4 folds less for C6 and C5 sugar, respectively, compared to what is produced by washed biomass, as illustrated in Figure 4-3. This

indicates that the existence of higher concentration of salts in the bioreactor might be increasing the pre-treatment severity contributing in higher sugar degradation.

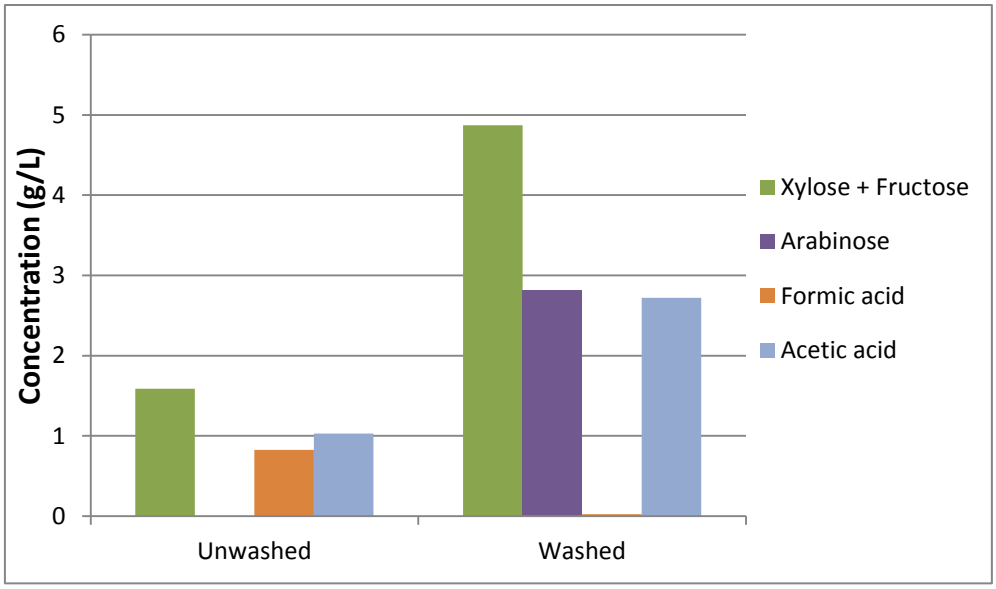


Figure 4-2. The sugar composition in the pre-treatment liquid for washed and unwashed *Salicornia bigelovii*.

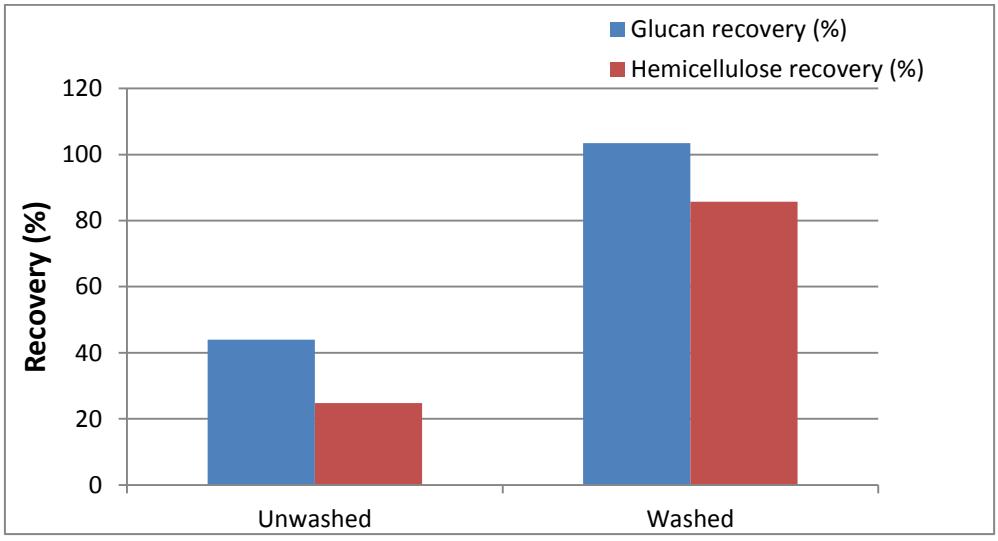


Figure 4-3. Sugar recovery of pre-treated *S. bigelovii* (with and without washing) obtained from solid and liquid fractions.

4.1.4. Fermentability of pre-treated Salicornia bigelovii

The fermentability of the pre-treated biomass was studied for 10% DM loading in water medium. Both washed and unwashed biomass showed ethanol yields of about 80% of the theoretical yield of glucose (unwashed: 82.47±2.00% and washed 78.22±10.96%), indicating similar values of what was previously obtained by using the external lab-bioreactor, where ethanol yields ranged between 87% and 95% of the theoretical yield.

4.2. Evaluation of Second Generation Biofuels from Fresh *Salicornia Sinus-Persica* (Native Species)

4.2.1. Fresh biomass

The fresh unwashed biomass of *Salicornia sinus-persica* was found to contain 22.42% dry matter (DM), of which 47.07% was ash.

After juicing the feedstock, mass distribution of each fraction (juice and pulp) was measured. The liquid fraction was found to represent 67.78 ± 6.57% of the biomass (unwashed batch) and 74.09 ± 3.68% of total biomass (washed batch); indicating that ~10% of water remains in the pulp fraction. The wet fiber fraction of fresh, unwashed salicornia was found to contain 38.88 ± 2.10% DM, of which 13.19 ± 1.15% is ash and 2.08% total nitrogen (representing 13.00% protein applying the factor 6.25 [246]). Nitrogen composition is comparable to what was reported for the halophyte *Salicornia gaudichaudiana* with total nitrogen of 2.2 ± 0.26% [247]. Dry matter content of the juice was found to be 13.53 ± 0.55% (unwashed) and 11.58 ± 0.01% (washed) of which 61.12±3.60% (unwashed) and 58.00±0.19% (washed) was ash. Fresh juice chemical composition (in % of DM of juice) is shown in Figure 4-4,

indicating that less than 50% of the DM of the juice is composed of protein, sugars and organic acids and the rest is ash.

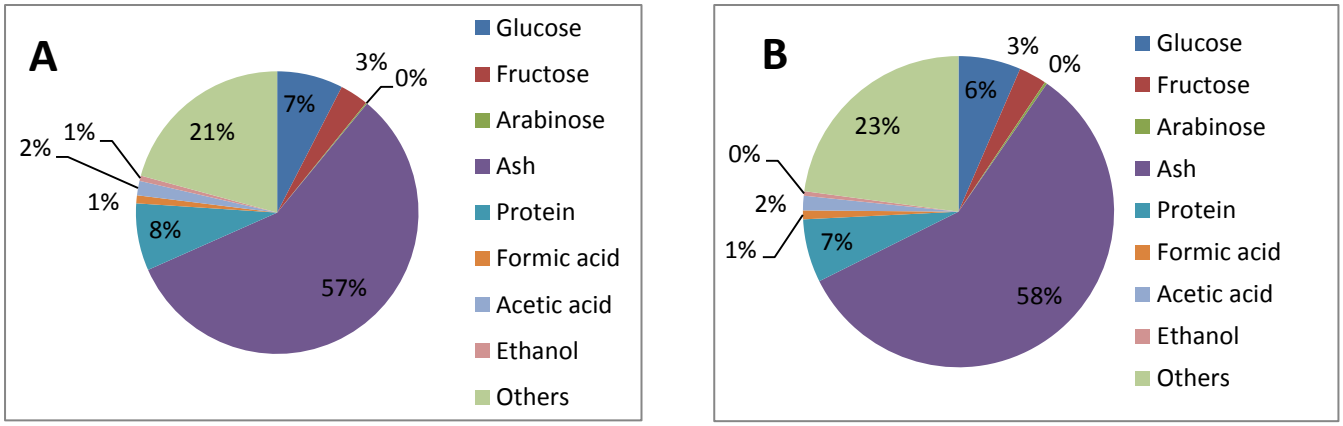


Figure 4-4. Salicornia fresh juice chemical composition (% of dry mass DM), left (A): washed biomass, right (B): unwashed biomass, indicating that the ash content is more than 50% of the DM of the juice.

The pulp of fresh *salicornia sinus-persica* as shown in Figure 4-5 contains 16.27 ± 1.47 g/100 g DM glucose, 11.57 ± 1.00 g/100 g DM fructose + xylose, and 11.04 ± 1.19 g/100 g DM arabinose for the washed biomass and 15.63 ± 0.50 g/100 g DM glucose, 10.68 ± 0.57 g/100 g DM fructose + xylose, and 11.08 ± 0.35 g/100 g DM arabinose for the unwashed biomass. This is comparable to the lignocellulose content of the mature (dry) plant [40].

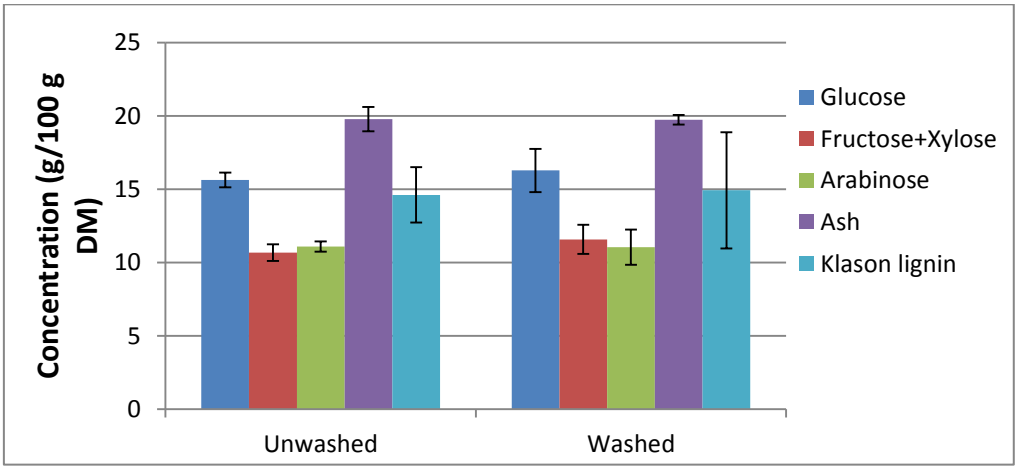


Figure 4-5. Sugar composition of the pulp fraction of freash *salicornia sinus-persica* (with and without washing)

4.2.2. Fiber fraction (pulp)

a. <u>Fresh pulp fermentation after mild pre-treatment</u>

Fresh pulp was pre-treated at mild conditions (i.e. 120 ˚C for 30 and 60 minutes) to study the impact of low severity pre-treatments on pulp fermentability. Generally, mild pre-treatment could increase the pulp's sugar concentration from about 16 g/100 g DM to a range of 20 – 28 g/100 g DM (depending on the pre-treatment duration; 30 or 60 minutes). Also, the enzymatic convertibility of the pulp increased from 7.35 ± 0.19% to values ranging between 46.42 ± 7.42% and 64.11 ± 12.92% (depending on the applied pre-treatment duration) as indicated in Figure 4-6. In order to obtain better hydrolysis yields, it is recommended to apply higher pre-treatment temperatures.

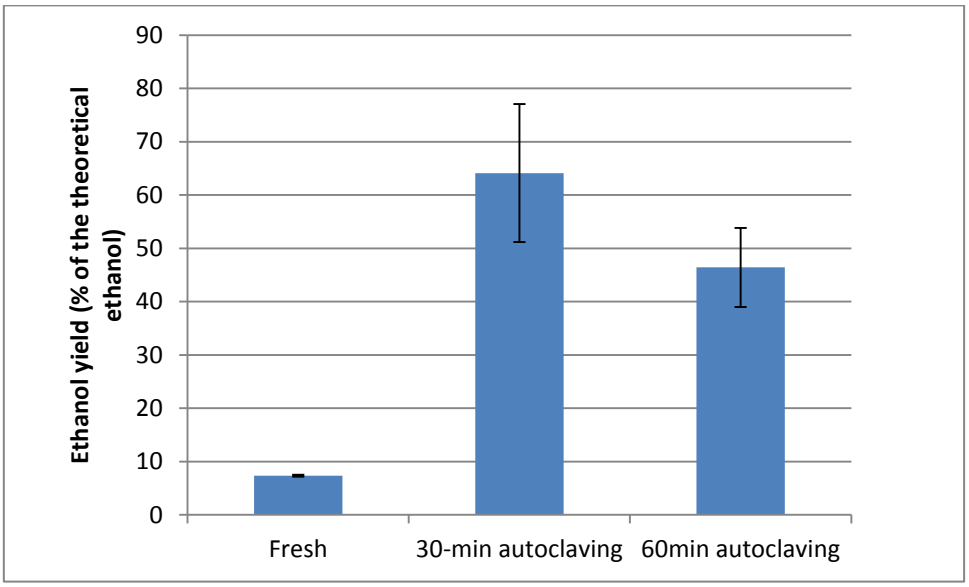


Figure 4-6. Ethanol yield (%) for fresh fibers compared to mildly pre-treated fibers at 120 ˚C for 30 minutes and 60 minutes.

Washed biomass obtained lower ethanol yields compared to unwashed biomass when treated at 120 ˚C for 30-minutes (~41% versus ~56%) when fermented in the pre-treatment liquid medium. However similar ethanol yields were obtained for the same pulp, when water was added as a fermentation medium, instead. Conflicting results

were obtained by fermenting the pulp pre-treated at 120 ˚C for 60 minutes; where washed biomass obtained higher ethanol yields in both fermentation media (water and pre-treatment liquid), as indicated in Figure 4-7. For unwashed biomass, increasing the pre-treatment time from 30 to 60 minutes showed about 30% decrease in ethanol yields in both water and pre-treatment media. Moreover, studying the pre-treatment liquid's inhibitory effects on the fermentation showed that the pre-treatment liquid exhibited inhibitory effects for all treatments except for the pulp pre-treated at 120 ˚C for 60 minutes (of washed biomass), as indicated in Figure 4-7. Mild pre-treatment managed to obtain ethanol yields that are about 6 to 10 times of what fresh untreated fibers could achieve, as shown in Figure 4-6.

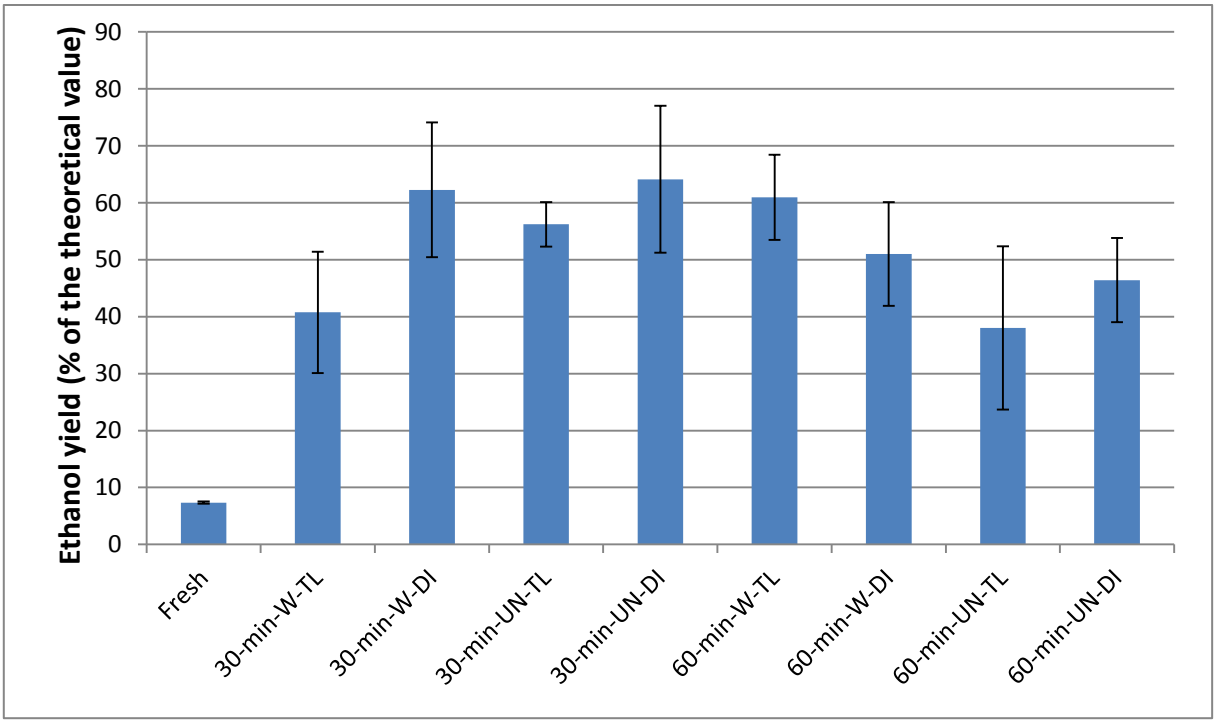


Figure 4-7. Ethanol yields (% to the theoretical ethanol) produced by fermenting mildly pre-treated 10% DM fibers at 120 ˚C for 30 minutes and 60 minutes, where dilution is done by both DI water and pre-treatment liquid for both washed and unwashed biomass.

- W: washed, UN: unwashed, TL: pre-treatment liquid, DI: deionized water.
- Error bars show standard deviation obtained from a duplicate of samples

b. Medium severity hydrothermal pre-treatment of green pulps

From the results obtained from the previous section, mild pre-treatment could achieve ethanol yields up to 66%. Nevertheless, the pre-treatment durations (30 and 60 minutes) are considered relatively long and need to be optimized. Generally, energy demands and related costs need to be minimized [131]. Furthermore, in a study done by Negro et al., 2003 [248], steam explosion at mild pre-treatment conditions indicated that the pre-treatment temperature has stronger hemicellulose hydrolysation effects than the pre-treatment residence times. Accordingly, low-severity hydrothermal pre-treatment of the pulp was studied aiming to increase convertibility and decrease biomass recalcitrance by applying different temperatures (150 ˚C and 170 ˚C in addition to 120 ˚C). The idea was to study the effect of increasing the pre-treatment temperature on the sugar composition and recovery, ash content and inhibitors' formation, in addition to the increase on the ethanol yields compared to those achieved by mild pre-treatment, while applying shorter residence times (10 minutes). As expected, glucose concentration for the pulp improved by increasing the pre-treatment temperature, due to the improved extraction of hemicalluloses and ash into the liquid fraction. The highest glucan content (i.e., 27.50 ± 1.77 g glucose/100g DM) was obtained at a pre-treatment temperature of 170 ˚C (Figure 4-8). The ash content of the untreated pulp is almost the double of that for the treated pulp; this is due to the washing taking place during pulp treatment. The results also show that the lignin content increased with increasing pre-treatment temperature. Enzymatic convertibility of cellulose (examined by SSF) was significantly improved after treating the pulp hydrothermally. The ethanol yield of the biomass pre-treated at 120 ˚C for 10 minutes was very low; it was comparable to what was obtained by the fresh, untreated biomass (about 6.83% ethanol yield). The yield increased 8 folds when the

pre-treatment temperature increased from 120 ˚C to 150 ˚C (comparable yields of the longer residence-time pre-treatments) and almost 12 folds when increased to 170 ˚C, to reach a yield of 76.91 ± 3.03%, as presented in Figure 4-9.

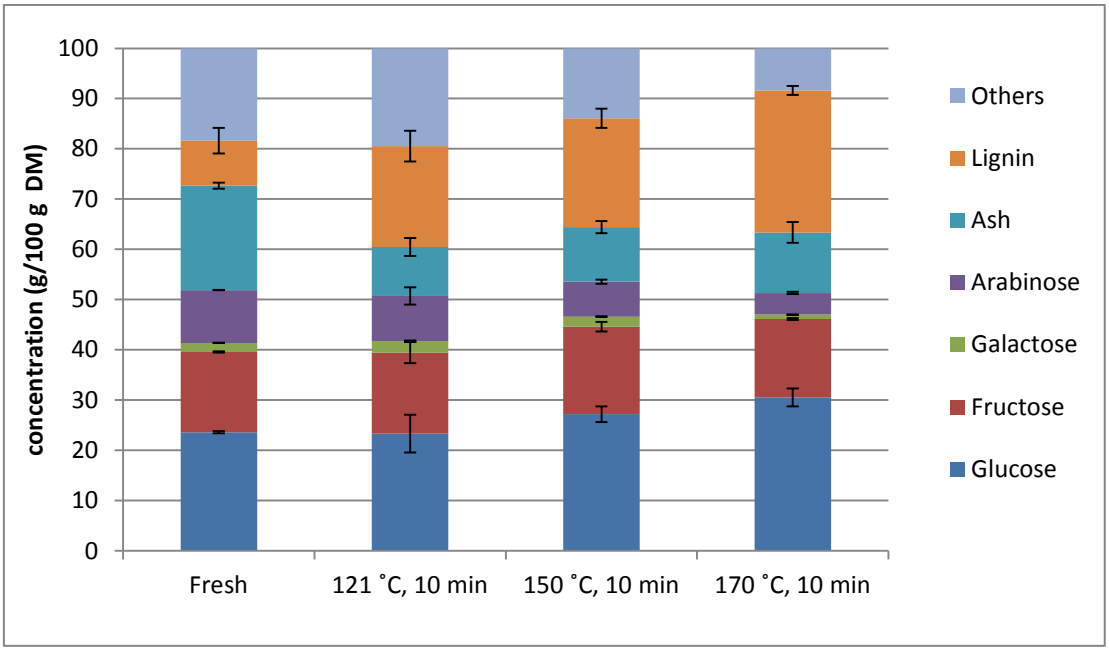


Figure 4-8. Fiber composition after low-severity pre-treatment at different temperatures.

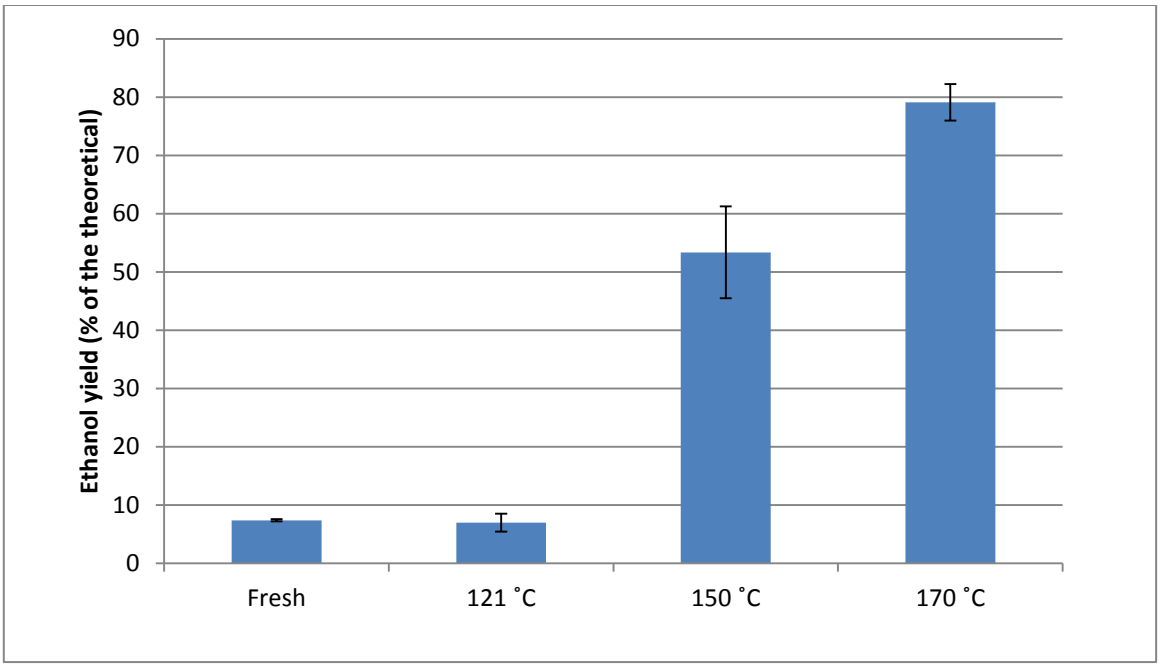


Figure 4-9. Fibers fermentability and ethanol yields after being pre-treated hydrothermally at different temperatures.

Sugar recovery

Sugar recovery was calculated for the pulp, which was pre-treated at 120 ˚C, 150 ˚C and 170 ˚C to understand the effect of increasing the temperature and residence time on sugar recovery/degradation, as described in Figure 4-10. Cellulose recovery was above 71% in all pretreatments, the highest obtained value was for biomass pre-treated at 120 ˚C for 60 minutes (94.6 ± 4.57%). The second highest glucan recovery was obtained by the pre-treatment done at 120 ˚C for 30 minutes (88.08 ± 11.63%), which decreased by applying shorter residence times or higher temperatures. Glucan recovery decreased to 77.49 ± 2.35% and to 74.47 ± 1.69% when the temperature increased to 150 ˚C and 170 ˚C respectively. The lowest recovery was obtained when 10 minutes of residence time at 120 ˚C was applied, to achieve a recovery of 71.56 ± 14.84%. Recovery of hemicellulose was relatively high for all pre-treatments, showing the lowest value for pre-treatment done at 120 ˚C for 10 minutes. In contrast with what is known from the literature, xylose recovery increased at higher pre-treatment severities (by increasing the treatment duration from 10 minutes to 60 minutes, or the pre-treatment temperature from 120 ˚C up to 170 ˚C), as shown in Figure 4-10.

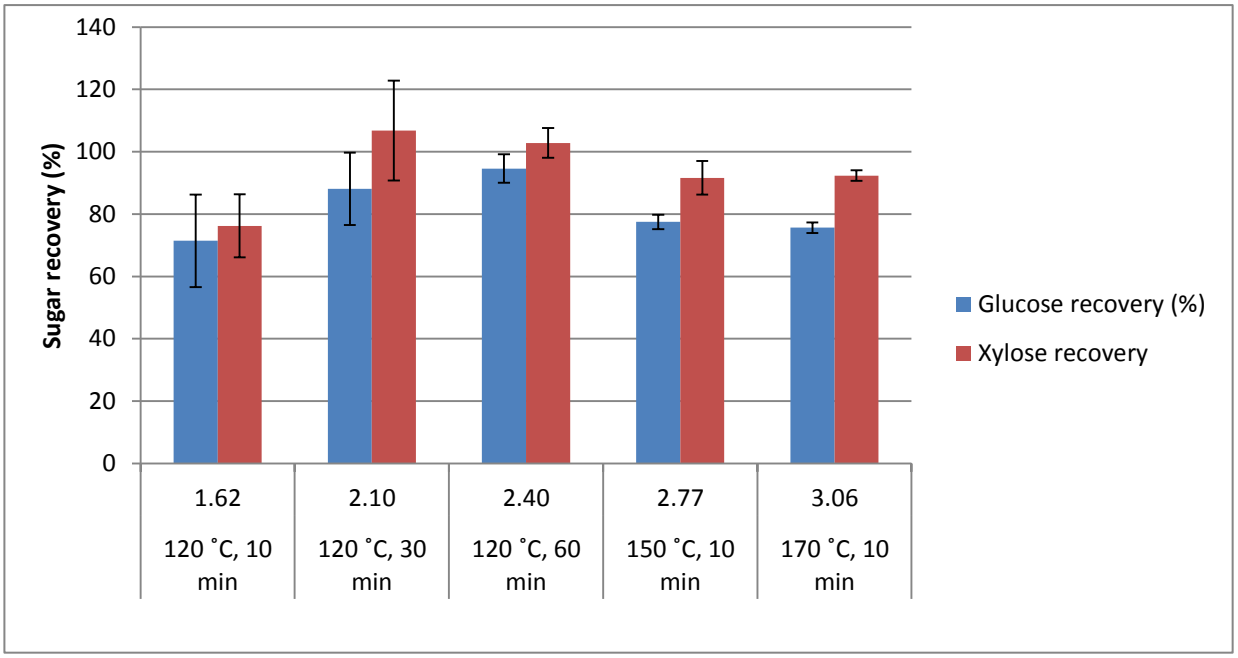


Figure 4-10. Glucan, xylan and galactan recovery (%) for pulp pre-treated at three different temperatures.

Pre-treatment severity

The pre-treatment severity was calculated for all pre-treatments using (Equation 18) [249], which showed that the temperature has a greater effect than the residence time (exponential versus linear). Figure 4-11 shows all experiments listed versus the severity factor. The residence time indicated a great influence on releasing sugars, as was observed in pre-treatments done at 120 ˚C (applying different residence times) when compared to the pre-treatment done at 150 ˚C. Ethanol yields obtained by pulp pre-treated at 120 ˚C for 30 and 60 minutes were similar to what was obtained by pulp treated at 150 ˚C for 10 minutes, despite the increase in the severity factor as shown in Figure 4-11. Comparing all treatments (mild and medium-severity) showed that the highest ethanol yield was obtained for the pre-treatment with the highest severity factor (at 170 ˚C for 10 minutes), which is achieved by applying higher temperatures.

$$\log(Ro)=\log(t\ \exp(\frac{T-100}{14.75})) \qquad \text{Equation 18}$$

Where T : pre-treatment temperature [°C]; t : pretreatment time [min]; R_o = severity factor [40].

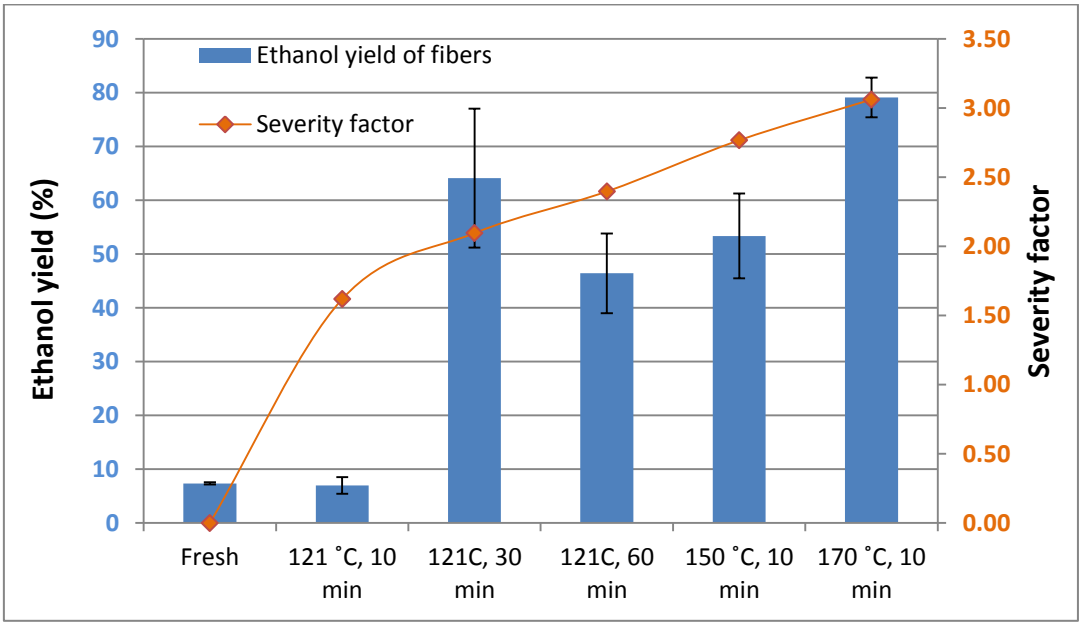


Figure 4-11. Ethanol yield and severity factor for pulp pre-treatments.

Pre-treated pulp fermentation

Adding the pre-treatment liquid instead of water as a fermentation medium (with 5% DM loading), showed a substantial increase in the ethanol yields obtained by pulp pre-treated at 120 ˚C for 10 minutes (ethanol yield increased from 6.97 ± 1.54% to 85.46 ± 0.94%), as shown in Figure 4-12. However, a decrease in the obtained ethanol yield was observed for all other pre-treatments. The slight decrease in the ethanol yield obtained indicates minor inhibitory effects produced by adding the pre-treatment liquid under the described pre-treatment conditions. This confirms that higher pre-treatment severity (weather by increasing the pre-treatment residence times or the pre-treatment temperature) results in sugar degradation and hence inhibitors formation. Higher inhibitory effects were detected by suspending the pulp treated at 170 ˚C in its pre-treatment liquid, where yields decreased from almost 80% to about 64%. Results are illustrated in Figure 4-12.

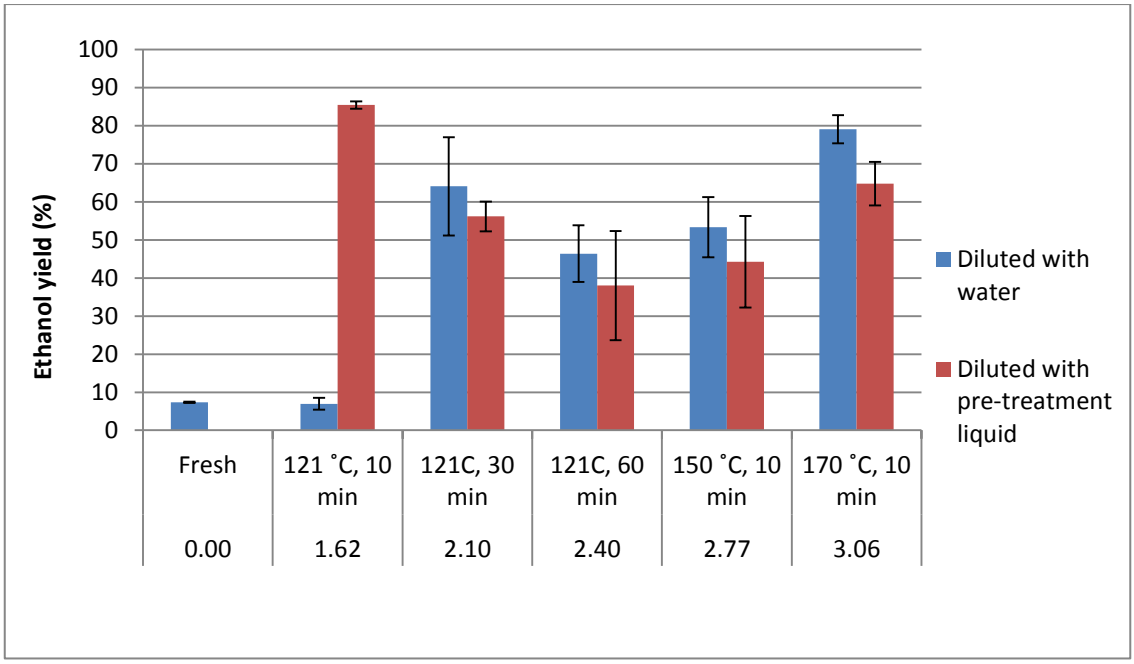


Figure 4-12. Ethanol yield obtained by pre-treated pulp suspended once in DI water and another in pre-treatment liquid.

4.2.3. Juice

a. <u>Chemical characterization of fresh juice</u>

Composition analysis of fresh untreated juice showed about 1.0 – 1.5% sugar, consisting of glucose, fructose and arabinose (i.e., 8.76 ± 0.39 g/L glucose, 3.80 ± 0.33 g/L fructose + xylose and 0.16 ± 0.03 g/L arabinose for washed and 8.78 ± 1.02 g/L glucose, 3.90 ± 0.79 g/L fructose + xylose, 0.35 ± 0.18 g/L arabinose for unwashed biomass). Inhibitors, organic acids and ethanol were detected in negligible concentrations and were not indicated in Figure 4-13. Acid hydrolysis of salicornia juice resulted in marginal change in the sugar composition; slightly lower for glucose and somewhat higher for fructose, xylose and arabinose (8.29 ± 0.08 g/L glucose, 5.83 ± 0.12 g/L fructose + xylose and 3.32 ± 0.02 g/L arabinose for unwashed and 7.40 ± 0.06 g/L glucose, 4.83 ± 0.28 g/L fructose + xylose, and 2.21 ± 0.01 g/L arabinose for washed biomass). This increased could be explained due the existence of residual pulp in the juice fraction, which released C5 sugars by acid hydrolysis.

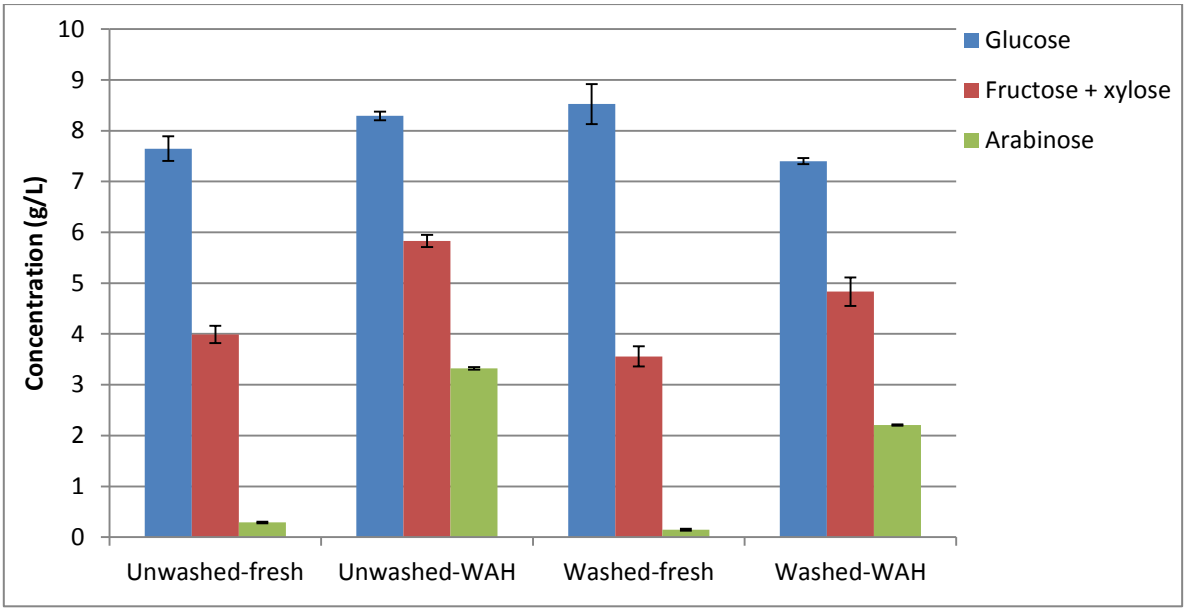


Figure 4-13. Composition analysis of fresh-untreated juice and acid hydrolyzed juice of *Salicornia sinus-persica* (of washed and unwashed biomass).

- WAH: weak acid hydrolyzed

According to the obtained results, juice sugar content is relatively low and biomass washing doesn't have any impact on the sugar composition except from decreasing the ash content of the juice (from 61.12 ± 3.60% to 58.00 ± 0.19%).

b. Sugar content of fresh, pH adjusted juice

Sugar composition, specifically glucose and xylose, was analysed for fresh juice, with and without pH adjustment. pH adjustment (pH value of 3.0) of the juice was done applying different acids (i.e., lactic, acetic, hydrochloric acid and sulphuric acid) to analyse the impact of the acid added on the monomeric sugar composition of the juice. As indicated in Figure 4-14, the highest glucose and xylose concentrations were encountered by the juice samples, which underwent pH adjustment using sulphuric acid (glucose: 5.90 ± 0.04 g/L and xylose: 2.67 ± 0.06 g/L). Similar glucose concentration (5.41 ± 0.37 g/L) was obtained by the fresh juice (without pH adjustment; pH ~7.0), where its xylose concentration was about the half of what was obtained by sulphuric acid-pH-adjusted samples (1.28 ± 0.08 g/L). A decrease of

about 40% of the glucose composition was obtained when pH adjustment was done using lactic acid and hydrochloric acid.

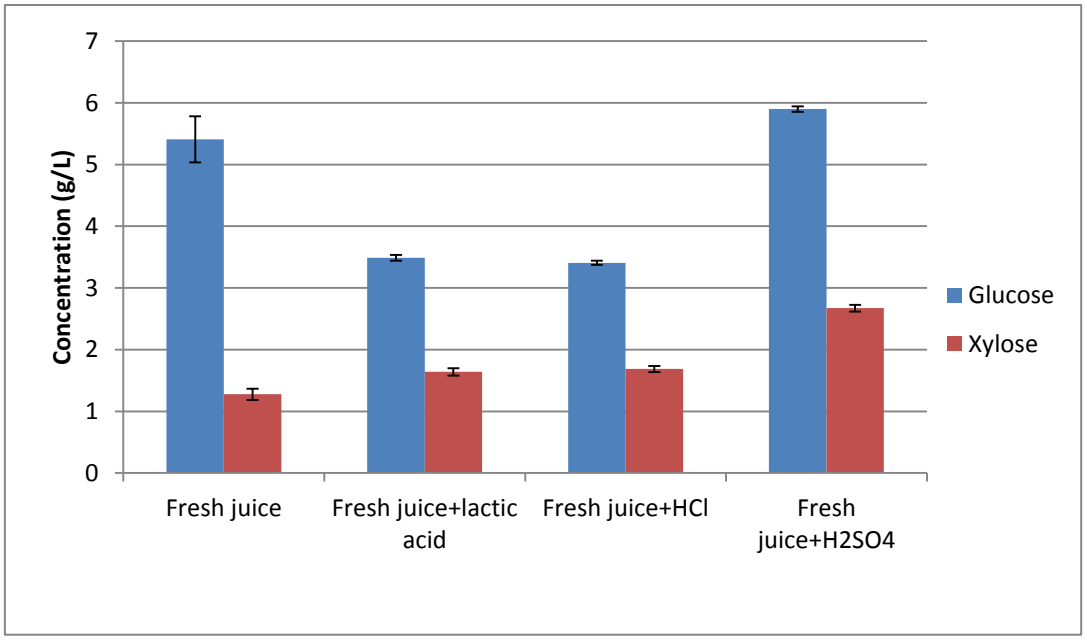


Figure 4-14. Monomeric sugar composition of fresh juice, with and without pH adjustment to 3.0, applying different acids.

c. Juice fermentation

Fermenting hydrolysed juice

Juice fermentation was studied on enzymatic hydrolyzed juice (as SSF), weak acid hydrolyzed juice, and also on fresh juice (as control). The results are shown in Figure 4-15. Ethanol yields slightly above 100% (based on glucan content) were identified for enzymatic hydrolyzed juice, while weak acid-hydrolyzed juice showed results that are slightly lower than the enzymatic hydrolyzed juice and slightly higher than the fresh juice as shown in Figure 4-15 and Figure 4-16. Nevertheless, the fresh juice showed ethanol concentrations of 3.55 ± 0.19 g/L (washed) and 3.39 ± 0.42 g/L (unwashed), indicating ethanol yields exceeding 75% of the theoretical ethanol yields (based on the available glucan content), as illustrated in Figure 4-16. The weight loss data (presented in Figure 4-17) show that fresh untreated juice underwent the greatest

weight-loss, which is equivalent to about 270% of the theoretical ethanol. Weight-loss measurements in enzyme hydrolysed juice also showed results that estimate more than the double amount of the theoretical ethanol yields (based on glucan content). However, weak-acid hydrolyzed samples showed weight-loss measurements, which are back-calculated to ~100% of ethanol yields, as indicated in Figure 4-17. Hence, the weight-loss in acid-hydrolyzed samples (which undergo autoclaving for 10 minutes) showed alliance with calculated data. This could be explained by the mild autoclaving step, which results in juice sterilization, minimizing the consumption of the sugar with microorganisms other than yeast.

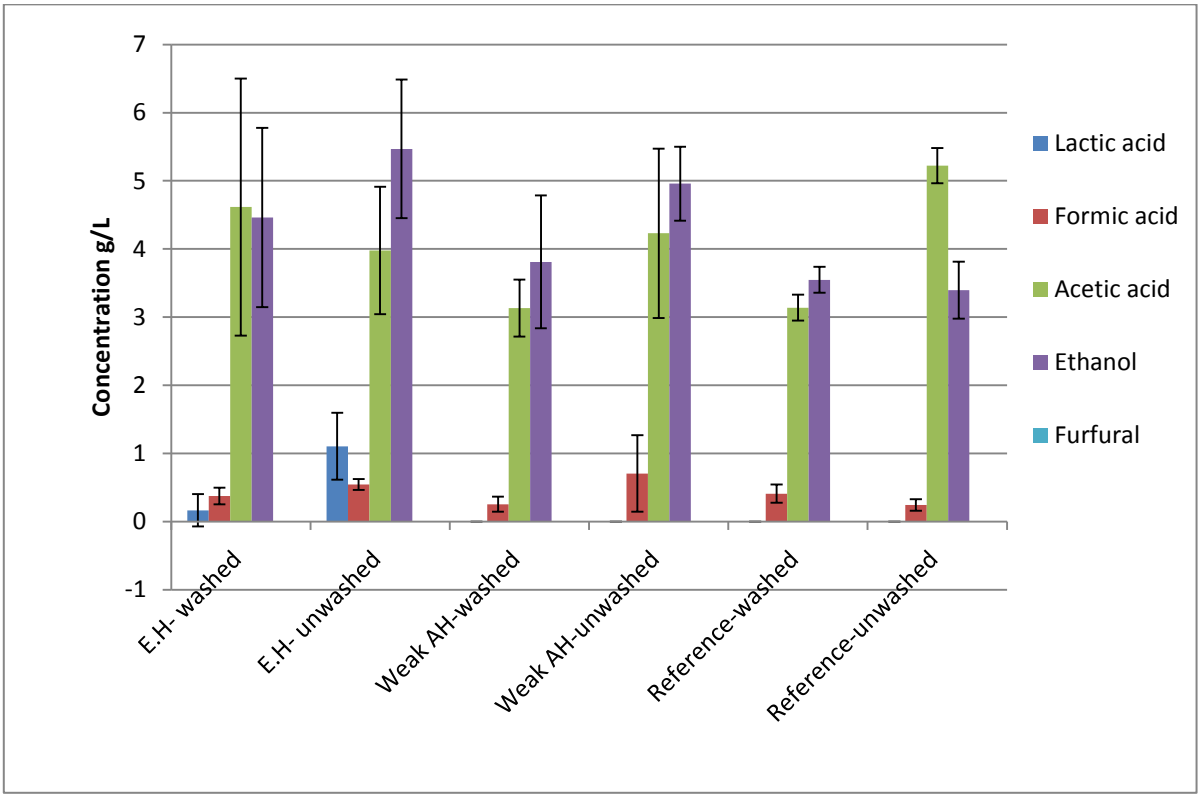


Figure 4-15. Ethanol production in yeast fermentation of fresh *Salicornia* juice studied after enzymatic hydrolysis (as SSF), weak acid hydrolysis followed by fermentation, and on fresh juice directly (reference).

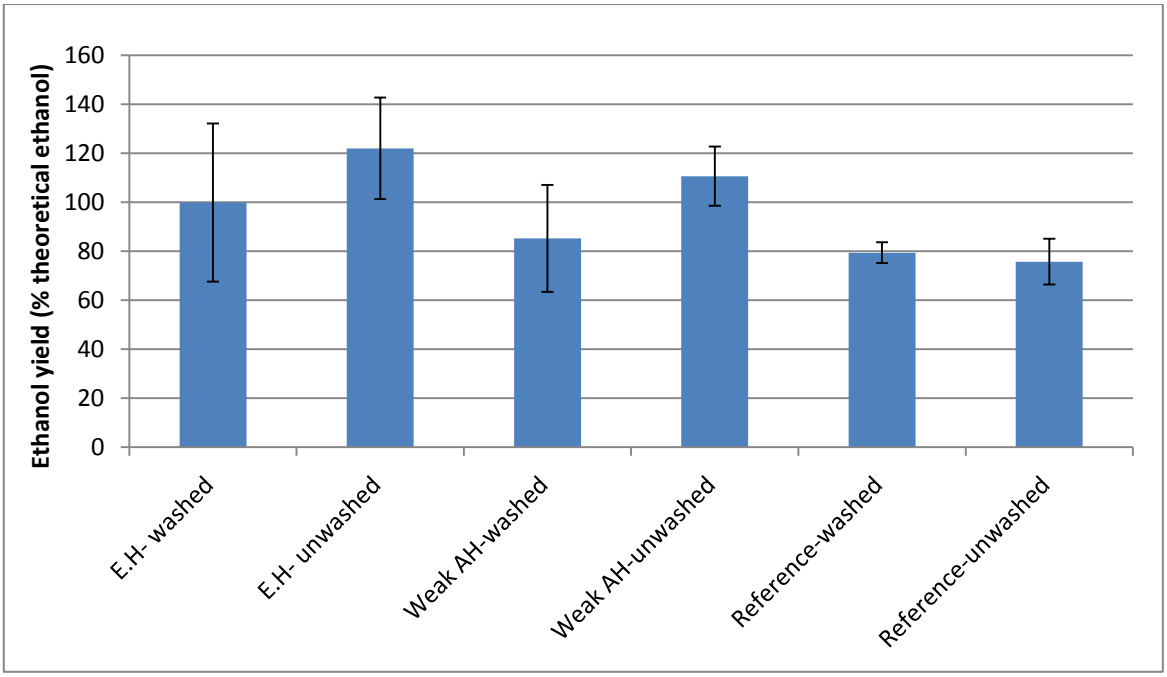


Figure 4-16. Ethanol yield (% of theoretical based on glucan calculations) for differently pre-treated salicornia juice.

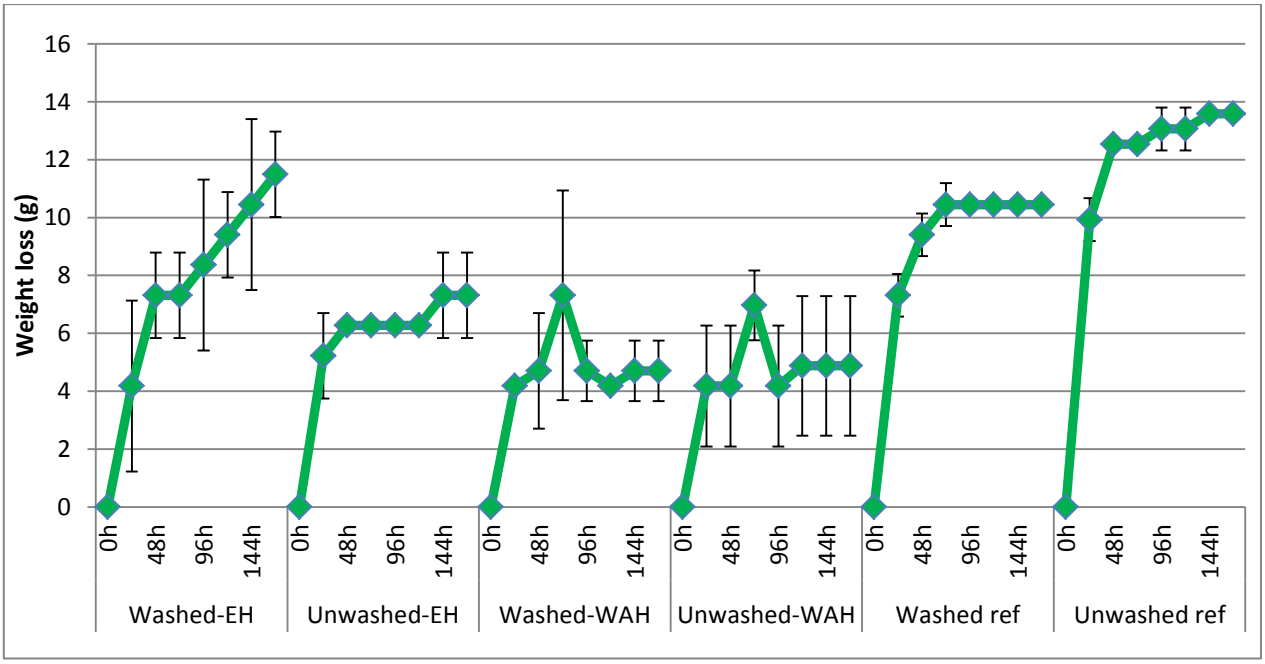


Figure 4-17. Ethanol (g/L) based on the weight-loss measurements.

Juice sugar content is negligible according to the sugar composition analysis, predicting a minimal production of ethanol. Hence, juice pre-processing by acid or

enzymatic hydrolysis or by sterilization will negatively influence a salicornia-based green biorefinery.

Fresh juice fermentation

Utilizing the fresh juice without a hydrolysis step increases process economics, especially with excluding enzymes application, which tends to be a significant part of the process cost [250]. Hence, untreated fresh juice fermentation was scaled up from shake flask to a 1.0 L bioreactor, where fermentation conditions could be controlled.

Throughout the juice fermentation process, glucose was being consumed completely after 18 hours of fermentation. Fructose and xylose were being consumed in parallel to glucose, however at slower rates, allowing complete consumption after 34 hours of fermentation, at which time the ethanol concentration reached its maximum. Arabinose, with its very minimal concentration (~0.5 g/L) was being consumed between 69 and 80 hours of fermentation. Around this point lactic acid started being generated to reach a concentration of 1.5 g/L after 170 hours of fermentation. Acetic acid was continuously produced throughout the fermentation process; the concentration doubled from 1.18 g/L (at 34 hours) to 2.31 g/L (at 80 hours) and reached the highest concentration (3.71 g/L) after 170 hours of fermentation. Production of acetic acid is probably obtained due to consumption of pentoses in this non-sterile medium (yeast is not capable of converting pentoses, hence no ethanol was formed during this period). Figure 4-18 shows the composition of the juice over 170 hours of fermentation.

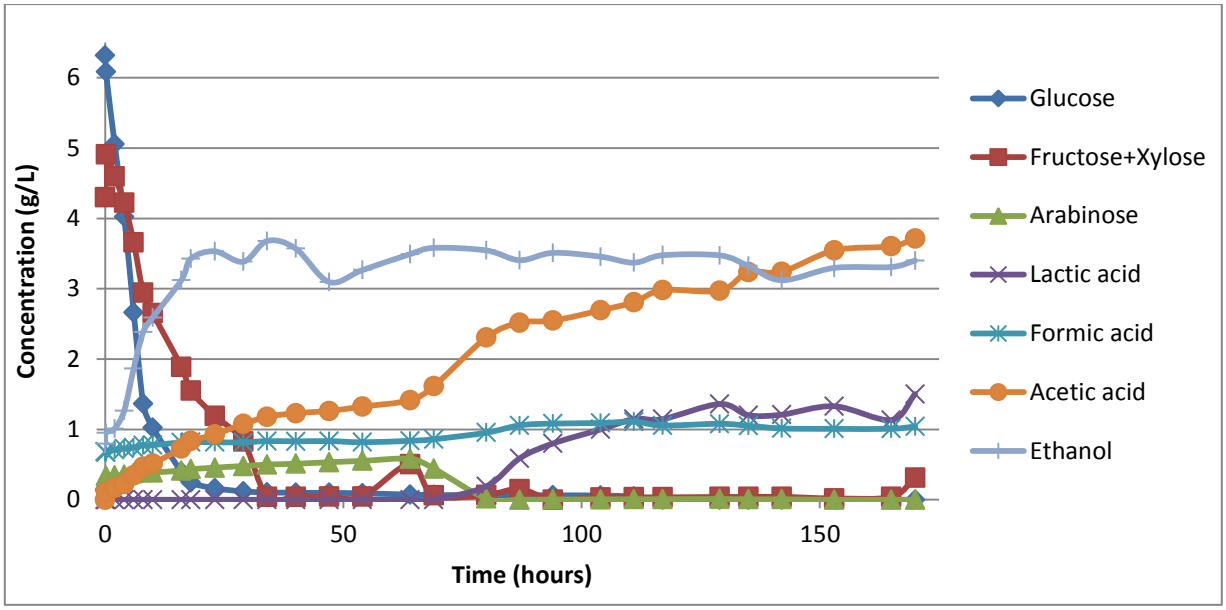


Figure 4-18. Fresh salicornia juice composition while being fermented by *saccharomyces sarevisiae*.

Experiments showed that fresh juice of unwashed biomass could obtain ethanol yields higher than 100% of the theoretical yield on available glucan (Figure 4-19), indicating that fructan could also have been utilized in ethanol production. Nevertheless, fructan concentration could not be determined, due to overlapping the two chromatograms of fructose with xylose (explained below). Generally, glucose is consumed at a higher rate than fructose during fermentation with *S. cerevisiae*, thus, fructose tends to be the main sugar source during late stages of alcoholic fermentation [251]. Also Wang et al., 2004 [252] discussed that *S. cerevisiae* tends to be glucophilic, although some strains have a clear preference for fructose.

Including the peak of fructan and xylan in calculating ethanol yields, in addition to the glucan content, a yield of 61-66% of ethanol was shown. However this result is not accurate due to including xylan (C5 sugar) in the calculation, where it cannot contribute in ethanol production.

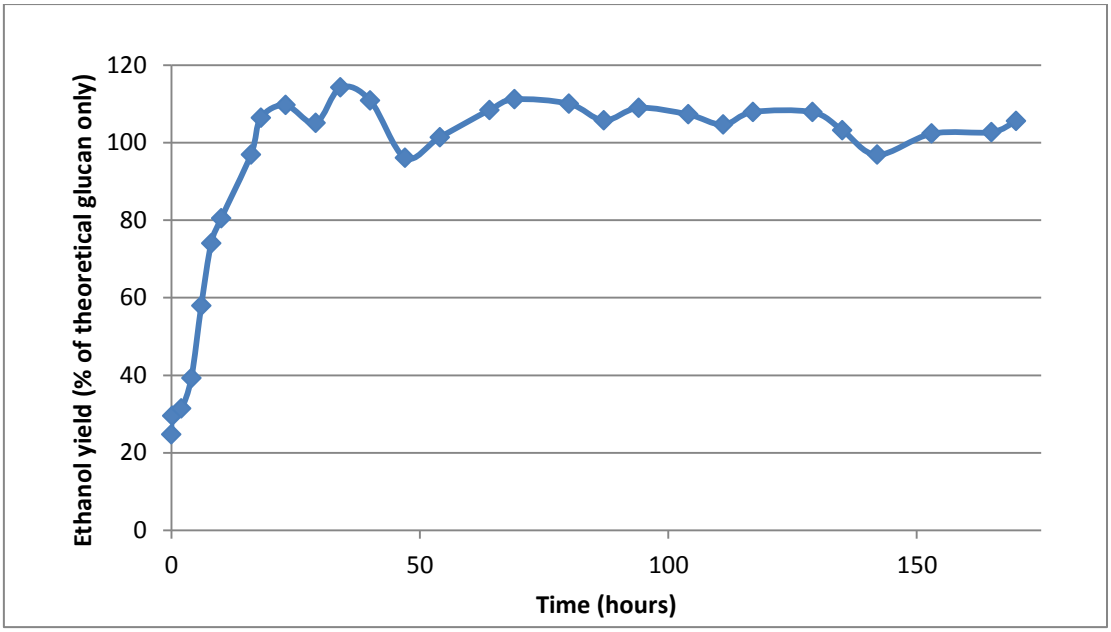


Figure 4-19. Ethanol yield obtained based on glucan composition.

Sugar analysis- chromatographic peaks

The chromatograms show one peak for both fructose and xylose (as indicated in Figure 4-20), hence ethanol yields obtained are falsely considering the xylose in the calculations and accordingly showing lower yields than it should be. In Figure 4-21, the chromatograms obtained from the Pb column confirm that xylose and fructose elution times are summed on the H column.

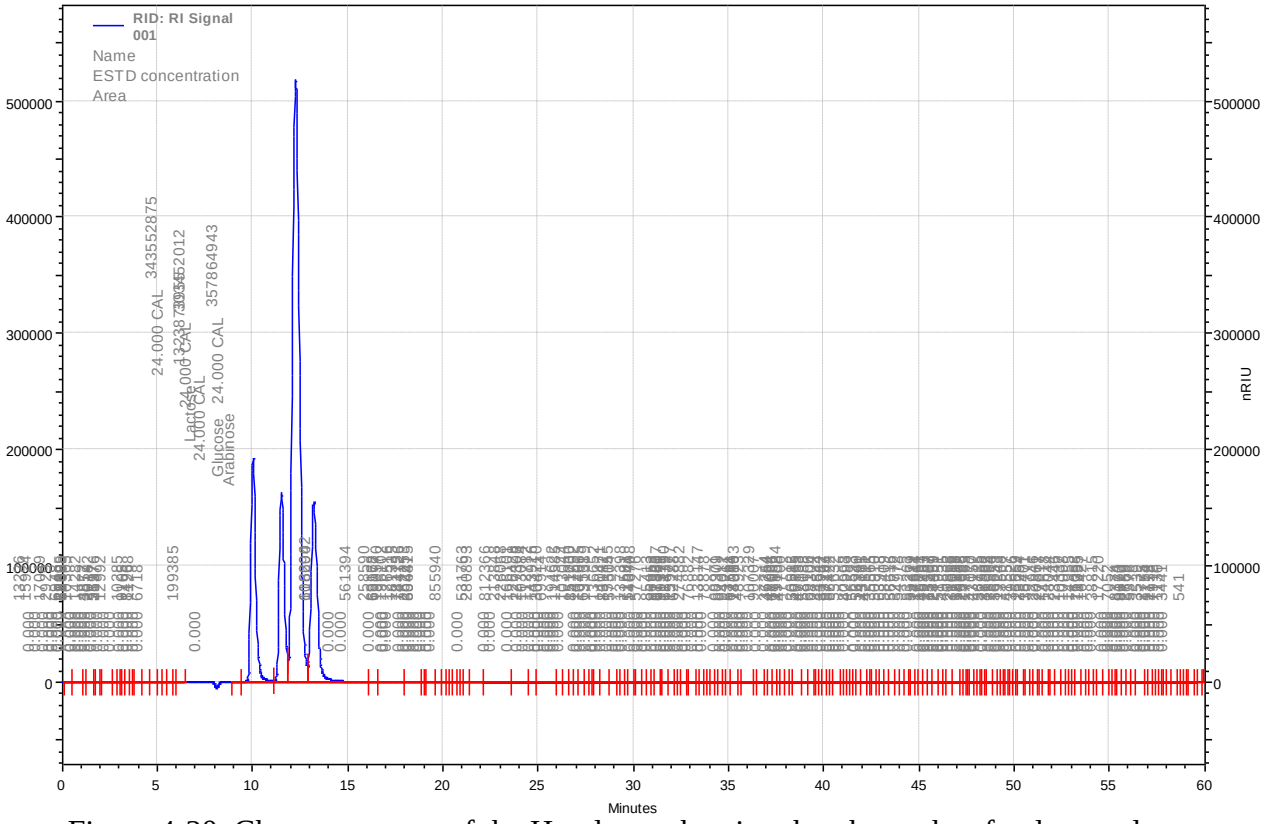


Figure 4-20. Chromatograms of the H-column showing that the peaks of xylose, galactose, mannose and fructose are overlapping and adding up into one peak.

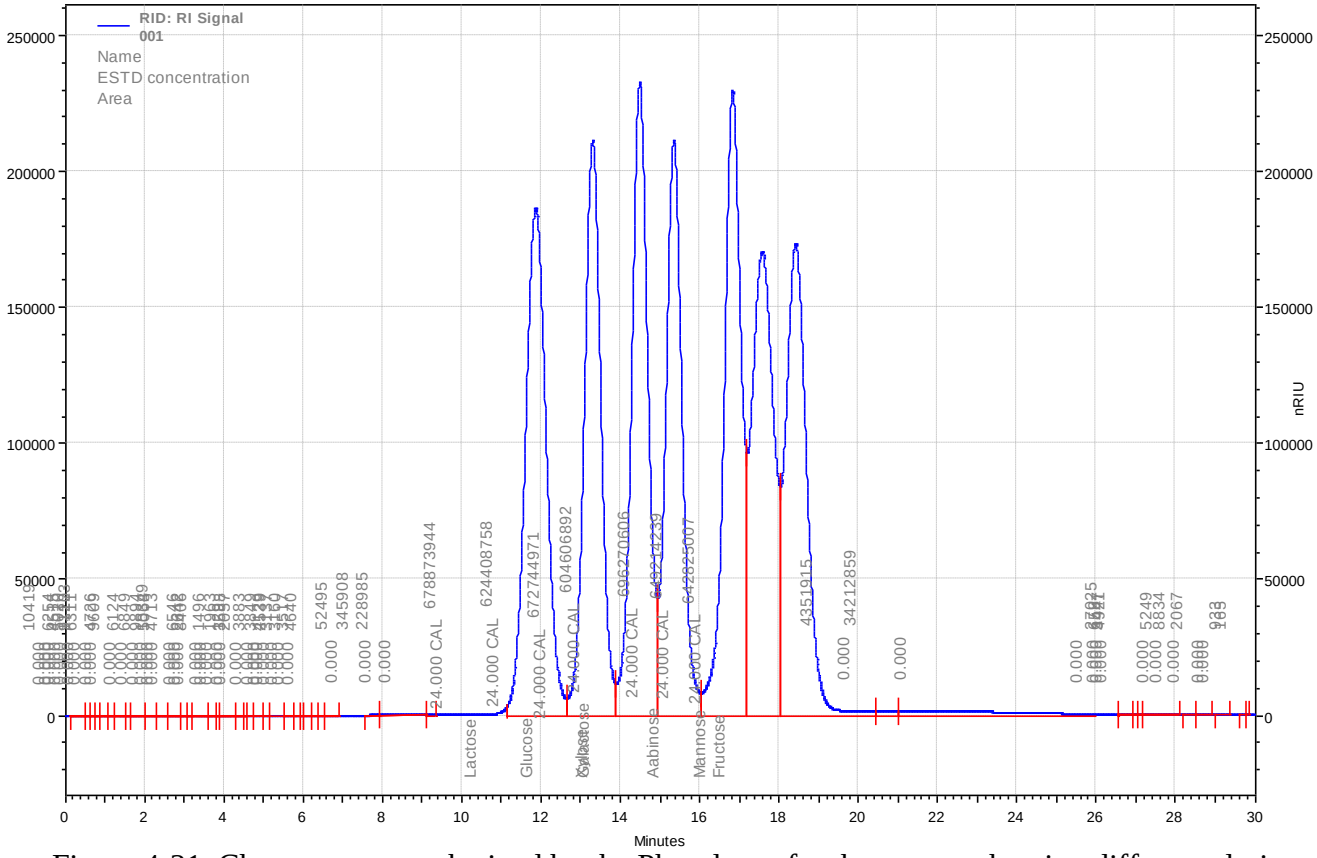


Figure 4-21. Chromatograms obtained by the Pb-column for the sugars, showing different elution times for xylose, fructose, galactose and mannose.

Effect of sterilization on juice fermentation

Juice sterilization did not enhance ethanol yields (3.76±0.2 g/L sterilized washed, 3.29±0.33 g/L sterilized unwashed versus 3.54±0.2 g/L unsterilized washed and 3.39±0.42 g/L unsterilized unwashed). However, sterilization obtained lower formic acid and acetic acid concentrations (0.11±0.08 g/L formic acid and 1.28±0.32 g/L acetic acid for sterilized washed, 0.18±0.05 g/L formic acid and 1.78±0.23 g/L acetic acid for sterilized unwashed against 0.41±0.13 g/L formic acid and 3.14±0.19 g/L acetic acid for unsterilized washed, 0.24±0.08 g/L formic acid and 5.22±0.26 g/L acetic acid for unsterilized unwashed). Results are illustrated in Figure 4-22. Measuring the weight-loss over 7 days of fermentation, the autoclaved samples showed lower weight-loss compared to unautoclaved samples, as shown in

Figure 4-23, meaning that the production of organic acids beside ethanol (by contaminating microorganism) contributes in more gas loss.

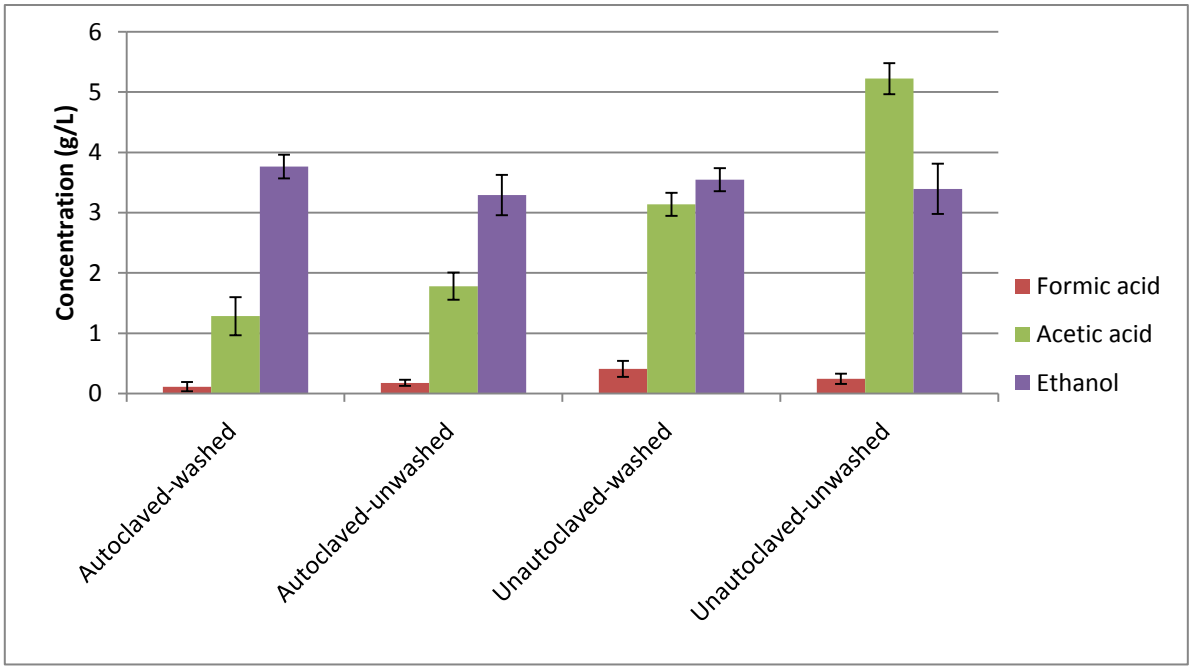


Figure 4-22. Ethanol and organic acid composition of salicornia juice with and without sterilization for both washed and unwashed biomass.

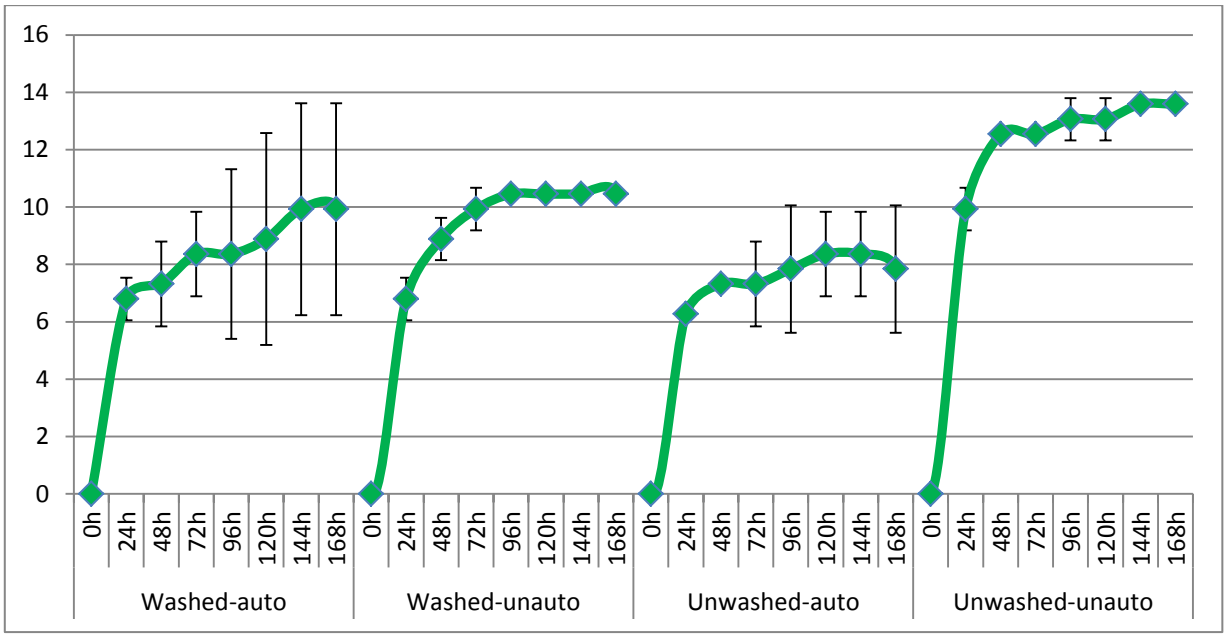


Figure 4-23. Weight-loss obtained by gas-loss obtained during juice fermentation for autoclaved and not-autoclaved samples

According to the results, sterilization did not enhance the fermentation process, where comparable ethanol concentrations were obtained. Normally, fresh-green juice, containing high value protein, phytochemcials, and easily accessible carbohydrates is used for direct lactic acid fermentation, which eliminates some of the challenges associated with pre-treatment processes and heat sterilization such as inhibitor formation and high energy usage [226], [227]. Another advantage of using fresh biomass in the green biorefinery is that the natural enzymes in the juice are still active after cropping, thereby helping the release of fermentable sugars [253], [254].

Heat sterilization could have negative effects if applied on the fresh juice, where the possibility of formation of Maillard reaction products between amino acids and carbohydrates increases when they are heated to a temperature of 120 ˚C. Maillard reactions' products are growth inhibitors and reduce the amount of available amino acids and carbohydrates. Furthermore, heat sterilisation significantly adds expenses to the low price end product.

4.2.4. Studying the acetic acid fermentation from fresh salicornia juice

a. Effects of diluting the fresh juice with citric acid before applying fermentation with *S. cerevisiae*

Juice sugar composition is fairly low (1.0-1.5%), hence we aimed to study the potential of producing other valuable products from the juice. In this experiment, juice samples were diluted by citric acid at a dilution factor of 2. Citric acid buffer was prepared in a concentration of 21 g/L (i. e., 0.19 M) and pH of the buffer was adjusted to 4.8. Fermentation was then conducted on diluted juice samples, with and without applying a pre-hydrolysis step. Initially the reason behind adding citric acid was to dilute the samples with an acidifying agent, pH-adjuster and antioxidant [194]. Citric

acid is highly soluble in water, it is characterized as a safe acid, which desplays chelating and buffering properties.

The effect of juice dilution with citric acid on juice fermentation was studied and showed that diluted samples have the potential of producing higher concentrations of acetic acid, especially for washed biomass. The fresh juice of unwashed biomass showed comparable acetic acid concentrations for both diluted and undiluted juice, knowing that the diluted juice contains half of the amount of the juice.

Generally, acetic acid is a typical co-product of the alcoholic fermentation carried out by *S. cerevisiae* and lactic and acetic acid bacteria contaminating the fermentation media [255]. Joyeux et al., 1984 [256] studied acetic acid bacteria existing in stored wine. Contaminating bacteria like *Gluconobacter oxydans*, *Acetobacter pasteurianus*, and *Acetobacter aceti* were found. *Acetobacter aceti*, specifically, can exhibit immediate propagation on short exposure of the wine to air and resulting in substantial increases in the concentration of acetic acid. Higher temperatures and higher pH values directly influence the development and metabolism of these species.

While calculating the concentrations of acetic acid and ethanol produced from juice fermentation, the dilution factor was not taken into consideration, since we were studying the solution as whole (juice + citric acid). The concentration of the ethanol produced by the diluted samples ranged between 0.3 and 0.4 of the amount produced by the undiluted juice. By considering the dilution factor, the ethanol produced by diluted samples is reduced by about 20% as shown in Figure 4-24. This can be explained by having lower pH values in the diluted samples, due to production of high concentrations of acetic acid, which results in yeast fermentation inhibition. Generally, acetate is considered cytotoxic, even at low concentrations (i.e. 0.5% (v/v))

[192]. The toxicity of acetic acid originates from the high lipophilic nature of the undissociated acetate, which can easily penetrate into the cytoplasm, leading in acetate anion accumulation and the consequent decrease in intracellular pH, which creates disruption in the proton-motive force [193]. Moreover, some of the ethanol produced could have been consumed by the existing, ethanol-resisting bacteria (e.g., acetic acid bacteria) [184]. Growth of acetic acid bacteria in ethanol containing media results in oxidation of ethanol to acetic acid (acidification process), in addition to formation of other odour- and flavor-active metabolites and possible generation of polysaccharides including dextrans and levans as was discussed by [190], [191], [175] and mentioned earlier (in section 2.4.4-b).

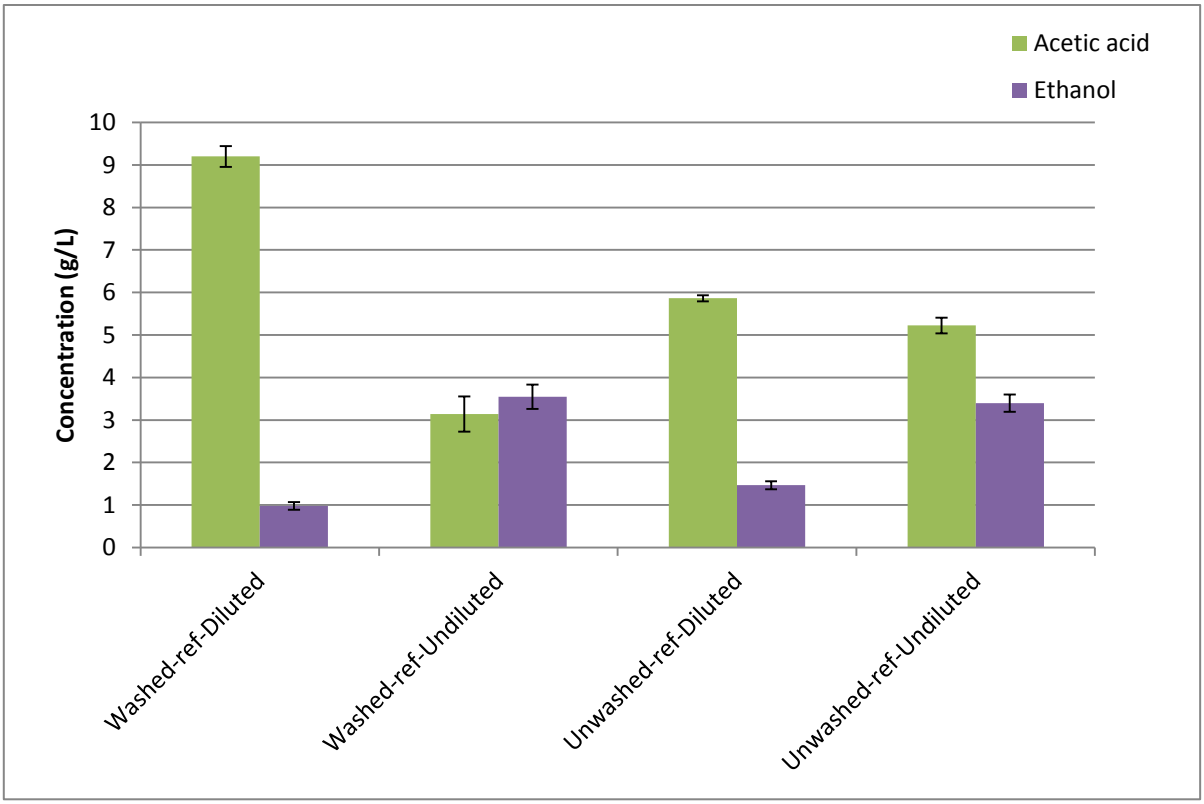


Figure 4-24. Ethanol and acetic acid produced in diluted and undiluted fresh juice samples (dilution is with citric acid, a factor of 2).

b. Effects of hydrolysing diluted juice (with citric acid) before applying fermentation with *S. cerevisiae*

The same trial was repeated for hydrolysed juice (enzymatic), in order to study the effect of enzymes on the produced ethanol and other organic acids. The concentrations of acetic acid of diluted, enzymatic hydrolysed juice increased by 3 - 4 folds in comparison to the undiluted, enzymatic hydrolysed juice, as shown in Figure 4-25. The ethanol produced in diluted samples ranged between 0.4 and 0.5 of the amounts produced by undiluted samples (Figure 4-25); hence, by considering the dilution factor, comparable amounts of ethanol are obtained for diluted- and undiluted-enzymatic hydrolysed juice. This indicates that the high concentrations of acetic acid don't solidly inhibit the yeast, which is conflicting to what was obtained by the fresh juice.

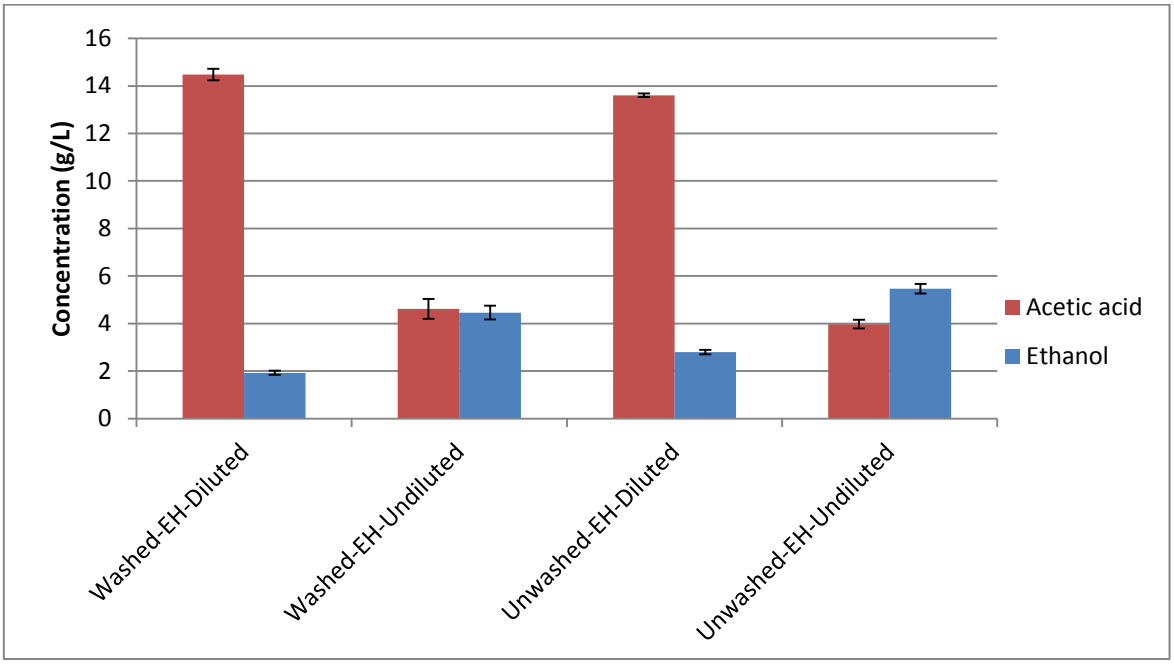


Figure 4-25. Ethanol and acetic acid produced for diluted and undiluted juice, while applying enzymatic hydrolysis (dilution is done with citric acid, a factor of 2).

c. Studying the differences between fresh and enzymatic hydrolysed juices, as a result of juice dilution with citric Acid

For diluted juice, ethanol produced by the enzymatic hydrolysed juice is almost the double of what was produced by the fresh juice. The same trend was obtained for acetic acid; the acetic acid produced by enzymatic hydrolysed juice was 3 – 4 times higher than that for fresh juice as indicated in figure Figure 4-26. For undiluted samples, the ethanol concentration of enzymatic hydrolysed juice was higher by a factor ranging between 1.2 and 1.5 (compared to fresh juice); nevertheless, comparable concentrations of acetic acid for fresh and enzymatic hydrolysed juices were obtained as shown in Figure 4-26. From these results we perceive the great impact generated by enzymes application on both ethanol and acetic acid yields. Moreover, enzymatic hydrolysed juice produced lactic acid, which was much higher for the unwashed biomass compared to the washed biomass (0.04 ± 0.01 g/L for diluted juice-washed biomass and 0.16 ± 0.05 g/L for undiluted juice-washed biomass versus 1.71 ± 0.2 g/L for diluted juice-unwashed biomass and 1.1 ± 0.1 g/L for undiluted juice-unwashed biomass). Lactic acid was not detected in the fresh juice, as indicated in Figure 4-26.

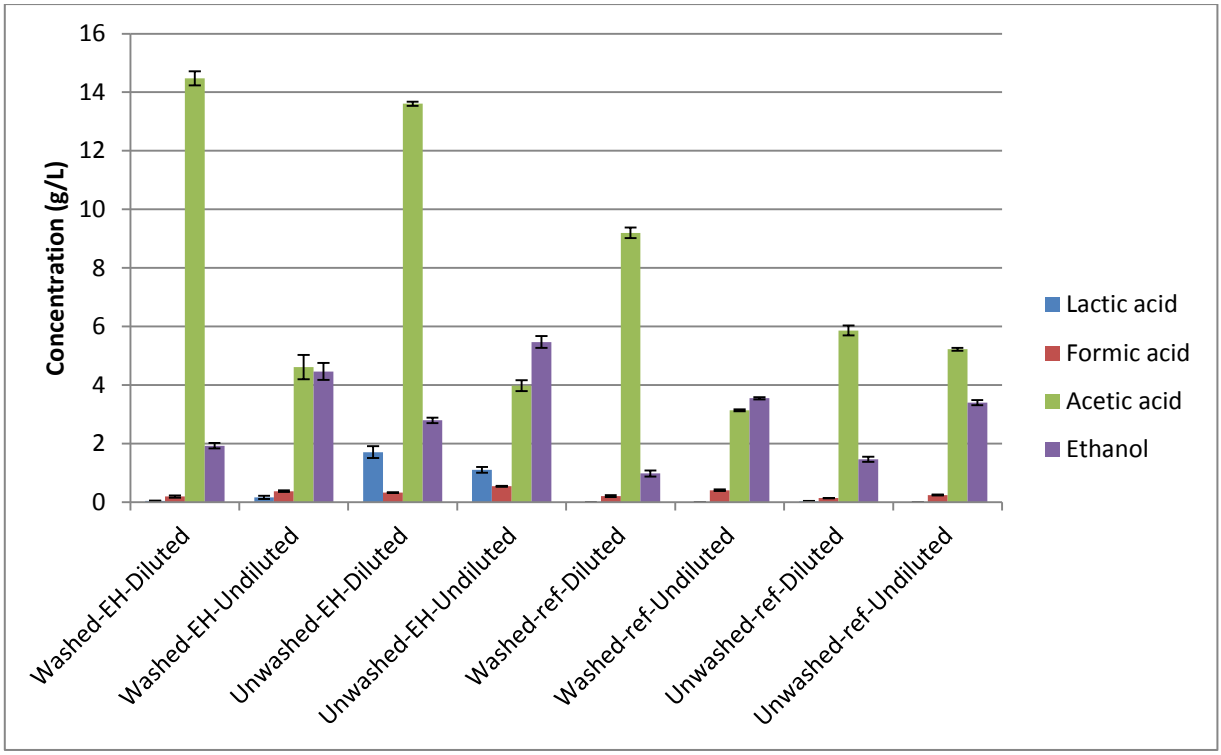


Figure 4-26. Comparison of ethanol, acetic acid, lactic acid and formic acid produced by diluted and undiluted salicornia juice for both fresh and enzymatic-hydrolyzed juices after 7 days of fermentation.

d. Mass balance calculations

Fresh juice fermentation without applying enzymatic pre-hydrolysis obtained a range of 3.0–5.0 g/L of acetic acid, which was increased to 6.0-9.0 g/L when fresh juice was diluted with citric acid (dilution factor is 2). Adding 0.109 M of citric acid and diluting it with juice to a final concentration of 0.055 M (i.e., 10.47 g/L) is expected to produce 0.123 M of acetic acid and 0.082 M carbon dioxide (i.e., 7.36 g/L and 3.61 g/L, respectively), if completely transformed into those two products as shown in (Equation 19). Measured acetic acid concentrations are comparable to the theoretically calculated values (indicated in Figure 4-24).

$$4C_6H_8O_7 + 2H_2O \rightarrow 9C_2H_4O_2\ C2H4O2 + 6CO_2 \qquad \text{Equation 19}$$

Fermenting C6 to produce ethanol will also contribute in generating carbon dioxide. With an average glucose content of about 8.76 g/L (i.e., 0.049 M) in the juice, which

was diluted by a factor of 2, we are expecting to produce about 2.24 g/L (i.e., 0.049 M) ethanol and 2.14 g/L (i.e., 0.049 M) carbon dioxide, according to Equation 20. Nevertheless, lower ethanol concentrations were obtained (1.0 - 1.5 g/L), indicating that one of the following has encountered: (i) either ethanol fermentation might have been inhibited, (ii) the acetic acid might have been produced at the expense of ethanol, (iii) or ethanol was converted into acetic acid after being produced.

$$4C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \qquad \text{Equation 20}$$

From mass and carbon balance calculations, we are expecting to lose an average weight of about 6 g for each L of diluted juice in the form of carbon dioxide gas. However, results showed greater weight-loss, ranging between 11.0 and 12.0 g/L. Figure 4-27 shows the weight loss throughout 168 hours of fermentation of fresh salicornia juice (washed and unwashed biomass), being diluted with citric acid.

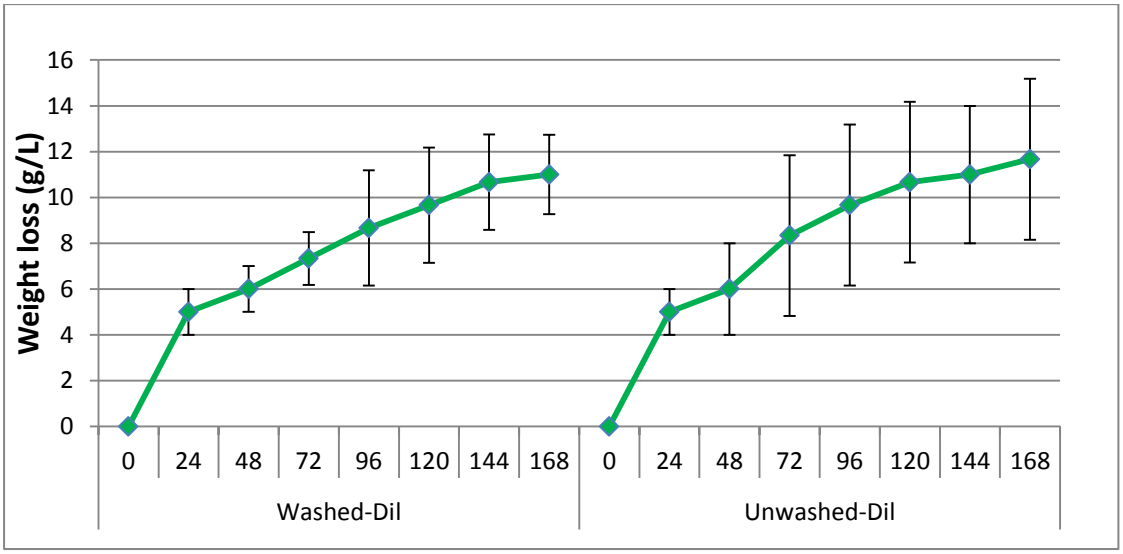


Figure 4-27. Weight-loss encountered by 1.0L diluted juice through 168 hours fermentation (without enzymatic pre-hydrolysis), which is suggested to be obtained due to losing CO_2 gas.

For enzymatic hydrolysed juice, undiluted samples produced about 4.5 g/L acetic acid compared to approximately 14.0 g/L for diluted samples. The amount of acetic acid

produced exceeds the theoretical amounts by almost the double. However, the fermented ethanol is comparable to the theoretical values (i.e., 2.24 g/L). Weight-loss results obtained by enzymatic-hydrolysed juice are similar to the theoretically calculated values; ranging between 6.0 and 8.0 g/L, as illustrated in Figure 4-28.

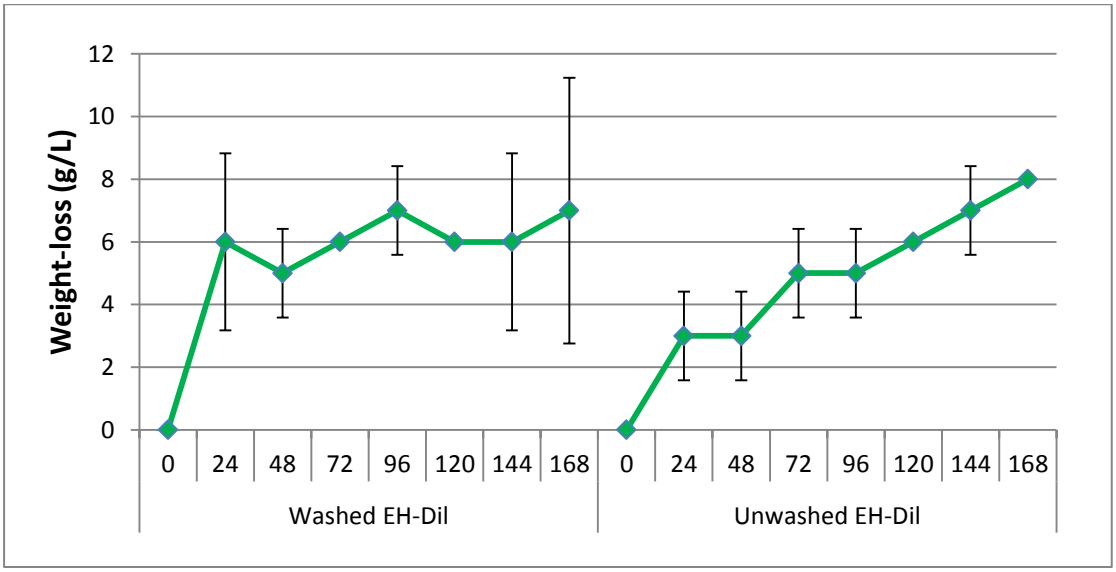


Figure 4-28. Weight-loss encountered by enzymatic-hydrolyzed juice being fermented for 168 hours. This weight-loss is calculated for 1.0L batch and suggested to be obtained by CO_2 gas loss.

e. Analyzing the changes of the chemical composition encountered by juice dilution

It was important to trace the consumption of citric acid in diluted juice samples of both washed and unwashed biomass, which was done by analysing for citric acid beside other components (ethanol, acetic acid and glycerol). Results were compared to undiluted juice. This experiment was conducted on enzymatic hydrolysed juice. After fermentation, samples diluted with citric acid showed almost zero citric acid concentrations (diluted samples: 0.04±0.02 g/L from washed biomass and 0.05±0.04 g/L from unwashed biomass versus undiluted: 0.47±0.6 g/L from washed biomass and 0.49±0.05 g/L from unwashed biomass), indicating that the added citric acid was

completely consumed (Figure 4-29). After fermentation, glycerol was completely absent in diluted samples, where it was detected in undiluted samples (1.38 ± 0.43 g/L for washed biomass and 1.91 ± 0.52 g/L for unwashed biomass) as indicated in Figure 4-29. The obtained results are conflicting with what is discussed in the literature; Omori, T. et al., 1995 discussed the enhancement in glycerol production by *S. cerevisiae* when adding citric acid [257]. Also, within the pH range from 4.0 to 4.5, *S. cerevisiae* showed stimulation in glycerol synthesis [258]. This trial showed that biomass washing results in inhibiting lactic acid production, since lactic acid was mainly detected in juice of the unwashed biomass.

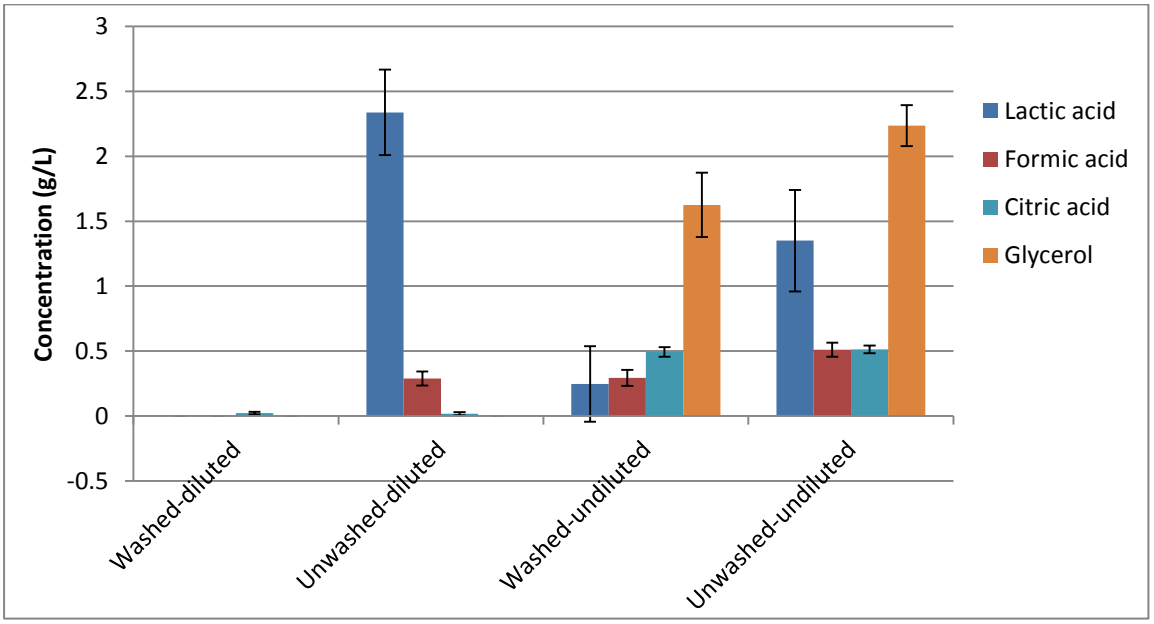


Figure 4-29. Citric acid, glycerol, formic acid and lactic acid concentrations in diluted and undiluted juices of both washed and unwashed biomass.

In order to assess whether acetic acid was generated due to contamination during the pre-hydrolysis (as described above), a trial of SSF was conducted on salicornia juice for both washed and unwashed biomass, after being diluted with citric acid (dilution factor of 2). The enzymatic hydrolysis step took place over 8 hours, during which sample analysis was done every two hours, to study the changes happening on the

chemical composition of the juice. Figure 4-30 shows that the concentration of the acetic acid in salicornia juice (for both washed and unwashed biomass) is constant over the 8 hours of pre-hydrolysis with negligible concentration (0.00 g/L for the washed biomass and ~0.45 g/L for the unwashed biomass). Accordingly, we conclude that the acetic acid was generated during the fermentation step only.

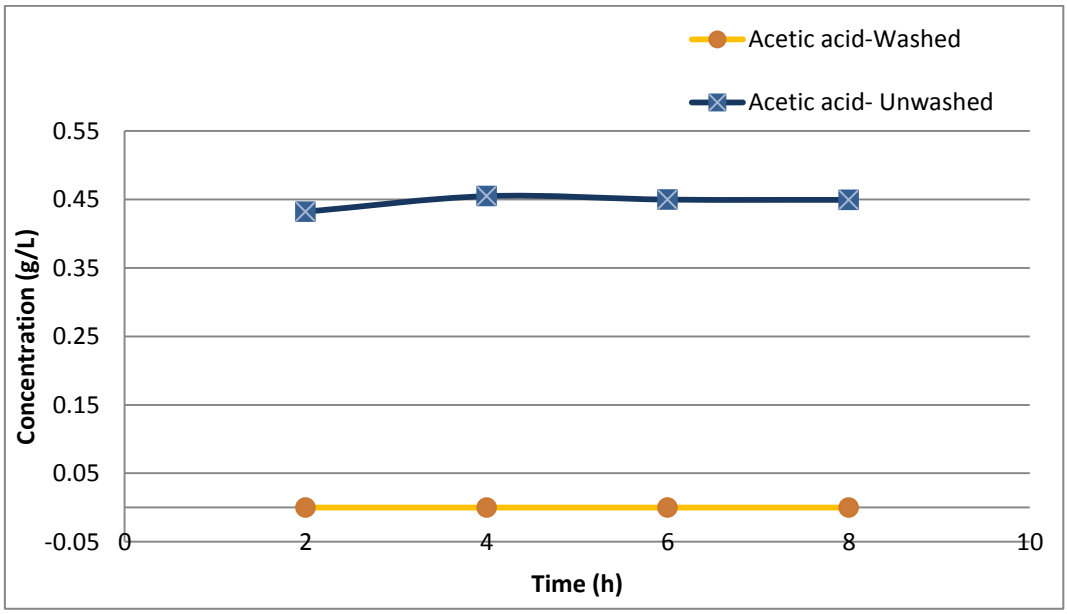


Figure 4-30. Change in acetic acid concentration over 8 hours of enzymatic hydrolysis of diluted juice (for washed and unwashed biomass).

f. The contribution of *Saccharomyces cerevisiae* in producing acetic acid in diluted juice

A trial was conducted, in which diluted and undiluted juice samples were incubated under the same fermentation conditions as for SSF, however no yeast was added. The aim of this experiment was to explore if the producer of acetic acid was *S. cereviciae* or other contaminating microorganisms. For the juice fermented with baking yeast, the undiluted juice showed marginal lower acetic acid concentrations (5.22 ± 0.05 g/L) compared to the diluted juice (5.86 ± 0.17 g/L), in addition to the ethanol

generated. Nevertheless, juice fermented without adding yeast showed minor acetic acid concentrations (0.78 ± 0.04 g/L for undiluted juice and 1.19 ± 1.20 g/L for diluted juice), as illustrated in Figure 4-31. Results show that the high acetic acid concentrations were produced by yeast containing samples; moreover, diluting the samples slightly increased the acetic acid production.

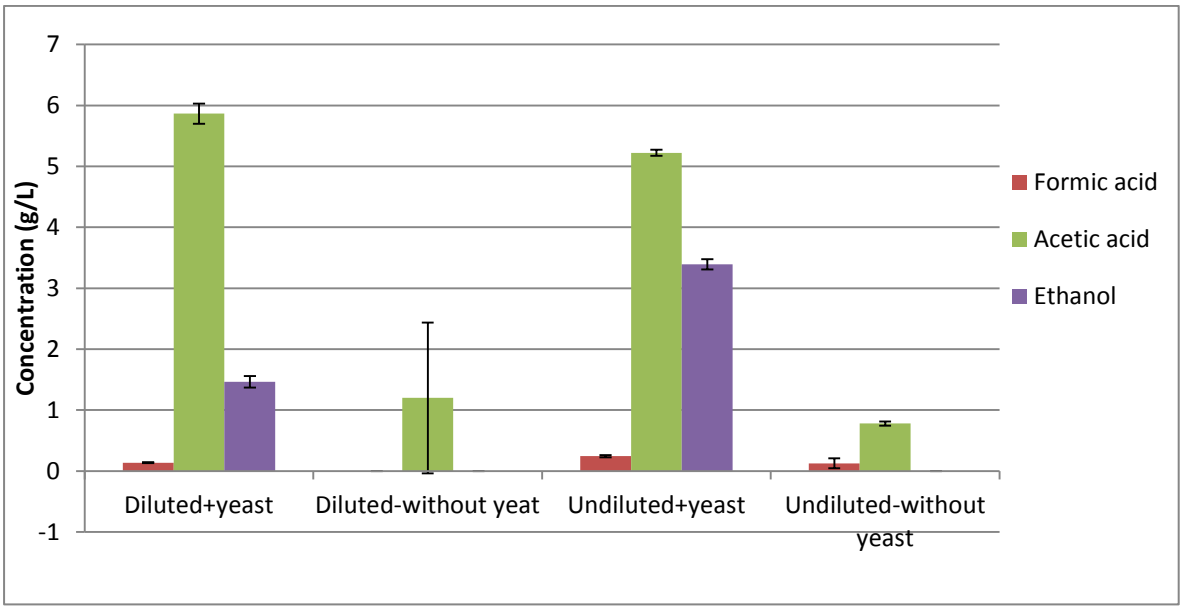


Figure 4-31. Comparing the chemical composition of fermented fresh juice, with and without adding *Saccharomyces cerevisiae*.

g. Evaluation of juice fermentation by *Debaryomyces hansenii*

D. hansenii is a highly heterogeneous, and thus adaptable, species with diverse optimal growth conditions. The ability of *D. hansinii* to grow at 10% NaCl or 5% glucose was discussed by Breuer et al., 2006 and hence showed advantageous discrimination from other ascomycetous yeasts [259]. In this study, *D. hansenii* was used to ferment fresh, sterilized salicornia juice. In order to boost the juice fermentability, glucose was added to leave the juice with glucose concentration of about 21 g/L. the idea behind sugar addition was to evaluate the juice as the

fermentation media, since it contains high salt content. Results in Figure 4-32 show that ethanol concentrations start to be significant after 100 hours of fermentation. Ethanol yield of only 8.2% was obtained after 180 hours of fermentation. It is recommended to have longer fermentation times, since the high salinity of the juice can be the reason behind the fermentation inhibition. Yeast needs time to adapt to the harsh conditions provided by the high-salt content.

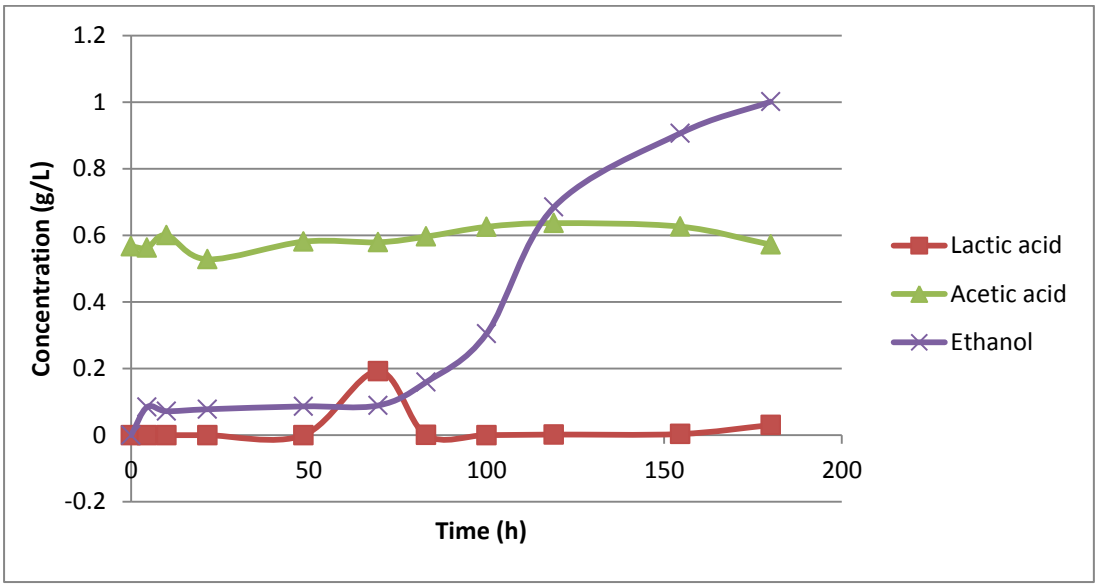


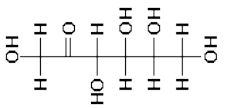
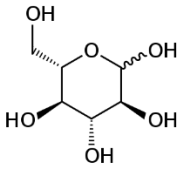
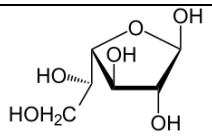
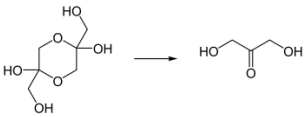
Figure 4-32. Ethanol produced by salicornia juice fermentation by *D. Hansinii*.

4.2.5. Salicornia sinus-persica juice analysis by GC-MS

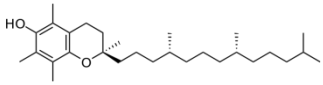
There is great of potential in utilizing green biomass in green biorefineries, since the green biomass contains significant amounts of high value protein (e.g., functional proteins such as RuBisCo), phytochemcials, and carbohydrates. The advantage of utilizing a biomass in a green biorefinery is to extract high-value chemicals. Moreover, the natural enzymes in the juice will still be active after cropping, helping the release of fermentable sugars [253], [254]. In order to evaluate the juice produced from wet-fractionation of *Salicornia sinus-persica*, GC-MS chemical analysis was carried out.

The GC-Ms analysis detected wide-range of added-value chemicals in *Salicornia sinus-persica* fresh juice, which are described in Table 4.

Table 4. Added-value chemical composition of *Salicornia sinus-persica* juice detected by GC-MS

Chemical	Description	Chemical structure
D-Fructose	• Sugar	
Glucofuranose (glucose)	• Sugar	
D-galactofuranose	• Sugar	
Dihydroxyacetone dimer	• DHA has **skin browning** effect which is non-toxic and similar to the Maillard reaction. • DHA reacts chemically with the amino acids in the skin, which are part of the protein containing keratin layer on the skin surface.	
Palmitic acid (hexadecanoic acid)	• Palmitic acid is mainly used to produce **soaps, cosmetics, and release agents**. These applications utilize sodium palmitate, which is commonly obtained by saponification of palm oil. • Recently, a long-acting antipsychotic medication, paliperidone palmitate	

	(marketed as INVEGA Sustenna), **used in the treatment of schizophrenia**, has been synthesized using the oily palmitate ester as a long-acting release carrier medium when injected intramuscularly.	
Stearic acid (octadecanoic acid)	• Stearic acid is mainly used in the production of detergents, soaps, and cosmetics such as shampoos and shaving cream products • Stearic acid finds many niche applications, for example, in making plaster castings from a plaster piece mold or waste mold and in making the mold from shellacked clay original. • Stearic acid is used to produce **dietary supplements**. • In **fireworks**, stearic acid is often used to coat metal powders such as aluminium and iron. This prevents oxidation, allowing compositions to be stored for a longer period of time. • Stearic acid is a **common lubricant** during injection molding and pressing of ceramic powders. It is also used as a mold release for foam latex that is baked in stone molds.	

Linoleic acid (octadecadienoic acid) – omega-6 acid	• Linoleic acid is an essential fatty acid that must be consumed for proper health. • Linoleic acid has become increasingly popular in the **beauty products** industry because of its beneficial properties on the skin. Research points to linoleic acid's **anti-inflammatory, acne reductive, and moisture retentive properties** when applied topically on the skin.	
α-Tocopherol (vitamin E)	○ Vitamin E has many biological functions, the **antioxidant** function being the most important and/or best known. Other functions include **enzymatic activities, gene expression, and neurological function(s)**.	

From the provided GC-MS analysis, added-value chemicals and nutraceuticals were detected in *Salicornia sinus-persica*, which could be extracted before being utilized in biofuels production. Nevertheless, further quantification and comprehensive composition analysis need to be conducted for feasibility assessment.

4.2.6. Salicornia green-biorefinery

Products of special interest like added-value extractives, proteins and nuraceutical chemicals (e.g. hormones, antibiotics) can substantially improve the economy of a biorefinery, making it more industrially attractive [225]. Generally, utilizing the green biomass in green biorefinery showed great potentials, allowing to avoid excessive

heat applications and energy-intensive mechanical pretreatment procedures. Accordingly, the activity of fresh biomass is maintained and inhibitors' formation is eliminated and/or minimized, besides decreasing the associated costs [226], [227].

Salicornia sinus-persica has succulent leafless stems [77], containing high moisture content (above 70%) and hence very suitable for juicing. Juicing of the biomass allows for water preservation in the system besides other advantages associated with wet fractionation. The aim of this work is to examine the fresh use of s*alicornia sinus-persica* in a green biorefinery as described in Figure 4-34. Salicornia plant needs to be disintegrated, as an initial step, to produce two fractions; pulp and juice. Pulp is rich in carbohydrates; hence it is a good candidate for bioethanol and/or biogas production by fermentation and/or digestion. Other by-products of the fermentation process are advantageous and needed to be considered in the study. Our set of experiments showed that *Salicornia sinus-persica* provides 19 - 25 kg glucose per 1.0 tonne of biomass with total sugar content ranging between 40 and 55 kg sugar per 1.0 tonne of biomass, as shown in Figure 4-33. This provides a theoretical ethanol yield of about 13 kg/tonne biomass.

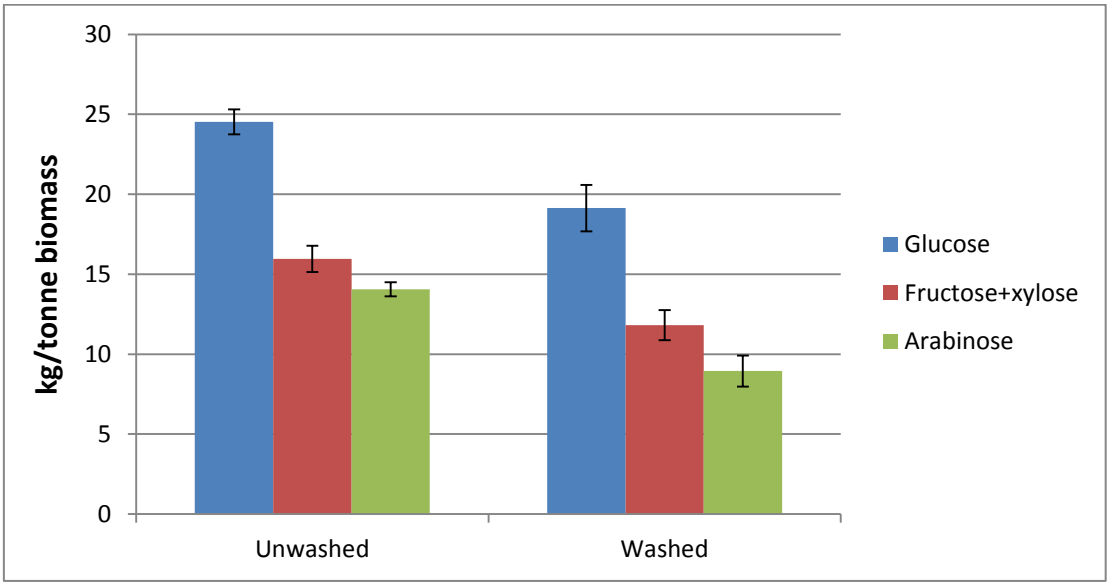


Figure 4-33. Amount of sugar provided from 1 tone of total biomass.

After exploiting the carbohydrates occurring in the pulp fraction, fermented pulp could be used as mineral-rich natural fertilizer, which needs to be assessed in terms of applicability. On the other hand, juice fraction is rich in water, which could be preserved in the system after extracting high-value chemicals (such as proteins, lipids, ascorbic acid, vitamins and chlorophyll). Also, plant's juice is a good fermentation medium. For this biomass, despite the high salt content, juice could show high sugar convertibility. Accordingly a green biorefinery is proposed to be assessed based on the main processes explained in Figure 4-34.

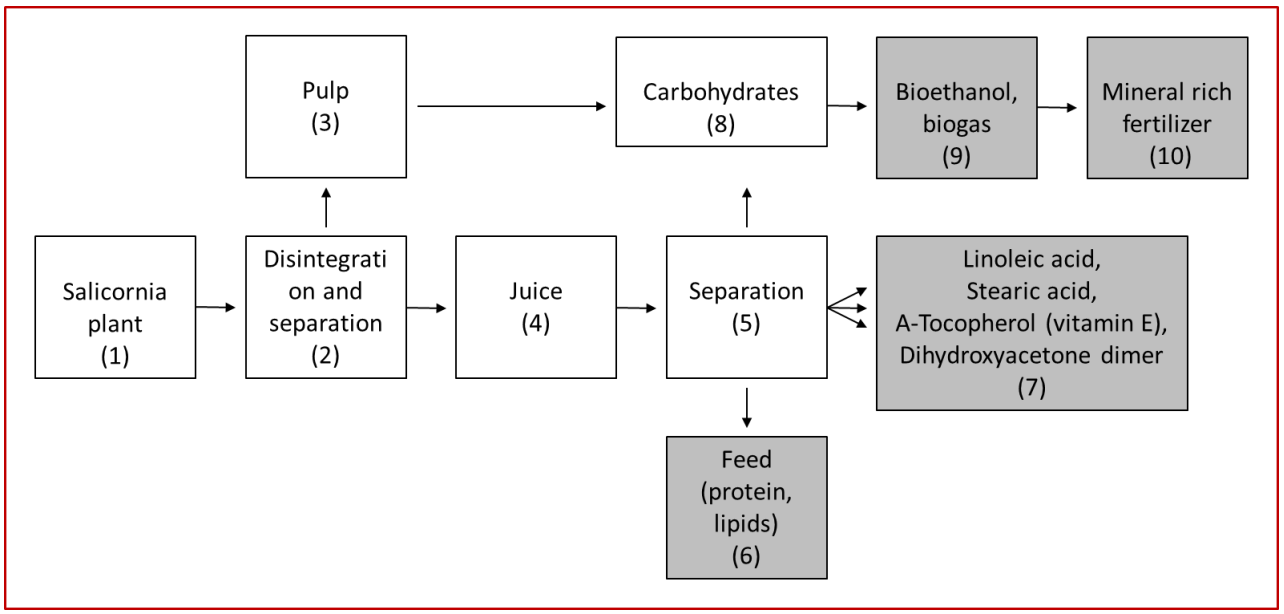


Figure 4-34. Fresh Salicornia plant in green biorefinery.

CHAPTER 5

5. Conclusion and Recommendations

5.1. Conclusion and Discussion

Salicornia sinus-persica, a native halophyte to the Arabian Gulf, could be cultivated in countries with limited fresh water supplies and arid lands. Fresh *Salicornia* biomass contains various added-value chemicals such as carbohydrates, proteins, lipids, oils, crude fibres and ash. It also contains 569, 159, and 58 mg/kg fresh weight of chlorophyll, β-caroten, and ascorbic acid, respectively [260].

In this study, *Salicornia sinus-persica* underwent wet-fractionation producing two main fractions; juice and pulp. Each fraction was assessed by studying its carbohydrate composition and its sugar convertibility by fermentation.

Wet-fractionation of this biomass is advantageous, since ~70% of the biomass is liquid, allowing for water preservation in the system besides the ability to run at lower dry matter in the fermentation step. Sugar composition of the juice is relatively low (1.0 – 1.5%), nevertheless, the fresh pulp shows fairly high sugar content (~50 w/w %).

Juice pre-processing by acid hydrolysis did not show any impact on the C6 sugar composition, where marginal increase in the released arabinose was observed. This

increase could be explained by hydrolyzing pulp residuals in the juice fraction, which were generated by the process of juicing. Juice sterilization at 121 ˚C for 20 minutes indicated lesser concentrations of the produced byproducts (i.e., acetic acid and formic acid), however the ethanol content was comparable. Hence fresh untreated juice (without any pre-processing) was directly fermented, where considerable amounts of ethanol as well as acetic acid were obtained (ranging from 3.0 to 4.0 g/L for each).

Fresh juice was also fermented after being diluted with citric acid. Citric acid was added as a buffering agent to maintain the pH at 4.8 and also to obtain lower dry matter content, allowing for minimized application of enzymes. Results showed noticeable formation of acetic acid at the expense of ethanol production, since marginal reduction in the ethanol produced was encountered.

Studying the effect of pulp pre-treatment on the sugar content, ash content, lignin content and also the sugar convertibility was assessed. Different temperatures and residence times were applied. The C6 sugar composition increased with increasing the pre-treatment temperature, while the C5 sugar content decreased accordingly.

Sugar recovery values showed that applying lower temperature, however, longer residence times could obtained higher glucose and xylose recoveries, where the highest sugar recoveries were encountered by treatments done at 120 ˚C for 30 and 60 minutes (88.08 ± 11.63% and 94.62 ± 5.57% respectively). Both glucose and xylose recoveries decreased with increased the pre-treatment temperature. On the other hand, the sugar convertibility increased with increasing pre-treatment temperature (ranging from 120 ˚C to 170 ˚C), while keeping the residence time constant at 10 minutes. The applied pre-treatment residence time showed a great influence on the obtained ethanol

yield when was increased from 10 to 30 minutes at a temperature of 120 ˚C (increased by almost 10 folds). Nevertheless, increasing the pre-treatment time from 30 to 60 minutes resulted in decreasing the ethanol yields obtained by about 28%. This indicates that increasing the pre-treatment durations result in a negative impact on the fermentation process due to increasing the possibility of inhibitors formation.

Fermenting the pulp fraction in the pre-treatment liquid medium instead of water showed some inhibitory effects for all pre-treatments, nonetheless it was advantageous for the pre-treatment done at 120 ˚C for 10 minutes, since the ethanol yield increased from 6.97 ± 1.54% to 85.46 ± 0.94% of the theoretical ethanol yield based of the glucan content.

GC-MS showed that the fresh juice of *Salicornia sinus-persica* contains organic acids and nutraceitical chemicals, which could bring a salicornia-based biorefinery into industrial application. However for such a conclusion further composition assessment is required.

5.2. Recommendations

- According to the findings of this study, it is recommended to use this halophyte freshly without any pre-washing, since washing did not show tangible impacts on the sugar composition or the sugar convertibility.
- The sugar composition of the juice is minimal; showing its infeasibility to be used for ethanol production, however diluting it with citric acid resulted in producing valuable amounts of acetic acid. It is recommended to apply further studies to understand the pathways followed, also to evaluate the optimal conditions to be applied.

- For juice fermentation with *D. Hansenii*, it is recommended to conduct a comprehensive study to study the optimum conditions and parameters to be applied.
- A general idea was obtained about pulp pre-treatment by applying mild to medium pre-treatments; nevertheless, it is suggested to optimize pulp pre-treatment by conducting an extensive study. Throughout this study, different temperatures and residence times to be applied aiming to optimize pulp pre-treatment while considering its impacts on the biorefinery's economics.
- To improve the economical implementation of salicornia-based biorefinery, it is important to evaluate other valuable by-products and their different applications.
- In order to evaluate a salicornia-based biorefinery and to assess its implementation, it is recommended to investigate the commercialization potential of *Salicornia sinus-persica* for biofuel and other byproducts production through a model.

APPENDIX A

Abbreviations

DI Deionized

DM Dry Matter

FPU Filter Paper Unit

HPLC High Performance Liquid Chromatography

NREL National Renewable Energy Laboratory

RID Refractive Index Detector

SSF Simultaneous Saccharification and Fermentation

TS Total Solid

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