Cascading Biorefinery Approaches for the Sustainable Fractionation of Macroalgae

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Conducted at
TNO Energy Transition

TU Delft

Cascading Biorefinery Approaches for the Sustainable Fractionation of Macroalgae

by

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in partial fulfillment of the requirements for the degree of

Master of Science
in Sustainable energy Technology

at the Delft University of Technology,
to be defended publicly on Monday October 19, 2020 at 1:00 PM.

Student number:	4807588	
Project duration:	January 27, 2020 – October 19, 2020	
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This thesis is confidential and cannot be made public until October 19, 2022.

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Abstract

The growing population is accompanied by an increase in the global energy and resource demand. Biorefining can be considered a means to help meet these growing demands through a range of bio-based materials. A biorefinery approach for developing an upstream process for the production of valuable products from macroalgae would be essential to realize a feasible valorisation chain downstream, wherein these products can be tailored to various applications. On a global scale, the biorefinery concept incorporates mainly, terrestrial first and second-generation biomass which compete with food and other energy applications, as well as involving sustainability issues such as land use change. Third generation macroalgae or seaweed thus has tremendous scope within the biorefinery concept. Seaweed has rapid reproduction rates and can be cultivated with minimal resource inputs thereby offering the attractiveness of high biomass yields. This study deals with the sustainable fractionation of two seaweeds, namely, *Ulva lactuca* (green seaweed) and *Palmaria palmata* (red seaweed) for the extraction of rhamnose and xylose, respectively. These sugars are of interest as they can be used to produce 5-methylfurfural and furfural serving as platform chemicals for various applications, including fuel production. Focus is laid on alternative hydrolysis methods of low severity for the extraction of the targeted sugars including enzymatic hydrolysis, hot water treatment and hydrolysis with organic acids and chelating salts. The characterization of the seaweeds and determination of their biochemical compositions are performed by acid hydrolysis, after the application of pre-treatment steps like washing with water and soxhlet extractions in order to maximize the sugars recovered. These compositions then form a basis for the study of low severity and more sustainable hydrolysis methods.

A series of low temperature enzymatic hydrolysis experiments are conducted in order to screen a number of potential enzymes for the two seaweed types, which led to enzyme selection for further investigation. It is followed by a study of the effect of operating conditions and parameters namely, the reaction temperature, the pH and enzyme dosage. Hydrolysis treatments with hot water, organic acids (acetic, formic, oxalic, citric) and chelating salts (sodium acetate, sodium citrate) are also conducted at different temperatures and for a selected range of reaction times. These are carried out in a multiclave, enabling the running of a large number of screening tests to determine the best combination of the hydrolysing agent, temperature and reaction time. The results from these low severity treatments and enzymatic hydrolysis tests are applied onto a larger reaction scale in order to implement a cascade biorefinery approach for the extraction of rhamnose and xylose. Hot water treatments at 120°C and 140°C for the *Ulva lactuca* and *Palmaria palmata*, respectively are chosen as the feasible routes. As for the enzymatic hydrolysis studies, hydrolysis with Cellic CTec2 enzyme cocktail and β-D-Xylanase enzyme (from *T.maritima*) at 50°C are selected. Enzyme dosages of 25% and 3%, respectively are deemed suitable for the release of the targeted sugars after 72 hours of hydrolysis.

Ultimately, the cascading approach is applied to a larger amount of seaweed (1.5 kg) where the biomass is washed followed by subsequent centrifugation in a decanter-centrifuge to separate the washed seaweed. The washing liquid fraction is further fractionated through membrane filtration to recover MW fractions of >100kDa, 100-1kDa and <1kDa. The separated solids are thereafter subjected to enzymatic hydrolysis in the bioreactor as well as autoclave treatments at a 2-3L scale, in accordance with the conclusions from the screening treatments in order to further evaluate the scalability of the process. In this cascading approach, a complete mass balance of the seaweed biomass components of interest (sugars, inorganics, protein) is carried out in order to evaluate the efficiency of the cascade approach and maximize the same by realising the potential of the by-product and residual streams.

Keywords: macroalgae; biorefinery; seaweed; fractionation; hydrolysis

Acknowledgements

The past few months at the Biomass and Energy Efficiency Group at TNO Energy Transition was a challenging, yet enjoyable experience for me. It was an exciting and wonderful opportunity for me to work in the field of biorefining. I would like to take this opportunity to sincerely thank everyone who helped me with this project and contributed to it.

First of all, I would like to thank my daily supervisor at TNO, Karla Dussan Rojas for providing me with this wonderful opportunity and also her insights, guidance and positive motivation. Her suggestions in times of need and her endearing approachability are what enabled me to successfully complete the project. Her mentorship is something I will always cherish. I would also like to extend my immense gratitude towards Esther Cobussen- Pool, for her equipment and apparatus training sessions and mentorship within the laboratory. Her insights on laboratory practices have been invaluable to my learning experience. I would also like to thank my supervisor, Prof.dr.ir. W. de Jong, for his much valued guidance.

I would like to thank my friend Anand for his inputs and insights from which I gained ideas on compiling and documenting my work. His experience was of great value to me.

Finally, I express my sincere gratitude to my family, whose constant love and support has been ever so important, more so over, the last few months. I am fortunate to have received the emotional and financial support from my parents and I dedicate this thesis to them.

N. U. Kodimaniyanda
Delft, October 2020

Contents

List of Figures

List of Tables

1 Introduction

Biorefining is the conversion of biomass into a wide range of bio-based materials including fuels, energy and energy carriers, chemicals, food and other materials. It is an alternate and sustainable means to alleviate the adverse environmental impacts brought about by the use of fossil fuels for the production of energy and industrially produced chemicals. The concept evolved in the late 1990s [1]. The essence of a biorefinery is to make advantageous use of the various components in biomass and valorize the conversion of biomass components and their intermediates to attain value-added products.

As defined by the International Energy Agency, biorefinery platforms are intermediates that link different biorefinery systems and their processes [2]. One such biorefinery platform is the sugar platform. These kinds of biorefineries work on biomass conversion into its different component sugars which can be further processed into fuel or chemical products [3]. The use of aquatic biomass like macroalgae can be seen as more favourable than terrestrial biomass as it can be made sustainably available without the need for deforestation, there is a large availability of marine water for cultivation, it is easy to cultivate and fast-growing [4].

As opposed to lignocellulosic biomass materials which are more resistant to pre-treatments and biochemical conversions, non-lignocellulosic macroalgae can be more easily pre-treated and fractionated into its constituent sugars. The composition of these kinds of biomass are quite different lignocellulosic biomass. In order to achieve highly economical and efficient biorefineries, it is important to develop more sustainable processes to obtain the platforms or intermediate compounds as ultimately, this would lead to better end-product generation and process intensification [5].

1.1. Scope of Seaweed Biorefining for the Extraction of Sugars

Seaweed is classified as brown, red and green seaweed [6]. This study includes work on *Ulva lactuca*, a green seaweed and *Palmaria palmata*, a red seaweed. Macroalgae has recently been identified as a renewable source for biofuel and green chemical production from its carbohydrate constituents. Thus, it is important to have an apt technique for the characterization of seaweed and the extraction of its carbohydrates.
Ulva lactuca and *Palmaria palmata* contain specialty carbohydrates like rhamnose and xylose, respectively. The dehydration of these carbohydrates results in the formation of furanic molecules. The most commonly seen dehydration conversions are acid-catalyzed. These furanic compounds find applications in products such as biofuels and bio-based chemicals. In addition to containing specialty carbohydrates, seaweed contains proteins and lipids, boosting the valorization potential of the biorefinery approach.

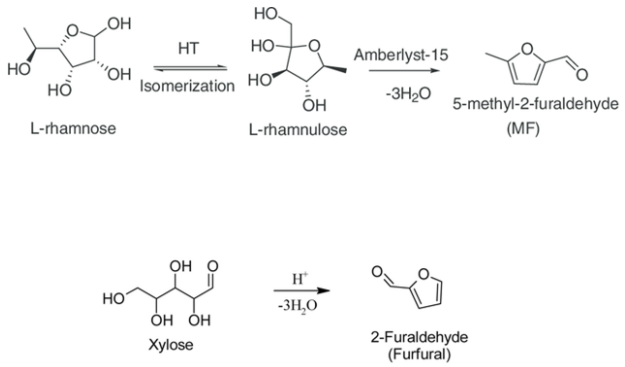


Figure 1.1: Dehydration of rhamnose and xylose to their respective degradation furanic molecules [7], [8].

1.2. Objectives

The aim of this project is to assess the cascading biorefining approach for the fractionation of macroalgae, namely green and red seaweeds - *Ulva lactuca* and *Palmaria palmata*, respectively - for the extraction of sugars. The targeted sugars are rhamnose from *Ulva lactuca* and xylose from *Palmaria palmata*. These sugars of interest account for a significant part of the composition of the seaweed they are extracted from. The sugars extracted can be utilized in the production of furanics downstream, which can serve as platform chemicals for fuel production, thereby, those originating from a biological source and a more sustainable approach. Furthermore, other components of these seaweeds, such as their inorganic content and nitrogen contents were also checked that can add value to the cascaded approach.
The experimentation and research route is conducted systematically as follows.

- The preparation and characterization of samples the above biomass types for the analysis of its composition, to set up a reliable mass balance by understanding the effects of pretreatments like washing and solvent extraction on the determination of the content of key seaweed components.
- To perform and investigate various low severity hydrolysis techniques for the extraction of the desired monomeric sugars as the hydrolysate. In doing so, the objective is to obtain a less chemically intensive and more efficient route, thereby making the extraction more sustainable. This is aimed to be done by the utilization of organic acids and alternatively, enzymes with the ability to target the desired sugars.
- The evaluation of the pre-extraction of other seaweed components (inorganics, minerals, protein), prior to hydrolysis will be carried out and the distribution of these components in the cascading and hydrolysis processes.
- From the outcomes of the above work, a suitable cascading approach will be set up for the biorefining treatment process of seaweed and validated at a 2L-3L scale.

2

Characterization of Seaweed

This chapter begins with an overview of the composition of *Ulva lactuca* and *Palmaria palmata* as well as the theoretical background of their characterization. The materials used in experiments and methods used in the analysis are dealt with in section 2.2. The results from the biochemical compositional analysis of the two seaweed types are discussed thereafter.

2.1. Theoretical background of seaweed composition

2.1.1. Composition of *Ulva lactuca* and *Palmaria palmata*

Macroalgae are a biomass source with a potential for the production of biofuels and biogas. Seaweed can especially be used for the development of a process for the extraction of carbohydrate sugars and their conversion to furanic products for application as fuels and other additives.

The green seaweed *Ulva lactuca* contains fibrous components, proteins lipids and minerals. The green colour is a result of chlorophyll *a* and *b*. The monosaccharide carbohydrates are primarily glucose, rhamnose and xylose, amongst others. In addition to cellulose, the cell walls are composed of a polysaccharide ulvan which contributes up to 36% of the dry weight of the biomass [9]. Ulvan is a sulphated polysaccharide whose main constituents are rhamnose, xylose, glucuronic acid and iduronic acid. These generally occur as repeating disaccharide units, the major ones being ulvanobiuronic acid 3-sulfate (A_3S) designated as type A and ulvanobiuronic acid 3-sulfate (B_3S) designated as type B as seen in figure 2.1. These are sulphated rhamnose linked to a uronic acid, which is glucuronic acid in the type A ulvanobiuronic acid and iduronic acid in the type B ulvanobiuronic acid. Sometimes, in the place of these uronic acids, partially sulfated xylose can be present [10], [11]. In figure 2.1, the minor repeating disaccharide units are ulvanobioses U_3S and $U'_2,_3S$ wherein the uronic acids are replaced by xylose and sulphated xylose, respectively. It can thus be expected that on hydrolysis of the ulvan polysaccharide, rhamnose, xylose, glucuronic acid and iduronic acid would be the major components in the hydrolysate. Furthermore, as cellulose is also a predominant polysaccharide in *Ulva lactuca*, glucose is another major component that can be expected in the hydrolysate. Henceforth, in this study, attention will be paid to these compounds as the key carbohydrates extracted from *Ulva lactuca*.

The red seaweed *Palmaria palmata* contains crude protein, high amounts of carbohydrates and some lipids. It also has mineral constituents like potassium, sodium and chlorine, amongst others. The cell walls primarily comprise of xylans, cellulose and floridoside [12]. On hydrolysis of this seaweed type, xylose and glucose are the expected carbohydrates to be detected from the hydrolysis of the xylan and cellulose, respectively and galactose and glycerol from the floridoside. In this study, attention will be paid tp these compounds and they will be referred to as the key carbohydrates from the hydrolysis of *Palmaria palmata*.

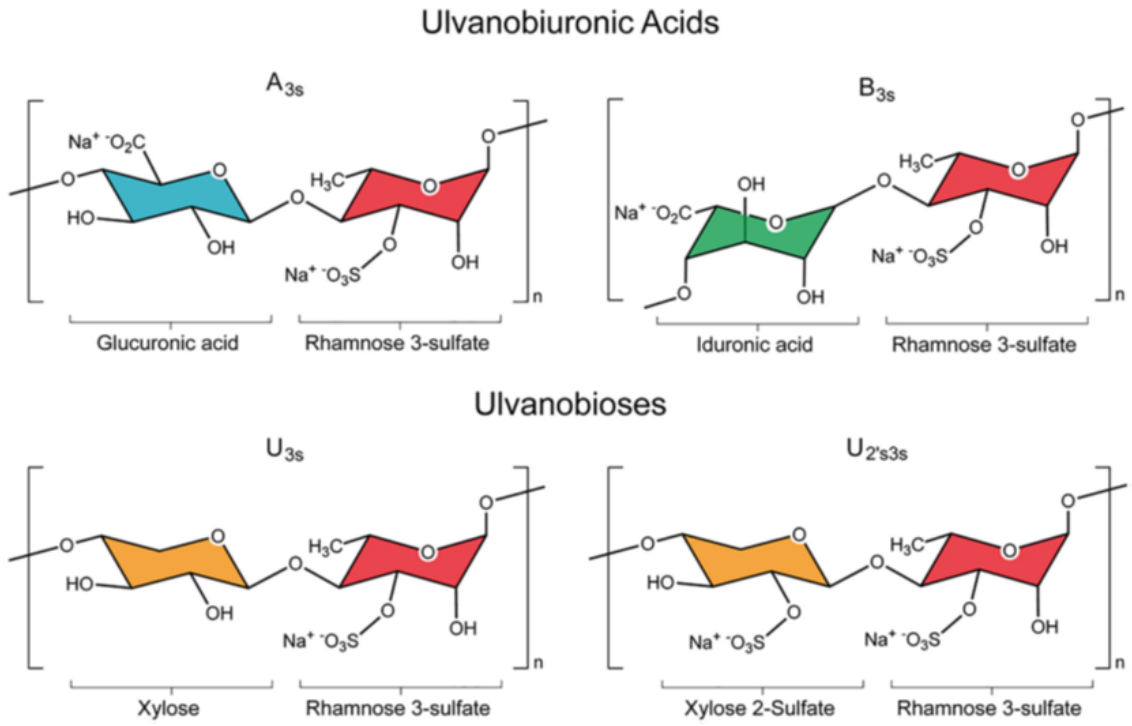


Figure 2.1: Structure of the main repeating disaccharides in polysaccharide ulvan, present in *Ulva*: ulvanobiuronic acids A_3S and B_3S and ulvanobioses U_3S and $U'_2,3S$ [9].

2.1.2. Determination of carbohydrate content in seaweed

Determination of the biochemical composition of seaweed is herein referred to as the determination of a spectrum of carbohydrates extracted from seaweed, by hydrolysis. extraction yield of carbohydrates can be influenced by biomass pre-treatment, particle size, temperature and duration of extraction and the kind of extractants used. The two types of seaweed are expected to contain some slats as inorganics. The reduction of salt content, prior to extraction has been found to enhance the extraction efficiency of carbohydrates. For this, a pre-treatment step of washing the seaweed with water can remove inorganics thereby causing lower interferences in carbohydrate extraction and the osmotic shock from water can also increase exposure of cell wall components of the seaweed to the extractant [9]. This is especially important as most of the rhamnose and xylose in *Ulva lactuca* and xylose in *Palmaria palmata* are present in the cell wall polysaccharides. This washing step can be carried out with demineralized water at room temperature as it can effectively remove salts from seaweed, with minimized temperature and time inputs [13].

The polysaccharides in seaweed are found bound to other components like lipids and pigments. In order to enhance the availability of these polysaccharides for their hydrolysis to obtain the targeted carbohydrates, these lipids and pigments can be removed by another pre-treatment step. Solvents like ethanol and acetone have been found to remove these lipids and proteins from seaweed as they are alcohol soluble [14], [15]. Soxhlet extractions performed on seaweed with these solvents can lead to removal of lipids and pigments.

Finally, the extraction of the targeted seaweed carbohydrates by hydrolysis of the polysaccharides are brought about by acid hydrolysis with sulphuric acid. For the adequate determination of the indi-

vidual carbohydrate content in *Ulva lactuca* and *Palmaria palmata*, the procedure put forth by Huijgen et al. is adopted as these are specific to the seaweed used. The method uses the High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) approach for the detection of monosaccharides. Hydrolysis is performed in two steps and the optimum hydrolysis time is specific to the seaweed [16].

2.2. Experimental Methods and Materials

This section entails a description on the materials and experimental approach employed.

2.2.1. Materials

The raw materials in the study are *Ulva lactuca* and *Palmaria palmata* which are green and red seaweeds, respectively. They were supplied from seaweed cultivation farms in Texel and Ireland in 2019, respectively. The seaweeds were obtained in cut and pre-dried form. Milling apparatus were utilised for further milling of the dry seaweed in order to obtain the required particle size for analytical characterization. Before milling, the *Palmaria palmata* was dried in an air-flow oven at 60°C.

(a) *Ulva lactuca* as received (dry flakes). (b) *Palmaria palmata*, pre-dried and milled to 1.5mm.

Figure 2.2: Raw material

Analyses were done in single replicates, unless otherwise specified. Demineralised water was used for the washing of the seaweed. A roller bank and centrifuge were used where mixing followed by solid residue and supernatant separation was required. The Soxhlet extractions were carried out using ethanol and acetone as solvents, with varying concentrations. For the preparation of the ethanol and acetone solvents, demineralised water was used. The setup for the Soxhlet extractions included the characteristic glassware, cellulose thimbles, a magnetic stirrer and a vacuum oven. An air-flow oven and a freeze-drier were utilized for drying of solid samples and glassware, when required. A Halogen Analyser was used for the rapid measurement of moisture content of various seaweed samples. For the biochemical compositional analysis, the hydrolysis was brought about by 72% w/w or 12M sulphuric acid. A vacuum pump was used to aid the filtration process for recovering any solid residue post hydrolysis. The measurement of monomeric carbohydrates in the liquor after hydrolysis was carried out by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

2.2.2. Pre-treatment and Extraction

For the determination of the composition of seaweed samples and extractives, certain treatments and extractions were carried out as seen in Figure 2.3. In order to assess the effect of each of these treat-

ment steps on the sugar quantification, sampling was done on the feed as well as on the extracts and residues of each step to obtain the composition of various seaweed constituents that may be extracted, lost or retained in the process.

Firstly, the seaweeds were milled to acquire a 0.2mm particle size for reaction. For the *Ulva lactuca*, this was done in two subsequent steps wherein it was first milled to <1.5mm and then to <0.2mm, to avoid heating up of the biomass and blocking of the milling system. In the case of the *Palmaria palmata*, an additional milling step to 0.5mm in between was added to avoid heating up and cluster formation of the biomass. Furthermore, it was dried for a day in an air-flow oven at 60°C, prior to milling wherein 10% of the moisture was removed. The moisture content of the milled feedstock was determined gravimetrically by drying in an air-flow oven at 105°C for 24 hours and the ash (inorganics) content by oxidation of the dried sample at 550°C for 6 hours in an electrical muffle furnace. The detailed procedure for the moisture and ash determination can be found in A.1

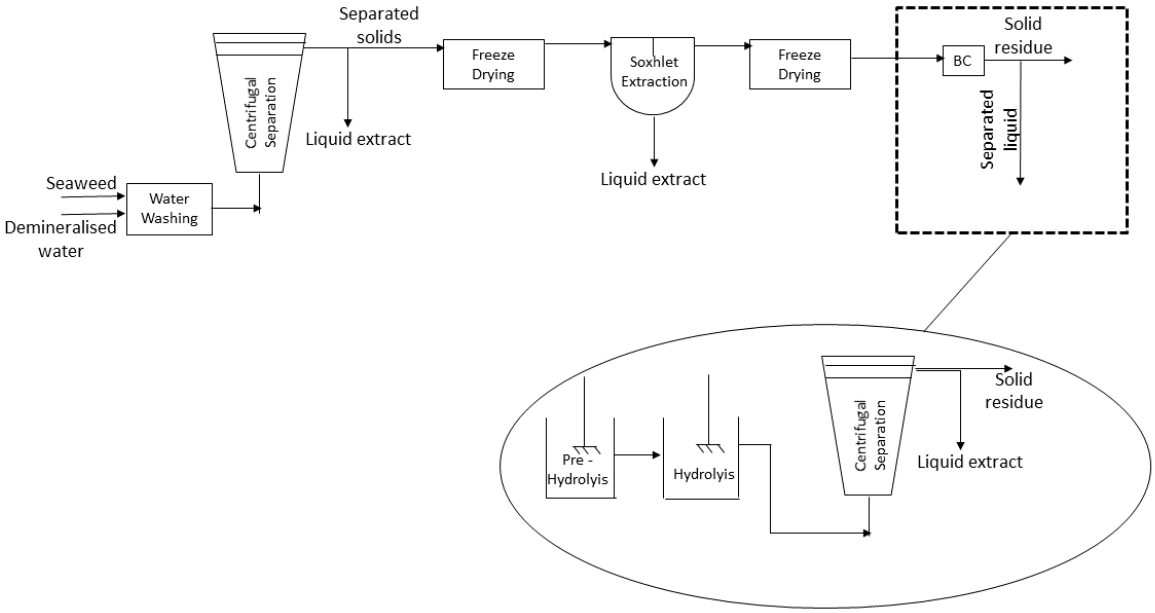


Figure 2.3: Block diagram of the treatment process for determination of seaweed composition.

100g of the seaweed sample was washed with demineralized water at room temperature. This treatment step is carried out in order to remove salts and water-soluble components present in the feedstock. This is especially applicable in biorefining of biomass for energy as the primary components of interest here are the organics like seaweed carbohydrates. For this treatment step, a consistency or loading factor of 10 (water to dry biomass ratio is 10:1 (v/w)) was chosen, similar to the works by Magnusson et al. in [13]. The container with the slurry mixture was placed on a roller bank for 1 hour for thorough mixing. The liquid was then separated as supernatant by centrifugal separation of the slurry at 4000 rpm for 20 minutes. The recovered solid was freeze-dried. The moisture and ash content of the dry solids were determined gravimetrically. Inductively Coupled Plasma (ICP) spectrometry and elemental analysis were also conducted to determine ionic and atomic (Na, Ca, K, S) content and other elemental (C, H, N) content, respectively.

An additional extraction step was performed on the solids obtained after freeze-drying, aiming to remove non-structural material that may cause interference in later analysis. This soxhlet pre-extraction step was carried out in various combinations of solvents with water-washing as seen in table 2.1, therefore a total of five soxhlet extractions per seaweed. The objective of experimentation with different combinations was to study the best fit for the treatment sequence, determined by the one that yields the highest quantity of the targeted sugars for the respective seaweeds. Through this treatment step, water-soluble as well as ethanol and acetone soluble material in the biomass may be removed,

such as inorganic and nitrogenous materials, non-structural materials, pigments, waxes and lipids and other minor components. The kind of solvent required may be depended on the type of seaweed, in the sense that some may not benefit in terms of quantification of sugars. The protocol followed for the soxhlet extractions can be found in appendix A.2. The solids are freeze-dried before further treatment.

Code	Sample	Solvent
WW/EE-100	water-washed	ethanol
WW/AE-100	water-washed	acetone
WW/EE-60	water-washed	60% ethanol
WW/AE-60	water-washed	60% acetone
EE-60	unwashed	60% ethanol

Table 2.1: Combination of solid and solvent for soxhlet extractions of seaweed type

For the determination of the biochemical composition of the two seaweed types, they were acid-hydrolysed as per the procedure put forth by Huijgen et al. in [16]. This was carried out on all dried the pre-extracted solids as well as on the untreated biomass. The hydrolysis was carried out with 12M H_2SO_4 in two stages:

1. Pre-hydrolysis at 30°C for 1 hour, 12M H_2SO_4.
2. Hydrolysis at 100°C for 2 or 3 hours, after dilution.

The optimum hydrolysis time for *Palmaria palmata* and *Ulva lactuca* are 2 hours and 3 hours, respectively [16]. The pre-hydrolysis of the seaweed is aimed at hydrolysing the structural polymeric carbohydrates into oligomeric carbohydrates which are further broken down during the hydrolysis step into their monomeric forms, making them detectable. Pre-hydrolysis is also required in order to facilitate the adequate hydrolysis of structural components like ulvan and cellulose. Furthermore, the two-stage hydrolysis allows for the higher recovery of some unstable carbohydrates like uronic acids which would have a higher recovery at lower temperatures. This is especially significant in the *Ulva lactuca* owing to its high uronic acid content [16].

2.2.3. Seaweed Carbohydrate Measurements

The monomeric carbohydrates released by the hydrolysis of the seaweed were analysed by HPAEC-PAD. This was chosen over Gas Chromatography (GC) and High-Performance Liquid Chromatography (HPLC) techniques as it was required to distinguish between all individual seaweed constituents and detect their concentrations. The sugars detected are seen in table 2.2. The liquors from the water-washing of the seaweeds were post hydrolysed with 2M H_2SO_4 at 100°C for 2 hours. The post hydrolysis of the liquors was carried out to facilitate the conversion of the sugars from oligomers to monomers which could be detected in the seaweed carbohydrate HPAEC analysis to obtain the total sugar content. The procedure for the post hydrolysis of liquors can be found in A.3. The extractives from the soxhlet pre-extraction were analysed both, directly (oligomeric content) and also after they were post hydrolysed (total sugar content).

2.3. Results and Discussion

This section begins with the results of the water-washing and soxhlet extraction treatment steps, followed by that of the biochemical composition tests.

2.3.1. Water washing of *Ulva lactuca* and *Palmaria palmata*

The washing of the seaweed resulted in the removal of sugars to different extents in *Ulva lactuca* and *Palmaria palmata*. The total seaweed carbohydrates in the liquor recovered from the water-washing for the two seaweeds are represented in table 2.4, in the form of a distribution into the various sugar

Carbohydrate group	Monomeric carbohydrates
Sugar alcohols	Glycerol Mannitol
Deoxy sugars	Fucose Rhamnose
Reducing sugars	Arabinose Galactose Glucose Xylose
Uronic acids	Galacturonic acid Guluronic acid Glucuronic acid Mannuronic acid

Table 2.2: Analysis of monomeric carbohydrates by HPAEC-PAD

categories that the detected sugars are classified under as in table 2.2. These are the total sugar contents of the liquors as they were post-hydrolysed before analysis as mentioned in section 2.2.3.

Sugar	Content (% dw *Ulva lactuca*)
Glycerol	0.13
Galactose	0.10
Glucose	0.59
Xylose	0.39
Rhamnose	2.026
Glucuronic acid	0.56
Total	3.78

Sugar	Content (% dw *Palmaria palmata*)
Glycerol	7.72
Mannitol	0.38
Fucose	0.06
Rhamnose	0.07
Galactose	13.63
Glucose	0.76
Xylose	12.62
Mannuronic acid	0.45
Total	35.72

Table 2.3: Carbohydrate content of the liquor from the water-washing of *Ulva lactuca* and *Palmaria palmata* in terms of dry matter of seaweed (dw).

In the case of *Ulva lactuca*, 3.8% of the dry matter of the seaweed was removed as sugars, the largest fraction being the deoxy sugars. This is owing to the removal of rhamnose, which is a major constituent of the ulvan that is present in green seaweeds[17]. 35.7% of the dry matter of *Palmaria palmata* was removed as sugars during the water washing treatment step. From Figure 2.4, we see that of this amount, 27.03 % and 8.1% are reducing sugars and alcohol sugars, respectively. The reducing sugars released here in galactose and xylose, resulting from the high floridoside and xylan contents in *Palmaria palmata*. Glycerol, an alcohol sugar, is also released here in significant amounts, again, owing to the floridoside present in this type of seaweed [12] [18].

The results from the characterization of the organic and inorganic elemental composition of the washing step original seaweed material as well as the solids post the washing step, are presented in Figure 2.5. All results here, are presented on the dry weight basis of the respective solids.

The ash content was estimated, from which the organic content (OC) was determined as OC% = 100 - ash%[19]. This treatment step resulted in a significant reduction in the inorganic (ash) content of the biomass. In the case of the *Ulva lactuca*, there was a reduction in ash content from 28.05% to 18.8% and in the case of the *Palmaria palmata*, the ash content dropped to 15.42% from that of original 20.07% in the starting material. The organic content of the raw material and the washed solid, respectively was thus 71.95% and 81.21% for the *Ulva lactuca* and 79.83% and 84.58% for the *Palmaria palmata*. This is analogous to the increase in the carbon content from 30.09% to 34.64% and the hydrogen content from 4.251% to 5.206%, as seen in Figure 2.5a and similarly, to the increase from

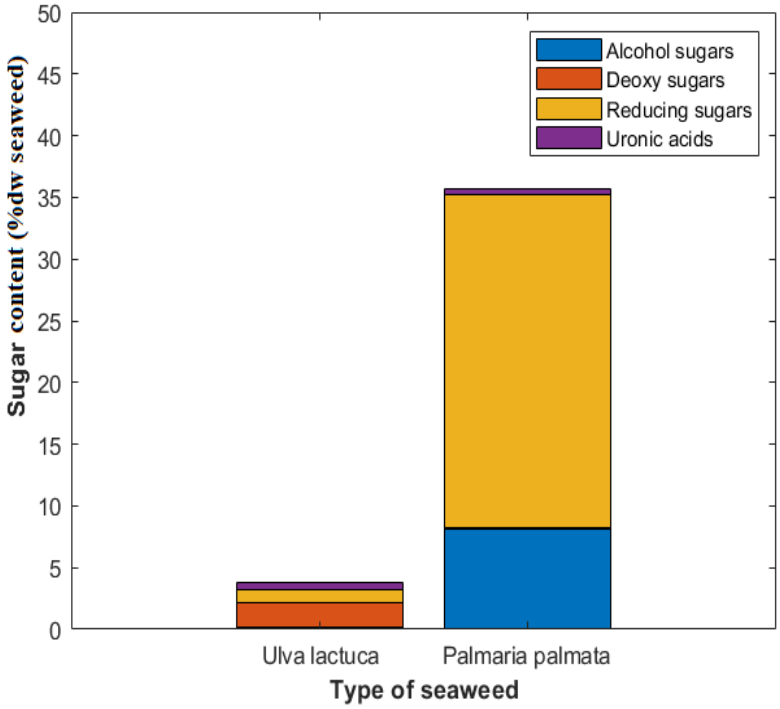


Figure 2.4: Seaweed carbohydrates in the washing liquor of *Ulva lactuca* and *Palmaria palmata*.

37.84% to 42.51% and 4.82% to 5.636% as observed in Figure 2.5b. As expected, the removal of salts and inorganic materials from the biomass is vividly observed by the reduction of the potassium and sodium contents through the washing, in both seaweed types. There is also a slight fall in the sulphur content from 6.19% to 4.84% in the *Ulva lactuca* whereas, in the case of the *Palmaria palmata*, the decrease is not so significant. This is expected as sulphur in *Ulva lactuca* is present as SO_3 groups in the water-soluble polysaccharide, ulvan.

Another interesting aspect is the increased nitrogen content as a result of the washing. Considering a protein conversion factor (k) of 5.65 for *Ulva lactuca* and 4.7 for *Palmaria palmata*, the protein content was estimated as N*k [20]. They are reported in table 2.4.

From the results of the ultimate analysis, the higher heating values (HHV) were estimated using the correlation deduced by Channiwala and Parikh. It is given by equation 2.1 [21]. The washing resulted in a 25.93% HHV increase in the *Ulva lactuca* and a 19.04% HHV increase in the *Palmaria palmata*.

$$HHV = 0.3491C + 1.1783H + 0.1005S - 0.1034O - 0.0151N - 0.0211A(MJ/kg) \tag{2.1}$$

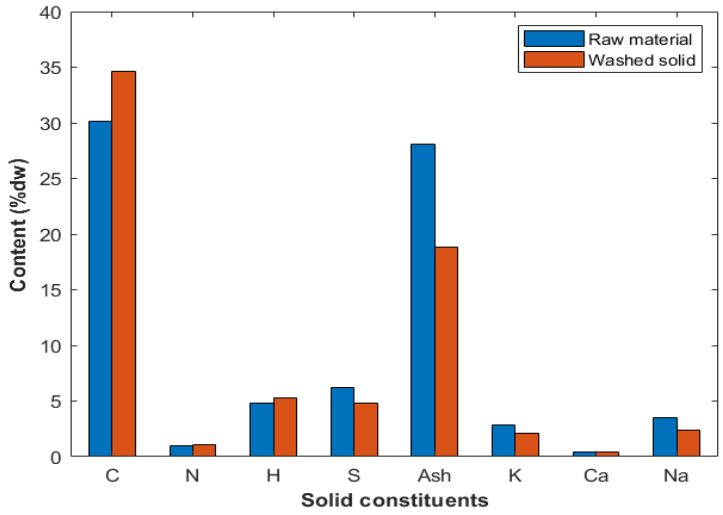
The oxygen content for HHV was estimated as in equation 2.2 [22].

$$O = 100\% - (C + H + N + S + Ash) \tag{2.2}$$

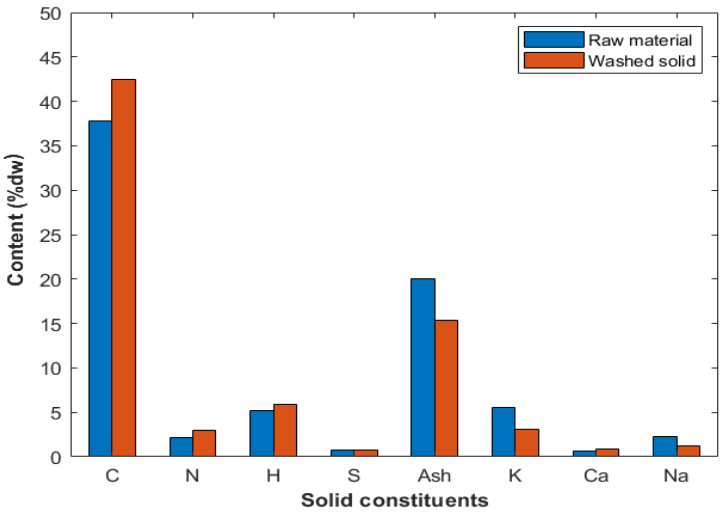
The overall mass recovery and solid yield of the water-washing treatment on the seaweeds, respectively are 103.21% and 82% for *Ulva lactuca* and 99.99% and 52% for *Palmaria palmata*.

		Ulva lactuca	***Palmaria palmata***
k		5.65	4.7
Protein content (%)	**Unwashed**	5.66	9.99
	Washed	6.28	14.16

Table 2.4: Protein content in seaweed before and after water-washing



(a) Organic and inorganic constituents of *Ulva lactuca*.



(b) Organic and inorganic constituents of *Palmaria palmata*.

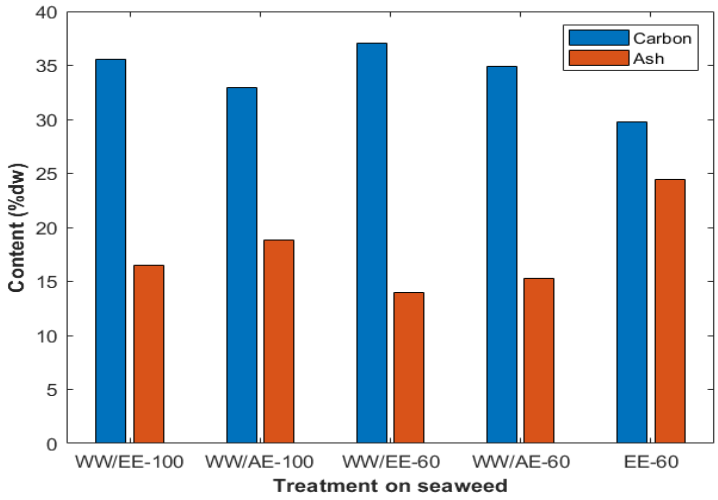
Figure 2.5: Results from the characterization of the seaweed solids prior to and post the water-washing treatment step.

2.3.2. Soxhlet extraction of *Ulva lactuca* and *Palmaria palmata*

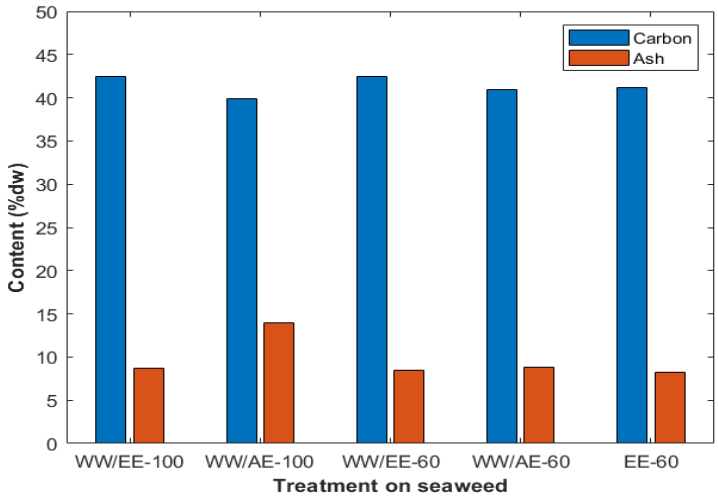
The soxhlet extractions on the washed as well as unwashed seaweeds with different solvents, resulted in sugar extraction in extremely low quantities. HPAEC analysis of the direct sugars could not detect any sugars in the extracts of both the seaweeds as they were either absent or present in very low amounts that were below the detection limits. The lower detection limits were 25 mg/kg of extract for all deoxy sugars and reducing sugars and 60mg/kg of extract for the uronic acids. On HPAEC analysis of the post hydrolysed extractives, all sugars except for galactose and glucose were again below detection limits. Hoer, these too were released in small quantities (see Appendix B).

The carbon and ash contents of the solids recovered from the extraction, post freeze-drying are presented in Figure 2.6. Freeze-drying is beneficial in the preservation of seaweed components [23], [24]. In the case of *Ulva lactuca*, it is clear that the pre-treatment steps resulted in the enhancement of the quality of the material as observed in Figure 2.6a. The carbon content, expressed as a percentage of the dry, pre-extracted seaweed, was the lowest when extraction with 60% ethanol was performed on the unwashed *Ulva lactuca*. Moreover, the ash content of this solid was determined to be the highest

(24.43%) amongst all the extracted solids. Amongst the four soxhlet extractions performed on the pre-washed sample, the ones with ethanol (100% and 60%) as the solvent resulted in solids with a higher carbon and lower ash content, when compared to the corresponding solids wherein acetone (100% and 60%) was used as the solvent for extraction. The extraction, when performed on pre-washed Ulva lactuca with 60% ethanol, yielded a solid with the highest carbon and lowest ash contents of 37.01% and 13.97%, respectively. It is noteworthy that the ash contents of the extracted solids are related to the polarity of the solvents [25] [26]. Water, being the most polar results in the pre-washed solids having the lowest ash contents than the directly extracted solid. Furthermore, the 60% ethanol and 60% acetone extractions on the pre-washed solids result in greater extents of ash removal than the extractions with 100% ethanol and 100% acetone.



(a) Carbon and ash contents of *Ulva lactuca*, post soxhlet extraction.



(b) Carbon and ash contents of *Palmaria palmata*, post soxhlet extraction.

Figure 2.6: Results from the characterization of the seaweed solids recovered from the soxhlet extraction treatment step. In the figure, 'WW' represents the solids that pre-washed with water, prior to soxhlet extraction; 'EE-100' and 'EE-60' represent extractions with ethanol (pure) and ethanol (60%), respectively; 'AE-100' and 'AE-60' represent extractions with acetone (pure) and acetone (60%), respectively;

In the case of the *Palmaria palmata*, it was the two extractions with ethanol (pure and 60%) on the pre-washed seaweed again, which yielded a solid with the highest carbon content as seen in Figure 2.6b. However, the extraction on the unwashed sample with 60% ethanol solution resulted in a solid with a similar quality to that on the pre-washed seaweed sample (C = -3.27% and ash = +2.24%). The

extraction with pure acetone which is the least polar solvent, results in a solids with the highest ash content.

The mass recovery and solid yield for each of the extractions, calculated from experimental data, is represented in table 2.5.

Extraction	Recovery (%)	Solid Yield (%)
washed/ ethanol (100%)	91.98%	96.50%
washed/ acetone (100%)	95.29%	97.59%
washed/ ethanol (60%)	98.93%	86.31%
washed/ acetone (60%)	97.32%	92.90%
unwashed/ ethanol (60%)	97.62%	82.37%

(a) Experimental mass recovery and solid yield of soxhlet extractions on *Ulva lactuca*

Extraction	Recovery (%)	Solid Yield (%)
washed/ ethanol (100%)	98.34%	83.72%
washed/ acetone (100%)	97.21%	98.13%
washed/ ethanol (60%)	98.27%	76.33%
washed/ acetone (60%)	80.19%	81.44%
unwashed/ ethanol (60%)	97.37%	57.67%

(b) Experimental mass recovery and solid yield of soxhlet extractions on *Palmaria palmata*

Table 2.5: Experimental mass recovery and solid yield of soxhlet extractions

2.3.3. Biochemical composition of *Ulva lactuca* and *Palmaria palmata*

In Figure 2.7 the sugars extracted by acid hydrolysis are represented on the dry weight basis of the starting biomass material. The biochemical compositional analysis on *Ulva lactuca* resulted in the extraction of slightly higher compositions of the significant sugars in the pre-treated material when compared to the untreated material. In the case of extractions with the ethanol-water and acetone-water mixtures, there was a slightly higher recovery of rhamnose and lower recovery of glucose when compared to extractions with pure ethanol and acetone. In the case of *Palmaria palmata*, acid hydrolysis on the untreated material and the unwashed, pre-extracted material results in the recovery of 49.01% and 36.4% of the significant sugars, respectively. The lower recovery of sugars through acid-hydrolysis of the washed and pre-extracted material as seen in 2.7b is lower here owing to the high quantities of reducing sugars that were removed through the initial water-washing and solvent extraction pre-treatment steps.

The biochemical composition analysis on the various pre-treated and the untreated seaweeds were each performed in duplicate, in order to obtain more reliable compositional results. Table 2.6 and table 2.7 gives the coefficient of variation between the duplicates for each biochemical composition test of *Ulva lactuca* and *Palmaria palmata*, respectively.

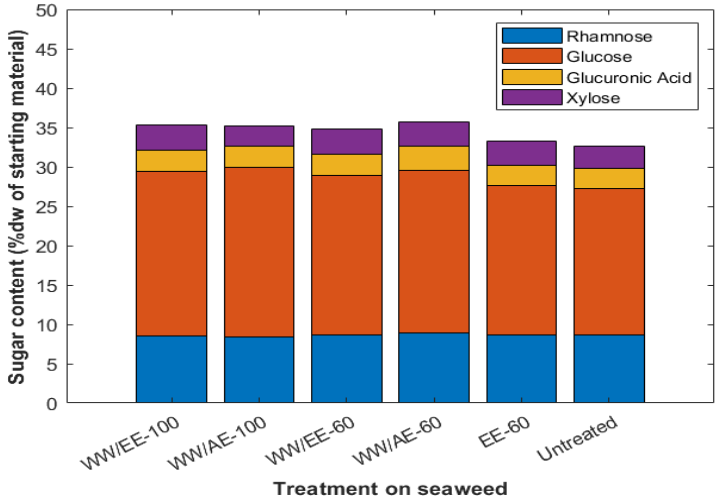
Pre-treatment	Coefficient of variance			
	Rhamnose	Glucose	Xylose	Glucuronic acid
Washed/ ethanol-100	1.21	1.6	5.41	0.31
Washed/ acetone-100	0.80	1.97	0.96	0.23
Washed/ ethanol-60	0.88	4.46	0.64	0.26
Washed/ acetone-60	7.05	4.06	0.69	13.48
Unwashed/ ethanol-60	1.22	0.07	0.01	1.308
Untreated	0.01	1.58	4.81	1.55

Table 2.6: Coefficient of variance for biochemical composition tests, shown for the significant sugars in *Ulva lactuca*.

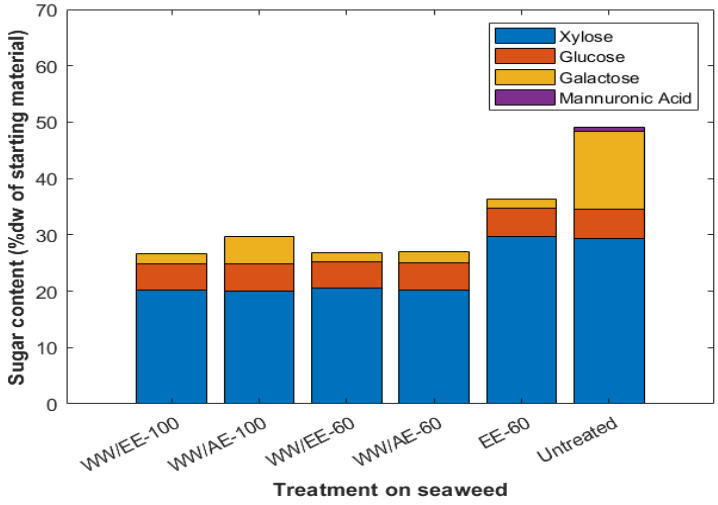
Pre-treatment	Coefficient of variance			
	Galactose	Glucose	Xylose	Mannuronic acid
Washed/ ethanol-100	2.93	3.98	0.54	0.14
Washed/ acetone-100	3.54	3.62	1.12	NA
Washed/ ethanol-60	0.41	0.32	0.86	141.42
Washed/ acetone-60	2.40	2.38	3.74	3.48
Unwashed/ ethanol-60	5.40	6.32	2.72	12.24
Untreated	0.97	1.34	0.19	9.66

Table 2.7: Coefficient of variance for biochemical composition tests, shown for the significant sugars in *Palmaria palmata*.

The seaweed carbohydrates measured by HPAEC analysis of the liquors are presented herein.



(a) Seaweed carbohydrates from the biochemical composition by acid hydrolysis tests of *Ulva lactuca*.

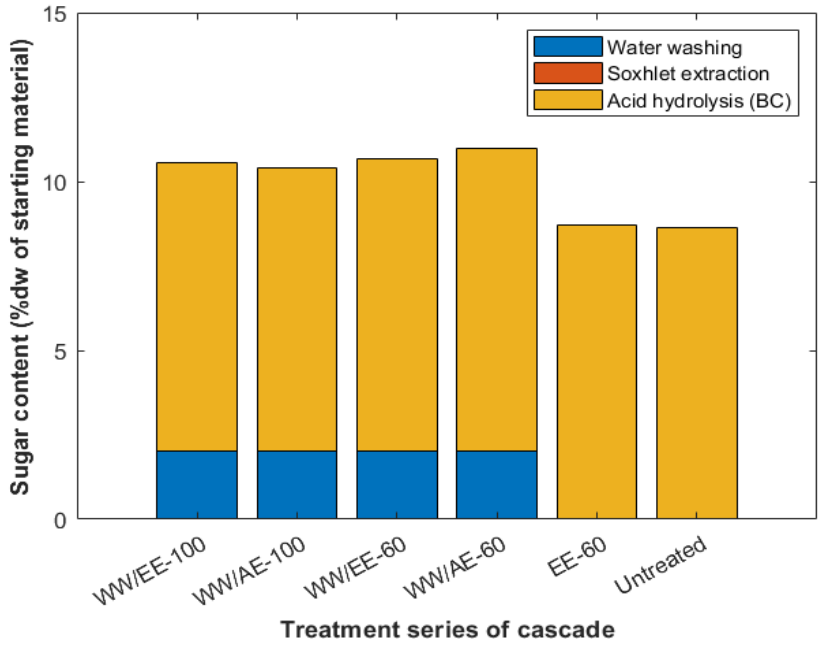


(b) Seaweed carbohydrates from the biochemical composition by acid hydrolysis tests of *Palmaria palmata*.

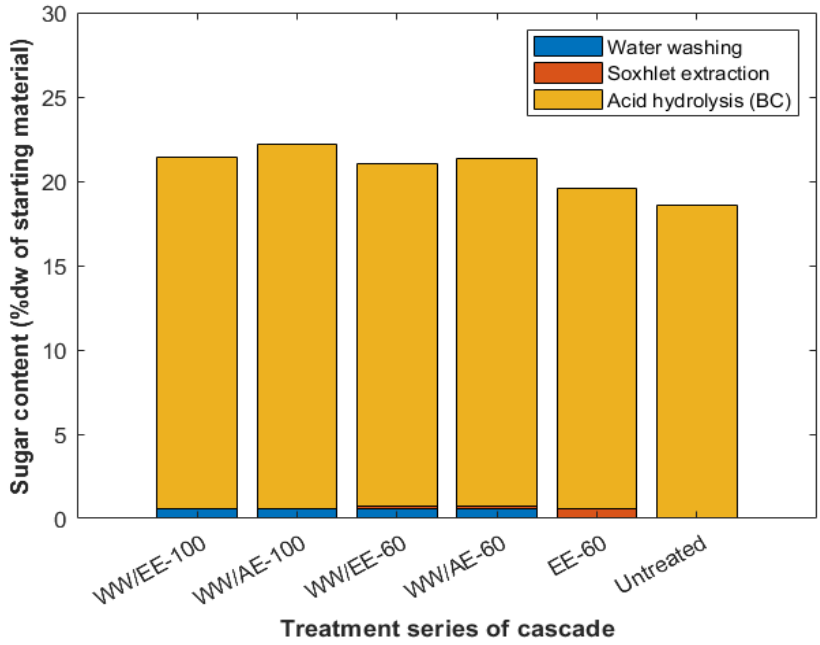
Figure 2.7: Results from the HPAEC analysis of the liquors of the biochemical composition tests on *Ulva lactuca* and *Palmaria palmata*.In the figure, 'WW' represents the solids that pre-washed with water, prior to soxhlet extraction; 'EE-100' and 'EE-60' represent extractions with ethanol (pure) and ethanol (60%), respectively; 'AE-100' and 'AE-60' represent extractions with acetone (pure) and acetone (60%), respectively;

2.3.4. Cascading of pre-treatment and extraction

Previously, the results from the acid-hydrolysis of seaweed with various pre-treatment combinations were presented. Herein, a representation of the overall treatment series is discussed in order to realize the best treatment series for each seaweed type. For this, the accumulative contents of key sugars in the water-washed liquor, the solvent extracts as well as from acid hydrolysis are considered.

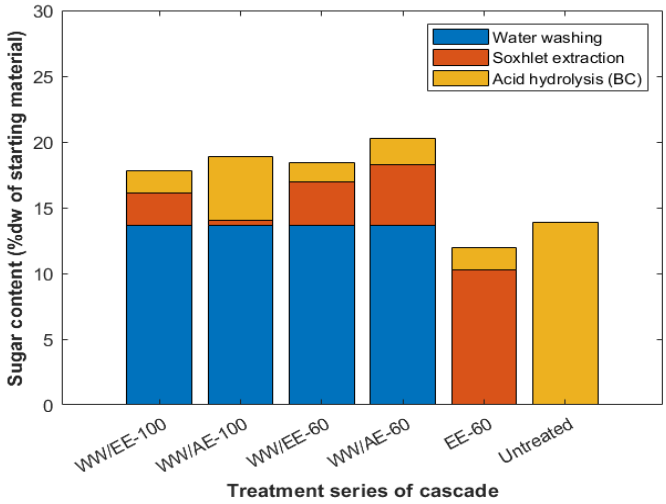


(a) Rhamnose yield from *Ulva lactuca* for the cascading approach.

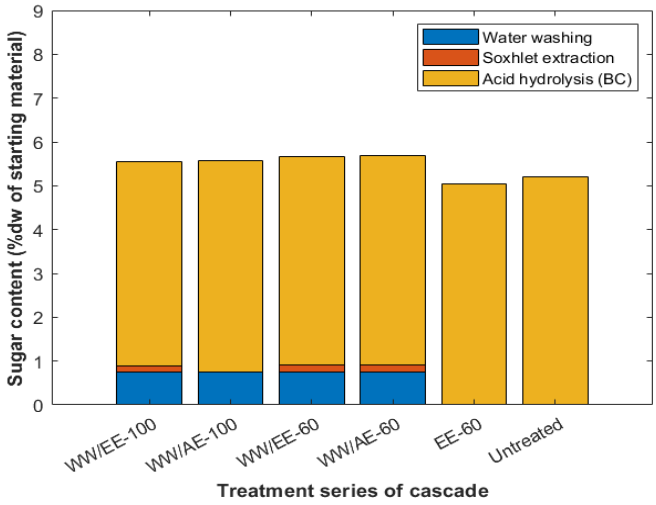


(b) Glucose yield from *Ulva lactuca* for the cascading approach.

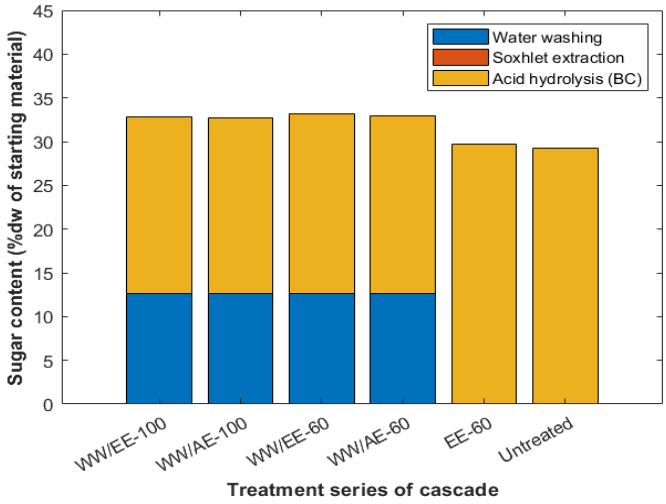
Figure 2.8: Representation of sugar contents of all treatment series for *Ulva lactuca*.In the figure, 'WW' represents the solids that pre-washed with water, prior to soxhlet extraction; 'EE-100' and 'EE-60' represent extractions with ethanol (pure) and ethanol (60%), respectively; 'AE-100' and 'AE-60' represent extractions with acetone (pure) and acetone (60%), respectively;



(a) Galactose yield from *Palmaria palmata* for the cascading approach.



(b) Glucose yield from *Palmaria palmata* for the cascading approach.



(c) Xylose yield from *Palmaria palmata* for the cascading approach.

Figure 2.9: Representation of sugar contents of all treatment series for *Palmaria palmata*.In the figure, 'WW' represents the solids that pre-washed with water, prior to soxhlet extraction; 'EE-100' and 'EE-60' represent extractions with ethanol (pure) and ethanol (60%), respectively; 'AE-100' and 'AE-60' represent extractions with acetone (pure) and acetone (60%), respectively;

From figure 2.8a, the cumulative rhamnose yield from the series of the pre-washed and extracted *Ulva lactuca* is higher than that from the untreated as well as from the unwashed and extracted solids. The rhamnose yield from the four series involving pre-washing and pre-extraction treatment steps are comparable but are slightly higher in the case of soxhlet extractions with 60% ethanol and 60% acetone as solvents. 10.67% and 10.97% rhamnose is extracted from these two treatment series, respectively. These also have a slightly lower glucose yield than the series' wherein pure ethanol and acetone are used as solvents for the soxhlet extraction treatment stage. As rhamnose is the sugar of interest in this work, the extractions with 60% ethanol and 60% acetone on pre-washed solids are deemed more suitable for the characterization of *Ulva lactuca*.

In the case of the *Palmaria palmata*, the galactose, glucose and xylose contents are each higher for the series of the pre-washed and extracted *Palmaria palmata* than that of the untreated as well as the unwashed and extracted solids, as seen in figure 2.9. Again, the xylose yield from the four series involving pre-washing and pre-extraction treatment steps are comparable but are slightly higher in the case of soxhlet extractions with 60% ethanol and 60% acetone as solvents. 33.09% and 32.82% xylose is extracted from these two treatment series, respectively. As xylose is the sugar of interest for this seaweed, the extraction with 60% ethanol on the pre-washed solid is deemed more suitable.

2.4. Conclusions

Water-washing is clearly a beneficial pre-treatment step in the extraction of sugars from *Ulva lactuca* and *Palmaria palmata*. It enhances treatability of the seaweed by removal of inorganics, seen as a reduction in the ash as well as K and Na contents. The soxhlet extraction treatment step also enhanced the quality of the biomass, lowering its ash content. The polarity of the solvent influenced the extent of ash removal through the soxhlet extraction, therefore the lowest ash content is determined in the solid treated with 60% ethanol. For this reason, this solvent is selected for the cascading approach. Furthermore, the coefficient of variance of the HPAEC analysis of rhamnose and xylose in the hydrolysates from the acid-hydrolysis of *Ulva lactuca* and *Palmaria palmata*, respectively, were lower for the series involving 60% ethanol pre-extractions on pre-washed solids when compared to that involving 60% acetone. Thus, pre-washing of the seaweed with water, followed by a soxhlet extraction with 60% ethanol as solvent, prior to acid-hydrolysis is the selected approach for the characterization of the seaweeds in order to guarantee highest rhamnose recovery from *Ulva lactuca* and highest xylose recovery from *Palmaria palmata*.

3

Extraction of valuable monosaccharide sugars from *Ulva lactuca* and *Palmaria palmata*

3.1. Low severity hydrolysis for carbohydrate extraction from seaweed

3.1.1. Potential of extraction

From literature, we see similarities between red and green seaweed groups in terms of characteristics of their water-soluble and insoluble components. The architecture of their cell walls is similar to that of land plants in the sense that structural support is provided by cellulose [27]. However, the absence of lignin in these seaweeds, as opposed to land plants and thereby low cellulose contents allow the polysaccharides in these seaweeds to be easily hydrolysed [28]. Owing to this, less energy-intensive and low severity hydrolysis techniques are applied in this study.

3.1.2. SOTA extraction of sugars from *Ulva lactuca* and *Palmaria palmata*

A generally accepted biorefinery approach for the extraction of carbohydrates from seaweed, specifically rhamnose from *Ulva lactuca* and xylose from *Palmaria palmara* is aimed to be put forth. A cascade of treatments with the objective of maximising the extraction of these sugars from their respective seaweed type is lacking. The U.S. National Renewable Agency Laboratory (NREL) has put forth protocols for the determination of carbohydrates from algal biomass which is based on hydrolysis by inorganic acids. The design of the process for dilute acid enzymatic deconstruction of biomass is customised to lignocellulosic feedstock [29], [30], [31]. The investigation of such low severity methods for macroalgae is approached in this study. The evaluation of the various routes and their treatment conditions are based on the factors of high sugar yield, selectivity in terms of sugar release and above all, extraction without extensive hydrolysis.

Enzymatic hydrolysis was carried out with a selection of enzymes designed to target xylanse, rhamnosidase or cellulase as these were the major components in the two types of seaweeds in this study. It was also found from literature that chelation by agents like sodium citrate with the ability to liberate certain polysaccharides wherein chelation is brought about by uronic acids and sulphated groups [32], [33]. Conventionally, chelation of the ions that crosslink ulvan strands favour the extraction of ulvan [34], [35].

3.2. Extraction of sugars by enzymatic hydrolysis

This section describes the approach for the experimentation with various enzymes as hydrolysing agents as well as their operating conditions.

3.2.1. Methods and Materials

The starting material for this series of tests was dry, untreated *Ulva lactuca* and *Palmaria palmata*, milled to 1.5mm. In the case of the *Palmaria palmata*, as mentioned in 2.2.1, it was dried overnight at 60°C prior to milling. The experiments were carried out in Erlenmeyer flasks mounted in a shaking incubator operable in the temperature range of 50°C to 80°C. A centrifuge was used for the separation of the solid and liquid fractions at the time of sampling. A halogen moisture analyser was used for the measurement of the moisture contents of the seaweed samples at the start of the experiment. The measurement of seaweed carbohydrates was performed with the HPAEC-PAD. The Knick Portamess pH meter was used for pH measurement during the experiments.1M sodium citrate (pH 5), 1M sodium acetate (pH 5) and 1M sodium phosphate (pH 6) were the buffers used. 1M NaOH solution and 1M H_2SO_4 solution were used as required to adjust the reaction mixtures to the desired pH, when applicable. 2M H_2SO_4 was used for the post-hydrolysis of the liquid hydrolysates. Cellic® CTEC2 (CTEC2), hemicellulase from Aspergillus niger (HCAN), β-D-xylanase from *T.maritima* (XYTM), endo-1,4-β-D-xylanase from *Neocallimastix patriciarum* (XYNP), endo-1,4-β-D-xylanase from *Trichoderma viride* (XYTV) and α-L–rhamnosidase from a prokaryote (RHPR) were the enzymes used in this study. CTEC2 is a commercially available enzyme made by Novozyme, HCAN is produced by Sigma-Aldrich and the rest was procured from Megazyme. These were used without further treatment.

The procedure followed for enzymatic hydrolysis is outlined herein. All buffers were prepared at the temperature at which the hydrolysis reaction was to take place. The moisture contents of the seaweeds were determined in the halogen analyser. A liquid:solid ratio of 10 was chosen for the reaction mixture. 5 grams of the sample (on a dry weight basis) was weighed into the Erlenmeyer flask. The required amounts of the respective buffer and demineralized water was added. In tests wherein the initial pH of the reaction mixture needed to be altered to a specific value or range of values, 1M NaOH and 1M H_2SO_4 was added. The flask was tightly sealed and then placed in the shaking incubator, set to operate at 140 rpm and the desired reaction temperature. When the set temperature was attained in the shaking incubator, the respective enzyme solution was added and the flask was placed back in the incubator again at the same settings for 72 hours (or 48 hours, as required). This was recorded as the start of the reaction (t=0). Samples were made at different time intervals, as and when required for the test in concern. The samples made were immediately cooled in a water bath to stop hydrolysis and then run in a centrifuge at 3000rpm for 10 minutes. The separated hydrolysate was stored frozen until HPAEC analysis for carbohydrates, in order to prevent microbial contamination.

3.2.2. Screening of enzymes

For the hydrolysis by enzyme approach for the extraction of sugars, particularly xylose and rhamnose, six enzymes were chosen based on their monosaccharide sugar of target and their specific activities defined with respect to these monosaccharides. Table 3.1 summarizes the specific activity as stated by the supplier as well as the optimum temperature and pH for enzyme performance. The specific activity of an enzyme is quantified in units per mL or mg where a unit (U) is defined as the amount of enzyme required to release one µmole of the respective reducing sugar per minute. These optimal temperatures are specific to the application they were developed for, which are not seaweed. They are considered as reference points, for the determination of the optimal temperature and pH conditions for enzyme activity with *Ulva lactuca* and *Palmaria palmata* as substrates.

Enzyme	Specific activity	Optimum Temperature (C)	Optimum pH
Cellic CTEC2 (CTEC2)	1500 U/mL	50	5
Hemicellulase from Aspergillus niger (HCAN)	300 - 3000 U/g	40	4.5
β-D-xylanase from *T.maritima* (XYTM)	100 U/mL	80	5
endo-1,4-β-D-xylanase from *Neocallimastix patriciarum* (XYNP)	900 U/mL	50	6
endo-1,4-β-D-xylanase from *Trichoderma viride* (XYTV)	230 U/mL	50	4.5
α-L–rhamnosidase from a prokaryote (RHPR)	190 U/mL	50	6

Table 3.1: Enzymes for screening and their optimal conditions of operation. All data in the table is provided by the suppliers.

Hydrolysis of *Ulva lactuca* by each of the enzymes in table 3.1 was carried out as described in section 3.2.2. In the case of *Palmaria palmata*, all enzymes, except for RHPR, were screened to determine their hydrolysing potential for the extraction of xylose. The hydrolysates were analysed in the HPAEC-PAD, in order to detect carbohydrate concentrations in them.

3.2.3. Assessment of conditions for enzymatic hydrolysis

From the results of the initial enzyme screening tests, two enzymes were selected for further investigation. These were then subjected to different hydrolysis conditions like hydrolysis time, reaction temperature, pH and enzyme dosage. The initial screening was carried out at 50°C for 72 hours as this was the optimum temperature for four of the enzymes and within the temperature stability range of the other two enzymes as well. Sampling was done after 48 and 72 hours of hydrolysis in order to determine the temperature effect on sugar release. Thereafter, a series of tests were carried out for a range of temperatures with the selected enzymes, to determine the effect of hydrolysis temperature on the extent of sugar extraction from seaweed. For each enzyme, the effect of pH on sugar extraction, at a selected temperature was investigated. Finally, the results from these screenings were incorporated in a test series aimed at studying the effect that enzyme dosage has on the release of the key sugars in this study from their respective seaweeds. In this case, samples were made after 5, 24, 48 and 72 hours of hydrolysis and the hydrolysates were analysed in the HPAEC-PAD, both directly and after post-hydrolysis with 2M H_2SO_4.

3.3. Extraction of sugars by treatments with organic agents and water

This section describes the approach for the experimentation with various hydrolysing agents such as organic acids, chelating salts and how water as well as their operating conditions.

3.3.1. Methods and materials

The starting material for this series of tests was dry, untreated *Ulva lactuca* and *Palmaria palmata*, milled to 1.5mm. In the case of the *Palmaria palmata*, as mentioned in 2.2.1, it was dried overnight at 60°C prior to milling. The experiments were carried out in a multiclave, within 100mL vessels. Formic acid (0.15M), acetic acid (0.15M), oxalic acid (0.15M) and citric acid (0.15M) were the organic acids used for hydrolysis. Sodium citrate (0.15M) and sodium acetate (0.15M) solutions were used for testing treatments with chelating salts. The hot water treatments were carried out with demineralized water. A centrifuge was used for the separation of the solid residue from the liquid hydrolysate. A halogen moisture analyser was used for the measurement of the moisture contents of the seaweed samples at the start of the experiment. The measurement of seaweed carbohydrates was performed with the

HPAEC-PAD. The Knick Portamess pH meter was used for pH measurement at the start and end of the experiments. Crucibles were prepared for the determination of the moisture and ash contents of the solid residues as in appendix A.1.

Around 6.5g of the sample was weighed into the Teflon container that fits into the multiclave vessel. The calculated amount of the hydrolysing agent was then added into the container. This was either an organic acid or chelating salt solution or in the case of the hot water treatment tests, demineralized water. A solid to liquid loading ratio of 10 was chosen. The pH of the reaction mixture was checked before sealing the vessel shut and fitting it in the multiclave. The multiclave was set to operate at the required temperature and for the duration required. At the end of the reaction time, the vessel was cooled by air draft before opening it up and recording the weights of the contents and the pH of the slurry, post-treatment. The contents were then transferred into centrifuge tubes and run in a centrifuge at 3000rpm for 15 minutes in order to separate the liquid hydrolysate from the solid residue. These fractions were weighed to calculate the overall mass recovery as well as the solid yield. A small, representative fraction of the solid residue was weighed into a prepared crucible to determine the moisture and ash contents of the solid residue as in appendix A.1. A small part of the liquid hydrolysates were post hydrolysed as per the procedure in appendix A.3 and then analysed by HPAEC for the detection of carbohydrates.

3.3.2. Assessment of treatment conditions

For the investigation of the extent of extraction of the respective key sugars from *Ulva lactuca* and *Palmaria palmata* by various agents, tests were conducted to find the temperature and time effects on sugar yields. The treatments with the four organic acids and two chelating salt solutions were conducted for 1, 2 and 3 hours at 120°C in a multiclave. A heating time of 80 minutes was required for each run, to allow for heating through the block and the vessel. This was obtained from previously conducted calibrations for the multiclave at TNO Energy Transition. A cooling time of 45-60 minutes followed each experiment run, wherein cooling was brought about by a draft of air. In the case of the organic acid treatments for both *Ulva lactuca* and *Palmaria palmata*, on separation of the hydrolysate from the solid residue, the hydrolysates recovered from the 3 hour tests were observed to be darker in colour and perceived as less viscous when compared to the corresponding 1 and 2 hour test hydrolysates. The higher viscosity and stickiness of the hydrolysates indicates the release of sugars in their oligomeric forms. Thus, in addition to analysing the post-hydrolysed hydrolysate samples from the 3 hour tests by HPAEC, analysis was also performed directly on these hydrolysates in order to estimate their monomeric sugar contents.
The treatments with hot water were conducted for 1, 2 and 3 hours, each at 100°C, 120°C and 140°C. Again, the same heating and cooling times were used.

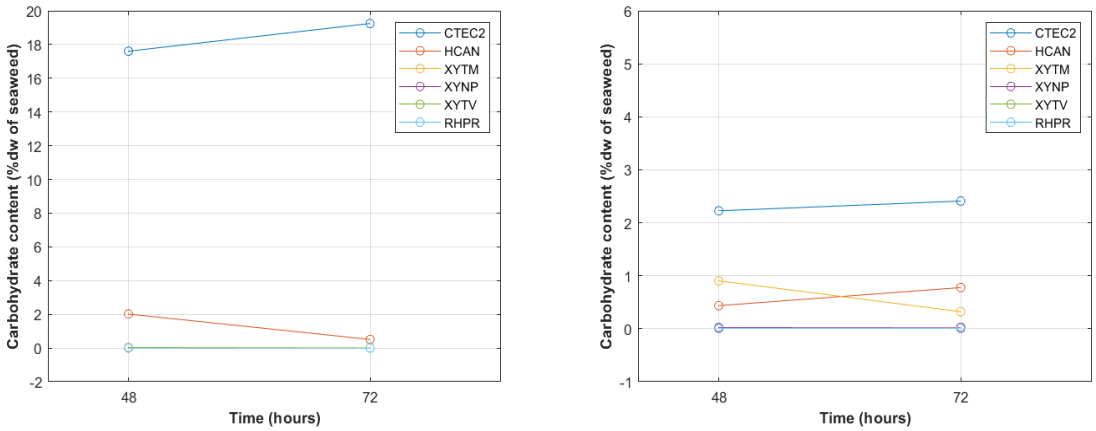
3.4. Results and discussion

This section begins with the results of the enzymatic hydrolysis tests, followed by those of the treatments with organic acids, chelating salts and hot water that were conducted in the multiclave.

3.4.1. Enzymatic hydrolysis

In figure 3.1, the content of sugars in the hydrolysates from the enzyme screening tests on *Ulva lactuca* are represented as the fraction of sugar extracted from the untreated seaweed on the basis of its dry weight. There was no rhamnose detected in any of the hydrolysates from the enzymatic hydrolysis of *Ulva lactuca* by these six enzymes. This does not necessarily mean that rhamnose was not released or extractable from the ulvan through hydrolysis by these enzymes. It is possible that rhamnose was released in oligomeric form which is undetectable in the HPAEC-PAD. From figures 3.1a and 3.1b, respectively, it is seen that both glucose and xylose were extractable from *Ulva lactuca* by hydrolysis with CTEC2 and HCAN. Hydrolysis with XYTM also resulted in the extraction of monomeric xylose in small amounts. From figure 3.1b, it is seen that the concentration of monomeric xylose in the hydrolysate

increased only by a small amount, from 2.22% to 2.42% on enzymatic hydrolysis with CTEC2 after 48 hours and 72 hours of hydrolysis, respectively. In the case of hydrolysis with XYTM, there was a slight drop in xylose concentration for longer hydrolysis times. This could be owing to the onset of xylose degradation between the 48^{th} and the 72^{nd} hour of hydrolysis or contamination by microorganisms leading to consumption of the released sugars.

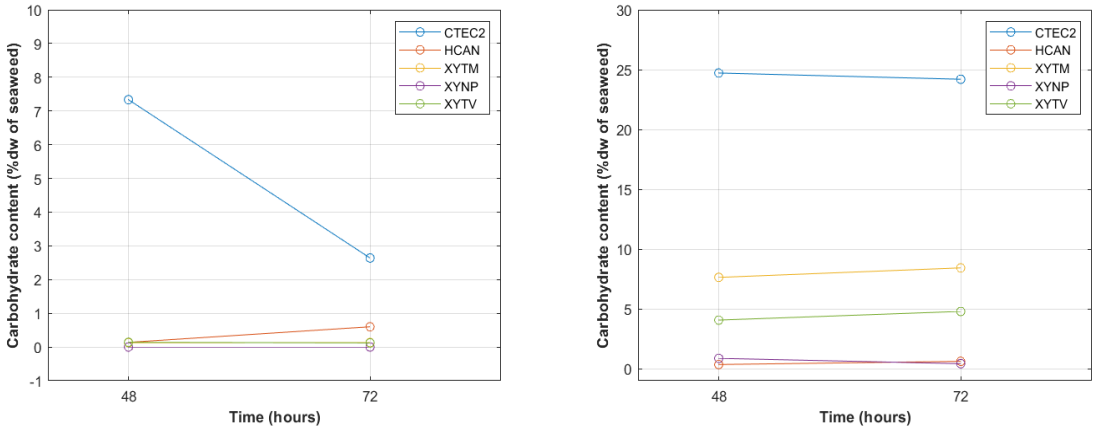


(a) Monomeric glucose extraction by enzymatic hydrolysis of *Ulva lactuca*.

(b) Monomeric xylose extraction by enzymatic hydrolysis of *Ulva lactuca*.

Figure 3.1: Carbohydrate extraction by enzymatic hydrolysis of *Ulva lactuca*.

From the enzyme screening tests on *Palmaria palmata*, figure 3.2b depicts that the xylose yield was highest for hydrolysis with CTEC2 and XYTM, as in the case of *Ulva lactuca*. By prolonging hydrolysis from 48hours to 72 hours, there was a slight decrease in xylose content from 24.72% to 24.19% for hydrolysis with CTEC2 and only a slight increase from 7.64% to 8.43% for hydrolysis with XYTM. For further investigation, CTEC2 was chosen as it resulted in the highest carbohydrate yields. Amongst HCAN and XYTM, XYTM was chosen for further investigation as it resulted in a higher xylose yield than HCAN after 48 hours of hydrolysis. Only two enzymes were chosen in order to facilitate further investigation of hydrolysis conditions effectively, keeping in mind the limited duration of the project. For the study on the effect of temperature and pH on extent sugar extraction, a hydrolysis time of 48 hours was chosen.



(a) Monomeric glucose extraction by enzymatic hydrolysis of *Palmaria palmata*

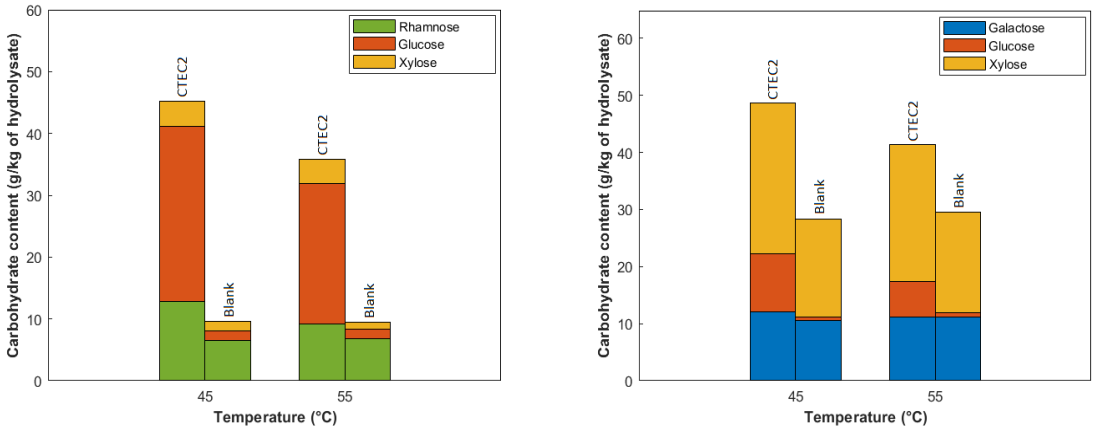
(b) Monomeric xylose extraction by enzymatic hydrolysis of *Palmaria palmata*.

Figure 3.2: Carbohydrate extraction by enzymatic hydrolysis of Palmaria palmata.

In order to study the effect of hydrolysis temperature on the extraction of sugars from the two sea-

weed types, enzymatic hydrolysis tests are run for a selected range of temperatures in the vicinity of the optimum temperature for each of the two enzymes. These enzymes were originally optimized for lignocellulosic biomass, hence the need for the determination of optimal conditions for seaweed. For CTEC2, whose optimum temperature is at 50°C, temperatures of 45°C and 55°C were chosen. For XYTM, in addition to these two, hydrolysis was also conducted at its optimum temperature of 80°C. Blank experiments were also conducted in the same way, but without the enzymes. After 48 hours of hydrolysis, the hydrolysate separated from the solid residue by means of a centrifuge was post-hydrolysed with 2M H_2SO_4 at 100°C for 2 hours and then analysed in the HPAEC-PAD.

As seen in figure 3.3a, the use of CTEC resulted in a substantial increase in glucose release when compared to the corresponding blank tests, for *Ulva lactuca*. There were also increases in xylose and rhamnose concentrations, however, these were not as substantial as in the case of glucose. The increase was more significant at 45°C for rhamnose and glucose and at 55°C for xylose.
As seen in figure 3.3b, similar to the case of the *Ulva lactuca*, at both 45°C and 55°C, the glucose yield increase substantially by the use of CTEC2 for *Palmaria palmata*. Benefits on xylose release was also seen, but in much smaller amounts. Both xylose and galactose increase are higher at 45°C than at 55°C.



(a) Total key sugar concentration in hydrolysates from enzymatic hydrolysis of *Ulva lactuca* with Cellic CTEC2.

(b) Total key sugar concentration in hydrolysates from enzymatic hydrolysis of *Palmaria palmata* with CTEC2.

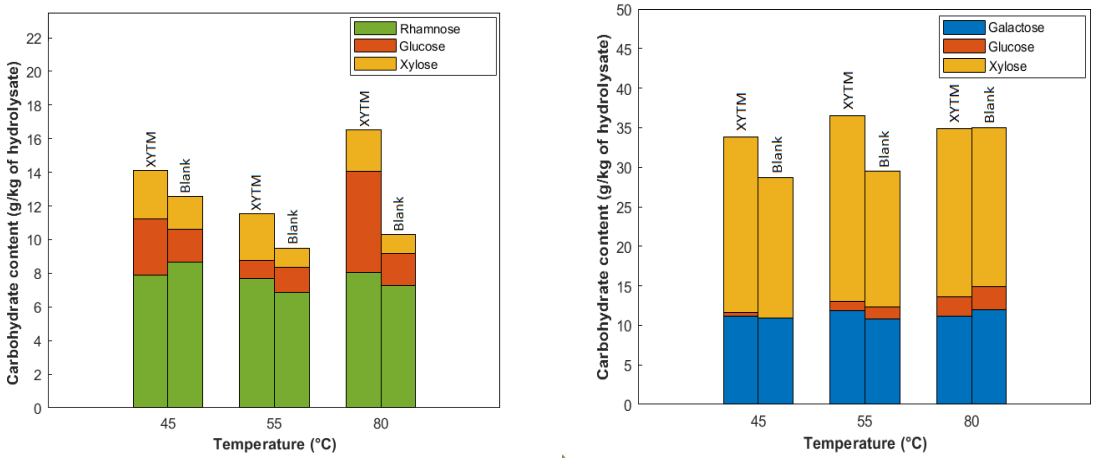
Figure 3.3: Total key sugar concentration in hydrolysates from enzymatic hydrolysis of *Ulva lactuca* and *Palmaria palmata* with Cellic CTEC2.

From figure 3.4a, hydrolysis of *Ulva lactuca* at the optimum temperature of XYTM (80°C), resulted only in a very small increase in rhamnose when compared to the blank sample. The increase for glucose and xylose at this temperature are significant. At 45°C and 55°C, the enzyme seems to inhibit the release of rhamnose.

Figure 3.4b presents the key sugars extracted from the *Palmaria palmata* on hydrolysis with XYTM. At lower temperatures, the benefits from enzyme use on xylose release are moderate compared to its release with CTEC2. Thus, hydrolysis at the elevated optimum temperature of 80°C for XYTM was not beneficial in the case of *Palmaria palmata* in terms of xylose release and neither for glucose and galactose.

As stated in the enzyme datasheet provided by the supplier, XYTM is used with an acetate buffer. In order to see if the process can be standardized in common with the more commercial CTEC2 enzyme which is used with citrate buffer, a check was done for the sugar quantification by the use of XYTM with a citrate buffer of pH 5. In figure 3.5, the total rhamnose extracted from *Ulva lactuca* and total xylose from *Palmaria palmata* are represented as grams of the respective sugar per kilogram of the hydrolysate. With the use of a citrate buffer instead of an acetate buffer, a 7.4% increase in rhamnose extraction from *Ulva lactuca* was achieved. The decrease in xylose extraction from *Palmaria palmata*

with the use of citrate buffer was only 0.92%. The buffer used thus does not have a substantial effect of sugar yield and can be used interchangeably.



(a) Total key sugar concentration in hydrolysates from enzymatic hydrolysis of *Ulva lactuca* with β-D-xylanase from XYTM

(b) Total key sugar concentration in hydrolysates from enzymatic hydrolysis of *Palmaria palmata* with XYTM.

Figure 3.4: Total key sugar concentration in hydrolysates from enzymatic hydrolysis of *Ulva lactuca* and *Palmaria palmata* with β-D-xylanase from *T.maritima*.

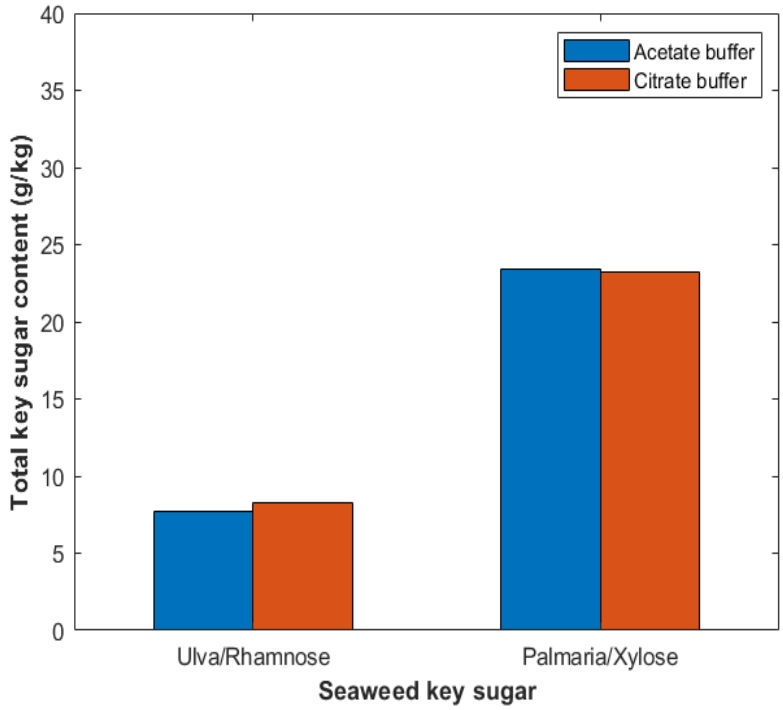


Figure 3.5: Total rhamnose and total xylose extracted from *Ulva lactuca* and *Palmaria palmata*, respectively, by hydrolysis with XYTM in the presence of acetate and citrate buffers.

The pH of the reaction mixture can influence the rate of enzymatic hydrolysis and in order to study these effects on the extraction of sugars from seaweed, tests were run for a selected range of pH conditions for each of the two enzymes. These were selected keeping in mind, the optimum pH for enzyme activity on the substrate it was originally optimised for as well as the pH conditions recorded

from previous tests in this study. Table 3.2 summarizes the pH conditions for the pH screening tests conducted on *Ulva lactuca* and *Palmaria palmata*.

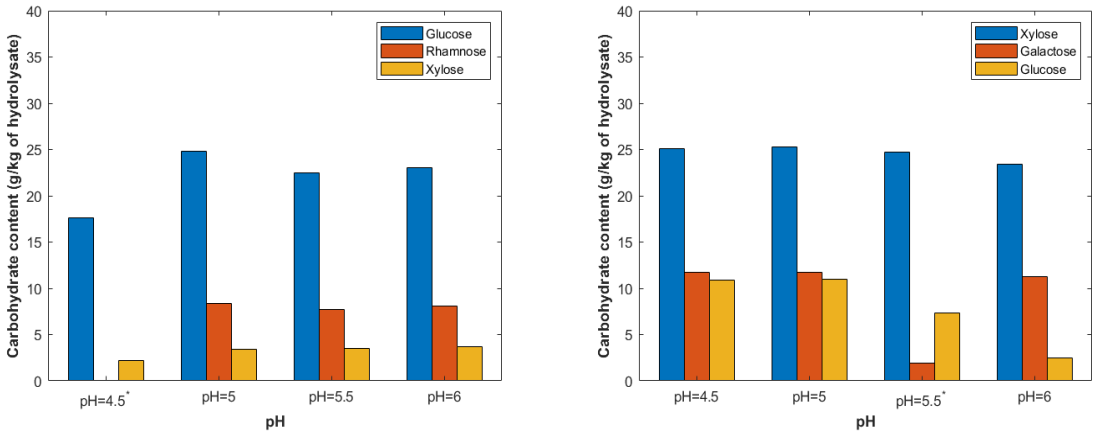
Seaweed	Enzyme	Original pH	pH for pH screening tests
Ulva lactuca	CTEC2	4.5	5, 5.5, 6
Ulva lactuca	XYTM	5	4.5, 5.5, 6
Palmaria palmata	CTEC2	5.5	4.5, 5, 6
Palmaria palmata	XYTM	5.5	4.5, 5, 6

Table 3.2: pH conditions for pH screening tests on *Ulva lactuca* and *Palmaria palmata*.

On HPAEC analysis of the post-hydrolysed hydrolysates from these screening tests, the total sugar contents in these hydrolysates were determined and are discussed below.

As seen in figure 3.6a, the release of rhamnose from enzymatic hydrolysis of *Ulva lactuca* with Cellic CTEC2 was comparable for a pH range of 5 to 6 but was slightly greater at 5. It is possible that rhamnose was released to a comparable or higher extent at a pH of 4.5, but was undetectable as the hydrolysate was not subjected to post-hydrolysis in order to further breakdown the oligomeric rhamnose. By the hydrolysis of *Palmaria palmata* with Cellic CTEC 2, highest amount of xylose was released at a pH of 5. At a pH of 5.5, the detection of a comparable amount of xylose in the hydrolysate implies that all or most of the xylose released from the *Palmaria palmata* could already be in the form of monomeric xylose.

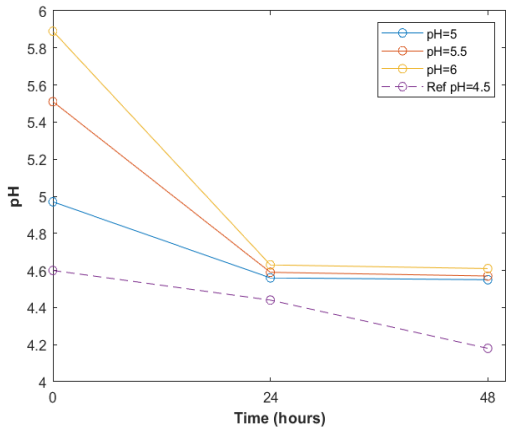
The variation of pH throughout the 48h hydrolysis of the two seaweed types by Cellic CTEC2 is plotted in figure 3.7. A drop in pH during the hydrolysis of *Ulva lactuca* by CTEC2 to a pH in the range of 4.2 to 4.7 was seen, irrespective of the pH that the reaction mixture is adjusted to at the time of enzyme addition. This could be owing to the uronic acid content in *Ulva lactuca*. Through the HPAEC analysis, low amounts of glucuronic acid ($\approx$ 2 g/kg) were detected in the hydrolysates from the enzymatic hydrolysis treatments on *Ulva lactuca*. In the case of *Palmaria palmata*, as seen in figure 3.6, there was a drop in the pH from 6 to 4.5, throughout the experiment as opposed to the trend of pH variation in the other three cases. The reason for this is unknown. In order to determine whether it was an experimental error that led to this pH drop, pH monitoring of further tests with a similar enzyme dosage will be done.



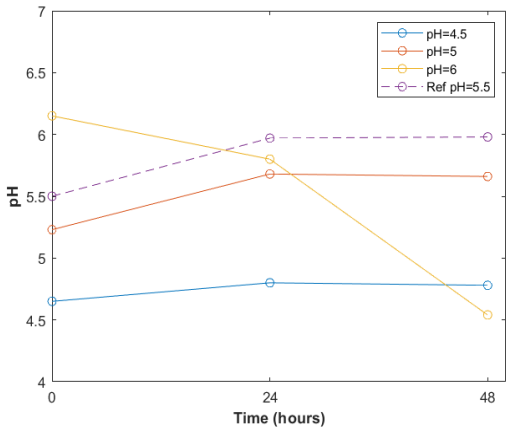
(a) Key sugar concentrations in hydrolysates from enzymatic hydrolysis of *Ulva lactuca* with Cellic CTEC2 at different pH conditions.

(b) Key sugar concentrations in hydrolysates from enzymatic hydrolysis of *Palmaria palmata* with Cellic CTEC2 at different pH conditions.

Figure 3.6: Key sugar concentrations in hydrolysates from the study of pH variation in the enzymatic hydrolysis of *Ulva lactuca* and *Palmaria palmata* with Cellic CTEC2. Values in * do not include oligomers.



(a) pH change during the 48h hydrolysis of *Ulva lactuca* with Cellic CTEC2.

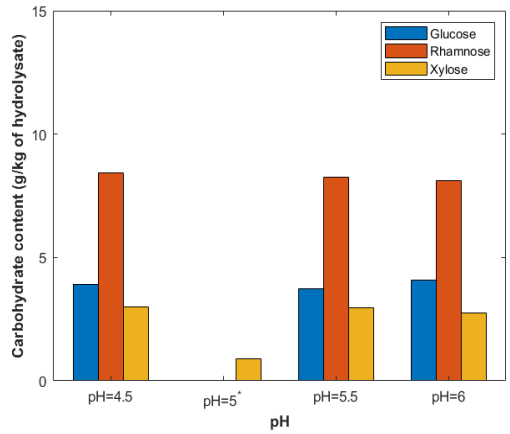


(b) pH change during the 48h hydrolysis of *Palmaria palmata* with Cellic CTEC2.

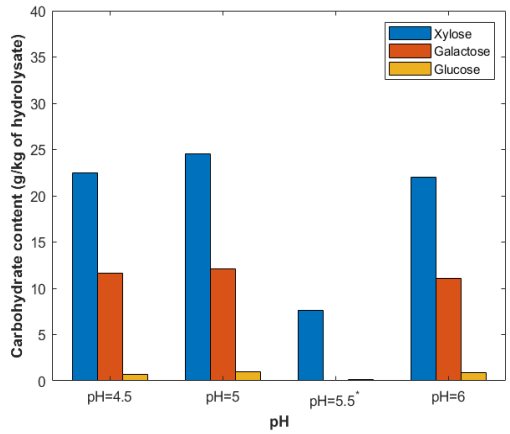
Figure 3.7: pH change during the 48h enzymatic hydrolysis of *Ulva lactuca* and *Palmaria palmata* with Cellic CTEC2.

As seen in figure 3.8a, the release of rhamnose from enzymatic hydrolysis of *Ulva lactuca* with XYTM was comparable for a pH range of 4.5 to 6 but was slightly greater at a lower pH of the reaction mixture, within the test range. It is possible that rhamnose was released to a comparable or higher extent at a pH of 5, but was undetectable as the hydrolysate of this test alone was not subjected to post-hydrolysis in order to further breakdown any oligomeric rhamnose that may have been released. It can be observed that above a pH of 5, there was a slight decrease in total rhamnose release at a higher pH of the reaction mixture. Thus, a pH in the range of 4.5 to 5 seems to be suitable. By the hydrolysis of *Palmaria palmata* with XYTM, highest amount of xylose was released at a pH of 5. At a pH of 5.5, it is possible that a comparable amount of xylose was released but was in its oligomeric form, making it undetectable through HPAEC analysis without further hydrolysis of the hydrolysate.

The variation of pH throughout the 48h hydrolysis of the two seaweed types by XYTM is plotted in figure 3.9. As opposed to the hydrolysis with CTEC2, in the hydrolysis of *Ulva lactuca* by XYTM, the pH was more steady without large variations and only slight reductions. This again, could be owing to the uronic acid content in *Ulva lactuca*. In the case of *Palmaria palmata*, as seen in figure 3.8, there was a drop in the pH only from 6.1 to 5.4, over the duration of the experiment as opposed to the trend of pH variation in the other three cases. Again, the reason for this is unknown.

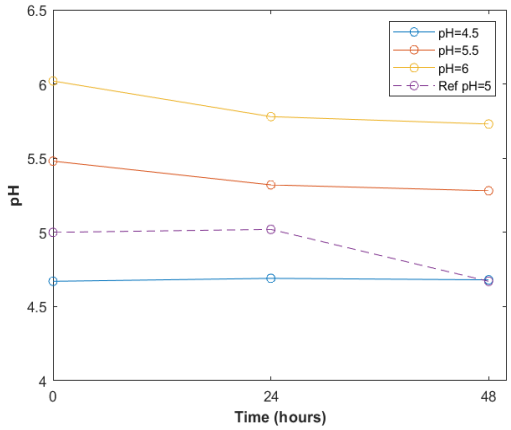


(a) Key sugar concentrations in hydrolysates from enzymatic hydrolysis of *Ulva lactuca* with XYTM at different pH conditions.

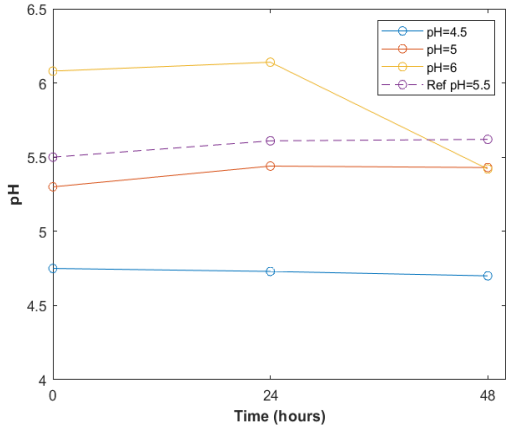


(b) Key sugar concentrations in hydrolysates from enzymatic hydrolysis of *Palmaria palmata* with XYTM at different pH conditions.

Figure 3.8: Key sugar concentrations in hydrolysates from the study of pH variation in enzymatic hydrolysis of *Ulva lactuca* and *Palmaria palmata* with XYTM. Values in * do not include oligomers.

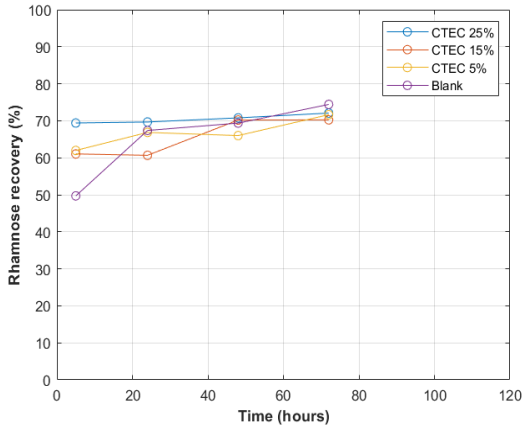


(a) pH change during the 48h hydrolysis of *Ulva lactuca* with XYTM.

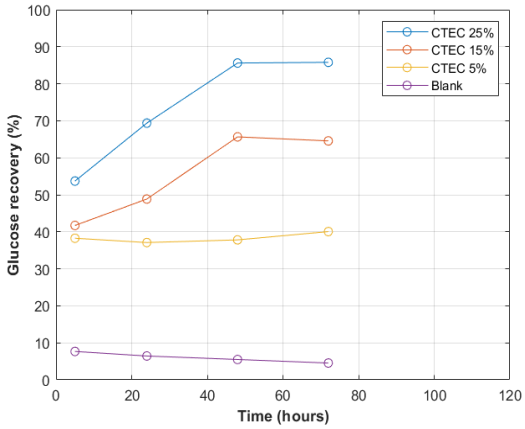


(b) pH change during the 48h hydrolysis of *Palmaria palmata* with XYTM.

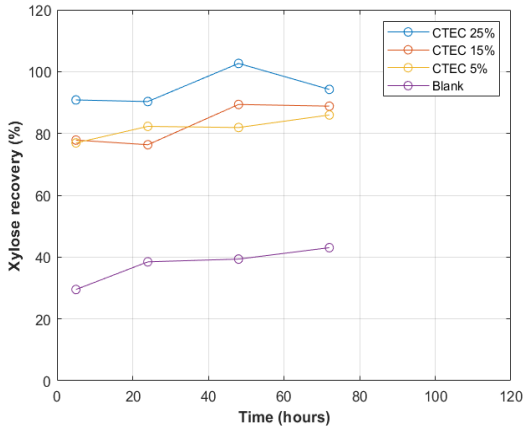
Figure 3.9: pH change during the 48h enzymatic hydrolysis of *Ulva lactuca* and *Palmaria palmata* with XYTM.



(a) Total rhamnose yield from *Ulva lactuca* by hydrolysis with CTEC2 at 50°C for different enzyme dosages.



(b) Total glucose yield from *Ulva lactuca* by hydrolysis with CTEC2 at 50°C for different enzyme dosages.

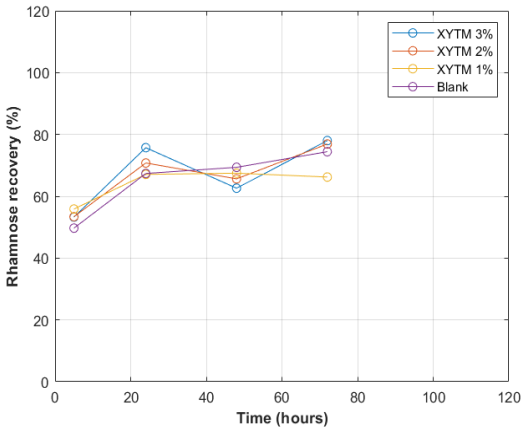


(c) Total xylose yield from *Ulva lactuca* by hydrolysis with CTEC2 at 50°C for different enzyme dosages.

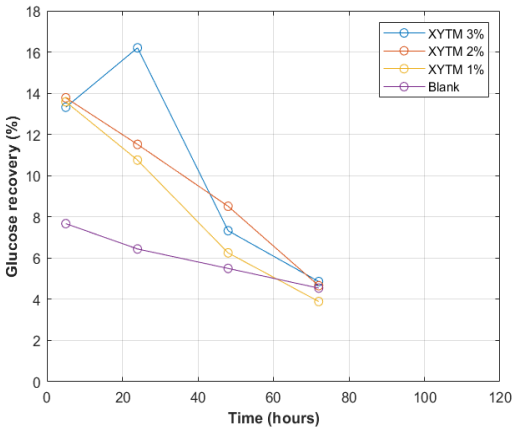
Figure 3.10: Total key sugar yields from the enzymatic hydrolysis of *Ulva lactuca* by CTEC2 at 50°C for different CTEC2 dosages.

Finally, a set of enzyme dosage screening tests were performed, in order to determine the effect of

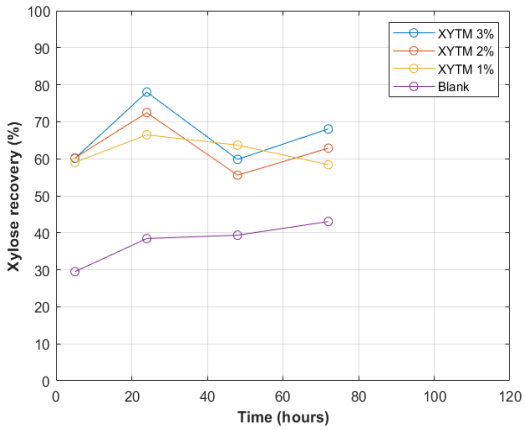
enzyme dosages on the release of key sugars from *Ulva lactuca* and *Palmaria palmata*. These were carried out at 50°C for 72 hours and at a starting pH of 5, with the use of a citrate buffer. Sampling of hydrolysates were done at t=0h, 5h, 24h, 48h and 72h. These hydrolysates were analysed in the HPAEC-PAD both, directly as well as after they were post-hydrolysed with 2M H_2SO_4 at 100°C for 2 hours, thus indicating the release of sugars as monomers and oligomers. The results from the analysis are represented herein as the recovered fraction of sugars on the basis of the characterization of the seaweed by the selected approach as in the previous chapter. A blank test was also conducted without seaweed to quantify the sugars in the enzymes themselves and incorporate these to correct for the sugars in the hydrolysates released from the seaweed alone. A blank test on each seaweed type, without the enzyme, was also conducted in order to estimate the effect of enzyme addition on sugar release.



(a) Total rhamnose yield from *Ulva lactuca* by hydrolysis with XYTM at 50°C for different enzyme dosages.



(b) Total glucose yield from *Ulva lactuca* by hydrolysis with XYTM at 50°C for different enzyme dosages.



(c) Total xylose yield from *Ulva lactuca* by hydrolysis with XYTM at 50°C for different enzyme dosages.

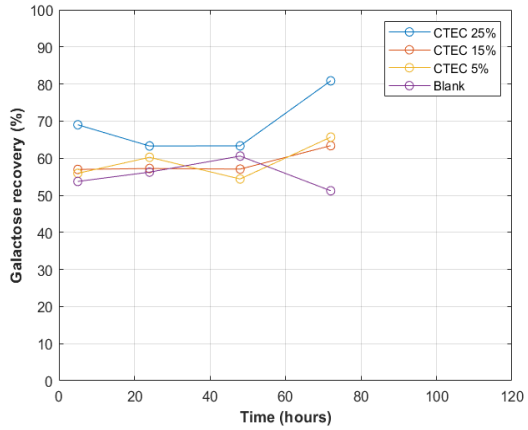
Figure 3.11: Total key sugar yields from the enzymatic hydrolysis of *Ulva lactuca* by XYTM at 50°C for different XYTM dosages.

Figure 3.10 shows the key sugars extracted from *Ulva lactuca* by hydrolysis with CTEC2 for an enzyme loading of 25%, 15% and 5% on a mass basis of dry seaweed. The use of CTEC2 as a hydrolysing agent for did not result in a significant increase in the yield of rhamnose after 72 hours as compared to the hydrolysate from the blank test on *Ulva lactuca* without enzyme addition, as seen in 3.10a. Increasing the CTEC2 dosage from 5% to 15% and 25% still showed no significant improvements in rhamnose release. An increase in glucose and xylose yields were seen with enzyme addition. This increase was proportional to the dosage of the enzyme, more so in the case of glucose than xylose as seen in figures 3.10b and 3.10c, respectively. An increase from 5% to 25% in CTEC2 dosage resulted in a 9.6% increase in xylose yield.

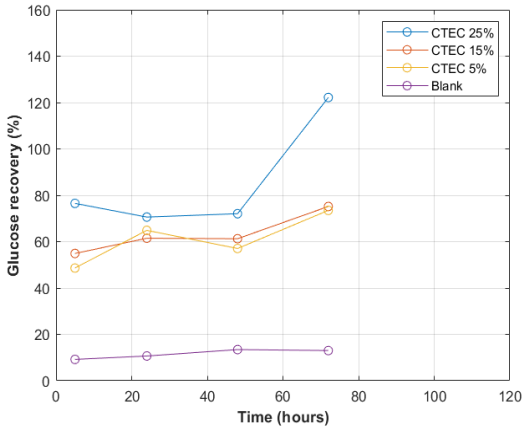
The key sugars extracted from *Ulva lactuca* by hydrolysis with XYTM for an enzyme loading of 3%, 2% and 1% on a mass basis of dry seaweed are represented in figure 3.11. The rhamnose yield increased with enzyme addition by a small amount, as in comparison with the blank. After 72 hours of hydrolysis with an XYTM dosage of 3%, there was a 4.8% increase in rhamnose yield. The XYTM seems to inhibit glucose release over time which is preferred as rhamnose is the sugar of interest, in this study. Furthermore, the xylose yield increased with XYTM dosage. An increase in the XYTM dosage from 1% to 3% resulted in a 16.5% increase in xylose yield.

The key sugars extracted from *Palmaria palmata* through enzymatic hydrolysis with a CTEC2 dosage of 25%, 15% and 5% are represented in figure 3.12. Increasing the CTEC2 dosage from 5% to 25% resulted in a 23% increase in galactose yield, a 66.28% in glucose yield and a 21.2% increase in xylose yield, after 72 hours of hydrolysis.

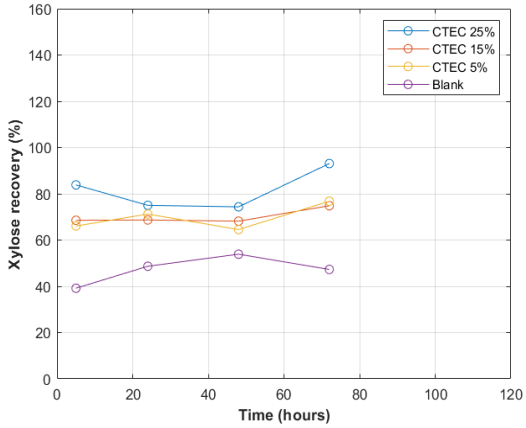
The extraction of key sugars extracted through hydrolysis of *Palmaria palmata* by XYTM for different dosages are represented in figure 3.13. In this case, an increase in XYTM dosage from 1% to 3% resulted in a 13.2% and 13.5% decrease in galactose and xylose as seen in figures 3.13a and 3.13b. There was an increase in glucose yield for higher enzyme dosages, however, as glucose is not a sugar of interest in this study, the increase in XYTM dosages for the hydrolysis of *Ulva lactuca* is not beneficial.



(a) Total galactose yield from *Palmaria palmata* by hydrolysis with CTEC2 at 50°C for different enzyme dosages.

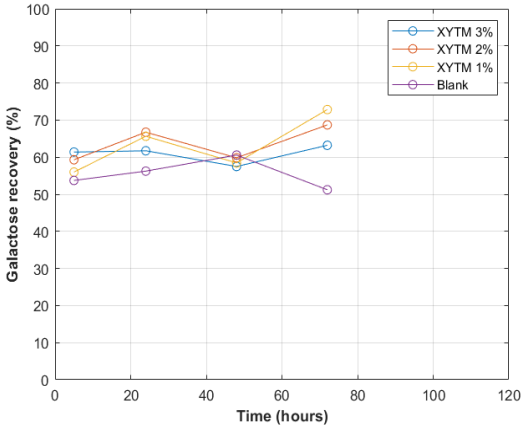


(b) Total glucose yield from *Palmaria palmata* by hydrolysis with CTEC2 at 50°C for different enzyme dosages.

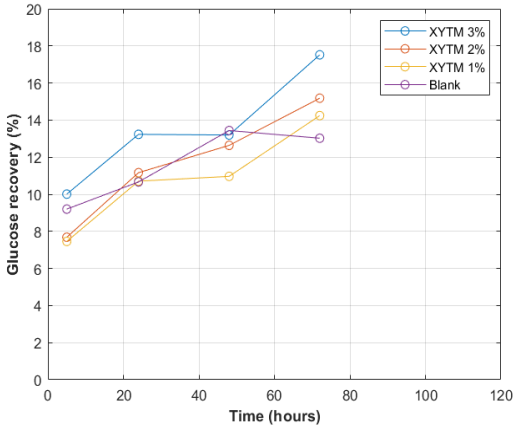


(c) Total xylose yield from *Palmaria palmata* by hydrolysis with CTEC2 at 50°C for different enzyme dosages.

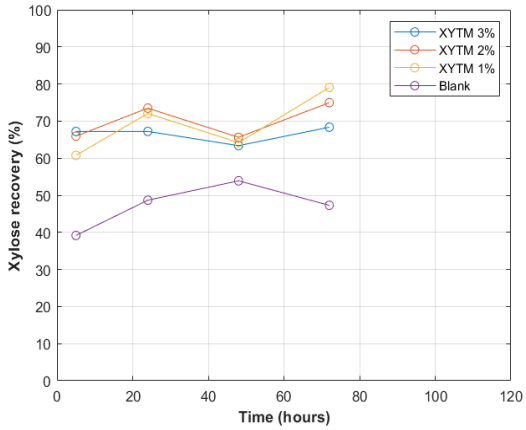
Figure 3.12: Total key sugar yields from the enzymatic hydrolysis of *Palmaria palmata* by CTEC2 at 50°C for different CTEC2 dosages.



(a) Total galactose yield from *Palmaria palmata* by hydrolysis with XYTM at 50°C for different enzyme dosages.



(b) Total glucose yield from *Palmaria palmata* by hydrolysis with XYTM at 50°C for different enzyme dosages.



(c) Total xylose yield from *Palmaria palmata* by hydrolysis with XYTM at 50°C for different enzyme dosages.

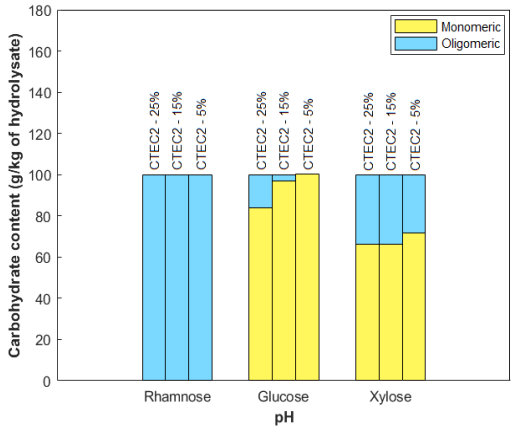
Figure 3.13: Total key sugar yields from the enzymatic hydrolysis of *Palmaria palmata* by XYTM at 50°C for different XYTM dosages.

Figures 3.10 to 3.13 all represent the key sugars extracted as the total quantified sugars, after post-hydrolysis of the hydrolysates that breaks the oligomeric sugars into monomers, thus making them detectable. To obtain a distribution of how these sugars are released, the hydrolysates were also analysed in the HPAEC-PAD directly thus not accounting for the sugars released as oligomers. It is important to take this into consideration while selecting the best pH conditions for the treatment series because not all sugars extracted in the enzyme pH screening tests are available as total sugars.

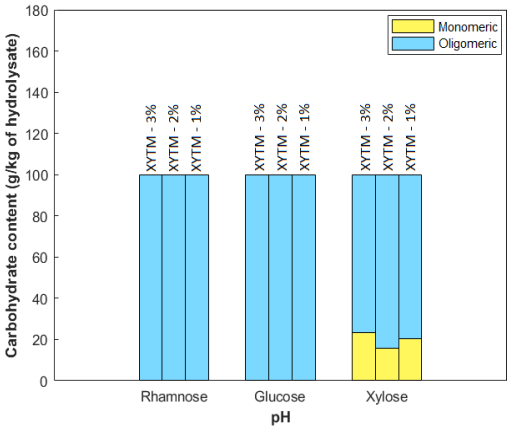
From figure 3.14, it is observed that all the rhamnose extracted from *Ulva lactuca* was released in its oligomeric form, irrespective of the enzyme used or its dosage strength. To the contrary, the glucose extracted by CTEC2 was released almost entirely as monomeric glucose and on post-hydrolysis of the hydrolysate, glucose degradation started to occur as observed in 3.14a. At lower CTEC2 dosages, a higher extent of monomeric release of glucose and xylose was observed. With the use of XYTM as the hydrolysing agent, 100% of the glucose extracted was released as oligomers and moreover, this amount was minimal. This is expected as XYTM inhibits the release of glucose, as seen in previous sections of this study.

In the case of *Palmaria palmata*, most of the xylose was released as monomers with the use of CTEC2 as the hydrolysing agent but not with XYTM, as seen in figures 3.15a and 3.15b, respectively. Galactose and glucose were predominantly released in their oligomeric forms when CTEC2 was employed as the hydrolysing enzyme and completely in their oligomeric forms with XYTM as the

hydrolysing enzyme.

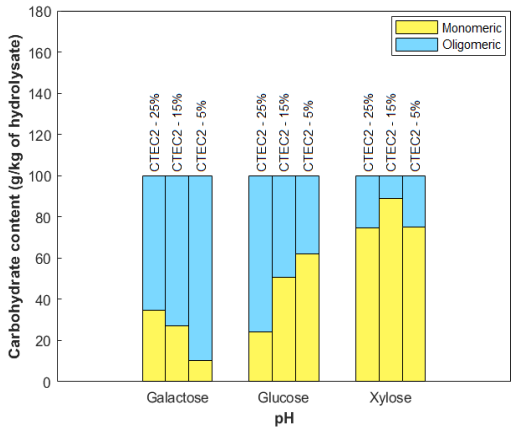


(a) Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of *Ulva lactuca* for different dosages of CTEC2.

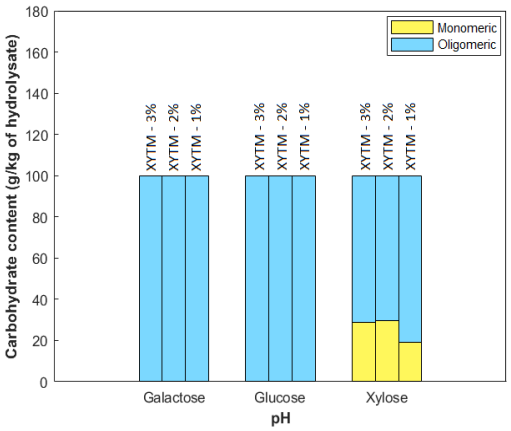


(b) Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of *Ulva lactuca* for different dosages of XYTM.

Figure 3.14: Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of *Ulva lactuca* for different enzyme dosages.



(a) Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of *Palmaria palmata* for different dosages of CTEC2.

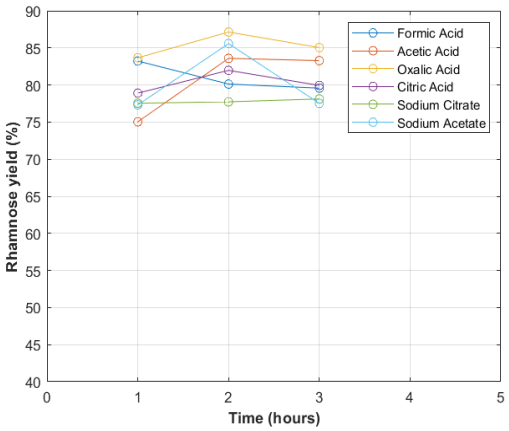


(b) Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of *Palmaria palmata* for different dosages of XYTM.

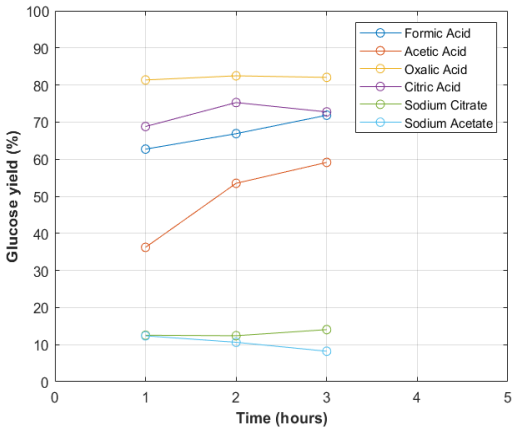
Figure 3.15: Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of *Palmaria palmata* for different enzyme dosages.

3.4.2. Organic acid and chelating salt hydrolysis

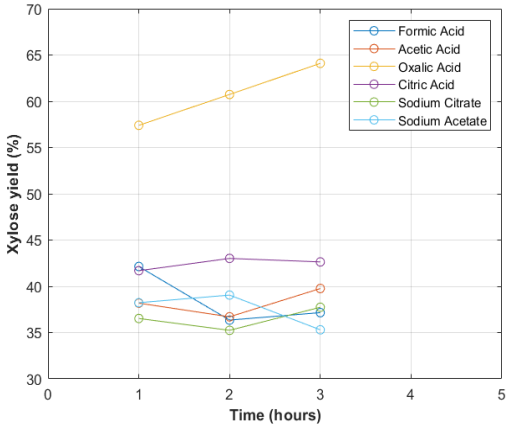
The seaweed carbohydrate analysis in the HPAEC-PAD were conducted on the post-hydrolysed liquid hydrolysate samples from the organic acid and chelating salt treatments to detect sugars as in table 2.2. The sugars extracted herein are presented as the recovered fraction of the sugar contents resulting from the characterization of the seaweed by the selected approach as in the previous chapter.



(a) Total rhamnose yield from *Ulva lactuca* for t= 1h, 2h, 3h at 120°C.



(b) Total glucose yield from *Ulva lactuca* for t= 1h, 2h, 3h at 120°C.



(c) Total xylose yield from *Ulva lactuca* for t= 1h, 2h, 3h at 120°C.

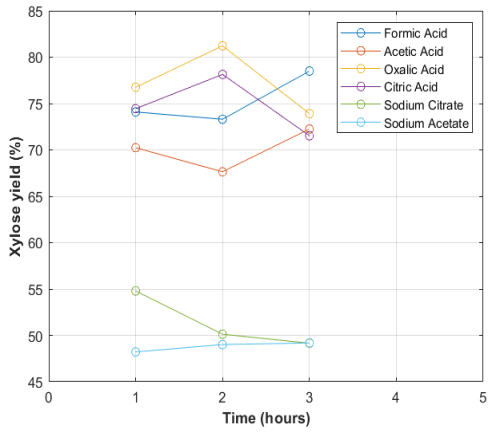
Figure 3.16: Total key sugar yields from the organic acid and chelating salt treatments of *Ulva lactuca* at 120°C in a multiclave, for different reaction times.

In the case of *Ulva lactuca*, the yield of all key sugars (rhamnose, glucose and xylose) was highest for the treatment with oxalic acid as seen in figure 3.17. The rhamnose yield was highest for the 2 hour treatments with acetic acid, oxalic acid, citric acid and sodium acetate as seen in figure 3.16a. An increased reaction time of 3 hours resulted in lower rhamnose yields. This could be a result of the possible degradation of rhamnose to 5-methylfurfural. With the use of sodium acetate as the hydrolysing agent, the degradation of rhamnose is already seen at 2 hours with its lower yield. In the case of treatment with sodium citrate, the reaction time is found to have a small effect on rhamnose yield. The glucose yield is observed to be higher at more acidic conditions, it is much higher in the hydrolysates from the organic acid treatments when compared to those from sodium acetate and sodium citrate. Furthermore, in figure 3.16b, a drop in glucose yields with time are seen in the case of oxalic acid, sodium acetate and citric acid, however, these are very minimal. This could be attributed to the degradation of glucose under these acidic conditions[36]. Furthermore, the low yield of xylose as seen in figure 3.16c, could be owing to the inhibition of its release by glucose [37].

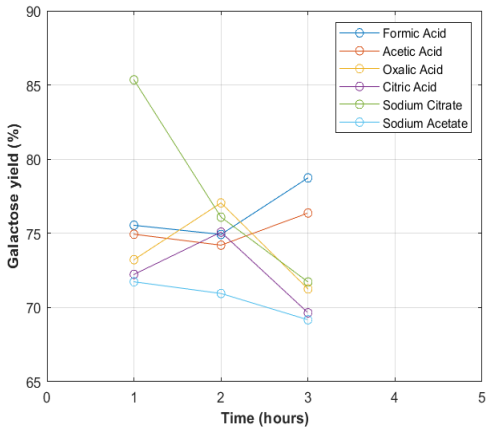
Treatment with oxalic acid resulted in the highest rhamnose and xylose yield for the 1 hour, 2 hour and 3 hour treatments. However, treatments with sodium acetate and sodium citrate also resulted in the high yield of rhamnose, only slightly lower than from the treatment with oxalic acid, but not accompanied by high glucose yields, unlike the oxalic acid treatment. As in this study, rhamnose extraction from *Ulva lactuca* is the sugar of interest, its extraction accompanied by a lower glucose yield is preferred.

In the case of *Palmaria palmata*, as seen in figure 3.17a, the total xylose yield was highest when

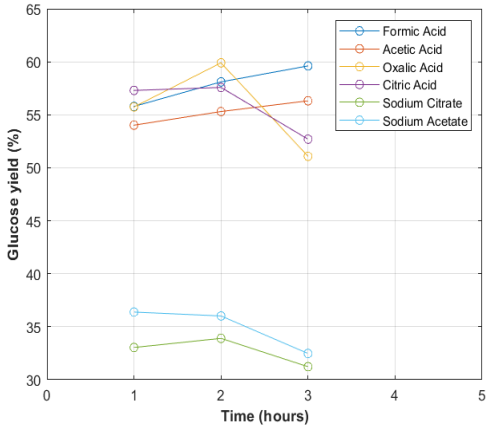
the seaweed was treated with oxalic acid at 120 °C for 2 hours and thereafter, and thereafter, its lowered yield may be due to its degradation to furfural. However, the increase in the total xylose yield by doubling the reaction time from 1 to 2 hours, is only 4%. This trend was also observed in the treatment with citric acid. In the treatments with formic acid and acetic acid, the total xylose yield was lower for a reaction time of 2 hours, than for 1 and 3 hours. A similar trend as in the xylose is also seen in the galactose yields, represented in figure 3.17b. In the case of the two chelating salt treatments, xylose yields were much lower when compared to the organic acid treatments, thus making them the less favourable choice. Amongst the organic acid treatments, oxalic acid is most favourable, as it resulted in a high xylose yield for the least reaction time. Moreover, an increase in the reaction time from 1 hour to 2 and 3 hours did not result in a significant increase in total sugar yields.



(a) Total xylose yield from *Palmaria palmata* for t= 1h, 2h, 3h at 120°C.



(b) Total galactose yield from *Palmaria palmata* for t= 1h, 2h, 3h at 120°C.

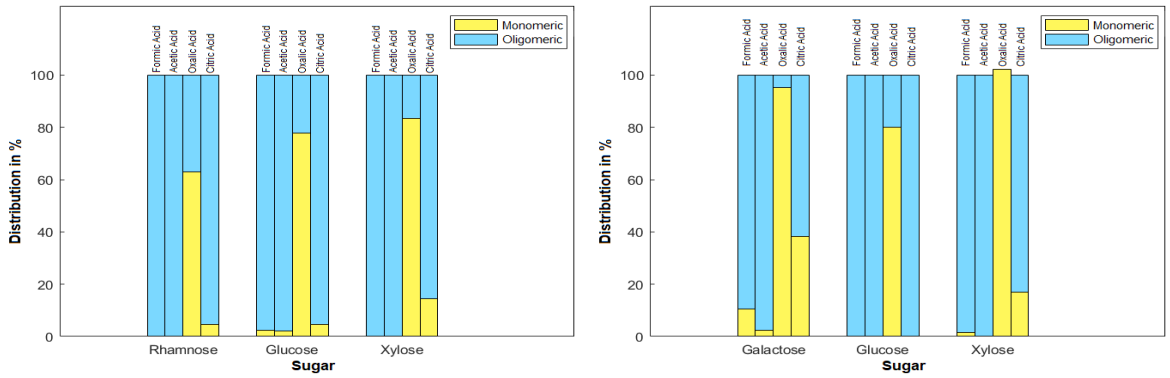


(c) Total glucose yield from *Palmaria palmata* for t= 1h, 2h, 3h at 120°C.

Figure 3.17: Total key sugar yields from the organic acid and chelating salt treatments of *Palmaria palmata* at 120°C in a multiclave, for different reaction times.

Figure 3.18a depicts the distribution of the release of rhamnose, glucose and xylose in their monomeric and oligomeric forms in the 3 hour organic acid treatments of *Ulva lactuca*. The monomeric sugars were quantified by a direct HPAEC analysis of the hydrolysates, without post-hydrolysing them as done for the detection of total sugars. Rhamnose and xylose are released solely in their oligomeric forms in the presence of formic and acetic acid. In the presence of oxalic acid, all three sugars are mainly released as monomers, however, in the case of rhamnose, 37% is still in its oligomeric form and thus post-hydrolysis of the hydrolysate with 2M H_2SO_4 at 100°C is necessary in order to make them detectable. This distribution in the release of sugars in their monomeric and oligomeric forms is associated with the pH of the reaction mixtures. Oxalic acid, being the most acidic with lower pH promotes sugar release

as monomers whereas acetic acid, being the weakest amongst the four with the highest pH, does not facilitate the further hydrolysis of released oligomeric sugars into monomers.

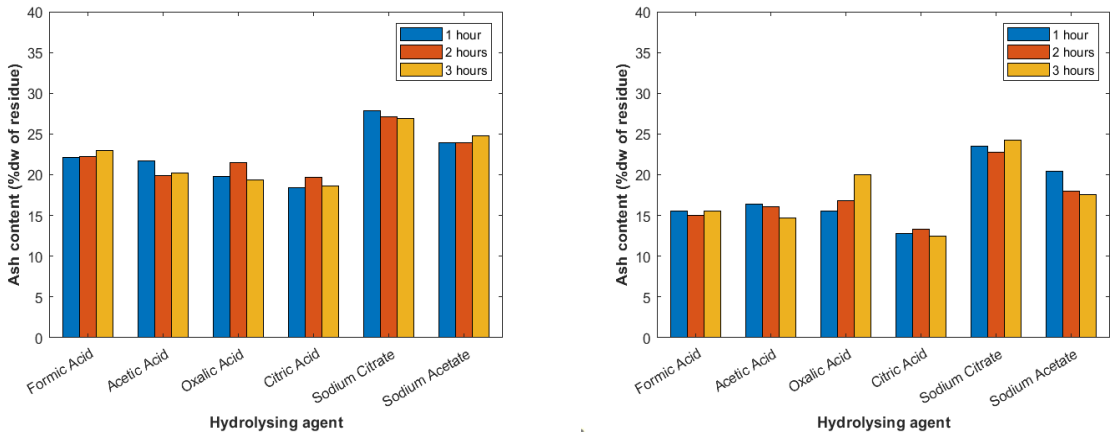


(a) Distribution of key sugars in their monomeric and oligomeric forms in the organic acid hydrolysis of *Ulva lactuca*.

(b) Distribution of key sugars in their monomeric and oligomeric forms in the organic acid hydrolysis of *Palmaria palmata*.

Figure 3.18: Distribution of key sugars in their monomeric and oligomeric forms in the organic acid hydrolysis of seaweed.

Figure 3.18b depicts the distribution of the release of galactose, glucose and xylose in their monomeric and oligomeric forms in the 3 hour organic acid treatments of *Palmaria palmata*. As in the case of the *Ulva lactuca*, here as well, on hydrolysis of *Palmaria palmata* by oxalic acid, all key sugars are detected primarily in their monomeric forms. In fact, xylan is hydrolysed to release xylose completely in its monomeric form. On post-hydrolysis of the hydrolysate with 2M H_2SO_4 at 100°C, monomeric xylose degradation occurs.



(a) Ash content of the solid residues from the organic acid and chelating salt treatments on *Ulva lactuca*.

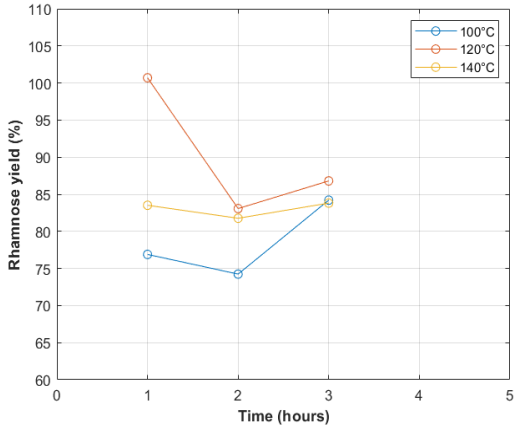
(b) Ash content of the solid residues from the organic acid and chelating salt treatments on *Palmaria palmata*.

Figure 3.19: Ash content of the solid residues from the organic acid and chelating salt treatments on seaweed.

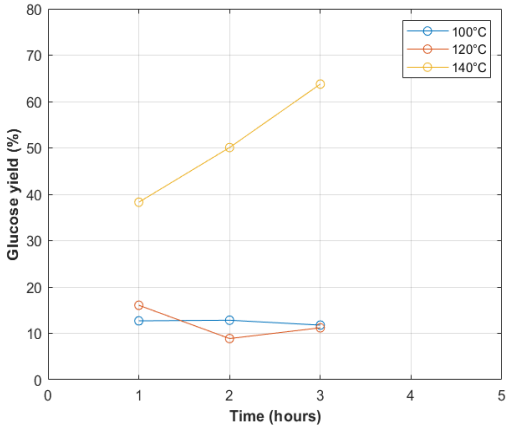
The ash contents of the solid residues from the treatments, determined gravimetrically are represented in figure 3.19. The ash contents are a representation of the inorganic and salt contents present in the residual solids from the treatments. It is noteworthy here that the treatments were conducted on seaweed without any pre-treatments such as washing to aid the removal of inorganics, prior to treatment. Ash in biomass is naturally alkaline owing to the high salt content of marine biomass like seaweed as seen from the fall in mineral contents in figure 2.5. This can hamper with the use of seaweed and residues in the reactor due to corrosion, fouling or slagging [38].

3.4.3. Hot water hydrolysis

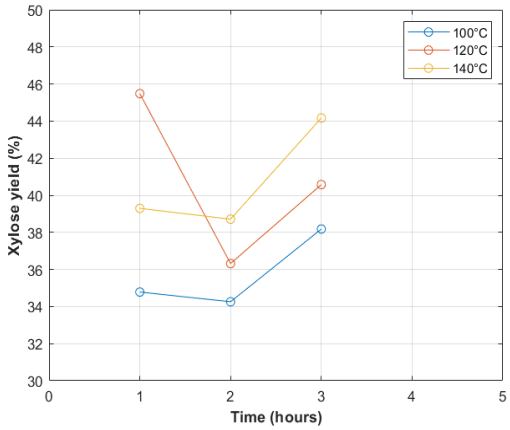
Analysis for seaweed carbohydrate contents in hydrolysates from the hot water treatments on seaweed, after their post-hydrolysis with 2M H_2SO_4 at 100°C was conducted in the HPAEC-PAD. The sugars extracted are presented as the recovered fraction of the sugar contents resulting from the characterization of the seaweed by the selected approach as in section 2.3.4 of the previous chapter.
In the case of *Ulva lactuca*, as seen in figure 3.20a, a 1 hour hot water treatment at 120°C resulted in a rhamnose yield of 100.7%. At these conditions, the xylose yield is also at its highest when compared to hot water hydrolysis treatments at all other temperature and time combinations for *Ulva lactuca*. Furthermore, the glucose yield at 120°C is very low (16.1 %), which makes these conditions more favourable in comparison to the rest.



(a) Total rhamnose yield from *Ulva lactuca* for t= 1h, 2h, 3h, each at T= 100°C, 120°C, 140°C.



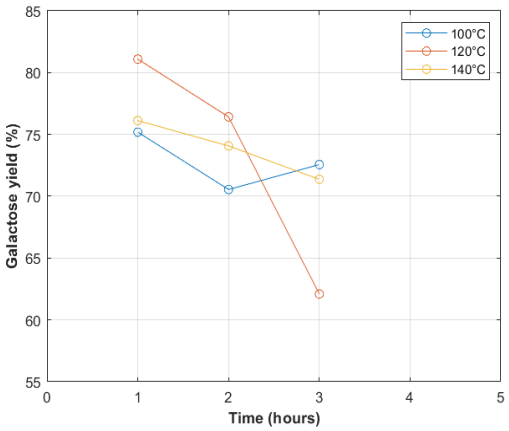
(b) Total glucose yield from *Ulva lactuca* for t= 1h, 2h, 3h, each at T= 100°C, 120°C, 140°C.



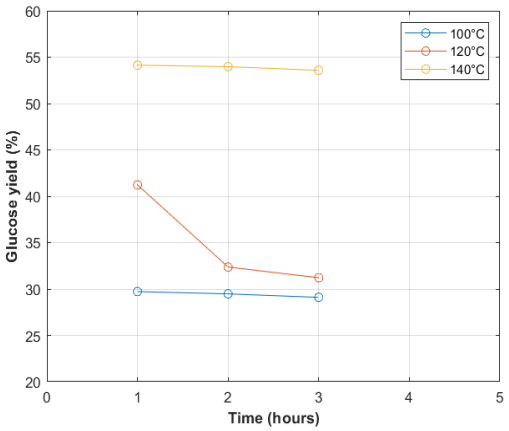
(c) Total xylose yield from *Ulva lactuca* for t= 1h, 2h, 3h, each at T= 100°C, 120°C, 140°C.

Figure 3.20: Total key sugar yields from the hot water treatment of *Ulva lactuca* in the multiclave for a range of reaction times and at different reaction temperatures.

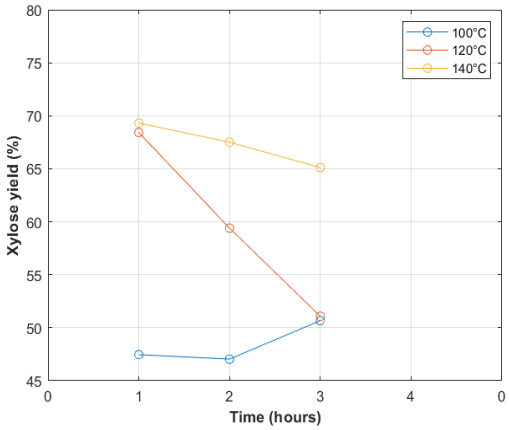
In the case of the *Palmaria palmata*, the xylose yield was 69.2% for the 1 hour hot water treatment at 140°C and 68.4% at 120°C, as seen in figure 3.21c. In both these cases, an increase in the reaction time to 2 hours and 3 hours resulted in xylose degradation. Thus, a reaction time of 1 hour is deemed sufficient. This trend is also observed in the galactose and glucose yields from figure 3.21a and figure 3.21b, respectively. Since for the 1 hour treatment at 140°C, the xylose yield is higher and the ash content of the residue is lower when compared to at 120°C, as seen in figure 3.22b, it is deemed to be the most suitable reaction temperature.



(a) Total galactose yield from *Palmaria palmata* for t= 1h, 2h, 3h, each at T= 100°C, 120°C, 140°C.

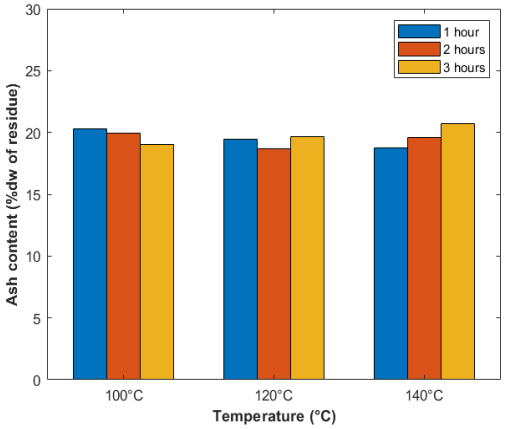


(b) Total glucose yield from *Palmaria palmata* for t= 1h, 2h, 3h, each at T= 100°C, 120°C, 140°C.

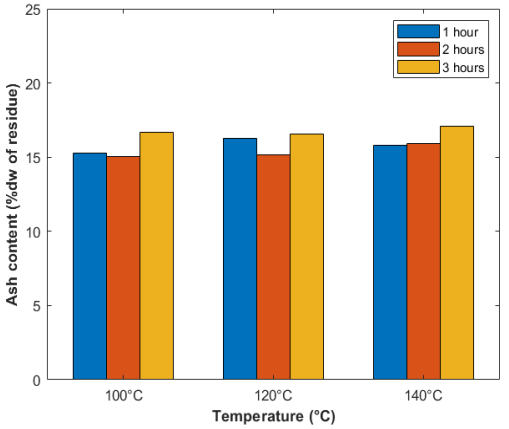


(c) Total xylose yield from *Palmaria palmata* for t= 1h, 2h, 3h, each at T= 100°C, 120°C, 140°C.

Figure 3.21: Total key sugar yields from the hot water treatment of *Palmaria palmata* in the multiclave for a range of reaction times and at different reaction temperatures.



(a) Ash content of the solid residues from the hot water treatments on *Ulva lactuca*.



(b) Ash content of the solid residues from the hot water treatments on *Palmaria palmata*.

Figure 3.22: Ash content of the solid residues from the hot water treatments on seaweed.

3.5. Conclusions

On comparison of the key sugar yields from the two seaweed types through the hot water treatments and the organic acid treatments, they were found to be better in the case of the hot water treatments. The rhamnose yield from the hydrolysis of *Ulva lactuca* with hot water was higher than from hydrolysis with organic acids and chelating salts and the xylose yields from the two were comparable. At 120°and for 1 hour of hydrolysis, hot water hydrolysis of *Ulva lactuca* resulted in a 100% rhamnose yield as opposed to the 84% yield with the use of oxalic, which was the highest amongst all the treatments with organic acid and chelating salt hydrolysing agents at this temperature. Furthermore, water is a more favourable hydrolysing agent. Thus, in the case of *Ulva lactuca*, hydrolysis by hot water is the more favourable approach. Similarly, in the case of *Palmaria palmata*, hydrolysis with hot water at 120°C for 1 hour resulted in a xylose yield of 69% as opposed to 77% from the oxalic acid treatment at the same conditions. Since the use of oxalic acid did not result in a yield much higher to that from the treatment with hot water, hydrolysis with hot water is preferred for the *Palmaria palmata* as well.

From the various time and temperature condition screenings for the hydrolysis of *Ulva lactuca* with hot water, the highest yields of rhamnose and xylose were obtained at 120°C for a hydrolysis time of 1 hour. Moreover, at 120°C, very low glucose yields are obtained as opposed to higher glucose yields at 140C. This is preferential in the context of this study. For hydrolysis of *Palmaria palmata* with hot water, the highest xylose yield is obtained with a reaction temperature of 140°C for 1 hour of hydrolysis. These conditions of hydrolysis will be employed for the larger scale treatments of the respective seaweed types in a 2L autoclave.

Amongst the various enzymes screened, CTEC2 and XYTM were selected for further investigation. The use of a citrate buffer instead of the recommended acetate buffer for hydrolysis of both *Ulva lactuca* and *Palmaria palmata* with XYTM resulted in comparable extractions of rhamnose and xylose, respectively, thereby standardizing the type of buffer used for enzymatic hydrolysis of seaweed in this study. Based on the rhamnose recovery from *Ulva lactuca*, hydrolysis with XYTM at a dosage of 3% was found to be the most suitable. A high rhamnose recovery via this approach was accompanied by an inhibition in glucose release over time which is preferred. In the case of *Palmaria palmata*, hydrolysis with CTEC2 of 25% dosage was the most favourable approach. This was based on higher xylose recoveries from *Palmaria palmata* when compared to its hydrolysis with XYTM wherein enzyme dosage seemed to have a slight negative influence on galactose and xylose yields. For both the chosen seaweed-enzyme combinations, a hydrolysis temperature of 50°C and a hydrolysis time of 72 hours was deemed to be the best fit.

4

Cascading of a treatment approach for the extraction of monosaccharide sugars

This chapter covers the treatment approach as a complete sequence, set up from the results and outcomes of the various pre-treatment and treatment strategies investigated in this study as in the previous chapters of the report.

4.1. Validation of biorefinery approach

The final objective of this research project was to evaluate experimentally a suitable cascade biorefinery approach for the extraction of rhamnose from *Ulva lactuca* and xylose from *Palmaria palmata*. From the experimental tests of various hydrolysis methods at varying operating conditions conducted in this study, the most favourable treatment methods for extraction of the targeted sugars were selected for each seaweed type and applied to a larger laboratory scale of treatment. The screening and evaluation of potential treatments in this study were carried out on a 5-7 g scale of dry seaweed. Validating the selected treatments at a 2-3L scale is necessary to assess the concept. Figure 4.1 is a representation of the chosen cascade of treatments wherein each of the pre-treatment and treatment steps are assessed on a larger scale.

The water-washing pre-treatment step was carried out with 1.5 kg of dry seaweed. A decanter centrifuge, flat-bed filtration setup and a membrane ultra-filtration unit were utilized for the separation of the product streams of the water-washing pre-treatment step.
The ethanol extraction pre-treatment step by means of the soxhlet apparatus is not implemented here as an alternative solvent extraction technique at the relevant kilogram scale is required and is underway. Although this pre-extraction step proved beneficial for the extraction of the key sugars from seaweed, the relevant extractives are not problematic for this specific application, that is the downstream production of furanics from these extracted sugars.
The selected combination of enzymatic hydrolysis treatments are carried out in a bioreactor of 3L capacity and their hot water treatments are carried out in a 2L autoclave.

4.2. Experimental Methods and Materials

This section covers the description of the materials as well as the approach employed in the cascade treatment approach.

4.2.1. Materials

The raw materials in this treatment series are the dry seaweeds *Ulva lactuca* and *Palmaria palmata*, milled to a size of 1.5mm. Demineralised water was used for the washing of the seaweed. The Lemitec decanter centrifuge was used for separation the washed seaweed and liquor. The separated liquor was further filtered using Sterlitech membrane filtration unit with Synder™ flat sheet membranes of types LY and NDX.
The hot water hydrolysis treatments were carried out in an autoclave reactor (2L, Büchi Glas Uster AG). The enzymatic hydrolysis treatments were carried out in a bioreactor (3L, INFORS HT Labfors). The enzyme Cellic® CTec2 was obtained from Novozymes and the β-D-xylanase from *T.maritima* (XYTM) from Megazyme. A citrate buffer of pH 5, 1M H_2SO_4, 1M NaOH and $Ca(OH)_2$ solutions of 0.02M and 0.015M are used to maintain the required pH during enzymatic hydrolysis. Whatman® glass microfiber filters of grade GF/D with a pore size of 2.7µm are used for filtrations.

4.2.2. Water washing of seaweed

The seaweed was milled to a size of 1.5mm for this treatment series. In the case of *Palmaria palmata*, drying in an airflow oven at 60°, prior to milling was necessary. Both seaweed materials were characterized according to the chosen method in chapter 2, to obtain the respective biochemical compositions of the feed for the treatment series. Figure 4.1 represents the cascade treatment approach for the extraction. 1.5 kg of the milled seaweed was washed with water. For this, a seaweed loading of 10% on the dry weight basis of seaweed was used. Washing was carried out overnight on a roller bank. The solid and liquid fractions from the washed material were separated by centrifugal separation in a decanter centrifuge, operated at 4000 rpm and a differential speed of 15 rpm. The small representative sample of the solid residue was freeze-dried in order to determine its biochemical composition by acid hydrolysis, as per the selected method as in chapter 2. To achieve better separation, vacuum-assisted flatbed filtration using a glass microfiber filter with a pore size of 2.7µm was carried out on the liquid fraction. Diatomaceous earth was used as a filter aid. Thereafter, further fractionation was conducted by means of two membrane ultra-filtration steps to recover fractions of molecular weights >100 kDa, 100-1 kDa and <1 kDa. Each membrane filtration step was run until attaining a permeation of 70% on a mass basis of the feed. The first ultrafiltration step was performed under a pressure of 3 bar on the membrane plates and recovered fractions of MW >100 kDa in the retentate. The second ultrafiltration step was performed under a plate pressure of 7bar and recovered fractions of MW 100-0.1 kDa and <1 kDa as retentate and permeate, respectively. The washed seaweed was subjected to extractions at different conditions. Samples from all liquid streams are post hydrolysed with 2M H_2SO_4 at 100°for 2 hours and thereafter analysed for the complete spectrum of seaweed carbohydrates in the HPAEC-PAD. The small amount of the washed solid was freeze-dried and its biochemical composition was determined after a pre-extraction with 60% ethanol. For the determination of the biochemical composition of solids, analysis was done in duplicates. The moisture and ash contents of the feed as well as the washed solid were determined gravimetrically. The total solid content and ash content of each liquid intermediate and stream was also determined gravimetrically.

4.2.3. Extraction of sugars by hot water

The pre-washed solids were subject to treatments with hot water in an autoclave. These were carried out at the conditions that were deemed most suitable for the respective seaweed type, from the temperature and time studies conducted in the multiclave. For this, the pre-washed seaweed and demineralized water were loaded into the autoclave to a total of 1.75 kg while maintaining a consistency of 10% on the dry weight basis of the washed seaweed. The *Ulva lactuca* was treated at 120°C and the *Palmaria palmata* at 140°C. The treatment time was 1 hour, along with some additional time for heating and cooling. The residual solid and liquid hydrolysate were separated by means of a centrifuge, operated at 4000 rpm for 15 minutes. The liquid hydrolysate was filtered by vacuum-assisted flatbed filtration using a glass microfiber filter with a pore size of 2.7µm. The hydrolysate was analysed in the HPAEC-PAD both directly and after post-hydrolysis, to determine both the monomeric and total sugar

content, respectively. A small part of the solid residue was freeze-dried for the determination of their biochemical composition by acid hydrolysis.

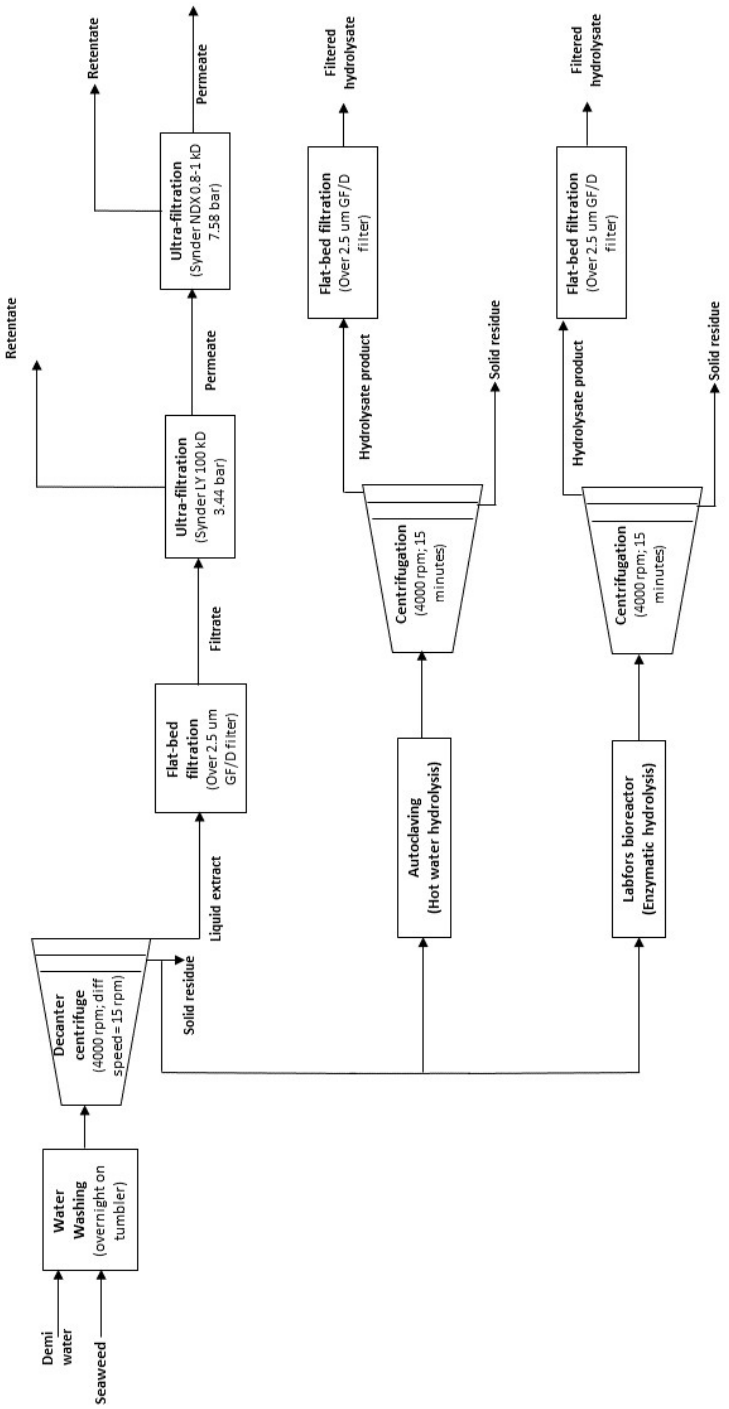


Figure 4.1: Block diagram for the two cascaded biorefinery approaches for the extraction of rhamnose and xylose from *Ulva lactuca* and *Palmaria palmata*.

4.2.4. Extraction of sugars by enzymatic hydrolysis

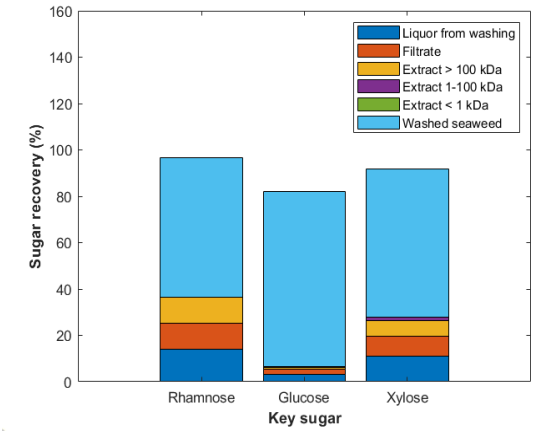
The pre-washed solids were subjected to enzymatic hydrolysis in the Labfors bioreactor. The conditions of these treatments were chosen based on the temperature, pH and dosage studies conducted for the enzymes on each seaweed. For *Ulva lactuca*, a 72 hour hydrolysis with XYTM at 50°C was chosen. In the case of *Palmaria palmata*, hydrolysis with CTEC2 at 50°C for 72 hours was selected. An XYTM dosage of 3% was used for hydrolysis of *Ulva lactuca* and a CTEC2 dosage of 25% was used for the hydrolysis of *Palmaria palmata*. A pH of 5 was desired for both seaweeds. The reactor has a PID system to control pH by gradual addition of acid and base, as required. The pH of the reaction mixture, however, needs to be in the vicinity of the desired pH, in order to avoid enzyme damage or inactivity. This was managed by the initial addition of an estimated amount of acid or base, manually. 200 g of pre-washed seaweed on a dry weight basis was loaded into the bioreactor. A 10% consistency of the reaction mixture at the start was intended and the required amounts of buffer, acid or base, enzyme and demineralized water were calculated accordingly. The buffer, acid or base and demineralized water were loaded into the bioreactor after which, heating and stirring of the contents were started. When the set temperature was attained in the bioreactor, the calculated amount of enzyme was loaded and this was recorded as the start of the reaction (t=0). The stirrer speed was maintained at 100 rpm throughout the experiment. The pH was monitored and adjusted by the PID system during the 72 hour hydrolysis in the bioreactor. Samples were made at 0, 6, 24, 48 and 72 hours of hydrolysis. On sampling from the bioreactor, the samples were immediately cooled in an ice bath to stop hydrolysis and then run in a centrifuge separator at 4000 rpm for 15 minutes. The separated hydrolysate was analysed for carbohydrates in the HPAEC-PAD, both directly and after post-hydrolysis with 2M H_2SO_4.

4.3. Results and Discussion

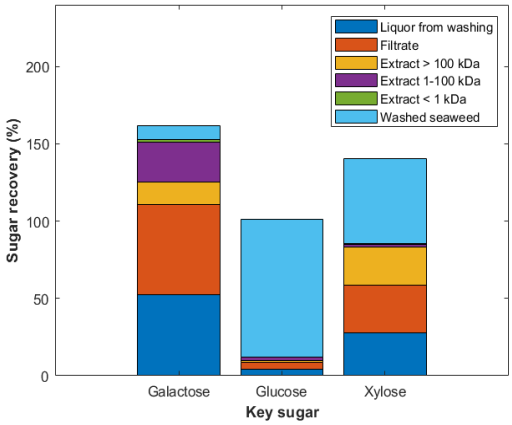
This section begins with the results of the larger scale water washing and subsequent filtrations, followed by those of extraction of sugars by the hot water treatment in the autoclave and enzymatic hydrolysis in the bioreactor. Thereafter, an outline of the sugar recoveries from the cascade treatment series as a whole are provided.

4.3.1. Water washing and filtration

The various post-hydrolysed liquid streams from the water washing of seaweed and subsequent filtration and ultra-filtration steps as well as the hydrolysate from the biochemical composition of the washed solids by acid hydrolysis were analysed in the HPAEC-PAD for the estimation of seaweed carbohydrates present. The carbohydrate contents are presented in figure 4.2 as a percentage of the respective sugar in the feed, which is obtained from the biochemical composition of the feed determined by the procedure put forth in chapter 2. From figure 4.2a it can be observed that after washing of the *Ulva lactuca*, the washed solid and the liquid fractions recovered by separation in the decanter centrifuge were found to contain 60.3% and 13.9% of the rhamnose contents of the *Ulva lactuca* used as the raw material or feed. The loss in rhamnose here may be due to some amount of the rhamnose being present in the wet solid residue which was not quantified as the determination of the biochemical composition of the solid residue was carried out on dried solid residue. Furthermore, all or some of the rhamnose not recovered herein may have been present in the form of oligomers released during the washing of the *Ulva lactuca*. It is possible that the intensive conditions imposed during the acid hydrolysis of the washed and dried *Ulva lactuca* brought about the degradation of the released oligomeric rhamnose. The subsequent filtration and ultra-filtration steps performed on the washing liquor resulted in a final rhamnose recovery of 11%. Similarly, a xylose recovery of 64% in the washed solid and 9% together in the retentate streams from the two ultra-filtration steps was obtained. In the case of glucose, 75% was recovered in the washed solid. The washing of *Palmaria palmata* resulted in xylose recoveries of 55% and 28% in the washed solid and washing liquor, respectively as seen in figure 4.2b. A high galactose recovery was obtained in the liquid intermediates. 9% and 42% of the galactose content of *Palmaria palmata* were recovered in the washed solid and retained liquid streams from the two step ultra-filtration of the washing liquor. A high glucose recovery of 90% in the washed solid was obtained.



(a) Total key sugar recovery from the water-washing and filtration of *Ulva lactuca*.



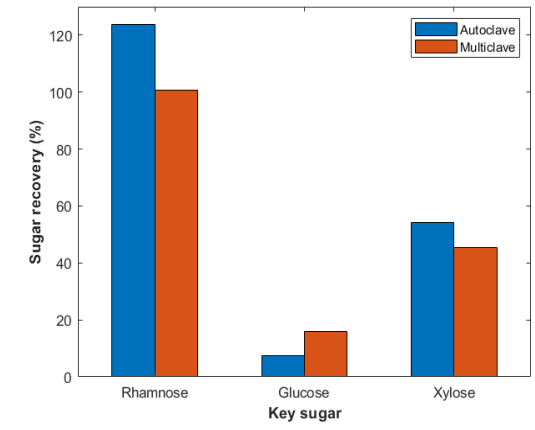
(b) Total key sugar recovery from the water-washing and filtration of *Palmaria palmata*.

Figure 4.2: Total key sugar recovery from the various streams of the large scale water-washing and subsequent filtrations performed on seaweed.

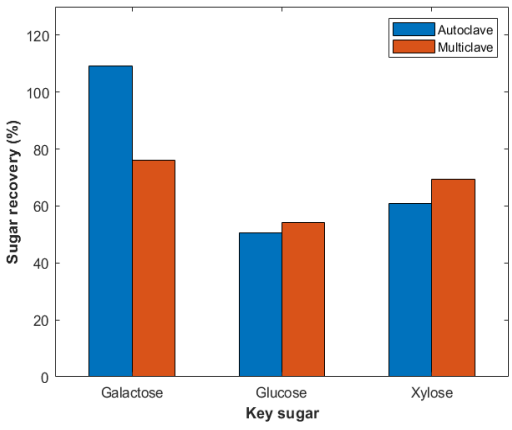
4.3.2. Extraction of sugars from seaweed by treatment with hot water

The extraction of total key sugars from *Ulva lactuca* and *Palmaria palmata* by treatment with hot water in an autoclave for 1 hour at 120°C and 140°C, respectively are represented in figures 4.3a and 4.3b. The total key sugars extracted are presented herein as a percentage of the respective sugar in the feed for the hot water hydrolysis in the autoclave. The feed herein was the washed seaweed. It was characterized to obtain its biochemical composition following the method put forth in chapter 2 and these compositions formed the basis for estimating the amount of the respective sugar extracted from the seaweed in the hydrolysate. The extraction is compared with the treatments performed on a smaller scale in the multiclave, in figure 4.3.

The hydrolysis of *Ulva lactuca* by hot water at 120°C in the autoclave resulted in the extraction of rhamnose in very high amounts. This was also accompanied by a moderately high recovery of xylose from the washed seaweed. Both, rhamnose and xylose were extracted to greater and comparable extents to that in the multiclave, thereby indicating the reliability of this low severity hydrolysis technique at this scale.



(a) Extraction of key sugars from *Ulva lactuca* by treatment with hot water in an autoclave and a multiclave at 120°C for 1 hour.



(b) Extraction of key sugars from *Palmaria palmata* by treatment with hot water in an autoclave and a multiclave at 140°C for 1 hour.

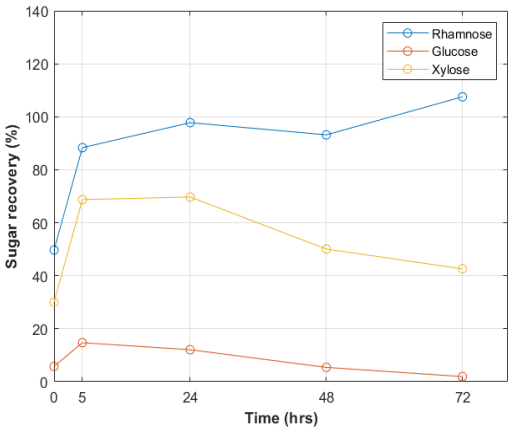
Figure 4.3: Comparison of extraction of key sugars from seaweed by treatment with hot water on a 2L scale in an autoclave and on a smaller scale in a multiclave.

In the case of hydrolysis of pre-washed *Palmaria palmata* at 140°C, 61% of the xylose present in the feed was extracted. This is comparable to the extent of xylose extraction in the treatment carried out in the multiclave for the same temperature and reaction time which was 69%, as seen in figure 4.3b.

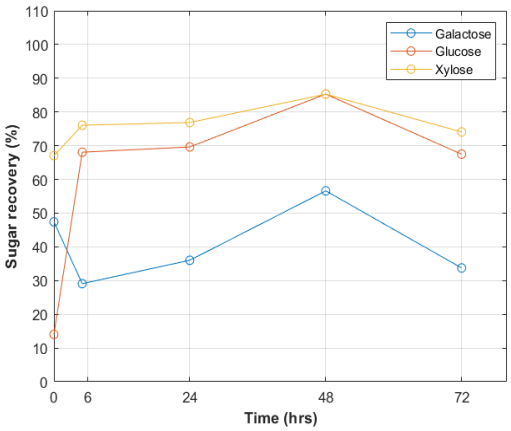
The hydrolysates from the treatments on both seaweeds were also analysed directly in the HPAEC-PAD, without post-hydrolysis in order to determine the form in which the key sugars were released. In the case of *Ulva lactuca*, all the rhamnose and xylose extracted were released in the form of oligomers. Furthermore, only 3% of the glucose was released as monomeric glucose and the rest, again, as oligomers. In the case of *Palmaria palmata*, galactose, glucose and xylose were all released as oligomers.

4.3.3. Extraction of sugars by the enzymatic hydrolysis of seaweed

The extraction of total key sugars by the enzymatic hydrolysis *Ulva lactuca* and *Palmaria palmata* by XYTM and CTEC2, respectively in the 3L bioreactor at 50°C are presented in figures 4.4a and 4.4b. From figure 4.4a, it is clear that all of the rhamnose from the washed seaweed was extracted. Furthermore, the inhibition of glucose release was seen just as in the case of the small scale (50 mL) hydrolysis. As in this study, rhamnose is the sugar of interest, lower glucose contents in the hydrolysate is preferential. From figure 4.4b, it is observed that 74% of the xylose in the pre-washed *Palmaria palmata* was extracted after 72 hours of hydrolysis.



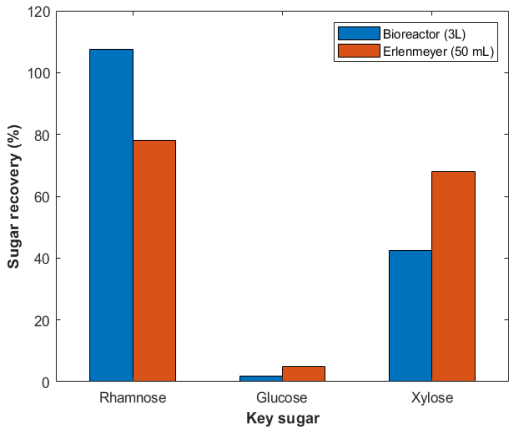
(a) Extraction of key sugars from *Ulva lactuca* by enzymatic hydrolysis with XYTM in a bioreactor at 50°C for 72 hours.



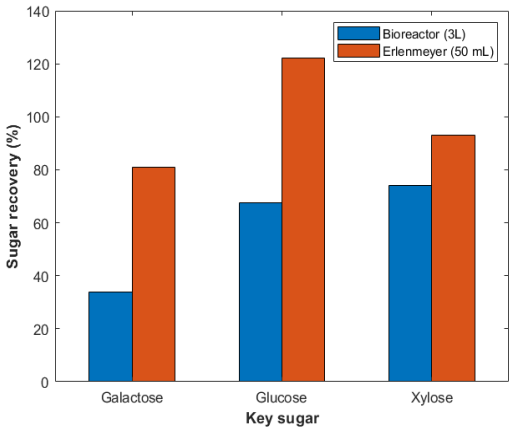
(b) Extraction of key sugars from *Palmaria palmata* by enzymatic hydrolysis with CTEC2 in a bioreactor at 50°C for 72 hours.

Figure 4.4: Extraction of key sugars from *Ulva lactuca* and *Palmaria palmata* by enzymatic hydrolysis in a bioreactor at 50°C for 72 hours.

In order to evaluate the upsizing of the enzymatic hydrolysis treatments in comparison with the smaller scale treatments at the same conditions of temperature, pH and enzyme dosages, the recovery of the respective key sugars from the two seaweed types after 72 hours of hydrolysis are presented in figure 4.5. It is to be noted that the recoveries here are on the basis of the seaweed used as feed for the respective tests. In the case of the bioreactor tests, the feed was pre-washed seaweed at the 15L scale and in the case of the Erlenmeyer tests, the feed was unwashed seaweed without any pre-treatments. From figure 4.5a, it can be observed that for both scales of treatment, a high rhamnose yield, moderate xylose yield and very low glucose yield was obtained from *Ulva lactuca*. The higher extent of extraction of the key sugars in this study which are rhamnose from *Ulva lactuca* and xylose from *Palmaria palmata* in the autoclave when compared with their respective counterparts at the smaller scale as seen clearly in the two figures reinforce the significance of washing of seaweed prior to extraction by hydrolysis.



(a) Extraction of key sugars from *Ulva lactuca* by enzymatic hydrolysis with XYTM in a bioreactor and in an Erlenmeyer flask at 50°C for 72 hours.

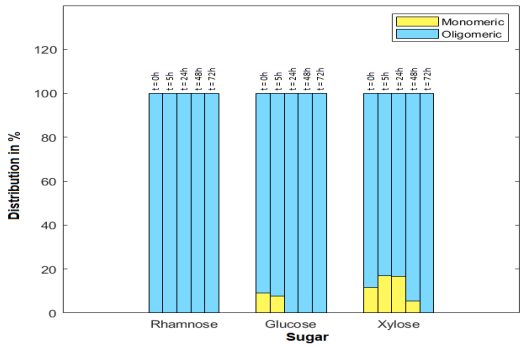


(b) Extraction of key sugars from *Palmaria palmata* by enzymatic hydrolysis with CTEC2 in a bioreactor and in an Erlenmeyer flask at 50°C for 72 hours.

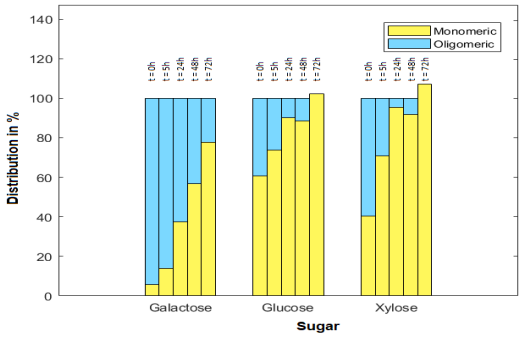
Figure 4.5: Extraction of key sugars from *Ulva lactuca* and *Palmaria palmata* by enzymatic hydrolysis in a bioreactor and in an Erlenmeyer flask at 50°C for 72 hours.

The hydrolysates from the bioreactor tests were also analysed directly in the HPAEC-PAD to determine only the sugars released as monomers. From the monomeric and total sugar contents in the hydrolysates, the distribution between the monomeric and oligomeric release of sugars were calculated and plotted in figure 4.6. All the rhamnose extracted from *Ulva lactuca* was released as oligomers. Xylose and glucose were released mainly as oligomers. The small amounts of xylose and glucose released as monomers, which were <20% and <10% respectively reduced over time and at 72 hours of hydrolysis, both these sugars were completely in the form of oligomers in the hydrolysate. This could be owing to the degradation of xylose and glucose over time or its consumption by microbial activity which could have possibly led to the drop in recoveries as seen in figure 4.4a.

In the case of the extraction of sugars from *Palmaria palmata*, the release of galactose, glucose and xylose as monomers increased over time. In figure 4.6b, a monomeric content of above 100% indicates a higher monomeric sugar content than the total sugar content in the hydrolysate. In these cases, all of the sugars were released as monomers and on post-hydrolysis of these hydrolysates, monomeric sugar degradation lower sugar contents in the hydrolysates. This is seen in the case of xylose and glucose in the hydrolysate from 72 hours of hydrolysis.



(a) Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of *Ulva lactuca* by XYTM.



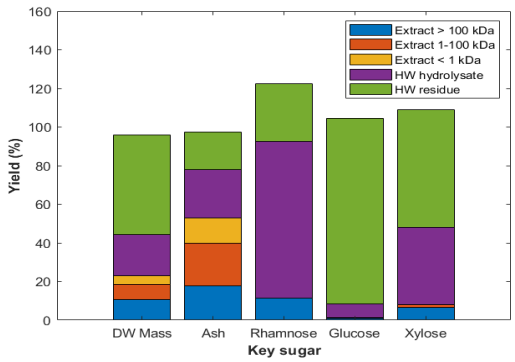
(b) Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of *Palmaria palmata* by CTEC2.

Figure 4.6: Distribution of key sugars in their monomeric and oligomeric forms in the enzymatic hydrolysis of seaweed.

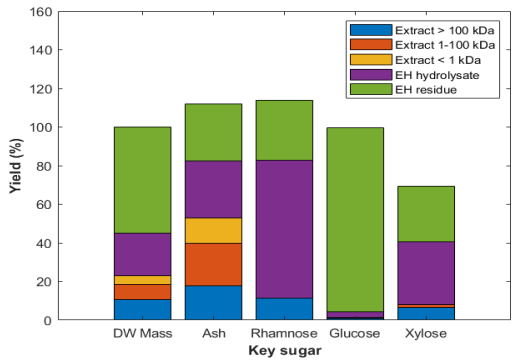
4.3.4. Cascade biorefinery approach for the extraction of key sugars from *Ulva lactuca* and *Palmaria palmata*

The final objective of this research project was to develop a suitable cascade of treatments for the fractionation of the two seaweed types in this study and validate the cascade approach at a 2L-3L scale. A high cumulative recovery of the total key sugars from each extraction step is the primary factor to assess the performance of the approach. Hence, in this section, the sugars of interest in this study that are extracted from *Ulva lactuca* and *Palmaria palmata* in each treatment step are presented as the fraction of the respective sugar in the raw material. Furthermore, a distribution of the ash content in the various product streams are presented as a fraction of the initial ash content in the raw material on a dry weight basis.

Figure 4.7a represents the performance of the hot water hydrolysis treatment approach on *Ulva lactuca*. Of the rhamnose removed from the seaweed by washing was recovered in the retentate stream from the first ultrafiltration step. This recovered fraction in the retentate stream of MW > 100 kDa was 11.2% of the total rhamnose content in the *Ulva lactuca* used as feed. The hydrolysis of the washed seaweed by hot water in the autoclave, resulted in a total rhamnose recovery of 81.4% in the hydrolysate. Thus, a rhamnose yield of 92.7% was achieved through this series of treatments. The residue from the hot water hydrolysis in the autoclave was freeze-dried and its biochemical composition was determined by acid hydrolysis to find that it contained 29% rhamnose on a dry weight basis of the original seaweed. It is possible that this was slightly overestimated owing to any rhamnose that was released as oligomers during the washing of seaweed that were retained in the residue. In the case of glucose, very low amounts were extracted which is preferred. A majority of the glucose (≈96%) was retained in the final residue from the autoclave. 48% of the xylose in *Ulva lactuca* was extracted together in the retentate streams of the two ultrafiltration steps and the hydrolysate from the hot water hydrolysis. From the ash distribution amongst various streams, it can be seen that nearly 53% of the inorganics present in the seaweed was removed by pre-washing.



(a) Overall mass balance and sugar yield from the various stages in the cascade biorefinery approach for the extraction of sugars from *Ulva lactuca* by hot water hydrolysis.



(b) Overall mass balance and sugar yield from the various stages in the cascade biorefinery approach for the extraction of sugars from *Ulva lactuca* by enzymatic hydrolysis with XYTM.

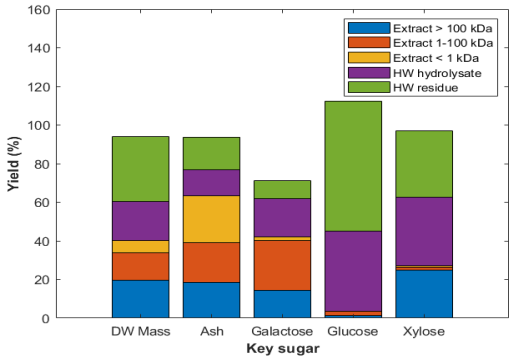
Figure 4.7: Overall mass balance and sugar yield from the various treatment steps in the cascade biorefinery approaches for the extraction of sugars from *Ulva lactuca* by low severity hydrolysis methods. In the figures, 'Extract >100 kDa' is the retentate stream from the first ultrafiltration step; 'Extract 1-100 kDa' is the retentate stream from the second ultrafiltration step and 'Extract <1 kDa' is the permeate stream from the second ultrafiltration step; 'HW hydrolysate' is the hydrolysate from the hot water hydrolysis in the autoclave; 'HW residue' is the solid residue from the hot water hydrolysis in the autoclave; 'EH hydrolysate' is the hydrolysate from the enzymatic hydrolysis in the bioreactor and 'EH residue' is the solid residue from the enzymatic hydrolysis in the bioreactor.

Figure 4.7b represents the performance of the enzymatic hydrolysis approach on *Ulva lactuca* with XYTM. The cumulative rhamnose yield from the series of treatments was nearly 83%. 72% was extracted from the washed seaweed during the enzymatic hydrolysis in the bioreactor and the other 11% was recovered in the retentate stream of the first ultrafiltration step. On characterization of the solid residue in the bioreactor, 31% of the rhamnose content in the original seaweed material was found

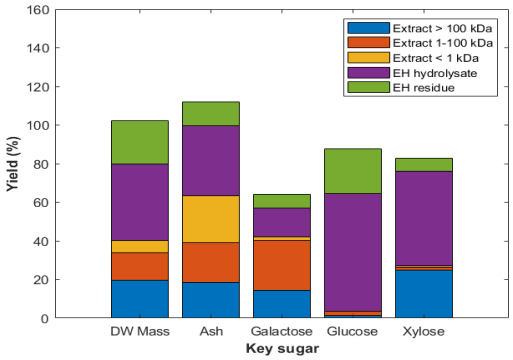
to be retained. Similar to the case of the hot water hydrolysis approach, very low amounts of glucose were extracted in the enzymatic hydrolysis approach as well. The final solid residue was found to retain over 99% of the total glucose content. 39% was extracted together in the retentate streams of the two ultrafiltration steps and the final hydrolysate.

Figure 4.8a represents the performance of the hot water hydrolysis treatment approach on *Palmaria palmata*. From the ash distribution amongst various streams, it can be seen that nearly 63% of the inorganics present in the seaweed was removed by pre-washing. The xylose yield in three streams from the ultrafiltration steps together amounted to 27%. Another 36% was recovered in the hydrolysate from the hot water treatment in the autoclave. In total, this amounted to a xylose yield of nearly 63% from this biorefining approach while the solid residue retained 34%. As opposed to the case of a similar treatment approach on the on *Ulva lactuca*, a moderate amount of glucose was extracted from *Palmaria palmata*, precisely 45%. This was extracted together in the retentate streams of the two ultrafiltration steps and the hydrolysate from the hot water hydrolysis. Galactose up to a total of 62% was extracted in the water-washing and hot water hydrolysis steps, together.

Figure 4.8b represents the performance of the enzymatic hydrolysis approach on *Palmaria palmata* with CTEC2. The cumulative xylose yield from this treatment approach was 76%. This was accompanied by a moderately high glucose and galactose yield of nearly 65% and 57%, respectively.



(a) Overall mass balance and sugar yield from the various stages in the cascade biorefinery approach for the extraction of sugars from *Palmaria palmata* by hot water hydrolysis.



(b) Overall mass balance and sugar yield from the various stages in the cascade biorefinery approach for the extraction of sugars from *Palmaria palmata* by enzymatic hydrolysis with CTEC2.

Figure 4.8: Overall mass balance and sugar yield from the various treatment steps in the cascade biorefinery approaches for the extraction of sugars from *Palmaria palmata* by low severity hydrolysis methods. In the figures, 'Extract >100 kDa' is the retentate stream from the first ultrafiltration step; 'Extract 1-100 kDa' is the retentate stream from the second ultrafiltration step and 'Extract <1 kDa' is the permeate stream from the second ultrafiltration step; 'HW hydrolysate' is the hydrolysate from the hot water hydrolysis in the autoclave; 'HW residue' is the solid residue from the hot water hydrolysis in the autoclave; 'EH hydrolysate' is the hydrolysate from the enzymatic hydrolysis in the bioreactor and 'EH residue' is the solid residue from the enzymatic hydrolysis in the bioreactor.

4.4. Conclusions

The upsizing of the pre-treatment and selected hydrolysis approaches on the seaweed were evaluated. It was found that the hot water hydrolysis of *Ulva lactuca* carried out in the 2L autoclave resulted in a higher rhamnose recovery from the feed as compared to the smaller scale tests. In the case of *Palmaria palmata*, a xylose recovery of 61% was obtained in the autoclave which is comparable with the 69% achieved in the multiclave. A similar trend was also seen in the enzymatic hydrolysis scale-up tests carried out in the bioreactor, in comparison with their respective counterparts at the smaller 50 mL scale. Both low severity hydrolysis methods can therefore be considered to be successfully validated at a 2-3L scale.

In the case of *Ulva lactuca*, slightly higher rhamnose and xylose yields were achieved with the hot water hydrolysis approach than with the enzymatic hydrolysis approach. The hydrolysate from the for-

mer was also found to have a lower amount of inorganics when compared with the hydrolysate from the latter. These two low severity hydrolysis methods for rhamnose extraction performed well in terms of rhamnose recovery. However, it is to be kept in mind that while the hot water approach requires a higher temperature than the enzymatic hydrolysis approach, the use of XYTM, which is not yet a commercially produced enzyme, is required for the enzymatic route. Thus, the comparison between the two is not within the scope of this study.

In the case of *Palmaria palmata*, the enzymatic route is significantly better in performance than the hot water route, with xylose recoveries of 74% and 61%, respectively. CTEC2 is a commercially produced enzyme and thus this low temperature hydrolysis route for extraction of xylose from *Palmaria palmata* is attractive.

All four cascade biorefinery approaches resulted in high sugar recoveries. 92.7% of the rhamnose in *Ulva lactuca* was extracted via the cascade biorefinery approach that employed the hot water treatment as the hydrolysis route and 83% via the approach that employed enzymatic treatment with XYTM as the hydrolysis route, post washing of the seaweed with water. 63% of the xylose in *Palmaria palmata* was extracted via the cascade biorefinery approach that employed the hot water treatment as the hydrolysis route and 76% via the approach that employed enzymatic treatment with CTEC2 as the hydrolysis route, post washing of the seaweed with water.

5

Discussion, Conclusions and Recommendations

In this chapter, the observations from the previous chapters are discussed and concluded based on the results and the existing literature.

5.1. Discussion and Conclusions

The biochemical composition of *Ulva lactuca* and *Palmaria palmata* was determined. The total rhamnose, glucose, xylose and glucuronic acid contents in **Ulva lactuca** were found to be 10.77%, 21%, 3.6% and 3.3% of the dry weight of the seaweed, respectively. The galactose, glucose and xylose contents of *Palmaria palmata* were estimated as 18.4%, 5.6% and 33% of the dry weight of the seaweed, respectively. This was obtained by pre-washing the seaweed with water and then performing a pre-extraction with 60% ethanol solvent with 16 hours of reflux in the soxhlet extraction. The solvent was selected over others as its use within the treatment series for seaweed characterization resulted in the highest total rhamnose and total xylose yields from textitUlva lactuca and *Palmaria palmata*, respectively. The pre-washing step resulted in the removal of inorganics from the seaweed, which is seen as a reduction in the ash content of the seaweed from 28% to 18.8% in the case of *Ulva lactuca* and from 20% to 15.4% in the case of *Palmaria palmata*. Furthermore, the washing pre-treatment step resulted in higher rhamnose and xylose extraction from *Ulva lactuca* and *Palmaria palmata*, respectively. Only 8.7% rhamnose on the dry weight basis of *Ulva lactuca* and 29.7% xylose on the dry weight basis of *Palmaria palmata* were extractible without pre-washing of the seaweed. Therefore, the most suitable pre-treatments, prior to hydrolysis, for determination of carbohydrate composition is pre-washing with water followed by soxhlet extraction of the washed seaweed with 60% ethanol as solvent.

Low severity hydrolysis routes for the extraction of the targeted sugars were investigated. For the enzymatic route, hydrolysis with CTEC2 and β-D-xylanase from T.maritima(XYTM) were found to be viable extraction routes. From temperature variation studies, it was found that XYTM activity is not restricted to operation in the vicinity of its optimum temperature of 80°C. The extraction key carbohydrates by enzymatic hydrolysis with XYTM at 50°C was comparable with that at its optimum temperature. Enzymatic hydrolysis by CTEC2 at 45°C resulted in higher rhamnose and xylose yields from *Ulva lactuca* and higher xylose yields from *Palmaria palmata* than at 55°C. Enzymatic hydrolysis by XYTM showed lesser significant temperature dependence. Within the pH range of 4.5 to 6, extraction of key carbohydrates did not vary drastically and thus the optimum pH of 5 as recommended by the enzyme producer is suitable for this application. Hydrolysis of *Ulva lactuca* by XYTM for 72 hours at 50°C with an enzyme dosage of 3% of the seaweed loading resulted in the highest rhamnose recovery and the added bonus of inhibited glucose release. Hydrolysis of *Palmaria palmata* by CTEC2 for 72 hours 50°C with an enzyme dosage of 25% of the seaweed loading resulted in the highest xylose recovery. The scale-up of

this hydrolysis route to a 3L bioreactor setup within the cascade approach resulted in an overall rhamnose recovery of 83% from *Ulva lactuca* and a xylose recovery 76% from *Palmaria palmata* through the series of treatments.

The use of organic acids and chelating salts as hydrolysing agents were found to have a lower extent of rhamnose extraction from *Ulva lactuca* when compared with hydrolysis by hot water at the same conditions. In the case of *Palmaria palmata*, the extent of xylose extraction was only slightly higher by oxalic acid hydrolysis than via hydrolysis with hot water. Hydrolysis with hot water is thus prefered, water being a more favourable reagent. Hydrolysis of *Ulva lactuca* at 120°C for 1 hour *Palmaria palmata* at 140°for 1 hour resulted in highest rhamnose and xylose yields, respectively. The scale-up of this hydrolysis route to a 2L autoclave setup within the cascade approach resulted in an overall rhamnose recovery of 92.7% from *Ulva lactuca* and a xylose recovery 63% from *Palmaria palmata* through the series of treatments.

5.2. Recommendations

- While a soxhlet pre-extraction step was found to be effective in maximising the release of key sugars during the hydrolysis of seaweed, an alternative to this conventional extraction technique that can be applied to a larger scale of treatment would be interesting to find.
- The extraction and recovery of value-added products like proteins, pigments, salts and lipids can contribute to the valorization of the biorefinery approach. Analyses such as determination of total solids, carbon, hydrogen, nitrogen, mineral (K, Ca, Na, S) and ash contents were conducted on most of the solid residues and dried liquid extracts and intermediates obtained in this project. The results from these analyses can serve as a starting point for developments on process valorization.
- The enzyme screening phase of the project included the selection of two of the six enzymes based on the monomeric sugars detected in the hydrolysates. A more reliable basis of comparison would be the total sugar contents in the hydrolysates.

A

Laboratory Analytical Procedures and Protocols

A.1. Determination of moisture and ash contents in biomass

This protocol is adopted from the U.S National Renewable Energy Laboratory (NREL) laboratory analytical procedures for biomass treatment [39] [40]

1. The required number of cleaned crucible filters were placed in an air-flow oven at 105°C overnight, for drying.
2. They were then let to cool in a desiccator for an hour after which the empty weight correct to 0.1mg, is recorded.
3. The crucible filters were placed back in the oven (at 105°C) for an hour to dry to constant weight. They were then let to cool in the desiccator for an hour and the empty weight of the of the crucible filter is checked again. Constant weight is defined as a standard deviation of lower than 0.0004. If this was not the case, then step 3 was repeated until so.
4. 1g ±0.2g of the respective samples were added into the prepared crucibles and they were placed in the oven at 105°C for 24 hours or overnight.
5. Thereafter, steps 2 through 4 were repeated to obtain the dry weight of the samples.
6. The crucibles with dry samples were placed in a muzzle furnace for ashing at 550°C for 6 hours. A ramping program was used as follows

    - Ramping from room temperature to 550°C in 1 hour.
    - Hold at 550°C for 6 hours.
    - Allow temperature to drop

7. The crucibles were then removed from the furnace into the oven at 105°C where they were held for an hour before cooling down in a desiccator and weighing the residue from ashing to constant weight as in step 3.
8. The residue was then discarded and the crucibles were then cleaned using a vacuum pump and demineralised water.

A.2. Protocol for soxhlet extraction

This protocol is adopted from the U.S National Renewable Energy Laboratory (NREL) laboratory analytical procedures for biomass treatment [41]

1. A round bottom boiling flask was dried in an oven at 105°C overnight and then weighed with a magnetic stirring stone inside.
2. A a cellulose thimble was dried in a vacuum oven at 50°overnight and its weight was noted down. The Whatman 10350241 (33mm x 90mm) thimbles were used.
3. The moisture content of the biomass sample was checked just before the extraction begins, by means of a halogen analyser.
4. The condenser was turned on and set to 6°C.
5. 5-7 g of the biomass sample was weighed into the thimble and the thimble was then fit into the extraction device.
6. 150ml of the solvent was added into the round bottom flask and the weight was noted down. The flask was wrapped with some aluminium foil to hold the heat and it was then attached to the soxhlet unit.
7. The heating jacket was turned on and set according to the boiling point of the respect solvent, to enable the process to begin.
8. The solvent was refluxed for 16 hours, after which, the heating was turned off and the apparatus was left to cool foe a while.
9. Thereafter, the condenser was also turned off and the extractives in the flask was weighed. The thimble was weighed and then allowed to stand covered in a fume hood for a few hours for the release of any volatile solvent retained in it.
10. The sample in the thimble was then freeze dried for further analysis.

A.3. Post hydrolysis of liquid extracts

1. 1mL of the sample was added to a 5mL glass culture tube with a screw cap. The weight of the sample was noted down.
2. 1mL of 2M H_2SO_4 was added and the weight was noted down again.
3. The contents of the tube were mixed and they were post hydrolysed in a water bath at 100°C for 2 hours. Mixing was done every 20 minutes to avoid any precipitate formation.
4. At the end of 2 hours, the tubes were immediately placed in an ice bath to stop the hydrolysis.
5. The tubes were weighed again, once cool.
6. By means of a pasture pipette, approximately 1.5 of the post-hydrolysed liquor was sampled out into a vial and stored frozen until sugar analysis.

B

Sugars in soxhlet extractives

The total sugars detected in the soxhlet extractives are expressed a percentge of the dry weight of the original seaweed, milled to 0.2mm. For this, the solvent:solid ratio of the respective soxhlet extractions and the solid yield of the water washing treatment step are used, where ever applicable.

Seaweed pre-treatment	Solvent for soxhlet extraction	Sugars in Ulva lactuca		Sugars in Palmaria palmata	
		Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)
water-washing	ethanol	0.000	0.000	2.489	0.134
water-washing	acetone	0.000	0.000	0.392	0.000
water-washing	ethanol (60%)	0.175	0.175	3.325	0.152
water-washing	acetone (60%)	0.000	0.156	4.62	0.14
None	ethanol (60%)	0.205	0.575	10.242	0.000

Table B.1: Sugars detected in the extractives from the soxhlet extraction treatment step of Ulva lactuca and Palmaria palmata. The sugars are expressed on the dry weight basis of the original seaweed, before any treatments were performed on it.

C

Recovery and yield of soxhlet extractions

The recovery and yield of each of the soxhlet extractions are presented in Table C.1.

Seaweed pre-treatment	Solvent for soxhlet extraction	Ulva lactuca		Palmaria palmata	
		Mass recovery (%)	Solid yield (%)	Mass recovery (%)	Solid yield (%)
water-washing	ethanol	91.98	96.50	98.34	83.72
water-washing	acetone	95.29	97.59	97.21	98.13
water-washing	ethanol (60%)	98.93	86.31	98.27	76.33
water-washing	acetone (60%)	97.32	92.90	80.19	81.44
None	ethanol (60%)	97.62	82.37	97.37	57.67

Table C.1: Recovery and solid yield of the soxhlet extraction treatment step, calculated from experimental data.

D

CHN Analysis

The procedure is adopted from the ISO 16948:2015 standard, 'Solid biofuels – Determination of total content of carbon, hydrogen and nitrogen'. A summary of the procedure is as below.

A known mass of the sample is burnt in a carrier gas mixture of oxygen/helium under conditions such that it is converted into ash and gaseous products of combustion using a reactor which is packed with quartz wool, reduced copper, chromium oxide and silvered cobalt oxide. The temperature of the reactor column is maintained at 975 °C. The products of combustion consist mainly of carbon dioxide, water vapour and nitrogen and/or oxides of nitrogen, oxides and oxyacids of sulfur and hydrogen halides. The products of combustion are treated to ensure that any hydrogen associated with sulfur or halides products are liberated as water vapour. Oxides of nitrogen are reduced to nitrogen, and those products of combustion which would interfere with the subsequent gas analysis procedure are removed. The carbon dioxide, water vapour and nitrogen mass fractions of the gas stream are then determined quantitatively by gas chromatography with thermal conductivity detection (TCD). Calibration of the instrument is performed using certified acetanilide (carbon content: 71.09%, hydrogen content: 6.71%, nitrogen content: 10.36%) [42].

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