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Lactic acid bacteria and yeast fermentation of *Ulva rigida* in bench-scale bioreactors to improve protein content and nutritional value

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Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The reported experimental studies in this dissertation were carried out at the Institute of Bioengineering and Biosciences (iBB) in Instituto Superior Tecnico (Lisbon, Portugal), under the supervision of Dr. Maria Teresa Ferreira Cesário Smolders and Dr. Marília Clemente Velez Mateus.

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Abstract

The increasing demand for sustainable and cost-effective nutritional sources has spurred intensive research into finding natural alternatives to fish meal. This study aims to explore the use of *Ulva* hydrolysates for such purpose. Green macroalgae are an abundant resource with rich nutritional profile, embodying immense potential for fish feed formulations. The analysis of the *U. rigida* whole biomass batches revealed carbohydrate and protein contents of 47% DW and 2% DW, respectively.

The seaweed biomass was subjected to a two-step treatment involving acid hydrolysis to release fermentable sugars and fed-batch fermentation to enrich the resulting product with increased protein content and improved nutritional quality. Bench-scale bioreactor assays were conducted with *Lactobacillus* consortium or *Saccharomyces cerevisiae* monocultures as well as mixed culture of LAB and yeast: (i) 3% sulfuric acid treatment was used, as it presented a suitable balance between sugar release (11 g/L glucose, 2 g/L xylose and 3 g/L rhamnose) and inhibitors generation (90 mg/L of both furfural and HMF);.ii) the mixed genus culture exhibited slightly superior concentrations of lactic acid (101 g/L) compared to lactobacillus culture (95 g/L), while yeast monoculture showed significantly higher levels of ethanol (105 g/L). The analysis of fermentation end products revealed a 3.2-fold protein increase in the fermentations with LAB (single 4LAB and LAB + yeast). However, the co-culture derived product exhibited additional benefits in terms of biological activity. These findings highlight the potential of utilizing *S. cerevisiae* and *Lactobacilli* as starter cultures in seaweed fermentation to produce high-value ingredients.

Keywords: macroalgae; *Ulva rigida*; lactic acid fermentation; acid hydrolysis; ethanol fermentation; nutritional value; fish feed

Resumo

A crescente demanda por fontes nutricionais mais sustentáveis e economicamente viáveis tem impulsionado a pesquisa em torno de alternativas naturais que complementem as proteínas de origem vegetal e animal atualmente disponíveis. As macroalgas verdes em concreto, constituem um recurso abundante e nutricionalmente rico, apresentando o potencial de se tornar numa importante matéria-prima. A caracterização dos lotes utilizados de *U. rigida* revelaram um teor aproximado de hidratos de carbono e proteína de 47% DW e 2% DW (peso seco), respetivamente. A biomassa foi sujeita a um tratamento de duas etapas, que consistiu numa hidrólise ácida para o fracionamento de polissacarídeos e enriquecimento via fermentação. Os ensaios em reator à escala de bancada foram conduzidos com um consórcio de *Lactobacillus* ou monoculturas de *S. cerevisiae* assim como com uma cultura mista de 4LAB e levedura: (i) o pretratamento de ácido sulfúrico a 3% (w/v) foi selecionado para produzir os hidrolisados de alga, uma vez que proporcionou um equilíbrio adequado entre a libertação de açucares (11 g/L de glucose, 2 g/L xilose e 3 g/L ramnose) e produção de inibidores (90 mg/L de furfural e HMF); ii) A cultura mista produziu atingiu uma concentração semelhante de ácido lático (100.85 g/L) em comparação com a monocultura de *Lactobacillus* (95 g/L), enquanto que a monocultura de levedura atingiu níveis significamente mais elevados de etanol (105 g/L). As análises dos produtos finais revelaram um aumento proteico de 3.2 nas fermentações de LAB (monocultura LAB e LAB + levedura). Contudo, os produtos derivados do processo de co-cultura apresentaram uma atividade biológica superior. Este projeto evidencia o potencial da utilização de *S. cerevisiae* e *Lactobacillus* na fermentação de algas marinhas para a produção de ingredientes de elevado valor nutricional.

Palavras-chave: macroalgas; *Ulva rigida;* hidrólise ácida*;* fermentação ácido lática; fermentação alcoólica; valor nutricional; ração aquática

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List of Abbreviatons

BAP - Bioactive Peptides
BSA - Bovine Serum Albumin
CFU - Colony Forming Units
CSL - Corn Steep Liquor
DW - Dry Weight
DH_2O - Distilled Water
DO% - Dissolved Oxygen Percentage
EAE - Enzyme Assisted Extraction
EDTA - Ethylenediaminetetraacetic Acid
EMP - Embden-Meyerhof-Parnas
FAO - Food and Agriculture Organization
FDA - Food and drug Administration
GRAS - Generally Recognized As Safe
HMF - Hydroxymethylfurfural
HPLC - High Performance Liquid Chromatography
IMTA - Integrated Multi-trophic Aquaculture
LA - Lactic Acid
LAB - Lactic Acid Bacteria
MAE - Microwave Assisted Extraction
MRS - De man, rogosa and shape
PUFA - Polyunsaturated Fatty Acid
SFE - Supercritical Fluid Extraction
UAE - Ultrasound Assisted Extraction
OD_{600nm} - Optical Density at 600 nm
YE - Yeast Extract
TCA - Trichloroacetic Acid
WHO - World Health Organization

1. Preamble

Food shortage is foreseen to define food production in the future. While population growth is one of the major drivers of the global food crisis, its impact is exacerbated by several factors, including climate change, arable land and freshwater scarcities. In this scenario, new sources of nutritional supply are required to sustain population growth. A paradigm shift towards a more sustainable global food system is mandatory to overcome the pressing problem of human population unsustained growth.

There has been a steady development in food technology to supply an ever-changing health-conscious consumption habits. This breakthrough opens the possibility to seaweed utilization for such a purpose. Macroalgae, commonly known as seaweeds, are regarded as a nutrient-dense food sources as they contain substantial amounts of proteins, carbohydrates, minerals, and fibers while being relatively low in lipids, making them desirable for balanced diets. Ergo, this marine resource has the potential to become an important feedstock, providing biomass for food and feed applications. Moreover, seaweed cultivation may contribute to carbon sequestration, buffer ocean acidification and climate change mitigation, all of which exert a positive impact on the earth's strained ecosystem. Specifically, *Ulva* spp. biomass is an abundant and low-cost resource, with only a small fraction being utilized in the production of low-value products such as fertilizers, composts, biofuels, biogas, and bioremediation (Farghali et al., 2022).

Without doubt, the establishment of macroalgae as a food source faces some challenges as its biomass is highly perishable, some species contain inferior protein content compared to grains and legumes, and its digestibility is rather low in raw, unprocessed form. Nonetheless, this substrate is suitable for the implementation of a cascading biorefinery that may counteract the earlier constraints while still being an economically feasible operation.

2. State of Art

The global population is expected to increase to nearly 9.7 billion people by 2050. As follows, food production must increase by 70% to feed all humans projected to inhabit Earth. In addition, available arable land is becoming scarce and will even decrease per capita and worldwide (FAO, 2017). Worse still, a portion of the population is already experiencing food shortage, with approximately one billion people worldwide unable to afford nutritious food. In opposition, some countries' high consumption of meat products is deemed as a serious problem in the context of climate change and sustainable production (FAO, 2017; FAO et al., 2018). Thus, the world is faced with the challenge of feeding people, making it critical to identify alternative food sources of nutritional benefit with the use of fewer resources. As a necessary consequence, the demand for novel protein sources is of immediate attention and must extend beyond traditional plant-based proteins such as soy, peas, and wheat (Salgado et al., 2021).

Seaweed resources have enormous potential to address the aforementioned gaps. The foremost benefit is that seaweeds grow in the marine environment, therefore its cultivation does not require fertile soil or freshwater, both of which are resources in increasingly short supply. Additionaly, seaweed productivity far exceeds that of terrestrial biomass (Bikker et al., 2016; H. Fernandes et al., 2019).

Macroalgae are an underexplored marine resource that hold boundless taxonomic diversity with high contents of protein, polysaccharides, and dietary fibers. Therefore, this material offers many opportunities for biorefineries, with algal proteins projected to become an important protein source in the ensuing decades (Wahlström, Nylander, et al., 2020). Although seaweed has been consumed for many millennia in south and east Asia due to its nutritious profile, they are still a largely underutilized food source in the Western countries, being mainly exploited for hydrocolloid extraction (Fleurence et al., 2018).

Green and red macroalgae are the most promising algal groups for food and feed utilization owing to their high value content of proteins (Arru et al., 2022). The natural digestibility and bioavailability of seaweed is undeniably impaired by the presence of anti-nutritional factors, phenolic compounds, and mucilaginous polysaccharides. Nevertheless, the impact of the latter components may be minimized through the application of processing steps including proteolysis, saccharification and fermentation (H. Fernandes et al., 2019). Moreover, additional benefits arise from seaweed fermentation by lactic acid bacteria and yeast, as this consortium enriches the algal material with vitamins, essential amino acids, and fatty acids, enhancing its nutritive value (Gänzle, 2015). In this context, the sequential processing of seaweeds has the potential to become a crucial tool in the discovery, extraction, and processing of novel products with industrial applications.

2.1 Macroalgae

Macroalgae encompass multicellular plant organisms, macroscopic, and eukaryotic, being extremely important primary producers of marine ecosystems (K. Sudhakar et al., 2018). Seaweeds still lack the complex structures found in higher plants, consisting of a leaf like thallus instead of roots, stems and leaves. Further, larger macroalgae have differentiated thalli that include organs for attachment, stem-like structures, and photosynthetic blades (Postma et al., 2018).

In their natural setting, macroalgae grow attached to stable substrates in seawaters, particularly in coastal areas, with some species adapted to proliferate in sedimentary environments while others exist unattached to a surface and float freely. The distribution and morphological characteristics of this group of organisms is primarily determined by a variety of environmental factors with most important of which being the quality and quantity of sunlight (K. Sudhakar et al., 2018).

In marine environments, these organisms are exposed to a variety of physical stressors including high salinity, wide temperature differences, large nutrient dynamics, and strong UV sunlight exposure. As a result, they can produce a vast range of secondary metabolites with specialized chemical and physical properties for their survival such as pigments, vitamins, phenolic compounds, and other active biomolecules (Leandro et al., 2020; Wan et al., 2019). Thus seaweeds, in addition to their ecological relevance, also have a prospective role in the worldwide market.

2.1.2 Classification

The term algae denote microscopic to macroscopic photosynthetic organisms, albeit being only distantly related. Briefly, macroalgae are subdivided in three lineages based on their chemical structure and pigment composition: red algae (*Rhodophyta*), green algae (*Chlorophyta*) and brown algae

(*Phaeophyceae*), each of which possesses unique pigmentation and ultrastructure (Mouritsen, 2013). The presence of chlorophyll α in macroalgae classes is a common feature, but presence of phytopigments other than chlorophyll α is specific to each algal division (Davis et al., 2003). These pigments reflect and absorb light in different wavelengths, as such seaweeds are found at various depths within marine ecosystems (K. Sudhakar et al., 2018).

Another key feature used in macroalgae classification is the nature of the reserve polymers synthesized by photosynthesis. Table 1 summarizes the most significant characteristics concerning the three macroalgal divisions.

Table 1. Criteria for macroalgae classification in green, brown or red. Adapted from (K. Sudhakar et al., 2018)

Subdivision	Characteristics	Photosynthetic Pigments	Genera
Green Algae (Chlorophyta)	Cell wall composed of cellulose (β1,4-glucopyroside) in many genera, xylans and mannans; Starch (amylose and amylopectin), oil in some as storage product	Chlorophyl a and b, carotene, xanthophylls	*Ulva* *Codium*
Brown algae (Phaeophyta)	Olive green to dark brown color; Cellulose in many genera, alginic acid and sulfated muco-polysaccharides (fucoidan) in cell walls; Laminarin (β1,3-glycopyranosidase, predominantly) and oils as storage products	Chlorophyll a, phycoerethrin, phycocyanin, xanthophylls	*Laminaria* *Fucus* *Sargassum*
Red Algae (Rhodophyta)	Cellulose, xylans, several sulfated polysaccharides (galactans) in cell wall; Starch (amylopectin-like) as storage product	Chlorophyll a and c, carotene, xanthophylls	*Gracilaria* *Gelidium* *Porphyra*

2.1.3 Features and Composition

Seaweeds are known to be rich sources of polysaccharides, proteins, minerals, vitamins, and several bioactive compounds, making them increasingly sought as functional ingredients to incorporate in products for different applications (Chan et al., 2017).

Macroalgae exhibit high variation in composition depending on the species, habitats, maturity, and environmental conditions (Chan et al., 2017; Marinho-Soriano et al., 2006). Moreover, the environmental parameters vary according to season and the elicited changes can either stimulate or inhibit the synthesis of certain nutrients (Marinho-Soriano et al., 2006).

Table 2 shows the biochemical compositions of different macroalgae species. Algae may present high moisture (70–90% fresh weight), being susceptible to both chemical and biological degradation. The high moisture content considerably impacts on biochemical reactions, as such after harvesting its biomass deteriorates rapidly. Therefore, post-harvest processing procedures such as lyophilization, drying, pasteurization, among others, are critical to preserve their quality (Gupta et al., 2011). Biomass composition is expressed as percentage of total biomass in dry weight (DW) with the purpose of establishing more robust comparisons between samples.

Table 2. Proximal biochemical composition of several seaweeds from the three macroalgae groups *Chlorophyta*, *Rhodophyta* and *Phaeophyta* expressed in % of algae dry weight (DW). Moisture values are expressed on wet weight basis and remaining values are dry weight based.

Macroalga	Biochemical Composition						Reference
	Moisture (%)	Ash (% DW)	Lipids (% DW)	Protein (% DW)	Fibers (% DW)	Carbohydrates (% DW)	
Chlorophyta							
U. lactuca	-	22.2	0.6	26.8	-	50.4	(Lee et al., 2014)
C. lentillifera	25.3	24.2	0.9	12.5	3.17	59.3	(Marquez et al., 2014)
U. reticulata	22.5	17.6	0.8	21.1	4.84	55.8	(Marquez et al., 2014)
Enteromorph a sp.	78.8	19.2	2.9	16.6	3.54	-	(Sivaramakris hnan et al. 2017)
Rhodophyta							
C. crispus	-	25.2	0.9	8.1	-	65.9	(Lee et al., 2014)
K. alvarezii	86.8 ± 0.7	16.3 ± 0.1	1.0 ± 0.6	6.2 ± 0.5	7.8 ± 0.6	35.9 ± 1.5	(Xiren et al. 2017)
E. cottonii	10.6 ± 1.6	46.3 ± 0.4	1.1 ± 0.1	9.8± 1.3	5.9 ± 1.2	26.5 ± 3.0	(Matanjun et al., 2009)
Phaeophyta							
U. pinnatifida	-	29.5	3.5	23.8	-	43.2	(Lee et al., 2014)
Sargassum s p	11.2	26.2	0.8	10.3	9.8	41.8	(Marquez et al., 2014)
S. polycystum	10.0 ± 0.6	42.4± 0.4	0.3 ± 0.0	5.4± 0.1	39.7 ± 0.6	33.5 ± 1.7	(Matanjun et al., 2009)
P. gymnospora	11.8 ± 0.1	28.3 ± 0.9	1.3 ± 0.0	10.0 ± 0.1	12.5 ± 0.0	36.2 ± 0,0	(Nazarudin et al., 2022)

2.1.4 Proteins

The most common types of proteins in seaweeds are lectins, glycoproteins, phycobiliproteins and mycosporine-like amino acids, many of which possess relevant bioactivities. Among the different classes of proteins identified up to date, it is important to emphasize lectins and phycobiliproteins. Lectins are a group of glycoproteins that exhibit antibacterial, antiviral, anticancer, anti-inflammatory, anti-HIV properties (Kim et al., 2015). Phycobiliproteins are fluorescent proteins that have received great attention due to their numerous medicinal uses, including as antioxidants, antiangiogenic, and neuroprotective (Cuellar-Bermudez et al., 2015).

Algal peptides may also have nutritional interest, and therefore enrich foods. The protein nutritional value is determined based on two parameters, protein digestibility and amino acid profiles (M. P. Sudhakar et al., 2019). Currently, a number of seaweeds have been utilized as food ingredients namely *Caulerpa racemose*, *Ulva intestinalis*, *Ulva lactuca*, and *Ulva rigida,* with the purpose of enhancing the antioxidant properties of cereal-based products (O' Brien et al., 2022).

The protein content in algal sources varies substantially across classes and is one of the major components of seaweeds (Table 2). Generally, red seaweeds are considered the most prominent among all classes, with a protein content up to 47% DW, being inclusively similar to traditional protein sources such as egg, meat, soybean and milk (Thiviya et al., 2022). Conversely, green (26±9% DW) and brown (15±3% DW) specimens exhibit lower protein load (Samarathunga et al., 2022). Yet, an exception was reported for *Undaria pinnatifida* species (wakame), a brown seaweed with a protein profile of 11%–24% of dry weight (Thiviya et al., 2022).

Seasonal cycles impact the protein fraction of algae with maximal values being reported during the period of winter–early spring and the lowest during summer–early autumn. For instance, Fleurence, (1999) showed that *Palmaria palmata* had significant variations in protein profile (9% -25% of proteins) with the highest values occurring during winter and spring months. Seasonal variation in algal protein content has also been reported in other red seaweeds including *Porphyra umbilicalis*, *Chondrus crispus*, *Gracilaria verrucosa*; in the brown seaweeds *Sargassum vulgare* and *L. digitata*; and in the green alga *Ulva lactuca* (Kim et al., 2015). Furthermore, it is noteworthy to mention that comparison of the protein content among algae is difficult because of the methodological differences especially for protein extraction, and the large number of species identified to date (Lourenço et al., 2002). One of the key challenges of most macroalgae purification techniques is to retain the protein structure intact, while also maximizing the extraction yields of added value products (Sadhukhan et al., 2019).

Functional qualities of specific seaweed proteins are defined by their physicochemical properties which include amino acid content, molecular weight, net charge, and surface hydrophobicity. Again, the extraction conditions and enzymatic treatments have an impact on the physicochemical qualities of protein extracts, due to their influence in protein solubility, yield, and purity (Samarathunga et al., 2022).

2.1.5 Carbohydrates and Fibers

Carbohydrates are the major components in algal biomass (1.8% to 66% DW), which are cell wall structural polysaccharides in the majority but also energy storage polysaccharides. Kelp species (*Phaeophyceae*) in particular possess some of the highest carbohydrate contents in any macroalgal group, ranging from 50% to 60% dry weight (Greetham et al., 2020; Wan et al., 2019).

In comparison to terrestrial plants, macroalgae carbohydrates have less cellulose, hemicellulose (2–10% and 9% DW respectively) and only rarely lignin is present (Bayu et al., 2021), which constitutes a major advantage for biorefinery purposes as there is no need for complex processes for carbohydrates extraction and lignin-originated inhibiting compounds detoxification.

Most seaweed taxa cell walls exhibit a microfibrillar skeleton network embedded in a gel-like matrix composed of several carboxylic and/ or sulfated polysaccharides, but other biopolymers such as proteins, proteoglycans and polymeric phenolics may also be present (Synytsya et al., 2015). The most prevalent component of the fibrillar skeleton is cellulose which imparts cell rigidity and mechanical strength. The most relevant matrix associated polysaccharides are ulvan in green seaweeds, alginate and fucoidan in brown seaweeds and agar and carrageenan in red seaweeds, although these components may suffer alterations in different phyla and genera. The main storage polysaccharides are

starch, laminarin and floridean starch in green, brown and red seaweeds respectively (Greetham et al., 2020). A large bulk of the previously referred carbohydrates are not digested by humans and therefore can be regarded as dietary fibers (Cesário et al., 2018).

Aside from a classification based on polysaccharide source, sulfation degree is also an important parameter to consider. In general terms, the degree of polymerization and sulfation of seaweed polysaccharides is related to the extent of their bioactivities, though this may also influence their hydrocolloid properties. Fucoidan and carrageenan are among the most sulfated polysaccharides, whereas the lower sulfated compounds of such nature comprise ulvan and agar (Otero et al., 2021).

The main seaweed polysaccharides and the simple sugars moieties in their biochemical composition are depicted in **Table 3**. Each macroalgae class holds distinct polysaccharides profiles, which influences their structural configuration and properties.

Table 3. Characteristic polysaccharides of each macroalgae subdivision and major sugar monomers resulting from complete hydrolysis. Adapted from (Postma et al., 2018)

Subdivision	Polysaccharides	Sugar Monomers
Red Macroalgae	Agar, carrageenan, agaropectin, cellulose, xylans, mannans	D-galactose, D-fructose, 3,6-anhydro-D-galactose, glucose
Green Macroalgae	Ulvan, starch, xylopyranose, glucopyranose, xyloglucan, glucuronan, cellulose, hemicellulose	Glucose, xylose, uronic acids, rhamnose, galactose
Brown Macroalgae	Fucoidan, laminaran, alginates, cellulose	Mannitol, glucose, guluronate, mannuronate, glucuronate, sulphated fucose

Multiple marine carbohydrates have recently come under the spotlight due to their chemical and physical properties. Agars, carrageenans and alginates, for example, have long been used in the food industry due to their gelling and thickening properties. Regarding galactans, these components are recognized for their anti-tumoral, anti-viral, immunomodulation, anticoagulant, anti-angiogenic, and anti-thrombotic properties. Alginate is also employed in non-food industries, including textile printing, immobilization of biocatalysts, and paper industries (Greetham et al., 2020).

2.1.6 Lipids, Minerals and Vitamins

Lipids are minor components of macroalgae (1 to 5% DW), with phospholipids and glycolipids being the most predominant classes (Filote et al., 2020). Macroalgal lipids are enclosed in small spherical droplets in the chloroplast, where they serve as structural support for the cell, metabolic organelles in photosynthesis, growth process of the cell and in synthesis of membrane lipoproteins (D. Rodrigues et al., 2015).

Despite their lower abundance compared to the other major metabolites, macroalgal lipids constitute a natural supply of polyunsaturated fatty acids (PUFA) that are generally within an optimum ω-6: ω-3 ratio (Lopes et al., 2020). According to the World Health Organization (WHO), this ratio value should not exceed the value 10 and marine macroalgae ratio has been routinely recorded around one.

As a result, macroalgae lipids are suitable and popular for usage in nutraceuticals, functional foods, pharmaceuticals, and cosmetics (Filote et al., 2020).

Seaweeds are also recognized as rich sources of elements as they can accumulate high concentrations of calcium, magnesium, sodium, phosphorus and potassium and trace elements such as zinc, iodine or manganese from their surroundings for up to 36% of dry matter in some species.

Seaweed contains water soluble vitamins namely B-complex, the largest group, C but also lipid soluble vitamins such as A and E at varying levels. Vitamin C and E both have important roles in promoting the immunological responses, but also have antioxidant activity, particularly vitamin E (D. Rodrigues et al., 2015).

2.1.7 Relevant species of the *Ulva* genus

Green macroalgae of the genus *Ulva* are cosmopolitan, tolerant in a wide range of salinities that can be found in freshwater, estuarine systems, and open coasts. Subsequently, these organisms are regarded as abundant sources of biomass (Wan et al., 2017). *Ulva* may even proliferate in the form of persistent blooms to the point of becoming an environmental hazard under favorable conditions such as reduced wave motion, elevated irradiance and raised water temperature. This seaweed is also successfully cultivated in integrated multitrophic aquaculture (IMTA) systems, for scalable and controlled cultivation conditions (Marinho et al., 2013). *Ulva* spp. are hence convenient raw materials for biorefinery purposes and have been utilized for food and feed, bioremediation, bioenergy, medicine, cosmetic, pharmaceutics and aquaculture (Mo'o et al., 2020).

To use macroalgal polysaccharides as carbon sources, their molecules need to be depolymerized into their sugar monomers. As previously mentioned, many of these polysaccharides are integrated into the cell walls, making their processing complex since they provide structural support to the cells, thus being more rigid and resistant to alterations of the network. Ulvan, the main polysaccharide of *Ulva* sp, ranges from 8% to 29% of the dry weight (DW) of the biomass (Wahlström, Nylander, et al., 2020). This sulfated polysaccharide is composed of variable amounts of rhamnose, glucuronic acid, xylose, sulfate and iduronic acid. Furthermore, ulvan structure is based on repeating sequences of two major disaccharides units designated as ulvanobiuronic type A (A3s) and type B (B3s), and by minor disaccharides units, ulvanobioses. Type A ulvanobiuronic acid is composed of D-glucuronic acid β (1→4) linked to L-rhamnose 3-sulfate, whereas the B type consists of L-iduronic acid α (1→4) linked to L-rhamnose 3-sulfate. The minor sulfated residues contain xylose in the place of uronic acids and the differentiation factor between the two existing types U3S and U2's3s is the sulfate group replacing the C2 hydroxyl group as it can be seen in Figure 1 (Figueira et al., 2020).

In respect to *Ulva* main storage polysaccharides, starch, is composed by two distinct glucose polymers: amylose which is a linear (1→4)-α-glucan, and amylopectin, a branched polymer consisting of linear (1→4)-α-glucan chains interlinked with (1→6)-α-glucosidic bonds (Zemke-White et al., 1999) .

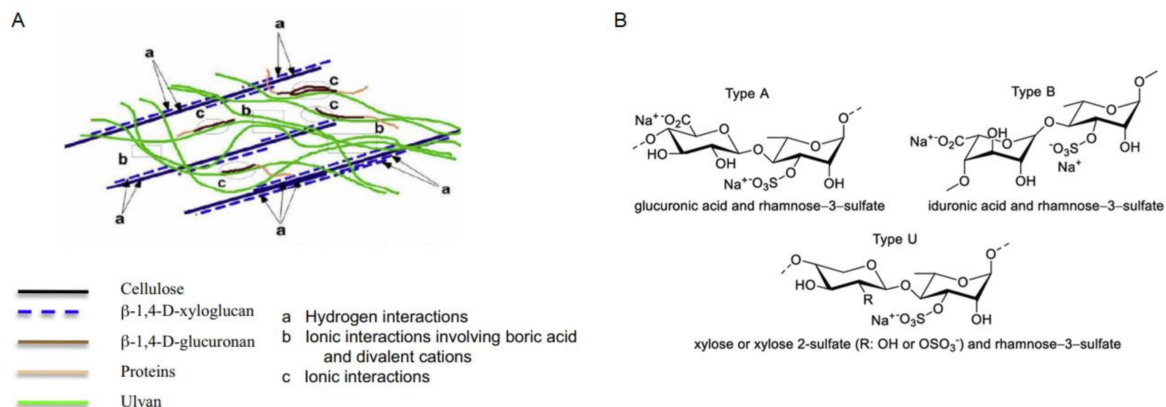

Figure 1. Schematic cell wall structure in green macroalgae. A) Components of the fibrillar and the matrix associated components of cell wall; B) Main repeating disaccharide units of ulvan. Adapted from Panggabean et al., 2022; Stiger-Pouvreau et al., 2016

2.2 Algae Industry

Seaweed's potential varies based on the type of algae, harvesting period, and environmental circumstances; hence, each species has peculiar compounds that can behave in a variety of ways, exhibiting diverse properties that condition its use in the industry (Lomartire et al., 2022).

The seaweed industry is based on the harvesting of either wild or cultivated resources, and it is far more developed in Asian countries, where most of the produced algae is obtained through aquaculture (Lomartire et al., 2022). In Europe, macroalgae is mostly harvested or imported; nevertheless, interest in seaweed cultivation is growing in the European countries, though it is still in its early stages. Such initiatives are being driven by both the demand for sustainable biomass resources for industrial applications and the growing emphasis on more sustainable food production and consumption (Jönsson et al., 2020).

As the popularity and applications of seaweeds increase, their asset value rises too. The global market of macroalgae value in 2024 (expected 9.98 million US dollars) will far exceed what was achieved in 2017 (4.1 million US dollars) (Leandro et al., 2019). In fact, Europe has recently been recognized as one of the most innovative regions in respect to the use of macroalgae as a food ingredient, increasing 1.47-fold the number of seaweed-based products on the European market between 2011 and 2015 (Cikoš et al., 2020).

2.2.1 Extraction Methods of Seaweed Polysaccharides

Despite having a high carbohydrate content, lignocellulosic biomass has numerous chemical and physical barriers that pose a challenge to its use as an industrial feedstock. Amidst their structural complexity, particular and heterogenous sugar composition, sulfation and other modifications, polysaccharides require a pretreatment stage to make sugars readily fermentable (Giacon et al., 2022). In this sense, macroalgal polysaccharides are acknowledged as unfavorable fermentation substrates since its sugars are embedded in complex polymers networks, thus not being available for microbial

action. Yet, the potential product range of seaweeds may surpass other biomass of comparable bulk and cultivation easiness (Jönsson et al., 2020).

In view to convert algal sugars into valuable chemicals by fermentation, the deconstruction of algal cell walls is crucial to retrieve these compounds (Poblete-Castro et al., 2020). The lower amount of lignin in seaweed specimens makes the pretreatment process easier and less expensive than lignocellulosic based biomass, as its rigid and complex structure require harsh treatments leading to partial cellulose degradation (Wahlström, Edlund, et al., 2020).

For this end, different extraction procedures are typically combined and preceded by a pretreatment to optimize the total yield of the process. The methods in use target either the swelling of fibers, enlargement of pore size or reduction of the material size. The most utilized pretreatment methods for macroalgae substrates are mechanical (size reduction, washing, grinding, and sonication), thermal (microwave, steam, oxidation, and plasma-assisted), chemical (acid or alkali, peroxide), and biological processes. Chemical hydrolysis, in particular, has been extensively used for depolymerization of various types of polysaccharides since it is a fast and highly cost-effective method.

Higher degree of depolymerization may be achieved through the use of enzymes after pretreatment, as polysaccharides are more accessible for the enzymatic hydrolysis reaction. For instance, hydrolysis of green algal biomass by carbohydrate-active enzymes (CAZymes) such as pectinases and cellulases was found to improve protein extraction for *Ulva lactuca, Ulva rigida*, and *Ulva rotundata* (M. Costa et al., 2022). Moreover, there are numerous reports on the use of cellulases for the degradation of *Ulva rigida*, *Ulva fasciata*, *Chaetomorpha linum*, and *Ulva lactuca*, all of which envision biotechnological applications (M. M. Costa et al., 2022).

2.3 Applications and Bioproducts from Macroalgae

Seaweeds and seaweed derived products have been used across the world since ancient times as whole food or ingredients, in agriculture to fertilize the soils and in traditional medicine even though its mechanisms of action were not known (Leandro et al., 2019; Lyu et al., 2017). The possible applications of seaweeds constitute a growing research field as their unique characteristics open the door wide for multidirectional biotechnological use (Michalak et al., 2018).

At present, macroalgae are involved in two major areas of industrial activity. The first, encompasses the use of intact macroalgal biomass or algal extracts that are a rich source of high-quality biological components such as polyphenols, phycobiliproteins among other valuable compounds. Food and feed applications represent the largest share of direct use, and the three seaweed groups can be ranked as follows: brown algae (65%), red algae with a 30% contribution and green algae representing 5%. The manufacturing of hydrocolloids, such as alginates, agar, and carrageenan holds the second largest share. A minute percentage of the production is utilized directly or processed through biorefinery for agronomics, bioenergy, as source of pigments and phenolic compounds (Álvarez-Viñas et al., 2019). The second area regards the exploration of the algae metabolisms to either transform already existing compounds, enriching its value as a product, or to produce *de novo* a vast array of components (Michalak et al., 2018).

2.3.1 As a Source of Feed, Food, and Nutraceuticals

As mentioned in section 2, humankind resorted to the use of algae as a food source, being a common practice since ancient times with strong roots in Asian countries. For instance, kelp was used in the 19th century to treat obesity and their crude extracts were also employed in brewing processes at the clarification stage (Kapur et al., 2013). Consumption of seaweed has been and continues to earn recognition as people become more aware of its benefits and properties.

There is a vast range of physicochemical and texturing properties that seaweeds impart in food products (Goff et al., 2019). Hydrocolloids, or more commonly gums, are mostly polysaccharides but also proteins that hold the capacity to retain or disperse water thus being chiefly utilized to modify the rheology of food systems, especially viscosity and texture (Sahin et al., 2004). Agar is highly sought due to its low gel strength matrix, a property that enables it as an ingredient for a variety of applications including spreadings, fat replacers, cryoprotectants to minimize the damage inflicted during freezing/ thawing processes and as edible films (Kowalski et al., 2011). This compound is mainly extracted from the red algae *Gelidium* and *Gracilaria* but also from genera such as *Pterocladia* and *Gelidiella*.

Another example of algae components used for this purpose are carrageenans. These sulfated polysaccharides are commonly employed in milk-based products such as ice cream, cheese, and yogurt, owing to their ability to form networks with milk proteins even at low concentrations (Roohinejad et al., 2017).

Seaweeds are also of nutritional interest since they are a low-calorie food but rich in both soluble and insoluble dietary fibers, vitamins (A, B1, B2, B9, B12, C, D, E, K), minerals (calcium, iron, iodine, magnesium, phosphorus, potassium, zinc, copper, manganese, selenium) and PUFAs, making its inclusion in the human diet and animal feed extremely attractive (Morais et al., 2020). For example, experiences with various seaweeds as aquafeed including *Ulva sp*, *Gracilaria sp*, *Sargassum sp* among others, enhances the growth, lipid metabolism, physiological activity, and meat quality of several fish species (Morais et al., 2020).

Aside from the nutritional benefits of algae, bioactive compounds play a pivotal role in the promotion of human health and illness prevention due to antiviral, antifungal, antibacterial, anti-inflammatory, antioxidative and anti-tumural properties (Michalak et al., 2018). Moreover, fucoidan holds a potential use as a gut microbiota modulator and treatment of intestinal dysbiosis. Although fucoidan cannot be fermented by intestinal microbes, it can alter the bacterial composition and fermentation ability of gut microbiota, thereby providing benefits to the host (Shannon et al., 2021).

Despite the huge scope for diet supplementation with edible seaweeds, there is still limited research on the improvement of food nutritional properties as well as the development of functional foods supplemented with this so-called sea-vegetable (Pandey et al., 2020).

2.3.2 As a Protein Source

The increased demand for protein sources, combined with dwindling land and water resources, has fueled interest in macroalgae as alternative protein sources (Gordalina et al., 2021). The analysis of concentration and quality of protein is crucial in determining the nutritional value of whole or fractioned seaweed biomass to be used as food ingredient. Furthermore, any new protein supply must be

evaluated in terms of providing essential amino acids on a whole-weight basis in comparison to the targeted product requirements (Hayes, 2020).

The protein quality is assessed through their amino acid composition and proportion, digestibility, and bioavailability (Thiviya et al., 2022). Almost all essential amino acids are found in seaweeds, particularly glycine, alanine, arginine, proline, glutamic, and aspartic acids. Furthermore, the levels of essential amino acids in macroalgae are compatible to both Food and Agriculture organization (FAO) and World Health Organization (WHO) requirements for dietary proteins. However, seaweeds are rather limited in terms of tryptophan, cysteine, lysine, threonine, and methionine in opposition to other protein-rich foods (Kim et al., 2015).

Aside from having proteins with excellent amino acid profiles, macroalgae are also rich in carbohydrates and poor in fats, making them excellent nutritional supplements (Yong et al., 2022). Still, even if a given protein has an excellent amino acid profile, it might be linked to a lower nutritional value if its digestibility is low. Corollary, protein bioavailability is also an essential factor in protein quality (Thiviya et al., 2022). The latter parameter can be defined as the extent of the ingested food element's that are available at the target site of action for utilization in various physiological functions. In other words, bioavailability entails the entire process starting from the point in which amino acids or short peptides are ingested, solubilized into the gastrointestinal tract until the absorption across the intestinal epithelial cells into the circulation system, and finally incorporation into the target site of utilization (Hayes, 2020).

The utilization of macroalgae as a protein source is indeed hampered by the high complexity of their cell walls and high carbohydrate content which negatively affect the activity of digestive enzymes, particularly in the vicinity of the intestinal epithelium. Likewise, the existence of indigestible fibers in macroalgae specimens impairs nutrient bioavailability, as most of these are resistant to digestive enzymes (Batista et al., 2020). Despite possessing rich amino acid profiles, seaweeds have poor protein digestibility in their raw, unprocessed form. Whilst natural seaweed bioavailability and digestibilities are low, processing methods such as saccharification, proteolysis, and fermentation can significantly improve these properties, transforming seaweeds into potential protein sources (Marrion et al., 2005).

2.4. Fermentation

Fermentation can be defined as a non-oxidative process in which a carbon source is dissimilated by microbes to produce energy (Ayivi et al., 2020). The fermentation products differ depending on the microorganisms, being governed mainly by enzyme cocktails of the cells and the environmental conditions (Doelle, 1969). Traditionally, fermentation processes have taken advantage of the general capacity of microorganisms to convert renewable feedstocks into valuable products including ethanol, organic acids (lactic, citric, itaconic), amino acids (lysine, threonine, glutamic acid) and antibiotics (penicillins) (Sanchez et al., 2008).

In recent years, the advent of powerful biotechnological techniques combined with more efficient engineering tools has culminated in the tremendous increase of both the range of products and the fermentation yield. This enabled the synthesis of new and added-value compounds namely tailored antibiotics, therapeutic drugs, heterologous enzymes, and polymers. One example relies in the

production of vitamin B12 by *Pseudomonas denitrificans*' aerobic metabolism for the inexpensive treatment of pernicious anemia (Reboleira et al., 2021).

For a successful fermentative process, is crucial to characterize the metabolic process involved in the synthesis of the desired product, to understand the physiological responses to the specific conditions of the process and the interactions of various physical and physiological variables. Among the physiologically most relevant parameters are pH, temperature, biomass, cell viability, concentrations of substrates, metabolites, products and possible inhibitors (Schmidt, 2005).

The global market for fermentation technology was appraised at approximately USD 1,573.15 million in 2017 and it is projected to generate revenues of the order of USD 2,244.20 million by the end of 2023 (Begum et al., 2021). Even so, fermentative processes still have a lot of margins for improvement, particularly in the yield, titer, and performance of the fermentation reaction itself. Except for therapeutic substances, industrial fermentation is still less competitive than chemical industries or agriculture.

Despite these challenges, industrial fermentation has been utilized for the production of an increasing number of products, due to its green and safety statuses, and garner increased attention seeing that sustainability is now a top investment concern (Chen et al., 2021). Moreover, fermentation processes are capable of converting perishable and low-value natural resources into valuable and stable commodities that are extremely difficult to replicate through alternative means (Reboleira et al., 2021). Thereafter, the modern food, feed and pharmaceutical sectors are forecast to unprecedentedly utilize fermentation processes.

Frequently, commercially adopted fermentative bacteria belong to the *Lactobacillales* order which include the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*. All are recognized for their fermentative potential as they enrich products by improving their organoleptic characteristics and health benefits, while also assuring food safety (Reboleira et al., 2021).

2.4.1 Modes of Fermenter Operation

Essentially, a fermentation process might be run in one out of three operation modes designated as batch, fed-batch and continuous albeit modifications of these exist to maximize microbial production such as repeated batch and repeated fed-batch. To develop and optimize biotechnological processes it is crucial to discern the latter three fermenter modes of operation.

Batch fermentation is regarded as the simpler system mode, in which all components required for the microorganism growth and product synthesis are added in the beginning of the process, apart from neutralizing and antifoam agents as well as oxygen supply (aeration in aerobic systems). Only once the reaction has exhausted one limiting nutrient, the reaction products are harvested. This closed system has the advantages of reduced contamination risk and operation reliability; however, reduced productivity may be a problem either because of substrate and/or product inhibition, apart from low cell concentration due to limited nutrients (Zhang et al., 2016). Generally, sugar concentration above a critical level in the system results in substrate inhibition fomenting prolonged lag phase and cell lysis, thereby, a sharp decrease of fermentation rate and sugar uptake is registered (Rawoof et al., 2020).

A fed-batch system is especially advantageous for microbial cultures in which high substrate concentrations promote undesirable metabolic pathways or when high growth rates are impaired by oxygen transfer constraints. In fed-batch fermentation, the vessel is fed with a low substrate concentration and as the reaction progresses, the substrate is added continuously or sequentially without the removal of fermentation broth. In a fed-batch configuration, the supply of substrate/ fresh media might be established in a variety of ways, constantly or intermittently over time, with volume supply of feed growing over time (exponentially or stepwise), etc., all of which could alter the efficacy of the fermentation. Moreover, this system is operated in bioreactor vessels which allow for control of other essential operation parameters such as dissolved oxygen, pH, temperature, among others (Yang et al., 2019) In addition to the previously mentioned advantages, fed-batch cultures extend the microorganisms' lifespan yet have the drawback of a higher contamination risk than batch mode.

In continuous fermentations, sterile medium is fed continuously into the vessel and the fermented broth (with cells and their metabolic products) is continuously withdrawn at equal flow rates, thus the bioreactor reaches a steady state i.e., reaction volume and concentration of broth components (cells and biochemicals) remain constant in time. But, then again, this operation mode might introduce cellular stress and an even higher risk of contamination.

2.4.2 Fermented Products

Fermented foods are prepared through the action of microorganisms in the food matrix, chiefly bacteria, yeasts, and mycelial fungi. These living beings may be indigenous in the substrate, introduced as a starter culture, or be present on the utilized ingredients and even in the environment (Melini et al., 2019). Contemporarily, fermented foods are regarded as a staple component of the human diet (Şanlier et al., 2017). This process is of great importance in the food sector as it serves as means to provide and preserve substantial volumes of nutritious food in a broad range of aromas, flavors and textures which enrich consumer's diet (Steinkraus, 1994). Moreover, fermentation is an inexpensive processing methodology that requires comparatively less energy, being regarded as the main strategy for food production in some cultures (Şanlier et al., 2017).

Even though fermented dairy products hold a great portion of the total economic value of fermented foods produced globally, the market share for other products has continued to grow as in the case of meat-based (sausages), vegetable based (mustard, pickles, and turnips) and grain based fermented products (Ray et al., 2015; Şanlier et al., 2017).

The fermentative process has been shown to improve food's nutritional quality in at least three different ways. Microorganisms are not only catabolic, metabolizing complex compounds into simpler molecules, but are also anabolic, synthesizing several vitamins, amino acids, organic acids among other beneficial compounds. Secondly, this process enables the release of nutrients locked into plant structures and cells. This is of major importance as the cellulosic and hemicellulosic structures are surrounded by endosperm, which is still rich in digestible carbohydrates and proteins that otherwise would be unavailable. A third mechanism that contributes to the nutritional value of food ingredients, particularly of plant-based products, is the enzymatic dissociation of cellulose, hemicellulose and related polymers that are not digestible into simpler sugars and sugar derivatives, enabling the use of such

sugars as substrate for the synthesis of microbial protein. Therefore, cellulosic materials' nutritional value can be considerably enhanced through fermentation (Potter et al., 1995)

There are several foods, particularly cereals, which are low in nutritional value but represent the core element of the low-income population's diet as the access to meat and fish is often limited. Hence, the implementation of fermentation processes in these countries is often sought to improve the overall nutritional quality of such products (Sandhu et al., 2017).

Even in Western countries where meat is currently the main protein source, there is a growing interest in developing alternatives to this resource as epidemiologic studies have linked the high consumption of animal products with escalating rates of chronic diseases. Moreover, the consumption of red and processed meats has been associated with increased risks of type 2 diabetes, stroke, heart diseases as well as certain cancers. At the same time, growing scientific consensus has established that significant adjustments must be established towards plant-based diets to accomplish climate change mitigation targets, particularly in countries with heavy meat consumption (Santo et al., 2020).

In all, more sustainable, healthy, and ethical alternatives to current protein sources are required and the implementation of the fermentation in vegetable substrates opens the door wide to novel products with increased protein content and quality.

2.4.3 Lactic Acid Fermentation

Lactic acid, a colorless and odorless monocarboxylic acid, has received considerable attention due to its numerous applications in food and non-food industries (Lin et al., 2020). Traditionally, lactic acid producing bacteria have been widely employed in fermentation processes as they can convert carbohydrate substrates into lactic acid (LA) as the main end product. Whilst during metabolism, these bacteria may synthesize several products as short chain fatty acids, amines, vitamins and exopolysaccharides (Y. Wang et al., 2021). The latter are categorized into four main groups, encompassing Lactic Acid Bacteria (LAB), *Bacillus* strains, *Escherichia coli* and *Corynebacterium glutamicum* (Budhavaram et al., 2009). These organisms are ubiquitous in fermented foods, non-fermented foods but also common components of the human commensal microflora.

A large bulk of LAB are “Generally Recognized as Safe” (GRAS) according to Food and Drug Administration (FDA). Furthermore, many of them have also been granted the status of “Qualified Presumption of Safety” (QPS) by the European Food Safety Authority (EFSA), including the genera *Lactococcus*, *Carnobacterium*, *Oenococcus*, *Leuconostoc*, *Streptococcus*, *Pediococcus* and the former *Lactobacillus* (Barcenilla et al., 2022).

LAB are capable of metabolizing glucose through three different pathways namely obligatory heterofermentative, obligatory homofermentative and facultative heterofermentative, depicted in Figure 2. The obligatory heterofermentative strains decompose glucose via the pentose–phosphoketolase pathway. As a result, through this pathway, also ethanol, acetic acid and carbon dioxide are synthesized in equimolar amounts reaction in addition to LA. Heterofermentative bacteria include *Lactobacillus pentosus*, *Lactobacillus bifermentans*, and *Lactobacillus brevis*, to name a few.

In the homofermentative cascade, glucose is metabolized via the Embden–Meyerhof–Parnas (EMP) pathway (glycolysis). As such, bacteria in this group are able to convert hexoses to LA but do not

hold the capacity to degrade gluconate or pentoses. As a direct consequence, LA is the only product formed and, thus, homofermentative strains are favored for the commercial production of the organic acid. Some of the employed homofermentative bacteria include *Lactobacillus delbrueckii*, *Lactococcus lactis*, *Lactobacillus casei*, and *Lactobacillus acidophilus* (Gänzle, 2015).

The facultative heterofermentative may occur under certain conditions, when some lactic acid bacteria regarded as homofermentative, use the pentose phosphate pathway to metabolize certain substrates. In this sense, a mixed fermentation may be conducted meaning that both pathways can occur in an alternated fashion depending on environmental cues suchlike temperature, pH, or nutrient availability (Pot et al., 2009).

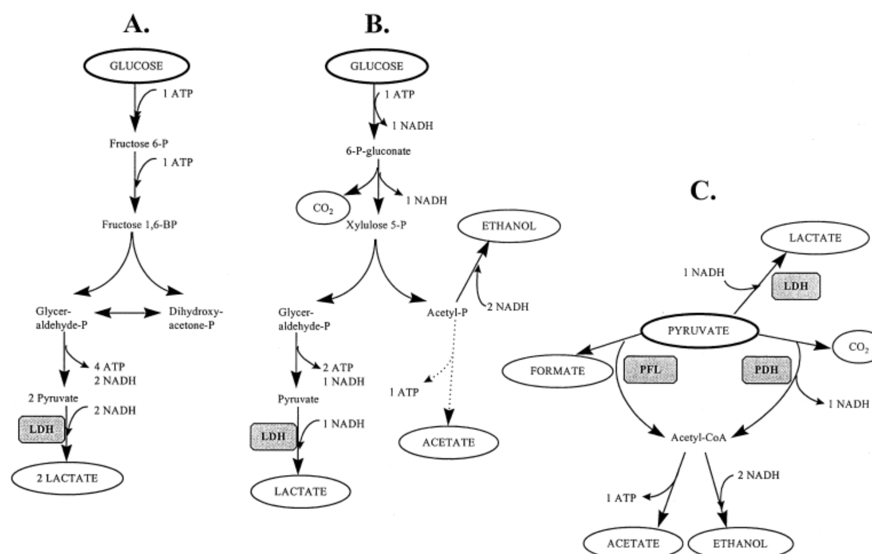

Figure 2. Metabolic pathways of LAB species for the synthesis of LA, homofermentative **(A)**, heterofermentative **(B)** and mixed acid fermentation **(C)**. Legend: LDH -lactate dehydrogenase; PFL -pyruvate formase lyase; PDH -pyruvate dehydrogenase; BP -biphosphate. Adapted from Hofvendahl et al., 2000

LAB producing organisms have fastidious nutrient requirements and as such the culture medium must be supplemented with a nitrogen source (peptides), amino acids (as they are auxotrophic for several amino acids), vitamins and minerals (to supply for trace elements), salts, fatty acids and fermentable carbohydrates. These demands are frequently met in the presence of fermentable carbohydrates, peptone meat and yeast extract (Sánchez et al., 2019).

The optimal pH value for a large bulk of LAB specimens ranges between 5.0 and 7.0 and must be maintained to prevent cell growth inhibition. In most LA production systems, $Ca(OH)_2$ is utilized as a neutralizing agent but alternative neutralizing agents such as ammonia and its hydroxide have also been reported (Djukić-Vuković et al., 2019).

One of the major bottlenecks of LA culture is product inhibition and in many lactic acid bacteria (LAB) species, LA concentration exceeding 50 g/ L elicits growth inhibition (Djukić-Vuković et al., 2019). Briefly, the accumulation of lactic acid elicits the medium pH decrease impairing the transmembrane pH gradient and ultimately disables the cellular functions. Secondly, as energy is primarily directed to maintain the transmembrane pH gradient, the amount of energy available for cell growth is limited (Othman et al., 2017). A conventional approach to surpass this limitation is the implementation of a fed-

batch regime. Lee et *al* reported the feasibility of fed batch in overcoming substrate limitation, product inhibition while improving the biomass yield of LAB.

In the context of lactic acid (LA) fermentation, fed-batch processes outperform batch, with higher LA concentrations and productivities in a variety of substrates including distillery stillage, cheese whey and corn stover. Even so, the accumulation of toxic or inhibitory chemicals may occur, as a result of repeated feeding, and impair the process productivity. Specifically, batch fermentation by *Lactobacillus pentosus*, the conversion of corn stover to lactic acid reached 38 g/L, while through the implementation of fed-batch operation, the concentration of lactic acid was raised to a maximum of 75 g/L (Zhu et al., 2007). In other study, the lactic acid production by *Lactobacillus rhamno*sus from cheese way reached the highest production of lactic acid (108.20 g/L) in fed-batch, while in batch the maximum production was 57 g/L (Bernardo et al., 2016).

Although extensive research has been conducted to efficiently improve lactic acid production through microbial fermentation, the high production cost of lactic acid remains a major limitation for its industrial application (Lin et al., 2020). Around 40 to 70% of total cost is attributed to the substrate, implying that the cost of fermentable biomass must be minimized (Rawoof et al., 2020). The main carbon sources within the scope of lactic acid fermentation are pure sugars as glucose. Regrettably, such sugars are very expensive substrates in addition to their negative impact on the human food chain, so attention has shifted to alternative biomass sources. All considered, whey and molasses are regarded as the most viable substrates for lactic acid production at present time, as these components are industrial byproducts from the manufacture process of cheese and sugar, respectively (Olszewska-Widdrat et al., 2020). Nonetheless, several studies have reported the use of alternative low-cost substrates including lignocellulosic biomass, seaweeds, and microalgae (Abedi et al., 2020). The employed conditions for the biological manufacture of lactic acid from different alternative substrates are displayed in Table 4.

Table 4. Lactic acid fermentation from different substrates in different conditions and operation modes oriented towards lactic acid production.

Raw Material	MO	Operation Mode	Culture Media	T (ºC)	pH	Mixing (rpm)	[LA] (g/L)	*References*
Cheese Whey	*L. rhamnosus*	Batch (48h)	Culture medium: 60 g/L of lactose from whey 45.mL/L of CSL, 1.00 mL/L of Tween 80 and 0.075 g/L of manganese sulfate, 10% (v/v) inoculum.	37	6.2	200	57	(Bernardo et al., 2016)
		Fed-Batch (95h)	Feed medium: 500 g/L of lactose (from whey) and 7.50% of CSL.	37	6.2	200	108.2	
Sugar Cane Molasses/ Glucose	*B. coagulans*	Fed-Batch	149 g/L cane molasses, 185 g/L glucose, 5 g/L (NH4)2SO4 and 1 g/L corn steep powder, 25% (v/v) inoculum	52	6.2	70	168.3	Xu et al (2014)
Sugar Molasses	*L. Lacti*	Fed Batch (72h)	5 g/l glucose, 5 g/l peptone, 5 g/l YE, 2.5 g/l xylose, 10% (v/v) inoculum	37	7.0	Static	10.6	(Jonglertjunya et al., 2012)

Legend: MO -microorganism; T- temperature; [LA] -lactic acid concentration; YE -yeast extract; *L.rhamnosus - Lactobacillus rhamnosus; L. B.coagulans -Bacillus coagulans*; *L.lacti -Lactococcus lacti*

2.4.4 Co-culture

Lactic acid fermentation is at its highest with the use of co-culture in which each microorganism exhibits its specific metabolic activity. Co-culture is a fermentation technique wherein two or more cell populations with a particular degree of interaction are inoculated as means to yield a given product (Eş et al., 2018). However, all the partaking microbes must have the same environmental and nutritional requirements (Rawoof et al., 2020).

Heterofermentative lactic acid bacteria synthesize by products such as acetic acid and ethanol, leading to reduced productivity and increased costs associated with lactic acid recovery. Instead, if hetero and homofermentative are combined, the glucose uptake is increased as the microbes follow different pathways for the bioconversion of sugars to lactic acid. As a natural consequence, by-product accumulation is reduced as homofermentative strains might outcompete heterofermentative bacteria. In 2015, Zhang et al. conducted a study with the purpose of producing LA from biomass derived sugars using both *L. brevis* and *L. plantarum* strains as co-cultures. In the case of *L. brevis* alone, a simultaneous uptake of glucose and xylose was registered but with low LA-to-substrate yield (0.5 g/ g). Conversely, when *L. brevis* was co-cultured together with *L. plantarum*, the yield was 1.6-fold increased (0.80 g/ g), whereas by-products accumulation was significantly reduced (Zhang et al., 2015). Similarly, Cui et al (2011) studied the metabolism of a mixed culture in sugars derived from cellulose and hemicellulose in complex media. The homofermentative *L. rhamnosus* efficiently converted glucose to lactic acid via the EMP pathway, but the leftover glucose and xylose were utilized by a *L. brevis* strain (heterofermentative) to produce LA and acetic acid.

In numerous fermented foods LAB and yeast are often self-assembled and the ensuing microbial interaction is critical for desired characteristics that improve food quality (Hirai et al., 2020). Yeasts secrete vitamins, essential amino acids, and other essential growth factors that sustain LAB growth, and yeast uses bacterial metabolic byproducts as energy sources. In contrast with other bacteria, *Lactobacilli* are known to be relatively resistant to ethanol and low concentrations of ethanol have even been shown to stimulate the metabolism of certain LAB (Mateo et al., 2010). Examples of mutualism between these species are found per instance in the manufacture of kefir, where yeasts supply crucial aminoacids and vitamins that are ideal for LAB conversion of carbohydrates, which by its turn lower the pH thereby establishing an environment that favors yeast growth (Cheirsilp et al., 2003). In the scope of bioethanol fermentation, *Lactobacillus plantarum* coexistence with *S. cerevisiae* improved ethanol tolerance by either promoting or inhibiting various metabolic processes in yeast cells. However, LAB-yeast association faces some challenges as there are also antagonistic interactions between these species since they compete for the same resources and due to the yeast sensibility to organic acids produced by bacterial cells, which can severely hinder yeast metabolism and decrease ethanol yield (Oliva-Neto et al., 2001). Moreover, in an experimental study Maiorella et al, 1983 revealed that yeast viability decreased 80% in the presence of 40 g/L of lactic acid.

There is indeed a complex relationship between *Lactobacillus spp.* and yeasts that requires further studies, but co-fermentation between these species may render a new product which combines the positive effects of these bacteria such as antibacterial activity and flavor compounds production, with the taste and aroma properties of yeast fermentation compounds.

2.4.5 Lactic Acid Applications

Lactic acid can be obtained either through chemical production or microbial fermentation routes. The former incorporates petroleum based-chemicals and yields a racemic mixture of D-LA and L-LA which is not adequate for the food, drink, and pharmaceutical industry due to the underlying metabolic problems that D-LA form may elicit. On the contrary, a fermentative-based production enables the synthesis of optically pure D- or L-lactic acid by selection of appropriate microorganisms (Jem et al., 2020). The fermentative pathway is gaining importance for lactic acid production, due to the increased volume of research in conjunction with a broad array of microorganisms that are capable of metabolizing lactic acid several alternative substrates to lactic acid, many of which are industrial residues.

Lactic acid holds a highly versatile profile as precursor compound thus many applications are found in the textile, chemical, food and pharmaceutical industries, including as preservative, acidulant among others (Rodrigues et al., 2017). Approximately 70% of the lactic acid produced is directed to the food sector due to its pivotal role in the manufacture of yogurt and cheese. In cheese manufacture, the pH decrease triggered by microbial LA release induces the aggregation of casein micelles. Moreover, direct acidification with LA is at times administered to mitigate the proliferation risk of undesirable microorganisms, depending on the sensory attributes sought for the final product (Castillo Martinez et al., 2013).

2.4.6 Ethanol Fermentation

Yeasts are unicellular ubiquitous eukaryotic fungi that are commonly found in ripe fruits, vegetables, and other plant materials (F. Fernandes et al., 2022; Maicas, 2020). These microorganisms play a crucial role in food fermentation by imparting distinctive flavors, textures and aromas through secondary metabolism, thereby modifying organoleptic properties of food. During ethanol fermentation there is production of bioactive peptide, vitamins while removing antinutritional-components, enhancing nutritional and functional value of food (Dussap et al., 2017; Kandasamy et al., 2018). Moreover, certain metabolites and preservatives are even linked to increased shelf life and potential health benefits (Kunyeit et al., 2023).

In the food sector, yeasts serve several important functions, including alcohol production, leavening to enhance the texture in bread, acidification of foods, and acting as natural preservatives. The genera *Saccharomyces contains* most of the favorable yeasts for desirable food fermentation and with a safe-status, particularly *Saccharomyces cerevisiae* (Belda et al., 2019; Kandasamy et al., 2018).

Ethanol fermentation, also referred to as alcohol fermentation, is one of the oldest and most important fermentation processes utilized in the biotechnology industry. In anoxygenic conditions, various microorganisms, including bacteria and yeasts, have the capability to convert sugars into ethanol and carbon dioxide, through a series of enzymatic reactions, generating energy (Maicas, 2020). In certain condition, the carbon flux may be redirected to produce smaller quantities of 2,3 butanediol, acetoin and acetate which contribute to the flavor characteristics of alcoholic beverages. For instance, glycerol is as important constituent of wine as it enriches its profile with sweetness, fullness, and smoothness (Brigham et al., 2014) .

Current industrial ethanol fermentation predominantly utilizes the yeast *Saccharomyces cerevisiae* due to its remarkable aptitude in the industrial context over other yeasts. The dominance of *S. cerevisiae* in ethanol fermentation is traditionally ascribed to its high ethanol production rates, rapid sugar consumption, fast growth, and aptitude to thrive in harsh environmental conditions including high tolerance to ethanol and organic acids, low pH values (3.0-3,5) and nutrient scarcity (Albergaria et al., 2016).

At the metabolic level, yeasts are characterized by their capacity to ferment a broad spectrum of sugars, among which glucose, fructose, sucrose, maltose and maltotriose predominate (Maicas, 2020). *S. cerevisiae* is mesophilic yeast that exhibits ethanol production within the temperature range of 20º to 35ºC, with an ideal temperature of 30ºC. The common agitation rate for yeast fermentation typically ranges from 150 to 200 rpm. Regarding pH, the optimum values fall between 4.0 to 5.0, with lower values resulting in longer incubation periods without a significant impact on ethanol concentration, while higher values considerably reduce ethanol production (Mohd Azhar et al., 2017).

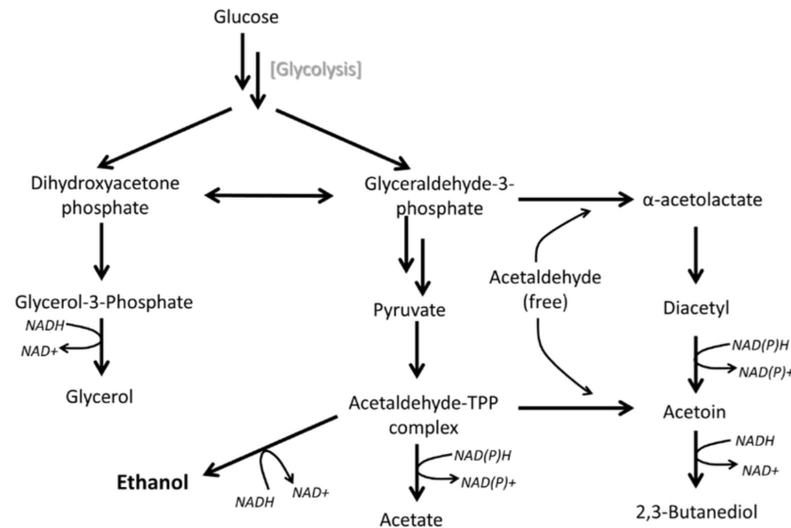

Figure 3. Ethanol biosynthesis route in *Saccharomyces cerevisiae.* The biochemical process wherein glucose *is* enzymatically converted to pyruvate is referred to glycolysis. Subsequently, pyruvate undergoes decarboxylation, leading to the formation of acetaldehyde. This compound is then reduced by NADH, resulting in the production of ethanol. Secondary fermentation metabolites encompass glycerol, acetate, 2-3 butanediol and acetoin which collectively act as flavor precursors. Adapted from Brigham et al., 2014

2.5 Seaweed as a Fermentation Substrate

Seaweed fermentation represents a potential cost-cutting solution while simultaneously encouraging the fabrication of novel food and feed products (Monteiro et al., 2021). Furthermore, conducting microbial seaweed-based fermentations has the added benefit of stabilizing this raw material while also modifying texture and flavor as well as improving their nutritional and functional properties, thus potentially increasing acceptability of seaweed products among consumers (Løvdal et al., 2022). Reported studies unveiled that freezing, blanching and fermentation had a relevant impact on kelp quality and consumer acceptance. Lactic acid fermentation specifically, stabilizes the seaweed biomass through LA production, which results in the abrupt pH decrease, thereby inhibiting the growth of pathogenic bacteria.

Multiple manufacturers have been investigating the use of seaweed in fermented cabbage products, with several products now on the market. However, in these products, seaweed is used as a seasoning ingredient rather than as a primary component of the formulation. This is most likely due to a combination of factors such as limited levels of LAB in seaweed microbiota, low levels of fermentable sugars, and high buffering capacity in comparison to land plants. Nonetheless, some researchers have overcome these obstacles by using lactic starter cultures, among other methods (Skonberg et al., 2021).

The use of seaweed as carbon source presents challenges the release of fermentable sugars often generates large amounts of inhibitory chemicals as result of the severity of most pretreatments. The nature and concentration of inhibitors is greatly dependent on the processing and operating conditions, and these may be categorized as phenolic compounds, furanic compounds and organic acids. Furanic compounds, which are dehydration by-products of hexoses and pentoses, have been linked with growth inhibition, DNA damage and activity loss of several enzymes involved in glycolysis, jeopardizing the viability of fermentation processes (Giacon et al., 2022).

2.5.1 Seaweed Lactic Acid Fermentation

The utilization of macroalgal biomass as an alternative source of nutrition for the growth of lactic acid bacteria was prompted by the possibility of hydrolyzing its complex polysaccharide molecules. Such discoveries enable the development of novel and improved seaweed products. Despite the potential of fermented seaweed for food and feed purposes, lactic acid fermentation of such biomass resources is still poorly reported in literature thus strategies to optimize this process must be investigated to ensure its economic viability (Uchida et al., 2017).

Lactic acid fermentation of seaweeds was first reported in a cellulase-treated *Ulva sp.* that underwent an incubation period of 17 months at 2ºC by autochthonous yeast and LAB strains. In these primary trials, an aliquot of the consortium present on the original fermented material was transferred to other *Ulva* sp. cultures and successfully induced fermentation. This native microflora was also isolated to test the feasibility of its use as a seed culture in different types of seaweeds. This study disclosed that the conjunction of glucose at a concentration of 0.1 to 1% (w/v) together with the addition of starter microorganisms at an initial concentration (expressed in colony forming units per unit volume) of 10^6 to 10^7 CFU/ mL are crucial to efficiently induce seaweed fermentation. Moreover, the use of salt at 2.5 to 5% (w/v) is further advantageous to mitigate the growth of contaminant bacteria and promote the dominance of lactic acid bacteria (Uchida et al, 2004).

Uchida et al., (2007) investigated the use of starter cultures in fermentation of non-sterilized brown algae *Undaria pinnatifida*. In all experiments relying on its native microorganisms developed contaminations whereas the use of *L. brevis*, *L. plantarum*, *L. casei* and *L. rhamnosu*s as a seed culture exhibited the greatest capacity of suppressing growth of spoilage microbiota among all *Lactobacillus* specimens evaluated. Subsequently, each of the detected LAB strains and yeast strains were tested for fermentation induction. LAB strains could initiate the fermentative process, however, yeast strains delivered mediocre results and bacterial contamination. Therefore, inoculation with yeast is not necessary (Ścieszka et al., 2019). When used as a starter culture, LAB outperforms other cultures in terms of growth but if not employed, there is not an apparent development of LAB specimens and the culture spoils, with dominance of *Bacillus*. Thus, *Bacillus* strains may synthesize deleterious compounds that interfere with the fermentation process.

Gupta et al (2011) brought to light that after conditioning *Laminaria digitata* and *Saccharina latissima* with pretreatment and *L. plantarum* inoculation, the latter seaweed biomass could be successfully fermented. Similarly, Bruhn et al., (2019) assessed whether heat treatment and *L. plantarum* inoculation affected the sensory and nutritional quality characteristics of lacto-fermented kelps. The authors reported that the heat-treated and inoculated kelps were stabilized within 48 hours and had a milder odor and flavor compared to the fresh samples of the same nature. These findings are crucial for the inclusion of seaweed products in the food industry, as such processing potentially increases consumer acceptability. Recently, more studies were performed to optimize this process and the conditions employed to this end are gathered in Table 5.

As aforementioned, due to the recalcitrant nature of lignocellulosic materials, harsher conditions are required for its depolymerization, which results in production of several by-products that may hold

an inhibitory effect on microbial metabolism. Zhang et al. (2016) investigated the toxicity limits of the latter compounds in the fermentation of hydrolysates to lactic acid by *Rhizopus oryzae*. The authors determined that furfural and hydroxymethylfurfural (HMF) were hazardous at concentrations of 0.6 and 1 g/L, respectively whereas formic and levulinic acid were not hazardous at concentrations below 4 and 10 g/L. Interestingly, ethanol production was even increased up until the inhibitory concentrations of the two carboxylic acids. For the first time, inhibitors were shown to shift the metabolic pathway from lactic acid to ethanol biosynthesis.

More recently, Giacon et al (2022) studied the impact of both furfural and HMF in the growth kinetics of heterofermentative and homofermentative LAB species. Such work revealed that the growth of heterofermentative LAB is enhanced; in opposition, the presence of these compounds is inhibitory to homofermentative species. This can be explained through the capability of heterofermentative bacterium to decrease furfural and HMF concentrations in the fermentation medium through detoxification mechanisms, while simultaneously producing lactic acid (Giacon et al., 2022).

Table 5. Lactic acid fermentation from different seaweed feedstocks under different conditions and operation modes with respective lactic acid production (g/L), yield (g/g sugar) and productivity (g/L/h).

Microorganism(s)	Substrate	Operation Mode	LA (g/ L)	Yield (g/ g sugar)	Productivity (g/L/h)	Reference
L. sakei and *W. paramesenteroides*	*Ulva* sp. acid hydrolysate	Batch	25.1	0.8	6.8	Nagarajan et al., (2022)
L. plantarum			25.0	0.7	6.3	
L. rhamnosus			28.8	0.8	7.2	
L. plantarum (immobilizied)	*Ulva* sp.acid hydrolysate	Continuous	36.8	0.9	12.3	Nagarajan et al., (2020)
B. coagulans	*E. denticulatum* microwave assisted enzyme hydrolysate	Solid State Fermentation	14.0	1.0	-	Chai et al., (2021)
L. acidophilus and *L. plantarum*	*Gracilaria* sp acid hydrolysate	Batch	19.3	-	-	Lin et al. (2020)

Legend: LA -lactic acid; *L.sakei* -*Lactobacillus sakei*; *W.paramesenteroides* -*Weissella paramesenteroides*; *L.plantarum* -*Lactobacillus plantarum; L.rhamnosus* -*Lactobacillus rhamnosus*; *L.acidophilus* -*Lactobacillus acidophilus*; *B. coagulans*- -*Bacillus coagulans*; *E.denticulatum* -*Eucheuma denticulatum*. – symbol represents not mentioned

2.5.2 Seaweed Fermented Products

Although pioneer, seaweed fermentation may have a pivotal role in the food sector. Seaweed fermentation is perceived as a valuable insight when attempting to develop value-added-products (Reboleira et al., 2021). In this context, several studies were conducted with prospects to either decrease the concentration of undesired compounds within seaweed biomass or alter its sensory profile as a food ingredient or additive. During fermentation, the microorganisms synthesize vitamins, proteins and essential amino acids while still improving protein quality and fiber digestibility.

Seaweeds, by themselves, hold multiple bioactive compounds that serve health-related purposes. LAB biomass is also largely recognized for its probiotic role with reported benefits of lowering the risk of diseases, regulating allergic response and improvement of the gastrointestinal consortia,

among others (Fijan, 2014). Moreover, these kinds of microbial specimens are assigned as protective starter cultures which have GRAS status, thus being reliable for food production (Michalak et al., 2016). To note that, the introduction of microorganisms in food products must upgrade the safety and quality of the final product. Furthermore, the most auspicious microorganisms for use as starter culture are those isolated from natural microflora of traditional fermented goods, as it is the case of *Lactobacillus* strains and *S. cerevisiae*. The underlying motive is that such microorganisms are well adapted to the selected food environment and vigorously compete with microbial pathogens (Ojha et al., 2016). For all these factors, LAB and yeast fermented seaweed-based products are compelling candidates for inclusion in feed and food.

2.5.3 In Human Nutrition

Fermented foods have long been established as more nutritious than their unfermented counterparts (Sharma et al., 2020). Coupling fermented products offering a high lactic bacteria content with algae that contain biologically active metabolites of natural origin enables not only for the development of nutritious products but also the establishment of a novel segment of fermented foods.

Preliminary studies on seaweed-based fermentation have expressed the potential that lies in the consumption of these fermented products due their functional properties granted by enriched bioactive compounds together with promising organoleptic features. Such finding culminated in one of the first seaweed fermented products, a sauce with a flavor profile resembling traditional soy sauce but with a lower sodium content 11% (w/v) versus 14% (w/v). The nori-sauce had a characteristic taste that can be traced to high concentrations of glutamic and aspartic acid, as well as an unusually high concentration of taurine. Also, there were no detectable allergens in fermented seaweed as those found in wheat, soybeans, or crustaceans (Uchida et al., 2017).

Other authors have contributed to the formulation of novel and beneficial seaweed-fermented products with potential inclusion in the food industry. Takei et al. (2017) upon fermenting a plethora of red seaweed residues with *L. plantarum*, reached the conclusion that the antioxidant potential of biomass had been increased substantially. Then, proposed that such processing might create a whole set of processed foods to meet current consumer trends.

The recently published work of Norakma et. al (2022) analyzed the physicochemical properties of *Kappaphycus* spp fermented extracts. A seaweed product of superior nutritional value, functionality, and sensory quality was discovered after thorough profiling of amino acids, phenolic, and volatile chemicals. In detail, concentrations of histidine, glutamic acid, and tyrosine reached levels of 0.44, 4.27, and 0.64 g/100g of seaweed DW, respectively, and an improvement in volatile content was confirmed in all fermented seaweed samples.

Despite seaweed fermented products exhibit many nutritional benefits, knowledge in this field is still rather limited, thereby further research needs to be conducted before such products are extensively introduced into the market.

2.5.4 In Animal Nutrition

Feed additives and antibiotics have been used in the livestock sector for more than 50 years to simultaneously improve growth performance and prevent pathogen and disease infection. However, dietary antibiotic use has resulted in widespread problems across the food chain such as the development of antibiotic-resistant bacteria. Whilst the use of antibiotics has been banned or narrowed, much research has been conducted with the aim of increasing animal immunity and productivity through feeding supplementation or additives (Choi et al., 2018).

In other standpoint, there is a need to suppress the high feed cost without compromising the quality of the feed itself. Consequently, much effort is directed to replace traditional protein sources of feed fish and soy meals with less expensive alternatives that must not directly compete with human food. Hence, there is a growing interest in the market for new livestock feed (Hua et al., 2019).

In line with this, scientific research has begun to unravel the seaweed potential as a commercial feed product for farm and domestic animals. Such findings suggest that the incorporation of seaweed in animal feed contributes to nutrient digestibility, health, growth performance and meat quality. While there are some studies in respect to fermented seaweeds in poultry, swine and laying hens diet (Balasubramanian et al., 2021; Choi et al., 2018; Hui et al., 2021), much of its volume is dedicated to aquaculture.

2.5.5 As Feed Ingredients in Aquaculture

The last few decades have seen tremendous growth in the aquaculture sector. The demand for nutrient-dense commercial and complex aquafeeds is rising while aquaculture is developing speedily (Ang et al., 2021). Aquaculture diet formulation, for carnivorous fish species, are almost entirely dependent upon the use of fishmeal and fish oil as a major source of proteins and lipids respectively (Tacon et al., 2008). Proteins including hormones, enzymes and immunoglobulins are required for normal bodily functions and deficiency of this compound compromises protein synthesis, which lead to a reduction in fish weight but also impart other symptoms (Gatlin III, 2010)

Despite fishmeal being an ideal source due to its high digestibility and good profile of essential amino acids, it is indeed a commodity of stagnated production with a rising demand which led to an overall cost increase in feed costs (Cho et al., 2011). Moreover, future environmental, economic, and sanitary legislations are expected to strongly limit fish meal utilization (Dawood & Koshio, 2020; Jennings et al., 2016). Therefore, the search for alternative ingredients to partially or totally substitute aquafeed dietary nutrients must be at the top of the world's agenda.

Within the scope of seaweed-based aquafeed, primary trials aimed to analyze the impacts of including raw seaweeds in fish diets. The effects of seaweed incorporation in aquafeed can be assessed by inspecting the growth performance of tested aquatic species. Gross growth can be monitored through increase in length, weight or by a combination of both parameters, in a relationship known as condition factor (Wilson et al ,. 1993).

In most studies, a performance comparable to that of traditional diets was reported in algal meal inclusion of 10 to 15%, whereas 10% substitution in shrimp diets led to improved growth and feed utilization. Yet, incorporations above these levels yielded a progressively declining performance of

consumer species (Cruz-Suarez et al., 2008; Hasan et al., 2009). These detrimental effects were mostly linked with high fiber and ash content of those diets. Further to that, complex and structural carbohydrates make up most of the high carbohydrate seaweed load that may act as chelators and barriers culminating in poor digestibility and utilization (Hasan et al., 2009).

Also, results from several studies suggest that the species of algae and fish feeding habits may influence the maximum levels of algae that are advantageous for inclusion (Wells et al., 2016). Fermentation presents itself as possible solution to the former problems since this process heightens crude protein content whilst also decreasing antinutritional factors (ANFs), crude fiber and toxic load in feed ingredients (Dawood et al., 2020). As a result, other experimental studies were carried out, which envisaged fermented seaweeds as a potential replacement protein element in aquafeeds, some of which are included in Table 6.

Table 6. Effects of the inclusion of different percentages of seaweed species, processed under different conditions, in aquatic animal species feed.

Macroalgae	Processing	Aquatic Animal Species	Inoculum	Operation Mode	Algae % in feed	Effect(s)	Reference
P.tetrastromatica	Raw Powder	*M. rosenbergii*	-	-	10, 20 and 30%	No significant changes	Felix et al. (2014)
	Fermentated		*Lactobacillus* spp. and *S. cerevisiae*	Batch		Higher weight gain and feed utilization at 10 and 20%	
P. Gymnospora	Fermented	*Catla catla*	*Lactobacillus* spp. and *S. cerevisiae*	Fed-Batch	10, 20 and 30%	Higher weight gain at 30%	Anthonyraj et al. (2018)
Sargassum polycystum	Raw Powder (2mm)	*Lates calcacifer*	-	-	1.5, 3.0 and 4.5 %	Higher carcass protein in 1.5 and 3 %, higher blood cell counts and general enhancement of carcass Fe	Nazarudin et al. (2022)

Legend: *P.tetrastromatica -Padina tetrastromatica*; *P. Gymnospora -Padina Gymnospora*; *S. polycystum - Sargassum polycystum*; *M. rosenbergii -Macrobrachium rosenbergii*; *S.cerevisiae -Saccharomyces cerevisiae;* * symbol represents not mentioned.

As depicted in Table 6, two studies employed a consortium of lactic acid bacteria and yeast. This is due to the discovery of a potential synergistic effect between both organisms, which leads to a lower risk of pathogenic microbe contamination in the production process (Filho-Lima et al., 2000). Furthermore, findings suggested that lactic acid bacteria and yeast may have a probiotic effect on aquatic animals (Gatesoupe, 1999).

The first study examined the incorporation of both raw and fermented *Padina tetrastomica* in the feed of *M. rosenbergii* (prawn). As expected, the fermentative process led to an improvement of the dietary nutritional seaweed profile, namely an increment of 5.4% and 2.1% of protein and lipid content respectively while the fiber contents experienced a drastic reduction of 21%. Further, the prawn fed with a formulation of 10 and 20% of raw and fermented algal substrate exhibited a higher mean weight gain

and feed efficiency than that of control. As such, the results suggest that fermentation is not required when *P. tetrastomica* is included at lower levels of 10% and 20% in the diets. Nonetheless, the fermentation of P. *tetrastomica* is required at 30% inclusion of seaweed substrate since the raw powder feed organisms had an inferior performance whereas the fermented powder did not negatively affect growth. Thus, inclusion levels higher than 30% of fermented *P. tetrastomica* may be viable.

In a separate investigation, an examination was conducted on the biochemical composition of *Catla catla* upon exposure to experimental diets. This study yielded analogous findings, since even at 30% inclusion of *T. ornata* in feed fish a nearly equal growth level was attained to the control feed. However, the maximum fraction of seaweed inclusion in fish diets is also contingent upon feeding habits of the fish and the specific species of algae chosen.

Carbohydrates are utilized as substrates for microorganism growth in fermentation, as such the different factors concerning monosaccharide release yield and uptake must be thoroughly investigated. As follows, an increased growth of the fermentative strains has the potential to increase the protein content, making fermented macroalgae a much more competitive alternative for aquafeed.

Other evidence suggests that the incorporation of this marine resource in aquafeeds can go beyond offering essential nutrients that are generally present in traditional fish formulations (Wan et al., 2019). The biochemical active compounds of seaweed may be responsible for generating responses other than gross growth of fish performance. For instance, the supplementation of *Sargassum* sp of between 1.5 and 3.0% into fingerling's diet led to increased feed efficiency, better survival, higher mineral absorption. enhanced immune functions and better fish flesh quality. The higher quality of fish carcass was attributed to the proliferation of good probiotic bacteria, *L. paracasei*, in the gastrointestinal tract along with higher protein and iron contents and lower lipid content.

3. Objectives and Deliverables

The current study aims to develop an added-value feed ingredient with increased nutritional value and protein content by means of fermentation technology, utilizing low-cost and carbon-rich *Ulva rigida* hydrolysates as biomass resource. Within this framework, the following deliverables have been defined:

- Selection of a batch of *Ulva* whole biomass with high carbohydrate content to use as standardized biomass source.
- Optimization of seaweed saccharification process, prompting at high monosaccharide release yields and low concentration of microbial growth-inhibiting compounds.
- Analysis of the fermentative performance of yeast and LAB consortia in an alternative medium under the presence of deleterious hydrolysis by-products.
- Development of a method to quantify protein content of microbial cultures in a seaweed complex medium.
- Scale-up the fermentation in a 2L bench-top bioreactor operating in fed-batch mode to increase microbial biomass concentration and total protein contents.

- Investigate the interactions between a consortium of four lactic acid bacteria (4LAB) and *S. cerevisiae* to develop a single product that embodies the beneficial characteristics conferred by both yeast and lactic acid bacteria fermentation in food matrices.
- Characterization of raw and fermented products nutritional profile via proximal composition and biological activities of processed *Ulva* (liquid-soluble plus insoluble residual biomass).
- Selection of the best inoculum and fermentation conditions to produce a fermented ingredient better suited for incorporation in aquafeeds.
- Substitute aquafeed traditionally derived protein source from fish meal with fermented seaweed

4. Materials and Methods

4.1 Raw Material

Ulva rigida batches U1.00820MB1002.15, U1.01021MB2201<1.5, U1.01121MB0201<1.5, U1.01021MB2202<1.5, U1.101021MB2301<1.5, U1.0101121MB2903<1.5 and U1.010121MB2901<1.5, were purchased at the Portuguese producer ALGAplus Lda. (10 kg bags). The macroalgae biomass was purchased already washed, dried, and milled in flakes measuring less than 1.5 mm. The final two digits of the code represent flake size, with all flakes being equal or smaller than 1.5 mm in size.

4.2 Enzymes and Chemicals

Enzymatic hydrolysis was performed using commercial enzyme cocktails, namely Celluclast BG and β-glucosidase NS 22118 from Novozymes (Bagsvaerd, Denmark), with specific activities of 700 endoglucanase units (EGU)/g and 250 cellobiase units (CBU)/g, respectively. Amylases were also used to target starch algal reserves. Glucoamylase NS 22035 from Novozymes with an activity of 750 AGU/g and α-amilase (A4862) from Sigma Aldrich with an activity of 250U/g were used. The chemicals used were sulfuric acid 95-97% (MERCK) hydrochloric acid 37% (Fisher Scientific), ammonium hydroxide (Fisher Scientific), calcium carbohydrate (Riedel-deHaen), sodium hydroxide -pellets 99.1% (Fisher Scientific), D(+)-glucose anhydrous 99.5 % (Thermo Fisher Scientific), peptone (Merk Millipore), BactoTM yeast extract (BD Biosciences), manganese sulfate monohydrate ≥ 99 % (Sigma), magnesium sulfate heptahydrate ≥ 99.5 % (LabChem), di-ammonium hydrogen citrate A.C.S. grade (Sigma-Aldrich), bovine serum albumin (Sigma-Aldrich), potassium sodium tartrate, ACS, ISO (Panreac AppliChem), sodium carbonate (Farma-Quimica), copper (II) sulfate anhydrous (Panreac AppliChem), Folin & Ciocalteu's phenol reagent (Sigma-Aldrich) and Phosphate Buffered Saline, 10x Solution (Fisher Scientific).

4.3 Fermentative Microorganisms

Four *Lactobacillus* strains were selected in virtue of their capability to metabolize the different monosaccharides released during algal biomass hydrolysis, namely: *L. casei* ATCC393, *L. rhamnosus* ATCC 7469, *L. brevis* DSM 20054 and *L. plantarum* ATCC 8014. The *S. cerevisiae* strain SafAle^{TM} US-05 was added to the microbial consortia due to its beneficial contribution in fermented foods. *Lactobacillus* strains and *S. cerevisiae* were kindly supplied by Prof. Gabriel Monteiro from BERG and Dr. Margarida Palma from BSRG, respectively, both from iBB-IST.

All microbial strains were stored at -80ºC in refrigeration chambers. Stock cultures were prepared in a laminar flow chamber (BioAir Instruments aura 2000 MAC 4 NF, Italy) under aseptic conditions by transferring 1.5 mL of inoculum in the exponential growth phase to 2 mL sterile cryovials with 300 µL of previously sterilized glycerol.

4.4 Culture Media

4.4.1 Inoculum Medium Composition

LAB inocula was grown in De Man, Rogosa and Sharpe (MRS) broth (20.0 g/L glucose, 10.0 g/L peptone, 4.0 g/L YE, 8 g/L meat extract, 5 g/L sodium acetate, 2.0 g/L ammonium citrate, 5 g/L K_2HPO_4, 0.1 g/L $MgSO_4$, 0.05 g/L $MnSO_4.4H_2O$) (PanReac AppliChem), and *S. cerevisae* in yeast extract-peptone-dextrose (YPD) broth (20 g/L glucose, 10 g/L yeast extract (YE), 20 g/L bactoreological peptone.

4.4.2 Shake Flask Medium Composition

An alternative medium based on MRS broth formulation was used, which is composed of 830ml/L of filtrated *Ulva* hydrolysate, 40 mL/L corn steep liquor (CSL; from a batch of 2021); 2 g/L di-ammonium hydrogen citrate; 0.2 g/L $MgSO_4$, and 0.05 g/L $MnSO_4$.

4.4.3 Bioreactor Medium Composition

The adopted fermentation medium (working volume of 1.3 L) consisted of the 3% H_2SO_4-treated seaweed slurry supplemented with concentrated nutrient solutions to achieve the following medium composition: 813 mL/L seaweed residues hydrolysate; 40 mL/L CSL (batch 2021); 2 g/L di-ammonium hydrogen citrate; 0.2 g/L $MgSO_4$ and 0.05 g/L $MnSO_4$. The volume of hydrolysate was calculated based on the difference between bioreactor working volume, medium components, and inoculum 10% (v/v).

4.5 Chemical Pre-Treatment

Ulva rigida treatments consisted of acid hydrolysis for which time and temperature were fixed (30 min at 121ºC incubation in autoclave) and acid type and concentration varied to find the adequate conditions for this catalysis. Diluted acidic treatments with H_2SO_4 and HCl at 0.5%,1%, 3%, and 5.0% (w/v) concentrations were implemented. To accomplish this, 5 g of dry and ground *Ulva rigida* were weighted in 50 mL stoppered glass containers and MiliQ® water was added for a final volume of 50 mL, yielding 10% (w/v) biomass load. These assays were performed in triplicates. After hydrolysis, the pH of the *Ulva* slurries was set to 4.8 and released sugar monomers along with inhibitory microbial compounds were quantified using HPLC.

To assess the efficiency of acid hydrolysis in *Ulva* biomass, both the yield of monosaccharide release and chemical treatment were calculated through Eq.1 and Eq.2 respectively:

$$\text{Yield sugar component release } (\%)= \frac{[\text{Sugar}]f}{[\text{Sugar}]0} \times 100 \qquad \textbf{Eq. 1}$$

$$\text{Yield chemical treatment}(\%)= \frac{[\text{Fermentable sugars}]f}{[\text{Carbohydrates}]} \times 100 \qquad \textbf{Eq. 2}$$

Where: sugar corresponds to either glucose, xylose and rhamnose released in the liquid-phase, supernatant to the residual biomass (g/L); fermentable sugars correspond to the sum of glucose, rhamnose and xylose (total sugars) released to the supernatant during the pretreatment (g/L); carbohydrates correspond to the sum of total sugars quantified trough means of NREL 60957 protocol; the subscripts f and 0 correspond to final and initial concentration

4.6 Enzymatic Treatment

Enzymatic hydrolysis was performed on 1% and 3% (w/v) H_2SO_4 pretreated *Ulva* slurries (in 6 Erlenmeyer flasks, 50 mL working volume, triplicates). Two different enzymatic cocktails were tested, one comprising cellulose degrading enzymes whilst the other consisted of enzymes that hydrolyzed both cellulose and starch from residual alga biomass. Table 7 presents a summary of the conducted enzymatic assays and their respective conditions.

Table 7. Enzymatic hydrolysis of acid pre-treated *Ulva* slurry (10% biomass load, 121ºC, 30 min)

Factor	Enzymatic Assay		
	Cellulose	Cellulose + Starch	
		Phase 1	Phase 2
Enzymes	β-glucosidase (0.75 mg/mL) and Celluclast (1.3 mg/mL)	α-amilase (9.45 U/mL)	β-glucosidase (0.75 mg/mL), Celluclast (1.3 mg/mL) and glucoamylase (7.5 AGU/mL)
Incubation Time (h)	30	2	30
pH	4.8	6	4.8
Temperature (ºC)	50	90	50

Under aseptic conditions, β-glucosidase and Celluclast enzymes were paired for cellulose hydrolysis, and the pH of the algal suspensions was adjusted to 4.8 using NaOH 8M, 4M and 2M. The assay was conducted in an orbital incubator at 50ºC with a stirring speed of 200 rpm. A sample was taken from each flask before and after enzyme addition, so that the contribution of the prior chemical treatment for sugar release is considered. Then, throughout 30 hours' time window, samples were collected at 2-hour intervals.

A two-step assay for cellulose and starch hydrolysis was carried out, to maximize the activity of all engaged enzymes. Initially, the hydrolysate pH was set to 6 was α-amylase is added. The solution was incubated at 90ºC for 2 hours, with samples collected every hour. The conditions implemented in the second phase of the assay were identical to those used in the enzymatic hydrolysis of cellulose, except that glucoamylase was also introduced in addition to glucosidase and celluclast. Released sugars were quantified and identified by HPLC.

The efficiency of the enzymatic treatment (Eq. 3) and of the combined treatment (Eq.4) were calculated as follows:

$$\text{Yield enzymatic hydrolysis (\%)} = \frac{[\text{Glucose}]\text{f} - [\text{Glucose}]0}{[\text{Glucose}]\text{T}} \times 100 \qquad \textbf{Eq. 3}$$

$$\text{Yield combined treatment (\%)} = \frac{[\text{Glucose}]T - [\text{Glucose}]\text{f}}{[\text{Glucose}]\text{T}} \times 100 \qquad \textbf{Eq. 4}$$

Where: the subscripts f and 0 and correspond to final and initial concentration of glucose in the enzymatic treatment; T represents the total glucose quantified to total glucose quantified trough means of NREL 60957 protocol;

4.7 Seed Medium and Inoculum Preparation

In the context of this work, two different inocula were prepared; a *Lactobacilli* consortium (4LAB) and an axenic *S. cerevisiae*. The four LAB strains were cultivated separately in MRS medium, in Erlenmeyer flasks with 80% of its nominal capacity under orbital agitator (Agitorb200 (ARALAB) at 37ºC and 100 rpm. The yeast *Saccharomyces cerevisae* was cultivated in YPD medium from in same type of flaks but filled only to 20% of nominal capacity, under orbital agitation (Agitorb200 ARALAB) at 30ºC and 250 rpm. Flasks were inoculated directly from 1.8 mL cryovials taken from the cell bank. Cells were harvested from 16h to 18h of growth corresponding to the end of the exponential growth phase.

For the shake flasks assays, each inoculum was prepared by transferring the necessary volume of a pre-inoculum to sterile Falcon tubes so that inoculated shake flasks cultures were started with an optical density (OD_{600nm}) of 0.5 for (measured at 600 nm with optical pass-length of 1cm and HITACHI U-200 Spectrophotometer)). The culture in Falcon tubes were centrifuged at 4ºC for 15 minutes at 6000×g (Centrifuge 5818 R, Eppendorf). Afterwards, the supernatants were discarded aseptically, and the pellets resuspended in 0.5% (w/v) NaCl solution to inoculate the medium.

In bioreactor experiments, each microbial species was inoculated with the respective axenic culture in exponential growth phase. For LAB strains, the volume pertaining to each strain was calculated with the objective of having the respective *Lactobacillus* strain at concentration of OD_{600}=0.2 (for each strain) at the beginning of the bioreactor fermentation assay; and for *S. cerevisiae*, an OD_{600}= 0.7 was chosen. The procedure for shake flask inoculation was replicated for bioreactor inoculation.

4.8 Shake Flask Assays

4.8.1 Filtration and Storage of Hydrolysates

To assess the effect of biomass pre-treatment on the cell growth by means of optical density readings, after the combined hydrolysis of the algae biomass, the retrieved suspension underwent additional processing to separate the biomass from the liquid hydrolysate. First, the algae suspension was centrifuged for 10 minutes at 6000×g to separate large solids and then vacuum filtered with the use of a Buchner funnel equipped with a 900 µm pore-sized membrane to remove residual particulates. The filtrates were stored in 500mL *Schott* flasks at 4ºC.

4.8.2 Microorganisms' Growth

Microorganisms' growth was compared among different *Ulva* slurry pretreatment conditions, aiming to assess the ideal method for seaweed saccharification considering not only sugar release but also microorganism growth (rate and maximum concentration) and putative process cost. To this end, three types of algae hydrolysates were tested for batch fermentation by LAB consortium and yeast inoculum: 1% H_2SO_4 and cellulose enzyme treatment and also simple 3% and 5% sulfuric acid liquors.

Prior to inoculation, the pH of the fermentation medium was adjusted to 6.2 - 6.5 using solutions of 1M HCl and 8M NaOH. The assays were run in duplicate for 50 hours under standard growth conditions, outlined in chapter 4.7. Samples were collected every 2 hours in aseptic conditions to monitor biomass concentration (OD_{660m}), pH, substrate (sugars), metabolic products (lactic acid, ethanol) and

inhibitory compounds (furfural and HMF). Sugars, metabolic products and inhibitory compounds were assessed through high performance liquid chromatography (HPLC).

4.9 Process Scale-up to a Bench Scale Bioreactor

The scale-up of *Ulva rigida* fermentation was conducted in 3L bioreactor vessel connected to a control unit cabinet and associated to BioCommand/SCADA software (BioFlo/CelliGen, EppendorfAG).

In respect to fermenter handling, the bioreactor was assembled, filled with 900 mL of distilled water, and autoclaved at 121ºC for 25 min. In a separate vessel, a new batch of *Ulva* hydrolysate produced after thermochemical hydrolysis with 3% H_2SO_4 was prepared as main component of the base culture medium (Section 4.4.3) and used in each scale up fermentation run. The *Ulva* slurry was pumped along with all the previously sterilized remaining elements of the culture medium, draining the sterilization water from the bioreactor. After correcting the pH of the media, the inoculum was added. All assays started with 1.3 L working volume of the base culture medium and after sugar exhaustion, were carried out in fed-batch mode, using 500 g/L sterile glucose solution as feed medium.

The pre-inoculum for bench-scale bioreactor was prepared as indicated in Section 4.7. Culture sampling was conducted periodically in aseptic conditions to monitor viable cells (CFU counts), protein content (Lowry method), sugar consumption and metabolite formation (HPLC). In the end of each experiment, a 200 mL sample of *Ulva* slurry was collected and kept at -80ºC before being freeze-dried for future characterization.

4.9.1 Lactic Acid Fermentation of Seaweed Hydrolysate

Lactic acid fermentation of *Ulva* biomass was carried out for 140h at 50 rpm agitation speed, 5% of dissolved oxygen (DO) and a 6.5 pH setpoint maintained with controlled addition of a 30% (w/v) NH_4OH solution. The agitation speed was set in cascade with the DO% to enable DO% control, with a lower and upper limit of 50 and 600 rpm. The air flow supply was first set to 0.65 L/min but lowered to 0.3 L/min at 46.4h due to high DO readings.

4.9.2 LAB and Yeast Co-Fermentation of Seaweed Hydrolysate

Mixed fermentation of *Ulva* hydrolysate was conducted by first inoculating *S. cerevisiae*. Initial working parameters were set to 0.65 (L/min) of air flow supply, 37ºC, 50 rpm agitation speed and pH 6.5, but at 14.8h, however, temperature and stirring speed were changed to 30ºC and 200 rpm, respectively, as the yeast inoculum was barely uptaking any carbon source. Once yeast growth took up, the initial bioreactor conditions were restored and, at 46.5h of fermentation the LAB consortium (LAB mix) was inoculated into the culture medium. Cascade mode of air supply was set with a lower and upper limit of 50 and 600 rpm.

4.9.3 Alcoholic Fermentation of Seaweed Hydrolysate

Yeast alcoholic fermentation was carried out for 141h at 200 rpm agitation speed, 5% DO, air supply of 0.65 (L/min) and a pH 5.5 setpoint maintained with 30% NH_4OH solution (w/v). The agitation speed was set with a lower and upper limit of 150 and 600 rpm in cascade mode.

4.10 Processing and Analysis of Fermented *Ulva*

The nutritional profile of fermented products was examined. Prior to characterization, the fermented *Ulva* slurries were lyophilized for 72 hours using a freeze dryer Alpha 1-2 LDplus (Martin Christ) to dehydrate the products with minimal degradation. After lyophilization, the recovered material was manually ground into powder and kept desiccated at room temperature until nutritional quality analysis.

4.11 Analytical Methods

4.11.1 Total Carbohydrates in Seaweed Biomass

Total carbohydrates within *U. rigida* flakes were identified and quantified based on a protocol adapted from NREL 60957 “Determination of Total Carbohydrates in Algal Biomass” (Wychen et al., 2013a), with an adjustment of the sample weight and subsequent process volume. The method described entails a two-step acid hydrolysis for the total hydrolysis of sample polysaccharides (starch, cellulose and ulvan) to monomeric constituents, enabling the analytical quantification of glucose, xylose and rhamnose in the *Ulva* supernatant solution by HPLC.

Firstly, 0.5 g of *Ulva* biomass were weighted into a 200 mL stoppered glass container in triplicate and 50 mL of a 72% (w/w) H_2SO_4 were added. Each analysis was done with independent triplicates. In the first hydrolysis step, the former suspension was incubated at 30ºC for the period of 1h under agitation conditions (100 rpm). Following, the hydrolysates were diluted with MiliQ® water to achieve a concentration of 4% (w/w) H_2SO_4. In the second hydrolysis step, the diluted samples were pretreated in an autoclave for 1h at 121ºC and then cooled to room temperature. Upon cooling, 3 mL aliquots from each sample were transferred to 50 mL Falcon tubes and neutralized with $CaCO_3$ to a pH of 6 to 8. To separate the suspended solids, the neutralized samples were centrifuged at 4000×g for 5 min in a 5810 R Eppendorf centrifuge using the rotor F-34-6-38. For quantification of glucose, xylose and rhamnose, the supernatant was recovered and subjected to HPLC analysis as described in quantification of sugar and organic acids.

4.11.2 Quantification of sugars and organic acids

HPLC was used for offline determination of algal compounds in an injection volume of 20 µL. The system (Hitachi LaChrome Elite) was equipped with a Rezex ROA-Organic acid H+ 8% (300 mm x 7.8 mm) column, an autosampler (Hitachi LaChrome Elite L-2200), a HPLC pump (Hitachi LaChrome Elite L-2130), plus Hitachi detectors L-2490 refraction index (RI) and a Hitachi L-2420 UV-Vis VIS detector. A column heater (Croco-CIL 100-040-220P, 40 cm x 8 cm x 8 cm, 30-99°C) was connected

externally to the HPLC system. The column was kept at 65°C. A mobile phase of 5 mM H_2SO_4 was used at an flow rate of 0.5 mL/min.

Samples for analysis of recovered supernatants of algal biomass -hydrolyzed or fermented, were diluted by 20-fold with a 50 mM H_2SO_4 solution. The concentrations of sugars and microbial metabolites in (or released from) the biomasses were estimated taking in consideration the algal mass subjected to hydrolysis, the implemented dilution of liquid samples and previously determined calibration curves.

4.11.3 Optical Density

In shake flask assays, *Lactobacillus* spp. and *S. cerevisiae* cellular growth was monitored by spectrophotometric measurement of optical density using U200 (HITACHI) spectrophotometer. Prior to the OD_{600nm} readings, distilled water was used as reference (blank solution). Microbial culture suspensions were diluted in order to obtain a culture measurement within the acceptable values range (0.1 - 0.8 OD_{600nmn}). Each sample was placed in a quartz cuvette with a path length of 1 cm, and the corresponding OD_{600nm} recorded.

4.11.4 Viable Cell Counting

Colony forming unit (CFU) method was used for viable cells count determination at several time points throughout bench-scale fermentations. Thereupon, 100 µL of homogeneous sample were diluted in 900 µL of 0.85% (w/v) NaCl and successive 1:10 dilutions were prepared in sterile conditions until 10^{-7} proportionality. For three selected dilutes, 50 µL of sample were transferred to MRS-agar Petri-dishes, in triplicates. For cell counting in LAB-containing cultures, agar plates were incubated for 48h at 37ºC and kept at 4ºC until counting. In axenic yeast cultures, MRS agar plates were incubated for 48h at 30ºC. The number of microbial cells per milliliter of sample was assessed through means of Eq.5:

$$\text{CFU/ mL} = \frac{\text{Number of Colonies}}{\frac{1}{\text{Dilution Factor}} \times \text{Volume in Culture Plate}} \qquad \textbf{Eq. 5}$$

4.11.5 Total Nitrogen and Protein Content

Two different methods were employed to determine protein content: Lowry and an elemental nitrogen analyses. The first method estimates total protein content, while the second determines total nitrogen content.

Lowry procedure is founded on both Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions, yielding Cu^{+}, and the Folin reaction where cuprous ions interact with Folin-Ciocalteu reagent. This phenomenon causes the formation of a colored compound with an absorbance from 650 to 750 nm, detectable with a spectrophotometer. A calibration curve was built enabling the calculation of protein concentration using bovine serum albumin as standard.

$$\text{ABS750nm} = 1.4574 \times [\text{protein}] - 0.028 \qquad \textbf{Eq. 6}$$

Where: ABS_{750nm} corresponds to sample absorbance measured after both reactions; [protein] corresponds to protein concentration in mg per liter of hydrolysate solution.

Pellet samples were prepared according to Lowry method for protein quantification protocol (Waterborg et al., 1996). BSA was used as the reference standard for protein quantification. The BSA standard samples (0 to 500 µg/mL) were prepared with deionized water as well as the inoculum samples of unknown protein concentration. The absorbance of samples was measured at 750 nm with the use of U200 (HITACHI) spectrophotometer. Two different approaches were used for protein extraction: NaOH hydrolysis and NaOH hydrolysis preceded by sonication. Sonication of *Ulva* samples consisted of pipetting 4 mL of *Ulva* slurry, which is next centrifuged at 8,900×g for 8 minutes, the supernatant discarded, and each cell pellet is re-suspended in phosphate buffer (20 mM, pH 7.0). Then, samples were re-centrifuged, and the second supernatant carefully removed. The washed pellet was re-suspended up to the initial volume, transferred into Falcon tubes and immersed in an ice bath for sonication. Cell disruption is performed in a Bandelin sonicator, model Sonoplus HD 3200, equipped with a UW3200 transducer and a MS73 tip. The process time per sample is 4 minutes with on-off cycles (10s on, 5s off) at a power of 40W.

At analysis laboratory of IST (LAIST) the elemental nitrogen analyzer (Fison Instruments, EA 1108 CHNS) was used to determine nitrogen content in both *Ulva* dry flakes of the selected batches and of the lyophilized aliquots resulting from the fermentation processes. This analytical method relies on the complete oxidation of the sample, converting all organic substances into combustion products. The resulting combustion gases are directed through a reduction furnace and swept into the chromatographic column by a helium gas carrier, where they are separated and detected quantitively by a thermal conductivity cell. The analytes are contained within a receptacle and subsequently introduced into a quartz tube furnace at approximately 1000ºC, in an oxygen stream to ensure complete oxidation. Any excess oxygen is removed by contact with copper, while nitrogen oxides are reduced to elemental nitrogen. A nitrogen to protein conversion factor of 5.45 was used since it provides a more accurate estimation of the protein content in *Ulva* species (Shuuluka et al., 2013).

4.11.6 Peptide Profile

The peptide profile of samples streaming from bioreactor assays were examined at Instituto Português do Mar e da Atmosfera (IPMA), in Algés by an expert researcher (project collaborator), using size exclusion chromatography with a fast protein liquid chromatography system (FPLC) AKTA (Amersham Biosciences, Sweden). Peptide detection was performed using a Monitor UPC-900 (AKTA) set at a wavelength of 254 nm (UV).

Bioreactor samples were suspended in a solution of 30% acetonitrile solution with 0.1% trifluoroacetic acid (v/v). For the analysis, gel filtration column Superdex 75 Increase 10/ 300 GL was used. This column has a separation range between 10 to 7000 Da that is suitable for the detection of small biomolecules including vitamins, organic acids, lipids, sugars, alcohols among other molecules, with a bed volume of 83 mL. For this analysis, a sample volume of 50µL and a flow rate of 0.45/min were utilized.

A calibration slope was established, relating molecules' Kav (calculating using Eq. 7) with the decimal logarithm of their molecular weight, log (MV). Known proteins, peptides and aminoacids were

used to construct this calibration curve (figure A.10 in the Appendix). The parameter Kav represents the correlation between the elution volume of each molecule and the total volume of the column.

$$Kav = \frac{Ve - V0}{Vi} = \frac{Ve - Vo}{Vt - Vo} \quad \textbf{Eq 7.}$$

Where: Ve represents the elution volume of the molecule; V_0 refers to the void volume of the column; V_i represents the volume within the beads; V_t corresponds to the total bed volume of the column.

4.11.7 Biological Activity

Assessment of antioxidant and chelating qualities of raw and fermented *Ulva* was conducted in the laboratory facilities IPMA with the objective of characterizing the biological activity of these materials. Dilutions of each sample were prepared in the concentration range of 1 to 20 mg/mL to determine the half maximum effective concentration (EC_{50}) for several biological effects. EC_{50} is defined as the sample concentration that causes a 50% decrease in the radical absorbance. The antioxidant activity was determined through EC_{50} values for 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) radicals. The same principle was applied to evaluate the reducing power and metal chelating activity of the samples, with copper and iron chelation serving as indicators of metal chelation. Detailed descriptions of the protocols followed to determine the biological activities can be found in the study by Henriques et al., (2021)

4.11.8 Total Solids and Ash Contents

The total solids and ash contents were determined using a protocol adapted from the analytical procedure NREL 560956 "Determination of Total Solids and Ash in Algal Biomass" of the National Renewable Energy Laboratory (Wychen et al., 2013b).

Crucibles was pre-conditioned at 575°C in a muffle furnace 3/11/B180 L-030K1CN (Nabertherm) overnight. Once conditioning of crucibles was complete, these were left to cool down to room temperature in a dessicator before being weighted. Samples of 100 ± 5 mg were put to pre-conditioned crucibles and then placed in a climate chamber at 60ºC until constant weight readings were achieved. Equations 8 and 9 were used, respectively, to calculate the total solids and moisture percentage of the samples. The oven-dry weight of algal biomass established as the weight of samples mathematically corrected for the solids content, was also derived through equation 10.

$$\text{\% Total Solids} = 100 \times \frac{\text{Weight crucible+dry sample-Weight crucible}}{\text{Weight sample as received}} \quad \textbf{Eq. 8}$$

$$\text{\% Moisture} = 100 \times \left(1 - \frac{\text{Weight crucible+dry sample-Weight crucible}}{\text{Weight sample as received}}\right) \quad \textbf{Eq. 9}$$

$$\text{ODW sample} = \frac{\text{Weight sample as received} \times \text{\% Total Solids}}{100} \quad \textbf{Eq. 10}$$

Following that, to determine the ash content of the samples, the cooled crucibles containing the dried samples were placed in the muffle furnace equipped with a ramping program. The temperature was first ramped to 105 ºC for 12 minutes, then increased to 250 ºC at a rate of 10 ºC/min for 30 minutes, and finally set to 600 ºC at a rate of 20 ºC/min and maintained at this temperature for 16 hours (instead of 180 minutes as referred in NREL protocol). Afterwards, the temperature was let to drop until 105ºC

to allow taking crucibles out of the muffle. After being taken out, these were placed in a desiccator to cool to room temperature before being weighed. Ash content was quantified via equation 10.

$$\% \text{ Ash} = 100 \times \frac{(\text{Weight crucible + ash - Weight crucible} \times 100)}{\text{ODW sample}} \qquad \textbf{Eq. 11}$$

5. Results

5.1 Total Monosaccharide Composition of *Ulva* Batches

First and foremost, the proximate monosaccharide composition of several *Ulva rigida* batches was assessed, aiming to select the batch with the highest carbohydrate content for further experiments. The obtained results are listed at Table 8.

Table 8. Proximate composition of *Ulva rigida* dry flakes. The presented values are expressed as mean ± standard deviation. Legend: TRS -total released sugars.

BATCH	Carbohydrates (g/ g DW)			
	Glucose	Xylose	Rhamnose	TRS
B_1 - U1.010820MB1002.15	0.15 ± 0.01	0.01 ± 0.00	0.10 ± 0.01	0.26 ± 0.01
B_2 - U1.01021MB2201<1.5	0.19 ± 0.00	0.03 ± 0.00	0.09 ± 0.00	0.31 ± 0.00
B_3 - U1.01121MB0201<1.5	0.21 ± 0.02	0.03 ± 0.01	0.07 ± 0.02	0.31 ± 0.02
B_4 - U1.01021MB2202<1.5	0.31 ± 0.01	0.04 ± 0.01	0.09 ± 0.03	0.43 ± 0.03
B_5 - *U1.01021MB2301<1.5	0.30 ± 0.04	0.09 ± 0.00	0.11 ± 0.00	0.50 ± 0.03
B_6 - U1.01021MB2903<1.5	0.10 ± 0.05	0.02 ± 0.01	0.04 ± 0.03	0.17 ± 0.05
B_7 - U1.1021MB2901<1.5	0.25 ± 0.03	0.02 ± 0.00	0.11± 0.01	0.39 ± 0.03

*This *Ulva* batch was purchased latter due to depletion of the content used as stock

In the present study, carbohydrate content was estimated considering that all *Ulva* polysaccharides were completely hydrolyzed into their monomeric sugars, using a two-step acid hydrolysis procedure. Among monosaccharides, glucose is dominating (0.15 to 0.31 g/g DW), rhamnose is the second largest component (0.04 to 0.11 g/g DW), and xylose only holds a small contribution to the total carbohydrate content (0.01 to 0.09 g/g DW). A study of the monosaccharide composition of two *Ulva* species revealed that both had more than 50% of glucose contributing to total sugars (54% ± 3%), followed by rhamnose (30% ± 6%) and xylose (9 ± 6%) (Tsubaki et al., 2014). The former percentages corroborate the retrieved results, as the same hierarchical pattern in sugar concentration is observed.

Although some studies report disparities in monosaccharide concentrations, differences in carbohydrate content are to be anticipated as algal composition is prone to fluctuation due to a variety of contributing factors including habitat, maturity, and environmental conditions. In a similar study, Yaich et al., (2011) reports different findings for the monosaccharide composition of *Ulva lactuca* with 0.17, 0.07 and 0.2 (g/g) for glucose, rhamnose and xylose respectively.

The most significant difference between the analyzed batches relies in the glucose fraction. The former possibly relates to different growth conditions imposed by ALGAplus to optimize the algae material for biorefinery cascades. At earlier experimental work for this thesis, batch 5 was not available; batch 4 (U1.01021MB22021.5) was the one with the highest amount of total released sugars, it was selected as feedstock for further assays.

5.2 The Effect of Acid Treatment in *Ulva* Saccharification

The pretreatment of seaweeds is regarded as indispensable as many sugars are not freely available but instead are found in structural and storage carbohydrates (El Harchi et al., 2018). The recovery rates of algal polysaccharides are constrained by the rigidity of the cell wall matrix (Ummat et al., 2021). By disorganizing the polysaccharide complex, the thermochemical treatment ensures the opening of the algal cell wall structure making cellulose more amenable for enzymatic treatment, resulting in a higher concentration of reducing sugars (El Harchi et al., 2018; Maneein et al., 2018). Concerning ulvan, the typical *Ulva's* complex polysaccharide, a particular enzymatic cocktail including ulvan lyases, would be needed to provide total ulvan hydrolysis to its rhamnose and xylose monomers. As this enzymatic cocktail is not yet a commercially available, hydrolysis of ulvan was partially attained with the thermochemical step.

In this experiment, overall sugar yield constitutes a key consideration in evaluating the performance of *Ulva* biomass hydrolysis as microbial growth should profit upon microbes' ability to utilize the available sugars. The current experimental design aimed to find the ideal conditions for dilute acid hydrolysis of *U. rigida's* carbohydrates. Chemical hydrolysis was evaluated based on the effects of two variables - acid nature and catalyst concentration for the degradation of *Ulva* polysaccharides. Solutions with varying concentrations of HCl and H_2SO_4 were prepared with MilliQ® water and used for this purpose.

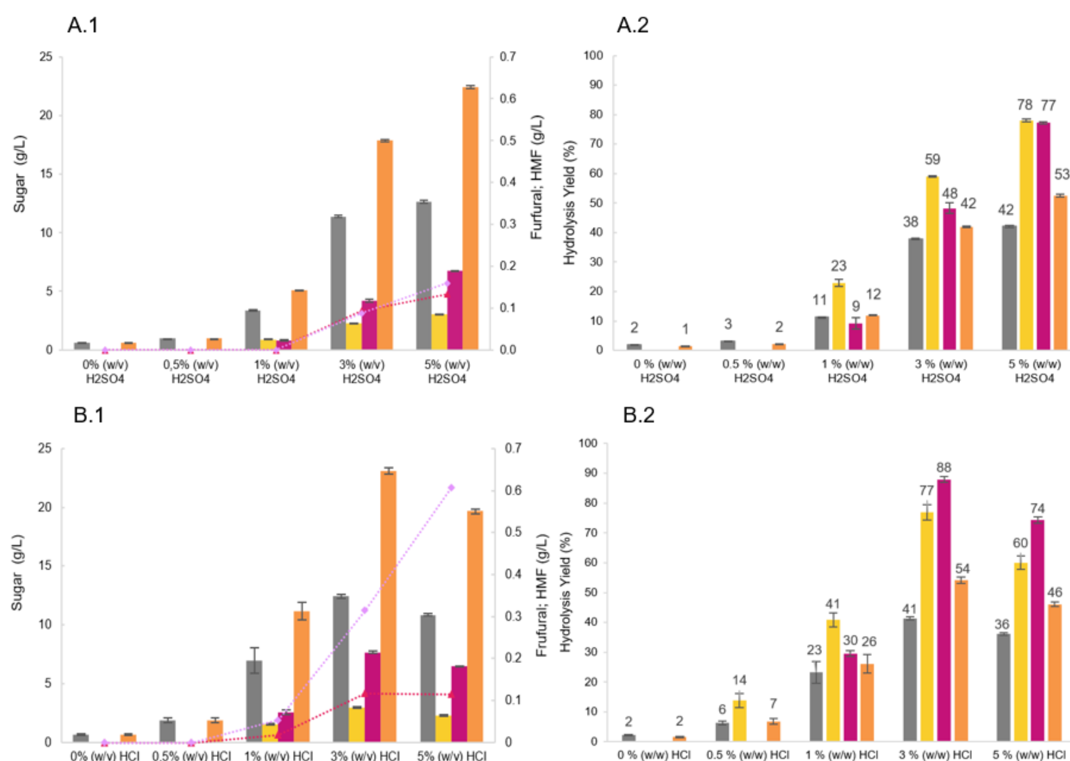

Figure 4. *Ulva* biomass chemical pre-treatment with H_2SO_4 (**A.1**) and HCL (**B.1**) of 0.5, 1, 3 and 5% (w/w) prepared with MilliQ® water for a biomass concentration of 100g/L, on the concentration of released algal monosaccharides of interest and potentital inhibitors; and hydrolysis release yield of each sugar component in H_2SO_4 treatment (**A.2**) and HCl treatment (**B.2**). All tested samples were in the autoclave for 30 min. Error bars indicate standard deviation of biological triplicate samples. Legend: Glucose (■); Xylose (■); Rhamnose (■); Total Released Sugars (■); HMF (♦); Furfural (▲)

As shown in Figure 4, the hydrolysis of carbohydrates to reducing sugars is correlated with acid concentration. In sulfuric acid treatment (A), all monosaccharides reached the highest concentrations at 5% of H_2SO_4, with xylose being the most released sugar in terms of yield (78%), but rhamnose follows closely behind (77%). Acid hydrolysis with 3% and 5% (w/v) led to a total release sugar of 18 g/L and 22 g/L, respectively, generating appreciable amounts of total sugars. The absence of rhamnose at 0% and 0.5% (w/v) conditions may be explained by the fact that the chemical bonds linking the respective monomers in the Ulvan require more severe hydrolysis conditions.

The use of higher acid concentrations is not only paired with higher sugar release yield but also with an increasing trend of furfural and 5-hydroxymethilfurfural (HMF), generated as byproducts of pentoses and hexoses degradation, respectivelly. At 5% sulfuric acid both furfural and HMF reach the maximum concentration of 0.16 g/L and 0.13 g/L respectively. Disclosed concentration of these aldehydes in lignocellulosic hydrolysates are ranged from 0.1 to 8.6 g/L of HMF and from 0.1 to 11g/L of furfural (Giacon et al., 2022).

Furans are known to be strong inhibitors of microorganism growth and sugar conversion, and their combination generally intensifies their inhibitory effect (Malav et al., 2017; L. Zhang et al., 2016).

A different behavior is observed in the profiling of reducing sugars with the use of hydrochloric acid: the concentration increases, then reaches a maximum and finally decreases. As follows, the employment of harsher pretreatment conditions does not consistently lead to higher sugar recoveries as a further increase in acid concentration from 3% to 5% (w/v) HCl led to a decrease of hydrolysis efficiency from 54% to 46%. This decreasing trend may be explained due to the higher susceptibility of sugar for thermal degradation to furan compounds (El Harchi et al., 2018). The maximum sugar release for HCl treatments was registered in 3% (w/v), with a sugar release yield comparable to the 5% (w/v) H_2SO_4 condition and even higher for rhamnose (88%).

Concentrations of these organic compounds near inhibitory levels found in literature must be avoided as they might jeopardize the microbial function. Concentrations between 0.5 to 1.0 g/L HMF and furfural were shown to be toxic in the lactic acid fermentation of hydrolysates by *Rhizopus oryzae* (Zhang et al., 2016).

The residence time of the acid reaction is an additional critical parameter in the pretreatment of seaweed materials. In a study focused on optimizing the saccharification of seaweed residues revealed that a reaction time of 1 hour had a significant effect on the productivity of glucose. Reaction times surpassing the 1-hour mark were found to hasten the degradation of cellulose, which led to the loss of glucose (Ge et al., 2011). Therefore, conducting assays with varying hydrolysis times would be of interest, as they have the potential to enhance sugar release. It is worth mentioning that the effectiveness of seaweed treatments is influenced by numerous factors, such as seaweed structure, chemical composition of the initial material, and processing methodology.

In all, even though HCl treatments yielded a higher total sugar release in comparison with H_2SO_4 at a lower concentration (3%), its use resulted in higher concentrations of inhibitory compounds. For this reason, HCl pre-treatment was discarded. Because a step increase of furfural and HMF were registered from 3% (w/v) to 5% (w/v) H_2SO_4, both 1%(w/v) and 3% (w/v) H_2SO_4 pretreatments were tested for subsequent enzymatic hydrolysis.

5.3 The Effect of Enzyme Hydrolysis in Chemically Pre-Treated *Ulva*

Enzymatic hydrolysis aims to extend the amount of released sugars from algal biomass by breaking down the residual poly- and oligosaccharides that were not hydrolyzed during chemical treatment.

5.3.1 Cellulose Hydrolysis

As glucose represents a significant portion of *Ulva*'s total sugars (Table 8) (Tsubaki et al., 2014), further increasing the release of this sugar may significantly increase the amount of total fermentable sugars. Additionally, glucose has the lowest release yield of all the analyzed components and is still low in the selected conditions (11% and 38%) as seen in Figure 4. Ergo, there is much prospect for an enzymatic assay that specifically targets glucose release. To this end, an enzymatic cocktail composed of β-glucosidase and cellulases was utilized, and subsequent results are shown in Figure 5. Cellulase was utilized for hydrolysis of the β-(1→4) glycosidic bond in cellulose molecules (Cavaco-Paulo, 1998); β-Glucosidase enzyme complements this process as they catalyze the hydrolysis of cellobiose to glucose (Keller et al., 2020).

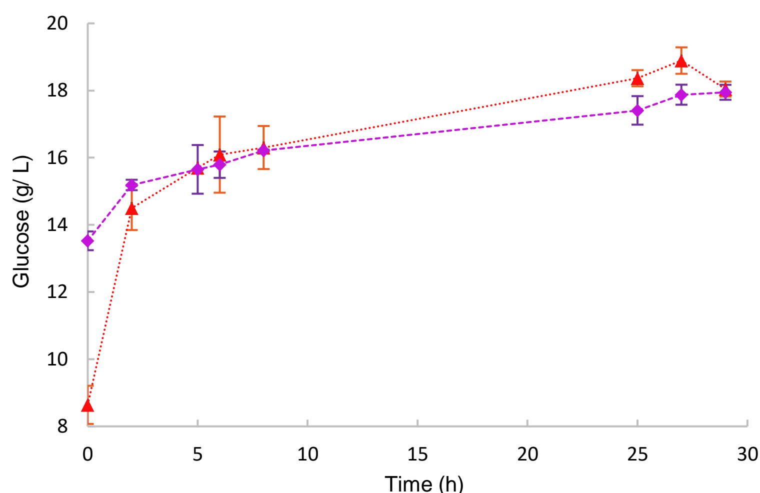

Figure 5. Enzymatic hydrolysis with Celluclast (1.3 mg/mL) and β-glucosidase (0.75 mg/mL) for chemically pre-treated *Ulva* suspensions (biomass load of 10% w/v) 1% (w/v) and 3% (w/v) H_2SO_4. The assay was conducted during 30h at pH 4.8 and temperature of 50ºC. Legend: 1% (w/v) H_2SO_4 (▲); 3% (w/v) H_2SO_4 (●).

The glucose concentration in solution was expected to rise as a result of the enzymatic mechanisms of action. A significant difference between the tested conditions at the start of the enzymatic assay is observable (Figure 5), with 1% (w/v) H_2SO_4 yielding lower fermentable sugars than 3% (w/v) H_2SO_4. Such results are to be expected as sugar content concentrations are directly attributable to the former heat treatment, since the enzymatic cocktail still did not have time to exert its function. Yet, throughout time, the disparity in glucose concentration profiles between both assays narrows, and by the fifth hour, the glucose concentration from the 1% (w/v) H_2SO_4 pre-treated surpasses that of 3% (w/v) acid pre-treatment.

Hydrothermal pre-treatments reduce the recalcitrance of lignocellulose by effectively removing a substantial portion of hemicellulose, consequently enhancing the accessibility of cellulases and the acid concentration has been shown to greatly impact the breakdown of seaweed cellular structure (Offei et al., 2019). Since the 3% treatment is potentially better at dissolving hemicellulose than the 1%

treatment, it was anticipated that the conversion of cellulose would increase. One hypothesis is that enzyme activity is impaired by the generated inhibitors which are more pronounced in 3% (w/v) H_2SO_4 condition. However, aldehydes have shown to have little impact on cellulases despite being potent inhibitors of yeast and bacterial metabolism. Instead, phenolic derivatives, were found to be significantly more inhibitory to cellulase enzymes that other hydrolysate compounds, as they might lead to precipitation and irreversible inactivation of cellulase enzymes (Y. Kim et al., 2011; Qin et al., 2016). Although phenols quantification was not performed, their presence in significantly distinct concentrations, could potentially justify the observed data.

The subsequent yield of dilute acid, enzymatic, and combined hydrolysis based on glucose and total sugar release are represented in Table 9 and 10 respectively.

Table 9. Reducing glucose yields from chemical treatment or enzymatic hydrolysis of cellulose, and from subsequent combined hydrolysis. Chemical hydrolysis of 100g/L *Ulva rigida*, carried with 1% (w/w) H_2SO_4 and 3% (w/w) H_2SO_4 for 30 min at 121ºC and then enzymatic hydrolysis at 50ºC and a pH 4.8 for 30h.

H_2SO_4 (w/v)	1%	3%
Thermal Treatment Yield %	11	38
Enzymatic Treatment Yield %	35	27
Total Yield %	63	60

Table 10. Reducing total sugar yields from chemical treatment or enzymatic hydrolysis of cellulose, and from subsequent combined hydrolysis. Chemical hydrolysis of 100g/L *Ulva rigida*, carried with 1% (w/w) H_2SO_4 and 3% (w/w) H_2SO_4 for 30 min at 121ºC and then enzymatic hydrolysis at 50ºC and a pH 4.8 for 30h.

H_2SO_4 (w/v)	1%	3%
Thermal Treatment Yield %	12	42
Enzymatic Treatment Yield %	25	20
Total Yield %	49	56

Regarding total process yield having as basis only glucose, using 1% H_2SO_4 (w/v) pretreatment is 63% while for 3% H_2SO_4 (w/v) treatment is 60%. Therefore, the first treatment is slightly more efficient in terms of this simple sugar release. However, when considering release of all monosaccharides (xylose, rhamnose and glucose) for the calculation of the total process yield, the 3% (w/v) sulfuric acid hydrolysis performs better (56%) then 1% hydrolysis. It is important to consider that through the employment of harsher acid conditions (3% H_2SO_4 (w/v)), the release of xylose and rhamnose are significantly increased (0.02 and 0.04 g/g respectively) in comparison to the use of 1% (w/v) of the same acid. This difference is expected since Ulvan hydrolysis was conducted through the thermo-chemical step and the enzymatic hydrolysis only targeted cellulose parcels.

5.3.2 Combined Hydrolysis of Starch and Cellulose

Glucose monomers are not only released from the hydrolysis of the cellulose in *Ulva* biomass but also from the hydrolysis of its starch. Numerous studies have demonstrated that when seaweeds are exposed to nutritional stress and blue light, starch concentration can be significantly increased (Prabhu et al., 2019). Such alterations are multiple times induced during algae cultivation to increase its carbohydrate content and thereby biorefinery potential.

In this scope, the possibility of further releasing glucose was explored by also adding α-amylase and glucoamylase into the previous enzymatic cocktail. Alpha-amylase is an endoamylase that randomly breaks down the starch chain's α (1→4) glycosidic bond whereas glucoamylase catalyzes the hydrolysis of α-(1 → 4) and α-(1 → 6) glycosidic bonds at the non-reducing end of starch molecule (Han et al., 2021). Since (1 → 6) glycosidic bonds remain even after exhaustive digestion with α-amylase (Zemke-White et al., 1999), glucoamylase exerts a complementary and essential role in starch hydrolysis. The results of starch and cellulose combined enzymatic assay are depicted in Figure 6.

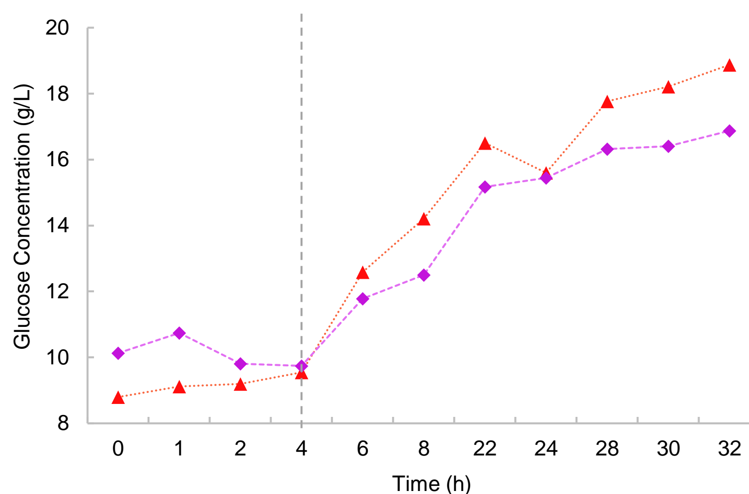

Figure 6. Combined starch and cellulose hydrolysis for 1% (w/v) and 3% (w/v) H_2SO_4 chemically pre-treated *Ulva* suspensions, with a biomass load of 10% (w/v). In the first two hours of the assay the conditions were set to pH 6 and temperature of 90ºC. From 4h onwards the conditions were shifted to a pH of 4.8 and temperature of 50ºC, to optimize the enzyme's mechanism of action. Legend: 1% (w/v) H_2SO_4 (▲); 3% (w/v) H_2SO_4 (●).

During the first 2 hours of the assay, the glucose concentration is rather constant in both studied conditions, which was to be expected given that at this stage only α-amylase is present in the algae solution. This enzyme requires the action of glucoamylase to further fragment the catalyzed starch dimers into glucose monomers, which were only detected in their monomeric form with the HPLC conditions imposed.

Once the remaining enzymatic components are added, the glucose concentration increases up to 16.9 and 18.9 (g/L) in 3% H_2SO_4 and 1% H_2SO_4, respectively, reaching nearly the same sugar concentrations as in the previous experiment (cellulose enzymatic treatment, Figure 5). These findings fell short considering that even higher amounts of glucose were projected to be recovered since starch and cellulose are both targeted in this experimental setup. In contrast to cellulose, starch possesses a structurally stable crystalline arrangement and exhibits an open, loosely bonded helical configuration. This characteristic renders starch a more amenable solubilization with chemicals or physical degradation processes (Offei et al., 2018). Along these lines it is possible to hypothesize that the glucose streaming from starch may be released in bulk during the chemical treatment, thus the use of enzymes that specifically target these parcels have little to no effect on the glucose yield. To test this hypothesis, an iodine reaction could have been performed in the *Ulva* suspension before and after the chemical treatment to quantify the solubilized starch in both circumstances and check for the concentration of the colored compound.

Cocktail A was selected for microorganisms' growth assays, as this process rendered a similar sugar release at a lower cost than cocktail B. Even still, hydrolyzing enzymes hold high costs, thus 3% and 5% H_2SO_4 pretreated *Ulva* were also selected for further experimentation.

5.4 Optimum *Ulva* Treatment for Microbial Growth

One of the largest constrains in the usage of hydrolysates is that the cheap pre-treatment procedures generate much higher levels of hazardous chemicals than the more expensive technologies (Vanmarcke et al., 2021). Although strategies for detoxifying lignocellulose hydrolysates and mild pretreatments have been developed, these protocols all add sizable amount of additional cost while also complicating biomass conversion processes and generating further waste (Brodeur et al., 2011; Chandra et al., 2007). In these circumstances, the answer appears to rely on the usage of inhibitor-tolerant specimens, making it critical to access microorganism growth under such harsh conditions.

Based on the results obtained thus far, three different pretreatment strategies of *Ulva*'s biomass have been identified as the most promising, allowing for the effective use of this material as substrate for microbial fermentation. Among the three pre-treatments displayed in Table 11, combined chemical and enzymatic treatment displays the highest glucose concentration, which is in general the most favored sugar for most lactic acid producing strains (Y. Wang et al., 2015). Withal, this treatment also has the highest cost and time requirements, both of which are imputable to the use of enzymes. While total sugar release is higher in 5% H_2SO_4 treated residues, is backed with the highest amount of inhibitory compounds which may hinder microbial fermentation. Additionally, 3% acid treatment exhibits a similar monosaccharide profile as in 5% treatment, except for rhamnose, but lower levels of furfural and HMF.

Table 11. Tested *Ulva rigida* hydrolysates for 4LAB consortium and *S. cerevisiae* growth, with detailed treatment and released molecule concentrations of interest. Experimental data obtained from a 100 g/L macroalgae concentration in a volume of 50 mL. Legend: TRS -total released sugars; - not detected.

Chemical Treatment	Enzymatic Treatment	Released Sugars (g/L)				Inhibitory Compounds (g/L)	
		Glucose	Xylose	Rhamnose	Total	Furfural	HMF
1% H_2SO_4	Cellulclast and β-glucosidase	18.04	2.25	0.63	20.92	-	-
3% H_2SO_4	-	11.37	2.29	3.03	16.69	0.09	0.09
5% H_2SO_4	-	12.65	3.03	6.74	22.42	0.16	0.13

Since each of the three treatments holds qualities that are prospective for its inclusion in culture medium, microbial growth in shake flasks was accessed in these conditions. The use of dilute acid pretreated substrates requires microorganisms that are not only capable to utilize the released sugars but to also withstand inhibitors. Furfural and HMF are among the most hazardous fermentation inhibitors found in lignocellulosic hydrolysates, severely jeopardizing optimal microorganism function (Becerra et al., 2022). Therefore, finding the right compromise between carbohydrate recovery and inhibitor generation is crucial.

5.4.1 *Lactobacillus* spp.

This section focuses on the combined growth of *Lactobacillus* (LAB) consortium in three different hydrolysates, operated in batch mode for 50h (approximately 2 days), in a 100 mL working volume. Since the main objective of this research work is to boost the nutritional and protein quality of seaweed fermented products, microbial growth and substrate transformation were determined at different time points and regarded as indicators of the performance of the fermentative process.

The results from microbial growth and pH are represented in Figure 7; data regarding sugar consumption and metabolite formation are depicted in Figure 8. It is worth mentioning that sugar levels were lower than those found in previous assays, reported in Table 11. This may be explained by volume scale-up of the hydrolysis reaction worsened by the absence of agitation. From this point forward, seaweed hydrolysis reaction was conducted in smaller reaction volumes per Erlenmeyer flasks (each with 100 g/L and 500 mL volume), but more shake flasks were utilized to achieve the desired working volume. This approach was outlined so that for scale-up, higher volumes of hydrolysate could be obtained without compromising sugar release yield.

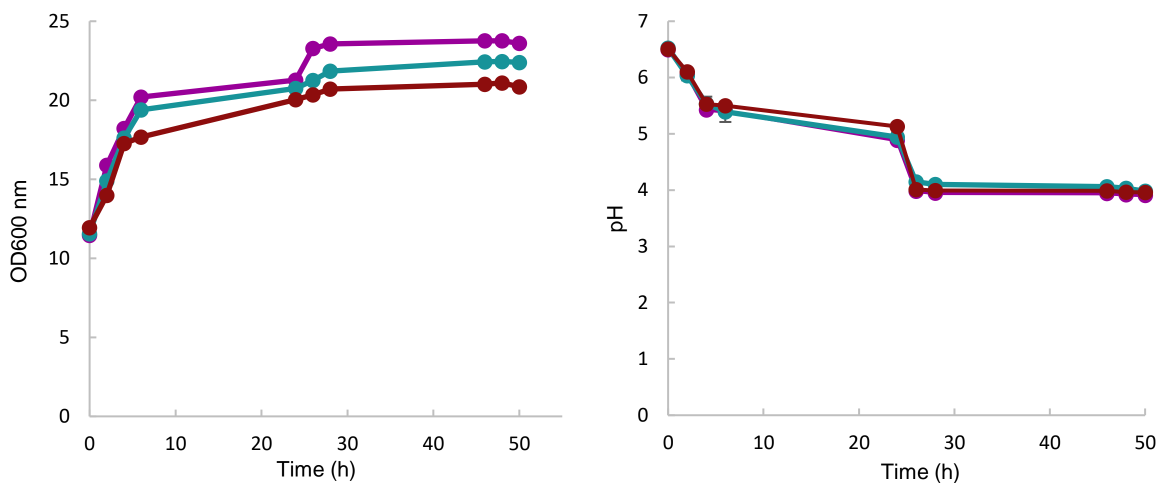

Figure 7. OD_{600nm} (**left**) and pH readings (**right**) for shake-flask co-culture of *L. casei* ATCC 393, *L. rhamnosus* ATCC 7469, *L. brevis* DSM 20054 and *L. plantarum* ATCC 8014 in *Ulva* hydrolysates at 37ºC, 100 rpm with initial DO_{600nm} of 0.5. Legend: 1% (w/v) H_2SO_4 (●); 3% (w/v) H_2SO_4 (●); 5% (w/v) H_2SO_4 (●)

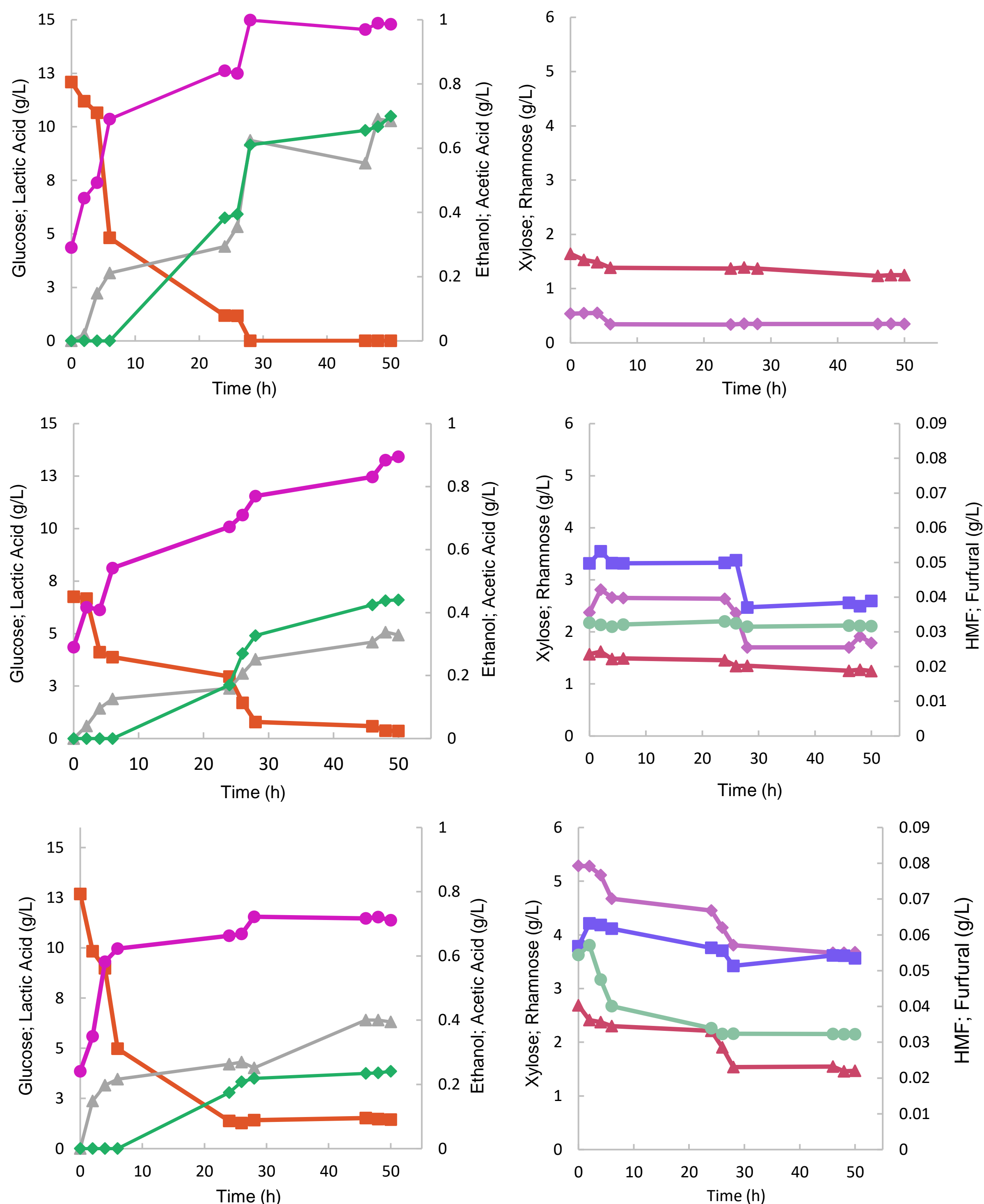

Figure 8. Concentration of glucose, lactic acid, ethanol, acetic acid **(left),** xylose, rhamnose, HMF and furfural **(right)** obtained by HPLC, for shake-flask co-culture of *L. casei* ATCC 393, *L. rhamnosus* ATCC 7469*, L. brevis* DSM 20054 and *L. plantarum* ATCC 8014 in tested *Ulva* hydrolysates at 37ºC, 100 rpm with initial DO_{600nm} of 0.5. Legend: Glucose (■); Lactic Acid (●); Acetic Acid (♦); Ethanol (▲); Xylose (▲); Rhamnose (♦); HMF (■); Furfural (●); data for *Ulva* pretreatments in 1% H_2SO_4 + celluclast and β-glucosidase **(top graphs)**; 3% H_2SO_4 (**middle graphs)**; 5% H_2SO_4 **(bottom graphs).**

Lactic acid, ethanol, and acetic acid were produced as metabolites, demonstrating the heterofermentative nature of some specimens present in the 4LAB consortium. In all tested conditions, lactic acid rapidly accumulated within the first 10h of the experiment and reached a plateau at 28 hours. The sharp drop in medium pH from 26 hours (pH 5.0) to 28 hours (pH 4.0) is likely what causes the

levels of lactic acid and growth to stabilize. As pH value decreases due to lactic acid accumulation, the undissociated form of lactic acid increases, which is responsible for cytoplasm acidification and failure of proton forces causing LAB fermentation inhibition (Othman et al., 2017).

The highest concentration of *Lactobacillus* metabolites was achieved in 1% H_2SO_4 solution combined with enzymes pre-treated *Ulva* suspension, while the lowest concentration was obtained in that of 5% H_2SO_4 pre-treatment, apart from ethanol which was slightly higher in the former condition (0.39 g/L) than in 3% H_2SO_4 condition (0.34 g/L).

In terms of sugar consumption, glucose was completely consumed at 48 hours in 1% H_2SO_4 plus enzymes treatment, while trace amounts were still remaining at the end of the assays for other two fermented hydrolysates (0.6 g/L and 1.45 g/L in 3% H_2SO_4 and 5% H_2SO_4, respectively). Besides glucose, *Ulva* hydrolysates also have a variety of minor quantities of other fermentable sugars, and from an economic standpoint, it would be highly beneficial if all sugar substrates would be utilized. Thus, consumption of xylose and rhamnose was also investigated. In the presence of 1% H_2SO_4 slurry xylose and rhamnose were barely utilized; in 3% H_2SO_4 there was some consumption of rhamnose but little of xylose; and in 5% H2SO4 both sugars were consumed. In all cases, xylose and rhamnose were uptaken by *L. brevis*, and *L. rhamnosus* (Maryanti et al., 2021) even at the beginning in the assays when glucose is still present in high concentrations, but there was never depletion of these monosaccharides. In LAB, the utilization of different carbohydrates is usually governed by the carbon catabolite repression (CCR) system including in *L. plantarum*, *L. casei*, *L. delbrueckii* and *L. pentosus* (Andreevskaya et al., 2016; Kim et al., 2009). Nonetheless, simultaneous carbohydrate utilization has been demonstrated in few species including *L. brevis* which exhibits simultaneous utilization of xylose and glucose through the heterofermentative pathway.

The optical density measurements are consistent with metabolite production (Figures 7 and 8), as the growth drops in the harsher acidic conditions. The high values of initial OD_{600nm} readings are due to the use of distilled water as reference-value for the spectrophotometric analysis. Even though the contribution of microbial cells was almost zero at the inoculation, suspended biomass in the hydrolysates together with the medium components ranked up the optical density. Moreover, throughout time deposits formed in the filtrated hydrolysates. As such this method does not provide a robust analysis for microbial growth in *Ulva* hydrolysates.

The experimental data indeed discloses the interference of furan compounds in microbial growth (Figure 8), since in substrates richer in inhibitory compounds, *Lactobacillus* consortium had weaker fermentation performances. Furfural and HMF have been shown to affect *Lactobacillus* species differently depending on their metabolism type (Giacon et al., 2022; W. Guo et al., 2010). Heterofermentative species have tendentially higher tolerance towards organic compounds than homofermentative *Lactobacillus*, whose growth rates are strongly hampered. In a study on this subject, Giacon et al. showed that the growth of *L. fermentum* E3 in the presence of only furfural and HMF was even faster and the lag phase shorter than in their absence. Yet, there are a number of toxic substances in seaweed hydrolysates, and their interaction exhibits a synergistic effect in microorganism-growth inhibition.

In both conditions in which furfural and HMF were quantifiable, their concentration decreased throughout the 50 hours of the assay. Despite the spectra of HPLC samples did not reveal the formation of any compound with the simultaneous decrease of the former compounds, the possibility of bioconversion to less toxic compounds was considered as these might not be detectable due to the low initial concentrations of HMF and furfural. Additionally, it has been demonstrated that some *Lactobacillus* strains can detoxify lignocellulosic liquors (Alves de Oliveira et al., 2018).

It is significant to note that while hydrolysates with 1% H_2SO_4 + enzymes and 5% H_2SO_4 start with an approximate concentration of around 12 g/L of glucose, the 3% H_2SO_4 rendered a much lower glucose concentration (7 g/L). As such, fermentative performance may be lower than in the weaker acid treatment, not only due to the inhibitor's negative contribution for microbial function but also because of the lower starter amount of glucose.

5.4.2 *Saccharomyces cerevisiae*

Yeast growth capacity was also compared in hydrolysates with different composition of sugars and toxic compounds, operated in batch mode for 50h (approximately 2 days), in a 100 mL working volume (Figures 9 and 10). Samples were withdrawn at different time intervals and analyzed for cell growth and metabolite formation.

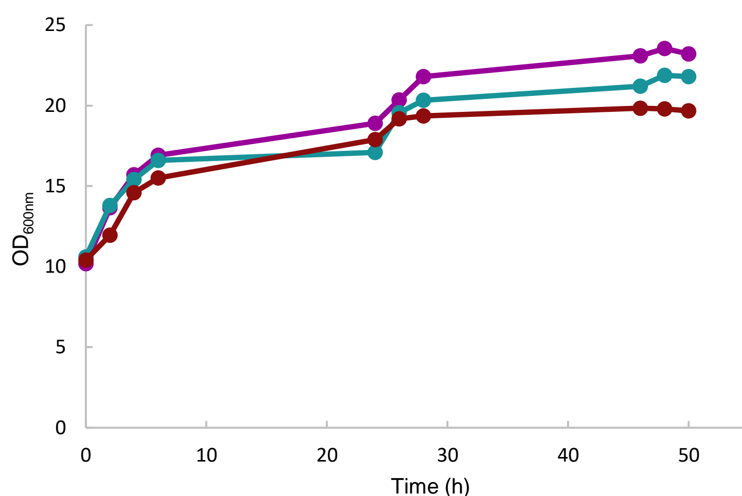

Figure 9. OD_{600nm} readings for *Sacharomyces cerevisiae* shake flask growth in *Ulva* hydrolysates at 30ºC, 200 rpm with initial OD_{600nm} of 0.5. Legend: 1% (w/v) H_2SO_4 (●); 3% (w/v) H_2SO_4 (●); 5% (w/v) H_2SO_4 (●)

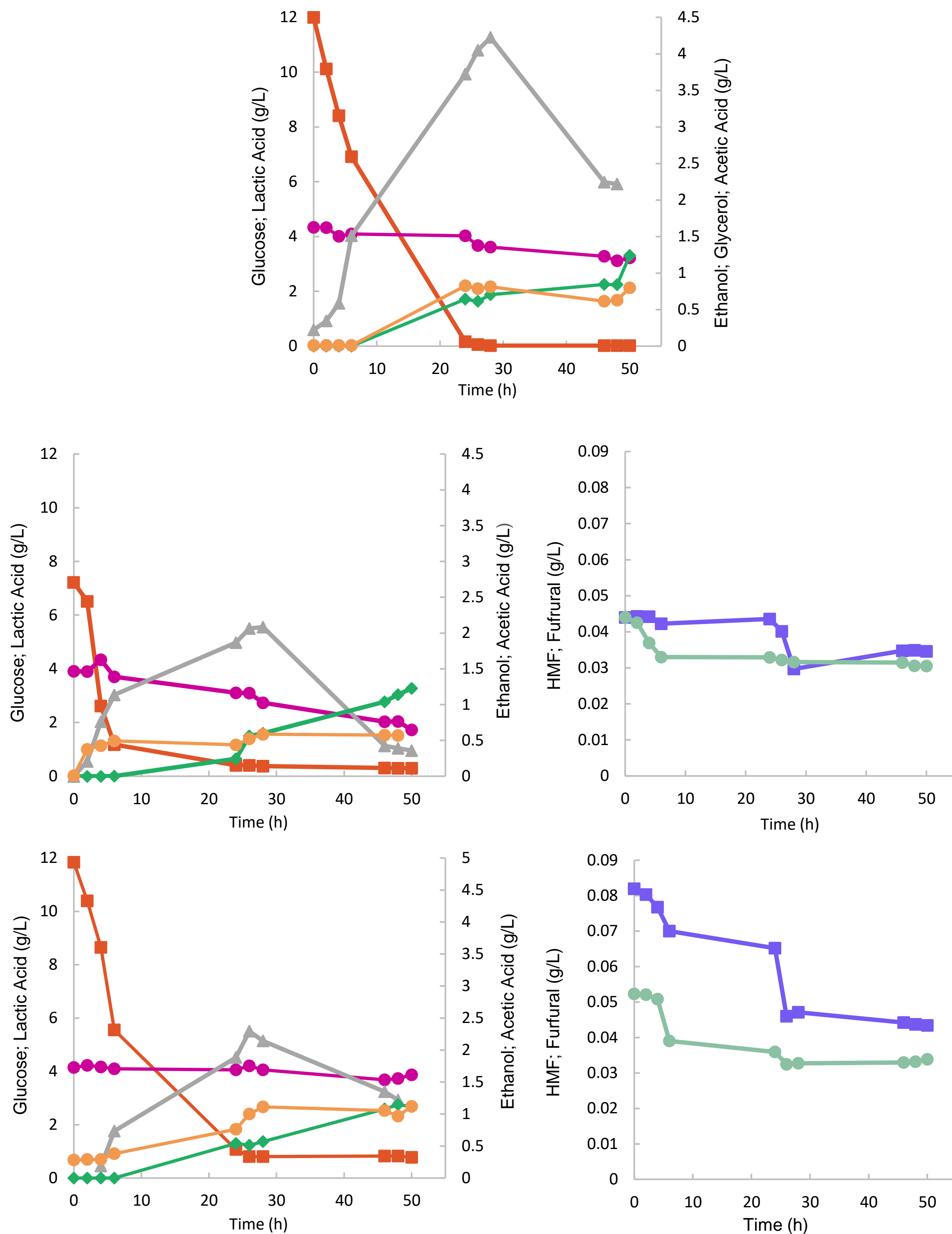

Figure 10. Concentration of glucose, lactic acid, ethanol, acetic acid, glycerol **(left),** HMF and furfural **(right)**, obtained by HPLC, for *Sacharomyces cerevisiae* growth in *Ulva* hydrolysates at 30ºC, 200 rpm with OD_{600nm} of 0.5 in *Ulva* hydrolysates. Legend: Glucose (■); Lactic Acid (●); Acetic Acid (♦); Ethanol (▲); Glycerol (●); HMF (■); Furfural (●). data for *Ulva* pretreatments in 1% H_2SO_4 + celluclast and β-glucosidase (**top graphs**); 3% H_2SO_4 (**middle graphs**); 5% H_2SO_4 (**bottom graphs**).

In the three studied conditions, ethanol concentration peaked at 28h and then began to decline until the end of the assay. Under high glucose conditions, *S. cerevisiae* primarily ferments pyruvate to ethanol, but once this source is depleted, it switches to aerobic respiration and uses ethanol as a carbon

source instead, a phenomenon known as diauxic shift (Galdieri et al., 2010). However, it is improbable that diauxic shift is responsible for the decrease in ethanol levels, as it would take some time for the yeast culture to transition to a respiration mechanism. In such scenario, it would be expected that the ethanol concentration would remain constant for a period until the required conditions are gathered to successfully shift to this metabolic route. Instead, a plausible explanation for the swift ethanol decline, observed after the absence of free glucose in the medium could be attributed to evaporation.

Maximum OD_{600nm} and ethanol production were registered in the absence of inhibitors and decreased significantly in both 3% and 5% H_2SO_4 treated hydrolysates. The discrepancy in fermentability between the hydrolysates is most likely due to a combination of total inhibitor concentration and initial glucose in solution. Natural *S. cerevisiae* do not have lactate dehydrogenase enzyme, but the detected amount of lactic acid was expected and attributable to the incorporation of corn steep liquor into the fermentation medium. Lactic acid makes up to 10%-30% dry basis in corn extractives, depending on the batch (Loy & Lundy, 2019). While the synthesis of acetic acid was very similar across treatments, glycerol concentration achieved higher values in the harsher chemical treatment. Glycerol is both a byproduct of ethanol production and a major osmolyte generated during hyperosmotic stress. This solute synthesis and accumulation allows yeast cells to avoid dehydration by balancing intracellular osmolarity with that of the environment (Aslankoohi et al., 2015). During the neutralization of hydrolysates, a higher volume of base was required in the 5% H_2SO_4 solution to attain the desired pH resulting in a higher osmotic pressure in the medium and subsequent higher production of glycerol as response.

Again, glucose was totally consumed with undetectable levels of furfural and HMF in the media, but it is still present at the end of the fermentation for the other conditions, suggesting that the inhibition effect is strong and that a longer fermentation may be required to uptake all of the glucose. The main drawback of using natural yeasts for fermentation of these hydrolysates is that they cannot metabolize existing C5 sugars, not taking advantage of its sugar-rich profile. As such, the co-fermentation with ethanol-tolerant microorganism capable of fermenting pentoses like *Lactobacillus*, opens an intriguing possibility for using hydrolysates as a carbon source.

In the context of ethanol production for the biofuels sector, extensive research on *S. cerevisiae* tolerance to inhibitors in hydrolysates has been conducted. Their toxic effects are related with the inhibition of several enzymes, such as pyruvate dehydrogenase, aldehyde dehydrogenase, and alcohol dehydrogenase (Giacon et al., 2022), the decrease of intracellular levels of ATP and NAD(P)H, and the divergence of cell energy towards damage repair at the expense of cell growth and metabolite production (Liu et al., 2021). The extent of these effects depends on the furan concentration as well as utilized yeast strain (Almeida et al., 2007). *S. cerevisiae* fermentation has been shown to be impaired in concentrations ranging from 1-5 g/L of HMF (Taherzadeh et al., 2000) and 0.5-4 g/L of furfural (Banerjee et al., 1981). Nonetheless, this microorganism has been demonstrated to be capable of converting HMF and furfural to less inhibitory compounds if the concentrations are below lethal levels (Ask et al., 2013). This capability is also demonstrated in the experimental data, as the concentration profile of both furans in 3% and 5% H_2SO_4 treatments decreases throughout both assays.

In general terms, all the selected microorganisms were able to grow despite the presence of inhibitors in the harsher fermentation conditions. Again, discrepancies between 1% and 3% H_2SO_4 are also attributable to initial concentration of sugar. Moreover, the significance of pH in lactic acid fermentation of hydrolysates has been established, wherein a decrease in pH below the pKa values of lactic acid (3.86) and acetic acid (4.76), causes the undissociation of these molecules. This phenomenon results in the formation of ions with a strong cytotoxic effect (Casey et al., 2010; Sjulander et al., 2020). The high toxicity of undissociated forms is assumed to be caused due to differences in membrane permeability or in inhibition potential of acids once within the cell (Casey et al., 2010). Both effects might be mitigated by fermenting *Ulva* slurries in a fed-batch regime with glucose pulses and pH control. In view to minimize procedure cost while still retaining efficient microbial function, 3% H_2SO_4 treatment was selected for bench-scale assays.

4.5 Protein Quantification

The use of *Ulva* seaweed as animal feed is a daunting task because, despite having a high nutritional value, the nutritional components in algal biomass exhibit a low bioavailability, making incorporation of these substances by animal metabolism an inefficient process. Bioconversion processes involving fungi, bacteria and yeast enrich the final product with enzymes, vitamins, proteins and decrease the presence of anti-nutritional factors and polysaccharides that impair the natural digestibility of seaweeds (Fernandes et al., 2019; Jamal et al., 2017). Moreover, research studies have shown that after a successful bioconversion process, fungi cells harbor high levels of vitamins and proteins (Dhanasekaran et al., 2011). The objective of this study is to examine microbial protein synthesis using *Ulva* seaweed as carbon source. To assess how fermentation affects the protein quality of seaweed slurries, the Lowry method was used to determine the total protein content of shake flask fermented samples.

4.5.1 *L.rhamnosus*

To optimize the protein extraction procedure, a preliminary experiment was performed with *L. rhamnosus* inoculum grown in MRS medium. The aim of this experiment was to see if sodium hydroxide (NaOH) hydrolysis included in the Lowry protocol was sufficient as an extraction step or if further processing would be required for protein recovery. Microbial pellets were either treated with NaOH hydrolysis or sonified prior to the hydrolysis stage. To the treated pellets and supernatant, Lowry procedure was conducted as detailed by Waterborg et al., 1996.

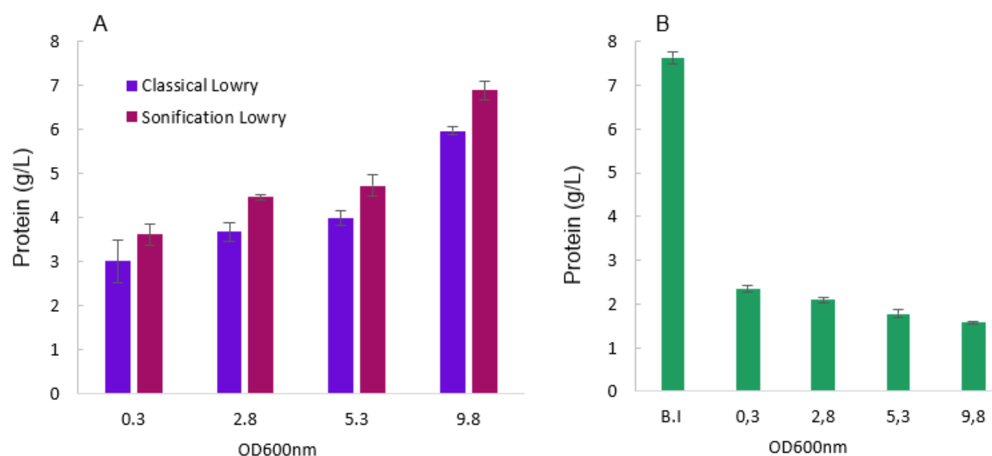

Figure 11. Protein contents (g/L of culture) estimated by the Lowry method in a *L. rhamnosus* inoculum in MRS medium of increasing optical density (OD_{600nm}). In (**A**) microbial pellets were treated with different approaches for protein extraction: Classical and Sonication Lowry; and in (**B**) just the sample supernatant was analyzed. Error bars indicate standard deviation of triplicate dependent samples. Legend: B.I -before medium inoculation

The graphs (in Figure 11) demonstrate that, with either approach, an increase of protein is associated with a rise in optical density, indicating that during microbial growth more protein is generated, from precursors in the culture medium. As protein accounts for approximately 55% of cell dry weight (Philips et al., 2015), an increase in protein was expected as outcome of microbial growth during the fermentation process. Furthermore, during fermentation microorganisms also synthesize enzymes to degrade complex materials and dissociate proteins into simple molecules like peptides and aminoacids, with different antioxidant activity level (Wang et al., 2021). Proteolytic enzymes are essential in LAB for supplying cells with the nitrogen compounds required for growth. Because many of the aminoacids they require are only found in trace amounts in their natural environment, the primary function of these enzymes is to retrieve components that can be absorbed by bacterial cells (Kieliszek et al., 2021).

Although all sampling points exhibit the same trend of protein increase, sonified samples have higher protein contents in comparison to non-sonified analytes, suggesting that NaOH hydrolysis alone is insufficient to efficiently rupture cells and to release/ expose their whole protein content. *Lactobacillus,* as Gram positive bacteria, have thick cell walls granting resistant to cell disruption techniques. Moreover, resistance varies even within Gram-positive bacteria, with previous research indicating that fungi and certain Gram-positive bacteria are more resistant to cell lysis than other Gram-positive bacteria or Gram-negative bacteria (Kelemen et al., 1979). A work on the same topic highlighted that even by applying ultrasonication bath for 10 min, the fungal and Gram-positive cultures tested remained almost intact, revealing a strong resistance (Starke et al., 2019). Thus, despite the increase in protein, there may be a lot more that is still encapsulated in the cell, and additional research would be needed to maximize protein extraction, which is critical step for representative and rigorous protein quantification. Nonetheless, from this point forward, samples were sonified prior to NaOH hydrolysis for Lowry method.

Analysis of the supernatant fraction were also conducted. The concentration of proteins within this phase drops significantly at the start of the fermentation but then gradually stabilizes until the very end. This strongly suggests that the microorganisms incorporated the proteins present in the medium.

4.5.2 *L. rhamnosus* growth in *Ulva* Slurry

L. rhamnosus was grown in 3% H_2SO_4 treated *Ulva* supernatant supplemented with other medium components (4.4.2) to access the viability of employing Lowry in a complex algae hydrolysate. During the 22-hour fermentation period, samples were taken, and the protein content of the microbial pellet and supernatant was examined.

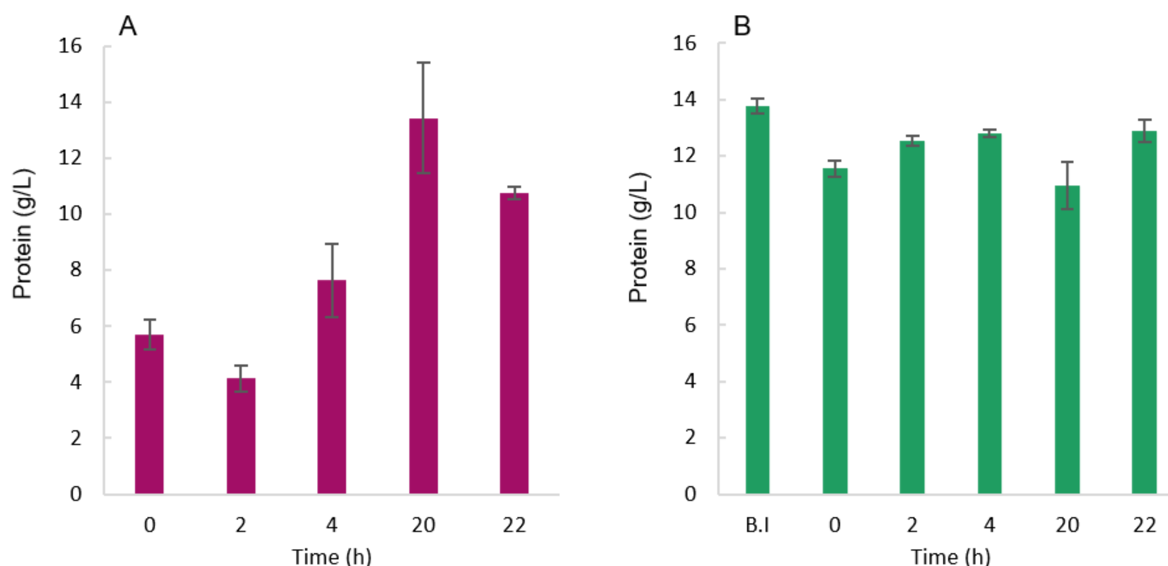

Figure 12. Protein content (g/L of culture) of microbial pellets **(A)** and liquid fraction **(B)** estimated by Lowry Sonication method in *L. rhamnosus* cultures in 3% H_2SO_4 *Ulva* treated liquor during 22h of fermentation. Error bars indicate standard deviation of triplicate dependent samples. Legend: B.I -before inoculation.

In these circumstances, there is no consistent growth of protein in the microbial pellet throughout the fermentation which would be expected as result of microbial function (Figure 12). Also in hydrolysate samples, little variation occurs during the 22h of the experiment. It is important to note that in this experimental set up both seaweed hydrolysate and microbial biomass are contained part of the pellet. As result, the process of extraction must be able to disintegrate cell walls of both the bacteria and the algae so that the contribution of both parts to total protein content is considered. In simpler terms, the protein quantification procedure must be adequate for seaweed-microbial samples.

Accurate protein estimation in algal samples is challenged by the recalcitrant nature of the cell wall. On top of that, algae contain several bioactive compounds that are co-extracted with proteins that interfere with protein measurements and may hinder protein solubility and reduction reactions with copper cations (Niemi et al., 2023). These interferences can cause an increase or reduction of the absorbance value of the sample. Considering that more complex samples were used in this experiment, it is reasonable to assume interferences in the retrieved data. Still, it is possible to get a rough indication of the protein increase in pellets during the fermentation course thus this method was utilized to analyze bioreactor samples. Even so, the retrieved protein data should be regarded as a qualitative information rather than quantitative measurement, given the presence of substances in seaweed that may interfere with the colorimetric reaction that constitutes the basis for Lowry's protein quantification.

4.6 Fermentation Scale Up

The conducted preliminary assays in shake flasks demonstrated the algal hydrolysate fermentability. Bench-bioreactors allow for a stricter control of key process parameters in the microbial culture and, in addition to retrieve real-time bioprocess information, enabling the optimization of *Ulva* fermentation. Scale-up to a 3L bioreactor was tested in fed-batch using three different inocula: 4LAB, 4LAB mixed with *S. cerevisiae,* and *S. cerevisiae* alone aiming to select the startup culture that renders a more interesting nutritional profile in the scope of aquaculture industry. This translates into an *Ulva* product with high contents biomass and consequently more protein and more biomass functional compounds but also more valuable microbial metabolite in the supernatant. These metabolites include food preservatives and possibly flavor compounds, which heighten the quality and stability of food products. All fermentations were carried until stabilization of metabolites production. To follow these processes specific sugars usage, metabolite production, cell count, agitation, dissolved oxygen, and pH were monitored.

Lignocellulosic hydrolysates are generally scarce in nutrients and in particular nitrogen supplementation has been reported to improve fermentation performance (Johansson et al., 2014). In the case of *Ulva* hydrolysates, a mineral solution and CSL were supplemented to the algae base medium, however those components might not be enough to meet microbial requirements during the whole fermentation process. To increase biomass and protein content in bioreactor fermentations, more nitrogen was added during the fermentation in the form of ammonium hydroxide. Pulses of this alkali were frequently added for pH control instead of sodium hydroxide as previously done in shake flasks fermentations. Ammonium hydroxide serves both as a protein source and pH regulator, possibly outperforming the nutrient scarcity concerns.

It should be noted that *Ulva rigida* batch U1.01021MB2202<1.5 was used as starting material in all studies except for yeast fermentation due to stock depletion. For this final experiment, a new batch with the code U1.01021MB2301<1.5 was used because it released similar glucose concentrations as the previously used batch upon hydrolysis, however, the levels of rhamnose and xylose were much higher, and this factor must be considered.

4.6.1 *Lactobacillus* Fermentation

The results presented in Figure 13 show that almost no glucose was consumed, and metabolite production was minimum during the first 16 hours of fermentation, accompanied by slow cell growth. This lag phase was not observed in shake flasks experiments and could be attributed to the higher levels of toxic compounds in the hydrolysate. Moreover, the initial decay of HMF (23h) is coincident with the rank up of microbial metabolites. To shorten the lag phase, an acclimatization process of 4LAB from MRS to the seaweed hydrolysate medium could be carried with increasing ratios of the hydrolysate fermentation medium for progressive consortium adaptation. Nonetheless, the LAB consortium was able to lower the contents of inhibitory compounds and to inclusively reduce furfural to negligible levels. Both lactic acid and acetic acid levels significantly increased starting at 23 hours, with lactic acid increasing gradually until reaching its peak at 113 hours. Moreover, the production of acetic acid nearly doubled in

just 17 hours, near the end of the process. The sharp increase of this constituent could be potentially linked to a cellular stress response, resulting in a metabolic shift that favors acetic acid production in detriment of lactic acid. In a study by Zhu et al., (2007) , acetic acid continued to increase while lactic acid leveled off at 72. The proposed hypothesis is that under high concentrations of acetic acid and lactate, a potential shift to xylose consumption might occur in such a way that acetic acid becomes the primary product of the fermentation. Nonetheless, since no replicates were performed, additional investigation is warranted to comprehensively elucidate the metabolic alterations occurring in *Lactobacillus* consortium during *Ulva* fermentation.

The abrupt consumption of xylose and rhamnose during the first hours of fermentation could be explained by low glucose availability (3.86 g/L) and high competition for carbohydrates, resulting in a shift to other sugars and even the depletion of rhamnose at 23 hours of the process. During the fermentation, 17 pulses of glucose were pumped and at 88 hours glucose concentrations reached higher concentrations probably due a decrease of the metabolic activity.

Growth was initially slow in the first 20 hours which may be due to either or a combination of high inhibitors and low glucose levels. From this point on, a rapid increase in colonies (CFU) was observed until 47 hours, after which a steady increase was observed until 95 hours, when they peaked, before declining until the end of the fermentation. In the beginning, lactic acid production is associated with microorganism growth, but the biomass then declines while the production of this metabolite continues to increase. The decoupling between cell growth and lactic acid production may be attributed to the redirection of the energy generated through fermentation processes, shifting from cellular growth to the maintenance of pH equilibrium, consequently resulting in the arrest of cell growth (de Oliveira et al., 2021). Furthermore, the ceasing of lactic acid fermentation in fed-batch regime has been associated to the inhibition of the cell growth induced by the high osmotic pressure caused by the accumulation of lactate in the medium(Cui et al., 2016).

The use of an alternative medium instead of MRS, did not appear to have a significant impact on bacterium growth as large metabolite production, high glucose consumption and substantial growth were achieved, even in the presence of microbial function inhibiting compounds. Given the shortage of nitrogen compounds in hydrolysates and the strict aminoacid requirements of *Lactobacillus* the addition of ammonium hydroxide base may have been extremely important to supplying crucial nutrients for microbial growth. This because, these microorganisms are not capable to synthesize a plethora of aminoacids that are required for their growth, thus they need to be present in the culture medium. In the case of MRS, the medium standardly utilized to grow *Lactobacillus* specimens, peptone is the source of nitrogen. The addition of CSL to the *Ulva*-based medium serves as a nitrogen source which potentially meets the amino acid requirements of LAB during the initial stages of fermentation. However, it may be insufficient for conducting a fed-batch fermentation using an alternative medium. In the fermentation of food waste, W. Zhang et al., 2020 research demonstrated that the addition of ammonium hydroxide is a cost-effective strategy to maintain stable L-LA production while providing favorable conditions for LAB development. In the concrete, ammonium promoted a stable reducing environment, supplied the vital nitrogen for LAB growth and triggered metabolic shifts that raised NADH intracellular levels for pyruvate reduction to L-lactate. Furthermore, due to the complex nutritional requirements of lactic acid bacteria

(LAB), there is a recognized correlation between the availability of nitrogen sources and the production of lactic acid.

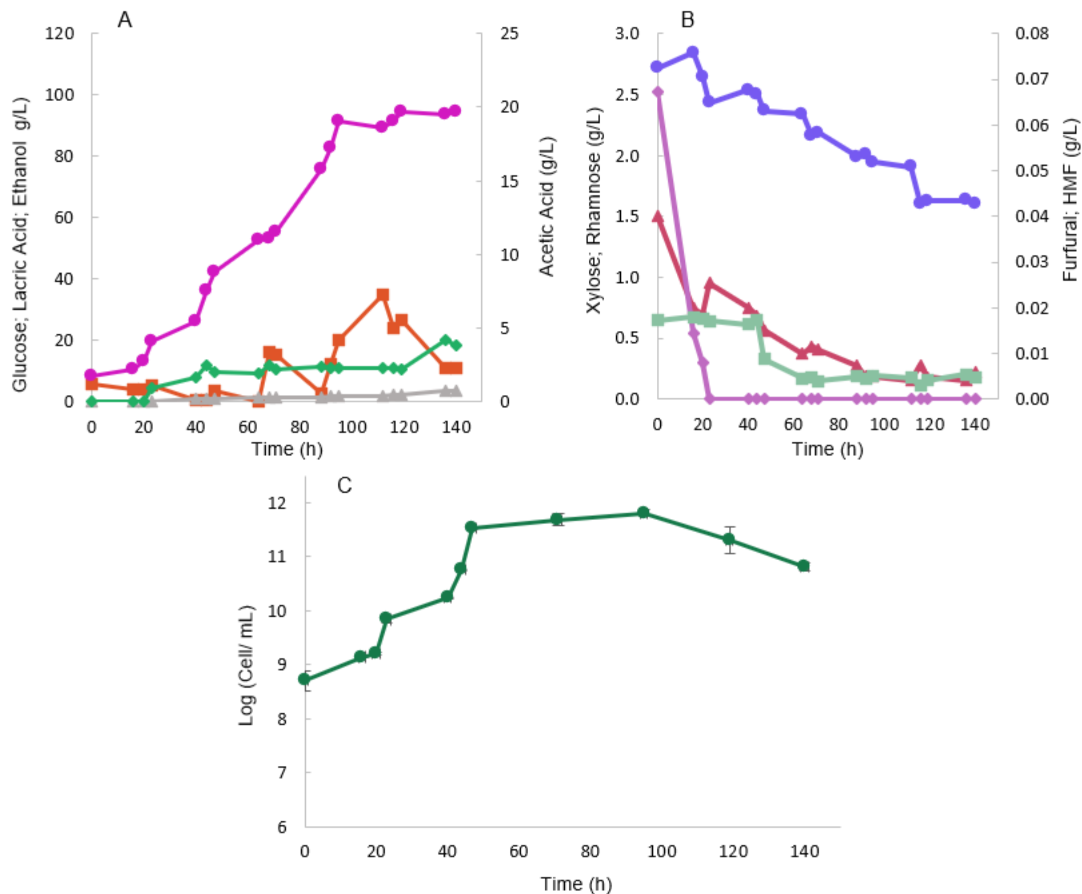

Figure 13. Bench-scale bioreactor culture results for metabolite concentration and log(CFU/mL) in fed-batch fermentation of 3% H_2SO_4 *Ulva* hydrolysate by 4LAB with initial DO_{600nm} of 0.2 of each bacterium at 37ºC, 50 rpm. Throughout the assay, 18 pulses of a 500 g/L glucose feeding were given (427 ml). Concentration of glucose, lactic acid, ethanol, acetic acid **(A),** xylose, rhamnose, HMF and furfural **(B)**, obtained by HPLC. Decimal logarithm of viable LAB cells' concentration (CFU/mL) (**C**). Legend: Glucose (■); Lactic Acid (●); Ethanol (▲); Acetic Acid (♦); Xylose (▲); Rhamnose (♦); HMF (●); Furfural (■); Log(cell/mL) (●).

Fermentation started with culture medium fully saturated with oxygen; an DO set point of 7.5% was reached 7.3h after fermentation started, never settling to the established 5% value for this parameter (Figure 14). Moreover, DO% was rather unstable throughout the process, even briefly reaching extremely high values of up to 97% (48h, 64.7h, 75.7h and 95.7h) suggesting low metabolic activity. However, this contradicts viable cells counts and data on metabolite production. A possibility is that due to low agitation regime algae fragments would attach to the oxygen sensor and interfere with measurements. However, the occurrence of elevated oxygen saturation in this context is not unexpected, considering that LAB are microaerophilic organisms capable of thriving in environments characterized by low oxygen levels.

The declines of pH value are coincident with higher production of acids, naturally eliciting a drop in the culture's pH and a possible slow response from the pH control system. There are several points starting from 82h in which pH rises beyond the desired value which may be explained by the abrupt increase of 26 g/L in lactic acid that is in the end accompanied by a 20 g/L increase in acetic acid. Large amounts of alkali may have been added to the culture medium to compensate for the acidification

caused by the production of these organic acids. Nevertheless, a likely alteration of the broth rheology must have led to increase of the mixing time in the bioreactor and to poor mixing conditions, causing more alkali to be added than was necessary to reestablish the pH set value.

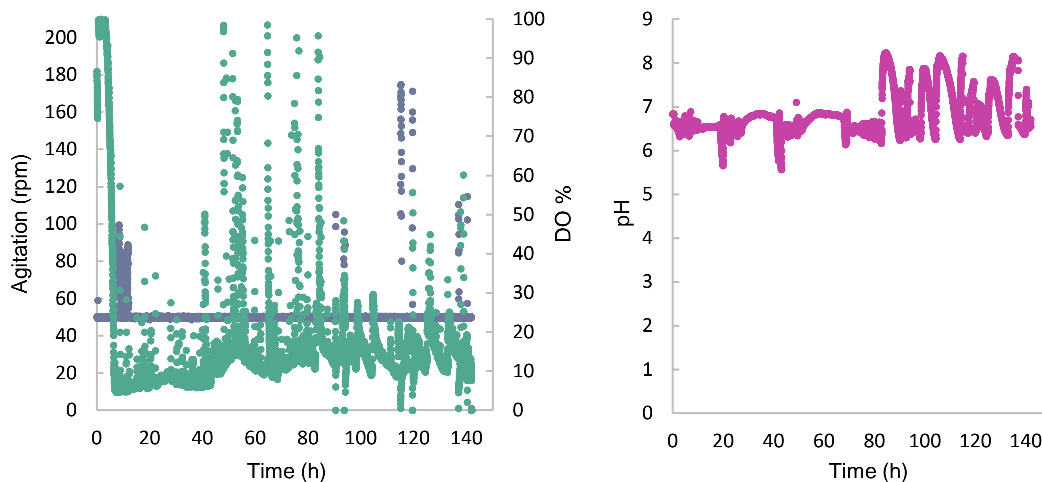

Figure 14. Real-time monitoring of DO% and agitation **(left)** and pH **(right)** data retrieved with BioCommand/SCADA software for fed-batch fermentation of 3% H_2SO_4 conditioned *Ulva* by 4LAB. Legend: Agitation (●); DO% (●); pH (●).

4.6.2 Yeast and *Lactobacillus* Co-fermentation

In this fermentation, different inoculation times for LAB and yeast were considered to maximize both species' metabolic activity for the co-fermentation of *Ulva* slurry. Because yeasts in general are sensitive to organic acid synthesized by *lactobacilli,* while LAB can proliferate in the presence of high ethanol concentration, it was decided to first inoculate yeast and latter proceed to the addition of *Lactobacillus* mix. Specifically, *L. brevis*, *L. casei*, *L. plantarum* and *L. rhamnosus* (all present in the LAB consortia) can still proliferate up to concentrations of up to 8, 15, 12 and 12% (v/v) respectively (Pittet et al., 2011).

The outputs resulting from fed-batch culture of *Ulva* derived substrates by co-culture of lactobacilli and yeast are shown in Figure 15. During the first 15 hours of the experiment there was a minor glucose consumption, and the production of yeast-specific metabolites was also minimal (Figure 16 A and B). The initial proliferation of yeast (Figure 15C) was difficult in the first 16h because, besides the presence of inhibitors, the temperature and agitation in the bioreactor was set at 37ºC and 50 rpms respectively, and the optimum growth conditions for yeast growth are 30ºC and 200 rpm. When this was corrected at 15 hours yeast cells were able to recover and resume ethanol fermentation and metabolite detoxification (shown by the reduction of HMF levels).

The *Lactobacillus* inoculum was added at 45 hours since the synthesis of ethanol and glycerol showed a fast rise tendency at 44 hours, accompanied by a further decrease in HMF levels. After the addition of LAB mix, lactic acid concentration increased significantly and even reached slightly higher levels (101 g/L) than in the single genus culture with 4LAB (94.5 g/L). The advantages of utilizing mixed cultures over pure culture in the fermentation process have been validated by several authors. Through an investigation into cocoa fermentation, it was determined that alcoholic conditions heightened the

yields of lactic and acetic acids from LAB, raising the possibility of an interaction between yeasts and LAB that may be crucial for acid synthesis (Ouattara et al., 2019). Furthermore, a study conducted by Tang et al., (2011) demonstrated that the co-cultivation of lactic acid bacteria and yeast in the simultaneous saccharification and fermentation process of furfural residues (from industrial furfural production) can lead to enhanced yields of lactic acid and ethyl lactate.

On the other hand, glycerol, acetic acid, and ethanol levels all stabilized at 70 hours which could indicate a decrease in yeast metabolic activity and growth arrest at this fermentation stage. To counteract medium high osmolarity conditions, *Saccharomyces cerevisiae* synthesizes glycerol and also acetic acid, as its production may balance the excess NAD+ generated from glycerol synthesis (Yang et al., 2017). Although lactic acid and acetic acid effects are lessened at higher pH levels, high concentrations of these acids are still capable of stressing yeast cells (Graves et al., 2006).

In comparison with the previous assay (Figure 13), more glucose pulses were given during the combined yeast and 4LAB co-culture process (40 pulses; Figure 15). Glucose pulses were administered every two hours starting at 44 hours and this abrupt consumption may be attributed to exponential yeast growth at this point as well as the contribution of *Lactobacillus* addition to the culture. Xylose and rhamnose are only metabolized upon *Lactobacillus* addition, emphasizing the importance of combining *Lactobacillus* with different metabolic routes to get the most out of the sugars released during the hydrolysis process.

It is important to note that MRS is a selective medium for lactic acid bacterium and using this medium for counting yeast colonies was not ideal; this may be the underlying reason for the lower cell counts 11.2 CFU/mL while in LAB monoculture 11.8 CFU/mL were achieved. From figure 9 it is possible to observe an initial increase in colonies, but by 20h, a dramatic decline is seen probably due to the inadaptation to initial temperature and agitation along with high inhibitor concentration. However, following this shortfall the total number of colonies increases throughout the process, reaching its maximum at 95 hours. Yet, yeast metabolite production seemed to imply yeast growth arrest starting from 70h. Since total cells are being counted, this number reflects a balance between the growth of both species; hence even if yeast cells are no longer viable this might be outweighed by *Lactobacillus* active growth. To better understand and monitor the growth dynamics during the co-fermentation process, plates for specific *S. cerevisiae* growth should be carried in parallel to MRS plates for LAB growth for a precise identification and quantification of total colonies.

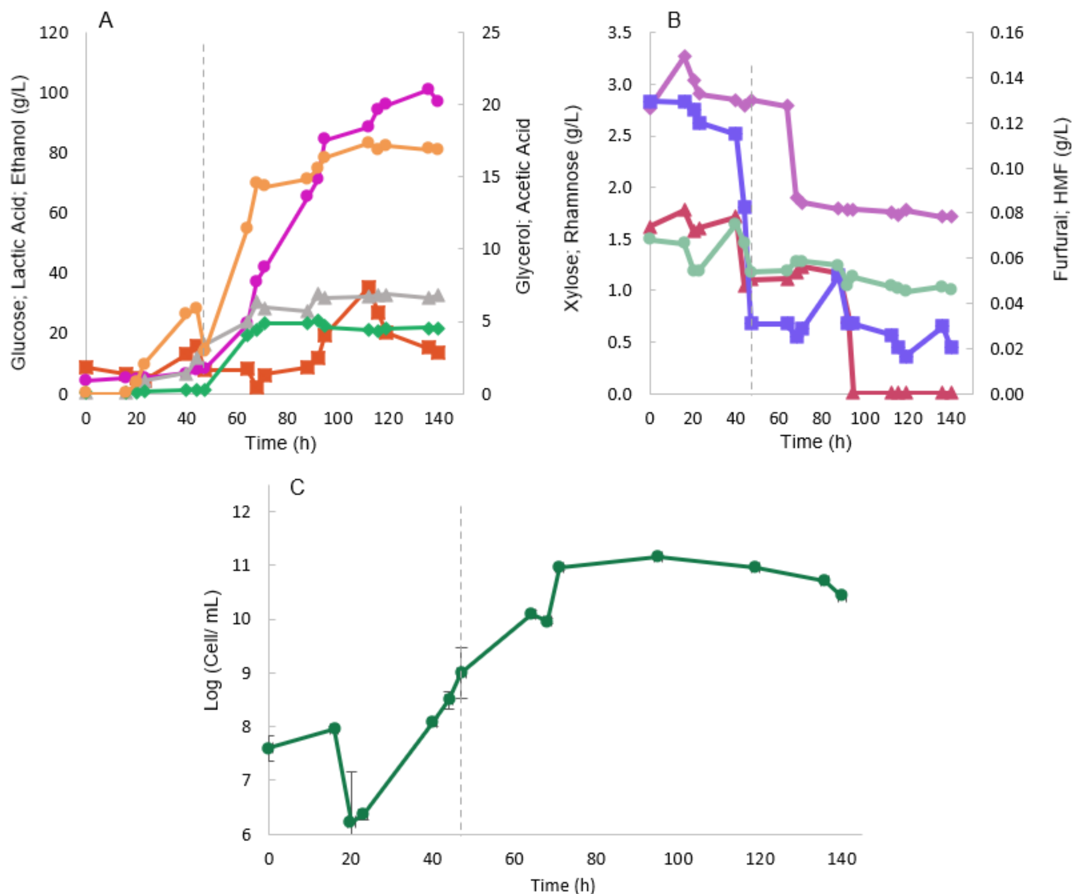

Figure 15. Bench-scale bioreactor culture for the concentration of glucose, lactic acid, ethanol, acetic acid, glycerol **(A),** rhamnose, xylose, HMF and furfural **(B)**, obtained in fed-batch fermentation of 3% H_2SO_4 treated *Ulva* hydrolysate by 4LAB and Yeast with 500 glucose (g/L) feeding (800 mL) and starting at OD_{600nm} of 0.2 for each bacterium and OD_{600nm} of 0.7 for yeast. Conditions were first set at 37ºC, 50 rpm but changed at 14h to 30ºC and 200 rpm. Before adding the LAB consortium (45h), the initial values for agitation and temperature were reset. Decimal logarithm of total viable cell's concentrations (CFU/mL) (**C**). Legend: Glucose (■); Lactic Acid (●); Ethanol (▲); Acetic Acid (♦); Glycerol (●); Xylose (▲); Rhamnose (♦); HMF (●); Furfural (■); Log(cell/mL) (●). Vertical dashed line indicates the 4LAB inoculation.

The initial increase of DO% was not expected and may be attributed to the yeast culture maladaptation to the experimental conditions. When conditions were modified to promote optimal yeast growth (14h), the curve declined, indicating that cells became able to thrive even despite the initial challenges. During the 40-to-50-hour interval, oxygen levels were near the setup value (5%), but quickly increased (Figure 15A). This phenomenon may be explained due to the deceleration of microbial growth at the 50-hour mark, until 90h where viable cells reached a plateau, indicating the onset of stationary phase. Towards the end of the experiment, a slight cell decline phase is observed, albeit not pronounced. Consequently, beginning at 50h and more even at 70h, the volumetric rate of oxygen consumption experienced a decrease, leading an excessive supply of oxygen through sparging and agitation conditions, surpassing the amount required by cells.

Regarding pH control (Figure 16B), little changes were visible in the first 30 hours due to low production of organic acids that cause a drop in culture's pH value. For approximately 7 hours, the yeast culture was exposed to pH 8 due to excessive alkali addition. Most yeasts species grow best withing a pH range of 4 to 6 and high pH levels can induce chemical stress, including glycerol production to balance the redox state of the cell (Yalcin et al., 2008). This prolonged exposure to a high pH level could

have negatively affected the yeast fermentation process, potentially leading to a decrease in fermentation efficiency.

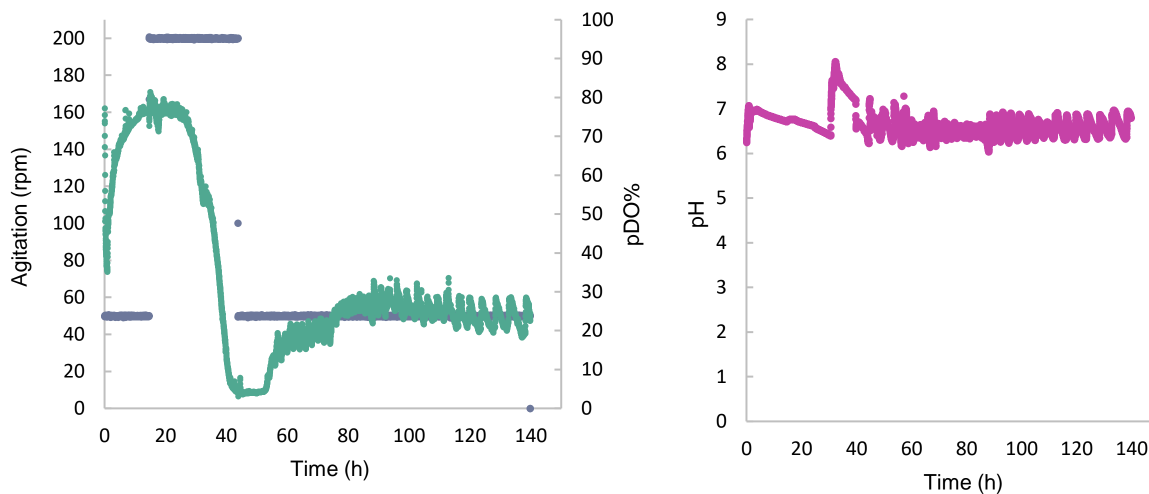

Figure 16. Real-time monitoring of DO% and agitation **(left)** and pH **(right)** data retrieved with BioCommand/SCADA software for fed-batch fermentation of 3% H_2SO_4 conditioned *Ulva* by 4LAB and yeast. Legend: Agitation (•); DO% (•); pH (•).

4.6.3 Yeast Fermentation

Sugar utilization rates, metabolite production and detoxification during the fermentation of the *Ulva* hydrolysate by a sole inoculum of *S. cerevisiae* are presented in Figure 17. It is observed that during the first 20h there is slow consumption of glucose which is probably attributable to the high levels of furfural and HMF. Given the higher content of xylose in this batch, it was expected to have a higher amount of furfural produced as a byproduct of sugar degradation. Considering the implementation of an acid hydrolysis pretreatment, using *Ulva* batches releasing high xylose levels is not the optimal strategy for cultivating wild *S. cerevisiae*, as yeasts cannot utilize this pentose as a carbon source. Nonetheless, yeast cells were able to reduce HMF and furfural concentrations from 0.1 and 0.14 g/L to 0.04 g/L and 0.06 g/L, respectively, demonstrating their capability to carry out the detoxification process.

Following the lag phase, there was an increase in metabolite production for all yeast metabolites investigated, which was accompanied by a corresponding increase in yeast biomass (colony counts; Figure 17C). The amount of ethanol produced during yeast and co-fermentation differed significantly, being 105 g/L (Figure 15A) and 33.1 g/L (Figure 17A), respectively. Although the goal is not to maximize extracellular metabolites production, this is also a metric of fermentation performance and of the process dynamics. Unfortunately, due to the plate culturing conditions, it is not possible to differentiate cell origin during the mixed fermentation (with two genera of microbes); thus, it is not possible to compare the growth of *S. cerevisiae* when in monoculture and mixed fermentation in terms of biomass. However, it is possible that the co-culture conditions are suboptimal for the development of both genera, resulting in a preference for lactobacilli growth. This is suggested by the minimal differences observed between growth of the sole LAB consortium and its behavior in co-culture with yeast, while yeast proliferation appears to be impacted.

As previously mentioned, substrate consumption in the *Ulva* hydrolysate inoculated just with yeast was initially slow but sharply increased at 40h, and a total of 50 pulses of glucose were

administered throughout the fermentation process, exceeding the pumped glucose volume in mixed culture conditions. This may be due to the higher sensibility of yeasts towards lignocellulosic degradation products than lactic acid bacteria (Gubelt et al., 2020). One possible explanation that given the lower tolerance of *S. cerevisiae* to these compounds, fermentation efficiency is further decreased, resulting in lower ATP production. As a result, yeast cells may require more glucose to obtain the energy necessary for growth and multiplication. Additionally, it should be noted that the hydrolysate used in this assay contained a higher concentration of inhibitors than the one used only with 4LAB (0.02 HMF g/L and 0.07 g/L furfural, Figure 13B) and 4LAB + yeast (0.07 g/L HMF and 0.13 g/L furfural, Figure 15B) assays, and thus more energy may have been required towards detoxification mechanisms.

The biomass production stabilized around 92 hours, indicating that there are no benefits of extending the fermentation beyond this time for the purpose of microbial growth. The microbial biomass concentration (in total number of colonies/ mL) is lower than in the first bioreactor, in spite of similar end-product concentrations (95 g/L lactic acid Figure 13A; 105 g/L ethanol, Figure 17A). This outcome may be explained by a combination of factors: first, the plate medium was not ideal for yeast growth; second, in the 4LAB-inoculated bioreactor four different species were used, whereas only one was inoculated for the alcoholic fermentation. Also, yeast cells are 3 μm - 5 μm in size, whereas lactobacill cells are 1 μm to 1.5 μm in size; so, despite having lower cells, yeast biomass concentration (g/L) may be equal to or even exceed that of whole lactobacilli (Aon et al., 2018; Schär-Zammaretti et al., 2003).

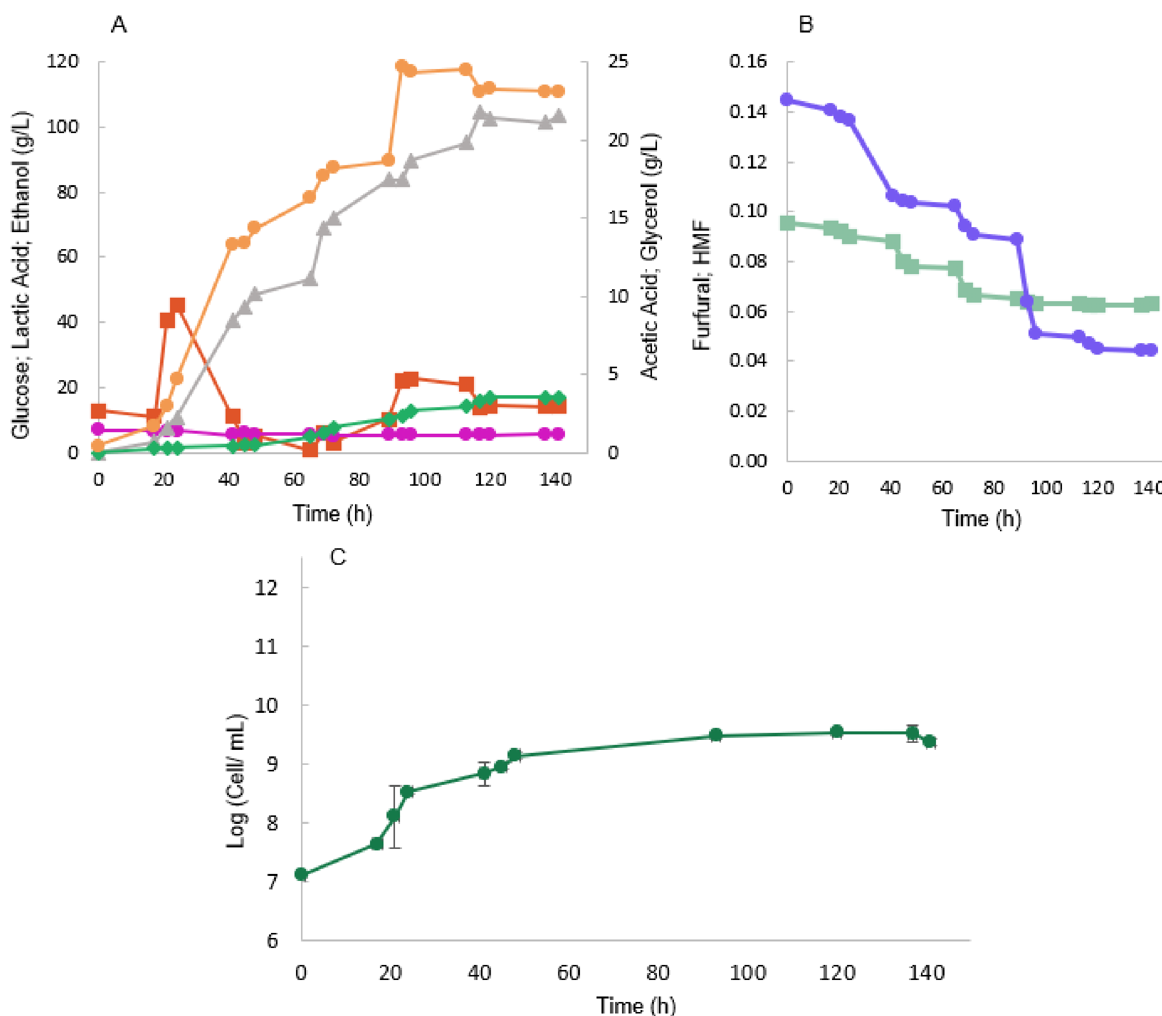

Figure 17. Bench-scale bioreactor culture for the concentration of glucose, lactic acid, ethanol, acetic acid, glycerol **(A),** HMF and furfural **(B)**, obtained in fed-batch fermentation of 3% H_2SO_4 treated *Ulva* hydrolysate by yeast at 30ºC, 200 rpm with 500 g/L glucose feeding (950 mL) and a starting OD_{600nm} of 0.7. Decimal logarithm of total viable cells's concentrations (**C**). Legend: Glucose (■); Lactic Acid (●); Ethanol (▲); Acetic Acid (♦); Glycerol (●); HMF (●); Furfural (■); Log(cell/mL) (●).

The dissolved oxygen percentages showed an unexpected trend (Figure 18A), since after DO 100% calibration, oxygen readings should decrease in virtue of microbial metabolism. This pattern could have resulted from technical issues with the oxygen electrode. Only after 30h, the dissolved oxygen reached the desired set-point which could be due to the presence of inhibitors, but this delay should be regarded carefully as oxygen data clearly deviated from the expected typical initial consumption pattern. Furthermore, according to registered dissolved oxygen levels yeast cell's function started to be compromised from around 90h; evolution of the metabolite synthesis indicates that growth arrest started at about 120 hours of fermentation since no more glucose was consumed from this point on (Figure 18A).

In respect to agitation, an increase is registered in the intervals of 40h to 42h and 52h to 61h which may have occurred due to temporary malfunctions of the oxygen sensor. Variations of the pH value occur throughout the fermentation process, which is attributed to the ongoing synthesis of acetic acid that only ceases in the last 4 hours of the experiment.

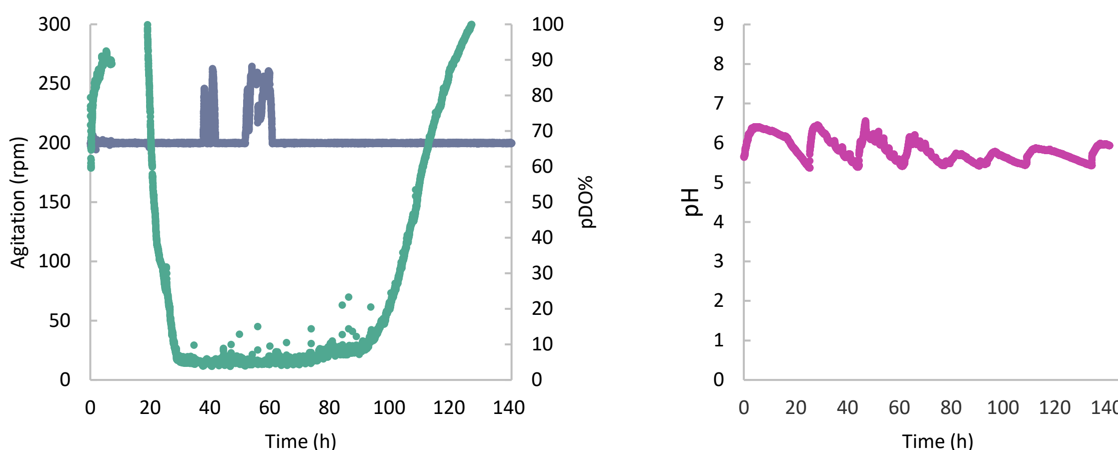

Figure 18. Real-time monitoring of DO% and agitation **(left)** and pH **(right)** data retrieved with BioCommand/SCADA software for fed-batch fermentation of 3% H_2SO_4 conditioned *Ulva* by yeast. Legend: Agitation (●); DO% (●); pH (●).

4.6.4 Review of Bench-Top Bioreactors Results

In all, the conversion of inhibitory chemicals was never complete, apart from furfural in LAB monoculture, likely due to its low initial concentration. Although the microorganisms used possess the enzymatic mechanisms to convert these compounds into less hazardous forms, these products are still toxic to the cells. Furthermore, it should be noted that the efficiency of HMF and furfural degradation routes may be affected by other toxic compounds, such as phenols and organic acids, that were not monitored in this study. The synergistic effects of these compounds could potentially overload the cell detoxification mechanisms, leading to incomplete degradation of HMF and furfural.

Despite the presence of inhibitors, all experiments yielded high cell growth and desired extracellular metabolites. One possible explanation is that nutrient supplementation of the culture medium with additional nutrients (corn steep liquor, glucose and ammonium hydroxide) may have improved the organism's tolerance to these compounds, leading to higher metabolite production or alternative metabolite routes might have been activated. Nevertheless, although external sources of carbon and nitrogen were supplied, the bulk of microelements required for the formation of new cells are derived from the biomass of hydrolyzed *Ulva*. Indeed, additional studies are required to validate these conjectures and acquire a better understanding of the underlying mechanisms. It is expected that fermented *Ulva* product has higher protein content, an increased bioacessibility and improved sensory products.

4.7 Fermented Product Characterization

Fermented products were freeze-dried for stability and characterized along with the unprocessed form of Ulva to examine the effect of fermentation on seaweed quality. Because protein determination is one of the main goals of the current project, a more in-depth analysis was performed. Unfortunately, the Lowry procedure led to inconclusive results, and more research needs to be conducted for the effective use of this methodology. Thus, analysis of the total nitrogen content was carried to complement the gathered data and gain a better insight into protein content.

4.7.1 Lowry Protein Determination

The protein content of *Ulva* species varies from 17.6 % to 20.1% (DW), making these algae suitable for feed production (Shuuluka et al., 2013). In this frame, protein quantification is a significant indicator of fermentation performance as well as the quality of end-algae product. Unfortunately, only the first and last samples from the sole 4LAB inoculated fermentation could be analyzed, making it impossible to monitor protein over the course of the whole bioprocess.

In the initial bioreactor samples (0h) the impact of microbial proteins is deemed negligible as the inoculum had just been introduced into the hydrolysate-based culture. However, differences between the feedstock and the initial samples are expected; attributable to the addition of the components of the culture medium, inoculum and seaweed hydrolysate that add to the total protein content (Figure 19). However, total protein contents were found rather similar between the first and last sampling points of all fermentations. Since sugar was greatly consumed and colony forming units (CFU) measurements support significant microbial growth in all studied conditions, a protein increase would be expected until the end of process. To overrule the possibility that this pattern is not visible due to the presence of interfering compounds, a precipitation protocol should have been conducted to mitigate their effects. Trichoroacetic acid (TCA) is often used for this purpose since this agent is capable of concentrating proteins while removing undesired substances, enabling a more accurate analysis (Barbarino et al., 2005). According with Niemi et al., 2023 colorimetric protein estimates in algae tend to deviate from aminoacid measurements, leading to either overestimating or underestimation depending on the species. Yet, the implementation of TCA or TCA/ acetone together with mercaptoethanol protocols are able to lessen the interference effects but not to eradicate them (Niemi et al., 2023).

Another possibility is that these substances interfere with the protein measurements but not to the extent of altering the protein evolution through the fermentation course. As so, it may be hypothesized that protein content reaches a maximum and then decreases due to conversion to other nitrogen compounds. Protein concentration increases during both co-fermentation and yeast fermentation, reaching a peak at 88h and 89h, respectively. These time points are close to the maximum biomass production in yeast (92h) and yeast with lab bioreactors (95h). A similar observation occured in a Brain-Isasi et al., 2021 study, in which protein production peaked at 72h of *T. reesei* growth in *Ulva rigida* and coincided with maximum biomass production. After registering maximum protein levels, the protein concentration severely decreased and stayed steady until the end of 288 hours of culture. This phenomenon was attributed to starvation of fungal metabolism. In a related work, Felix et al., 2014 were able to successfully enrich *Ulva lactuca* from 21% to 30 % protein (DW) by co-fermenting *Lactobacillus* sp. and *Saccharomyces cerevisiae* for almost 72h. This could imply that shorter fermentation times are needed for protein-enriched cultures to prevent protein loss.

Since the Lowry analysis yielded ambiguous findings regarding the protein content, a subset of samples was chosen from all carried bioreactors (4LAB; 4LAB + yeast; Yeast) for the purpose of evaluating total nitrogen levels. Based on nitrogen assessment, protein values were estimated. This supplementary analysis was conducted to acquire a more profound understanding of the fluctuations in protein content during the fermentation process.

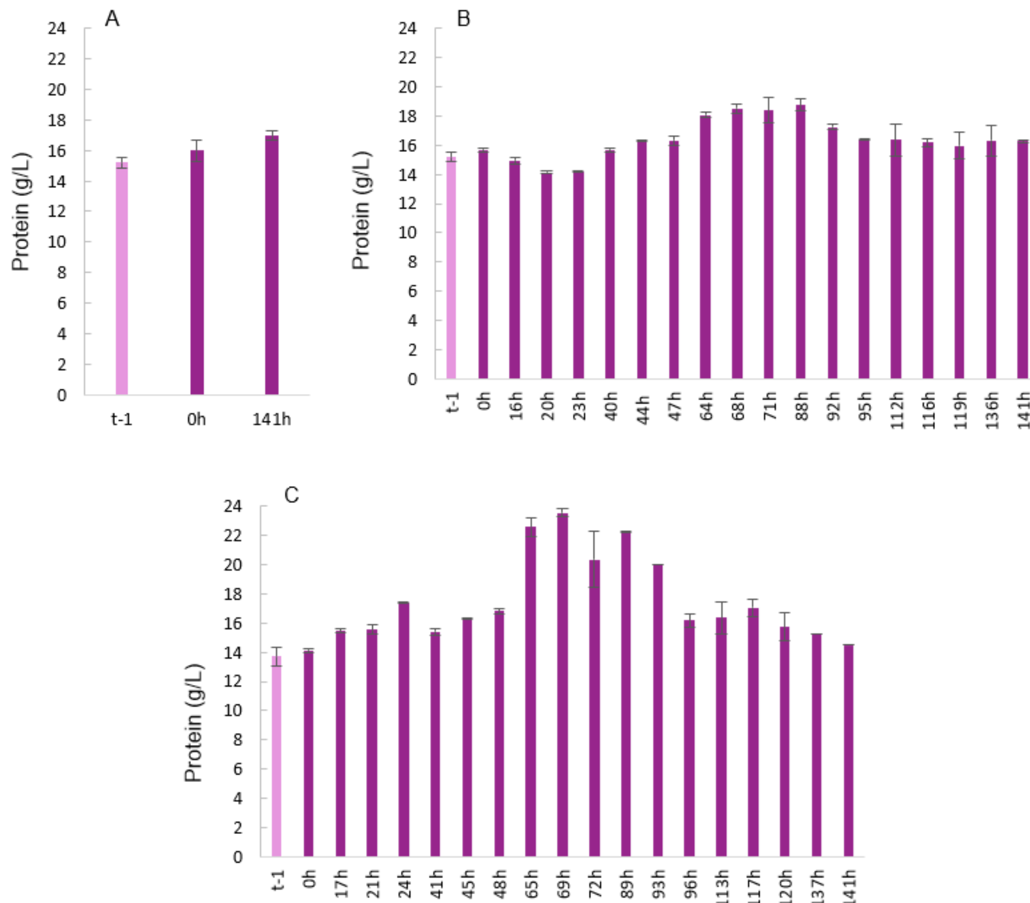

Figure 19. Lowry protein quantification of the sonified biomass (dry centrifuged pellet samples) from 4LAB **(A)**; 4LAB + Yeast **(B)**; Yeast **(C)** fermentation. Fermentations were carried in fed-batch regime for 141h, with 3% H_2SO_4 chemically treated *Ulva* slurry (100 g/L) together with CSL and mineral medium were used as substrate for microbial growth, in a 1.3L bioreactor working volume. Legend: t_{-1} -raw seaweed.

4.7.2 Total Nitrogen Content Analysis

Given the significance of protein as a primary growth factor in fish diets, additional efforts were undertaken to quantify this parameter in fermented products for the purpose of assessing their suitability for aquaculture application. In light of the inconsistent results obtained through Lowry, the determination of total nitrogen content was carried as an alternative approach (Figure 20).

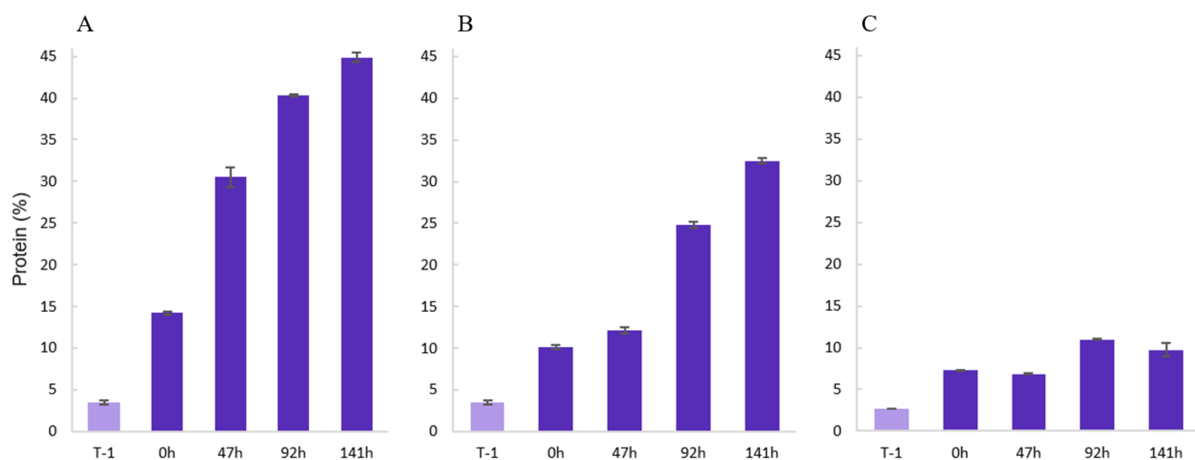

Figure 20. Determined protein content % in raw seaweed and whole fermented samples from 4LAB **(A)**; 4LAB and Yeast **(B)**; Yeast **(C)** fed-batch fermentations, collected at 4 time points. A sample volume was retrieved from

the end of each fermentation for the purpose of lyophilization. The standard deviation was calculated based on technical replicates. A bioconversion factor of 5.45 was utilized to derive protein values. Legend: T_{-1} represents the raw unprocessed *U. rigida*

As previously mentioned, the typical protein content of *Ulva* sp. typically ranges from 17.6 to 20.1 DW%. However, considerably lower values were detected in the raw specimens in this study, specifically 3.5 DW% for batch 4 (used in lab monoculture and yeast plus LAB co-culture) and for batch 5 it was not possible to determine DW% as the protein content was below the detection limit of the method (used in yeast fermentation). This deviation from the literature references for *Ulva* can potentially be attributed to the poor digestibility of seaweed leading to a low recovery of the algal proteins. For a more robust analysis of initial protein content, a more effective protein extraction protocol should have been studied and applied beforehand. Nevertheless, to visualize the effect of fermentation, a comparison of protein values between the initial sampling point (0h) and subsequent sampling points can still provide valuable insights. Both yeast and LAB monocultures inoculated were initiated with ≈ 0.8 OD_{600nm} and 0.7 OD_{600nm} microbial load, respectively; however, in the case of co-fermentation, the yeast started with a 0.7 OD_{600nm} and after a period of time an equivalent amount of 0.8 OD_{600nm} LAB biomass was introduced, increasing the total load of introduced microbiota in comparison with the other practiced fermentations. This factor should be taken into consideration when analyzing the evolution of protein content as an increase is naturally attributable not only to fermentation efficiency but also due to the higher biomass in the culture.

The introduction of ammonium hydroxide as a nitrogen source will naturally lead to an increase in nitrogen values throughout the fermentation since whole samples were analyzed. However, the form and distribution of nitrogen are expected to undergo alterations as a result of microbial function, growth and seaweed transformation. The results show a 3.2-fold increase of protein content in *Lactobacillus* consortia and mixed fermentation while in yeast a 1.3-fold increase was registered, considering fermentation start as a point of reference (0h). Although higher protein values were observed at the end of the LAB fermentation (44.8% DW) this culture exhibited higher values of protein at the fermentation onset (14.22% DW), while co-fermentation exhibited lower values in both instants (3.49% and 32.48% DW). However, the increase ratio is the same.

It is important to note that the process of nitrogen conversion into microbial protein may vary among the organisms under study. Moreover, the introduction of ammonium hydroxide contributes to the presence of nitrogen in the form of NH_4 that serves as a neutralizing agent of lactic acid. Regrettably, no distinction was made between protein nitrogen and ammonia nitrogen during the analysis, biasing the results. This bias arises from the fact that more alkali was added in LAB culture (520 mL) and with yeast co-culture (600 mL) than in yeast single-culture (300 mL). In line with this, there is a higher amount of nitrogen erroneously accounted as protein. As such, it is natural to observe lower nitrogen values in the global medium of yeast cultivation. In the future, it is important to distinguish between ammonia nitrogen and protein nitrogen forms.

Based on the evaluated colony-forming unit (CFU) measurements, it was anticipated that the protein content in lactic acid fermentation would escalate from 40 hours to 95 hours due to microbial proliferation. However, it should be noted that a portion of this increase can also be ascribed to the introduction of substantial amounts of NH_4 to uphold the pH of the medium. Despite a decrease in

viable cells towards the end of the fermentation (Figure 13), the protein content continued to increase. Considering the absence of substantial lactate synthesis and the decline in viable cell count between 90 and 140 hours, the rise in nitrogen content is likely linked to an elevation in protein content, possibly attributable to a metabolic shift.

A similar scenario is observed in terms of colony pattern in mixed fermentation; however, there was a significant decrease in total number of colonies at 20 hours. From 47h to 92h, the protein content nearly doubles which could be attributed to the inoculation of LAB consortia into the fermentation medium. In the yeast-derived product, it is conceivable that nonviable cells are accumulating in the bioreactor while the number of viable cells remains steady. This phenomenon could explain the sustained protein synthesis observed. In this case, the synthesis of ethanol and acetic acid do not lead to a significant addition of NH_4^+ to uphold the pH value, thus the contribution of ammonia-nitrogen is likely diminished when compared to LAB fermentations.

4.7.3 Peptide Profile

The microbial activity influences the bioconversion of macromolecules present both in the algae matrix and the supernatant, into other substances during fermentation processes. Consequently, the implementation of different fermentation conditions is anticipated to yield different effects on the composition of proteins and peptides. Bioactive peptides (BAPs) are initially inert within their precursor molecules but can be activated after release through processes such as *in vivo* gastrointestinal digestion, *in vitro* hydrolysis, or microbial fermentation (Q. Guo et al., 2023; Jakubczyk et al., 2020). These peptides generally consist of 2 to 20 aminoacid residues, that exhibit diverse biological properties depending on their specific aminoacid composition, sequence, and structure (Du et al., 2022). Considering that the average aminoacid molecular weight ranging is 100 Da, then peptides within the size range of 200 to 2000 might present beneficial biological effects (Philips et al., 2015). Moreover, LAB, hold considerable potential for the production of a substantial quantity of bioactive peptides due to their proteolytic system (Q. Guo et al., 2023).
The molecular weight size for fermented samples is shown in Figure 21.

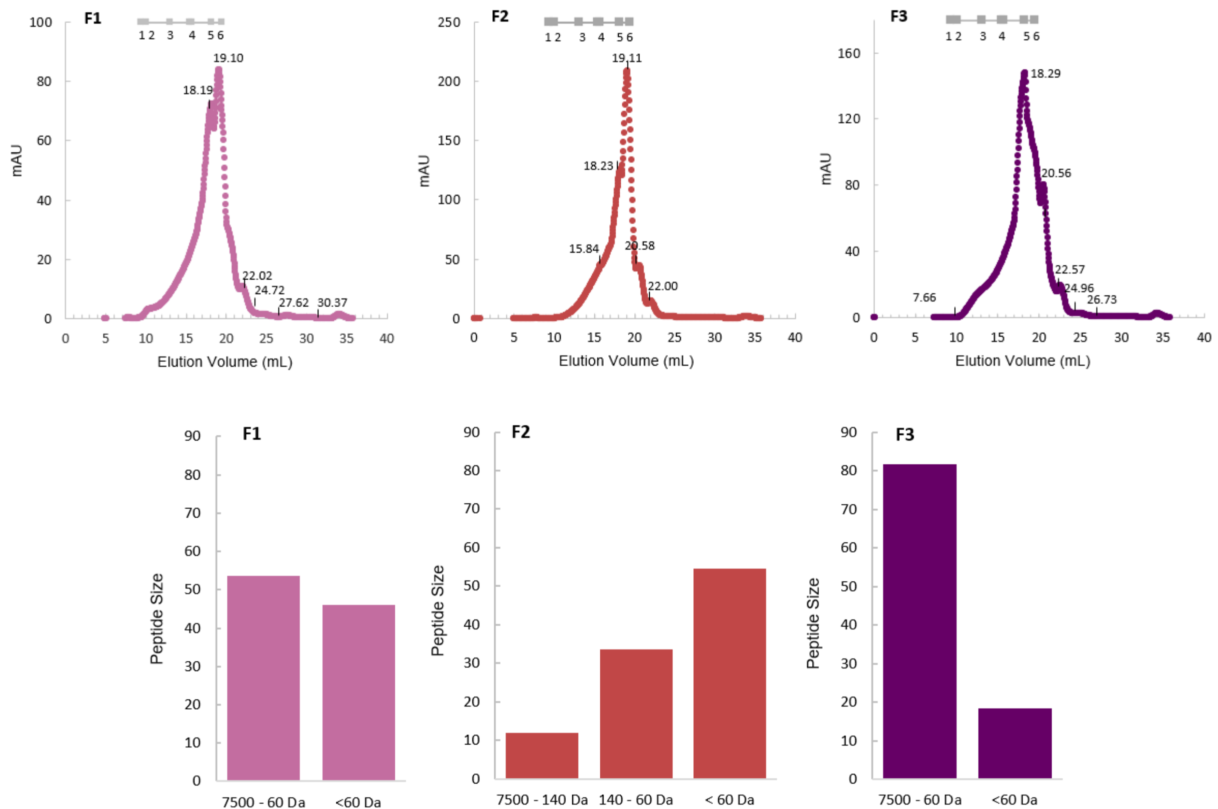

Figure 21. Peptide profile evaluated at IPMA obtained by size exclusion chromatography with column Superdex Peptide (10 to 7000 Da) for 4LAB fermentation (**F1**); 4LAB and yeast co-fermentation (**F2**); yeast fermentation (**F3**). Legend top graph: in grey the elution volumes of the biomolecules utilized for the calibration curve: 1. Cytochrome C (12384 Da); 2. Aprotenine (6512 Da); 3. Angiotensin I (1296 Da); 4. (Gly)6 (360 Da); 5. (Gly)3 (189 Da); 6. Gly (75 Da). Legend bottom graph: peptide size distribution (%) calculated based on peak area.

The separation of components in this chromatographic analysis was not very efficient, as it was not possible to discern clearly between peaks. Nonetheless, it is possible to compare among fermented samples. In all samples, the presence of components was observed throughout the entire separation range of the column (Figure 21). The products derived from both LAB fermentations (mono and co-culture) exhibit very similar profiles, although combined fermentation allowed for better differentiation of peptides size. The major difference between samples was the absence of a peak at 19 mL (elution volume) in yeast fermented product which corresponds to di and/or tripeptides in terms of molecular weight.

In the range of 7500 to 60 Da molecules, F3 accounted for the higher percentage at approximately 82%, followed by F2 with 58%, and finally F1 with 54%. However, due to the broad range of this interval, it is not possible to conclude through this analysis which product is potentially richer in bioactive peptides. Additionally, all samples presented molecules with a molecular weight below 60 Da. These molecules do not correspond to aminoacids, as the smallest aminoacid, glycine, has a molecular weight of 75 Da. Hence, it is plausible that substances other than peptides, proteins and aminoacids were detected at this wavelength.

4.7.4 Proximate Composition

A comprehensive analysis of utilized *Ulva* batches and respective fermented products was undertaken to determine their potential for feed purposes and the results are presented at Table 12.

Table 12. Proximate composition of unprocessed and fermented *Ulva* in 3L BioFlo/ CelliGen 115 bioreactor. All fermented products were synthesized through fed-batch fermentation of 813 ml/L 3% H_2SO_4 conditioned seaweed slurry, 40 mL/ L CSL (batch 2021), 2 g/L di-ammonium hydrogen citrate; 0.2 g/L $MgSO_4$, and 0.05 g/L $MnSO_4$. The presented values are expressed as mean ± standard deviation.

Parameter	**B1** *Ulva* batch U1.01021MB2202<1.5	**B2** *Ulva* batch U101021MB2301<1.5	**R_1 L** 141h fed-batch fermentation with 4LAB	**R_2 LY** 141h fed-batch fermentation with 4LAB and yeast	**R_3 Y** 141h fed-batch fermentation with yeast
Total solids (%)	82.5 ± 0.5	79.3 ± 2.2	89.7 ± 1.8	88.9 ± 0.5	84.0 ± 0.9
Moisture (%)	17.5 ± 0.5	20.7 ± 2.2	10.3 ± 1.8	11.1 ± 0.4	16.0 ± 0.9
Ashes (%)	26.0 ± 1.0	21.0 ± 1.8	15.0 ± 0.8	10.0 ± 2.2	42.0 ± 1.4
Total carbohydrates DW (%)	43.4 ± 0.6	50.2 ± 0.5	45.3 ± 0.3	45.9 ± 0.7	24.7 ± 0.4
Glucose	31.0 ± 1.0	29.6 ± 3.1	3.2 ± 0.8	2.7 ± 0.5	8.0 ± 0.9
Xylose	3.6 ± 0.8	9.3 ± 0.3	-	-	7.0 ± 0.1
Rhamnose	8.9 ± 3.2	11.3 ± 0.2	-	-	9.7 ± 0.3
Lactic acid	-	-	42.1 ± 0.6	43.2 ± 0.3	-
Protein	15.2 ± 0.3[a] 3.4 ± 0.2[b]	13.7 ± 0.6[a] <0.5*[b]	17.0 ± 0.1[a] 44.9 ± 0.6[b]	16.3 ± 0.3[a] 32.5 ± 0.3[b]	14.5 ± 0.0[a] 9.7 ± 0.8[b]

Where: R_1 L represents bioreactor fermentation with 4LAB; R^2 LY represents bioreactor fermentation with 4LAB and yeast; R_3 Y represents bioreactor fermentation with yeast; * represents below quantification limit; - represents not detected; [a] Based on the Lowry analysis expressed in (g/ L) of culture; [b] Based on the nitrogen content accessed by the elemental nitrogen analyzer LECO, using a 5.45 bioconversion factor expressed in DW%.

There was no discernible trend observed in the carbohydrate content at the end of the fermentation process. This can be attributed to the fact that carbohydrate content is a function of the balance between metabolites produced and monosaccharide consumption. Nevertheless, it was observed that glucose consumption was high in all fermentations, while xylose and rhamnose were also efficiently utilized by microorganisms in LAB fermentations for growth and metabolite synthesis. Despite the presence of high concentrations of ethanol in yeast fermentation, it was not detected in any of the processed samples, possibly due to its high volatility and subsequent evaporation during the lyophilization process. The elevated lactic acid contents may raise concerns about the effect of this compound when incorporated in aquafeeds, but it is important to remember only a small percentage of the herein described ingredients (R_1 - R_3) would be incorporated in fish feed. Moreover, the limits of lactic acid in fish-feeds vary according to the fish species, culturing conditions, size, and age (Ng et al., 2017). Thus, it would be important to conduct feeding trials in fish to test the suitability of these fermented products.

The level of moisture is a key factor in determining the shelf-life and overall quality of processed seaweeds, as high moisture might hasten microbial growth (Rohani-Ghadikolaei et al., 2012). Although the decrease of moisture is attributable to the lyophilization rather than the treatment applied, a low moisture content is imperative to ensure the preservation of the product. Furthermore, the production of

lactic acid is linked with inhibition of putrefactive bacteria, and high concentrations of this metabolite are present in the final formula. This feature may exert a significant inhibitory effect, thereby also contributing to prolong product's shelf life. Moreover, the preservation of organic materials is not only based on lactic acid synthesis but also on the production of bacteriocins, which show a wide range of antibacterial effects on several Gram-positive bacteria such as *Clostridium* and *Staphylococcus* (Q. Wang et al., 2001). Antimicrobial activity studies of the fermented samples were however out of the scope of the present thesis (not conducted).

Ash content reflects the quantity of mineral elements present in a sample, which consequently provides the principal source of mineral elements essential for microbial metabolism. The raw *Ulva* batches utilized in this study exhibited high proportion of ashes, specifically 26% and 21%. These values are consistent with those reported for other species of the *Ulva* genus (Bobin-Dubigeon et al., 1997). The high ash content in *Ulva* spp has been linked to the presence of minerals and sulfates derived from sulfated rhamnose moieties in ulvan (Brain-Isasi et al., 2021). A decrease in the ashes content is registered in LAB fermentation and mixed fermentation. This may be attributable to the hydrolysis of Ulvan during acid-treatment and the conversion of insoluble organic matter to other products. In contrast, high ash content was observed in the yeast fermented *Ulva* and this might be related with the lowest carbohydrate and protein levels among products.

Owing to the unreliability of Lowry data and the absence of bioreactor replicates, the results of total nitrogen quantification were considered for the discussion of protein content of the seaweed-derived products (in future fermentations, the ammoniacal-nitrogen analysis should be performed though, to confirm the allegation). In all conditions, a protein increase was registered in fermented end products in comparison to the beginning of the biological process (0h). Both 4LAB + *S. cerevisiae* and 4LAB fermentations showed increase of protein contents of 22% and 31% DW. Despite the higher microbial load in mixed fermentation, this process did not present the higher value of protein content indicating that the fermentation efficiency might be suboptimal in the conditions tested. This analysis could have been done in the solid fraction to mitigate the contribution of ammonium hydroxide to the protein content, however such approach would exclude any proteins present in the supernatant phase. Nonetheless, a comprehensive examination of the amino acid composition of the biomass would be necessary to fully comprehend the influence of each type of fermentation on the nutritional value of the final product.

4.7.5 Biological Activity

Fermentation may also be employed for the improvement of functional properties of non-conventional feed ingredients, including seaweed. This section regards the data analysis of antioxidant and quelating ("anti-pro-oxidant") properties of final fermented samples to evaluate the potential benefits of including these substrates in aquafeeds. The collected data is presented in Table 13, and it is possible to infer that the utilized fermentation conditions influence the biological activities of the analytes, particularly in terms of ABTS and DPPH antioxidant activity.

Table 13. The antioxidant activities (DPPH, ABTS radical scavenging activities and reducing power) and chelating activities (Cu^{2+} and Fe^{2+}) of final products derived from *U. rigida* fed-batch fermentation, expressed as the concentration of sample needed to decrease to half the concentration of radical/ion in each method (EC_{50}, mg/mL).

Sample	ABTS EC_{50} (mg/mL)	DPPH EC_{50} (mg/mL)	Reducing power Abs=0.5(mg/mL)	QCu EC_{50} (mg/mL)	QFe EC_{50} (mg/mL)
R_1: 4LAB	16.5 ± 0.7	8.4 ± 0.0	5.1 ± 0.2	3.5 ± 0.1	-
R_2: 4LAB + yeast	10.1 ± 0.8	6.0 ± 0.3	3.8 ± 0.0	3.0 ± 0.0	-
R_3: Yeast	10.4 ± 0.5	9.2 ± 0.0	3.1 ± 0.0	2.3 ± 0.1	11.0 ± 0.5

Where: - represents that there was no activity detected for the tested concentrations; RP represents reducing power; R_1 represents fermentation by 4LAB; R_2 represents fermentation by 4LAB and yeast; R_3 represents fermentation by yeast.

Regrettably, the analysis of the biological properties of non-fermented *Ulva* was not feasible, as the sample was not water soluble. In both mixed and yeast fermentation, the ABTS activity and reducing power showed a similar profile, whereas the LAB-yeast consortia demonstrated significantly higher DPPH scavenging activities. Comparing both monocultures, yeast cells fermentation exhibited superior ABTS, reducing power activities and chelating activities, but performed the worst in terms of DPPH, whereas LAB fermentation products exhibited a slightly stronger efficacy. Consequently, the combination of both microorganisms appears to generate a synergistic effect in terms of biological activity that may arise from complementary metabolic activities, harnessing the beneficial attributes of each microorganism in a singular product. Yet, in terms of chelating properties and reducing power, these were still stronger in yeast fermented seaweed than in mixed fermentation. Moreover, only the product derived from yeast exhibited detectable chelating activity towards iron ions.

The enhanced antioxidant activities may be attributed to the microbial hydrolysis or breakdown of algae cell walls, resulting in the release of various antioxidant compounds such as phenolics and flavonoids. Furthermore, the production of microbial secondary metabolites can contribute to beneficial biological effects since several biochemical reactions that take place during fermentation such as decarboxylation, hydrolysis and esterification processes, which potentially generate active ingredients, (Hur et al., 2014). Numerous studies concerning the production of antioxidant LAB-fermented products have indicated that the development of radical scavenging activity is a strain-specific attribute, with radical scavengers being associated with proteolysis (Faraki et al., 2021). Fermentation also induces the structural degradation of proteins, resulting in the release or synthesis of various compounds that exhibit iron chelating activity.

A study conducted by García-Moreno et al., (2014) examined the biological properties of protein hydrolysates derived from several discarded fish species. The results revealed that the DPPH radical scavenging EC_{50} values ranged from 0.091 ± 0.02 to 4.45 ± 0.06 mg/mL and Fe^{2+} chelating activity varied from 0.32 ± 0.01 to 0.63 ± 0.03. In a similar study, Henriques et al. (2021), observed that ABTS EC_{50} and Cu^{2+} values varied between 1.47 ± 0.02 to 4.93 ± 0.02 and 2.49 ± 0.02 to 5.66 ± 0.10 mg/mL respectively, while the reducing power ($A_{0.5}$) fell within the range of 3.19 ± 0.06 to 6.35 ± 0.04 mg/mL.

ABTS and DPH radical scavenging activities coupled with Fe^{2+} chelation values are much higher in fish hydrolysates that in our seaweed-derived fermented products (lower concentrations to achieve the objectives), while reducing power and Cu^{2+} chelating activity are within the range.

In all, fermentation conditions clearly influence the potential biological impact of the utilized substrate due to the specific type and extent of modifications to the bioactive compounds. It is crucial to analyze the nature of the compounds that exhibit natural antioxidant and chelating properties, particularly when considering the application of these products as feed supplement. For example, as previously mentioned phenolic compounds are known to possess antioxidant effects; however, these chemicals tend to form complexes with proteins and inhibit digestive enzymes, impairing the functional and nutritional properties of proteins. Consequently, these compounds are generally undesirable in food and feed products (Sim et al., 2021).

It is also important to acknowledge that comparison between biological activities of feed products, is challenging owing to several factors such as the variability in the utilized methods, starting materials and processing methodologies. However, the findings of this study indicate that mixed fermentation has great potential regarding biological activity. Moreover, further refinement of the processes developed herein could still enhance these activities towards a valuable and commercial feed source.

5. Conclusions and future prospects

In recent years, there has been a growing interest in exploring eco-friendly and cost-effective alternatives to conventional protein sources. Concurrently, advancements in food technology have opened up opportunities to use untapped resources such as seaweed. Seaweeds possess a rich nutritional profile that encompasses various essential elements vital for healthy living, making them a potential sustainable source of food and feed.

Fermentation is a low-cost technique to enrich *Ulva* biomass that also imparts food materials with unique aromas, flavors, and advantageous nutritional qualities. The use of seaweeds as a fermentation substrate primarily depends on the content of fermentable sugars in the raw material. Thus, it is crucial to quantify algal carbohydrates to determine the degree to which the algae can be hydrolyzed to generate fermentable sugars. However, seaweed carbohydrates are not easily accessed due to the cell structure. This because sugars are either in the cell wall, are connected to cellulose fibers and covered by a mucilaginous polysaccharide layer or contained in cells as storage polysaccharides. The effect of varied concentrations of sulfuric and of hydrochloric acid pretreatment as well as enzymatic treatments in *U. rigida* were analyzed to find a suitable saccharification process of seaweed biomass for further use as a fermentation substrate. The pre-treatment of seaweed material is critical for the effective release of sugar components, however overprocessing must be avoided to prevent the production of toxic compounds that can severely hinder the growth and metabolism of microorganisms. After several hydrolysis tests, a few pre-treatments were selected for fermentations: 1% (w/v) sulfuric acid at 121ºC for 30h followed by the enzymatic hydrolysis of Celluclast and β-glucosidase at 90ºC and pH 4.8 for 30h; and acid hydrolysis with acid loadings of 3% (w/v) and 5% (w/v). Shake flask fermentations of *Lactobacillus* consortium and *S. cerevisiae* revealed the influence of hydrolysate inhibitors. Thus, in future work developments, would be valuable to subject the microorganisms to serial adaptation with

escalating concentrations of hydrolysate medium or to employ genetically engineered strains with greater resistance to furfural and HMF.

In shake flask assays, microbial growth was observed in all conditions, pointing to the fermentability of the hydrolysates. The *Lactobacillus* consortium used, consisting of *L. brevis*, *L. rhamnosus*, *L. plantarum*, and *L. casei*, was selected for their different metabolisms, with the aim of maximizing the utilization of hydrolysate sugars. Additionally, heterofermentative bacteria have greater resistance to toxic products in hydrolysates due to enzymatic degradation mechanisms, which may contribute to the success of the bacterial consortium. Hence, additional studies to assess whether this bacterial consortium is indeed the most effective under the established conditions would be of considerable interest.

Fed-batch fermentations were carried out using as substrate in the batch phase the *Ulva* hydrolysate produced after thermochemical acid hydrolysis with 3% H_2SO_4 supplemented with salts and corn-steep liquor. Upon glucose exhaustion, a concentrated glucose solution was used as feed. Despite the use of an alternative base-medium and economical feedstock, and the presence of inhibitors, both metabolites and biomass production reached high levels. *Lactobacilli* have complex nutritional requirements and hydrolysates are typically low in nitrogen, but the latter limitation was surpassed through the addition of corn steep liquor to the base medium and ammonium hydroxide for medium neutralization purpose. An additional approach that merits future exploration and would contribute to reduce process expenses is the utilization of concentrated hydrolysate as feed instead of glucose solution. In all, bioreactor experimental results unveil a feasible process for lactic acid and ethanol production using a hydrolysate derived from an abundant and renewable feedstock - *Ulva*. Future experiments should explore new conditions that promote biomass production routes and enhance the protein content of the final product. Also, replicates of the bioreactions should be performed since biological processes exhibit inherent variability, and it is imperative to ensure the consistency of the obtained production for commercial application. More detailed studies of microorganism population activities and relevant enzymes during fermentation of *Ulva* are required to establish precise mechanisms causing fermented seaweed to further improve their nutritional value.

The application of Lowry quantification for protein measurement yielded inconclusive results. Hence, improving this protocol and investigating the use of protein precipitation techniques, such as TCA, is warranted. Protein quantification is not only pivotal for evaluating the nutritional value of the ultimate product but also serves as a monitoring parameter for microorganism behavior. The findings from the analysis of nitrogen content indicated that the *Lactobacilli* derived product exhibited a higher protein content in the end of the process compared to the other samples. Nonetheless, it is still needed to account for ammoniacal-nitrogen present in the product sample and refine the protein titer obtained. Furthermore, the consideration of protein content alone is insufficient to assess the nutritional quality of a certain product, and it should be complemented with the evaluation of protein quality including bioaccessibility, and peptide profile along with other indicators of nutritional value such as product's composition, stability, minerals and heavy metals are extremely relevant for the comprehensive analysis of these products, particularly when their application within the food industry is intended. Moreover, toxic contaminants including minerals and heavy metals generally limit their application as human food and

animal feed ingredients, as such quantification of these compounds should also be performed prior to *in vivo* testing trials (Gunathilake et al., 2022).

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Appendix

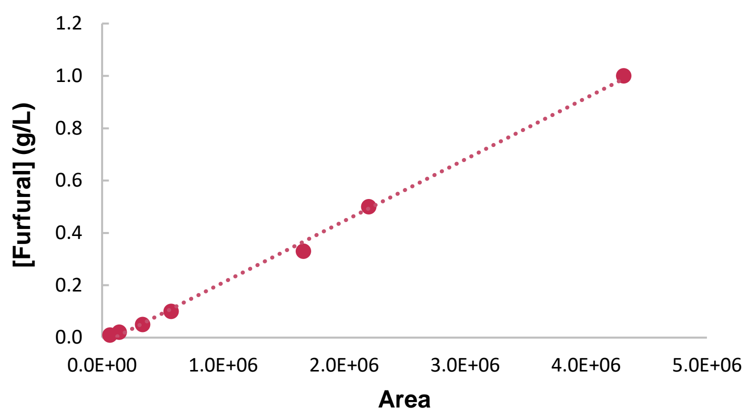

Figure A1. Calibration curve for furfural (g/L) correlating peak area with concentration, as determined by HPLC in the ultraviolet (UV) spectrum, with a retention time of 57 minutes. Slope equation: [Furfural] (g/L) = $2.07 \times 10^{-7} \times$ Area – 0.0247 ($R^2 = 0.9973$).

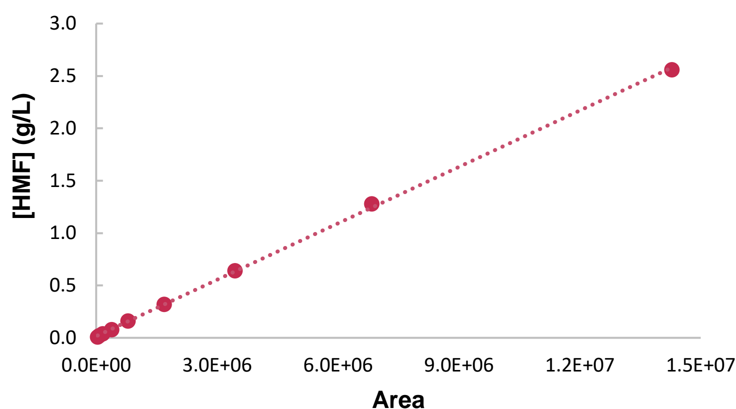

Figure A2. Calibration curve for HMF (g/L) correlating peak area with concentration, as determined by HPLC in the ultraviolet (UV) spectrum, with a retention time of 38 minutes. Slope equation: [HMF] (g/L) = $2.07 \times 10^{-7} \times$ Area + 0.0157 ($R^2 = 0.9996$).

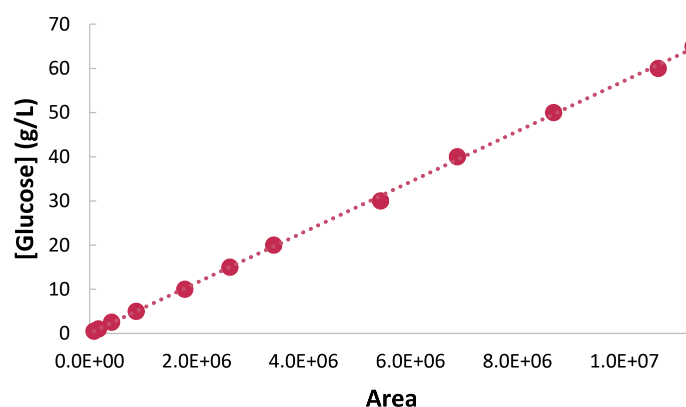

Figure A3. Calibration curve for glucose (g/L) correlating peak area with concentration, as determined by HPLC in the refractive index (RI) spectrum, with a retention time of 13.4 minutes. Slope equation: [glucose] (g/L) = $6.06 \times 10^{-6} \times$ Area + 0.0252 ($R^2 = 0.9995$).

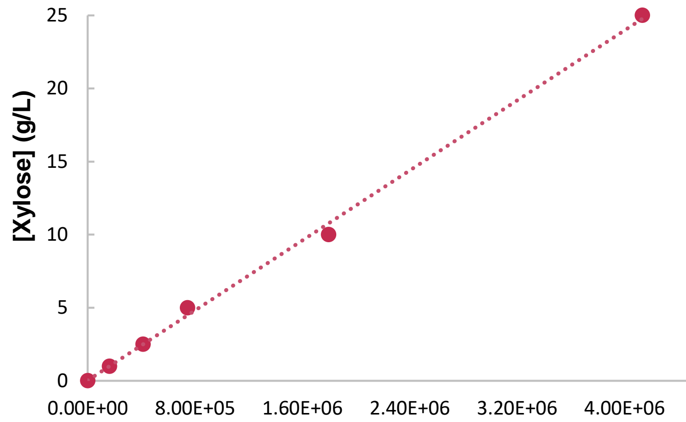

Figure A4. Calibration curve for xylose (g/L) correlating peak area with concentration, as determined by HPLC in the refractive index (RI) spectrum, with a retention time of 14.2 minutes. Slope equation: [xylose] (g/L) = 6.06 × 10^{-6} × Area + 0.0245 (R^2 = 0.9979).

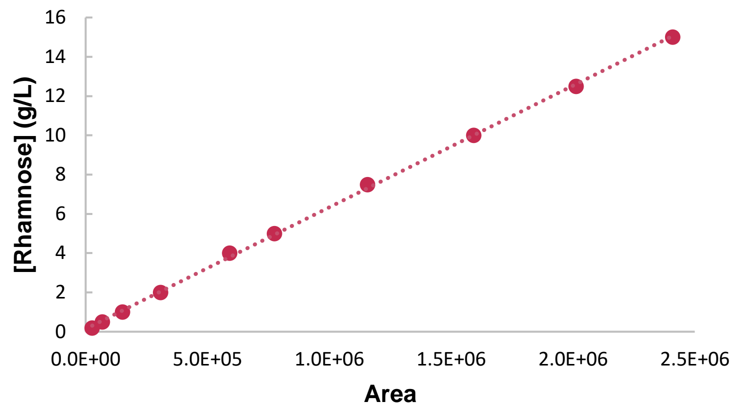

Figure A5. Calibration curve for rhamnose (g/L) correlating peak area with concentration, as determined by HPLC in the refractive index (RI) spectrum, with a retention time of 14.9 minutes. Slope equation: [rhamnose] (g/L) = 6.02 × 10^{-6} × Area + 0.1429 (R^2 = 0.9995).

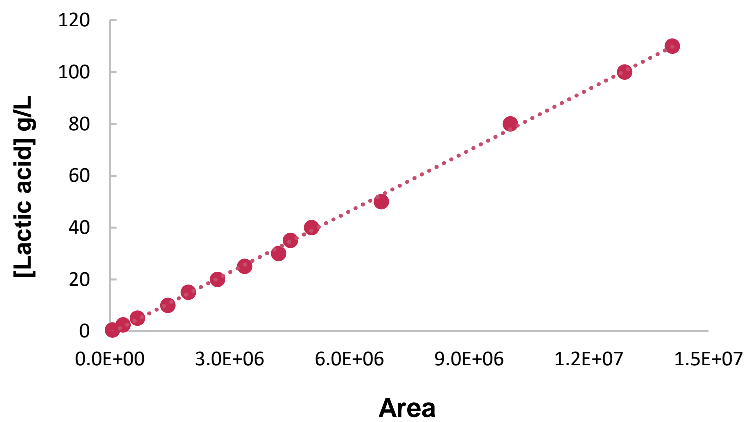

Figure A6. Calibration curve for lactic acid (g/L) correlating peak area with concentration, as determined by HPLC in the refractive index (RI) spectrum, with a retention time of 18.2 minutes. Slope equation: [lactic acid] (g/L) = 8.06 × 10^{-6} × Area - 0.08742 (R^2 = 0.9988).

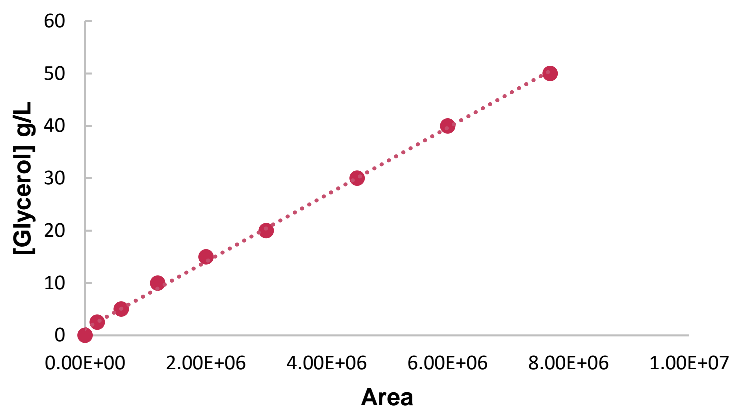

Figure A7. Calibration curve for glycerol (g/L) correlating peak area with concentration, as determined by HPLC in the refractive index (RI) spectrum, with a retention time of 18.8 minutes. Slope equation: [glycerol] (g/L) = 6.80 × 10^{-6} × Area - 0.08797 (R^2 = 0.9983).

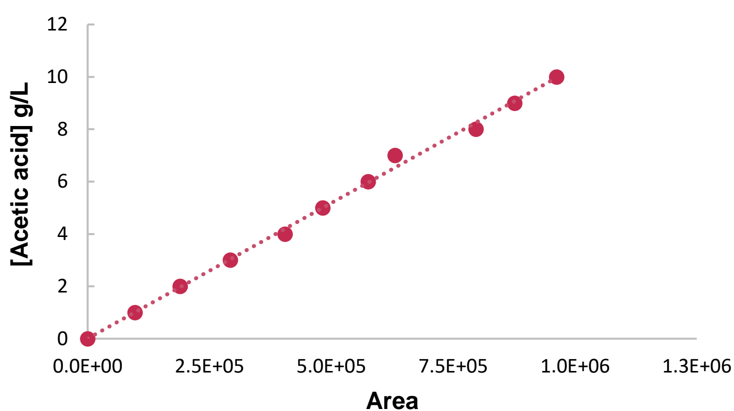

Figure A8. Calibration curve for acetic acid (g/L) correlating peak area with concentration, as determined by HPLC in the refractive index (RI) spectrum, with a retention time of 20.2 minutes. Slope equation: [glycerol] (g/L) = 1.04 × 10^{-5} × Area + 0.05 (R^2 = 0.9977).

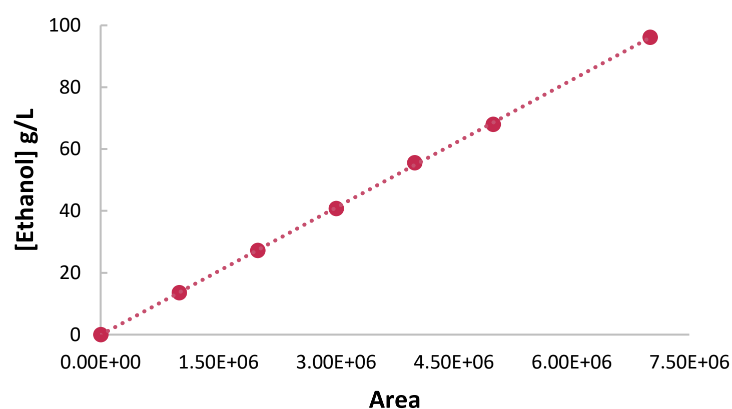

Figure A9. Calibration curve for ethanol (g/L) correlating peak area with concentration, as determined by HPLC in the refractive index (RI) spectrum, with a retention time of 27.2 minutes. Slope equation: [ethanol] (g/L) = 1.41 × 10^{-5} × Area - 0.129 (R^2 = 0.9988).

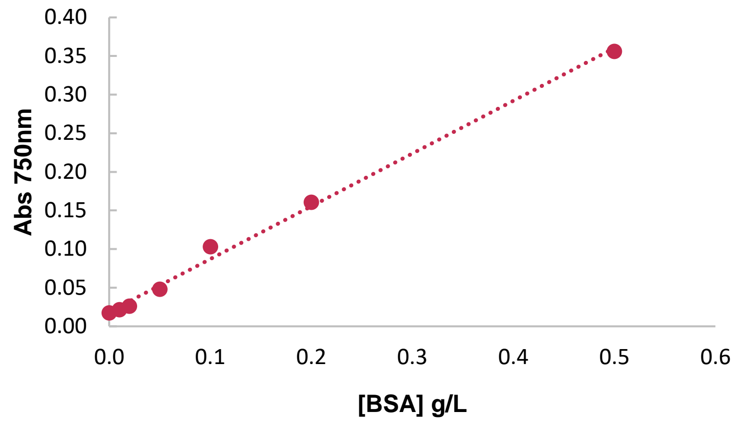

Figure A10. Calibration curve for bovine serum albumin (BSA) correlating absorbance at 750_{nm} with concentration. Slope equation: Abs_{750nm} = 0.6832[BSA] + 0.0187 (R^2 = 0.9958).

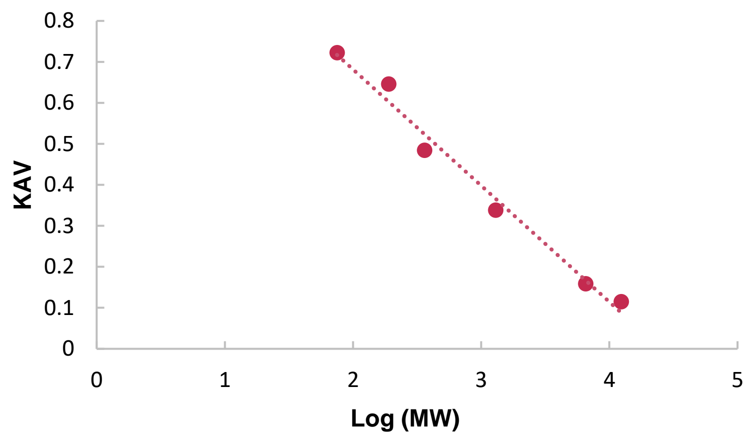

Figure A11. Calibration slope size exclusion chromatography equipped with Superdex Peptide 10/300 GL column, correlating the Log (MW) of known proteins, peptides, and AA with their calculated KAV. The biomolecules used for the construction of the calibration slope were: 1. Cytochrome C (12384 Da); 2. Aprotenine (6512 Da); 3. Angiotensin I (1296 Da); 4. (Gly)6 (360 Da); 5. (Gly)3 (189 Da); 6. Gly (75 Da). Slope equation: [KAV] = -0.283 6× Log(MW) + 1.2488 (R^2 = 0.9841).