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

Marta Costa Campos Brandão
Supervisors: Dr. Maria Teresa Smolders and Dr. Marilia Clemente Velez Mateus

Abstract

Current animal and plant proteins are insufficient to meet the nutritional requirements of human's population; thus, a paradigm shift towards a more sustainable global food system is mandatory to address this pressing issue. Aquaculture reliance in fish meal feed ingredients has become a bottleneck, due to long term sustainability concerns and increasing costs. Green macroalgae emerge as promising alternative resources, as they are abundant and have nutrient-rich profiles, offering potential as valuable feedstock for feed applications. The analysis of the *U. rigida* whole biomass batches revealed carbohydrate and protein contents of approximately 47% DW and 2% DW, respectively. These seaweed biomasses were processed into sugar-rich syrups through acid hydrolysis to be then used as substrate for microbial fermentation. 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 culture exhibited similar concentration of lactic acid (101 g/L) compared to mixed 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 4LAB + 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; ethanol fermentation; nutritional value; fish feed

1. Introduction

There has been a steady development in food technology to supply an ever-changing health-conscious consumption habits necessary to address the current food shortage scenario. This breakthrough opens the possibility to seaweed utilization for such a purpose. Macroalgae, commonly known as seaweeds, are regarded as a nutrient-rich food source as it contains substantial amounts of proteins, carbohydrates, minerals, and fibers while being relatively low in lipids (Yong et al., 2022). This nutritional profile makes their inclusion desirable for balanced diets. Furthermore, macroalgae offer additional benefits such as high productivity that far exceeds that of terrestrial biomass, require no agricultural input as fertilizers as they naturally grow in the marine environment. Consequently, their cultivation does not rely on fertile soil or freshwater, both of which are increasingly scarce resources (Fernandes et al., 2019). Ergo, this marine resource has the potential to become a crucial feedstock, providing biomass for food and feed applications. Regrettably, the utilization of macroalgae as a protein source faces some challenges due to complex structure of their cell wall and high carbohydrate content, which result in low natural digestibility and bioavailability, challenging its use as an industrial feedstock. Likewise, the presence of indigestible fibers offer resistance to digestive enzymes. However, processing methods such as saccharification, proteolysis, and fermentation can significantly improve these properties, transforming seaweeds into high value ingredients (Reboleira et al., 2021). In this regard, saccharification and fermentation of seaweed biomass might not only counteract the former constraints but also further enhance product's nutritional and functional qualities. Since most algal polysaccharides are enclosed within cellular walls, the deconstruction of these complexes is crucial to release and fractionate fermentable sugars for the fermentative process. Compared to lignocellulosic biomass, seaweed biomass can be hydrolyzed under milder conditions due to the absence of lignin. Acid hydrolysis

efficiently renders sugar-rich syrups, but often generates inhibitor compounds as by-product of sugar degradation. These components are known to be detrimental for microbial growth and metabolite production, decreasing fermentation yields (Giacon et al., 2022).

Ethanol and lactic acid fermentations are well-established core processes in the food sector, as they produce a broad range of aromas, flavors, that are extremely difficult to replicate through alternative means while still ensuring food safety. Furthermore, the growth of the fermentative strains has the potential to increase the protein content of algal substrates, making fermented macroalgae a highly competitive alternative for aquafeed. It is imperative to improve seaweed fermentation conditions for the successful transition of this process to large scale, ensuring the economic viability in the industrial setting. This work intends to establish an optimized and scalable method for the saccharification of *U. rigida* whole biomass, with a primary focus on glucose release through acid hydrolysis. Also, fermentation conditions must be tuned for increased biomass and protein content. Once fully optimized, the potential impact of macroalgal on global industry could be transformative, contributing towards pressing societal problems including climate change, freshwater expropriation, and arable land scarcity.

2. Materials and Methods

2.1 Chemical Pretreatment

Ulva rigida treatment consisted of acid hydrolysis for which residence time (30 min) and temperature (121ºC) were fixed and acid type and concentration varied to find the adequate conditions for this catalysis. Dilute acid treatments with H_2SO_4 and HCl at 0.5%, 1%, 3% and 5% (w/v) were implemented for a 10% (w/v) seaweed biomass load. After hydrolysis, the pH of *Ulva* slurries was adjusted to 4.8, and released sugar monomers and inhibitory microbial compounds were quantified using high-performance liquid chromatography (HPLC).

2.2 Enzymatic Treatment

Enzymatic hydrolysis was performed on 1% and 3% (w/v) H_2SO_4 pretreated *Ulva* slurries. For cellulose hydrolysis, β-glucosidase (0.75 mg/mL) and Celluclast (1.3 mg/mL) enzymes were combined. Under aseptic conditions, the pH of the algal suspensions was adjusted to 4.8 using 8M, 4M and 2 M NaOH solutions. The assay was conducted in an orbital incubator, with a stirring speed of 200 rpm at 50ºC for 30 hours. A sample was taken from each flask, before and after enzyme addition, so that the contribution of the previous chemical treatment to sugar release is considered. Then, throughout 30 hours' time window, samples were collected at 2-hours intervals.

2.3 Seed Medium and Inoculum Preparation

Two different inocula were prepared: *Lactobacilli* consortium (4LAB) encompassing *L. rhamnosus*, *L. brevis*, *L. casei* and *L. plantarum* strains (4LAB) and an axenic *Saccharomyces cerevisiae.* The four LAB strains were cultivated separately in MRS medium from under orbital agitation at 37ºC and 100 rpm. *S. cerevisiae* was cultivated in YPD medium 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 shake flask assays, each inoculum was prepared by transferring the necessary volume of pre-inoculum so that shake flasks cultures were started with an optical density (OD_{600nm}) of 0.5. In bioreactor experiments, the volume pertaining to each LAB strain was calculated to achieve an OD_{600nm}= 0.2, at the beginning of the bioreactor fermentation assay; and for the yeast an OD_{600nm}= 0.7 was chosen. For both assays, the required culture volume was collected in Falcon tubes and centrifuged at 6000×g at 4ºC for 15 minutes. Afterwards, supernatants were discarded asseptically, and the pellets resuspended in 0.5% (w/v) NaCl solution to inoculate the medium.

2.4 Shake Flask Fermentations

Three types of alga hydrolysis were tested in fermentations - 1% sulfuric acid and cellulose enzymes treatment, and also simple 3% and 5% acid hydrolysates, were centrifuged (6000×g, 10 min) and vacuum filtered using a Buchner funnel equipped with a 900 µm pore-sized membrane. Mineral components were added to these algal filtrates, resulting in the following formulation: 830 mL/L *Ulva* filtrate, 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 pH of the culture medium was adjusted to 6.2 - 6.5 using solutions of 1M HCl and 8M NaOH before inoculation. The growth of LAB consortium and *S. cerevisiae* in the different test

conditions was conducted in duplicates for 50 hours.

2.5 Bench-Scale Fermentations

The scale-up of *Ulva rigida* fermentation was conducted in 3L bioreactor, 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. A new batch of *Ulva* 3% H_2SO_4 treated hydrolysate was prepared and used in each scale up fermentation run, as main component of the base culture medium. The *Ulva* slurry was pumped along with all the previously sterilized remaining elements of the culture medium. All assays started with 1.3 L working volume of the base culture medium, and after sugar exhaustion, were carried in fed-batch mode, using a 500 g/L sterile glucose solution as the feed medium.

2.5.1 Lactic Acid Fermentation

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

2.5.2 Co-Fermentation

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 was inoculated into the culture medium. Cascade mode of air supply was set with a lower and upper limit of 50 and 600 rpm.

2.5.3 Alcoholic Fermentation

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

2.6 Processing and Analysis of Fermented *Ulva*

The fermented *Ulva* slurries were lyophilized for 72 hours. Afterwards, the recovered material was manually ground into powder and kept desiccated at room temperature until chemical and nutritional quality analysis.

2.7 Analytical Methods

2.7.1 Total Carbohydrates in Seaweed Biomass

Total carbohydrates within *U. rigida* flakes were quantified in triplicaye through NREL protocol “Determination of Total Carbohydrates in Algal Biomass” (Wychen et al., 2013a), with an adjustment of the sample weight. Firstly, 0.5 g of *Ulva* biomass were weighted and 50 mL of a 72% (w/w) H_2SO_4 were added. The resulting suspension was incubated at 30ºC at 100 rpm for 1 hour. Following, the hydrolysates were diluted to achieve a concentration of 4% (w/w) H_2SO_4 and pretreated in autoclave at 121ºC for 1 hour. Upon cooling to room temperature, 3 mL aliquots from each sample were neutralized with $CaCO_3$ to a pH of 6 to 8. Next, samples were centrifuged at 4000×g for 5 min. For quantification of glucose, xylose and rhamnose, the supernatant was subjected to HPLC analysis as described in 2.7.3 section.

2.7.3 Quantification of sugars and organic acids

Offline determination of algal compounds was done by HPLC in an injection volume of 20 µL. The Hitachi LaChrome Elite system was equipped with a Rezex ROA-Organic acid H+ 8% column, an autosampler, a HPLC pump, a Hitachi L-2490 refraction index (RI) and a Hitachi L-2420 UV-Vis VIS detector. A column heater 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 the flow rate of 0.5 mL/min. Sample of recovered algae supernatants (from hydrolysates and fermentation runs) were diluted by 20-fold in a 50 mM H_2SO_4 solution. The concentrations of sugars and microbial metabolites were derived using previously determined calibration curves.

2.7.4 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 OD600nm 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 OD600nmn). Each sample was placed in a quartz cuvette with a path length of 1 cm, and the corresponding OD600nm recorded.

2.7.5 Viable Cell Counting

Colony forming unit (CFU) method was used for viable cells count determination at several time points throughout bench-scale fermentation. Thereupon, 100 µL of homogenized samples were diluted with 900 µL of NaCl 0.85% (w/v) and successive 1:10 dilutions were prepared under sterile conditions. 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.

2.7.6 Total Nitrogen and Protein Content

Two different methods were employed to determine protein content: Lowry and elemental nitrogen analyses. For Lowry method, pellet samples were prepared according to protein quantification protocol (Waterborg & Matthhews., 1996), but NaOH hydrolysis was preceded by sonification. Sonification was conducted in 4 mL of sample volume and processed for 4 min at 40W.

At LAIST, the Fison Instruments EA 1108 CHNS elemental nitrogen analyzer was utilized to determine nitrogen content of *Ulva* dry flakes and of the lyophilized fermented samples. This analytical method involves complete oxidation of the sample, converting organic substances into combustion products. The resulting gases are directed through a reduction furnace and carried by helium gas into a chromatographic column, where they are separated and quantitatively detected using a thermal conductivity cell. The analytes undergo oxidation in a quartz tube furnace in the presence of an oxygen stream. Excess oxygen is eliminated with copper, and nitrogen oxides are reduced to elemental nitrogen. A nitrogen to protein mass conversion factor of 5.45 was used (Henriques et al., 2021).

2.7.7 Total Solids and Ash Content

Total solids and ash contents were determined using an adapted protocol based on the "Determination of total solids and ash in algal biomass" analytical procedure. Crucibles were pre-conditioned at 575°C in a Nabertherm muffle furnace, cooled in a dessicator, and weighed. Approximately 100 ± 5 mg of the sample were put in the pre-conditioned crucibles and placed in a constant climate chamber at 60°C until a constant weight was achieved. To determine the ash content, the crucibles were placed in the muffle furnace and subjected to a temperature ramping program, reaching a maximum temperature of 600°C. The temperature was then allowed to decrease before removing the crucibles and weighed. The total solids and ash contents of the samples were calculated using the equations described by Wychen et al. (2013b).

2.7.8 Biological Activity of Fermented Products

Assessment of antioxidant and chelating qualities of raw and fermented Ulva by a project partner (IPMA IP). 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. The antioxidant activity was determined through EC50 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 ions chelation serving as indicators of metal chelation.

3. Results and Discussion

3.1 Total Carbohydrate Quantification

The monosaccharide composition of various *U. rigida* batches was evaluated to select the batch with the highest carbohydrate content for further experiments. The results are provided in Table 1.

Table 1. Proximate composition of *U. rigida* dry flakes.

BATCH	Carbohydrates (g/g dw)		
	Glucose	Xylose	Rhamnose
B_3	0.21 ± 0.02	0.03 ± 0.01	0.07 ± 0.02
B_4	0.31 ± 0.01	0.04 ± 0.01	0.09 ± 0.03
B_5*	0.30 ± 0.04	0.09 ± 0.00	0.11 ± 0.00

Where: B_3 corresponds to batch U1.01121MB0201<1.5; B_4 to U1.01021MB2202<1.5; B_5 U1.101021MB2301<1.5. *This *Ulva* batch was purchased later due to stock depletion

Among monosaccharides, glucose is dominating (0.21 to 0.31 g/g DW), rhamnose is the second largest component (0.07 to 0.11 g/g DW), and xylose only holds a small contribution to the

total carbohydrate content (0.03 to 0.09 g/g DW). This hierarchical pattern in terms of sugar concentration is also observed in other studies and typical of *Ulva* species. 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 environmental and physiological conditions (Chan et al., 2017). The most significant difference relies in glucose concentration and is probably a result of imposed growth conditions. 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 released glucose thus it was selected as feedstock for further assays.

3.2 Acid Hydrolysis for *Ulva* Saccharification

Acid hydrolysis was evaluated based on the effect of two variables 2: acid type and concentration for the degradation of *Ulva* polysaccharides.

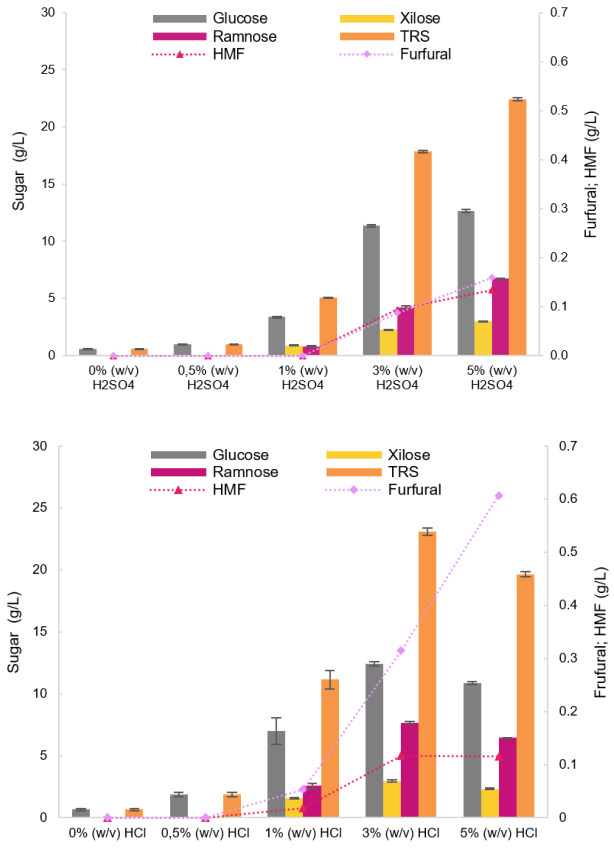

Figure 1. *U.rigida* dilute acid hydrolysis with H_2SO_4 (**on top**) and HCl (**on bottom**) of 0.5, 1, 3 and 5% for 30 minutes, at 121ºC.

As shown in Figure 1, the hydrolysis of carbohydrates into sugars is influenced by acid concentration. In sulfuric acid treatment all monosaccharides reached the highest concentrations at 5% (w/v) while in the hydrochloric treatment the best performance was achieved at 3% (w/v). As follows, harsher pretreatment conditions do not always correlate with higher sugar recoveries. This phenomenon can be attributed due to higher susceptibility of sugar for thermal degradation to furan compounds. Furans compounds are known to be inhibit microorganism growth and hinder biomass conversion, and their combined presence generally amplifies their inhibitory effects. Thus, concentrations near the inhibitory levels must be avoided. Furthermore, concentrations of 0.5 to 1 g/L for 5-hydroxymethilfurfural (HMF) and furfural were found to be toxic in hydrolysates lactic acid fermentation (Zhang et al., 2016). Even though HCl treatments yielded higher total sugar release in comparison with H_2SO_4 at lower concentration (3%), its use resulted in higher concentrations of inhibitory compounds. For this reason, HCl pre-treatments were discarded. Because a step increase of furfural and HMF was registered from 3% (w/v) to 5% (w/v) H_2SO_4, both 1% (w/v) and 3% (w/v) H_2SO_4 were tested for enzymatic hydrolysis.

3.3 Enzymatic Hydrolysis

An enzymatic cocktail targeting cellulose degradation with β-glucosidases and cellulase enzymes, was utilized for further release of glucose. The subsequent results are depicted in Figure 2.

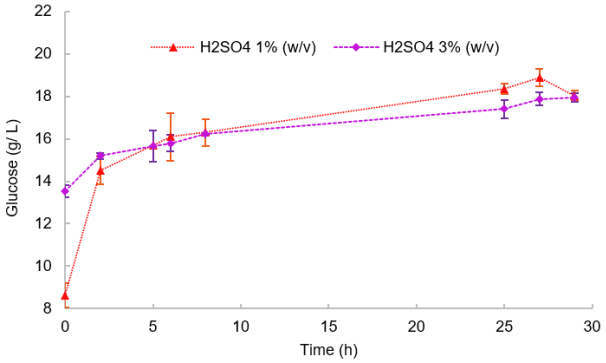

Figure 2. Enzymatic hydrolysis for pre-treated *Ulva* suspensions at pH 4.8, temperature of 50ºC during 30h.

Initially, there is a significant difference between the two conditions, with 1% (w/v) H_2SO_4 producing lower fermentable sugars than 3% (w/v) H_2SO_4 (Figure 2) and the direct result of heat treatment applied prior. However, throughout time, the disparity between glucose concentration profile

between both assays narrows, and beyond the fifth hour, the glucose concentration from 1% (w/v) H_2SO_4 treatment exceeds that of the 3% (w/v) acid treatment. Acid concentration has been shown to significantly enhance the breakdown of seaweed cellular structure. Due to the potential higher efficiency of hemicellulose solubilization in the 3% treatment compared to the 1% treatment, an enhancement in cellulose conversion was anticipated. 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 (Qin et al., 2016). Although phenol quantification was not performed, the presence of significantly distinct concentrations, if detected, could potentially justify the observed data.

3.4 Optimum *Ulva* treatment for microorganism growth

Based on the obtained results, different pretreatment strategies of *Ulva* biomass were identified as promising. Combined chemical and enzymatic treatment holds the highest glucose release but is costly and time-consuming. *Ulva* 3% H_2SO_4 exhibits a similar monosaccharide profile as in 5% treatment, except for rhamnose, but lower levels of furfural and HMF. Consequently, these hydrolysates were utilized for yeast and 4LAB consortium growth.

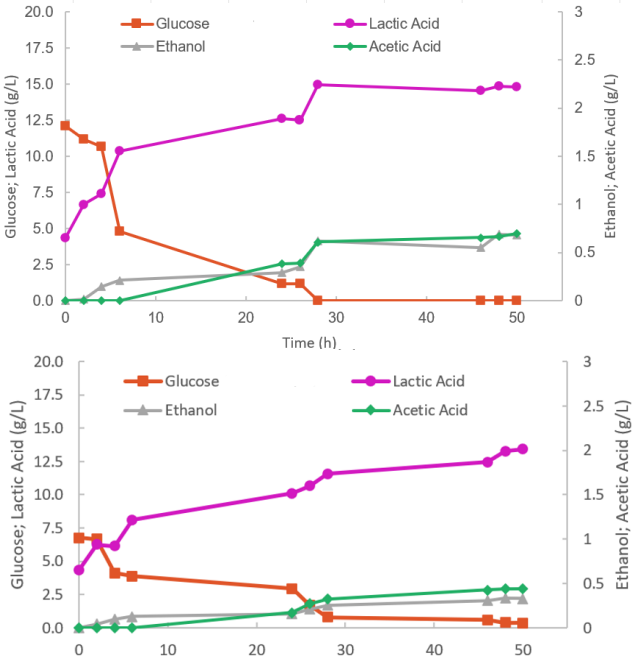

Figure 3. Progress of *Lactobacilli* consortium fermentation in 1% H_2SO_4 + cellulases (**top**) and 3% H_2SO_4 (**bottom**) *Ulva* hydrolysate based medium.

As observed in Figure 3, the highest metabolite concentrations of *Lactobacilli's* shake flask fermentation was achieved in 1% H_2SO_4 and enzyme treatment. Moreover, OD_{600nm} measurements revealed a difference in terms of growth-promoting capacity (23.6 and 22.5 OD_{600nm} at 50h of shake flask fermentation). The experimental data indeed discloses the interference of furan compounds in microbial growth, since in a substrate richer in inhibitory compounds, *Lactobacilli* consortium had weaker fermentation performances. Nonetheless, it is significant to note that while hydrolysates with 1% H_2SO_4 + cellulases 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.

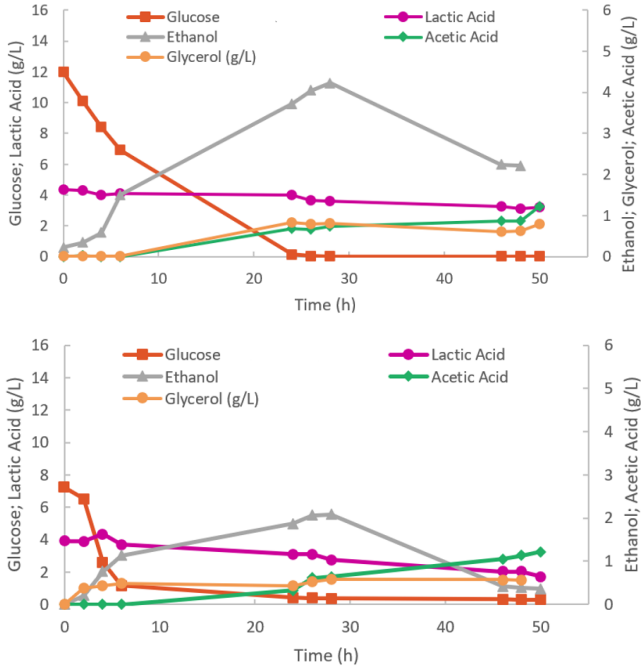

Figure 4. Progress of *S. cerevisiae* fermentation in 1% H_2SO_4 + cellulases (**top**) and 3% H_2SO_4 (**bottom**) *Ulva* hydrolysate based medium.

Ethanol concentration reached its peak at 28 hours, after which decreased until the end of the assay. This decline in ethanol coincides with the depletion of glucose, as result of diauxic shift. Maximum OD_{600nm} (23.5) and ethanol production (4.22 g/L) were registered in the absence of inhibitors and decreased significantly in 3% H_2SO_4 treated hydrolysates (21.9 OD_{600nm}; 2.06 g/L). The discrepancy in fermentability between the hydrolysates is most likely due to differences in available glucose.

In general terms, all the selected microorganisms were able to grow despite the

presence of inhibitors in the harsher settings. Again, discrepancies between 1% and 3% H_2SO_4 are also attributable to initial concentration of sugars. Additionally, pH value plays a vital role in hydrolysate fermentation, as reducing pH may cause the dissociation of inhibitory chemicals such as lactic acid, intensifying their cytotoxic effects. Thus, the employment of a fed-batch regime may mitigate these effects. For bench-scale assays, 3% H_2SO_4 treatment was selected based on its cost-effectiveness, while still retaining efficient microbial function.

3.5 Fermentation Scale-Up

Scale up to a 3L bioreactor was tested in fed-batch regime. The objective was to identify a startup culture that would yield a more compelling nutritional profile for the aquaculture industry. Aside from growth inhibitors, lignocellulosic hydrolysates are generally scarce in nutrients and in particular nitrogen supplementation has been reported to improve fermentation performance (Johansson et al., 2014). To increase biomass and protein content in bioreactor fermentations, neutralization of the fermentation medium was carried with ammonium hydroxide instead of sodium hydroxide as it serves as both a protein source and pH regulator, possibly outperforming the nutrient scarcity concerns. Except for yeast fermentation, all studies utilized *Ulva rigida* batch 4 as the starting material. For the final experiment, batch 5 was used due to stock depletion.

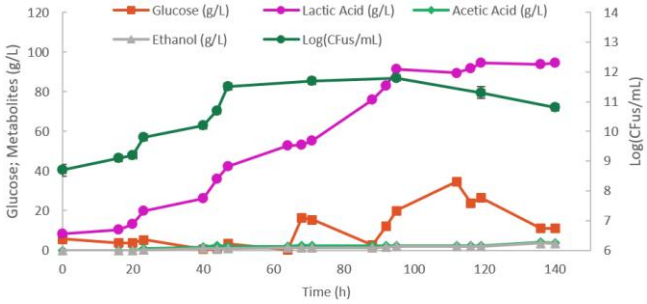

Figure 5. Glucose substrate, metabolites and biomass concentration (as log(CFUs/mL)) in fed-batch fermentation by 4LAB.

Figure 5 shows the glucose consumption and metabolite production was minimal, accompanied by slow cell growth during the first 16 hours of fermentation. This lag phase, not observed in shake flasks experiments, may be attributed to higher levels of toxic compounds in the hydrolysate. To shorten the lag phase, an acclimatization process of 4LAB from MRS to the seaweed hydrolysate medium could be carried out. Lactic acid and acetic acid levels significantly increased starting at 23 hours, with lactic acid gradually rising at 113 hours and acetic acid till the end of the process. Colony numbers experienced a rapid increase until 47 hours, followed by growth deacceleration until 95 hours, before declining towards the end of the fermentation. The decoupling between cell growth and lactic acid production towards the end of the process, may be attributed to the shift of energy from cellular growth to the maintenance of pH equilibrium, resulting in arrested cell growth. The use of an alternative medium instead of MRS, did not appear to have a significant impact on bacterial growth as large metabolite production, high glucose consumption and substantial growth were achieved

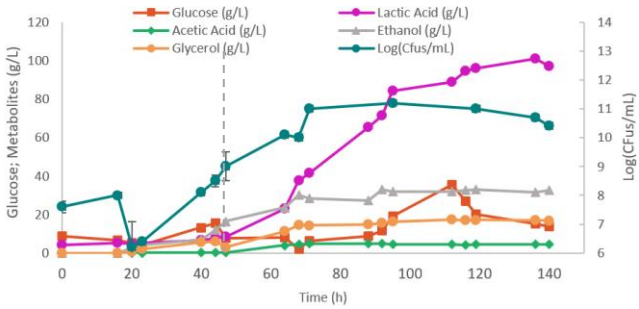

Figure 6. Glycose substrate, metabolites and biomass concentration (as log(CFUs/mL) in fed-batch fermentation by yeast + 4LAB. Vertical dashed line indicates the 4LAB inoculation.

Regarding mixed yeast + 4LAB culture in the alga hydrolysate base medium (Figure 6), the initial proliferation of yeast 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 yeast growth conditions are 30ºC and 200 rpm. When this was corrected at 15 hours yeast cells were able to recover and resume ethanol fermentation. At 45 hours *Lactobacilli* were introduced to the bioreactor since ethanol and glycerol showed a fast rise tendency at 44 hours. Following, lactic acid concentration increased significantly and even reached slightly higher levels (101 g/L) than in the LAB monoculture (94.5 g/L). Several studies have demonstrated the significant role of yeast and LAB interaction for acid synthesis (Tang et al., 2011). Glycerol, acetic acid and ethanol levels stabilized at 70 hours, suggesting a decline in yeast activity and growth arrest. Despite the diminished impact of lactic acid and acetic acid at higher pH levels, their concentration can still stress yeast cells, potentially leading to the decline of ethanol fermentation. In terms of growth, after an initial rise in colonies, there is a sharp decline by 20 hours. Following, the total number of colonies gradually increases, peaking at 95 hours. Yeast metabolite production suggests growth arrest from 70 hours onwards. As total cell count represents a

balance between both species, the active growth of lactobacillus may outweigh the decline of yeast viable cells. To better understand the growth dynamics during co-fermentation, specific plates for *S. cerevisiae* growth should be used alongside MRS plates for LAB growth.

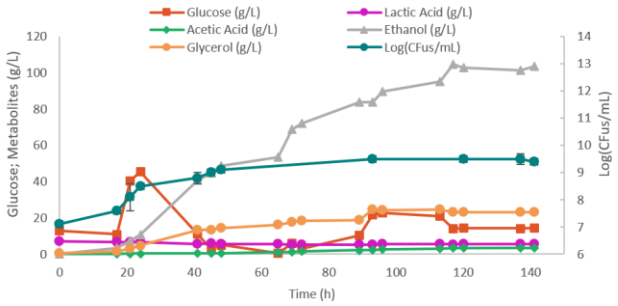

Figure 7. Glucose substrate, metabolites and biomass concentration and (as log(CFUs/mL) in fed-batch fermentation by yeast.

Following the lag phase (first 20 hours), both yeast metabolite production and colony counts increased (Figure 6). Ethanol production differed significantly between yeast fermentation (105 g/L) and co-fermentation (33.1 g/L) Regrettably, due to plate culturing conditions, it is not possible to distinguish the origin of cells in mixed fermentation, hindering a direct comparison of *S. cerevisiae* growth in monoculture and mixed fermentation in terms of biomass. It is possible that suboptimal conditions in co-culture favor *Lactobacilli* growth over yeast proliferation, as suggested by minimal differences in metabolites between isolated LAB growth and their behavior in co-culture with yeast. The biomass production stabilized around 92 hours, indicating that no benefits arise from prolonging fermentation for the purpose of microbial growth. The total number of colonies was lower than 4LAB fermentation in the spite of similar end-product concentrations (95 g/L of lactic acid, Figure 5; 105 g/L of ethanol, Figure 7). This outcome may be explained by a combination of factors: the plate medium being suboptimal for yeast growth; secondly, four different strains were used in the lab bioreactor whereas only one was inoculated in alcoholic fermentation. Nonetheless, yeast cells are larger in size compared to *Lactobacillus* cells thus despite fewer cells, yeast biomass may be equal to or even exceed that of lactobacillus

In all, despite inhibitors presence, the experiments yielded high cell growth and metabolites concentration. Nutrient supplementation with glucose and ammonium hydroxide may have enhanced organism tolerance to these compounds or alternative metabolite routes might have been activated. Further studies are needed to confirm these conjectures and acquire better understanding of the underlying mechanisms.

3.6 Protein Content of Fermented Products

Protein quantification of the feedstock and fermented products was conducted and presented in Figure 8.

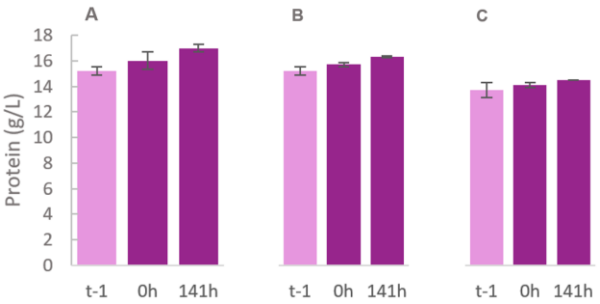

Figure 8. Lowry protein quantification (g/L) of bioreactor products by 4LAB **(A)**; 4LAB and Yeast **(B)**; Yeast **(C)**. Legend: t_{-1} refers to raw *Ulva*; 0h refers to beginning of the fermentation; 141h refers to the end of fermentation

In the initial bioreactor samples (0h) the effect of microbial proteins is deemed negligible as the inoculum has just been introduced into the hydrolysate-based culture. However, differences between 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. Moreover, initial and final fermentation samples show minimal variation. This outcome was unexpected since significant microbial growth occurred, thus an increase of protein content was anticipated. One possibility is that other components that are being co-extracted with proteins are hindering the quantification process (Niemi et al., 2023).

3.7 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 2.

Table 2 Proximate composition of unprocessed and fermented *Ulva*.

Parameter	B_3	B_4	R_1	R_2	R_3
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 ±1.8	15 ±0.8	10 ±2.2	42 ±1.4
TC % (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 ±0.34[a]	13.7 ±0.60[a]	17.0 ±0.0[a]	16.3 ±0.28[a]	14.5 ±0.0[a]
	3.4 ± 0.22[b]	<0.5*[b]	44.9 ±0.5[b]	32.5 ±0.3[b]	9.7 ±0.8[b]

[a] Based on the Lowry analysis; (g/L) [b] Based on the nitrogen content accessed by the elemental nitrogen analyzer LECO (% DW); - Not detected; * below quantification limit

Where: TC represents total carbohydrates; B3 and B4 are batch U1.01021MB2202<1.5 and U1.01121MB0201<1.5 respectively; R_1 represents bioreactor fermentation by 4LAB; R_2 represents bioreactor fermentation by 4LAB and yeast; R_3 represents fermentation by yeast.

Glucose was consumed extensively in all fermentation, and LAB efficiently utilized xylose and rhamnose for growth and metabolite synthesis since there are no significant traces of these compounds in the respective fermented samples. Ethanol, despite being present in high concentrations during yeast fermentation, was not detected in the processed samples, possibly due to its high volatility and subsequent evaporation during lyophilization process. Despite elevated lactic acid contents in aquafeeds may raise concerns, it is important to consider only a small percentage of the herein ingredients (F1-F3) would be incorporated. The raw *Ulva* batches had high ash content, around 26% and 21%, which is typical of species from *Ulva* genus (Bobin-Dubigeon et al., 1997). Ash content decreased in LAB fermentation and mixed fermentation, likely due to ulvan hydrolysis during acid treatment and the conversion of insoluble organic matter into other products. In contrast, *Ulva* samples fermented with yeast showed an increase in ash content 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, fermentation was able to increase the protein content. Both 4LAB + *S. cerevisiae* and 4LAB fermentations showed increase of protein contents of 22. % and 31% DW, respectively, considering fermentation start as a point of reference (t_0). Despite the higher microbial load in mixed fermentation, this process did not led to the product with the highest protein content indicating that the fermentation efficiency might be suboptimal in the conditions tested. 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.

3.8 Biological Activity of Fermented Products

The biological activity data of fermented products is presented in Table 3.

Table 3. The antioxidant activities (DPPH, ABTS radical scavenging activities and reducing power) and chelating activities (Cu^{2+} and Fe^{2+}) of final products derived from bioreactor fermentation, expressed as EC_{50} (mg/mL) values.

	ABTS EC_{50} (mg/mL)	DPPH EC_{50} (mg/mL)	RP Abs=0.5 (mg/mL)	QCu EC_{50} (mg/mL)	QFe EC_{50} (mg/mL)
R1	16.48 ±0.68	8.42 ±0.03	5.08 ±0.17	3.46 ±0.12	-
R2	10.11 ±0.77	6.02 ±0.26	3.81 ± 0.00	3.01 ±0.02	-
R3	10.41 ±0.52	9.22 ±0.04	3.10 ±0.03	2.29 ±0.09	11.01 ±0.51

Where: RP represents reducing power; R1 represents fermentation by 4LAB; R2 represents fermentation by 4LAB and yeast; R3 represents fermentation by yeast.

In mixed and yeast fermentation, ABTS activity, reducing power, and DPPH scavenging activities showed similar profiles. Comparing yeast and LAB products, yeast fermentation exhibited superior ABTS and reducing power activities, but performed poorly in terms of DPPH scavenging, whereas LAB fermentation led to a slightly stronger efficacy. The combination of both microorganisms seems to generate a synergistic effect in the biological effects. However, yeast fermented seaweed still showed stronger chelating properties

and reducing power compared to mixed fermentation. Additionally, only the product derived from yeast exhibited chelating activity towards iron ions. In García-Moreno et al., (2014) study on fish protein hydrolysates, 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 Henriques et al., (2021) study, 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. The reducing power of the hydrolysates fell within the range of 3.19 ± 0.06 to 6.35 ± 0.04 mg/mL. Comparing with the products developed in the present study, fish hydrolysates exhibit higher ABTS and DPPH radical scavenging and Fe^{2+} chelating activities, while their reducing power and Cu^{2+} chelating activity fall within the same range.

4. Conclusion

Ulva biomass is an abundant and cost-effective resource with untapped potential due to its high carbohydrates content. Acid hydrolysis effectively saccharifies this seaweed, making it suitable for microbial fermentation. Despite the presence of inhibitory compounds, both *Lactobacilli* consortium and axenic yeast culture demonstrated significant growth and microbial function, producing high quantities of lactic acid and ethanol respectively. Moreover, mixed fermentation showed synergistic effects between yeast and Lactobacilli, resulting in slightly increased lactic acid production in comparison to 4LAB cultures and beneficial biological activities. Further refinement of the processes developed herein could still potentially enhance biomass, protein content and biological activities towards a valuable and commercial feed source. Future research should focus in improving total nitrogen as a tool for protein quantification, specifically conducting analysis that distinguish protein nitrogen from ammoniacal nitrogen and the use of different plate media to better understand the co-culture dynamics of the LAB mix and yeast.

5. References

Bikker, P., et al. (2016). Biorefinery of the green seaweed *Ulva lactuca* to produce animal feed, chemicals and biofuels. Journal of Applied Phycology, 28(6), 3511–3525. https://doi.org/10.1007/S10811-016-0842-3

Bobin-Dubigeon, C., Lahaye, & M., Barry, J.-L. (1997). Human colonic bacterial degradability of dietary fibres from sea-lettuce (*Ulva* sp). JSFA, 73(2), 149–159. https://doi.org/10.1002/(SICI)1097-0010(199702)73:2<149::AID-JSFA685>3.0.CO;2-~

Brain-Isasi, S., Carú, C., & Lienqueo, M. E. (2021). Valorization of the green seaweed *Ulva rigida* for production of fungal biomass protein using a hypercellulolytic terrestrial fungus. Algal Research, 59. https://doi.org/10.1016/J.ALGAL.2021.102457

Chan, P. T., & Matanjun, P. (2017). Chemical composition and physicochemical properties of tropical red seaweed, *Gracilaria changii*. Food Chemistry, 221, 302–310. https://doi.org/10.1016/J.FOODCHEM.2016.10.066https://doi.org/10.1016/J.FOODCHEM.2016.10.06

Felix, N., & Brindo, R. A. (2014). Evaluation of raw and fermented seaweed, *Ulva lactuca* as feed ingredient in giant freshwater prawn *Macrobrachium rosenbergii*. International Journal of Fisheries and Aquatic Studies, 1(3), 199–204. www.fisheriesjournal.com

García-Moreno et al. (2014). Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species. Food Research International, 65(PC), 469–476. https://doi.org/10.1016/J.FOODRES.2014.03.061

Giacon, Thiago et al. (2022). Homo- and heterofermentative *Lactobacilli* are distinctly affected by furanic compounds. Biotechnology Letters, 44(12), 1431–1445. https://doi.org/10.1007/s10529-022-03310-6

Henriques, A., Vázquez, J. A., Valcarcel, J., Mendes, R., Bandarra, N. M., & Pires, C. (2021). Characterization of protein hydrolysates from fish discards and by-products from the North-West Spain fishing fleet as potential sources of bioactive peptides. Marine Drugs, 19(6). https://doi.org/10.3390/MD19060338

Johansson, E., Xiros, C., & Larsson, C. (2014). Fermentation performance and physiology of two strains of *Saccharomyces cerevisiae* during growth in high gravity spruce hydrolysate and spent sulphite liquor. BMC Biotechnology, 14(1), 1–9. https://doi.org/10.1186/1472-6750-14-47

Niemi, C., et al. (2023). Rapid and accurate determination of protein content in North Atlantic seaweed by NIR and FTIR spectroscopies. Food Chemistry, 404, 134700. https://doi.org/10.1016/J.FOODCHEM.2022.134700

Qin, L., et al. (2016). Inhibition of lignin-derived phenolic compounds to cellulase. Biotechnology for Biofuels, 9(1), 1–10. https://doi.org/10.1186/S13068-016-0485-2

Reboleira, J., et al. (2021). Seaweed fermentation within the fields of food and natural products. Trends in Food Science & Technology, 116, 1056–1073. https://doi.org/10.1016/J.TIFS.2021.08.018

Tang, Y., Zhao, D., Liwei, Z., & Jiang, J. (2011). Simultaneous saccharification and fermentation of furfural residues by mixed cultures of lactic acid bacteria and yeast to produce lactic acid and ethanol. European Food Research and Technology, 483–489. https://doi.org/10.1007/s00217-011-1542-5

Waterborg, J. H., & Matthews, H. R. (1996). The Lowry Method for Protein Quantitation. The Protein Protocols Handbook, 7–9. https://doi.org/10.1007/978-1-60327-259-9_2

Wychen, S. Van, & Laurens. (2013a). Determination of total carbohydrates in algal biomass: Laboratory analytical procedure

Wychen, S. Van, & Laurens. (2013b). Determination of total solids and ash in algal biomass*:* Laboratory analytical procedure

Yong, W. T. L., Thien, V. Y., Rupert, R., & Rodrigues, K. F. (2022). Seaweed: A potential climate change solution. Renewable and Sustainable Energy Reviews, 159, 112222. https://doi.org/10.1016/J.RSER.2022.112222

Zhang, L., Li, X., Yong, Q., Yang, S. T., Ouyang, J., & Yu, S. (2016). Impacts of lignocellulose-derived inhibitors on l-lactic

acid fermentation by *Rhizopus oryzae*. Bioresource Technology, 203,173–180. https://doi.org/10.1016/J.BIORTECH.2015.12.014