Fermentation of *Porphyra umbilicalis* hydrolysate by lactic acid bacteria and *Saccharomyces cerevisiae* to improve protein content and nutritional value

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Abstract

Seaweed has been gaining attention as a potential source of nutrients for fish. In particular, *Porphyra umbilicalis*, characterised in this work with a content of 51.6±1.7% DW of carbohydrates, 34.5±0.3% of protein, 1.4±0.1% of total lipids and 10.8±0.3% DW of ash, shows a great potential for fermentation and incorporation in aquafeed. Thermal acidic hydrolysis with sulfuric acid (100 g/L *P. umbilicalis*, H_2SO_4 5% w/w, 121℃, 30 minutes) led to the release of 37.9±1.1% of the total available sugars of the seaweed, producing an hydrolysate with 14.7±0.4, 1.1±0.04 and 0.9±0.04 g/L of galactose, glucose and 5-hydroxymethylfurfural, respectively. Fermentation of the hydrolysate with *Lactobacillus brevis*, *L. plantarum*, *L. rhamnosus* and *L. casei* (LAB mix) in a fed-batch operated reactor produced the highest concentration of lactic acid (65.0 g/L), measured in the supernatant. Acetic acid, ethanol and glycerol were maximum in fermentation with *Saccharomyces cerevisiae* and LAB mix inoculated separately, reaching 3.2, 7.5 and 7.8 g/L, respectively, in the supernatant. In terms of nutritional quality, batch fermentation with *Lactobacillus* in small-scale had higher protein content (21.7±0.3% of the lyophilised fermented product) and showed better bioaccessibility (85.9±1.0% of the total protein in the sample). Scaling up the processed rendered similar values of bioaccessibility. After scale-up to a 2 L bioreacor, all fermented products showed great antioxidant potential and Cu^{2+} and Fe^{2+}-chelating ability.
Keywords: *Porphyra umbilicalis*, acid hydrolysis, lactic fermentation, ethanol fermentation, nutritional value

1. Introduction

As the world's population increases and food security becomes increasingly threatened for larger numbers of people, the search for new sources of nutrition, as well as for better practices to apply to the already existing food industries is mandatory to avoid over exploration of land and sea resources.[1]

Responsible for suppressing part of the world's nutritional needs, the aquaculture sector has been steadily developing. However, more sustainable sources of protein for aquafeed need to be found, in order to decrease the impact this sector has on the marine fish stocks used to produce fishmeal, as well as on the prices of fish available in the market[2, 3]. Due to their protein composition and amino acids profile[4, 5], seaweeds have been considered a potential alternative to the the fishmeal currently in use in aquafeed. Furthermore, macroalgae do not require arable land, freshwater or fertilisers, thus being a potential substitute for terrestrial plants protein concentrates used in the aquaculture industry[6, 7]. However, due to their high content in carbohydrates, seaweeds need to undergo pre-treatment in order to improve nutritional quality. For this reason, several methods of hydrolysis of macroalgal polysaccharides and macroalgal fermentation have been studied in recent years[8, 9].

Thermal-acid hydrolysis is one of the most commonly used pre-treatment methods for seaweeds. Despite their cost-efficient character, these treatments often lead to the production of compounds such as 5-hydroxymethylfurfural (5-HMF) and furfural, which are cell growth inhibitors[7, 8]. In studies with *G. amansii*, Jang et al.[10] reported a 80.7% saccharification efficiency through the use of 3% sulphuric acid (121℃ for 30 minutes), while Greetham et al.[11] attained a saccharification yield of 63% of *P. umbilicalis* treated with 5% sulphuric acid (121℃ for 15 minutes). Combination of thermal-acid hydrolysis with enzymatic treatment is also common, with reported sugar recovery yields of up to 84.2% for *G. verucosa*

treated with an enzymatic cocktail (Celluclast 1.5L plus Viscozyme L) after hydrolysis with sulphuric acid (270 mM, 121℃ for 60 minutes)[12].

Fermentation of seaweeds has been studied mainly from the perspective of biofuel production. From that point of view, *Saccharomyces cerevisiae* has been used to convert the monosaccharides obtained from the hydrolysis of algal biomass into ethanol with promising yields[13, 14]. The lactic acid associated industries have also used seaweed hydrolysates to produce lactic acid with great yields[15, 16] for application as a precursor of the biopolymer PLA (polylactic acid) or for incorporation in food as a preservative[17], for example. Studies on the fermentation of seaweed for incorporation in aquafeed are scarce. Even so, Felix and Brindo have showed that incorporation of *K. alvarezii*, *U. lactuca* or *P. tetrastomatica* fermented with *Lactobacillus* and *S. cerevisiae* improve protein content and decrease the amount of dietary fibre present in the algae, resulting in improved fish growth parameters[18–20]. It is still important to mention that fermentation with LAB and/or yeasts is related with the production of molecules with bioactive properties, such as anti-inflammatory[21], anti-cancer, antimicrobial, antioxidant[22, 23] activities. Moreover, these microorganisms can also be added to animal feed as probiotics to aid nutrient digestion, improve gastro-intestinal microflora and stimulate enzyme production, resulting in increased weight gain and improvement of overall animal health[24].

Porphyra umbilicalis, a red seaweed (Rhodophyta) commonly known as "laver", has high carbohydrates content (up to 50%[25]), consisting mainly on porphyran, an agar-like polysaccharide rich in galactose[26, 27]. Combined with a protein content that can reach 37%[25], this seaweed constitutes a potential substrate for fermentation to attain products rich in protein that can be incorporated in aquafeed. For this reason, this study focused on the fermentation of *P. umbilicalis* to improve the protein content and nutritional quality of the seaweed.

2. Implementation

2.1. Algal biomass

Porphyra umblicalis, was obtained from ALGAS ATLÁNTICAS ALGAMAR, S.L. (1 kg bags). According to the information provided by the supplier, *P. umbilicalis* was harvested manually along the coast of Galicia and dryed at low temperature. The granulometry of the algae powder was 1.0 mm.

2.2. Bacterial and yeast strains

Four different species of lactobacilli (*Lactobacillus brevis* DSM 20054, *Lactobacillus casei* ATCC393, *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus rhamnosus* ATCC 7469) were kindly supplied by Prof. Gabriel Monteiro (iBB-IST). *Saccharomyces cerevisiae* SafAle^{TM} US-05 was kindly supplied by Dr. Margarida Palma (iBB-IST).

2.3. Culture media

Lactobacillus and *S. cerevisiae* inocula were cultivated in De Man, Rogosa and Sharpe (MRS) broth (PanReac AppliChem) and Yeast extract-Peptone-Dextrose (YPD) broth, respectively. In growth assays, the medium contained 50 mL/L culture medium of corn steep liquor (COPAM, Portugal), 2 g/L di-ammonium hydrogen citrate, 0.05 g/L manganese (II) sulphate and 0.4 g/L magnesium sulphate heptahydrate and a buffer solution (4.5 g/L di-sodium hydrogen phosphate dihydrate and 1.5 g/L potassium di-hydrogen phosphate). D(+)-galactose (15 g/L) was used as the carbon source (from a stock solution of 100 g/L). 5-HMF (1 g/L) was added from a 21.2 g/L stock solution. Fermentations were done in media containing corn steep liquor (COPAM, Portugal; 40 mL/L culture medium), di-ammonium hydrogen citrate (2 g/L), manganese (II) sulphate (0.05 g/L) and magnesium sulphate heptahydrate (0.4 g/L). Carbon sources consisted on D(+)-galactose (approximately 31.8 mL/L culture medium from a 100 g/L stock solution) and algal hydrolysate containing 1.1 ± 0.1 g/L glucose and 14.9 ± 1.2 g/L galactose (approximately 838 mL/L culture medium). When fed-batch cultures were carried out, the feeding during the fed-batch phase was composed of either powdered D(+)-galactose (fermentation with LAB mix) or a combination of powdered and dissolved (100 g/L) D(+)-galactose (fermentation with LAB mix and *S. cerevisiae*).

2.4. Characterisation of *P. umbilicalis*

Moisture, total solids and ash, as well as total carbohydrate content were determined using the protocols provided by the National Renewable Energy Laboratory (USA)[28, 29]. Protein content was determined by project partner IPMA via the elemental nitrogen analyser FP-528 DSP (LECO), with a nitrogen to protein conversion factor of 4.59[30]. Lipid content was determined based on the experimental work of Cohen, Vonshak and Richmond[31], through gas chromatography of fatty acid methyl esters.

2.5. Hydrolysis

Hydrolysis of *P. umbilicalis* was optimised in 100 mL flasks containing 100 g/L of the seaweed for a temperature of 121℃. The studied concentrations of sulphuric acid were 0, 1, 3and 5% (% w/w), in distilled deionised water or in a NaCl solution (3.5% w/w). Reaction times were either 15 or 30 minutes. A combined treatment was performed with

Viscoyme L (2.2 FBGU/mL) for 30 hours (magnetic stirring of 600 rpm, 50 ℃, pH 4.5-5.0), after acid hydrolysis with 5% H_2SO_4 for 30 minutes at 121 ℃.

2.6. Growth assays

Culture media were inoculated with each of the *Lactobacillus* species, a mixture of all LAB (LAB mix) or *S. cerevisiae* to obtain an initial $OD_{600\ nm}$ of 0.5 of each microorganism. The cultures were performed for 26.2 to 27.4 hours at 37 ℃ and 100 rpm (Agitorb200, ARALAB). Optical densities of the culture were measured every hour (UH5300, HITACHI), as well as galactose and 5-HMF consumption and lactic acid, acetic acid and ethanol production (via High Performance Liquid Chromatography).

2.7. Bioreactor fermentations

Fermentations were prformed in a B.Braun Biostat MD 2 L fermenter and associated control system, with a maximum working volume of 1.8 L. Data acquisition and conversion was done via a MICRO-MFCS (IFB RS-422) and respective software. In batch and fed-batch fermentations with LAB mix, after calibration the pO2 probe and stabilisation of pH and temperature at 6.2 and 37 ℃, respectively, the medium was inoculated with *Lactobacillus* to obtain an initial $OD_{600\ nm}$ of 0.2 for each species. Fermentation occurred for 46.3 hours (batch) or 116.7 hours (fed-batch). In fed-batch mode, galactose was fed to the reactor at 28.2 and 50.0 hours. The aeration conditions were maintained at 1 vvm and minimum stirring speed of 100 rpm (in cascade with oxygen saturation, with a set-point of 5% sat) for batch culture and 0.5 vvm and 50 rpm (in cascade with oxygen saturation, with a set-point of 5% sat) for fed-batch culture. In the last fed-batch assay, the medium was inoculated with *S. cerevisiae* to obtain an initial $OD_{600\ nm}$ of 0.8, after temperature and pH were stabilised at 28 ℃ and 5.5, respectively. LAB mix was added to the medium (initial $OD_{600\ nm}$ of 0.2 for each species) with the first galactose feed at 28.5 hours. Two additional feeds of galactose were given to the reactor at 51.9 and 71.2 hours. Fermentation was performed for 145.7 hours, with aeration conditions maintained at 1 vvm and 50 rpm (in cascade with oxygen saturation, with a set-point of 5% sat).

In all assays, glucose, galactose, 5-HMF, lactic acid, acetic acid, ethanol and glycerol concentrations were followed via HPLC analysis of samples harvested every 2 hours. The final products of fermentation were kept (200 mL) for analysis of nutritional quality.

2.8. Nutritional quality of fermented products

Nutritional quality of fermented products was assessed through determination of the protein content and protein bioaccessibility, as well as through determination of antioxidant potential (ABTS, DPPH and FRAP) and cupric and ferrous ions chelating ability. All determinations were performed by project partner IPMA.

2.9. Sample analysis via HPLC

Quantification of glucose, galactose, lactic acid, acetic acid, glycerol, ethanol and 5-HMF was done via HPLC (Hitachi LaChrom Elite) with a Rezex™ ROA-Organic acid H+ 8% (30x7.8 mm) column, Hitachi LaChrom Elite L-2130 pump (0.5 mL/min) and L-2200 autosampler (injection volume of 20 μL), a Hitachi L-2490 refraction index detector and a Hitachi L-2420 UV-Vis detector (210 nm). The column was kept at 65 ℃ with a Croco-CIL 100-040-220P (40x8x8 cm, 30-99 ℃) external heater. Elution of injected samples was done with 5 mM H_2SO_4. Samples were prepared with two consecutive centrifugations and dilution of supernatant (1:20) in 50 mM H_2SO_4.

3. Results and Discussion

3.1. Characterisation of *P. umbilicalis*

The proximate composition of *Porphyra umbilicalis* was determined in order to evaluate its potential as a source of fermentable sugars, as well as of protein, lipids and minerals.

The content of total carbohydrates determined through NREL 60967 showed similar results (51.6±1.7% DW) to those reported by Morrissey et al.[25] (50-76% DW), as well as to the values attained by Dawczynski et al.[4] and Murata and Nakazoe[5], who reported total carbohydrates contents of 48.6±5.9% and 46.5%, respectively, in dry weight. Similarly, the protein contents determined by IPMA (34.5±0.3% DW) were consistent with those reported by the aforementioned authors for *Porphyra* spp., which varied between 15% and 38.8% DW[4, 5, 25]. These results evidenced the potential of *Porphyra umbilicalis* as a source of fermentable monosaccharides, namely glucose and galactose, in addition to being a viable starting point for protein enrichment.

Note that the determined ash and lipid contents, 10.8±0.3% and 1.4±0.1%, respectively, were also consistent with those found in *Porphyra* genus, which typically are between 7-21%[32] and up to 2.5%[4, 5], respectively.

3.2. Hydrolysis

Hydrolysis was tested under 13 different conditions, which included four different concentrations of sulphuric acid (0, 1, 3 and 5% w/w), different reaction times (15 and 30 minutes), the presence of salt (NaCl 3.5% w/w) and combination with enzymatic hydrolysis (Viscozyme L, 2.2 FBGU/mL). The final concentrations of monosaccharides and

Table 1. Final concentrations of total sugars and 5-HMF, and saccharification yields attained after hydrolysis following the specified method (time, salt concentration and H_2SO_4 %w/w). Results expressed as average±standard deviation (n=2).

Method		Sugars (g/L)	5-HMF (g/L)	η[a] (%)
15 min	0%	1.4±0.5	0.00	2.9±1.1
	1%	2.4±0.1	0.4±0.02	5.3±0.2
	3%	6.2±0.5	1.0±0.1	14.4±1.1
	5%	11.4±0.5	0.9±0.1	27.6±1.3
30 min	0%	1.3±0.4	0.0±0.0	2.6±0.8
	1%	2.7±0.03	0.7±0.1	5.9±0.1
	3%	11.8±0.3	1.0±0.02	27.5±0.8
	5%	15.7±0.5	0.9±0.04	37.9±1.1
30 min + NaCl (3.5%)	0%	1.1±0.04	0.0±0.0	2.2±0.1
	1%	3.3±0.2	0.9±0.1	7.3±0.4
	3%	10.9±0.7	1.1±0.1	25.3±1.6
	5%	15.0±0.3	1.0±0.02	36.2±0.7
Combined[b]		17.3±0.1	0.9±0.01	41.7±0.2

[a]Yield calculated as $100 \times g_{\text{sugars recovered}}/g_{\text{total carbohydrates}}$; [b]Combination of 5% H_2SO_4 %w/w, 121 ℃, 30 minutes and Viscozyme L (2.2 FBGU/mL).

saccharification yields are presented in table 1.

As seen in table 1, the total concentration of sugars released in the hydrolysate is consistently higher for a reaction time of 30 minutes (at 121 ℃), and increases significantly with the increase in the concentration of sulphuric acid. The utilisation of saltwater solution instead of distilled deionised water during the process of hydrolysis was showed to increase the saccharification yield of *P. umbilicalis* carbohydrates from 48.6% to 63.0%[11] when similar hydrolysis conditions were applied (5% H_2SO_4, 121 °, 30 minutes). However, in this study, the replacement of distilled deionised water with a sodium chloride solution (3.5% w/w) resulted in lower total monosaccharides (15.0±0.3 g/L) and higher 5-HMF (1.0±0.02 g/L) concentrations than those obtained for the same conditions without the addition of salt. Therefore, the best thermal-acid pre-treatment condition was determined to be 5% H_2SO_4 (% w/w) at 121 ℃ for 30 minutes (in distilled deionised water). Note that the saccharification yield attained in these conditions was 37.9±1.1%, which is lower than that reported by Greetham et al.[11] (48.6%). This might be explained by seasonal fluctuations in the composition of the algae, as well as by the different range of monosaccarides identified by Greetham et al., which besides glucose and galactose included xylose, arabinose, fucose, rhamnose and mannitol.

Combination of acid hydrolysis with an enzymatic treatment with Viscozyme L increased the saccharification yield in 12.7% after an incubation time of 30 hours (data from prior acid hydrolysis not shown). Although the final yield (41.7±0.2%) is consistent with others found in literature regarding red macroalgae[33–35], this treatment was not considered sufficiently effective, since a great part of the galactose present in *P. umbilicalis* remained to be extracted (61.5%). The low degree of extraction of galactose is possibly related to the structure of porphyran, which cannot be completely digested by the agarases present in the enzymatic cocktail, despite its similarity with agarose[36, 37].

3.3. Growth of fermentative microorganisms on galactose and 5-HMF

The tolerance of the four *Lactobacillus* species and *S. cerevisiae* to 5-HMF at a concentration of 1 g/L was studied, in order to understand how the presence of this compound in the algal hydrolysates could affect the following fermentations. The specific growth rates determined after cultivation of these microorganisms in the presence and absence of 5-HMF are presented in table 2.

Table 2. Specific growth rates, μ (h^{-1}), of *L. brevis*, *L. rhamnosus*, *L. casei*, *L. plantarum* and LAB mix cultivated in the absence or presence of 5-HMF (1 g/L), determined through the linearization of the optical densities (600 nm) of the cultures across time (h).

	Specific growth rate, μ (h^{-1})	
	No 5-HMF	With 5-HMF
L. brevis	0.37	0.37
L. rhamnosus	0.24	0.27
L. casei	0.17	0.17
L. plantarum	0.20[a], 0.27[b]	0.20[a], 0.28[b]
LAB mix	0.25	0.23

[a]μ determined between between 0 and 6.8 hours; [b]μ determined between 9.7 and 11.7 hours.

Despite inducing a lag-phase of up to two hours in *L. rhamnosus*, *L. casei* and *L. plantarum* (data not shown), the presence of 5-HMF did not affect substantially the overall growth of the bacteria. At the studied concentration, 5-HMF was not expected to cause a decrease in specific growth rate, since 1 g/L is a value 5- to 5-fold lower than those reported to impact cell growth[38, 39]. However, the increase verified for *L. rhamnosus* (0.24 to 0.27 h^{-1}) and *L. plantarum* (0.27 to 0.28 h^{-1}) was not expected either. In the case of *L. rhamnosus*, this might be explained by the disparity in the number of points considered to calculate the specific growth rate.

Assays with *S. cerevisiae* (not shown) showed

that the yeast had the ability to grow on medium containing 1 g/L of 5-HMF. Even so, a lag-phase of more than 9.7 hours was observed. This delay in growth was a result of the presence of 5-HMF and lactic acid (a component of corn steep liquor), both yeast growth inhibitors[40–42], as well as of the change in the carbon source (from glucose in the inoculum to galactose in the medium of the growth assay), which required the yeast to adapt by activating the Leloir pathway, previously reppressed.

In all assays the concentration of 5-HMF decreased significantly, which is likely a result of the conversion of the inhibitor into a less harmful compound, such as 2,5-bis-hydroxymethylfuran[43, 44]. However, this was not possible to verify in the HPLC spectra.

3.4. Fermentation of *P. umbilicalis* hydrolysates

Fermentation in a bench-top reactor was performed: 1) in batch mode using a LAB mix as inoculum, 2) in fed-batch mode using a LAB mix as inoculum and 3) in fed-batch mode using as inoculum *S. cerevisiae* and a LAB mix added to the medium at different moments during the fermentation. Monosaccharides, metabolites and 5-HMF concentrations during the cultivation time are represented in figures 1 to 3.

Performing a batch fermentation with LAB mix alone in a bioreactor with controlled pH and aeration to led final concentrations of all the analysed compounds similar to those obtained in shake flask (data not shown), reaching 18.0 g/L of lactic acid, 1.7 g/L of acetic acid and 0.5 g/L of 5-HMF. However, two latency periods were observed between 0 and 17 hours and between 24 and 41 hours, where the consumption of glucose and galactose and production of lactic acid occurred at slow rates (figure 1 A). The first lag period was expected, since *Lactobacillus* spp. are either facultative or strictly anaerobic, and the inoculum for this assay was prepared under anaerobic conditions, which would require the bacteria to undergo an adaptation period when in contact with the aerated environment inside the reactor (initial pO_2 of 64% and an aeration rate of 1 vvm, see figure 1 C). The second latency period coincided with the decrease of dissolved oxygen below 20%.

Fed-batch fermentation was done by addition of solid galactose as feed instead of the usual concentrated sugar solution, to avoid diluting other components of the medium. This process is especially necessary when performing fed-batch cultivations with galactose because of its low solubility in water (100 g/L). Over 115.5 hours of cultivation, two feeds of solid galactose were given (at 28.2 and 50.0 hours). Even so, the last one was excessive, resulting in a galactose surplus of 29.1 g/L at

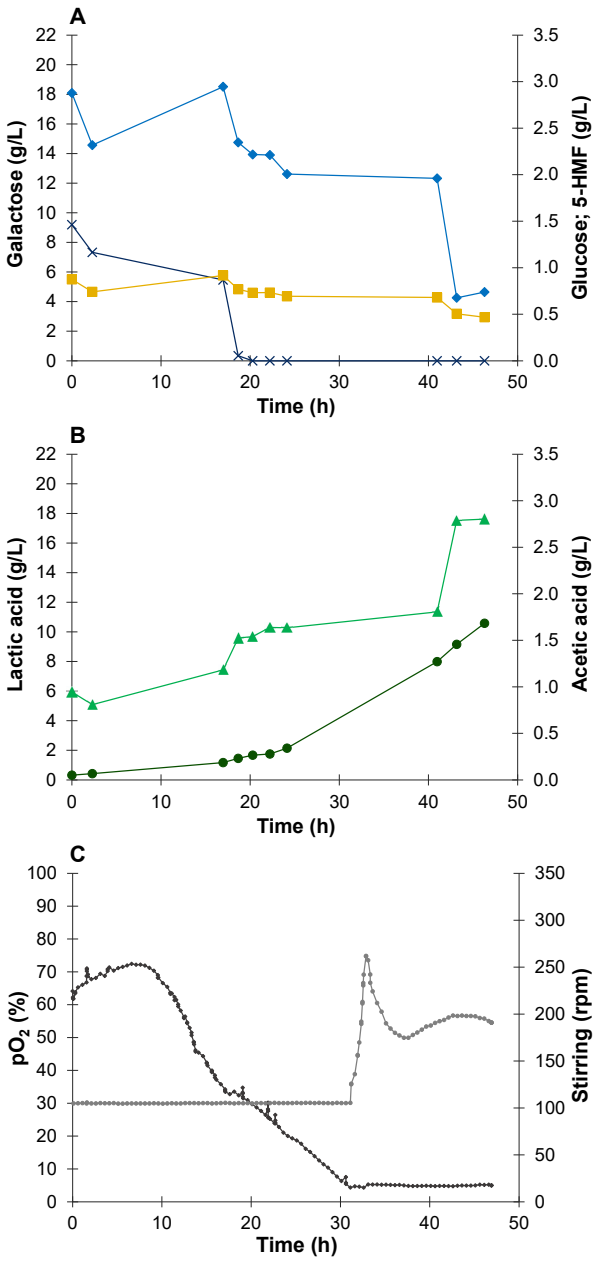

Figure 1. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲) and acetic acid (●) during the batch fermentation of the hydrolysate of *P. umbilicalis* (working volume of 1.8 L, 37℃, pH 6.2, 1 vvm and 5% pO_2 setpoint), for a period of 46.3 hours. Evolution of dissolved oxygen (%, ◆) and stirring (rpm, ●) are also represented.

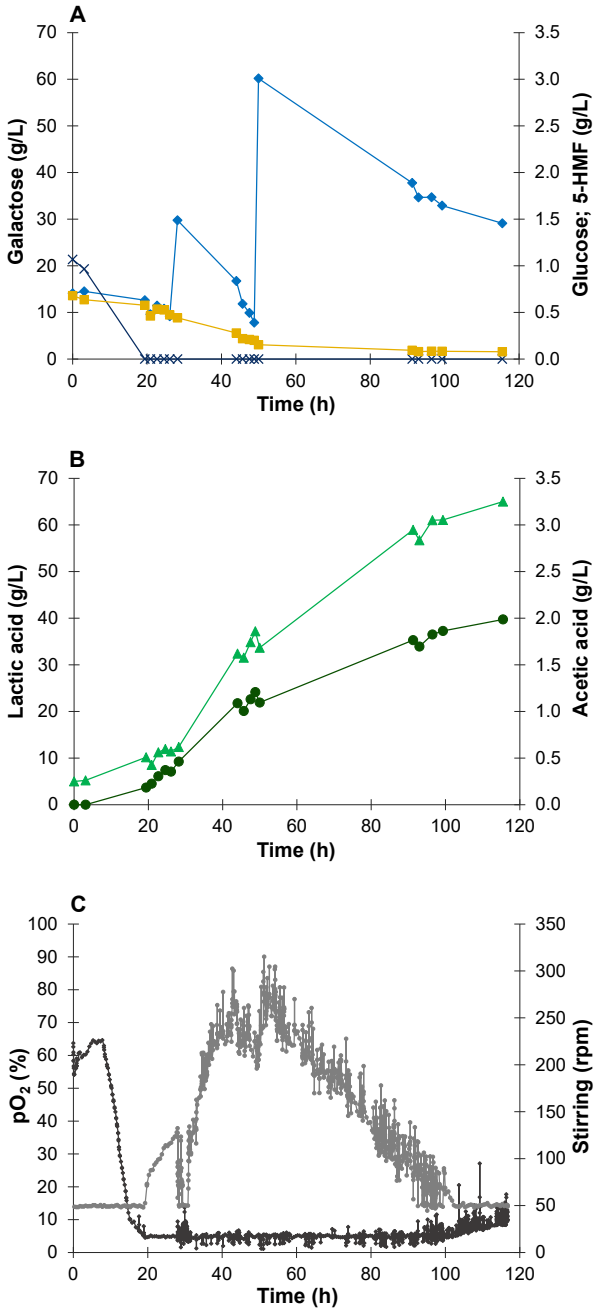

Figure 2. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲) and acetic acid (●) during the fed-batch (B) fermentation of an hydrolysate of *P. umbilicalis* (working volume of 1.8 L, 37℃, pH 6.2, 0.5 vvm and 5% pO_2 setpoint), for a period of 115.5 hours (fed-batch). Fed-batch fermentation was given two feeds of galactose at 28.2 and 50.0 hours. Evolution of dissolved oxygen (%, ◆) and stirring (rpm, ●) are also represented.

the end of the fermentation (figure 2 A). It is possible that the bacteria exhausted other essential nutrients or growth factors, causing the decreased activity observed in the last two days of the assay.

In order to avoid the 17-hour lag-phase observed in the previous batch fermentation, the aeration rate was decreased to 0.5 vvm, with a minimum stirring speed of 50 rpm. These changes allowed a quicker adaptation of the lactobacilli to the conditions in the reactor, resulting in the immediate consumption of glucose and galactose by the bacteria upon inoculation of the medium (figure 2 A). Such changes justify the lower amount of time needed to reach the established oxygen saturation set-point (pO_2=5% sat). Moreover, the maintenance of a pH of 6.2 allowed the production of a copious amount of lactic acid (65.0 g/L), since the organic acid remained in its dissociated form, which did not compromise lactobacilli viability.

The last fermentation tested in this scale was done by inoculation of *S. cerevisiae* before LAB mix, which was only added at the time of the first feed. The yeast adapted rapidly to the aeration conditions in the reactor, resulting in a rapid consumption of the sources of carbon (figure 3 A). Prior to the first addition of galactose to the reactor, the concentrations of ethanol, glycerol and acetic acid had reached 3.5, 2.2 and 0.3 g/L, respectively. Note that the membrane of the dissolved oxygen probe was ruptured at the beginning of the fermentation (see figure 3 C), therefore forbidding control over the dissolved of oxygen at 5% sat. The aeration conditions were maintained all throughout the experiment at 1 vvm and 50 rpm.

The first feed of galactose led to a sharp increase in the concentration of ethanol to 21.1 g/L at 68.6 hours of fermentation (figure 3 B), which indicates that the yeast was active after lactobacilli were added to the medium. Although the presence of ethanol at this concentration raised questions about the maintenance of lactobacilli viability, according to Gold et al.[45], it was not high enough to inhibit the growth of the lactic acid bacteria in LAB mix, which can tolerate concentrations of the alcohol between 10-16% (%v/v). After inoculation (at 28.5 hours), the bacteria still underwent a latency period of approximately 23 hours where no lactic acid was produced. After that period, lactobacilli appeared to develop steadily, as lactic acid concentration started to increase. However, it only reached 21.0 g/L. It is possible that a great part of the sugar fed to the bioreactor after inoculation with LAB mix was consumed by *S. cerevisiae* or that the yeast depleted growth factors essential for lactobacilli. Another possibility for the lower activity of *Lactobacillus* might be the inadequacy of the aeration conditions.

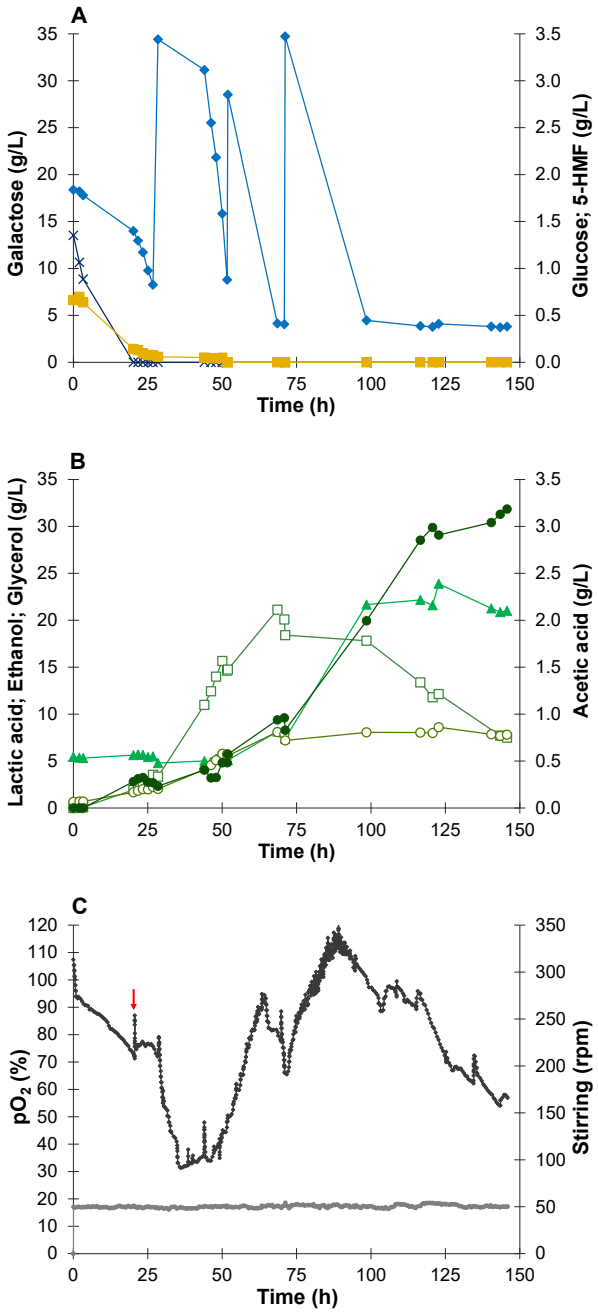

Figure 3. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲), acetic acid (●), ethanol (□) and glycerol (○) during the fed-batch fermentation of hydrolysate of *P. umbilicalis* with *S. cerevisiae* and LAB mix (inoculation simultaneous with the first galactose feed), for a period of 145.7 hours, with addition of galactose at 28.5, 51.9 and 71.2 hours. A temperature of 28 ℃ and pH of 5.5 were maintained for the first 28.5 hours of assay and then changed to 37 ℃ and 6.2 until the end of fermentation. Evolution of dissolved oxygen (%, ◆) and stirring (rpm, ●) are also represented. ↓ - Time at which pO_2 sensor failed.

A relevant aspect to this fermentation was the formation of glycerol, which was not present in any other fermentations nor in the medium during the determination of the growth curves of *S. cerevisiae*. Glycerol is produced by the yeast under osmotic stress to avoid cellular dehydration[46, 47], which is a result of the salt (sodium sulphate) produced during the neutralization of the hydrolysate prior to fermentation.

Note that in all fermentations the concentration of 5-HMF decreased over time, being that in fed-batch mode, it was completely depleted (figures 2 A and 3 A). This depletion occurred significantly faster when *S. cerevisae* was present, which was expected, as the yeast is known to convert 5-HMF into its less harmful derivative, as mentioned in section 3.3.

Lastly, despite the presence of lactic acid and glycerol at the attained concentrations was a source of concern regarding the incorporation of the fermented products in aquafeed, these compounds have been shown to improve fish growth and health by acting as an alternative to antibiotics[48] and allowing a more efficient use of the available amino acids[49], respectively.

3.5. Nutritional quality of fermented *P. umbilicalis*

The bioaccessibility of the protein and biological activity of the final product of all three fermentations were determined by project partner IPMA using lyophilised 200 mL samples of the fermented products and are presented in table 3.

The highest protein content was determined in small-scale batch fermentation with LAB mix (21.7±0.3% DW, data not shown). Even so, scaling up the same fermentation rendered a protein content of 21.1±0.1% DW. Since the media were prepared with the same proportions of algal hydrolysate and CSL, similar protein contents were expected at the end of all fermentations, which was not observed. The lowest protein content (12.1±0.2% DW) might be explained by the excessive addition of galactose to the fermentation. Since it was not completely consumed, the excess was present in the lyophilised sample along with metabolites produced during fermentation, thus contributing to the lower relative quantity of protein. This issue did not occur in the fermentation with *S. cerevisiae* and LAB mix added to the medium at different times of the assay (Y + LAB). In this fermentation, the major differences were found in the final concentrations of ethanol, acetic acid and glycerol, which reached values slightly higher than in other assays (see figures 1 to 3). Although these compounds could evaporate during the process of lyophilisation, their quantification in the lyophilised products should be performed, in order to evaluate

Table 3. Protein content (% in dry weight), bioaccessible fraction (%), antioxidant activity measured by ABTS, DPPH and FRAP methods and cupric (Q-Cu^{2+}) and ferrous Q-Fe^{2+} ions chelating ability, expressed as the concentration of sample needed to decrease to half the concentration of radical/ion in each method (EC_{50}, mg/mL), in *P. umbilicalis* and *P. umbilicalis* hydrolysates fermented in bioreactor.

		2L bioreactor		
	Pu	**LAB(B)**	**LAB(FB)**	**Y+LAB**
Prot.	34.5±0.3	21.1±0.1	12.1±0.2	17.4±0.3
Bioac.	77.8±1.8	84.4±2.1	79.7±5.2	73.7±5.9
Biological activity (mg/mL)				
ABTS	—	6.9±0.1	10.5±0.2	5.6±0.3
DPPH	—	5.5±0.2	8.3±0.3	5.8±0.4
FRAP	—	2.5±0.1	1.8±0.1	2.5±0.1
Q-Cu^{2+}	—	2.2±0.1	2.7±0.1	2.3±0.1
Q-Fe^{2+}	—	9.8±1.3	11.1±1.1	5.9±0.4

Pu - *Porphyra umbilicalis*; LAB(B) - batch fermentation with LAB mix (bioreactor); LAB(FB) - fed-batch fermentation with LAB mix (bioreactor); Y + LAB - fermentation with *S. cerevisiae* inoculated first and LAB mix added with the first galactose feed (bioreactor).

the influence they might have in protein content determination.

In terms of bioaccessibility, protein obtained from batch fermentation with LAB mix in flask showed the best results (85.9±1.0%, not shown), despite scale-up of the same fermentation rendered very similar results (84.4±2.1%). Contrary to what was expected, the presence of *S. cerevisiae* did not increase protein bioaccessibility (see table 3). This result might be justified by the nature of *S. cerevisiae*, which has a thicker cell wall that shows some degree of resistance to enzymatic digestion. For this reason, whole yeast cells have a lower degree of digestibility than its protein extracts[50, 51], which directly affects protein bioaccessibility. Despite these less promising results, further study of this specific type of fermentation could still be done to understand if the utilisation of the yeast could bring any benefits in terms of nutritional quality of the fermented product, since addition of *Lactobacillus* and *S. cerevisiae* to animal feed is related with improvements in animal growth[52, 53].

Note that, overall, products of fermentation in bioreactor showed promising antioxidant and chelating properties (table 3). These results might be a consequence of the aerated environment in the vessel, especially at the beginning of the fermentation. These conditions subjected the lactic acid bacteria to oxidative stress, which likely triggered the production of compounds with antioxidant properties.

4. Conclusions

Aiming at *Porphyra umbilicalis* fermentation, the carbohydrate fraction was hydrolysed to monosaccharides. Among the 13 hydrolysis conditions studied, the most effective was pre-treatment with sulphuric acid (5% w/w, 121ºC, 30 minutes), rendering a hydrolysate with a concentration of 1.1±0.04 g/L of glucose and 14.7±0.4 g/L of galactose, while producing 0.9±0.04 g/L of 5-HMF.

The metabolism of the selected four species of *Lactobacillus* (LAB mix) was not inhibited in the presence of 1 g/L 5-HMF in the culture medium, while a period of adaptation of a few hours was needed with *Saccharomyces cerevisiae*. Fermentations of the *Porphyra* hydrolysate using a bench-scale bioreactor working in fed-batch mode and in microaerophilic conditions were carried out using only lactobacilli or using a mixture of *S. cerevisiae* and lactobacilli. The lactic acid fermentation rendered the highest lactic acid concentration (65.0 g/L), while in the fermentation using yeast and lactobacilli (inoculated at different moments during the cultivation) other metabolites were produced, namely acetic acid, ethanol and glycerol, at concentrations of 3.2, 7.5 and 7.8 g/L, respectively. Apart from metabolite production, protein content and nutritional quality of the fermented *Porphyra* were evaluated. Tests of protein bioaccessibility revealed that utilisation of only *Lactobacillus* rendered a fermented product with a higher fraction of protein available for absorption after digestion. In terms of antioxidant and Cu^{2+} and Fe^{2+}-chelating properties, all products of fermentation with LAB and LAB plus *S. cerevisiae* presented promising results.

In the future, it is possible that fermented seaweeds become an important part of aquafeed. With that aim, the conditions in which these fermentations were performed should be optimised to increase biomass productivity and, therefore, protein content in the fermented product. In addition, it would be relevant to find a method that allows the evaluation of microbial growth during fermentation, thus allowing the optimisation of the previously mentioned conditions in a more adequate timeframe. More importantly, hydrolysis methods that are able to release monosaccharides with higher yields should be studied. Lastly, the replacement of whole *P. umbilicalis* biomass with residues from the seaweed aquaculture and food processing sectors should be considered, as to decrease the impact of the utilisation of laver for aquafeed purposes.

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