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Fermentation of *Porphyra umbilicalis* hydrolysate by lactic acid bacteria and *Saccharomyces cerevisiae* 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 work presented in this thesis was performed at iBB - Institute for Bioengineering and Biosciences of Instituto Superior Técnico (Lisbon, Portugal), and developed within the scope of the Smart Valorization of Macroalgae project (FA_05_2017_033) financed by Fundo Azul - Direcção Geral da Política do Mar, during the period from March to October 2021, under the supervision of Doctor Maria Teresa Ferreira Cesário Smolders and Professor Marília Clemente Velez Mateus.

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Resumo

A necessidade de fontes de nutrição mais sustentáveis tem conduzido o setor da aquicultura à procura de alternativas para a farinha de peixe, de modo a que o aumento da procura por alimentos de origem aquática seja suprido sem que os preços dos mesmos sejam comprometidos. Com vista a melhorar o conteúdo proteico e a qualidade nutricional, em termos de biodigestibilidade e bioatividade do produto final, para posterior incorporação em ração para peixes, a macroalga *Porphyra umbilicalis* (51,6±1,7% DW carboidratos totais, 34,5±0,3% proteína, 1,4±0,1% lípidos totais e 10,8±0,3% DW cinzas) foi hidrolisada e fermentada. A hidrólise ácida a alta temperatura com ácido sulfúrico (100 g/L *P. umbilicalis*, H_2SO_4 5% m/m, 121° C, 30 minutos) provocou a libertação de 37,9±1,1% dos açúcares totais disponíveis na alga, produzindo um hidrolisado com 14,7±0,4, 1,1±0,04 e 0,9±0,04 g/L de galactose, glucose e 5-hidroximetilfurfural, respetivamente. Fermentação deste hidrolisado com *Lactobacillus brevis*, *L. plantarum*, *L. rhamnosus* e *L. casei* (LAB mix) num reator operado em fed-batch produziu a concentração mais alta de ácido láctico (65,0 g/L) no sobrenadante à biomassa. As concentrações de ácido acético, etanol e glicerol foram máximas para um reator operado em fed-batch em que *Saccharomyces cerevisiae* e *Lactobacillus* foram inoculados separadamente, atingindo concentrações de 3,2, 7,5 e 7,8 g/L, respetivamente, no sobrenadante. Em termos de qualidade nutricional, fermentação em batch (flask) com apenas *Lactobacillus* revelou ter maior conteúdo proteico (21,7±0,3% DW do fermentado liofilizado) e maior biodisponibilidade (85,9±1.0% da proteína total da amostra). Todos os produtos de fermentação em bioreator apresentaram bom potencial antioxidante ($EC_{50,ABTS}$=5,6±0,3–10,5±0,2 mg/mL, $EC_{50,DPPH}$=5,5±0,2–8,3±0,3 mg/mL e $EC_{50,FRAP}$=1,8±0,1–2,5±0,1 mg/mL), bem como boa atividade quelante de iões Cu^{2+} e Fe^{2+} ($EC_{50,Cu^{2+}}$=2,2±0,1–2,7±0,1 mg/mL e $EC_{50,Fe^{2+}}$=5,9±0,4–11,1±1,1 mg/mL).

Palavras-chave: *Porphyra umbilicalis*, hidrólise ácida, fermentação láctica, fermentação alcoólica, valor nutricional

Abstract

The need for more sustainable sources of nutrition has led the aquaculture sector to search for alternatives to fishmeal, in order to comply with the increasing demand for seafood, while not compromising its prices. Seaweed has, for that reason, been gaining attention as a potential source of nutrients for fish. In particular, *Porphyra umbilicalis*, characterized 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° C, 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 (flask) with *Lactobacillus* 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). All products obtained from scaled-up fermentations presented good antioxidant potential ($EC_{50,ABTS}$=5.6±0.3–10.5±0.2 mg/mL, $EC_{50,DPPH}$=5.5±0.2–8.3±0.3 mg/mL and $EC_{50,FRAP}$=1.8±0.1–2.5±0.1 mg/mL) and Cu^{2+} and Fe^{2+} chelating ability ($EC_{50,Cu^{2+}}$=2.2±0.1–2.7±0.1 mg/mL and $EC_{50,Fe^{2+}}$=5.9±0.4–11.1±1.1 mg/mL).

Keywords: *Porphyra umbilicalis*, acid hydrolysis, lactic fermentation, ethanol fermentation, nutritional value

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Chapter 1

Project motivation and aim

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 exploitation of land and sea resources. The aquaculture sector has great potential to fulfil part of the world's nutritional needs. However, it is still dependent on sources of feed whose demand cannot be suppressed in a sustainable manner, as is the case of fishmeal and fish oil. Production of these compounds still relies mostly on captured fish, which has been leading to a steady increase of the feed components prices due to their high demand and limited supply. Seaweeds come as a prospective ingredient to include in aquafeed, due to their exceptional nutritional profile. In particular, *Porphyra umblicalis*, with a substantial carbohydrates content and great amino acids profile, is a promising component to be incorporated in aquafeed, especially if its properties are improved through lactic and alcoholic fermentation.

Lactic acid and ethanol fermentation are promising methods to improve the nutritional quality of seaweed products. After hydrolysis of the complex polysaccharides present in the macroalgae cell walls, lactic acid bacteria and yeasts are able to convert the sugars to biomass, increasing the protein content of the final product. Moreover, these microorganisms are probiotics, which may contribute to fish health and growth by improving gut microbiome. Lastly, fermentation might also improve the organoleptic properties of the algal hydrolysates, which would contribute to a better acceptance of the final product by fish when incorporated in aquafeed.

Aiming for the improvement of nutritional quality and protein content, hydrolysis of *P. umbilicalis* biomass was studied in several conditions, including different acid concentrations and reaction times, presence of salt, and enzymatic treatment. The most effective method was chosen and the tolerance of *Lactobacillus* and *Saccharomyces cerevisiae* to inhibitors present in the hydrolysates was determined. Fermentation of the hydrolysates was studied in batch and fed-batch mode and with a mixture of four species of *Lactobacillus* (lactic acid fermentation) or with lactobacilli plus *S. cerevisiae* (lactic and ethanol fermentation). Lastly, the most relevant fermentations were scaled-up to a bench-top reactor, where the culture conditions were controlled to improve microbial growth. Protein content and nutritional quality of the fermented *Porphyra* were analysed to determine which conditions provided the more promising final

product for incorporation in aquafeed.

Chapter 2

Literature review

2.1 The future of food security

> "Food security [is] a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life." (The State of Food Insecurity, 2001)[1]

In the next ten years, the world's population is expected to reach 8.5×10^9 (8.5 billion) people, possibly rising to 10.1 and 12.7 billion in 2050 and 2100, respectively, if trends in fertility, mortality and migration favour population growth[2]. According to the latest FAO report on food security[3], the goals set to 2030 with the perspective of extinguishing hunger and malnutrition will not be met, as food insecurity has been rising in the past 6 years. This regression has its roots on the high cost of adequate diets, which cannot be met by more than 40% of the world's population, and as a consequence of environmental changes coupled with inefficient food supply chains. Therefore, concerns over food security have been pressing the food sector to find new and sustainable alternatives to increase the availability of nutritional goods while avoiding the escalation of prices[3].

The supply of protein can be increased, either by increasing the volume of the commonly consumed sources, such as fish, meat, plants and dairy, or by finding improved replacements for those sources. Both approaches are under development. Insect protein or whole insect consumption (entomophagy) is one of the alternatives supported by FAO, due to its highly digestible and highly proteinaceous character. However, acceptance of these products by the Western consumer is low, and traditional sources are still preferred[4, 5]. For this reason, improvements in crop maintenance and growth, protein meal substitutes in animal feed and enhancement of animal health have been the target of numerous studies.

2.2 Macroalgae

The terms seaweed and macroalgae refer to a diverse group of organisms unrelated phylogenetically that are described as oxygen-producing photosynthetic multicellular entities visible to the naked eye, that do not have roots, stems or leaves[4, 6, 7]. Seaweeds have been used as food since ancient

times, especially in coastal communities, and are a great source of several types of nutrients, especially protein, fibre, vitamins and minerals[8, 9]. In addition to human and animal nutrition, macroalgae have also been applied to fertilisation[10] and, more recently, to the production of biofuels and the extraction of bioactive compounds, which are used in a wide range of industries (food, pharma and cosmetics, among others)[6].

Seaweeds are divided into three distinct classes: Chlorophyta (green), Phaeophyta (brown) and Rhodophyta (red). These differ from each other in their main pigments, structural polysaccharides, and protein content. Green seaweeds, such as *Ulva* spp. and *Enteromorpha* spp., are mostly found in shallow waters where light is abundant, are rich in chlorophyll a and b, despite carotenes and xanthophyll also being present. Although variety within this class of seaweeds is high, the majority has cellulose and hemicellulose as structural polysaccharides and starch as storage polysaccharide[6, 11]. Brown macroalgae, whose colour is a result of the abundance of fucoxanthin, are typically found from equatorial to subpolar regions. Similarly to Chlorophyta, cellulose is present as a structural polysaccharide, along with algin and fucoidan, which are exclusive to brown seaweeds[6, 11]. Some of the most consumed algae belong to this class, as is the case of *Saccharina japonica* (Japanese kelp), *Undaria pinnatifida* (Japanese wakame or miyeok) and *Sargassum fusiforme* (Japanese hiziki)[10]. Rhodophyta, or red macroalgae, occur all throughout the globe, especially in temperate and tropical environments. Moreover, phycobiliproteins, which are the main pigments in these seaweeds, allow their survival in deep waters, where light is dimmer than in the upmost layers of the oceans[6, 11]. This class of seaweeds, along with Phaeophyta, represents a large part of the macroalgae market for human consumption with species such as *Porphyra* (nori or laver), *Gelidium*, *Kappaphycus alvarezii* and *Eucheuma*, which are also explored for carrageenan, an hydrocolloid widely used in the pharmaceutical, cosmetics and food processing industries as a thickening agent[10].

2.2.1 Nutritional profile of macroalgae

Owing to the fact that macroalgae are highly diverse, have a wide geographical distribution and are subjected to seasonal variations of temperature, currents, light, salinity and nutrient availability, the compositions in terms of protein, carbohydrates, lipids and ash are not fixed and suffer great variations[12, 13].

Protein, one of the main constituents of seaweeds, is present in higher contents in green and red macroalgae (up to 47% DW in *Porphyra umbilicalis*)[6], with brown seaweeds reaching only as high as 21% (table 2.1). Furthermore, macroalgae contain all the essential amino acids required by fish[14], thus being comparable to animal sources of protein[8, 12].

Carbohydrates, the most abundant components in seaweed, are present in contents as high as 76%, in a dry weight base[15] (table 2.1), rendering macroalgae comparable to terrestrial crops such as soybean and pulses[4, 6]. The majority of these carbohydrates are in the form of structural polysaccharides, such as cellulose, agar, alginate and carrageenan, which are extracted for different applications in several industries. For instance, agar and carrageenan, extracted from red macroalgae, are used not only

Table 2.1. Proximate composition of red, brown and green seaweeds, reported in a dry weight base (g/100 g dry seaweed).

Seaweed	Carbohydrates (%)	Protein (%)	Lipids (%)	Ash (%)	Ref.
Red seaweed					
Gracilaria sp.	64.69±0.32	11.86±0.16	1.42±0.05	14.49±1.23	[16][b]
	46.9±0.4	23.6±0.2	0.7±0.1	28.9±0.2	[17]
G. lemaneiformis	61.61	20.87	0.87	16.66	[18]
K. alvarezii	40.39 (25.87±1.64 CH + 14.52±0.11 DF)[a]	19.25±0.15	0.64±0.08	27.0±1.62	[19]
Palmaria palmata	71.0	12.3	1.2	11.9	[20]
	50 - 76	12 - 21	0.7 - 3	—	[21]
Porphyra sp.	48.6±5.9	31.3±7.3	2.1±1.2	—	[8]
	41.3 (39.5 CH + 1.8 DF)[a]	38.8	1.9	6.9	[12][b]
P. umbilicalis	46 - 50	15 - 37	0.12 - 2.48	—	[21]
P. yezoensis	54.08	27.72	0.62	17.57	[18]
Brown seaweed					
F. vesiculosus	56.4±0.4	15.1±0.2	3.0±0.3	25.5±0.2	[17]
Laminaria sp.	36.0±5.7	7.5±1.9	1.0±0.3	—	[8]
L. digitata	48	8 - 14	1	—	[21]
L. japonica	37.5	21.43	1.79	39.29	[18]
Sargassum powder	50.7	7.0	1.33	27.0	[22]
S. latissima	68.9±0.3	10.2±0.3	0.5±0.1	20.4±0.1	[17]
S. siliquosum	63.92±0.33	9.61±0.35	0.56±0.05	12.08±0.85	[16][b]
Green seaweed					
Enteromorpha sp.	60.2[a]	18.1	0.3	14.1	[12][b]
U. lactuca	43.91±0.42	21.54±0.06	0.51±0.02	22.75±0.37	[16][b]
U. pinnatifida	38[a]	15	3.2	30.8	[12][b]
	45.9±1.5	19.8±1.4	4.5±0.7	—	[8]
U. rigida	58.1±0.7	9.3±0.5	0.9±0.1	31.7±0.6	[17]

[a] Carbohydrate content calculated as the sum of carbohydrates (CH) and dietary fibre (DF);
[b] Proximate composition reported on a wet base (g/100 g seaweed).

as gelatine-like substances and thickening agents during food preparation[10], but also as a support for microbial growth[12]. Moreover, these water-soluble polysaccharides also present anticoagulant, anti-hypercholesterolimic and anti-tumoural effects, besides playing a role in cancer, diabetes and obesity prevention[15].

Furthermore, macroalgae are also a great source of minerals, vitamins and, although in lower quantities, polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)[6, 8]. Despite the fluctuations induced by seasonal and environmental variations, the content of minerals, especially calcium, magnesium, potassium, phosphorus, sodium, iron and iodine, is still generally higher than those observed in terrestrial crops[13, 15].

2.2.2 *Porphyra umbilicalis*

Porphyra umbilicalis is a red seaweed that is part of the group of macroalgae called “laver” (“nori” in Japan, “kim” in the Republic of Korea and “folhuda” in Portugal)[23], which comprises several species of

Porphyra and *Pyropia*[24]. With high growth rates, these seaweeds require only 45 days from seeding of conchospores (entities produced by sexual reproduction that develop into blades) in adequate substrates to the time of harvest[6] and, according to FAO[25], its production takes place, mostly, in the Republic of Korea and China, which in 2019 contributed with 20% and 71% of the world production of these seaweeds, respectively.

As mentioned in the previous section (table 2.1), *P. umbilicalis* is rich in carbohydrates, and its content depends on the geographical location and the environmental conditions. This genus in particular is characterised by the lack of cellulose as a structural polysaccharide, which is replaced by mannan and xylan, while carrageenan is replaced by porphyran[26, 27], an agar-like polysaccharide whose structure consists of a linear backbone of 3-linked β-(D)-galactopyranosyl with alternating units of 4-linked α-L-galactosyl 6-sulfate, as well as a low amount of 3,6-anhydro-α-L-galactosyl[28]. Porphyran, the major component of the extracellular matrix in these macroalgae prevents algae desiccation during low tide[27]. It has been gaining attention as a bioactive macromolecule due to its anti-cancer, anti-inflammatory and antioxidant[29] properties, as well as for its ability to participate in the prevention of diseases related to the cardiovascular and the nervous systems. In addition, *Porphyra* spp. is also recognised by its abundance in vitamins from the B complex, in particular vitamin B12, rendering these species a good source of this nutrient in vegan lifestyles[24]. Due to these characteristics, lavers, in general, are primarily considered as a source of food, therefore there is still a reduced number of reports on the effect of the bioactive compounds that can be extracted in the health of humans and animals.

In addition, considering the relatively high protein content of *Porphyra umbilicalis* (table 2.1) and the availability of polysaccharides that can be degraded into digestible monosaccharides and functional oligosaccharides[29], this seaweed may be considered as a promising source of nutrition to include in aquafeed.

2.3 Seaweed pre-treatment

Seaweeds are possible substitutes of terrestrial biomass in the fermentation industry, such as corn and sugar cane, owing to the fact that their content is high on fermentable sugars and low on lignin (a constriction in terrestrial biomass), and that they do not require the utilisation of fresh water, arable land, fertilisers or pesticides, in addition to contributing to the decrease of high nitrogen and potassium levels in eutrophic waters and to carbon sequestration and oxygen release[30, 31]. However, the majority of the algal carbohydrates are in the form of structural, namely cellulose, agar or carrageenan, or reserve polysaccharides, such as starch. Therefore, hydrolysis is a crucial step to obtain fermentable monosaccharides, mainly glucose and galactose, from those complex polysaccharides[32]. This process is usually done resorting to chemical or enzymatic treatments, being that the use of acidic solutions is less costly, while the use of enzymes renders a higher final concentration of sugars[31]. Note that the efficiency of the hydrolysis step depends strongly on the type of reagent, its concentration, reaction conditions as well as the concentration of algae, and the type of polysaccharide itself[31].

2.3.1 Mechanical treatment

Mechanical (or physical) pre-treatment is applied, generally, before any other step of hydrolysis, in order to alter the structure of the seaweed and increase its contact with reagents or biological agents[32, 33]. This class of pre-treatments includes washing, which aims to decrease the concentration of salts and other impurities, and size reduction (e.g. milling). However, the effectiveness of these treatments in different seaweeds, as reported by several authors, is dependent on the structure of the alga itself and the type of method utilised to perform size reduction. In general, however, mechanical treatment does not have the same high impact on sugar hydrolysis yields when compared to chemical and biochemical treatments[34]. Vanegas et al.[35] reported that although sonication prior to thermal-acid treatment (4% nitric acid at 120° C for 60 minutes) of *Laminaria digitata* and *Saccharina latissima* did not have an impact in terms of the release of reducing sugars, milling of these macroalgae (from a particle size of 1 mm to 0.1 mm) prior to the same thermal-acid treatment increased the yield of sugar extraction in approximately 1%. Application of the same type of milling prior to thermal-acid treatment with 3% hydrochloric acid (120° C for 60 minutes) increased reducing sugars recovery in approximately 5% for both algae.

2.3.2 Chemical treatment

Chemical treatment is widely used to degrade polysaccharides due to its fastest and less expensive character. The processes included in this class use acids, alkali, organic solvents, ionic liquids and other compounds that change the chemical structure of the macromolecules, resulting in their degradation[32].

Acid hydrolysis is performed, mainly, with inorganic acids such as hydrochloric or sulfuric acid, or with phosphoric and nitric acid in fewer instances. Note that the neutralization of said reagents results in the accumulation of the respective salts[36]. Despite cheaper, this process has the disadvantage of producing inhibitory compounds to cell growth, such as furfural and 5-hydroxymethylfurfural (5-HMF), as a result of the decomposition of pentoses and hexoses, respectively. These compounds have a negative effect on key enzymes in ethanol production and cell growth[31, 32, 37].

Even so, due to its cost-efficient character, acid pre-treatment has been studied extensively with seaweeds from all three classes. Jang et al.[38] reported that *Gelidium amansii* (3-5% solid to liquid ratio, SLR) subjected to 3% sulfuric acid at 121° C for 30 minutes rendered a monosaccharide recovery yield of 80.7%, while Nguyen et al.[39], using a SLR of 12% and a concentration of H_2SO_4 of 180 mM (approximately 1.8%) for 45 minutes at 121° C, reached a yield of 37.2%. Also belonging to Rhodophyta, *Porphyra umbilicalis* was treated with 5% sulfuric acid (15 minutes, 121° C), in a study by Greetham et al.[40], rendering a monosaccharide conversion yield of 63% (in a final concentration of 24 g/L). In the same study, brown seaweed *L. digitata* and green algae *Ulva linza* were subjected to the same treatment, yielding reducing sugar recoveries of 52.8% and 69.1%, respectively. Further results are presented in table 2.2.

The use of alkali has also been studied as a means to degrade seaweed polysaccharides, although to a lesser extent. Despite showing good results in the pre-treatment of lignocellulosic material, alkali

do not perform as well with macroalgae, requiring larger amounts of reagents and yielding consistently worse monosaccharide recoveries than those obtained via acid hydrolysis of the same substrates. According to Greetham et al.[40], the utilisation of 5% NaOH for 15 minutes at 121° C in *U. linza*, *P. umbilicalis* and *L. digitata* slurries (10% w/v), only rendered saccharification yields of 10.3%, 7.4% and 10.2%, respectively. An additional constriction to this method was reported by Kim et al.[41] when it is used with red algae, leading to the formation of gels via the interaction of hydroxide ions with the typical red seaweed polysaccharides, thus converting L-galactose-6-sulphate to 3,6-anhydro-L-galactose, which is a known means to improve gelling properties[42]. As discussed by Vanegas et al.[35], it is most likely that alkali act more effectively on the hydrolysis of lignin, while acids hydrolyse cellulose and hemicellulose, the main polysaccharides in seaweeds. Nonetheless, alkaline thermal treatment of macroalgae does not produce the inhibitors that occur in acid hydrolysis[43], which may come as an advantage for fermentations with microorganisms which are intolerant to those compounds.

Less common chemical pre-treatments of macroalgal biomass include ionic liquids and oxidation through the use of hydrogen peroxide. In both methods the chemical compounds are used either to directly hydrolyse the complex carbohydrates to smaller molecules (oligo and monosaccharides) or to weaken the structure of the polysaccharides as a means to increase the area available for enzymatic activity. In studies with ionic liquids, Bodachivskyi et al.[44] reported a 43% saccharification yield, obtaining mostly glucose, from polysaccharides of *Ulva lactuca*, through the combination of 1-butyl-3-methylimidazolium chloride ([C4mim][Cl], ionic liquid) and choline chloride and oxalic acid (deep eutectic solvent), whereas Uju et al.[45] resorted to the combination of paracetic acid and 1-hexylpyridinium chloride ([Hpy][Cl], ionic liquid) pre-treatment with enzymatic saccharification of seaweed waste from the carrageenan industry, rendering a 91% conversion of cellulose in mainly mono- and disaccharides. Processes with hydrogen peroxide have also been studied in association with ascorbic acid (vitamin C) to treat carbohydrate extracts, such as porphyran, proving its ability to decompose complex polysaccharides to low molecular weight fractions[29, 46]. It was also used as a pre-treatment of residues of green seaweed *Ulva prolifera* prior to enzymatic hydrolysis, increasing the reducing sugar yield in 80%[47] (table 2.2) .

2.3.3 Thermal treatment

Thermal treatment, which includes processes such as heating, autoclaving, microwaving, wet oxidation and steam explosion, may also be performed for monosaccharide extraction from seaweed biomass, although it is commonly used before or alongside other type of hydrolysis in order to improve the final yield of extraction and saccharification[32].

The most commonly used thermal pre-treatments referred in literature are heating and autoclaving, which often precede biological treatment or are performed simultaneously with chemical treatments (table 2.2). Even so, microwave-assisted methods have been gaining attention due to their ability to disrupt cell walls and polysaccharide structures effectively without the formation of inhibitors such as furfural and 5-hydroxymethylfurfural[32]. Yuan et al.[48] reported a 74.1% solubilisation of *U. prolifera*

biomass, after treating a 5% (w/v) slurry in 0.1 M hydrochloric acid at 150° C under microwave irradiation (2.54 GHz) for 15 minutes. Methods involving steam explosion, wet oxidation and plasma-assisted processes are not widely represented in the literature regarding seaweed pre-treatment, since these were shown to be more energy-demanding and to produce more compounds which may inhibit microbial growth, such as furan derivatives and acetic acid[32].

2.3.4 Biological and enzymatic treatments

Biological treatment is performed with either enzymes extracted from living organisms or commercially available cocktails, or with microorganisms or fungi that are able to degrade seaweed polysaccharides. Although the efficiency of processes involving microorganisms is generally measured in terms of ethanol or methane production, while enzymatic saccharification efficiency may be quantified in terms of whole carbohydrates conversion into short chain saccharides and monosaccharides, results still show that such processes are promising. Yahmed et al.[49] showed that direct solid-state fermentation of washed *Ulva* spp. improved the biochemical methane potential (BMP) of the product when compared to washed untreated biomass from 138 to 153 mL CH_4/g volatile solids. In a similar study, Tapia-Tussell et al.[50] reported that transformation of a macroalgal consortium (mainly *Ulva* spp. and *Sargassum* spp.) with fungi (*Trametes hirsuta* Bm-2) resulted in a product with higher BMP than the same algal consortium treated with enzymes (laccase 7000 U/mL) alone, corresponding to 104 and 86 L CH_4/kg volatile solids, respectively. The combination of acid hydrolysis and bacterial treatment has also been studied by Sudhakar et al.[51], which showed that pre-treating biomass with 1% sulfuric acid (15 minutes, 121° C) before bacterial saccharification led to increased conversion of sugars to ethanol when compared with direct bacterial saccharification: an increase of 0.78 g/L with *Gracilaria corticata* subjected to biosaccharification with *Ensifer adhaerens* and *Pseudomonas geniculata* and an increase of 0.62 g/L with *Sargassum ilicifolium* subjected to biosaccharification with *Ensifer adhaerens*, *Pseudomonas geniculata* and *Sinomicrobium oceani*.

Although enzymes require adequate pH and temperatures to perform optimally, they do render high monosaccharide extraction yields, especially when paired with some of the aforementioned methods that either do a prior decomposition of the polysaccharides to smaller units or weaken the structure of said molecules and increase the area exposed to the biological treatment[43]. Note that enzymatic hydrolysis is not usually done without resorting to acid or alkaline treatment, thus the studies in this specific area are scarce. One of the few reports on this subject was done by Manns et al.[52] using cellulase (10% enzyme to substrate by weight) and alginate lyase (2% enzyme to substrate by weight) in a suspension of 5% (w/w) of *Laminaria digitata* for 8 hours, which rendered a 48% glucose extraction yield.

Nevertheless, the production of enzymatic cocktails and their use is economically challenging and time consuming, which, to this day, is not a competitive scenario when compared with thermal acid pre-treatments, for example. For this reason, methods such as mild acid hydrolysis and acid-enzyme combined methods are still in use[33], despite being far from ideal from an environmental point of

Table 2.2. Thermal, acid, alkaline, enzymatic, biological and novel methods used in the pre-treatment of red, green and brown seaweed, and final concentrations (g/L) and yield (%) of monosaccharides release. Yields are expressed as ($m_{extracted\ monosaccharides}/m_{total\ carbohydrates}$x100).

	SLR (%w/v)	Physical/chemical treatment	Saccharides (g/L)	Yield (%)	Biological treatment	Saccharides (g/L)	Yield (%)	Ref.
Thermal treatments								
L. digitata	10	121° C 20 min	—	20.1	—	—	—	[53]
U. linza	10	121° C, 15 min in seawater	4.87	2.19	—	—	—	[40]
P. umbilicalis	10	121° C, 15 min in seawater	4.69	2.12	—	—	—	[40]
Thermal acid treatments								
K. alvarezii	10	0.2 M H_2SO_4, 130° C, 15 min	38.45	—	—	—	—	[31]
G. amansii	3/5	3% H_2SO_4, 121° C, 30 min	—	80.7	—	—	—	[38]
P. umbilicalis	10	5% H_2SO_4, 121° C, 15 min	24.02 (RS)	63	—	—	—	[40]
U. linza	10	5% H_2SO_4 in seawater, 121° C, 15 min	26.23	69.2	—	—	—	[40]
L. digitata	10	5% H_2SO_4, 121° C, 15 min	20	52.8	—	—	—	[40]
L. digitata	10	5% H_2SO_4 in seawater, 121° C, 15 min	20.81	64.6	—	—	—	[40]
L. digitata	20	4% HNO_3, 130° C, 2h	33	—	—	—	—	[35]
L. digitata	10	0.1 M HCl, 2 h room temperature + 65° C, 2h	—	12.4	—	—	—	[53]
S. latissima	20	4% HNO_3, 130° C, 2h	46	—	—	—	—	[35]
Thermal acid combined with enzymatic/biological treatment								
G. amansii	12	180 mM H_2SO_4, 121° C, 45 min	32.89 (RS)	37.2	Celluclast 1.5L 16 U/mL, 36 h	45.2 (RS)	50.8	[39]
G. verrucosa	2	0.05 M H_2SO_4, 121° C, 15 min	7.47	37.4	Cellic CTec2 10% w/w, 48 h	8.53	47.2	[54]
G. verrucosa	12	270 mM H_2SO_4, 121° C, 60 min			Celluclast 1.5L + Viscozyme L 16 U/mL, 24 h	21.7 (glu)	84.2	[55]
G. verrucosa	10	0.1 M citric acid, 150° C, 60 min	—	50.9	Viscozyme L + Cellic CTec2 + Cellic HTec2 (20 FBG/g biomass, 28 FPU/g biomass), 24 h	—	57.8	[36]
G. amansii	10	0.1 N HCl, 121° C, 15 min	—	11.6	Celluclast 1.5L + Viscozyme L (0.01 g/g biomass, 1:1)	—	56.6	[41]
G. edulis	—	1% v/v H_2SO_4, 121° C, 15 min	—	—	Bacterial consortia	13.88	57.3	[51]
G. corticata	—	1% v/v H_2SO_4, 121ºC, 15 min	—	—	Bacterial consortia	9.25	30.1	[51]
S. ilicifolium	—	1% v/v H_2SO_4, 121° C, 15 min	—	—	Bacterial consortia	11.44	38.7	[51]
Sargassum spp.	10	4% H_2SO_4, 115° C, 90 min	—	—	Cellulase + β-glucosidase (50 FPU/g biomass, 250 CBU/g biomass)	17.65	42.0	[56]
Thermal alkaline treatment								
P. umbilicalis	10	5% NaOH, 50° C, 12h	3.31	7.4	—	—	—	[40]
P. palmata	5	0.04 g NaOH/g biomass	7.7	35.4	—	—	—	[57]
U. linza	10	5% NaOH, 50° C, 12h	4.63	10.3	—	—	—	[40]
L. digitata	10	5% NaOH, 50° C, 12h	3.93	10.2	—	—	—	[40]
Other treatments								
L. digitata	5	—	—	—	Cellulase (0.1 g/g biomass) + alginate lyase (0.02 g/g biomass), 8 h	—	48 (glu)	[52]
U. prolifera	5	0.1 M HCl, 150° C, 2.45 GHz (microwaves), 15 min	—	74.1	—	—	—	[48]
U. prolifera residue	—	0.2% H_2O_2, 50° C, 12 h	—	—	Cellulase + cellobiase (7.5 FPU/g biomass and 4.5 U/g biomass), 48 h	0.42 g/g biomass	—	[47]
U. lactuca	—	[C4min][Cl] + choline chloride + oxalic acid, 120° C, 6 h + 30% water 120° C, 4 h	—	40 (glu)	—	—	—	[44]
Seaweed waste	—	1.9% v/v paracetic acid, 80° C, 3h + 300 mL [Hpy][Cl], 100° C, 30 min	0.17	—	Cellulase (0.05 mg/mL), 48 h	—	91	[45]

RS - Reducing sugars; glu - Glucose; FBG - Fungal β-glucanase unit; FPU - Filter paper unit; CBU - Cellobiase unit; [C4min][Cl] - 1-butyl-3-methylimidazolium chloride; [Hpy][Cl] - 1-hexylpyridinium chloride.

view. Moreover, the concentration of monosaccharides obtained in final hydrolysates are, in general, not enough to guarantee high final product yields[40], especially from a biofuel perspective, as this is the main reported term of comparison. For a great part of the bioprocesses, hydrolysates with the reported monosaccharide concentrations would still be low on sugars and would not be a viable source of carbon if not concentrated or further processed. Therefore, there is the need to explore new hydrolysis conditions and new combinations of methods to ensure sufficient saccharification efficiencies[51].

2.4 Fermentation

Fermentation, defined as the generation of energy through anaerobic metabolism, is a technology that is known to have been used by several ancient communities, such as in Babylon and Egypt, over 7000 and 5000 years ago, respectively, for the production of beverages, as well as in the production of bread, in Egypt, 3500 years ago. Since then, fermentation has been applied to numerous sources of food, both from vegetable and animal origin, in order to preserve those that otherwise would spoil easily or to change their organoleptic properties[58, 59]. Although early fermentations were, most likely, accidental, eventually the use of starter cultures of organisms native to specific substrates would be developed, namely the isolation of pure starter cultures of *Saccharomyces cerevisiae* in British breweries and of lactic acid bacteria for milk fermentation, followed by the engineered microorganisms utilised nowadays. Along with the starter cultures, the fermentation technology concerning bioreactors, modes of operation and control of fermentation conditions were also developed to what we are familiar with today[59–61].

A wide variety of biological cultivations is now applied to the production of a wide range of compounds, such as antibiotics, hormones, growth factors and recombinant vaccines. Even so, fermentations with yeast and lactic acid bacteria remain two of the most important methods to process food all around the world[60].

2.4.1 Lactic acid fermentation

Lactic acid fermentation is performed by lactic acid bacteria (LAB), a group that includes *Lactobacillus*, *Lactococcus* and *Streptococcus*, among other genera, which convert their preferred source of carbon to pyruvate which is then converted into lactic acid. While the majority of the microorganisms in this group can be characterised as mesophilic with a preference for acidic pH values (4.0-4.5)[61], the manner in which the carbon source is metabolised allows their classification into two main categories: homofermentative and heterofermentative[59, 62]. In microorganisms that are exclusively homofermentative, the source of carbon is converted to lactic acid alone, in a ratio of 1:2 (equation 2.1); on the other hand, heterofermentative bacteria are able to metabolise the carbon source to a mixture of lactic acid, ethanol (or acetic acid) and carbon dioxide (equation 2.2). Note, however, that some LAB may be facultatively homo- or heterofermentative and that in the presence of carbon sources other than glucose the final products of fermentation will not be restricted to those mentioned above, being possible the

formation of diacetyl, acetoin and 2,3-butanediol[63, 64].

$$1\,\text{mol Glucose} \longrightarrow 2\,\text{mol Lactic acid} \tag{2.1}$$

$$1\,\text{mol Glucose} \longrightarrow 1\,\text{mol Lactic acid} + 1\,\text{mol Ethanol} + 1\,\text{mol Carbon dioxide} \tag{2.2}$$

In the food industry, lactic acid plays an important role as a generally recognised as safe (GRAS) preservative and acidulant in processed foods, added to avoid bacterial spoilage. In addition, lactate is also applied in the cosmetics industry due to its moisture retaining properties, as well as in the pharmaceutical industries due to its biocompatibility[65]. Lactic acid is also employed to synthesise the biopolymer polylactic acid (PLA). Besides lactic acid, LAB are also capable of producing other antimicrobial agents, although in lesser amounts. Acetic and propionic acids are also important additives in several food related industries, because they also act against microbial growth through the acidification of the cytoplasm, affecting transmembrane potential and, consequently, active transport of molecules across the cellular membrane. Morevover, some bacteriocins produced by this group of bacteria, such as nisin and pediocin-like molecules are also used to prevent the growth of moulds, yeasts and pathogenic bacteria, including *Clostridium* spp., *Bacillus* spp. and *Listeria monocytogenes*[61, 66].

Apart from the production of molecules that act as preservatives, lactic acid fermentation also improves the nutritional quality of the substrate. During the fermentation of vegetable feedstocks, *Lactobacillus* have been reported to reduce the concentrations of anti-nutritional factors, such as phytic acid present in flour from wheat and rye used in sourdough[66]. In addition, the decrease of the symptoms associated with lactose intolerance through digestion of this disaccharide[67], and of the gluten sensitivity in celiac disease patients through inhibition of influx of gliadin into epithelial cells, which is associated with inflammatory response[68], were also reported. Overall, lactic acid fermentation has been reported to present numerous health benefits, as reviewed by Sanlier et al.[67], which are dependent on the microorganisms performing the fermentation, as well as on the substrate being metabolised.

2.4.2 Yeast fermentation

Yeasts, available all throughout nature, have been used for millennia for baking and brewing various types of beverages (alcoholic and non-alcoholic), and more recently for nutraceuticals and biofuel production, as well as bioremediation[69, 70]. Even so, despite being widely available in nature, not all yeasts participate in fermentation for food purposes, with this role being fulfilled mainly by ascomycetous yeasts, such as *Saccharomyces* spp., and microorganisms belonging to the genus *Candida*, which are, in general, mesophiles with optimal growth in the range of 20° C to 30° C with a preference for slightly acidic environments[58]. From this group of yeasts, *Saccharomyces cerevisiae* is one that presents a high fermentative capacity and, to this day, is the most studied organism and thus the most commercially available[69].

Yeast fermentation pertains to the process that converts a carbon source, preferably glucose, fruc-

tose, sucrose, maltose or maltotriose, into ethanol and carbon dioxide (equation 2.3)[63, 69]. Alike lactic acid fermentation, this process has for long been used to both preserve and alter the palatability of food, owing to the fact that compounds such as ethanol and acetic acid, with well known antimicrobial properties, amino acid-derived alcohols (fusel alcohols), e.g. 2-phenylethanol, responsible for flowery flavours, and isoamyl alcohol, responsible for fruity flavours, acetaldehyde, esters and phenolic molecules are produced[71]. Moreover, metabolites produced during yeast fermentation have also been related to health benefits. Apart from the moderate consumption of wine being linked to reduced cardiovascular mortality, several molecules present interesting activities from a health point of view. Resulting from the conversion of tryptophan by yeasts, melatonin and serotonin play a role in circadian rhythm and sleep regulation, as well as in prevention of neurodegenerative diseases. Tyrosol, produced from tyrosine, and glutathione, abundantly present in yeasts to inactivate reactive oxygen species, present antioxidant and anti-tumoral activities[72].

$$1\,\mathrm{mol\,Glucose} \longrightarrow 2\,\mathrm{mol\,Ethanol} + 2\,\mathrm{mol\,Carbon\,dioxide} \tag{2.3}$$

Yeasts and yeast-derived products have also been utilised in animal feed for over a century for promoting growth and health. Addition of these organisms to feed has been done under the form of live yeasts (probiotics) and yeast cell wall. When alive, yeasts have been shown to improve digestibility of nutrients, gastro-intestinal microflora or stimulating enzyme production, among other effects. Yeast cell wall components showed similar results, with reported decreased mortality of populations and positive weight gains, as well as antioxidant, antimutagenic and immunostimulant activities[70].

2.4.3 Seaweed fermentation

Seaweeds, as a promising source of carbohydrates than can be converted into fermentable saccharides, have been the target of numerous studies that focus on bioethanol and biomethane production, as well as on lactic acid production as the precursor of polylactic acid (PLA), a type of biodegradable plastic, as both fuels and plastic industries are aiming to decrease their dependency on petrochemicals[34, 65].

The potential of seaweeds as a source of saccharides for lactic fermentation was studied by Hwang et al.[73], that reported the ability of seven species of *Lactobacillus* to metabolise sugars typically obtained from the hydrolysis of such biomass, namely glucose, galactose, mannose, mannitol, xylose, rhamnose, fucose and gluconate. The authors found that lactobacilli were able to use seaweed monosaccharides to produce lactic and acetic acids through fermentation with yields that are comparable to those obtained from terrestrial monosaccharide sources. Studies reporting the use of algal hydrolysates as a substrate for lactic acid are summarized in table 2.3.

Alcoholic fermentation, as mentioned before, can be carried out by a wide variety of organisms, in which yeasts are included. Even so, yeasts have a narrow range of saccharides that can be used as sources of carbon. Nevertheless, *Saccharomyces cerevisiae* has been widely applied to diverse algal substrates, to study its ability to produce bioethanol. Those studies are presented in table 2.4.

Table 2.3. Concentration of lactic acid (g/L) and lactic acid yield ($g/g_{sugars\ consumed}$) resulting from the fermentation of red, green and brown seaweeds hydrolysates with lactobacilli.

Seaweed	Pre-treatment	Fermentation	Lactic acid (g/L)	Yield (g/g)	Ref.
Gracilaria sp.	4% H_2SO_4, 120° C, 20 min	*L. rhamnosus*, 30° C, 24 h	22.50-33.82	0.79-0.90	[74]
Gracilaria sp.	0.4 N HCl, 121° C, 30 min + cellulase (7.6 U/mL), 37° C, 48 h	*L. acidophilus* and *L. plantarum* (6% v/v), 30° C, 72 h	19.32	0.19	[16]
G. amansii	3% v/v H_2SO_4, 140° C, 15 min	*L. rhamnosus*, 37° C, 6 days	12.53	0.42	[75]
Ulva sp.	4% H_2SO_4, 120° C, 20 min	Immobilized *L. plantarum*, 30° C, continuous fermentation (3 h retention time)	36.8	0.91	[76]
Ulva sp.	4% H_2SO_4, 120° C, 20 min	Immobilized *L. plantarum*, 30° C, 30 h	30.9	0.77	[76]
E. prolifera	0.5 M H_2SO_4, 120° C, 2 h	*Lactobacillus* spp., 30-37° C, 48 h	5.4-7.8	0.51-0.69	[77]
S. cristaefolium	4% H_2SO_4, 120° C, 20 min	*L. rhamnosus* and *L. plantarum*, 30° C, 24 h	9.02-11.65	0.83-0.89	[74]
Algal cake (de-oiled)	15% algal cake + α-amylase, pepsin and cellulase	*L. casei* (1% v/v), 37° C, 24 h	11.17	—	[78]

Table 2.4. Concentration of ethanol (g/L) and ethanol yield ($g/g_{sugars\ consumed}$) resulting from the fermentation of red, green and brown seaweeds hydrolysates with *Saccharomyces cerevisiae*.

Seaweed	Pre-treatment	Fermentation	Ethanol (g/L)	Yield (g/g)	Ref.
G. elegans	2% H_2SO_4, 121ºC, 30 min + meicelase, 50° C, 120 h	*S. cerevisiae*, 30° C, 24 h	55	—	[79]
Gracilaria sp.	4% H_2SO_4, 121° C, 30 min + cellulase (53 FPU/g) and β-glucosidase (30 U/g), 50° C, 4 h	*S. cerevisiae* (2% v/v), 30° C, 96 h	19.9	—	[80]
G. verrucosa pulp	cellulase (20 FPU/g) and β-glucosidase (60 U/g), 50° C, 36 h	*S. cerevisiae* (6% v/v), 30° C, 20 h	14.89	0.43	[81]
U. pertusa	meicelase, 50° C, 120 h, twice	*S. cerevisiae*, 30° C, 24 h	30	—	[79]
E. intestinalis	72mM H_2SO_4, 121° C, 60 min + Celluclast 1.5L (8.4 U/mL) and Viscozyme L (1.2 U/mL), 36 h	*S. cerevisiae* (0.2 g/L), 30° C, 48 h	8.6	—	[82]
A. crassifolia	meicelase, 50° C, 120 h, twice	*S. cerevisiae*, 30° C, 24 h	34.4	—	[79]
Sargassum sp.	3% H_2SO_4, 121° C, 30 min + cellulase (53 FPU/g) and pectinase (20 U), 50° C, 4 h	*S. cerevisiae* (2% v/v), 30° C, 96 h	28.7	—	[80]
Sargassum spp.	4% w/v H_2SO_4, 115° C, 1.5 h + cellulase (10 FPU/g) and cellobiase (250 CBU/g), 48 h	*S. cerevisiae* (0.5 g/L), 40° C, 48 h	2.79	—	[56]

2.5 Aquafeed: a developing industry

As the world's need for sources of protein increases along with the concern of a healthy lifestyle, so does the average intake of seafood. So much so that the traditional fish capture would not be able to suppress the demand for seafood without over exploring fish stocks[83].

The aquaculture sector has been under constant development since the last half of the XX century. In 2018, a total of 179 million tonnes of fish (finfish, molluscs, crustaceans, bivalves and aquatic animals excluding mammals and reptiles) were produced, from which 156 million tonnes went towards human consumption and 18 million tonnes were processed for fishmeal and fish oil. The aquaculture sector alone was responsible for 46% of total fish production on that year, with China being the main contributor, whereas Europe relied mainly on imported seafood. Along with the development of this activity, fed aquaculture became prevalent over non-fed and in 2018 was responsible for 69.4% of the total fish production by the sector[25].

According to the latest report issued by FAO[25], considering the predicted rises in population and food demand, as well as socioeconomic development, fish consumption is expected to reach 21.5 kg per capita in 2030, corresponding to 89% of the yearly production, which is expected to rise to 204 million tonnes in the same year, with aquaculture being responsible for 109 million tonnes. Although such development has been the target in the last decades, it raises concerns over the availability and price of fishmeal and fish oil.

2.5.1 Fishmeal and protein meal alternatives

Fish meal, the product from drying and milling whole fish or fish waste and by-products[25], was initially a product obtained exclusively from pelagic fish (top to mid layers of the sea), which includes several species that are part of the human diet. Despite its utilisation for fertilisation purposes, fishmeal was mainly used as a component of the feed for terrestrial animals, as it is a very nutritionally complete product, rich in essential amino acids, omega fatty acids such as DHA and EPA and a wide variety of vitamins. However, part of the captured species of pelagic fish was redirected towards the human food market, in order to both fulfil the nutritional needs of lower income individuals, which needed cheaper alternatives to the usually expensive fish, and to increase profitability. As a result, the fishmeal industry was led towards the exploitation of by-products from capture and culture fisheries that could be processed to obtain oil and protein concentrates[83]. For this reason, 25 to 35% of the world's fishmeal and fish oil stock is now obtained after repurposing those by-products (scales and skin, viscera, heads or bones)[25]. Other alternative sources of protein that have been studied over the years include plant protein, animal protein from properly processed wastes of the meat industry, such as blood, meat and bone protein, and insect protein. These might also be combined with protein from sources such as brewer's yeast[83, 84].

Plant protein concentrates have been in wide use despite their limitations, namely the presence of anti-nutrients that lead to defficient nutrient absorption and not being well-accepted by carnivorous fish, which consequently lead to a decline of both growth and health of these populations. Even so, through

careful and extensive species-specific testing, it is possible to obtain protein concentrates with a rich amino acid profile that may replace part of the fishmeal present in the feed. Nevertheless, the feedstocks utilised in the production of these concentrates are also a part of human and livestock nutrition, as well as raw materials for biofuel production, thus increasing the competition and, consequently, the price of such plants[84]. Therefore, there is still a need to either find new, more sustainable sources of protein or reach a sustainable compromise in the proportions and types of components included in aquafeed. In fact, although fishmeal was once incorporated in aquafeed in large proportions (up to 50% for marine fish, 45% for salmon and 28% for marine shrimp), the advances made in the aquafeed sector allowed to decrease the proportion in which it was incorporated in feed, reaching values as low as 26% for marine fish, 22% for salmon and 16% for marine shrimp (in 2010)[83].

2.5.2 Seaweed and fermented seaweed in aquafeed

Despite the alluring character of the aforementioned alternatives to fishmeal, limitations such as competition over arable land, fresh water and fertilisers in the case of plant protein, and insufficient availability or strict legislation forbidding the use of part of the animal by-products in aquaculture feed, led the industry to explore the potential of seaweeds as a source of nutrition in culture fishery. Considering the characteristics described in section 2.2, seaweeds gained a new appeal as an alternative protein source. In the last 40 years, a plethora of studies with several species of fish whose feed was supplemented with seaweed from various species instead of fishmeal were conducted. Kamunde et al.[85] showed that a feed containing 3% and 10% of brown seaweed *Laminaria* spp. improved not only the final weight, but also the specific growth rate, the daily weight gain and the plasma total antioxidant capacity of juveline Atlantic salmon. Abdel-Warith et al.[86], Choi et al.[87] and Ragaza et al.[88] reached similar findings through the supplementation of *Ulva lactuca* to African catfish, *Pyropia yezoensis* to olive flounder and *Eucheuma denticulatum* to Japanese flounder, respectively. A more extensive review of the studies carried out until recently has been done by Wan et al.[89]. Overall, addition of seaweed to aquafeed in a proportion of up to 15% leads to an improvement of fish growth and protein efficiency ratio, as well as of fish health, despite this improvements being highly species dependent.

The major issue raised by the utilisation of seaweeds is, in fact, the abundancy of complex carbohydrates that especially carnivorous species do not have the enzymatic machinery to digest. As mentioned in section 2.3, it is possible to process algal biomass in order to break down complex polysaccharides to mono- and oligosaccharides. These molecules can then be used by the fish for energy or play a role in health through their bioactive properties, namely anti-inflammatory, anti-oxidant, anti-tumoral and anti-cholesterol activities, among many others[9]. Even so, a better exploitation of the available carbohydrates and improvement the nutritional quality of the macroalgae can be achieved through fermentation with bacteria, yeast or fungi[89]. Nonetheless, the number of studies concerning this particular subject is limited, owing to the fact that algal fermentation has been explored mainly from the perspective of biofuel production.

The available research reports similar results in terms of improvement of nutritional quality of sea-

weed. Felix and Brindo[90] showed that fermentation of *Kappaphycus alvarezii* with *Lactibacillus* spp. and *S. cerevisiae* resulted in an increase of protein content (14.0% to 23.9%) and decrease in dietary fibre (18.5% to 5.2%), when compared to the raw seaweed. In addition, replacement of 10% of the feed with the fermented red macroalgae was proven to improve digestibility, as well as the intake of feed, consequently increasing the specific growth rate, weight gain and protein efficiency in giant freshwater prawn (*Macrobrachium rosenbergii*) when compared to a control feed or a replacement of feed with raw seaweed in the same proportions. The same authors used a similar method to explore the potential of green seaweed *Ulva lactuca*[91] and brown seaweed *Padina tetrastomatica*[92]. After fermentation, the protein and fibre content of *U. lactuca* increased from 21.0% to 30.4% and decreased from 19.6% to 2.1%, respectively, while for *P. tetrastomatica* protein content increased from 10.5% to 15.9% and fibre content decreased from 24.0% to 3.6%. Similarly to what was observed with *K. alvarezii*, digestibility, feed intake, weight gain, specific growth rate and protein efficiency improved when feed was replaced with 30% fermented *U. lactuca* or 10% fermented *P. tetrastomatica*. Note, in addition, that although the referred proportions are those that resulted in better fish growth, incorporation of up to 30% of all three fermented seaweeds showed better growth parameters than the control feed and raw seaweed incorporated feeds. Hardjani et al.[93] showed that fermentation of *K. alvarezii* with *S. cerevisiae* increased protein and lipid content from 8.24% to 9.13% and 0.27% to 0.29%, respectively, despite no significant change was found in the amount of carrageenan, which may be of interest as an immunostimulant for white shrimp (*Litopenaeus vannamei*). Ilias et al.[94] explored solid state fermentation of *Sargassum fulvellum* and palm kernel cake with the fungi *Phanerochaete chrysosporium* and *Candida utilis*, which rendered an increase of protein content from 37.5 mg/g substrate to 53.3 mg/g substrate, in addition to a decrease of 445.9 mg/g substrate in carbohydrates.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Chemicals and enzymes

The chemicals used were calcium carbonate (Merck), dextrose monohydrate (COPAM, Portugal), D(+)-galactose (>98%, Carl Roth GmbH + Co. KG), di-ammonium hydrogen citrate (Chem-Lab), di-sodium hydrogen phosphate anhydrous (PanReac AppliChem), glycerol (ACROS Organics), hydrochloric acid 37% (w/w) (Honeywell Fluka), 5-hydroxymethylfurfural (Biosynth Carbosynth), magnesium sulfate heptahydrate (LabChem), manganese (II) sulfate monohydrate (Sigma-Aldrich), phenol (Merck), potassium di-hydrogen phosphate (PanReac), sodium chloride (Fisher Scientific) and sodium hydroxide (Fisher Scientific).

The enzymatic cocktail utilised was Viscozyme L, from Sigma-Aldrich (130.7 FBGU/mL).

3.1.2 Algal biomass

The seaweed used in this project, milled *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.

3.1.3 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), kindly supplied by Prof. Gabriel Monteiro (iBB-IST), were utilised for their capability to metabolise different monosaccharides released during hydrolysis of algal biomass. *Saccharomyces cerevisiae* SafAle^{TM} US-05, kindly supplied by Dr. Margarida Palma (iBB-IST), was added to part of the fermentations to possibly improve protein content.

All microbial strains were maintained in cryovials at -80° C. Cryovials were restocked as needed by culturing each individual strain in its preferred growth medium (MRS broth for *Lactobacillus* and YPD medium for *S. cerevisiae* - see section 3.2.1) for 16 to 18 hours and distributing the cell broth through 2 mL cryovials containing 0.3 mL of sterile glycerol. The final optical density (600 nm) of the culture was registered in all occasions.

3.2 Culture media

3.2.1 Medium composition for the inoculum

De Man, Rogosa and Sharpe (MRS) broth (20 g/L D(+)-glucose, 2 g/L di-ammonium hydrogen citrate, 0.2 g/L magnesium sulphate, 0.05 g/L manganese (II) sulphate, 2 g/L di-potassium hydrogen phosphate, 5 g/L sodium acetate, 1 g/L Tween 80, 8 g/L meat extract, 4 g/L yeast extract and 10 g/L bacteriological peptone) was obtained from PanReac AppliChem, ITW Reagents.

Yeast extract-Peptone-Dextrose (YPD) broth was made as needed with 20 g/L bacteriological peptone, 10 g/L yeast extract (ThermoFisher Scientific) and 20 g/L dextrose (COPAM, Portugal).

3.2.2 Medium composition for growth assays

The media prepared to follow the growth of *Lactobacillus* spp. and *S. cerevisiae* was composed of 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, 0.4 g/L magnesium sulphate heptahydrate, a buffer solution (4.5 g/L di-sodium hydrogen phosphate dihydrate and 1.5 g/L potassium di-hydrogen phosphate). D(+)-galactose at a concentration of 15 g/L was used as the carbon source (from a stock solution of 100 g/L).

Addition of 5-hydroxymethylfurfural to obtain a final concentration of 1 g/L in assays with *Lactobacillus* or 0.5 and 1 g/L in assays with *S. cerevisiae* was done from a previously sterilised stock solution (21.15 g/L).

3.2.3 Medium composition in shake flask cultivations

An alternative medium, based on MRS broth, was formulated to eliminate the presence of meat extract and peptone extract and thus decrease costs when scaling up the process. This medium was composed of 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, 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). An algal hydrolysate (approximately 838 mL/L culture medium, containing 1.1±0.1 g/L glucose and 14.9±1.2 g/L galactose) and D(+)-galactose (approximately 31.8 mL/L culture medium from a 100 g/L stock solution) were used as a carbon sources.

3.2.4 Medium composition for the bioreactor cultivations

In experiments with 2 L bioreactor, the culture medium was composed of: 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*).

3.3 Biomass characterisation

3.3.1 Total solids, moisture and ash

The determination of the seaweed's composition in total solids and ash was done, in triplicate, according to the protocol provided by the National Renewable Energy Laboratory (USA), NREL 60956[95]. Crucibles were conditioned at 60° C for 24 hours and weighed (HR-251AZ, A&D). *Porphyra umbilicalis* was weighed in the crucibles (100±5 mg), which were placed in a constant climate chamber at 60° C during 24 hours, period after which they were weighed again. The composition in total solids (TS) and moisture (M), as well as the samples oven dry weight (ODW) were determined through equations 3.1, 3.2 e 3.3.

$$\%TS = \frac{m_{crucible+dry\ sample} - m_{crucible}}{m_{initial\ sample}} \times 100 \tag{3.1}$$

$$\%M = 100 - \%TS \tag{3.2}$$

$$ODW = \frac{m_{sample} \times \%ST}{100} \tag{3.3}$$

The ash content of the samples was determined according to the same protocol, with alterations to the temperature program. To that end, the crucibles containing the dried seaweed were inserted into a furnace (3/11/B180 L-030K1CN, Nabertherm) and submitted to the following ramp temperature program: (1) heating to 105° C for 30 minutes, (2) maintain 105° C for 12 minutes, (3) heating to 250° C for 15 minutes, (4) maintain 250° C for 30 minutes, (5) heating to 600° C for 25 minutes, (6) maintain 600° C for 16 hours. The crucibles were weighed after cooling down to room temperature in a desiccator. The seaweed's content in ash was determined through equation 3.4.

$$\%Ash = \frac{m_{crucible+ash} - m_{crucible}}{ODW_{sample}} \tag{3.4}$$

3.3.2 Total carbohydrates

The quantification of the total carbohydrates content was perfomed using two distinct methods.

For the first method, based on the protocol provided by the National Renewable Energy Laboratory (USA), NREL 60957[96], 0.5 g of *P. umbilicalis* were weighed (HR-251AZ, A&D) into a 100 mL Schott, in triplicate, to which were added 2.5 mL of 72% (w/w) sulphuric acid. The suspension was incubated at 30° C and 120 rpm (orbital agitation in Agitorb200, ARALAB) for 1 hour. Next, 69.2 mL of distilled deionized water were added to dilute the acid to 4%, and the suspension was autoclaved at 121° C for 1 hour. After cooling to room temperature, the pH of 10 mL aliquots of the suspensions was adjusted to a value between 6 and 8 (Metrohm pH meter) with calcium carbonate, followed by centrifugation at 1932×g (5810R with F-34-6-38 rotor, Eppendorf) for 10 minutes and samples were collected and processed to undergo HPLC analysis.

For the second method, based on the phenol-sulphuric acid assay developed by Du Bois[97] and its variation used by Alavijeh et al.[98] for the analysis of microalgae, 10 mg of *P. umbilicalis* were weighed (HR-251AZ, A&D) into test tubes, in triplicate, to which were added 5 mL of 2.5 M hydrochloric acid. The suspensions were incubated at 100° C for 3 hours and were then neutralized using 5 mL of 2.5 M sodium hydroxide. Samples of 500, 250 and 167 μL were collected to obtain dilutions of 1:2, 1:4 and 1:6 in a total volume of 1 mL. Next, 500 μL of a solution of 5% phenol and 2.5 mL of concentrated sulphuric acid were added to the samples and these were let rest for 10 minutes and cooled to room temperature. The absorbances of the samples were read at 430 nm (UH5300, HITACHI) and the concentrations of total sugars were determined through a calibration curve established for glucose with concentrations between 0 and 0.1 g/L. The calibration curve was obtained through dilution of a 1 g/L standard solution of glucose into five 1 mL samples with the concentrations 0.01, 0.02, 0.05, 0.08 and 0.1 g/L, in test tubes, which were processed as described above for absorbance reading. The calibration curve can be consulted in Appendix A (section A).

3.3.3 Total nitrogen and protein content

Total nitrogen of *Porphyra umbilicalis* hydrolysates was determined in the laboratory using Laton LCK 338 cuvette tests 20-100 mg/L (Hach Lange™), which are based on a first step of Koroleff digestion with peroxydisulphate for 15 minutes in an HT200S thermostat (Hach Lange™), followed by reaction of nitrate ions with 2,6-dimethylphenol and photometric detection with a DR3900 spectrophotometer (Hach Lange™). Hydrolysates of *Porphyra umbilicalis* were diluted 80 times prior to digestion in the kit. This dilution factor was calculated based on the total nitrogen content of the seaweed, previously determined as 6.87 $mg_{nitrogen}/g_{seaweed}$, to obtain a total nitrogen concentration within the range of detection of the kit (20-100 mg_N/L). It is important to refer that dilutions were carried out with sterilised demineralised water to avoid quantification of nitrogen from organic matter other than from the sample. Total nitrogen at the end of fermentation was determined after a 1:160 dilution of the sample. In the cases where the sample was a pellet, obtained after centrifugation (Sigma 1-15P, 9168×g, 5 minutes) and washing with sterilised distilled de-ionized water (ddH_2O), 15±0.1 mg were suspended in 5 mL of ddH_2O before

digestion. In parallel, the dry weight of the pellet was determined following the protocol in section 3.3.1, with conditioned weighed eppendorfs and approximately 50 mg of sample pellet.

Protein determination in *Porphyra umblicalis* was also performed at *Instituto Português do Mar e da Atmosfera* (IPMA), partner in the project, via the elemental nitrogen analyser FP-528 DSP (LECO), using a nitrogen to protein conversion factor of 4.59[99]. Through this method, total nitrogen in *Porphyra umbilicalis* was determined to be 6.87 mg/$g_{seaweed}$. Additional 200 mL samples of two small-scale fermentations and of the three fermentations in reactor were sent to IPMA to undergo protein quantification, bioaccessibility and bioactivity analysis (see section 3.3.4).

3.3.4 Protein quality

Protein quality was assessed by IPMA in terms of bioaccessibility (digestibility) and antioxidant, reducing power and chelating properties. Bioaccessibility was determined *in vitro* through digestion of 1.5 to 2.0 g of a sample in conditions similar to those found in the digestive tract of fish, in terms of digestive fluids compositon, retention times in pivotal organs and temperature. The digested samples were placed in ice and centrifuged (2750×g for 10 minutes at 10° C) to separate the bioaccesible (BIO) and non-bioaccessible fractions, which were subjected to total protein determination in a FP-528 DSP analyser (LECO)[100]. Protein bioaccessibility was then determined through equation 3.5.

$$\%Bioaccessibility = \frac{Total\,protein\,in\,BIO\,fraction}{Total\,protein\,before\,digestion} \times 100 \tag{3.5}$$

Antioxidant activity of the samples was determined via DPPH and ABTS radical scavenging assays, reducing power was determined in a ferric reducing antioxidant power (FRAP) assay, and chelating activity was determined for ferrous (Fe^{2+}) and copper (Cu^{2+}) ions.

3.3.5 Lipid content and profile

Lipid content and profile was determined by project partner IPMA, based on the experimental work of Cohen, Vonshak and Richmond[101]. Dried algal biomass samples (300 mg) were transmethylated with 5 mL of an acetyl chloride:methanol (1:19 v/v) solution, heated to 80° C for 1 hour. Distilled deionised water (1 mL) and n-heptane (2 mL) were added prior to centrifugation (5000 rpm for 5 minutes) and the moisture from the resulting supernatant was removed with anhydrous sodium sulphate. A sample of the supernatant was collected and subjected to gas chromatography for fatty acid methyl ester (FAME) analysis. Fatty acid methyl esters were identified through comparison of retention times with those of Sigma standards and quantified through peak area ratio and adequate software (Varian)[102].

3.4 Hydrolysis

3.4.1 Optimization of hydrolysis conditions

The hydrolysis of *Porphyra umbilicalis* was tested under several conditions, which included variable time and sulphuric acid concentration, as well as in the presence or absence of salt (sodium chloride). The concentration of seaweed was maintained at 100 g/L (10% SLR) in a volume of 50 mL. All the assays were performed in duplicate.

First, 5 g of *P. umbilicalis* were weighed into 100 mL Erlenmeyers, to which were added 50 mL of either distilled deionised water or sulphuric acid (1%, 3% or 5% (w/w)). The Erlenmeyers were closed with rubber stoppers (perforated with a needle to alleviate pressure) and the suspensions were autoclaved at 121° C for 15 minutes. This procedure was repeated, increasing the time of hydrolysis to 30 minutes. The influence of the presence of salt (35 g/L NaCl) was studied for an hydrolysis time of 30 minutes, maintaining all the remaining conditions.

The best hydrolysate (5% sulphuric acid, 30 minutes, no salt) was then submitted to enzymatic hydrolysis with Viscozyme L. For that purpose, the pH of the acid hydrolysate was adjusted to a value between 4.5 and 5 and the enzymatic cocktail was added in a ratio of 0.2 $g_{cocktail}/g_{seaweed}$ (826 μL Viscozyme L), corresponding to an activity of 2.16 $FBGU/mL_{reaction\ medium}$, and the suspensions were incubated at 50° C and 600 rpm (magnetic agitation) for 30 hours.

In the acid hydrolysis assays, samples of 1 mL were collected immediately after the addition of water or sulphuric acid solutions to the seaweed (t=0 h), and upon reaching room temperature after exiting the autoclave, in order to determine the concentrations of monosaccharides and any other identifiable compounds via HPLC. During the assay of enzymatic hydrolysis, samples were collected after the addition of Viscozyme L and every 2 hours, except during the night. These samples were collected and immediately iced and processed for HPLC analysis in order to avoid further polysaccharide degradation by the enzymes.

3.4.2 Hydrolysis for medium preparation

Hydrolysates of *P. umbilicalis* to be used as a component of fermentation broth were prepared using the most favourable conditions determined in section 3.4.1, namely 100 g/L of algal biomass in a solution of 5% (w/w) H_2SO_4 (without salt) subjected to 121° C for 30 minutes. For small scale batch and fed-batch fermentations, 167.5 mL or 251.3 mL of hydrolysate, respectively, were prepared. Two 200 mL flasks or one 500 mL flask were used, respectively, in order to maintain enough headspace in the vessel during the autoclaving step. Similarly, 1507.5 mL of hydrolysate for the bioreactor assays were prepared in a 3 L Erlenmeyer flask.

The adjustment of hydrolysates pH was done either with a 15 M NaOH solution, in the case of small scale assays, or with NaOH pellets, in the case of the scaled-up hydrolysis. In order to obtain hydrolysates with pH similar to that required at the beginning of each fermentation and avoid dilution of the culture media by addition of acid or base solutions, any small adjustments were done with a 1 M

NaOH solution when pH was close to a value of 6.2 (starting pH for cultures with *Lactobacillus*) or 5.5 (initial pH of cultures where *S. cerevisiae* was added prior to *Lactobacillus*).

3.5 Growth curves of fermentative microorganisms

Growth of *L. brevis*, *L. casei*, *L. plantarum* and *L. rhamnosus*, as well as of a mixture of the four *Lactobacillus* (LAB mix), in medium containing 15 g/L of galactose or 15 g/L of galactose and 1 g/L of 5-hydroxymethylfurfural were followed.

Pre-inocula (PI) were prepared for each bacterial strain using a 50 mL flask containing 37.5 mL MRS broth and 4 cryovials of *Lactobacillus brevis* and 25 mL flasks containing 19 mL MRS broth and 2 cryovials of *Lactobacillus casei* or 1 cryovial of *Lactobacillus plantarum* or *Lactobacillus rhamnosus*. These were incubated at 37° C and 100 rpm (orbital agitation, Agitorb200, ARALAB) for 16 to 18 hours. The optical density of each culture was measured at 600 nm (UH5300, HITACHI) and the needed volume of each PI was harvested to start the growth assay with a $OD_{600\ nm}$ of 0.5 (for each strain). The needed volume of each inoculum was transferred to a common sterile Falcon, that was then centrifuged at 4347×g (5810R with F-34-6-38 rotor, Eppendorf) and 4° C for 15 minutes. The pellet was kept in ice until further use.

Media prepared as described in section 3.2.2, containing galactose and 5-HMF in the aforementioned concentrations were inoculated with the pelleted cells and incubated at 37° C and 100 rpm (orbital agitation, Agitorb200, ARALAB) for approximately 26 hours. Samples (800 μL) were collected every hour and the pH (Metrohm pH meter), $OD_{600\ nm}$, sugar consumption and lactic acid production followed.

The growth curves for *Saccharomyces cerevisiae* in medium containing 15 g/L of galactose and either 0, 0.5 or 1 g/L of 5-HMF were obtained after preparing the respective pre-inocula in 250 mL flasks containing 50 mL YPD broth and 5 cryovials, which were incubated at 28° C and 200 rpm (orbital agitation, Agitorb200, ARALAB) for 16 to 18 hours. The culture medium utilised for the cultures, as well as all the procedures and analytical methods, were the same as the ones used for the *Lactobacillus* spp. growth curves.

3.6 Shake flask fermentations

3.6.1 Batch fermentation

Batch fermentations were performed for 69 hours at 37° C and 100 rpm (orbital agitation, Agitorb200, ARALAB) in 250 mL flasks with a working volume of 200 mL, using the alternative medium described in section 3.2.3, with an initial pH of approximately 6.2, and an inoculum containing a mixture of *L. brevis*, *L. casei*, *L. plantarum* and *L. rhamnosus* (LAB mix) or LAB mix combined with *S. cerevisiae*. In both cases, after inoculation of the culture medium, each species presented an $OD_{600\ nm}$ of 0.5.

Samples (850μL) were collected from the hydrolysate, from the medium prior to inoculation and several times during the assay to determine pH (Metrohm pH meter), as well as sugar consumption

and organic acids and ethanol production via HPLC. In addition, the total nitrogen content of the whole sample and of the solid phase of the culture was determined at 0 and 69 hours (additional sample of 2 mL).

3.6.2 Fed-batch fermentations

Fed-batch fermentations were done in 500 mL flasks with a working volume of 300 mL, to minimise the change of the gas/liquid area when addind pulses of a concentrated galactose solution. The culture was performed for 69 hours at 37° C and 100 rpm (orbital agitation, Agitorb200, ARALAB) using the same medium and the same type of inocula described for batch fermentation. A single pulse of 17 mL D(+)-galactose at a concentration of 100 g/L was given at 25 hours of fermentation, when galactose consumption hit a plateau, as to increase its concentration to approximately 10 g/L in the broth.

Because the growth of yeast was limited in the assay described above, another fed-batch culture was carried out, where yeast was inoculated first and aftersome time LAB inoculum was added. This strategy allowed the yeast to grow without being inhibited by the metabolites produced by the bacteria. The same culture medium, with an initial pH of approximately 5.5, was inoculated with *S. cerevisiae* (to meet an $OD_{600\,nm}$ of 0.5), and the flasks were maintained at 28° C and 200 rpm (orbital agitation). At 25 hours of fermentation, when galactose concentration was estimated to be close to 5 g/L, the medium was inoculated with LAB mix (to meet an $OD_{600\,nm}$ of 0.5 for each strain) and a pulse of 40 mL of galactose (100 g/L) was given. The assay continued until no more galactose was being consummed.

In both assays samples (850 μL) were collected from the hydrolysate, from the medium prior to inoculation and several times during the assay to determine pH (Metrohm pH meter), as well as sugar consumption and organic acids and ethanol production via HPLC. In addition, the total nitrogen content of the whole sample and the solid phase of the culture was determined at the time of inoculation and the final time of culture (additional 2 mL sample).

3.7 Fermentation scale-up

Scale-up was done using 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.

The inoculum for these fermentations was prepared by growing the four LAB separately. One cryovial of each lactobacilli was added to 19 mL of MRS broth and allowed to grow overnight. The cell concentration ($OD_{600\,nm}$) of each culture was measured and these cultures were used to inoculate other flasks with fresh medium (76 mL or 190 mL, for strains that had higher and lower OD at 600 nm, respectively). A 10% (v/v) inoculum was used. After 16 to 18 hours, the optical densities (600 nm) of all four cultures were registered and the volume necessary to obtain an $OD_{600\,nm}$ of 0.2 after inoculation of the reactor was collected into 50 mL Falcon, which were kept in ice until further use. The tubes were then centrifuged at 4° C and 4355×g for 15 minutes, the supernatant was carefully discarded, and the pellets

dissolved in a total of 39 mL of 0.85% (w/w) NaCl. All the Falcon content was tranferred into a sterile syringe attached to a sterile clamped tube. The same process was done for the yeast inoculum, using the adequate culture medium (YPD broth) for the assays in which the microorganism was used.

The pH probe was calibrated, the reactor was assembled, filled with 1.5 L of distilled water and autoclaved along with all the additional material (tubes, connectors, syringes and flasks) for 20 minutes at 121° C. The hydrolysate prepared beforehand as described in section 3.4.2 was pumped into the reactor along with all the remaining components of the culture medium (see section 3.2.4) after the reactor-filling water was removed. The control loops for pH (set-point of 6.2 maintained with 5 M NaOH and 5 M HCl), stirring (set-point of 200 rpm in cascade with pO_2) and dissolved oxygen (set-point of 5%) were defined. Lastly, when fermentation pH and temperature (37° C) were stable and the medium saturated with oxygen, the dissolved oxygen probe was calibrated to 100%. The temperature during the fermentation was maintained at 37° C using a jacket with circulating water at approximately the same temperature (B. Braun Melsungen AG Thermomix^{R}BU circulator coupled with a Frilabo water bath) and the aeration was constant all throughout the assay at either 1 vvm for batch or 0.5 vvm for fed-batch procedures. A sample of the culture medium was collected.

The reactor was inoculated using the previously prepared syringe. A sample of the culture medium was collected immediately after inoculation. Culture sampling occurred every two hours during the fermentation time to analyse sugar consumption and metabolite formation.

Batch fermentation was performed for a period of 46.3 hours while fed-batch fermentation occured for 116.7 hours, until no further galactose consumption was detected. In the last case, solid galactose was fed to the bioreactor at 28.2 hours (54.5 g) and at 50 hours (112.3 g) of culture. This occurred when the sugar concentration was below 10 g/L and in such a way to guarantee that cells would have enough galactose overnight. Note that solid galactose (sterilised with UV light) was used instead of a concentrated galactose solution (maximum galactose solubility = 100 g/L) to avoid diluting the bioreactor culture.

An additional fed-batch fermentation with *S. cerevisiae* and LAB mix added to the reactor at different times was performed in a total of 145.7 hours. The reactor was prepared as described above and the initial conditions were changed to a pH of 5.5, 28° C and aeration at 1 vvm, while maintaining other parameters unaltered. The inoculum of *S. cerevisiae* was prepared following the same process as above, in order to obtain an initial $OD_{600\,nm}$ of 0.8 in the reactor. The first feed of galactose (80.6 g of powdered galactose simultaneously to 110 mL of a 100 g/L galactose solution) was done at 28.45 hours, concurrently with the inoculation of LAB mix (to meet an $OD_{600\,nm}$ of 0.2 for each strain of *Lactobacillus* sp.); the second and third supplementations of galactose were done at 51.9 and 71.2 hours, respectively, with 50 g of galactose and 100 mL of a galactose solution. Note that during this fermentation feeds were given as a mixture of powdered and dissolved galactose to avoid an excessive decrease in the volume of the fermentation broth caused by the collection of samples, which could compromise the pH and pO_2 probes.

In all fermentations sample harvesting was done by discarding 5 mL of the fermentation broth, followed by collection of 5 mL of the fermented product. From those 5 mL, 850μL were processed for HPLC

analysis and 2.5 mL (2 mL + 1.5 mL eppendorfs) were kept at -20° C for possible further total nitrogen analysis.

3.8 Sample analysis via High Performance Liquid Chromatography (HPLC)

Quantification of glucose, galactose, lactic acid, acetic acid, glycerol, ethanol and 5-HMF was done via HPLC (Hitachi LaChrom Elite) with a Rezex^{TM} 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° C with a Croco-CIL 100-040-220P (40x8x8 cm, 30-99° C) external heater. Elution of injected samples was done with 5 mM H_2SO_4.

Samples collected for HPLC analysis were centrifuged (1-15P minicentrifuge, Sigma) at 9168×g for 5 minutes and the supernatant was collected - if not processed immediately, it was frozen (-20° C) until further use. In order to avoid injection of proteins into the equipment, 200 μL of the supernatant were added to 200 μL 50 mM H_2SO_4, vortexed and centrifuged again in the same conditions. The new supernatant was then diluted in a ratio of 1:10 with 50 mM H_2SO_4 in HPLC vials (100μL supernatant and 900μL acid).

Conversion of the areas of the identified peaks into concentrations was done through calibration curves obtained prior to sample analysis. An example chromatogram, compound retention times and calibration curves can be found in Appendix B.

Chapter 4

Results and Discussion

4.1 Characterisation of *Porphyra umbilicalis*

The algae, obtained from the supplier already dried and reduced to fine particles, was characterised in terms of moisture, total solids and ash content, as well as in terms of carbohydrates, protein and lipid content. The results for the mentioned analyses are presented in table 4.1.

Table 4.1. Proximate composition of dried *P. umbilicalis*. [a]Total carbohydrates determined based by phenol-sulphuric acid method; [b]Total carbohydrates determined based on the protocol NREL 60957; [c]Protein content determined by IPMA as total nitrogen, converted using a factor of 4.59; [d] Lipid content determined by IPMA. The results are expressed as average±standard deviation (n=3).

	Content
Moisture (%)	6.2±0.3
Total solids (%)	93.9±0.3
Ash (%DW)	10.8±0.3
Total carbohydrates (%DW)	27.6±1.7 [a]
	51.6±1.7 [b]
Galactose	46.8±1.3 [b]
Glucose	3.1±0.2 [b]
Protein (%DW)	34.5±0.3 [c]
Lipids (%)	1.4±0.1 [d]

The content of total carbohydrates of the seaweed was determined using two distinct methods: total hydrolysis followed by HPLC analysis and phenol-sulphuric acid method. Although the latter is widely used, the amount of total sugars quantified through this procedure was nearly half (27.6±1.7% DW) of those quantified via the protocol provided by NREL (51.6±1.7% DW). Both assays were repeated and the results remained consistent. Due to the complex character of the hydrolysate of *Porphyra umbilicalis*, it is possible that some interference occurred in the form of parallel reactions. In addition, the product of hydrolysis is a mixture of monosaccharides and, most likely, di- and oligosaccharides, which do not react with sulphuric acid and phenol in the same manner, leading to either underestimation or overestimation of the total carbohydrate content. Lastly, the monosaccharide that was utilised for the calibration curve (glucose) might not be the most adequate for the sample under analysis. Although the incomplete

degradation of polysaccharides also represents an issue for HPLC quantification of monosaccharides, the total carbohydrate content of *P. umbilicalis* calculated through this procedure was closer to the values indicated by Morrissey et al.[21] for this algae species (50 to 76% in a dry weight base), by Dawczynski et al.[8] for *Porphyra spp.* (48.6±5.9% DW) and by Murata and Nakazoe[12] for the *Porphyra* complex, which reported that the dried seaweed contained 39.5% carbohydrates and 1.8% fibre in its composition, corresponding to a total sugar content of 46.5%DW. Note, however, that the value reached in this study might be underestimated, since only the peaks for glucose and galactose were identified. Mannose and xylose are also reported to be present in the cell walls of *Porphyra* under the form of β-1,4-linked mannan and β-1,3-linked xylan[27]. These sugars might also be present in the hydrolysate although in lower concentrations. Even so, the prevalence of galactose (46.8±1.3% DW) over any other monosaccharides translated the presence of copious amounts of porphyran in the constitution of the red seaweed.

The protein content of dried macroalgae, analysed by IPMA, was similar to that reported in the aforementioned literature: 15-37% indicated by Morrissey et al.[21] specifically for *Porphyra umbilicalis*, and similar to the protein contents reported by Dawczynski et al.[8] and Murata and Nakazoe[12] for species of the genus (31.3±7.3% and 38.8%, respectively, in a dry weight base). Regarding the determined ash content of 10.8±0.3%, despite lower than that reported on other seaweeds (see table 2.1 in section 2.2.1), it is within the values reported for *Porphyra* spp. (7 to 21%[15]). Even so, the mineral content of seaweed is dependent of the conditions surrounding the alga during its development, rendering these fluctuations foreseeable. Lastly, according to the same studies, the determined lipid content (1.4±0.1%) is also within the values usually found in *Porphyra* genus, which are generally below 2.5%.

4.2 Hydrolysis

4.2.1 Acid hydrolysis

The acid hydrolysis performed with different concentrations of sulphuric acid rendered, after HPLC analysis, several peaks with significant areas. The concentrations of the identified monosaccharides that were calculated through calibration curves obtained for each one (Appendix B), are presented in figure 4.1. It is also important to compare the total concentration of sugars released during hydrolysis (sum of the detected and identified monosaccharides) with the concentration of inhibitors formed in the process, namely 5-HMF (figure 4.1).

Considering the results obtained for seaweed hydrolysis in distilled deionized water, the total concentration of sugars released in the hydrolysate is consistently higher for hydrolysis performed for 30 minutes at 121° C, and increases significantly with the increase in the concentration of acid. Therefore, the best condition in terms of monosaccharide concentration was 30 minutes and 5% sulphuric acid, which rendered a final concentration of glucose, galactose and 5-HMF of 1.05±0.04, 14.7±0.4 and 0.88±0.04 g/L.

The effect of the presence of salt at a concentration similar to that of seawater (3.5% w/w) was tested, since this condition had recently been reported to increase significantly the yield of monosaccharide

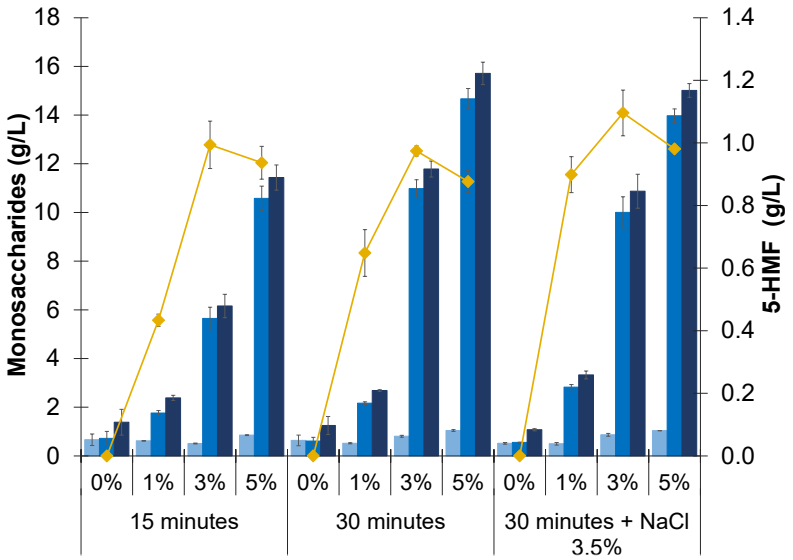

Figure 4.1. Concentration of glucose (■), galactose (■), total sugars (■) and 5-HMF (◆) obtained after acid hydrolysis with sulphuric acid (0%, 1%, 3% or 5% (w/w)) for a period of either 15 or 30 minutes, in the presence and absence of salt (3.5% (w/w) NaCl). The results are expressed as average±standard deviation (n=2).

release during dilute acid hydrolysis [40]. Apart from possibly increasing sugar release, the success of this condition could also suggest that seaweed could conceivably be hydrolysed after less steps of washing to remove salt, which would result in a more sustainable scenario from a perspective of fresh water utilisation. However, the presence of sodium chloride at a concentration of 3.5% not only decreased the amount of glucose and galactose released, but also increased the amount of 5-HMF formed during the process. In what was determined as the best condition (5% sulphuric acid, for 30 minutes at 121° C), the final concentrations of glucose and galactose decreased from 1.05±0.04 to 1.04±0.00 g/L and from 14.7±0.4 to 14.0±0.3 g/L, respectively, while 5-HMF increased from 0.9±0.04 to 1.0±0.02 g/L. For this reason, this condition was not applied in further hydrolyses.

Additionally, for concentrations of sulphuric acid higher than 3%, the concentration of 5-HMF formed during the process does not increase, as opposed to what happens for lower concentrations. It was expected that the concentration of the inhibitor would be greater for more aggressive acidic conditions, however it was not possible to identify a peak in the obtained HPLC spectra. The apparent decrease of 5-HMF release could be explained by its conversion to levulinic acid, one of the products of decomposition of 5-HMF when exposed to high temperatures and acidic conditions [103, 104].

4.2.2 Acid hydrolysis and enzymatic pre-treatment

In order to possibly increase the concentration of monosaccharides in the hydrolysate, an additional step of enzymatic treatment after acid hydrolysis (5% sulphuric acid for 30 minutes at 121° C) was tested with Viscoyme L, a carbohydrase cocktail composed of cellulase, hemicellulase, β-glucanase, arabanase and xylanase. This additional step resulted in an increase of 33.0% and 11.8% in the concentrations of glucose and galactose, respectively, after 30 hours of enzymatic hydrolysis, which translates to a 12.7% increase in the release yield of total sugars. Although such results are similar to

those registered in the literature (table 4.2) for red seaweed, most of the galactose (61.5%), considering the composition determined in section 4.1, remained to be extracted. For this reason, the enzymatic treatment was not considered sufficiently effective. The low degree of extraction of galactose is most likely related to the structure of porphyran, the main structural polysaccharide in *P. umbilicalis*, which, although similar to agarose, cannot be digested completely by agarases, rendering several sulphated and non-sulphated oligosaccharides [105, 106]. Note that the presence of cellulase in the cocktail was not expected to be effective, since this *Porphyra* sp. does not have cellulose as a major component, which is often replaced by xylan and/or mannan [27, 107, 108]. The most efficient enzyme cocktail in this process would ideally contain β-porphyranase, which proved to produce the shortest saccharides and to degrade the majority of porphyran structure [106].

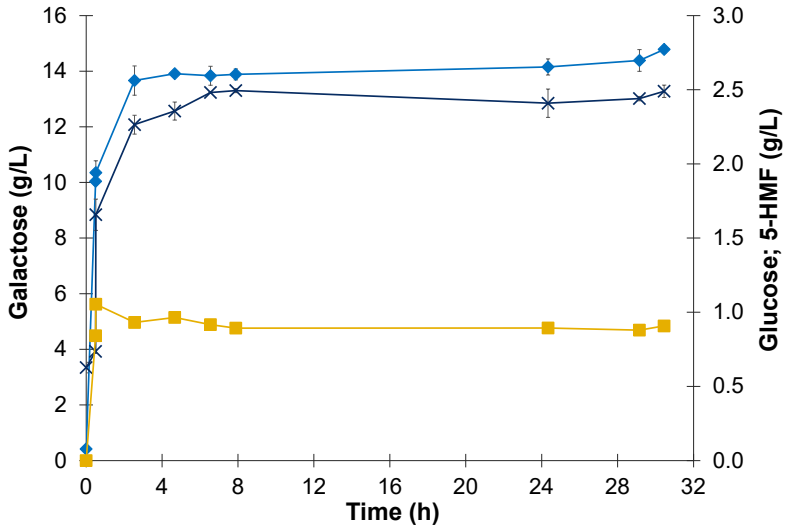

Figure 4.2. Evolution of the concentration of glucose (×), galactose (◆) and 5-HMF (■), in g/L, during thermal-acidic hydrolysis with 5% H_2SO_4 at 121° C for 30 minutes (t=0 and t=0.5 h) followed by enzymatic hydrolysis performed with Viscozyme L (0.2 $g_{enzyme}/g_{seaweed}$) of *P. umbilicalis* biomass. The results are expressed as average±standard deviation (n=2).

Overall, the method used for the hydrolysis of *P. umbilicalis* rendered similar yields of monosaccharides recovery, when compared to those found in literature for red seaweeds (table 4.2). Note that, in the present study, the sugar release yield is slightly lower (37.9%) than that obtained by Greetham et al.[40] (48.6%) in similar conditions. This might be explained by the seasonal fluctuations in the composition of algae, as well as by the different range of sugars identified by the authors, which besides glucose and galactose included xylose, arabinose, fucose, rhamnose and mannitol.

Table 4.2. Concentrations of sugars (g/L) and sugar extraction yields (%) resulting from the thermal acid and enzymatic hydrolysis of the biomass of red macroalgae (adapted from table 2.2). Yields are expressed as $g_{sugars\ recovered}/g_{total\ carbohydrates}$x100.

Seaweed	Seaweed content (%w/w)	Acid concentration	T (° C)	Time (min)	Sugar yield (%)	Enzymatic treatment	Sugar yield (%)	Ref.
G. amansii	3, 5	3% H_2SO_4	121	30	80.7	—	—	[38]
	12	180 mM H_2SO_4 (1.76%w/w)	121	45	37.2	Celluclast (16 U/mL)	50.8	[39]
Gracilaria sp.	10	0.4 N HCl (1.5% w/w)	121	30	33.6	cellulase (7.6 U/mL, 48 h)	48.1	[16]
G. verrucosa	12	H_2SO_4 270 mM (2.6% w/w)	121	60	—	Celluclast 1.5L Viscozyme L (16 U/mL,24 h)	84.2	[55]
	2	0.1 N H_2SO_4 (0.5% w/w)	121	15	37.4	cellulase (10% w/w)	47.2	[54]
P. umbilicalis	10	5% H_2SO_4	121	15	48.6	—	—	[40]
	10	5% H_2SO_4	121	30	37.9	Viscozyme L (2.2 FBGU/mL, 30 h)	41.7	This study

FBGU - Fungal β-glucanase unit.

4.3 Growth of fermentative microorganisms on galactose and 5-hydroxymethylfurfural

To determine how the microorganisms to be used in the fermentation of *P. umbilicalis* hydrolysate are affected by the type and concentration of monosaccharides and inhibitors released during acid hydrolysis in the chosen conditions (5% sulphuric acid for 30 minutes at 121° C), the growth curves of each lactic acid bacterium, of the LAB mix and of the yeast were determined. For this purpose, the media under comparison were based on the medium described in section 3.2.2 and contained either galactose or galactose plus 5-HMF in concentrations similar to those identified in the hydrolysate.

4.3.1 *Lactobacillus* spp.

For the assay with *Lactobacillus* spp., the presence of 5-HMF at a concentration of approximately 1 g/L was shown to induce a lag-phase of up to two hours in *L. rhamnosus*, *L. casei* and *L. plantarum*. Nonetheless, there was no substantial effect on the overall growth of the bacteria, as a similar optical density was reached at the end of the experiment for both conditions (figures 4.3 A1, B1, C1, D1 and E1).

Comparison of the specific growth rates of each lactobacilli culture showed that only LAB mix was affected by the presence of 5-HMF (table 4.3). For the pure cultures, the presence of 5-HMF at a concentration of 1 g/L was not expected to cause a significant decrease in growth rate, as it is a value 5- to 8-fold lower to those reported to impact cell growth [109, 110]. Even so, the increase of the specific growth rate of *L. rhamnosus* and *L. plantarum* was not expected either. In the case of *L. rhamnosus* (figure 4.3 B1), the more noticeable increase from 0.24 to 0.27 h^{-1} might be related to the disparity in the number of points considered as part of the exponential growth phase, which was a result of performing the assays (no 5-HMF and 1 g/L 5-HMF) in two different days.

In terms of galactose consumption and lactic acid production, the lactobacilli presented similar be-

haviours in both conditions, reaching close final concentrations of the monosaccharide and of the organic acid at the end of the assay (figures 4.3 A2, B2, C2, D2 and E2). Note that only *L. rhamnosus* showed a different behaviour in the presence of 5-HMF, apparently beginning the production of lactic acid later. However, the pH did decrease (data not shown) while no increase in the concentration of the organic acid was detected and no other acid was identified in the HPLC spectra. This led to the assumption that some acid was actually being produced, despite not being correctly quantified.

Table 4.3. 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-hydroxymethylfurfural (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.

Finally, it is still important to notice that in all experiments the concentration of 5-HMF decreased: 0.72 g/L for *L. brevis* (figure 4.3 A2), 0.39 g/L for *L. rhamnosus* (figure 4.3 B2), 0.45 g/L for *L. casei* (figure 4.3 C2) and 0.25 g/L for *L. plantarum* and LAB mix (figure 4.3 D2 and E2). Although examination of the spectra obtained via HPLC analysis of the samples collected from the cultures did not reveal the formation of any compound simultaneously with the decrease of 5-HMF, the hypothesis of either chemical degradation or metabolization by the bacteria were considered. In fact, despite being a growth inhibitor, 5-HMF may be converted into less harmful compounds [109, 111], which, given the initial concentration of 5-HMF, might not have been detectable.

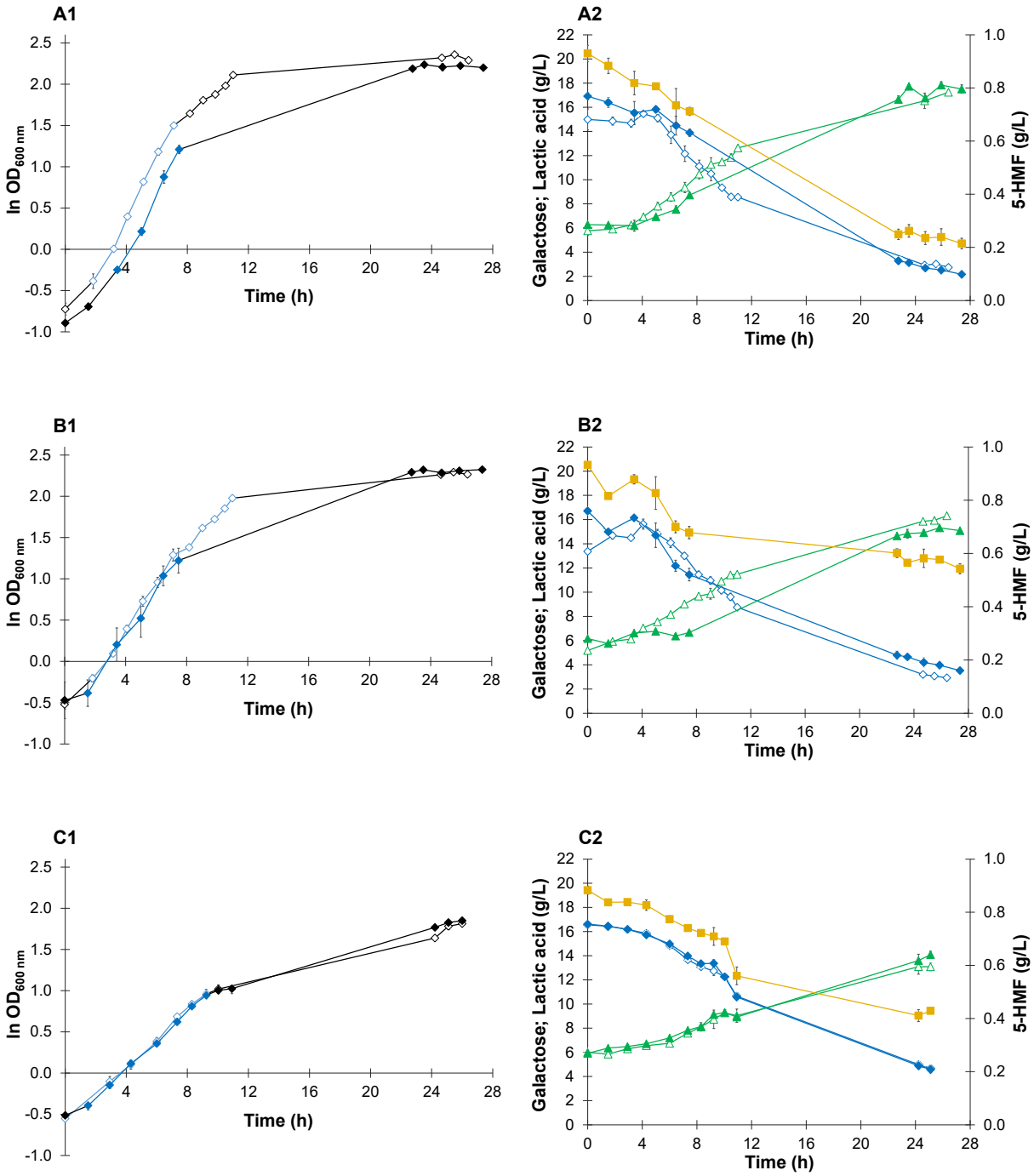

Figure 4.3. A1 to E1 - Growth curves of LAB with galactose as source of carbon, in the absence (◇) and presence (◆) of 5-HMF, expressed in terms of the natural logarithm of the optical density measured at 600 nm over time, in hours. Values used to determine the specific growth rate in each condition are highlighted (◇/◆). A2 to E2 - Galactose (◇/◆), lactic acid (△/▲) and 5-HMF (■), in g/L, over the cultivation time, in hours. A - *L. brevis*, B - *L. rhamnosus*, C - *L. casei*, D - *L. plantarum*, E - LAB mix. The results are expressed as average±standard deviation (n=2).

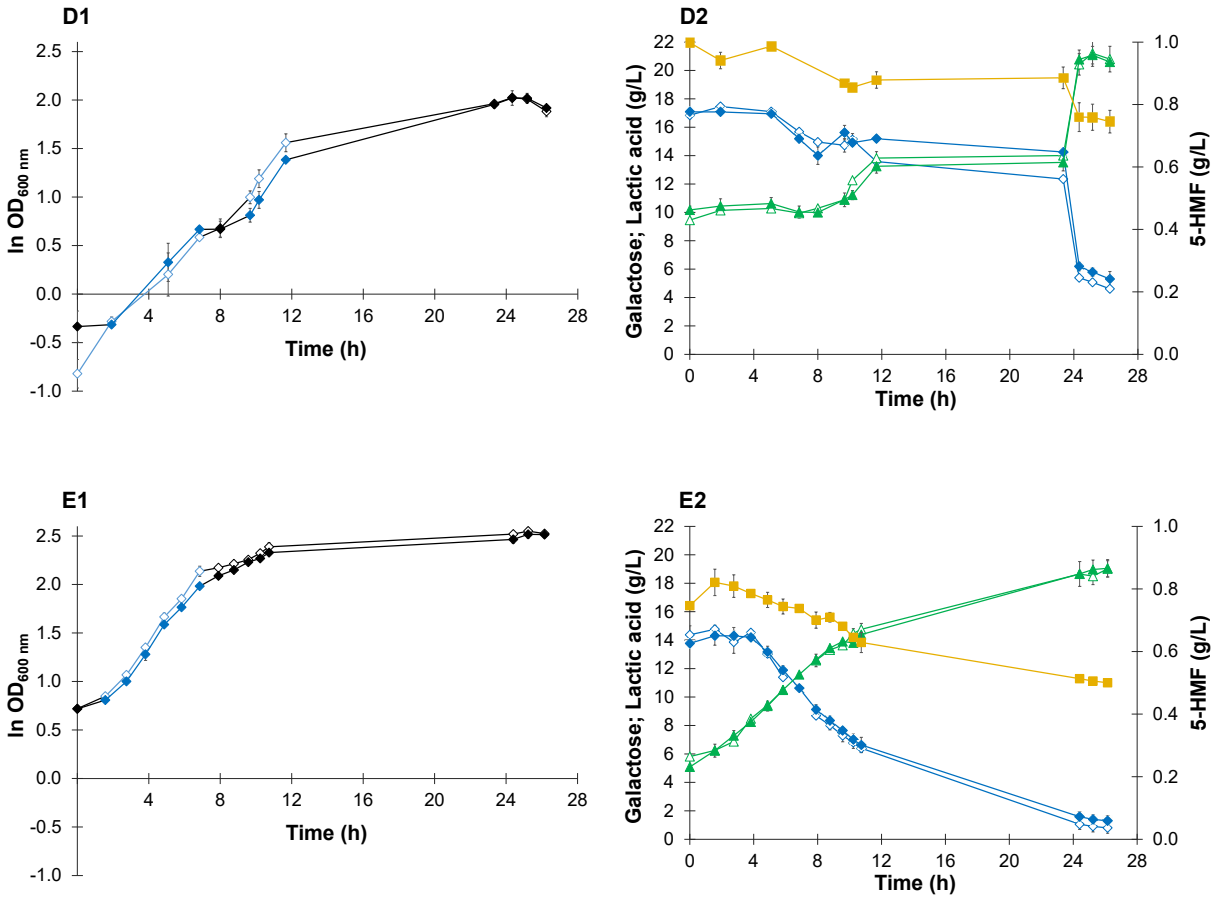

Figure 4.3. (cont.) A1 to E1 - Growth curves of LAB with galactose as source of carbon, in the absence (◇) and presence (◆) of 5-HMF, expressed in terms of the natural logarithm of the optical density measured at 600 nm over time, in hours. Values used to determine the specific growth rate in each condition are highlighted (◇/◆). A2 to E2 - Galactose (◇/◆), lactic acid (△/▲) and 5-HMF (■), in g/L, over the cultivation time, in hours. A - *L. brevis*, B - *L. rhamnosus*, C - *L. casei*, D - *L. plantarum*, E - LAB mix. The results are expressed as average±standard deviation (n=2).

4.3.2 *Saccharomyces cerevisiae*

The yeast growth behaviour was studied in the same conditions as the lactobacilli (15 g/L galactose or 15 g/L galactose plus 1 g/L 5-HMF) and, additionally, in medium containing 15 g/L galactose and 0.5 g/L 5-HMF, as the tolerance to the inhibitor was expected to be lower in *S. cerevisiae*. These three conditions allowed the determination of the feasibility of fermentations of hydrolysate using the yeast.

As described in section 3.2.2, the media utilised in this assay contained corn steep liquor (50 mL/L culture medium), which is a by-product of corn wet-milling and that may contain fragments and particles of the raw material. For this specific experiment, a new batch of CSL was used, where more fragments were visible to the naked eye. Although after inoculation the media in all three conditions was opaque, as the experiment progressed the fragments appeared to flocculate and settle in the bottom of the flasks, decreasing the turbidity of the media. For this reason, despite the fact that galactose was consumed and metabolites were produced (figure 4.4), the measured optical densities were not consistent with cellular growth. Consequently, no growth curves and specific growth rates were determined and the evaluation of the effect of the presence of 5-HMF in the medium was based only on galactose consumption and production of ethanol and acetic acid.

The tolerance of *S. cerevisiae* towards furan compounds, such as 5-hydroxymethylfurfural and furfural, has been studied mainly from the perspective of ethanol production for the biofuel industry. These compounds were found to inhibit enzymatic activity, namely of alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase[37], damage DNA and impair both mRNA and protein synthesis [112], leading to a deviation of energy towards cellular repair in detriment of cell growth and metabolite production[113, 114]. Even so, several authors point out that this tolerance is both dose and strain dependent[112, 114]. Consequently, in a range from 1 to 7.6 g/L 5-HMF, effects such as prolonged lag phase[112, 113], delayed ethanol production[112, 115], lower biomass and ethanol productivity[114, 115] and total inhibition of cellular growth[112], are all observed. Despite the lower tolerance when compared with lactobacilli, *Saccharomyces cerevisiae* has been proven to convert 5-HMF into 2,5-bis-hydroxymethylfuran (or furan-2,5-dimethanol), which is less harmful to the cell. This conversion allows the yeast to resume sugar consumption and metabolite production when the levels of 5-HMF are sufficiently low[112, 114].

In this assay, under quasi-anaerobic conditions and with galactose as the carbon source, *S. cerevisiae* suffered a lag phase of 7 hours (figure 4.4 A), considering that carbon consumption and ethanol and acetic acid production mark the start of cell growth. Such a long period of latency was expected, since the yeast was cultivated in YPD broth, where the source of carbon was glucose, before being transferred to a medium containing exclusively galactose, thus having to activate the Leloir pathway that was previously repressed[116]. Besides, due to the utilisation of corn steep liquor, the medium used in this assay also has an initial concentration of lactic acid that ranges from 5.5 to 7 g/L, which behaves as another yeast inhibitor (lag phase increases and specific growth rate decreases), thus contributing to the long lag phase in the experiment[117, 118]. In the flasks with either 0.5 g/L or 1 g/L of 5-HMF (figures 4.4 B and C, respectively), the lag phase took more than 9.7 hours, although it was not possible to determine it exactly because no samples were harvested in the night period. Even so, considering the effects of

the inhibitor in the growth of *S. cerevisiae*, it is possible to infer that the addition of 5-HMF caused this delay.

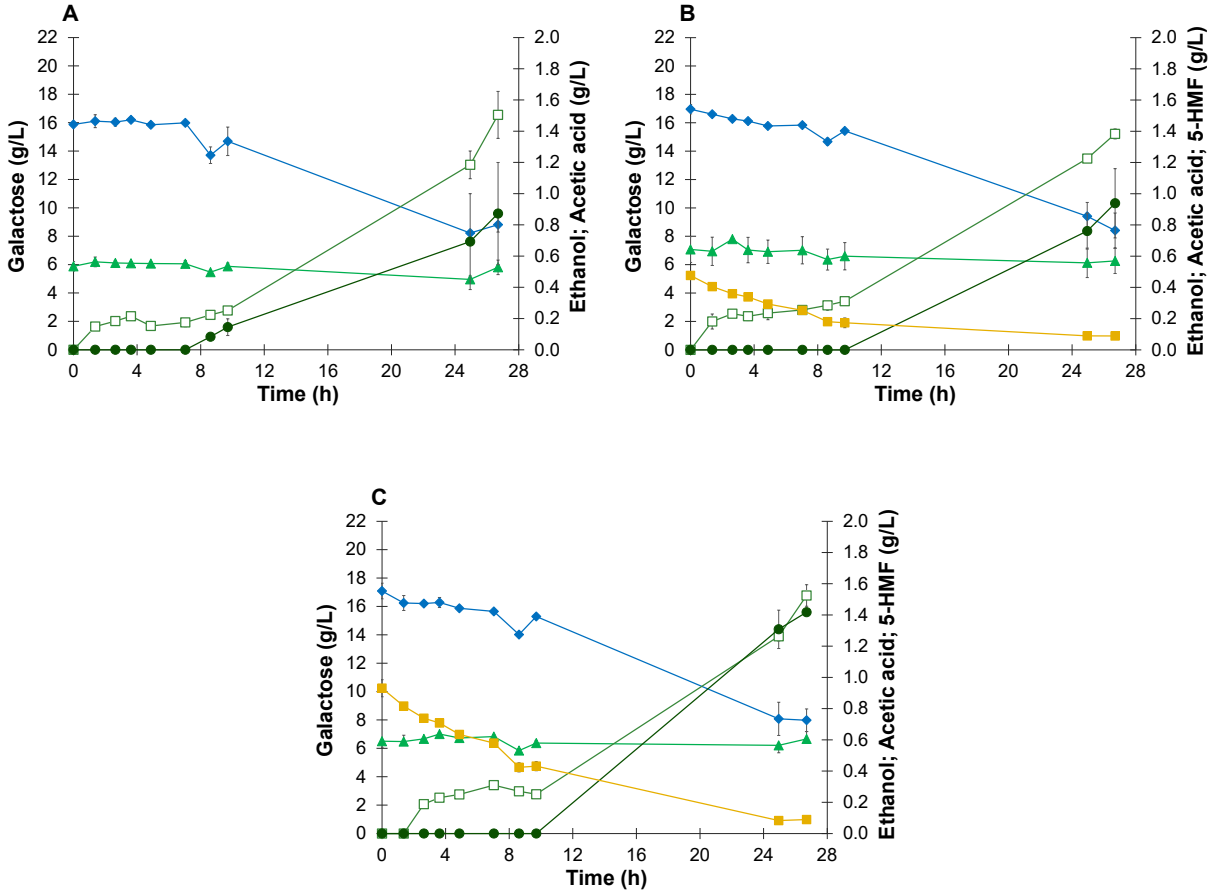

Figure 4.4. Evolution of the concentrations (g/L) of galactose (◆), 5-HMF (■), lactic acid (▲), acetic acid (●) and ethanol (□) over cultivation time in an assay with *Saccharomyces cerevisiae* in the absence of 5-HMF (A) and in the presence of 0.5 g/L (B) or 1 g/L (C) of 5-HMF. The results are expressed as average±standard deviation (n=2).

Since the experiment was not carried out until galactose was depleted, it is not possible to compare final ethanol and acetic acid concentrations. However, apart from the delayed metabolite production in the presence of 5-HMF, the metabolism of galactose was affected by the higher inhibitor concentrations: the concentration of produced acetic acid increased from 0.9 g/L in the absence of 5-HMF to 1.4 g/L when the concentration of the inhibitor was 1 g/L (1.6-fold increase) at 26.7 hours. This result is the consequence of the inhibition of alcohol dehydrogenase by 5-HMF, which renders the yeast unable to reduce acetaldehyde to ethanol. Moreover, since this specific inhibitor does not completely impair the activity of aldehyde dehydrogenase, it is able to successfully convert acetaldehyde into the carboxylic acid[37, 64].

Note that in both the media containing the inhibitor, either at 0.5 g/L or 1 g/L, the concentration of 5-HMF decreased to 0.09 g/L, a value which appeared to be stable at least in the last 2 hours of culture. Although it is not possible to determine if there is a specific concentration of the inhibitor below which

the yeast is able to grow, it is shown that only after its decrease *S. cerevisiae* can metabolize the carbon source (figures 4.4 B and C).

Overall, the yeast showed ability to grow in medium containing 1 g/L of 5-HMF and with galactose as the carbon source. However, due to the extended lag phase, there were concerns about this microorganism not being able to adapt fast enough when in co-culture with LAB mix, which adapt to the same conditions instantly and produce lactic acid, an efficient and widely used preservative in the food industry.

4.4 Fermentation of acid hydrolysates of *Porphyra umbilicalis*

4.4.1 Small scale batch fermentations

Batch fermentation of acid hydrolysates of *P. umbilicalis* was done in 250 mL flasks with a working volume of 200 mL, as described in section 3.6.1, for 69.2 hours at 37° C and 100 rpm. Through the spectra generated via HPLC analysis of the samples collected during the assay, the evolution of the concentrations of monosaccharides, organic acids, ethanol and 5-HMF were obtained (figure 4.5).

Similarly to what was observed in section 4.3.1, the LAB mix did not undergo a latency period after inoculation of the culture medium, in spite that the pre-inoculum had been cultivated in MRS broth (optimal medium for growth of most *Lactobacillus* spp.) and the medium had an initial concentration of lactic acid and 5-HMF of 6.78±0.04 and 0.72±0.02 g/L, respectively (figure 4.5 A1). Even so, since the concentration of the furan derivative was below the previously mentioned inhibitory concentrations and the pH at the beginning of the fermentation was 6.13±0.01, which is well above the pKa of the acid, no inhibition was observed. Note that, despite the presence of glucose at a low concentration (1.20±0.01 g/L), this monosaccharide was consumed before galactose, which was available at a higher initial concentration (15.44±0.01 g/L), evincing the preference of *Lactobacillus* spp. for glucose over galactose.

In terms of metabolite production, lactic and acetic acid were both produced, showing the heterofermentative character of the lactobacilli used. With final concentrations of 19.6±0.6 and 1.06±0.01 g/L for lactic acid and acetic acid (figure 4.5 A2), respectively, molar ratios of metabolite to carbon source of 1.8 and 0.2 were reached. Since the expected ratio of lactic acid to glucose (or galactose) is 1:1 during heterofermentative metabolism and 2:1 during homofermentative metabolism, while that of acetic acid is 1:1 in heterofermentative metabolism[63], then it is possible to infer that during this fermentation there was a mixture of both, with prevalence of the homofermentative pathway.

The addition of *Saccharomyces cerevisiae* to the culture (figures 4.5 B1 and B2) did not cause a significant change in monosaccharide consumption and metabolite production, despite glucose was consumed at a slightly higher rate and a slight decrease on lactic acid final concentration was observed, from 19.6±0.6 to 18.7±0.5 g/L, which was accompanied by an increase in acetic acid concentration, from 1.06±0.01 to 1.71±0.04 g/L, and ethanol production (0.14±0.03 g/L). As expected, the molar ratio of produced lactic acid to consumed monosaccharides decreased to 1.6, while that of acetic acid was

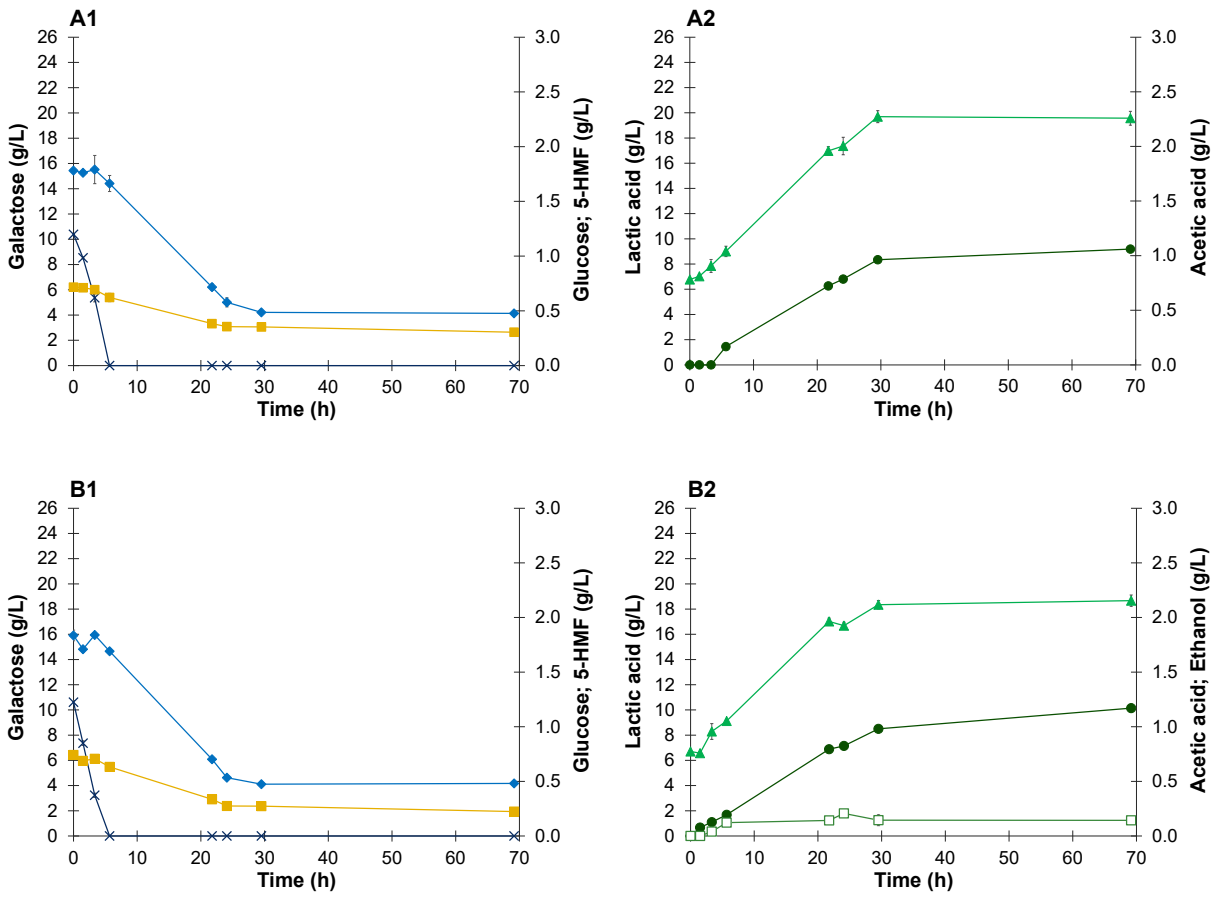

Figure 4.5. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲), acetic acid (●) and ethanol (□) during the fermentation of hydrolysates of *P. umbilicalis* (working volume of 200 mL, 37° C, 100 rpm), for a period of 69.2 hours. A - Fermentation with LAB mix; B - Fermentation with LAB mix and *S. cerevisiae*. Results are expressed as average±standard deviation (n=2).

maintained. The lack of effect of the addition of *S. cerevisiae* to the culture may be explained by two main reasons. First, once glucose was depleted, the yeast had to adapt to galactose metabolism - similarly to what is described in section 4.3.2-, leading to a latency period in which the lactobacilli consumed the majority of the carbon source and produced lactic acid, which inhibited any further possible yeast growth. Another possibility is that the conditions imposed during this fermentation (37° C and 100 rpm orbital agitation) were not optimal to yeast growth, which according to the provider would be between 25 and 30° C at a pH of 5.5.

4.4.2 Small scale fed-batch fermentations

Fed-batch fermentation was tested in 500 mL flasks with a working volume of 300 mL, for 69 hours in the conditions described in section 3.6.2. The evolution of the concentrations of monosaccharides, 5-hydroxymethylfurfural and metabolites is represented in figure 4.6.

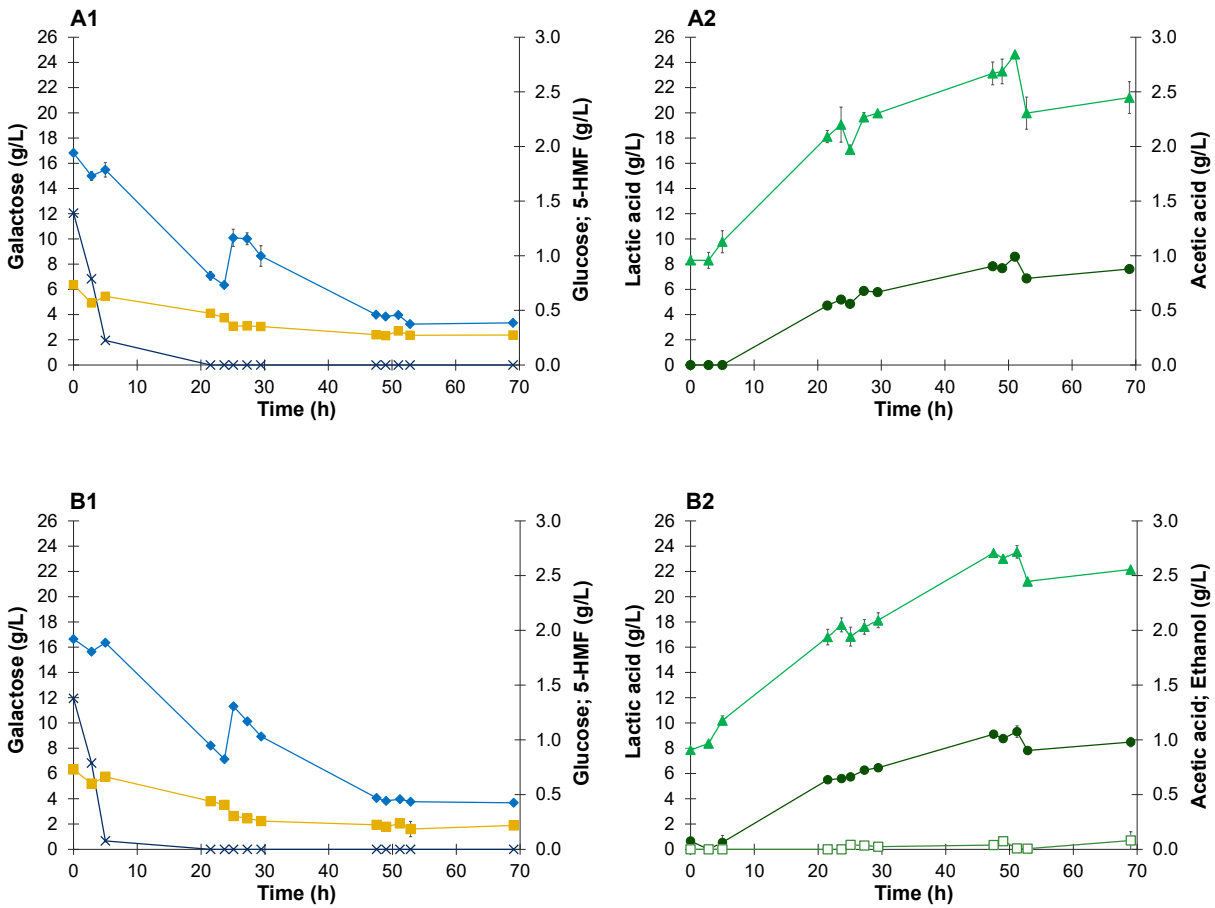

Figure 4.6. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲), acetic acid (●) and ethanol (□) during the fed-batch fermentation of hydrolysates of *P. umbilicalis* (working volume of 300 mL, 37° C, 100 rpm), for a period of 69.0 hours, with one addition of galactose at 25.1 hours. A - Fermentation with LAB mix; B - Fermentation with LAB mix and *S. cerevisiae*. Results are expressed as average±standard deviation (n=2).

Similarly to batch fermentations with the same combinations of microorganisms, the behaviours shown in terms of sugar consumption and metabolite production were similar when cultures with LAB mix and *S. cerevisiae* were compared with those done using LAB mix alone. Fed-batch fermentation with LAB mix rendered a higher final concentration of lactic acid (21.2±1.3 g/L) which was to be expected, since more galactose was consumed. Even so, the molar ratio between produced lactic acid and consumed monosaccharides decreased more than 2-fold to 0.8, accompanied by a similar decrease in the molar ratio of acetic acid to sugar. Despite no concrete reason for this decrease was found, since all conditions were maintained, one possibility could be an increased production of biomass in detriment of metabolites, which would have to be quantified through methods such as sample dry weight or optical density, or total nitrogen determination in the solid phase of the fermented product. On the other hand, fermentation with *S. cerevisiae* did not show any significant difference from the one with LAB mix only, reinforcing that the conditions in use were not adequate.

In order to test if it was, in fact, possible to use yeast to ferment *P. umbilicalis* hydrolysates alongside lactic acid bacteria, an additional fed-batch assay was performed where LAB mix was added to

the fermentation broth only when *S. cerevisae* had consumed the initially available sugars under optimal conditions. The evolution of monosaccharides, inhibitor and metabolites concentrations over the 55.5 hours of fermentation is represented in figure 4.7.

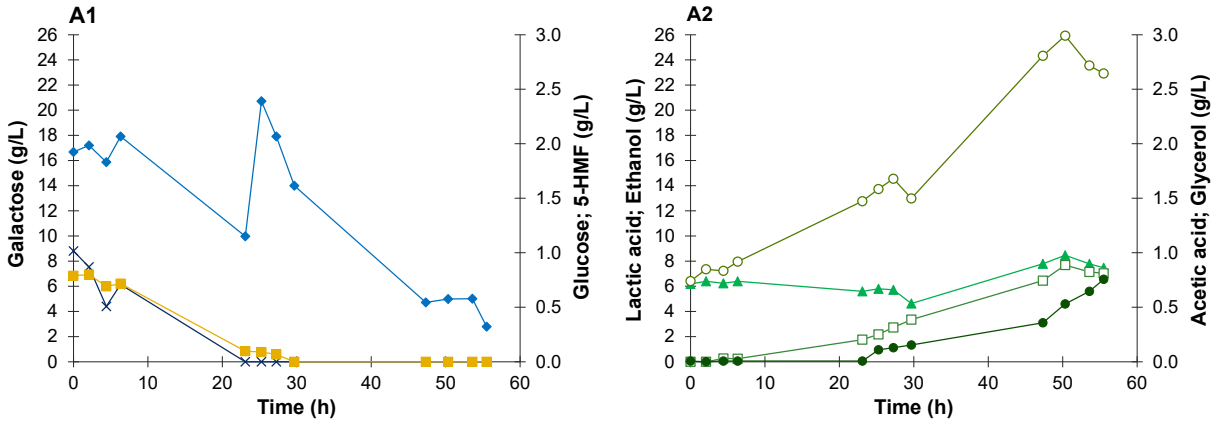

Figure 4.7. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲), acetic acid (●), ethanol (□) and glycerol (○) during the fed-batch fermentation of hydrolysates of *P. umbilicalis* (300 mL working volume) with *S. cerevisiae* and LAB mix with different inoculation times, for a period of 55.5 hours. One addition of galactose (40 mL, 100 g/L stock solution) and addition of LAB mix were done at 25.3 hours. Temperature and agitation were maintained at 28° C and 200 rpm for the first 25.3 hours of assay and then changed to 37° C and 100 rpm until the end of fermentation.

Although yeast growth was initially slow, as shown by the low rate of glucose and galactose consumption (figure 4.7 A1) when compared to that of cultures with LAB mix, the microorganism was able to metabolise the monosaccharides present in the algal hydrolysate to produce ethanol and glycerol, which reached concentrations of 2.48 and 1.80 g/L, respectively, at 25.3 hours (concentrations before a 40 mL galactose feed). Note that an initial latency period was expected, since the yeast not only had to adapt to the new source of carbon, but also to the presence of 5-HMF, which had to be converted to its less inhibiting derivative, as discussed in section 4.3.2. However, this period was not as prolonged as that verified during the assays in section 4.3.2 (figure 4.4), owing to the fact that both a lower temperature and a lower pH were used during the fermentation. Regarding the produced metabolite to consumed monosaccharide molar ratios, this fermentation performed worse in terms of lactic acid, which reached only 0.2:1, while maintaining that of acetic acid (0.1:1). The ratio of ethanol to sugars reached a final value of 0.5:1, which was expected due to this being the final product from yeast fermentation, despite part of the sugar was directed towards glycerol synthesis.

Inoculation of LAB mix at 25.3 hours of fermentation, simultaneously with a 40 mL galactose feed (100 g/L) and the alteration of culture conditions (temperature and agitation) to those optimal for lactobacilli, allowed the yeast to develop without being inhibited by the fast increase in lactic acid concentration and consequent pH decrease. In previous assays, the production of organic acids by lactobacilli led to a rapid decrease of pH to values between 4.0 and 4.4, while in this specific fermentation pH was maintained above 5.0 at all times. 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[119, 120], which is a result of the salt (sodium sulphate) produced during the neutralization of the hydrolysate prior to fermentation.

Despite allowing the development of yeast, this fermentation appeared to have an impact on the metabolism of LAB mix, with lactic acid production reaching extremely low values, only increasing from 6.18 to 7.47 g/L. The possibility of the growth and metabolism of *Lactobacillus* strains being inhibited by either ethanol or glycerol was proposed, as these were the major differences when this fermentation was compared to those performed previously. However, Gold et al.[121] reported that the majority of the 31 strains of *Lactobacillus* tested for ethanol tolerance were able to grow on medium containing 4% (per volume) of the alcohol, with *L. brevis*, *L. casei* and *L. plantarum* tolerating ethanol concentrations up to 10%, 14% and 16%, respectively, which excluded the possibility of this compound affecting the LAB mix to a great extent even at its maximum concentration during the assay (1% v/v at 53.3 hours). The presence of glycerol, according to studies performed on the ability of lactobacilli to use this compound as a carbon source[122, 123], should also not be a hindrance to bacterial growth and metabolism, since some strains of *Lactobacillus* were proved to be able to grow either on glucose and glycerol or glycerol alone, yielding mainly lactate and acetate. Even so, those results were obtained under aerobic metabolism and resorting to different media, reason by which this specific subject would need further study.

One additional possibility taken into consideration was the negative impact of the coexistence of lactobacilli and yeast. Narendranath et al.[124] showed that the combination of ethanol and lactic acid in the fermentation broth had a negative synergistic effect on the bacteria, resulting in increased cell death when concentrations of lactic acid increased towards the end of the fermentation, despite the combination of the microorganisms had initially been beneficial for lactobacilli growth. In addition, the authors emphasised the fact that this type of co-culture not only leads to competition over the carbon source, but also over essential growth factors. In the specific context of this assay, where *S. cerevisiae* and LAB mix were not inoculated simultaneously, it is possible that the yeast consumed a great part of the nutrients initially available in the medium, therefore not allowing lactobacilli to grow and metabolise galactose as well as previously observed.

4.4.3 Total nitrogen content in small scale fermentations

The total nitrogen content in small scale fermentations was analysed in the laboratory as described in section 2.2.1 for the first and last samples collected from each fermentation. Note that since there were no additions of nitrogen to the media and the method determines all organic and inorganic nitrogen, total nitrogen of the whole samples was only measured in one fermentation (small scale, batch with LAB mix) in order to estimate the quantities of nitrogen present in the hydrolysate and in the remaining medium. This analysis rendered a nitrogen concentration of 6.0 g/L in the hydrolysate and 7.7 g/L at the beginning of the fermentation. Therefore, the majority of the nitrogen has its origin in the algal biomass.

Analysis of the pellets was done to detect any possible increase in microbial biomass, which is

Table 4.4. Total nitrogen content ($mg_N/g_{dried\ pellet}$) and nitrogen increase, ΔTN (%), in the solid fraction of the samples collected at the beginning (TN_0) and at the end (TN_f) of small scale fermentations. Nitrogen increase defined as $100x(TN_f\text{-}TN_0)/TN_0$.

Fermentation	TN_0 (mg/g)	TN_f (mg/g)	ΔTN (%)
Batch, LAB	73.5	94.4	28.4
Batch, LAB/Y[a]	83.5	84.5	1.2
Fed-batch, LAB	91.5	72	-21.0
Fed-batch, LAB/Y	68.2	73	6.7
Fed-batch, Y + LAB[b]	81.4	83.3	2.4

[a] LAB/Y - fermentation with LAB mix and *S. cerevisiae* inoculated simultaneously; [b] Y + LAB - fermentation with *S. cerevisiae* inoculated first and LAB mix added with the first galactose feed

separated along with algal biomass through centrifugation of the samples harvested at the beginning and at the end of each fermentation. The results obtained from this quantification are presented in table 4.4. In general, fermentation appears to increase nitrogen content in the solid fraction of the samples (ΔTN), with the higher variation verified in small-scale fermentation with LAB mix (28.4%). However, these increases are lower than expected, since microbial activity was observed through the production of organic acids, ethanol and glycerol. Note that as the sediments present in the sample were a complex mixture of suspended solids constituted by algal and microbial biomass, it is possible that the 15 mg that were resuspended in water for total nitrogen analysis were not representative of the fermentation broth. For this reason, the total nitrogen content in the sediment of each sample might have been overestimated or underestimated, which could explain the values obtained for ΔTN. Owing to the fact that this method was not considered a reliable measurement of the total nitrogen present in microbial biomass from the samples, it was not carried out for any further fermentations.

4.4.4 Fermentation scale-up

Scale-up to a 2L bioreactor was tested in batch and fed-batch using LAB mix, as described in section 3.7. The evolution of the concentrations of monosaccharides, 5-hydroxymethylfurfural and metabolites are represented in figures 4.8 and 4.9, respectively.

Performing a batch fermentation with LAB mix alone in a bioreactor with controlled pH and aeration led to similar final concentrations of all the analysed compounds as those obtained in flasks, reaching 18.0 g/L of lactic acid, 1.7 g/L of acetic acid and 0.5 g/L of 5-HMF, despite the longer period needed to reach said concentrations. However, scaling-up the fermentation led to an increased latency period of approximately 17 hours, in which the lactobacilli did not consume galactose nor glucose at the previously observed rate, in addition to the appearance of a period between 24 and 41 hours where no galactose was consumed or lactic acid was produced (figure 4.8 A and B). The initial lag phase was predicted, since *Lactobacillus* spp. are either facultative or strictly anaerobic[125], 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 4.8 C). As anticipated, the consumption of monosaccharides and the decrease of dissolved oxygen were concurrent. Regarding the 17-hour period mentioned above, it coincides with

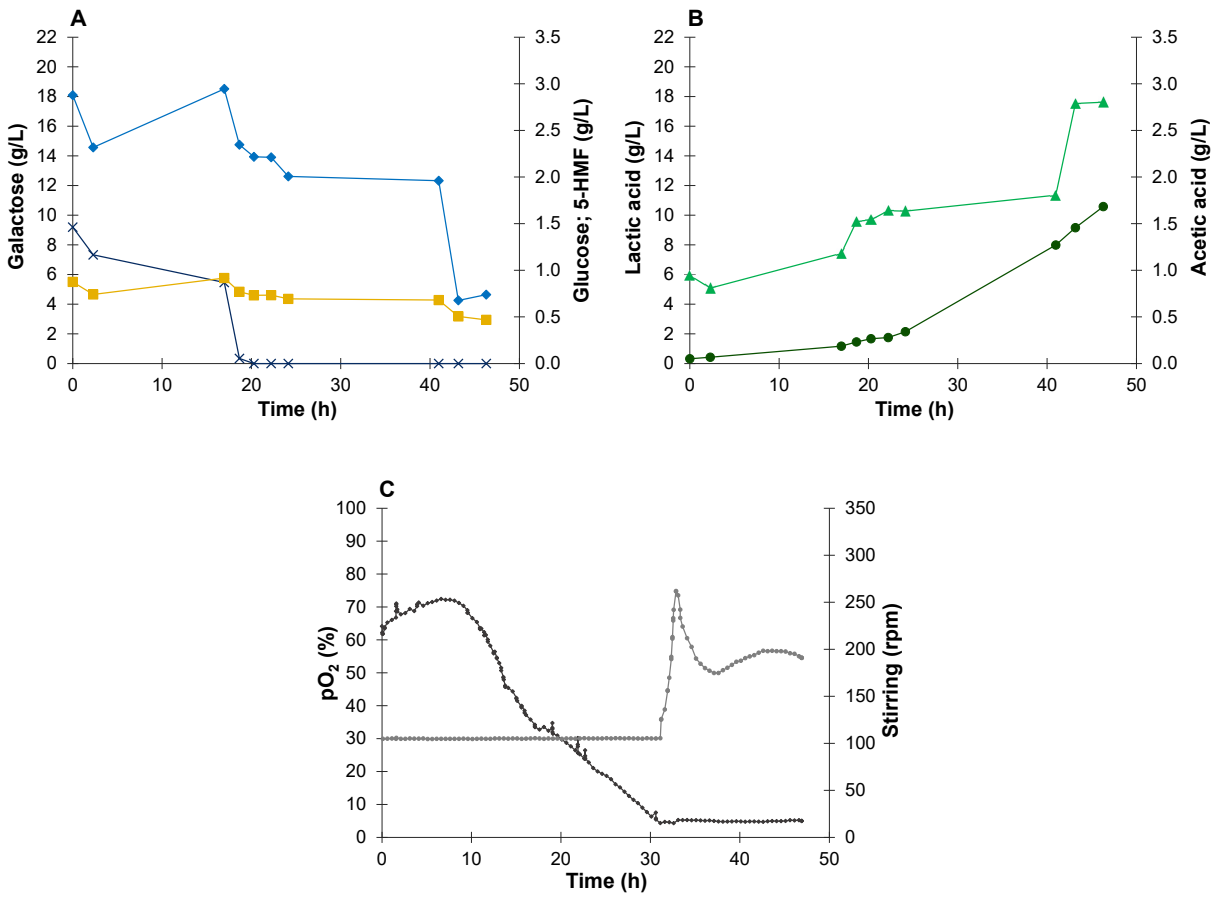

Figure 4.8. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲) and acetic acid (●) during the batch fermentation of the hydrolysate of *P. umbilicalis* (working volume of 1.8 L, 37° C, pH 6.2, 1 vvm and 5% pO_2 setpoint), for a period of 46.3 hours. Evolution of dissolved oxygen (%, ◆) and stirring (rpm, ●) are also represented.

the decrease of dissolved oxygen below 20% (at 24 hours). It is important to note, however, that the sample taken at 41 hours was the first harvested in the second day of fermentation. It is possible that the volume of broth discarded prior to sample harvesting was not enough to clean the sample collection tubing, leading to concentrations of galactose, lactic acid and acetic that were not accurate.

Fed-batch fermentation was done over 115.5 hours, until the rate of galactose consumption started to decrease. Note that since the feeds were given in the form of solid galactose, it was possible to reach higher concentrations of the monosaccharide without diluting other components of the medium or surpassing the maximum volume of the reactor, which allowed one additional feed. Addition of solid galactose as feed instead of the usual concentrated sugar solution is especially necessary when performing fed-batch cultivations with this sugar because of its low solubility in water (100 g/L). Even so, this last addition of galactose at 50 hours was excessive, resulting in a galactose surplus of 29.1 g/L at the end of the fermentation (figure 4.9 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.

As discussed previously, lactobacilli went through an initial latency period induced by the aerated environment. Aiming for the decrease of this period, at the beginning of the fed-batch assay the aera-

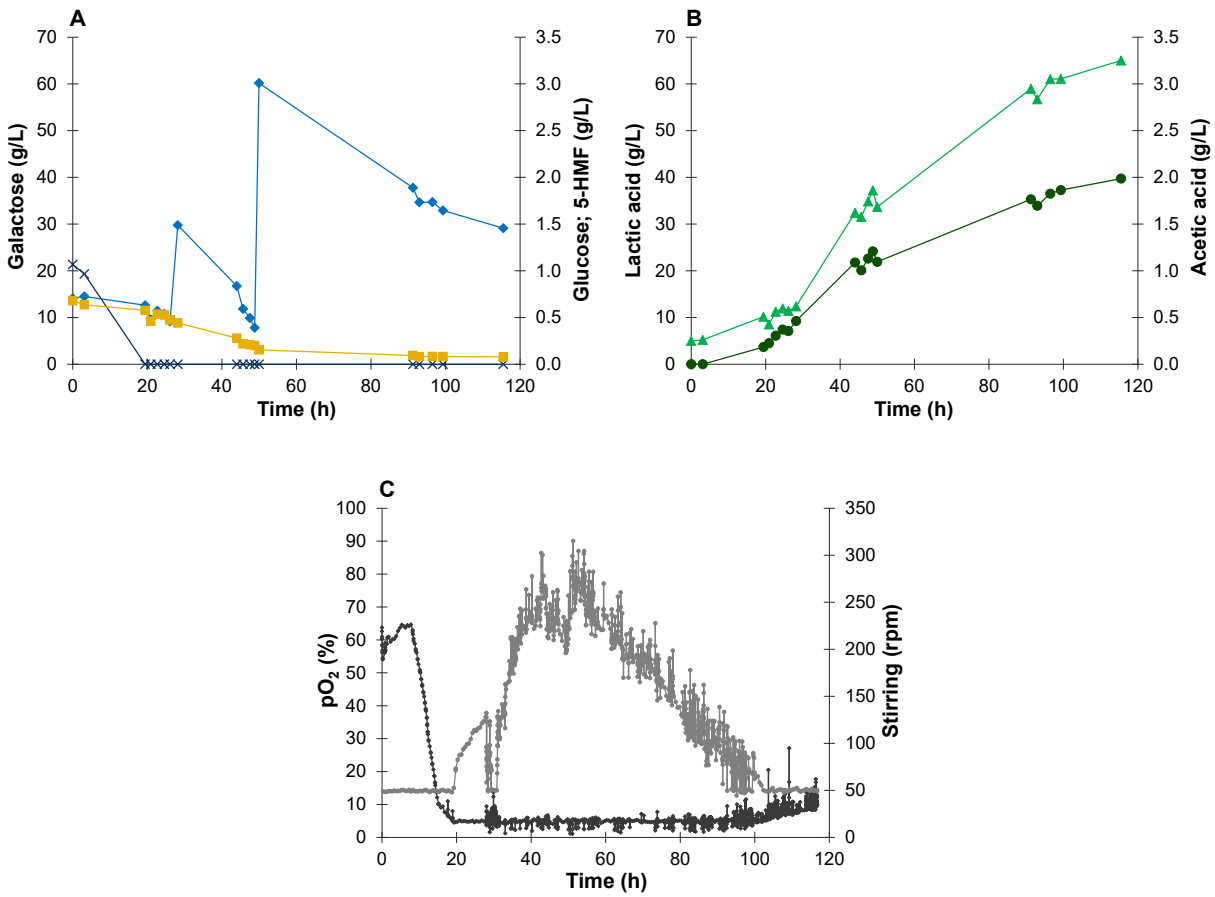

Figure 4.9. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲) and acetic acid (●) during the fed-batch (B) fermentation of an hydrolysate of *P. umbilicalis* (working volume of 1.8 L, 37° C, pH 6.2, 0.5 vvm and 5% pO_2 setpoint), for a period of 115.5 hours (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.

tion rate was lowered to 0.5 vvm and the minimum agitation speed to 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 4.9 A). Such changes justify the lower amount of time needed to reach the established oxygen saturation set-point (pO_2=5% sat), which decreased from 23.5 hours to 12 hours (see figures 4.8 C and 4.9 C).

Although not all the provided galactose was consumed, lactic acid reached a concentration as high as 65.0 g/L (figure 4.9 B), due to the maintenance of a pH of 6.2, which guaranteed that the organic acid remained in its dissociated form, therefore ensuring lactobacilli viability for an extended period of time. The final concentration of acetic acid reached only 2.0 g/L (4.9 B), despite its steady increase throughout the time of the assay. Considering both results, it is possible to infer that although 3 out of the 4 strains of *Lactobacillus* sp. are facultatively heterofermentative, homolactic fermentation was the overall preferred metabolism.

Regarding the molar ratios of lactic or acetic acids to monosaccharides, both batch and fed-batch fermentations performed similarly, reaching proportions of 1.4 and 1.2 moles of lactic acid formed per

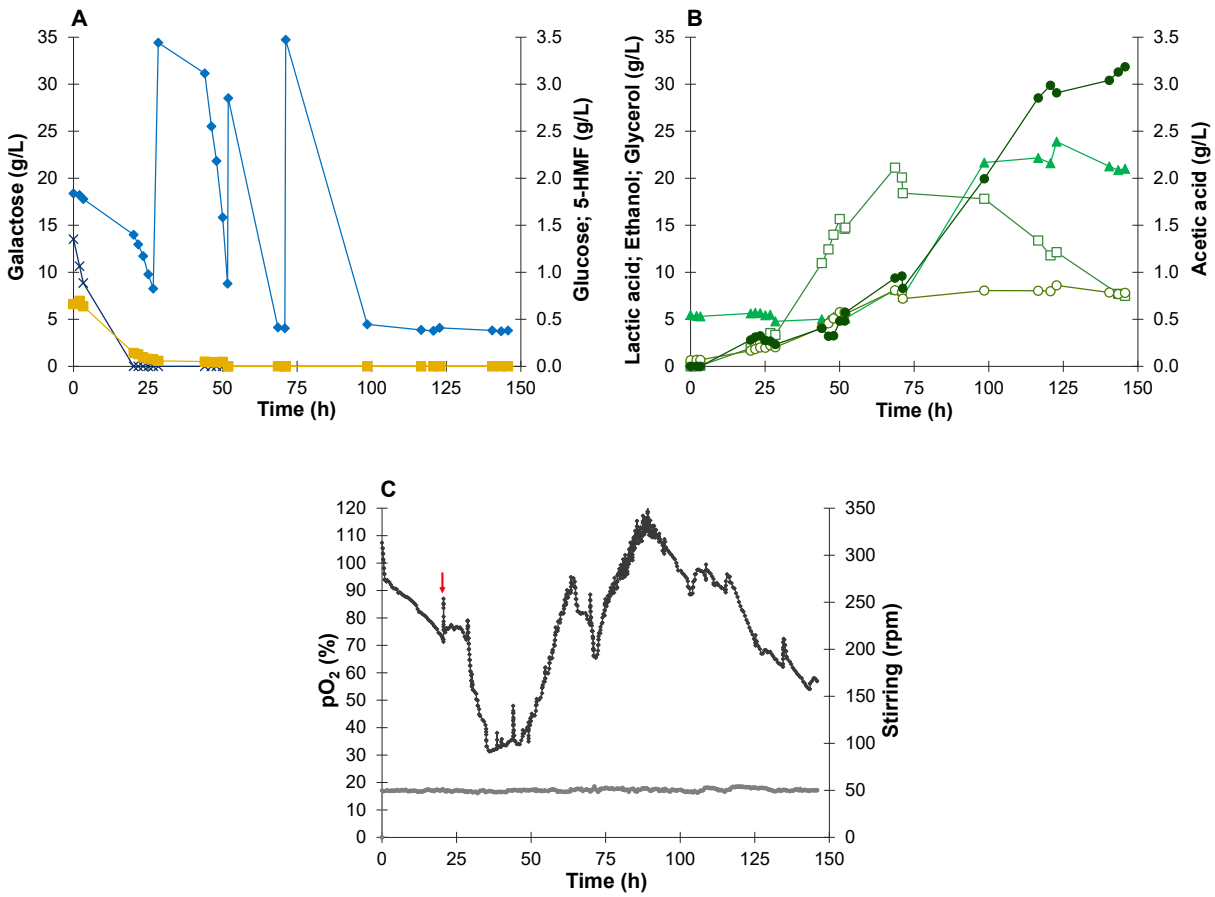

Figure 4.10. Evolution of the concentrations (g/L) of glucose (×), galactose (◆), 5-HMF (■), lactic acid (▲), acetic acid (●), ethanol (□) and glycerol (○) during the fed-batch fermentation of hydrolysate of *P. umbilicalis* with *S. cerevisiae* and LAB mix (inoculation simultaneous with the first galactose 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° C and pH of 5.5 were maintained for the first 28.5 hours of assay and then changed to 37° C 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.

mole of glucose (or galactose) consumed, respectively, and 0.2 and 0.1 moles of acetic acid per mole of sugar, respectively. These values fit the overall homofermentative metabolism more adequately, as the amount of produced acetic acid is low. Nevertheless, the theoretical ratio would be 2 moles of lactate per mole of sugar, which indicates that part of the galactose was likely diverted to others ends, such as cellular maintenance and biomass formation.

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. Once again, the concentrations of glucose, galactose, 5-HMF and of the identified products of the metabolism of both the yeast and the lactobacilli were followed throughout the duration of the assay and are represented in figure 4.10.

Similarly to what was observed at a smaller scale, glucose was rapidly depleted along with 5-HMF, while galactose was only consumed at a high rate after no glucose was available (figure 4.10 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 4.10 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 (figure 4.10 C).

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 4.10 B), which indicates that the yeast was active after lactobacilli were added to the medium. Even so, according to what was already discussed in section 4.4.2, this concentration of ethanol was not high enough to inhibit the growth of the lactobacilli in LAB mix. 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, a value similar to that observed in the batch fermentation at this scale and in fed-batch fermentations in flask, where lower amounts of galactose were fed to the culture. 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.

As observed in small-scale fermentation (figure 4.7 A2), there was production of glycerol, likely due to the presence of salt in the medium, which resulted from the neutralisation of the hydrolysate. An additional point to mention is the consumption of ethanol that occurred between 68.6 and 71.2 hours and between 98.5 and 145.7 hours, as a result of the depletion of glucose and galactose as carbon sources[126].

Note that the concentrations of galactose measured via HPLC for the first two feeds did not correspond to those predicted. Addition of 110 mL of a galactose solution (100 g/L) along with 80.6 g of solid galactose would generate an increase of 51.6 g/L of the monosaccharide. However, after the first feed, the measured concentration of the sugar was only 34.4 g/L. Similarly, while after the second feed an increase of 33.6 g/L was predicted, the concentration of galactose was determined as 28.5 g/L. These differences are owed to the fact that powdered galactose was not immediately dissolved or was deposited at the bottom of the vessel.

If the total amount of sugar supplied to the fermentation is used to determine the molar ratios of produced metabolites to consumed monosaccharides, proportions of 0.2:1, 0.1:1 and 0.2:1 are obtained for lactic acid, acetic acid and ethanol, respectively, which are the lowest among all the three fermentations performed at this scale. The possibility of carbon being converted into biomass in detriment of these compounds would have to be determined through adequate methods, such as evolution of sample dry weight and determination of the number of colony forming units (CFU) in several points of the fermentation or protein quantification.

4.4.5 Protein bioaccessibility and biological activity of fermented *P. umbilicalis*

The bioaccessibility and biological activity of the protein present at the end of small-scale batch fermentation with LAB mix and LAB mix plus yeast, as well as of all the fermentations performed in

Table 4.5. Protein content (% in dry weight), bioaccessible fraction (%), antioxidant activity measured by ABTS, DPPH and FRAP methods and cupric and ferrous 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 *Porphyra umbilicalis* and in *P. umbilicalis* hydrolysates fermented in flask and bioreactor.

		Shake flask		2 L bioreactor		
	P. umbilicalis	**LAB**	**LAB/Y**	**LAB (B)**	**LAB (FB)**	**Y + LAB**
Protein content (% DW)	34.5±0.3	21.7±0.3	21.6±0.1	21.1±0.1	12.1±0.2	17.4±0.3
Bioaccessible fraction (%)	77.8±1.8	85.9±1.0	74.8±2.4	84.4±2.1	79.7±5.2	73.7±5.9
Biological activity						
ABTS (mg/mL)	—	9.5±0.8	12.5±0.6	6.9±0.1	10.5±0.2	5.6±0.3
DPPH (mg/mL)	—	9.3±0.1	9.8±0.4	5.5±0.2	8.3±0.3	5.8±0.4
FRAP (mg/mL)	—	6.7±0.1	6.6±0.1	2.5±0.1	1.8±0.1	2.5±0.1
Cu^{2+} quelation (mg/mL)	—	1.4±0.1	1.8±0.03	2.2±0.1	2.7±0.1	2.3±0.1
Fe^{2+} quelation (mg/mL)	—	—	—	9.8±1.3	11.1±1.1	5.9±0.4

LAB - batch fermentation with LAB mix (shake flask); LAB/Y - batch fermentation with LAB mix and *S. cerevisiae* inoculated simultaneously (shake flask); 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).

a bench-top reactor, were determined by IPMA using lyophilised 200 mL samples of the fermented products and are presented in table 4.5.

At the end of all the fermentations, protein content was lower than that of the seaweed prior to pretreatment and fermentation. Note, however, that the method used for protein determination is based on the quantification of total nitrogen in the samples, which might under or overestimate the amount of protein present both in the dried and in the fermented alga. Furthermore, the evaluation protein increase during fermentation cannot be measured by quantification of total nitrogen in a sample of the whole fermentation broth. Even so, no significant difference was found between batch fermentations, in small-scale, with LAB mix or LAB mix plus yeast, despite the latter had been inoculated with more biomass. Additionally, scale-up of batch fermentation with LAB mix rendered a similar protein content (21.1±0.1% DW) to that attained in shake flask (21.7±0.3% DW), which was expected since the medium was prepared with the same proportions of algal hydrolysate and CSL. The major differences in protein content were found for products obtained from fed-batch fermentations. 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 section 4.4.4). Although these compounds could evaporate during the process of lyophilisation, their quantification in the lyophilised products should be performed, in order to evaluate the influence they might have in protein content determination.

The bioaccessible fractions of the protein present in the tested samples are consistent when fermentations performed in bioreactor are compared with those done in shake flask (table 4.5). The high-

est protein bioaccessibility was found in the fermentation performed only with LAB mix in small-scale (85.9±1.0%), although its scaled-up equivalent presented a close result (84.4±2.1%). Note that, contrary to what was expected, the presence of *S. cerevisiae* did not increase protein bioaccessibility. In the assay where the yeast was able to grow (Y + LAB), bioacessibility was the lowest (73.7±5.91%). 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[127, 128], 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[129, 130].

In terms of biological activity, algal hydrolysate fermented with LAB mix and *S. cerevisiae* added to the medium at different times showed better results in terms of ABTS and DPPH radical scavenging ability, which required 5.6±0.3 and 5.8±0.4 mg_{sample}/mL, respectively, to decrease to half the concentration of the radicals. all the products obtained from fermentations in bioreactor showed similar results in terms of cupric ion-chelating properties, needing between 1.8±0.1 and 2.7±0.1 mg_{sample}/mL to decrease to half the concentration of free Cu^{2+} ions. Chelation on ferrous ions was performed more effectively with samples from fermentation with yeast and LAB mix (Y + LAB), which required 5.6±0.4 mg_{sample}/mL. The fermentation product of LAB mix (bioreactor) performed better in terms of total reducing power, measured in the FRAP assay. Note that Fe^{2+}-chelating ability was also studied for the fermented products obtained in small-scale, but none of the tested sample concentrations were sufficient to chelate 50% of the free ferrous ions.

The same properties were determined for a sample concentration of 1 mg/mL, which showed that the fermentation products were promising as cupric and ferrous ion chelators. At that concentration, *P. umbilicalis* hydrolysate fermented with LAB mix was able to chelate 40% and 15% of free Cu^{2+} and Fe^{2+} ions, respectively, while the product of fermentation with LAB mix and *S. cerevisiae* chelated 35% and 20%, respectively.

Note that, overall, products of fermentation in bioreactor performed better regarding antioxidant and chelating properties. 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.

Although these results cannot be compared with those found in literature, all fermented products obtained at the larger scale were shown have great potential against oxidative stress, as they were able to act by scavenging compounds that generate reactive oxygen species. Taking into consideration the protein bioaccessiblity in each of these products, batch fermentation with *Lactobacillus* appears to be the most promising for inclusion in aquafeed. Even so, this product could be compared with the one obtained from fermentation with yeast and LAB mix (Y + LAB) in *in vivo* assays, to determine how the presence of different metabolites and different microorganisms might affect feed acceptablity and fish growth performance.

4.4.6 Fermentation metabolites for incorporation in aquafeed

The presence of lactic acid and glycerol in the fermented products in concentrations up to 65.0 and 7.8 g/L, respectively, raised concerns about the effects these compounds might have when incorporated in aquafeed, despite incorporation of fermented seaweed broth will only account for 2 to 10% of the feed (information provided by SPAROS, Portugal).

In addition to its preservative properties, lactic acid, which has been regarded as a possible alternative the antibiotics usually incorporated in animal feed, was shown to increase growth and weight gain of finfish whose feed was supplemented with up to 15.0 g lactate/kg feed[131]. Similarly, glycerol has been shown to not impair fish growth when supplemented to feed (up to 15%). Moreover, it is thought to affect the metabolism of carnivorous fish in a positive manner: glycerol competes with amino acids as a substrate for gluconeogenesis, therefore avoiding their conversion into glucose. For this reason, more amino acids become available for protein synthesis and biomass formation[132, 133]. Even so, there is no information on how the inclusion of these compounds might affect the organoleptic properties of the feed, thus acceptance would have to be studied in a species-dependent manner.

Chapter 5

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, 121° C, 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. Antioxidant and chelating properties were also shown to be better in products fermented with lactobacilli, despite both types of fermentation rendered products with excellent Cu^{2+} and Fe^{2+}-chelating properties.

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 improved. 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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Appendix A

Phenol-sulphuric acid method

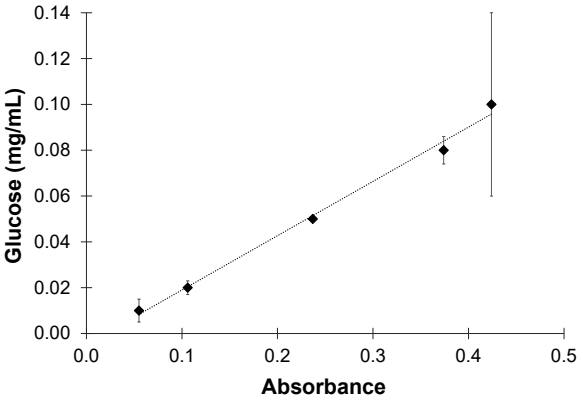

Figure A.1. Calibration curve of glucose (0 to 0.1 mg/mL) for the phenol-sulphuric acid method. Standards analysed in duplicate. Glucose (mg/mL) = 0.237×Absorbance - 0.00469, R^2=0.993

Appendix B

HPLC

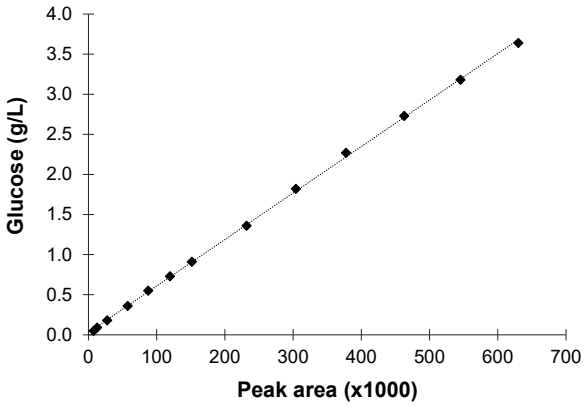

Figure B.1. Calibration curve of glucose (0 to 3.64 g/L) for HPLC analysis (RI). Glucose (g/L) = $5.799\times10^{-6}\times$Area + 2.908×10^{-2}, R^2=0.9996; Retention time = 13.33 minutes.

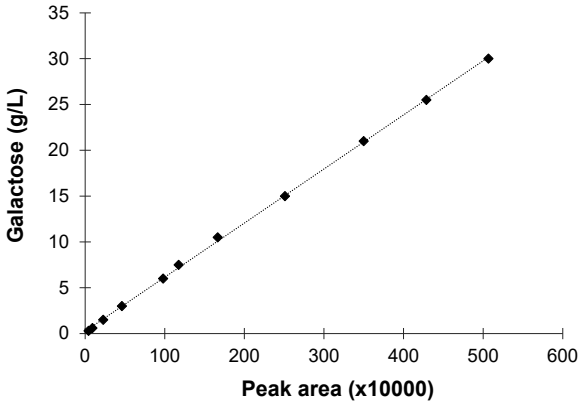

Figure B.2. Calibration curve of galactose (0 to 30 g/L) for HPLC analysis (RI). Galactose (g/L) = $5.903\times10^{-6}\times$Area + 2.562×10^{-1}, R^2=0.9996; Retention time = 14.18 minutes.

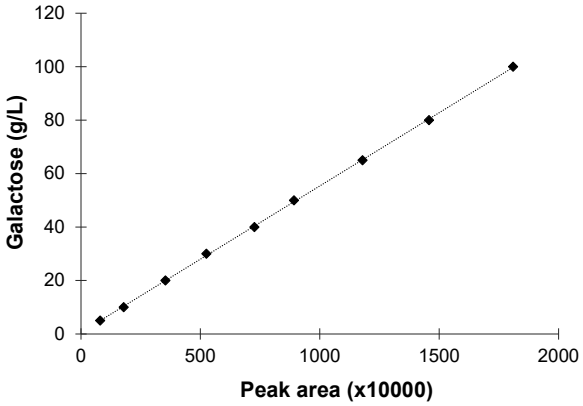

Figure B.3. Calibration curve of galactose (5 to 100 g/L) for HPLC analysis (RI). Galactose (g/L) = $5.480\times10^{-6}\times$Area + 5.874×10^{-1}, R^2=0.9998; Retention time = 14.18 minutes.

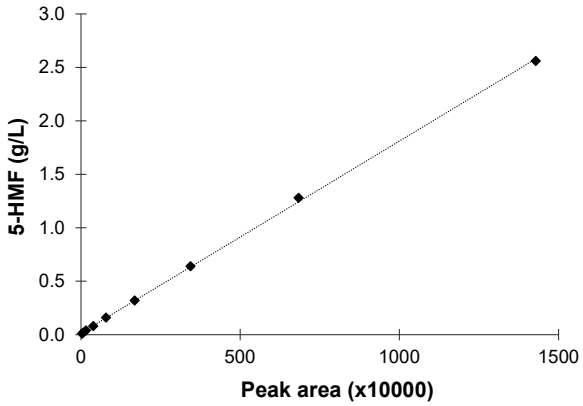

Figure B.4. Calibration curve of 5-HMF (0 to 2.56 g/L) for HPLC analysis (UV-Vis). 5-HMF (g/L) = $1.795\times10^{-7}\times$Area + 1.568×10^{-2}, R^2=0.9996; Retention time = 36.70 minutes.

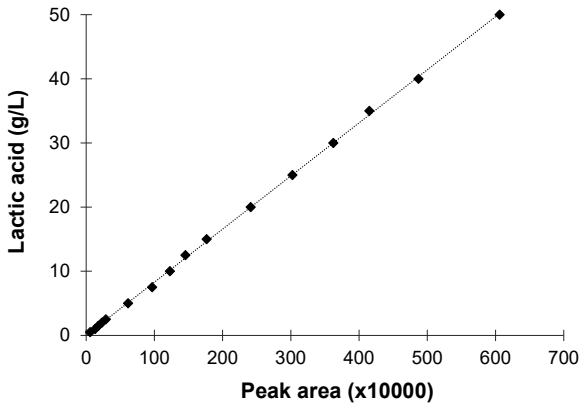

Figure B.5. Calibration curve of lactic acid (0 to 50 g/L) for HPLC analysis (RI). Lactic acid (g/L) = $8.278\times10^{-6}\times$Area + 2.578×10^{-2}, R^2=0.9997; Retention time = 17.63 minutes.

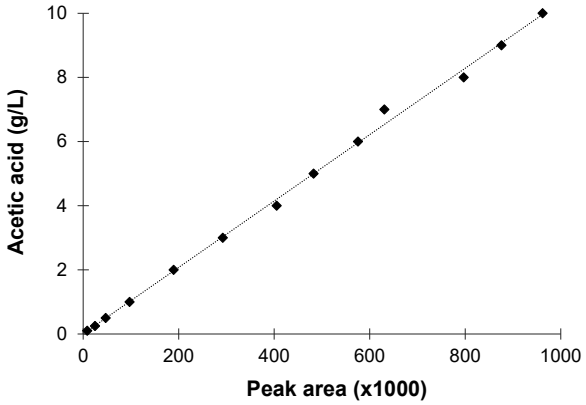

Figure B.6. Calibration curve of acetic acid (0 to 10 g/L) for HPLC analysis (RI). Acetic acid (g/L) = $1.035\times10^{-5}\times$Area + 6.627×10^{-3}, R^2=0.9977; Retention time = 19.97-19.99 minutes.

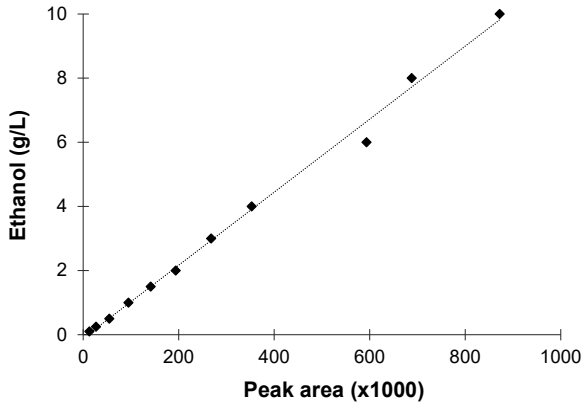

Figure B.7. Calibration curve of ethanol (0 to 10 g/L) for HPLC analysis (RI). Ethanol (g/L) = $1.139\times10^{-5}\times$Area - 1.099×10^{-1}, R^2=0.9996; Retention time = 27.34-27.39.

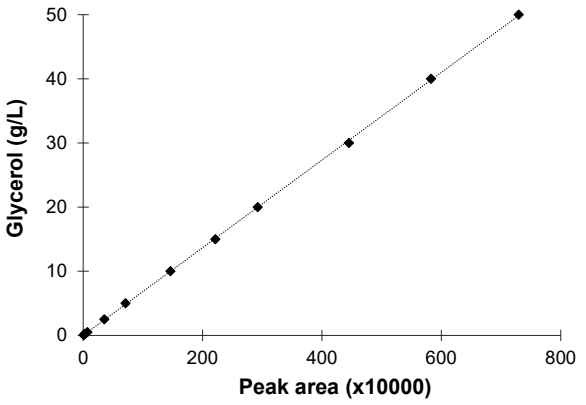

Figure B.8. Calibration curve of glycerol (0 to 50 g/L) for HPLC analysis (RI). Glycerol (g/L) = $6.833\times10^{-6}\times$Area + 8.797×10^{-3}, R^2=0.9999; Retention time = 18.63.

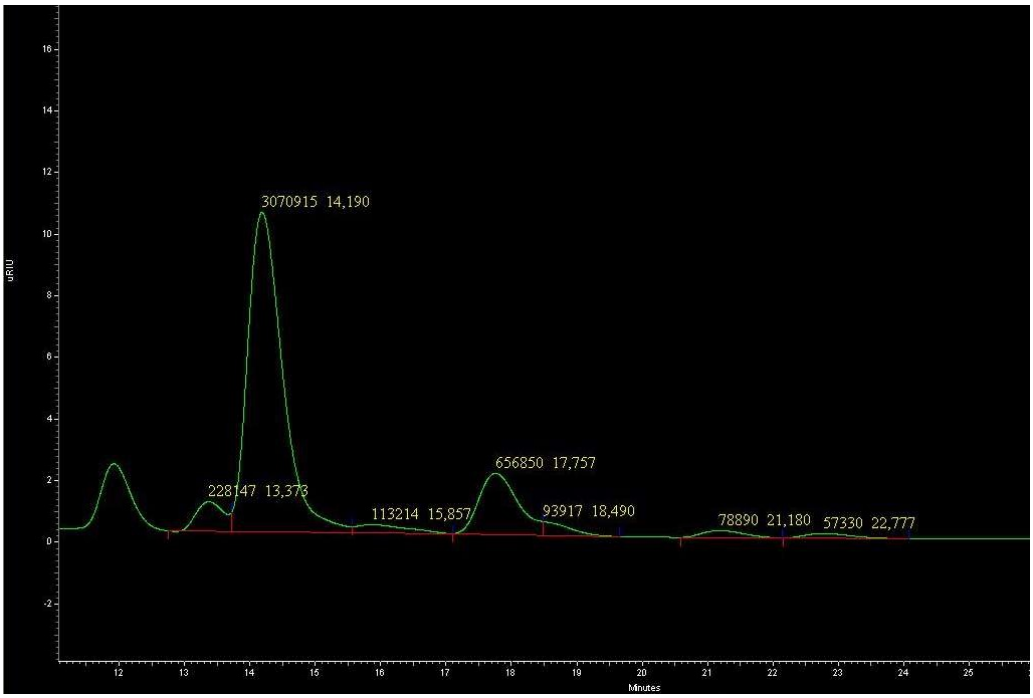

Figure B.9. Example of a typical HPLC spectrum (RI) from a sample collected at the beginning of fermentation. The spectrum was redimensioned to present relevant peaks from 11 to 26 minutes. Peaks are identified with (Area, Retention time). In order, from left to right, glucose (13.373 min), galactose (14.190 min), unknown (15.857), lactic acid (17.757 min), glycerol (18.490 min), unknown (21.180) and unknown (22.777 min).

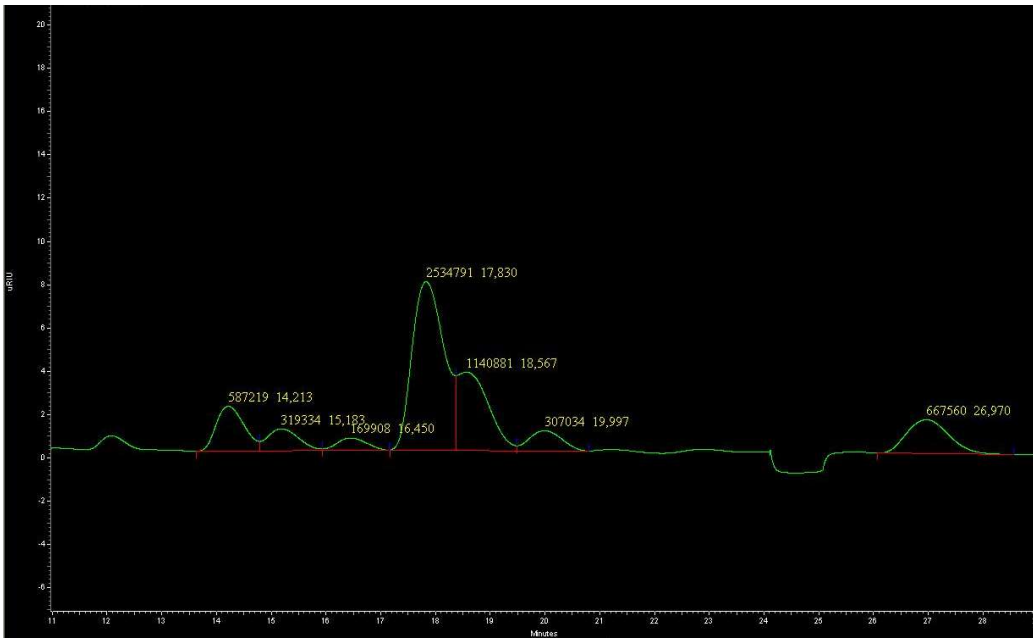

Figure B.10. Example of a typical HPLC spectrum (RI) from a sample collected at the end of fermentation. The spectrum was redimensioned to present relevant peaks from 11 to 29 minutes. Peaks are identified with (Area, Retention time). In order, from left to right, galactose (14.213 min), unknown (15.183), unknown (16.450), lactic acid (17.830 min), glycerol (18.567 min), acetic acid (19.997 min) and ethanol (26.970).