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Third Generation Lactic Acid Production by *Lactobacillus pentosus* from the Macroalgae *Kappaphycus alvarezii* Hydrolysates

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Abstract: The evaluation of macroalgae as a new raw material for diverse bioprocesses is of great interest due to their fast growth rate and low environmental impact. Lactic acid has a high value in the bio-based industry and is mainly produced via fermentation. The anaerobic lactic acid fermentation of *Kappaphycus alvarezii* hydrolysates using the high-producing strain *Lactobacillus pentosus* was evaluated for detoxified and non-treated hydrolysates prepared from concentrated algal biomass and dilute acid solution mixtures. A novel hydrolysate detoxification procedure, combining activated charcoal and over-liming, for 5-hydroxymethylfurfural (HMF) removal was used. *L. pentosus* was found to successfully ferment detoxified and untreated hydrolysates produced in up to 30% and 20% *w*/*v* solutions, respectively. Significant production rates (1.88 g/L.h) and short lag phases were achieved in bioreactor fermentation operating at 37 °C and pH 6 with 150 rpm impeller velocity. A 0.94 g/g yield from fermentable sugars (galactose and glucose) was achieved, indicating that *K. alvarezii* could be used as a raw material for lactic acid production, within the context of Third Generation (3G) biorefinery.

Keywords: seaweed; lactic acid fermentation; 5-hydroxymethylforfural; biorefinery; detoxification

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1. Introduction

In recent decades, the search for new renewable biomass sources that could, in the future, serve as feedstock for a bio-based industry which may provide an alternative for our fossil-based products has attracted great interest. With the rise of global awareness for environmental issues, governments, industry, and the scientific community alike are gradually aligning on the path to a more sustainable future.

Macroalgae (seaweed), for their fast growth rate and high fixation of carbon dioxide emissions, are a promising low carbon footprint feedstock for diverse bioprocesses. Furthermore, the cultivation of macroalgae does not require land use and does not affect terrestrial fauna and flora, and promoting algae cultivation can boost life quality in coastline communities [1]. If need be, macroalgae can be cultivated not only in open sea, but in artificial bodies of water [2]. Macroalgae biomass can be used in a wide array of industrial purposes, including food, cosmetic, pharmaceutic, and plastic industries [3].

According to specialists, macroalgae is more preferable than other feedstock regarding social, economic, and environmental scenarios [4]. In the European Union, research and development resources are most likely to be invested in algal biomass as raw material for biorefineries more than any other alternative feedstock between 2010 and 2030 [5].

In the context of a 3G biorefinery, seaweed is a resourceful feedstock that can yield a wide array of products for different uses. Macroalgae are mainly composed of polysaccharides, which make the production of ethanol and butanol feasible via fermentation [6]. Most species are rich in proteins, salts, anti-oxidants, and minerals that are absorbed from their cultivation environment, and could be extracted in a sustainable and environ-beneficial way [7–9]. In a SWOT analysis, Balina et al. [10] list the strengths and opportunities for the

seaweed biorefinery concept, mentioning the added value products, environment-friendly processes without significant need for land use, fresh water, and raw material expenses. Weakness and threats in SWOT analysis mainly include lack of governmental policies and economic stability. Although seaweed has a relatively low content of lipids, it is still mentioned alongside microalgae as an option for biodiesel production in The Handbook of Algal Biofuels [11], and in the extensive review by Torres et al. [6].

Kappaphycus alvarezii, being a red seaweed (Rhodophyta), is rich in the galactan polymer, carrageenan, a galactose-rich polysaccharide [12,13]. The carrageenan market has been steadily growing since 1980, and its use has more than doubled in the last two decades [14]. The farming of seaweed such as *K. alvarezii* is mainly cultivated for its high carrageenan content, which, according to Hung et al. [15], can reach up to 49%. The hydro-soluble content of *K. alvarezii*, which consists mostly of carrageenan, contains an average of 31.6 (%mol) of galactose and 25 (%mol) of 3,6-anhydrogalactose [16].

With a restricted natural growing region between 20° N and 20° S latitudes, *K. alvarezii* can be easily controlled as to not invade local flora outside of its farming areas if cultivated out of its naturally occurring zones [17]. It can be easily cultivated via fragmentation, and can be farmed jointly with other seafood products such as fish, mussels, and shrimp [18].

Due to the high demand of carrageenan, *K. alvarezii* is currently not cost effective when compared to other agricultural biomasses. Fresh *K. alvarezii* seaweed is sold at about 120 USD per ton, and dry weight seaweed can reach up to ten times the production cost [19]. For comparison, another widely available biomass in Brazil, sugarcane bagasse, has a price of 33 USD per ton [20]. Although *K. alvarezii* biomass is not yet competitive economically, the steady growth of its demand in the food, pharmaceutical, and plastic industries, with joint environmental concerns and the creation of government incentives should lead to a decrease in its overall cost and financial viability. The galactose content of *K. alvarezii* can be released from the galactan polymer by acid and enzymatic hydrolysis [13,21–23]. The sugar content of the carrageenan could be explored for the production of various products through fermentation processes.

A byproduct of acid hydrolysis of galactans is the formation of 5-hydroxymethylfurfural (HMF), which can be inhibitory to fermentation processes [24]. Although it restricts fermentative activity, HMF could be also used as a building block platform and intermediate in chemical industries [25]. HMF can be removed by activated charcoal and overliming treatments [26–28].

As a versatile building block molecule and natural acidulant, lactic acid has significant value for food, pharmaceutical, cosmetics, and chemical industries. Close to a third of its production is diverted for the production of the biodegradable polymer, polylactic acid (PLA), which has gained interest in the last decade [29].

Lactic acid is manufactured mainly via fermentation, in which, its two distinct isomers, L(+) or D(−), can be yielded purely [30]. Biological production of lactic acid uses cheap and natural substrates and less severe thermal conditions, thus reducing its energetic requirements. Chemical production by catalysis has its advantages, the most evident being the low-cost separation of the final product [31].

The strain *Lactobacillus pentosus* belongs to the polygenetic group *plantarum*. It is facultatively heterofermentative and facultatively anaerobic, and grows in a wide temperature range, from 15 °C up to 40 °C. As its name suggests, this microorganism can successfully produce lactic acid from pentoses. Zanoni et al. [32] reported that all *L. pentosus* strains in their study successfully grew on five carbon sugars, such as xylose and arabinose. Pot et al. [33], reported that it also consumes ribose. Pentose sugars are metabolized by *L. pentosus* via the EMP (Embden–Meyerhoff–Parnas) pathway. *L. pentosus* metabolizes hexoses via the PK (phosphoketolase) pathway. Studied *L. pentosus* strains are also known to consume galactose [33], the main sugar product of carrageenan hydrolysis. *L. pentosus* produces both L-lactic acid and D-lactic acid, the percentage of each isomer are 40 and 60%, respectively [34].

Lactic acid production with *L. pentosus* was already studied using renewable biomass such as sugarcane bagasse [34–36], vine shoots [37], microalgae [38], wood extract [30], corn stover [39,40], and wheat straw [41,42], as carbon sources. Hydrolysates of these biomass contain HMF which shows the adaptation of this culture to different hydrolysate and its resistance to fermentation inhibitors.

For more than a decade, the investigation of seaweed as raw material for lactic acid production has taken place around the globe, utilizing different seaweed species and a variety of lactic acid bacteria [43]. Dilute acid hydrolysis is widely used for obtaining fermentable sugars. Successful fermentation of *Enteromorpha prolifera* with *Lactobacillus rhamnosus* and *Lactobacillus salivarius* was done by Hwang et al. [44]. The microorganism *Lactobacillus plantarum* was able to ferment hydrolysates from *Gracillaria* sp. [45], *Ulva* sp. [46], *Ulva fasciata*, *Gracilaria corticata*, *Kappaphycus alvarezii* [47], and *Sargassum cristaefolium* [48]. Ramnose is a fermentable sugar found in green seaweed and the lactic acid bacteria, *Lactobacillus rhamnosus*, was used to ferment *Laminaria japonica* [49], *Gelidium amansii* [50], and *Ulva* sp. [48].

High titers of lactic acid have been obtained from fermentation in flasks ranging from 37.7 to 12.5 g/L [45,46,48,50–52], the highest concentration being from *Lamniaria japonica* [52]. Jang et al. [49] executed fermentation in a 1-L working volume bioreactor reaching 14.5 g/L of lactic acid, with a 0.9 (g/g) yield. A study of the potential for using marine biomass in bioreactor fermentation was done by Mwiti et al. [53], where hydrolysates of commercial agar were fermented in a 2-L working volume fed batch reactor, in this case, reaching 31.9 g/L of lactic acid.

Recently, it was shown that *L. plantarum* was able to ferment diluted hydrolyzed *K. alvarezii* solutions [47]; nevertheless, implementing bioprocesses for high concentration hydrolysates can prove arduous due to the use of non-preferred carbon sources and the presence of compounds that could prove toxic, and inhibit cell growth and product formation. Short term adaptation of microorganisms to media can be done via acclimatization processes, in which, cultures are gradually exposed to a new substrate or toxin-containing environment. The mechanisms of short-term cell adaptation are complex and differ for each strain. Mechanisms for adaptation include long-term retention proteins, epigenetic modifications, and cross-stress protection as explained by Tan et al. [54]. Species of *Lactobacillus* grow in suboptimal conditions during food fermentation. Huang et al. [55] described genetic cross-stress protection strategies of *L. plantarum* strains.

The fermentability of hydrolyzed *K. alvarezii* biomass has principally been studied in the last decade for bioethanol production [13,22,23,56]. In their review of successful approaches for red seaweed biorefineries, Álvarez-Viñas et al. [9] describe a refinery scheme for *K. alvarezii*, including only ethanol as a fermentation product.

The use of seaweed as raw material does not require land use and acts as a natural filter for sea pollution, and could, if implemented on a large scale, have a positive impact on the environment. This current study investigates the potential of using the macroalga *K. alvarezii* as raw material for lactic acid production via fermentation with *L. pentosus*, an efficient fermenting bacterium, which produces lactic acid in high concentrations. The possible production of lactic acid could add another valuable product to a *K. alvarezii*-based biorefinery.

2. Materials and Methods

2.1. Algal Biomass and Processing

2.1.1. Initial Processing

Dry *K. alvarezii* algae grown in the southern coastline of Rio de Janeiro was purchased for processing. The macroalgae biomass was washed in deionized water three times over in order to remove salt and small impurities. Shortly after, the biomass was placed in a drying oven set to 50 °C and left until apparently fully dry. The newly dehydrated biomass was then grinded in a small-scale Wiley-type mill, and thereafter, in an analytical mill for fine grain production. Monosaccharide compositions of dry processed algae and

pre-treatment residue were determined by a two-step hydrolysis procedure described by Hoebler et al. [57].

2.1.2. Acid Pretreatment

Dilute acid-thermic hydrolysis took place in 1-L conical flasks, containing 500 mL of a 1 % (*v*/*v*) sulfuric acid solution and different amounts of algal biomass, with thermal conditions of 111 °C (0.5 bar gauge) in an autoclave for a 45-min period. The resulting material was then press-filtered, and the hydrolysate subjected to clarifying/detoxifying treatments. From recent studies it was shown that to achieve above 30 g/L of fermentable sugars, a 20–30% (*w*/*w*) ratio should be used [13,21–23]. To reach the highest fermentable galactose-containing media, a range of 20–40% algae–dilute acid solution was chosen for evaluation in this study.

2.1.3. Hydrolysate Clarification Procedures

First, the hydrolysates were over-limed with calcium hydroxide, adjusting pH to 12 for 30 min. Sedimentation was vacuum filtered through a qualitative 150 mm diameter, 25–40 µm pore paper filter, and fine powdered activated charcoal was added to reach a 25% *w*/*w* ratio and left for one hour, as suggested by Hargreaves et al. [13]. During the charcoal treatment flasks were agitated in a shaker at 150 rpm at 30 °C, and the final hydrolysate solution was obtained by vacuum filtering at between −200 to −400 mmHg gauge using the aforementioned paper filter.

2.2. Microorganism, Media Culture

The strain of *L. pentosus* ATCC 8041 was obtained from the American Type Culture Collection. The strain was grown, and later fermented in a Man–Rogosa–Sharpe medium (MRS), which is traditionally used for *Lactobacillus* ssp. fermentations. The composition of culture media was as follows: 20 g/L galactose, 10 g/L peptone, 5 g/L yeast extract, 10 g/L beef extract, 1mL/L Tween 80, 2 g/L ammonium citrate, 0.1 g/L $MgSO_4$, 0.05 g/L $MnSO_4$ and 2 g/L K_2HPO_4. Note that glucose was substituted by galactose in the original media formula due to the rich galactose content obtained by carrageenan hydrolysis.

2.3. Culture Adaptation and Fermentation

Propagation and initial fermentation assays were carried out in 100 mL serum flasks filled with 70 mL, while fermentation experiments in higher volumes were run in 1-L conical flask filled to 400 mL and a 2-L stirred tank BioFlo III bioreactor manufactured by Brunswick with a 1000 mL working volume. All propagation and fermentation systems were stirred at 150 rpm (via shaker for serum and conical flasks, and rotor-impeller for bioreactor) and kept at 37 °C.

As a means for achieving better anaerobic conditions, both clasped serum flasks and bioreactor were sparged with nitrogen and sterilized at 121 °C for 20 min. Whether fermentation took place in serum flasks or in conical flasks, 20 g/L of calcium carbonate were added as a pH buffer and to slightly promote anaerobic conditions.

Current literature does not provide a clear consensus regarding the exact pH level to use for optimal lactic acid production. Wang et al. [58] reported that optimum production was achieved with a pH 5.6, while other authors reported successful lactic acid production with slightly higher pH, 6 [30], and 6.5 [34,35]. For a rigorous pH control during fermentation in the bioreactor, a 1.5 M NaOH solution was used to maintain the pH of the fermentation media at 6.

To ensure the survival rate of cells and improve galactose intake, *L. pentosus* was gradually introduced to the hydrolysate media prior to fermentation, as shown in Figure 1, starting at 20% (*v*/*v*) hydrolysate–MRS mixtures and up to a 60% volumetric hydrolysate–MRS ratio before hydrolysates were inoculated.

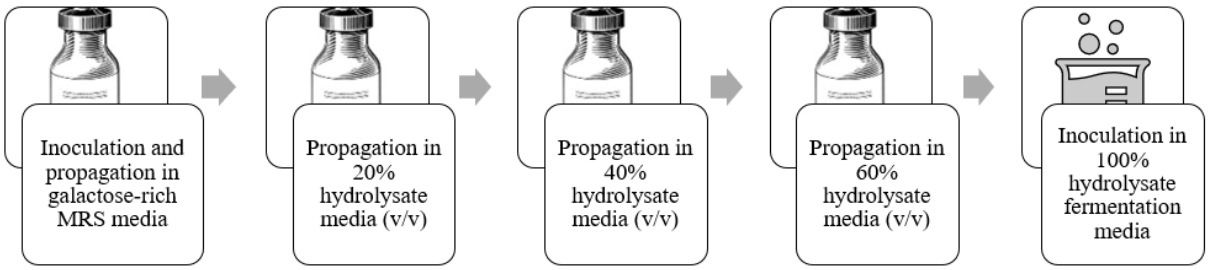

Figure 1. Acclimatization scheme for *L. pentosus* preparation in increasing ratios (% *v*/*v*) of *K. alvarezii* hydrolysate in galactose rich MRS—Man–Rogosa–Sharpe medium.

For evaluating the possibility of strain growth in hydrolyzed *K. alvarezii*, the algal biomass-acid solution ratio used to prepare hydrolysates varied from 20% (*w*/*v*) to 40% (*w*/*v*). For each concentration a treated and not treated for HMF removal was evaluated. After the strain adaptation, hydrolysates were inoculated with a 25% (*v*/*v*) cell suspension grown in serum flasks. To investigate the production capacity under non ideal anaerobic conditions, a 25% cell suspension of non-acclimatized *L. pentosus* were inoculated in conical flasks with synthetic MRS media with a 50 g/L galactose concentration. The fermented media were analyzed after a 72-h incubation period. After resistance trials, 30% (*w*/*v*) and 25% (*w*/*v*) hydrolysates were fermented in conical flasks and a bioreactor, respectively. In conical flasks, a cell suspension of 1.2 g/L of acclimatized and non-acclimatized cultures were inoculated to test acclimation effects on fermentation kinetics. A cell suspension of 30% (*v*/*v*) was used in bioreactor as inoculum.

A standard optical density (OD absorbance at 600 nm) to dry weight calibration curve was used to determine cell concentration during the bioreactor experiments. Glucose, galactose, lactic acid, and HMF were analyzed by High Performance Liquid Chromatography (HPLC) Shimadzu using a Hi-Plex H (8 µm) column with RID and UV 210 nm detectors. A H_2SO_4 0.005 mol/L mobile phase at a flow rate of 0.6 mL/min at 60 °C.

3. Results

3.1. Composition of the Algae, and Algal Hydrolysates

The composition of sugars and HMF in dry algal mass and the remaining residue after pretreatment are shown in Table 1. The sum of carbohydrates present was of 61.31% of total dry weight of *K. alvarezii* dry weight, composed mostly of galactose. Pretreatment residue had low HMF content, with glucose as the main component. Combining galactose and HMF content in dry weight *K. alvarezii* biomass, carrageenan can be estimated to be of 41%.

Table 1. Monosaccharide composition of *K. alvarezii* used in this project.

Component	% of Dry Weight Algae Biomass	% of Dry Weight of Residue after Acid Pretreatment
Galactose	33.36 ± 3.71	15.27 ± 1.53
Glucose	15.72 ± 2.17	30.36 ± 0.24
HMF	12.24 ± 0.55	0.49 ± 0.21

The hydrolysate compositions obtained after hydrolysis and treatment are shown in Table 2. Galactose concentration achieved in the most concentrated hydrolysate (40% algal biomass) was of 64.33 g/L. HMF/galactose formation ratio during hydrolysis reached between 25 to 35%. HMF was totally removed by detoxification procedures, and alongside detoxification, there was also loss of sugar content, between 5 and 15%. It is also worth mentioning that lactic acid was detected for most of the dilute acid pretreated algal biomass, excepted for the detoxified hydrolysates generated with the ratios of 20–30% algal mass/acid solution.

Table 2. Composition of detoxified and non-detoxified *K. alvarezii* hydrolysates.

Ratio of Algal Mass/Acid Solution (*w*/*v*)	Detoxification	Galactose (g/L)	Glucose (g/L)	Lactic Acid (g/L)	HMF (g/L)
20%	Detoxified	19.06 ± 2.12	1.83 ± 0.45	-	-
30%	Detoxified	45.67 ± 0.44	4.76 ± 0.01	-	-
40%	Detoxified	60.53 ± 1.08	9.14 ± 0.24	4.34 ± 0.14	-
20%	Non-treated	19.97 ± 0.96	1.40 ± 0.01	5.08 ± 0.73	6.73 ± 0.84
30%	Non-treated	54.29 ± 1.50	4.82 ± 0.31	5.74 ± 0.18	12.51 ± 0.84
40%	Non-treated	64.33 ± 0.40	10.27 ± 0.08	3.71 ± 0.07	21.97 ± 0.05

An additional batch of 30% (*v*/*v*) hydrolysate was produced and galactose and HMF were quantified showing respective output of each treatment step for *K. alvarezii* (Table 3). Based on estimated carrageenan content, and overall galactose present in biomass used in this study recovery of total galactose, given full theoretic solution recovery, is of 44% after dilute acid pretreatment and is diminished to 41% after detoxification. About 40% of solution was lost during laboratory-scale treatment process, though it should it noted that using proper industrial machinery could lead to much higher recovery of fermentable sugars from wet solid residue.

Table 3. Input and output of *K. alvarezii* pretreatment.

Step	Input	Output
Dilute acid hydrolysis	300 g DSW + 1 L 1% (*v*/*v*) H_2SO_4	0.7 L hydrolysate (44 g/L galactose + 24 g/L HMF)
Overliming detoxification	0.7 L hydrolysate + $CA(OH)_2$	0.65 L hydrolysate (43 g/L galactose + 9 g/L HMF)
Activated charcoal clarification	0.65 L hydrolysate + 100 g activated charcoal	0.6 L hydrolysate (41 g/L galactose)

HMF, 5-hydroxymethylfurfural.

3.2. Algal Hydrolysate Fermentability

Preliminary fermentations of hydrolysates are summarized in Table 4. No uptake of fermentable sugars was detected in 40% algal mass concentration, as well as in non-treated (non-detoxified) 30% algal biomass hydrolysate. At the end of the fermentations, there still remained galactose and glucose, though high lactic acid yields were obtained. These results also showed that *L. pentosus* used other more complex organic compounds to produce lactic acid. In the less strict anaerobic assay that took place in conical flasks with MRS media, galactose was almost totally consumed.

The acclimatized *L. pentosus* ATCC 8041 strain successfully fermented a medium containing detoxified 30% (*w*/*v*) algae hydrolysate added with MRS solution in conical flasks, in aerobic conditions. With non-acclimatized cells, a 72-h lag phase was observed during cultivation, and for acclimatized cells, the lag phase was 24 h (Figure 2).

The results clearly show the essentialness of acclimatization, which led the bacterial cells to perform much better, yielding higher rates of galactose consumption and lactic acid production when compared to the non-acclimatized culture for the first 72 h after inoculation (Table 5).

3.3. Fermentation in Batch Bioreactor

For bioreactor fermentation, cell growth, consumption of carbohydrates and lactic acid production kinetics were observed in regular 4-h intervals for 48 h (Figure 3). It should be noted that the hydrolysate used in the bioreactor was prepared separately with a 25% (*w*/*v*), and in larger volumes, which resulted in lower galactose content of 27.5 g/L, reaching 29.4 g/L of lactic acid.

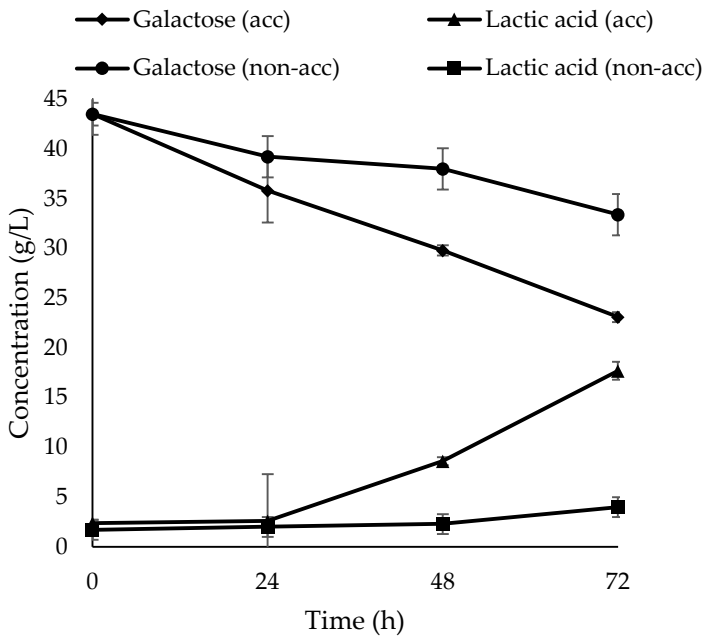

Figure 2. Batch fermentation kinetics of lactic acid by acclimatized (acc) and non-acclimatized (non-acc) cultures of *Lactobacillus pentosus* from *Kappaphycus alvarezii* hydrolysates in conical flask with 20 g/L $CaCO_3$ buffer, using a 1.2 g/L inoculum, at 37 °C, and 150 rpm.

Table 4. Fermentability of *K. alvarezii* hydrolysates by acclimatized *L. pentosus* for lactic acid production carried out in 100-mL serum flasks with a 70 mL working volume of hydrolysate media with a 72-h incubation period.

Hydrolysate Media	$Galactose_{Initial}$ (g/L)	$Glucose_{Initial}$ (g/L)	$Galactose_{Final}$ (g/L)	$Glucose_{Final}$ (g/L)	Lactic Acid (g/L)	Yield (g/g)
Detoxified 20% (*w*/*v*)	19.06 ± 2.12	1.83 ± 0.45	2.47 ± 1.25	1.09 ± 0.58	20.24 ± 0.22	1.27
Non-treated 20% (*w*/*v*)	19.97 ± 0.96	1.40 ± 0.01	7.31 ± 0.16	1.39 ± 0.01	16.12 ± 1.76	1.17
Detoxified 30% (*w*/*v*)	45.67 ± 0.44	4.76 ± 0.01	4.32 ± 0.23	2.26 ± 0.14	57.61 ± 2.23	1.31
Non-treated 30% (*w*/*v*)	54.29 ± 1.50	4.82 ± 0.31	No consumption of fermentable sugars and production of lactic acid detected			
Detoxified 40% (*w*/*v*)	60.53 ± 1.08	9.14 ± 0.24	No consumption of fermentable sugars and production of lactic acid detected			
Non-treated 40% (*w*/*v*)	64.33 ± 0.40	10.27 ± 0.08	No consumption of fermentable sugars and production of lactic acid detected			
MRS 50 g/L galactose *	50.00 ± 0.01	-	0.80 ± 0.01	-	55.30 ± 1.10	1.12

* Carried out in a 1-L conical flask with a 400 mL working volume of Man–Rogosa–Sharpe medium (MRS).

Table 5. Batch 72-h fermentation kinetics of lactic acid by acclimatized and non-acclimatized cultures of *Lactobacillus pentosus*, from detoxified *Kappaphycus alvarezii* hydrolysates in conical flask with 20 g/L $CaCO_3$ buffer, using a 1.2 g/L inoculum, at 37 °C, and 150 rpm.

Bacterial Culture	$Gal_{Initial}$ (g/L)	PRS * (%)	Lag Phase (h)	LA Produced (g/L)	dGal/dt (g/L.h)	dLA/dt (g/L.h)	$Y_{P/S}$ (g/g)
Acclimated	43.5 ± 0.21	48.3	24	18.6	0.29	0.32	0.79
Non-Acclimated	43.5 ± 0.21	23.2	72	2.0	0.13	0.04	0.20

* PRS: percentage reduction of substrate.

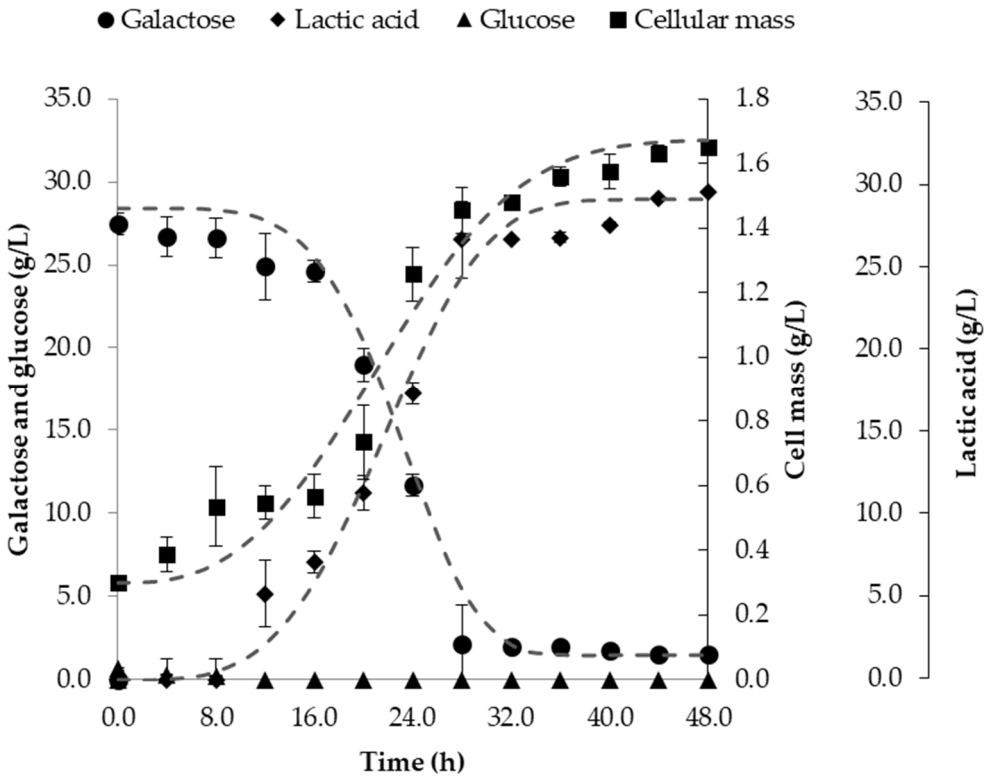

Figure 3. Batch fermentation kinetics of lactic acid by acclimatized *Lactobacillus pentosus* from detoxified *Kappaphycus alvarezii* hydrolysates in 2-L bioreactor with a 1-L working volume at pH 6, 37 °C, and 150 rpm.

Upon inoculation, the low glucose content present in media was immediately consumed in the first 8 h, accompanied by slight cell growth. Henceforward, galactose was consumed, and significant amounts of lactic acid were produced with cell mass multiplying more than 5 times over. The lactic acid production rate during galactose consumption was of 1.88 g/L.h, with the corresponding global yield, YP/S, and percentage reduction of substrate, PRS, of 1.16 g/g and 94%, respectively. Nonetheless, maximum lactic acid content was reached in less than 48 h, leading to a high volumetric productivity, because of the more controlled process conditions in the bioreactor.

4. Discussion

According to the results in this study, galactose-rich concentrated *K. alvarezii* hydrolysates can be used for lactic acid production, previously reported only in dilute solutions [47]. The hydrolysis procedure resulted in galactose concentrations which were compatible with previously reported procedures [13,21–23], and the combined overliming and charcoal detoxification method removed the fermentation inibitor HMF completely from the fermentation media, which, up to date, was not totally removed by using only activated charcoal [13].

4.1. Acid Pretreatment and Detoxification

Reports made in the last decade show that fermentable galactose and glucose can be readily obtained from *K. alvarezii* biomass via dilute acid hydrolysis using either hydrochloric or sulfuric acid (Table 6), both of which lead to similar results. Sulfuric acid is easier to separate from the hydrolysate because of its neutralization and sulfate sedimentation with calcium hydroxide during pH adjustment [13].

One of the main hurdles of hydrolysate fermentability is the formation of HMF, which inhibits fermentation and bacterial growth [33]. Acid hydrolysis of *K. alvarezii* results in up

to ten or even fifty times the amount of HMF than other raw materials submitted to similar acidic conditions [34,35,59].

The galactan polymer, κ-carrageenan, that is present in *K. alvarezii* is made up of alternating units of 3,6-Anydro D-galactose and D-galactose 4-sulfate [60]. HMF is formed by dehydration of the 3,6-Anydro D-galactose molecule by acid catalysis at high temperatures [61], which explains the high HMF concentration in hydrolysates.

Several strategies can be applied to significantly reduce or eliminate HMF presence in hydrolysates, the first of which is to optimize hydrolysis conditions, manipulating temperature, acid concentration, reaction time, and algal biomass:acid solution ratio. A fast (5 min) hyperthermic reaction (140 °C) was done by Ra et al. [23] yielding high galactose content and less than 5 g/L of HMF. Similar results to those obtained in this study can be seen in the work of Hargreaves et al. [13], with high HMF formation during hydrolysis.

In order to achieve more sugar content from the cellulose content of *K. alvarezii*, one can combine acid hydrolysis with enzymatic hydrolysis, as demonstrated by Abd-Rahim et al. [21]; with acid hydrolysis followed by enzymatic hydrolysis, the yield of reducing sugars increased from 42.8 to 62.4% after enzymatic hydrolysis. Another way to better exploit *K. alvarezii* for fermentable sugar production is shown by Paz-Cadeno et al. [62], where after carrageenan removal by KOH treatment, glucose and galactose were obtained, initiating the hydrolysis process enzymatically, using commercial extract Cellic CTec II, and then using mild-acid conditions with lower temperatures (no more than 80 °C). This way, additional fermentable sugars are obtained without diminishing the commercially valued carrageenan.

Table 6. Acid pretreatment conditions reported in literature for *K. alvarezii*.

Research Group	Temperature	Acid Concentration	Hydrolysis Time	Algal Biomass Hydrated Triturate Ratio	Galactose (g/L)	HMF (g/L)
Ra et al. [23]	140 °C	180 mM	5 min	12% (*w*/*v*)	38.3 *	<5.0
Hargreaves et al. [13]	121 °C	1.0% *v*/*v*	60 min	50% (*w*/*v*)	81.6	20.7
Khambhaty et al. [22]	100 °C	0.9 N	1 h	26.2–30.6% (*w*/*w*)	30.6 *	Not reported
Abd-Rahim et al. [21]	110 °C	0.2 M	90 min	8.0 g/100 mL	34.27 *	Not reported
This study	111 °C	1.0% *v*/*v*	45 min	30% (*w*/*v*)	54.23	12.51

* Total of reducing sugars, should include glucose, galactose, and 5-hydroxymethylfurfural.

If low enough values of HMF for fermentation are not attained upon acid hydrolysis, cleansing methods can be applied. Khambhaty et al. [22] and Hargreaves et al. [13] both successfully fermented hydrolysates inoculating *Saccharomyces cerevisiae* in fermentation media and producing 3G bioethanol, the former without using detoxification procedures and the latter after using activated charcoal for efficient HMF removal.

The formation of organic acids during hydrolyzation was detected by HPLC, including lactic acid, which does should not form during dilute acid hydrolyzation. This phenomenon could have occurred during neutralization and overliming procedures. Alkaline catalysis can transform the main constituents of carrageenan and cellulose to lactic acid [63], and the $Ca(OH)_2$ used for neutralization and overliming was shown to convert carbohydrate to lactic acid by Onda et al. [64], explaining the low content of lactic acid found in the fermentation media. In any case, detoxification with activated charcoal removed organic acids from the fermentation media. As can be observed in Table 2, HMF was not detected via HPLC in treated hydrolysates due to the combined overliming treatments with activated charcoal in this study, showing the promise of this kind of procedure in fermentation applications.

The total amount of reducing sugars in this study (Table 1) was similar to those reported by Abd-Rahim et al. [21]. Again, the presence of HMF was due to the dehydration of 3,6-Anydro D-galactose, during the acid pretreatment. The galactose content in pretreatment residue represents the unattained percentage from dilute acid hydrolysis, where total yield amounted to around 45% of galactose present in dry mass of *K. alvarezii*. In general,

the yield of galactose in this study stands within the range of previously reported contents of hydrolysate production (Table 6).

4.2. Fermentability of K. alvarezii Hydrolysates by L. pentosus

Lactic acid bacteria (LAB) can metabolize galactose via the Leiloir pathway, using galactose permease for intake transportation or through a galactose phosphotransferase in the tagatose phosphate pathways [65]. It is reported in literature that *L. plantarum* possesses only the Leloir pathway [66]. Being facultatively heterofermentative, *L. pentosus* successfully produced lactate exclusively via the EMP pathway, with no ethanol and acetic acid byproducts. Lack of growth in untreated hydrolysate from 30% algal biomass concentration and treated 40% algal biomass concentration indicates the presence of other unknown fermentation and growth inhibitors in hydrolysates, a factor which should be further investigated in order to bring up the tolerance of microorganisms to high titer solutions.

Best result for fermentation of concentrated hydrolysates was obtained with the detoxified 30% (*w*/*v*) mixture of biomass and dilute acid solution. Fermentation of the detoxified 20% (*w*/*v*) hydrolysate resulted in a 25% increase in lactic acid production when compared with non-treated hydrolysate of the same biomass to dilute acid solution ratio, emphasizing the advantages of detoxification for processing *K. alvarezii* hydrolysates.

L. pentosus was able to ferment galactose in synthetic MRS media in non-strict anaerobic conditions as reported by Cubas-Cano et al. [42], which shows that this microorganism is able to ferment galactose under such non-strict anaerobic conditions as well as glucose and xylose. Fermentation in non-strict anaerobic conditions resulted in much longer lag phases and reduced lactic acid production. Again, no ethanol and acetic acid were detected showing the high specificity to lactic acid production by this species.

The data presented in Table 5 show the benefits of acclimation procedures. An addition of a 2-day lag phase was seen in fermentation with non-acclimated bacteria. Lactic acid productivity and $Y_{P/S}$ with acclimatized cells were of 0.32 g/L.h, and 0.79, respectively, compared to a mere 0.04 g/L.h and 0.20 with non-acclimated cultures. PRS was double when acclimated cultures were applied. Implementing acclimation procedures on *L. pentosus* for bioreactor fermentation proved to be a sound choice since acclimated cells showed better suited for efficient lactic acid production.

It should be noted that although assays took place with rigorous temperature control, maintaining stable levels of pH was achieved by adding calcium carbonate, which releases CO_2 upon reaction with produced acid. Although calcium carbonate buffers were reported to augment lactic acid production in *Lactobacillus rhamnosus* and *Lactobacillus fermentum* [67], it acts as a buffer with close to 7 pH values, which are not optimal for cell growth [58].

As reported by Cubas-Cano et al. [42], strict anaerobic conditions play a crucial role in efficient and productive lactic acid fermentation. In their study lag phases of *L. pentosus* reached around 15 h in aerobiosis (unclamped flasks), and almost 10 h in anaerobiosis (clamped flasks). When using helium to reach even stricter anaerobic conditions, lactic acid production started with negligible lag phases. The results in the present work corroborate with these findings, having negligible lag phases in bioreactor fermentation where the fermentation media is better shielded from the oxygen-rich external environment.

The results obtained in the bioreactor clearly show the benefits of rigorous pH control for lactic acid fermentation by *L. pentosus*. The tolerance of *L. pentosus* is around pH 4, though optimal values reported and those used in recent studies are around 6 pH [58].

As reported in literature, *L. pentosus* is able to ferment hydrolysates from a variety of raw materials. Table 7 exhibits recent studies of lactic acid fermentation with *L. pentosus*. Overall productivity in this study was of 0.90 g/L.h, and 94% of fermentable sugars were consumed, which can be considered more than average in comparison to the use of other raw materials. As aforementioned, due to the exclusivity of hexoses as a carbon source, no byproducts were produced during fermentation, yielding lactic acid as the sole product of

not only fermentable glucose and galactose, but other organic compounds present in the fermentation media.

As reported by Wischral et al. [34], working with process development to produce lactic acid by simultaneous saccharification and co-fermentation from hemicellulose and cellulose hydrolysates of sugar cane bagasse showed that *L. pentosus* was able to efficiently consume both sugars, though glucose was first totally fermented before the fermentation of xylose. This denotes the notable versatility of this species as far as the fermentation of several sugars. Wang et al. [58] reported that production of lactic acid with free *L. pentosus* cells was significantly lower than immobilized cells with a production rate of 1.323 g/L.h and 83% consumption of glucose in 72 h. In the present work, *L. pentosus* consumed 94% of galactose in hydrolysates in less than 48 h, giving a 0.9 g/L.h production rate. Though production in K. alvarezii hydrolysates took a day less, Wang et al. [58] initiated production with a 120 g/L glucose titer, producing approximately 100 g/L of lactic acid.

Table 7. Lactic acid production by *L. pentosus* from different raw material reported in literature.

Research Group	Carbon Source	Yield (g/g) *	Overall Productivity (g/L.h)
Wischral et al. [34]	Sugarcane bagasse	0.93	1.01
Unrean [36]	Sugarcane bagasse	0.61	1.01
Talukder et al. [38]	Microalgae (*Nannochloropsis salina*)	0.93	0.45
Bustos et al. [37]	Vine shoots	0.78	0.93
Hu et al. [39]	Corn stover	0.66	1.92
Buyondo & Liu [30]	Wood extract	0.80	1.53
Wang et al. [58]	Synthetic glucose MRS media	0.83	1.33
This study	Macroalgae (*Kappaphycus alvarezii*)	0.94	0.90

* Yields for g(fermentable sugars)/g(lactic acid).

Lactic acid fermentation of seaweed was accomplished using a variety of green, brown, and red algae (Table 8), the most comparable to this current study being the galactose-rich Rhodophyte (red seaweed), from which, lactic acid fermentation yielded up to 29.0 g/L of lactic acid [48]. Utilizing *L. pentosus* in a high titer hydrolysate resulted in more than 25 g/L more than highest titer reported in literature, with a similar initial fermentable sugar concentration, demonstrating the productiveness of this versatile microorganism [45].

Table 8. Serum flask scale production of lactic acid from seaweed hydrolysates reported in literature.

Macroalga	Microorganism	Hydrolysis Conditions	Carbon Source	Initial Titer (g/L)	Yield (g/g) *	Final LA Product (g/L)	Ref.
Kappaphycus alvarezii	*Lactobacillus pentosus*	1% H_2SO_4 at 120 °C, 30% (*w*/*v*)	Galactose, glucose	45	1.3	57.6	This study
Laminaria japonica	*Escherichia coli*	0.1 N HCl at 120 °C, 10% (*w*/*v*)	Glucose, mannitol	50	0.8	37.7	[52]
Ulva sp.	*Lactobacillus plantarum*	4% H_2SO_4 at 120 °C	Rhamnose, glucose, xylose	40	0.9	36.8	[46]
Ulva sp.	*Lactobacillus rhamnosus*	4% H_2SO_4 at 120 °C	Rhamnose, glucose, xylose	40	0.8	30.9	[48]

Table 8. *Cont.*

Macroalga	Microorganism	Hydrolysis Conditions	Carbon Source	Initial Titer (g/L)	Yield (g/g) *	Final LA Product (g/L)	Ref.
Gracilaria sp.	*Weissella paramesenteroides*	3% H_2SO_4 at 120 °C	Galactose, glucose	40	0.9	29.0	[48]
Sargassum cristaefolium	*Lactobacillus plantarum*	5% H_2SO_4 at 120 °C	Fructose, xylose, rhamnose, glucose	40	0.8	26.0	[48]
Eucheuma denticulatum	*Bacillus coagulans*	0.1 M H_2SO_4, 120 °C, 6% (*w*/*v*)	Galactose	25	0.9	22.0	[51]
Gracillaria sp.	*Lactobacillus acidophilus* and *Lactobacillus plantarum*	0.4 N HCl at 120 °C, 10% (*w*/*v*)	Galactose	30	0.8	19.3	[47]
Gelidium amansii	*Lactobacillus rhamnosus*	3% H_2SO_4 at 120 °C, 5% (*w*/*v*)	Glucose, galactose	-	0.4	12.5	[50]

* Yields for g(fermentable sugars)/g(lactic acid).

Fermentation of marine biomass in bioreactors reported in literature are shown in Table 9. Results of fermentation of agar by *Lactobacillus brevis* reached a final product of 31.9 g/L of lactic acid with 17.2 g/L of ethanol as a byproduct [53]. In this case, glucose was required to activate galactose consumption by *L. brevis*, which was not necessary when fermenting *K. alvarezii* hydrolysates that already contain a low quantity of glucose. Results for bioreactor fermentation in this study were of double the lactic acid final concentration when compared to similar working volume of *Laminaria japonica* fermented by *L. rhamnosus* [49].

Table 9. Bioreactor scale production of lactic acid from seaweed hydrolysates reported in literature.

Macroalga	Microorganism	Bioreactor System	Yield (g/g) *	Final LA Product (g/L)	Reference
Laminaria japonica	*Lactobacillus rhamnosus*	1-L Batch	0.9	14.4	[49]
Agar hydrolysate	*Lactobacillus brevis*	2-L Fed batch	1.0	31.9	[53]
Kappaphycus alvarezii	*Lactobacillus pentosus*	1-L Batch	0.9	29.4	This study

* Yields for g(fermentable sugars)/g(lactic acid).

Sudhakar and Dharani [47] successfully fermented *K. alvarezii* hydrolysates alongside hydrolysates of *Ulva fasciata* and *Gracilaria corticata* to lactic acid with *L. plantarum*, in which case, a 2% (*w*/*v*) of algae to dilute acid solution was used.

Up to date, the study of biorefinery products from *K. alvarezii* was limited to the production of bioethanol and biogas alongside the production of carrageenan. Lactic acid has a wide-range use in multiple industries and it is a building block molecule for bioplastics. This study endeavored to study the production of lactic acid with *K. alvarezii* as biomass feedstock on a broader scale, showing its potential for up-scaling and implementation within the concept of a biorefinery.

5. Conclusions

Seaweed, as a fast-growing biomass, requires negligible land use, and can be better to the environment through CO_2 fixation and absorption of chemicals. Potential bio-refinery implementations can boost coastline communities' income and are beneficial to the bio-based economy. The macroalga, *K. alvarezii*, has yet to be fully explored for all potential products that can be derived from its high fermentable sugar content. This study shows

that *K. alvarezii* hydrolysates could be used as feedstock for lactic acid production by *L. pentosus*. The strain was able to convert galactose to lactic acid with high productive rates and achieve short lag phases and yields when suitable anaerobic conditions and pH control were applied. *L. pentosus* successfully processed hydrolysates prepared with up to 30% of algal biomass when treated for HMF removal. Bioreactor simple batch kinetics show an efficient production, and with proper upscaling, 3G lactic acid production from *K. alvarezii* could prove to be a promising bio-based industrial project. Nonetheless, further investigations should be carried out, particularly related to other modes of process operation for increasing volumetric productivity of 3G lactic acid, as well as the production of other chemicals from this interesting marine biomass.

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