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Lactic Acid Fermentation of Carrageenan Hydrolysates from the Macroalga *Kappaphycus alvarezii*: Evaluating Different Bioreactor Operation Modes

Adam Tabacof [1], Verônica Calado [2] and Nei Pereira, Jr. [2,*]

[1] Chemical Processes Technologies Department, Federal Institute of Rio de Janeiro for Education, Science and Technology, Rio de Janeiro 20270-021, RJ, Brazil; adam@eq.ufrj.br
[2] Center of Biofuels, Petroleum and Derivatives, School of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro 21941-972, RJ, Brazil; calado@eq.ufrj.br
* Correspondence: nei@eq.ufrj.br

Abstract: Lactic acid is a molecule used abundantly in the food, cosmetic, and pharmaceutical industries. It is also the building block for polylactic acid, a biodegradable polymer which has gained interest over the last decade. Seaweeds are fast growing, environmentally friendly, and economically beneficial. The Rhodophyta, *Kappaphycus alvarezii*, is a carrageenan-rich alga, which can be successfully fermented into lactic acid using lactic acid bacteria. *Lactobacillus pentosus* is a versatile and robust bacterium and an efficient producer of lactic acid from many different raw materials. Bioreactor strategies for lactic acid fermentation of *K. alvarezii* hydrolysate were tested in 2-L stirred-tank bioreactor fermentations, operating at 37 °C, pH 6, and 150 rpm. Productivity and yields were 1.37 g/(L.h) and 1.17 g/g for the pulse fed-batch, and 1.10 g/(L.h) and 1.04 g/g for extended fed-batch systems. A 3.57 g/(L.h) production rate and a 1.37 g/g yield for batch fermentation operating with an inoculum size of 0.6 g/L was recorded. When applying fed-batch strategies, fermentation products reached 91 g/L with pulse feed and 133 g/L with constant continuous feed. For control and comparison, a simple batch of synthetic galactose-rich Man-Sharpe-Rugosa (MRS) media was fermented at the same conditions. A short study of charcoal regenerability is shown. A scheme for a third-generation lactic acid biorefinery is proposed, envisioning a future sustainable large-scale production of this important organic acid.

Keywords: seaweed; biorefinery; third generation biomass; galactose; *Lactobacillus pentosus*

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1. Introduction

The industrial scale microbial fermentation for the production of organic acids dates back over a century [1]. They play a role in the conservation and production of day-to-day products such as food, beverages, cosmetics, pharmaceuticals, and plastics. Moreover, most commercially produced organic acids serve as intermediates for added value products as well as being chemical platforms for the production of these valuable materials [1,2]. Since most organic acids can be readily produced using bacteria, yeast, or fungi, the search for suitable and/or genetically engineered microorganisms for high production rates, titer, and yield is constantly on the agenda of academic and industrial sectors [3].

Lactic acid has the fourth largest global market size (USD 2.7 billion by 2020), topped only by valeric, acetic, and citric acid, among which it has the highest compound annual growth rate of 8% [4]. It is traditionally used in food preservation, having much less negative health and environmental effects than chemical preservatives [5]. In 2017, Komesu et al. [6] reported that 39% of lactic acid production is used for polymer manufacturing, 35% for the food and beverage industries, and the remaining 26% is equally divided between solvent production and personal care products. The production of lactic acid

can be carried out using chemical methods, which involve the hydrogenation of acetaldehyde obtained from petrochemical resources with a hydrogen-cyanide catalyst, followed by hydrolysis with sulfuric acid to obtain a racemic DL-lactic acid mixture [7]. Due to the importance of the optical purity of lactic acid for more thermostable polylactic acid biodegradable plastics, obtaining it via bioprocesses is a more preferable method despite the elevated separation costs [8]. The optical purity of lactic acid from a wide array of microorganisms can reach up to 99.9%, and a suitable efficient lactic acid producer could be made to produce L(+) or D(−) lactic acid by modification of inducible gene expression systems [9].

Various microorganisms have been at the center of studies for lactic acid production. *Lactobacillus* strains are widely explored for their ability to prosper in a wide range of pH and temperature conditions. *Bacillus* spp. strains can be alkaliphilic and thermophilic, conditions which can make for higher production; additionally, these bacteria can process mineral salt mediums with minimal nitrogen sources. Engineered *E. coli* strains have piqued interest for their genetic malleability, thus removing screening processes for finding new strains [10]. Metabolically engineered yeasts such as *Saccharomyces* have been studied in recent years for easier product recovery. Fungi, especially *R. oryzae*, are extensively researched for their high productivity and yield of lactic acid [11]. In the focus of many studies in last years is the lactic acid bacteria, *Lactobacillus pentosus*, a heterofermentative, facultative anaerobic bacterium that can consume a wide array of carbohydrates [12], them being pentoses or hexoses concurrently, producing high-titer lactic acid solutions with high productivity rates [13–15], indicating its potential for use with different biomasses as raw materials. Pentoses are metabolized via the phosphoketolase (PK) pathway, resulting in an iso-molar ethanol and acetic acid production alongside the desired lactic acid. When the fermentation media contains solely hexoses, carbohydrates catabolize into lactic acid as the sole product through the Embden-Meyerhoff-Parnas pathway (EMP) [16]. When fermenting a xylose and glucose mixture, *L. pentosus* produced both enantiomers, D(−) and L(+) lactic acid, at a percentage of 60 and 40%, respectively [13]. Variations of enantiomer ratios may occur due to the use of different metabolic pathways for hexoses and pentoses metabolism when different substrates are available for the microorganism.

With the rise of global environmental awareness, lactic acid production from different renewable resources is gradually intensifying. Reports of lactic acid production from food waste and a variety of industrial crops are available in literature [17]. As the use of high-end PLA is rising steadily, cheap and sustainable ways for lactic acid production need to be found [18]. An emerging alternative for lactic acid production is seaweeds (macroalgae), which exhibit high carbon dioxide fixation and fast growth rates, making them a promising and environmentally friendly feedstock [19]. In comparison with other sustainable options such as lignocellulosic material, seaweed requires almost no land use, and can be beneficial for biomes as a natural absorbent of harmful wastes [20]. *Kappaphycus alvarezii* is a red seaweed (Rhodophyta) rich in the galactose-containing gel, carrageenan, a polysaccharide whose use in end-markets is steadily growing, leading to an increase in its farming due to industrial interest [21].

The concept of a seaweed biorefinery has already been discussed by various authors over the last years [22–25]. Seaweeds are composed mainly of polysaccharides, and the gelatinous portion is easily extracted from the biomass using simple treatment methods. Minerals, proteins, and lipids can also be separated and used in industrial applications. Some possible products envisioned in third-generation seaweed refineries, besides agar and carrageenan, are ethanol from fermentation, biogas from anaerobic digestion, and biochar and biooil from pyrolysis. Lange et al. [23] emphasize the potential of extracting medicinal components from seaweed.

In their extended review, Álvarez-Viñas et al. [24] describe four configurations of a *K. alvarezii*-based biorefinery, one for the extraction of plant bio-stimulants and carrageenan, the second for production of carrageenan and ethanol, and the third for production of fertilizers jointly with carrageenan, ethanol and biogas. The last configuration showed a thermal

treatment biorefinery for the production of 5-hydroxymethylfurfural (HMF), levulinic acid, and formic acids, using the algae residue for combustion and energy generation. Torres et al. [22] also described an ethanol-producing scheme for *K. alvarezii* alongside carrageenan extraction. Using the sugar platform available in macroalgae polysaccharides for purposes other than ethanol production was contemplated by Sadhukhan et al. [26] in 2019, where they mentioned fermentation of succinic and lactic acid from sugars derived from seaweed. A recent publication by Chung et al. [27] spotlighting lactic acid production in seaweed biorefineries discussed carbon lifecycles and exergy-related themes for a sustainable and environmentally friendly process for obtaining lactic acid.

Most modern industrial fermentation processes are operated using fed-batch systems [28]. Algorithmized fine tuning and optimization of substrate feeding rates is the fundamental tool used for augmenting productivity and yields in such processes, mainly relying upon the kinetics of cellular growth in fermentation media [29]. Application of fed-batch strategies upon lactic acid fermentation has been shown to improve production results in almost all cases reported in the literature, reaching higher overall production rates and higher final lactic acid concentrations. Higher yields can be observed in most cases as well. One obstacle resolved by gradually feeding the substrate is inhibition by substrate as reported by Bai et al. [30], using constant feeding to significantly reduce fermentation time. Oliveira et al. [31] also achieved better results when *L. casei* was introduced with a lower titer of fermentable sugar in pulse and continuous fed-batch fermentations. Elevated concentrations of lactic acid can be obtained when fed-batch strategies are implemented, as investigated by Abdel-Rahman et al. [32], where lactic acid concentration was almost doubled using two pulse feedings during fermentation. Ding et al. [33] compared pulse, continuous, and exponential feeding strategies, obtaining the best results with exponential feeding strategies with an up to 60% improvement in both lactic acid concentration and overall productivity. Comparing fed-batch strategies, Oliveira et al. [31] reached a 40 and 75% increase in lactic acid titers utilizing exponential feeding when compared to continuous and pulse strategies, respectively. In the case study performed by Ding et al. [33], these same differences were reported to be around 20 and 40%. Comparing a fed-batch after 24 h in fermentation systems with simple batch fermentation of lactic acid by *L. plantartum* (of which *L. pentosus* is a sub-species), Machado et al. [34] found that lactate concentration was about 20 g/L higher when feeding glucose and xylose to the medium after all glucose was depleted. Investigating the potential of lactic acid production of *L. pentosus*, Lobeda et al. [35] reached a high 157 g/L of lactic acid solution using three pulses of a 400 g/L solution of glucose and fructose.

Macroalgae bioreactor scale experiments reported in the literature are limited. Jang et al. [36] evaluated hydrolysates brown seaweed, using *Laminaria japonica* hydrolysate on a bioreactor scale, reaching 14.4 g/L of lactic acid. Mwiti et al. [37] fermented galactose derived from agar hydrolysate in a pulse fed-batch reactor resulting in 31.9 g/L of lactic acid. In a recent study [14], batch bioreactor fermentation of *K. alvarezii* hydrolysates reached 29.4 g/L.

Seaweed biomass is one of the most promising sustainable feedstocks for bioprocesses. *K. alvarezii*, for its rich saccharide content, is an excellent choice as a raw material for biorefinery fermentation processes. Recent reports in the literature indicate that *L. pentosus* is an efficient, robust, and versatile lactic acid producer. This study explores bioreactor feeding strategies for *K. alvarezii* hydrolysates for lactic acid fermentation by *L. pentosus*, applying new bioreactor operation modes for fermenting *K. alvarezii* hydrolysates, and a larger inoculum size in order to achieve higher lactic acid concentrations, production rates and yields. Potential implications of results within the biorefinery and industrial concepts are also discussed, aiming to envision a third-generation algal mass biorefinery.

2. Material and Methods

2.1. Microorganism, Propagation and Fermentation Media

The American Type Culture Collection *L. pentosus* (ATCC 8041) was propagated and stocked after growing in a galactose-rich Man-Rugosa-Sharpe (MRS) medium with the following composition: 20 g/L galactose, 10 g/L bacterial peptone, 5 g/L yeast extract, 10 g/L beef extract, 1mL/L Tween 80, 2 g/L ammonium citrate, 0.1 g/L magnesium sulphate heptahydrate, 0.05 g/L $MnSO_4$, and 2 g/L K_2HPO_4. The same enriched MRS medium was used for acclimatization with partial or no synthetic galactose.

2.2. Hydrolysate and Acclimation Procedures

K. alvarezii hydrolysate was produced and detoxified for HMF removal following the method described by Tabacof et al. [14]. A 30% (*w*/*v*) was hydrolyzed in a 1% (*v*/*v*) sulfuric acid solution at 110 °C for 45min, followed by overliming and activated charcoal treatment, reaching a 40 g/L galactose solution with negligible amounts of HMF. Small amounts of glucose and organic acids were detected using HPLC analysis as well.

The hydrolysate was used to help the microorganism adapt to the media by gradual exposure to mixed hydrolysate and synthetic MRS media. Acclimation procedures are also detailed in Tabacof et al. [14].

2.3. Bioreactor Fermentation Essays

A versatile Electrolab 360 benchtop fermenter controller was used for parameter and feed control during fermentations in a mechanically agitated 2L bioreactor. All bioreactor experiments were operated at 37 °C, pH 6, and at a 150 rpm agitation speed. The pH values were adjusted with a 3M NaOH solution.

The hydrolysate fermentation media was inoculated with cells produced in the last step of the acclimation procedure, using an inoculum size of 100% of the bioreactor working volume, which was equivalent to a concentration of 0.6 g/L of *L. pentosus* cells upon fermentation assay inoculation, a value slightly below a third of the maximum cell mass observed in hydrolysate bioreactor assays. These lower cell concentrations were due to lack of pH control in penicillin bottles during acclimation.

For pulse fed-batch operation, a 1L hydrolysate medium was fed using a 400 g/L galactose solution upon carbohydrate depletion after 16 h of fermentation as to elevate the substrate concentration back to its initial titer.

Extended fed-batch operation was carried out injecting a constant 2 mL/h flow of a 400 g/L galactose solution when galactose levels were detected to be around 5 g/L, which occurred 14 h after initiating the fermentation process.

For better evaluation of the performance of acclimated *L. pentosus* strains in hydrolyzed media, a synthetic MRS media containing similar amounts of galactose and glucose found in *K. alvarezii* hydrolysates were fermented in a 1L working volume bioreactor, then inoculated with 0.4 g/L of non-acclimatized cells.

2.4. Regeneration of Activated Charcoal

From an industrial point of view, the regenerability of the activated charcoal is an interesting aspect. The charcoal used for detoxification was recuperated and treated based on the work of Carratalá-Abril et al. [38] in a muffle at 450 °C for 3 h, where temperatures were increased at a 1 °C per minute rate until reaching the final target temperature. The overlimed hydrolysate was subjected to detoxification using regenerated charcoal. The process was subsequently repeated three times, and the detoxified hydrolysates were analyzed for monosaccharides and HMF content.

2.5. Analytical Methods

Cell mass was detected using standardized 600 nm OD absorbance. Monosaccharides were analyzed in a Waters 2707 HPLC injector with a Hi-plex column (8 µm) and a RID detector, along with a 0.6 mL/min of 5 mM of H_2SO_4 solution. Detection time for glucose

was 10.379 min and detection time for galactose was 10.975 min. For HMF and organic acids, a Shimadzu injector with an HPX-87 column and a 210 nm UV detector was used with the same mobile phase. Lactic acid was detected at 13.048 min, and the HMF peak was detected at 32.720 min. The total HPLC running time for carbohydrates, lactic acid, and HMF were 45 min. To determine the Chiral isomers of lactic acid—L(+) and D(−) lactic acid in the final fermentation products—a CHIREX 3126 column was used with a 1 mL/min flux of 1 mM of $CuSO_4 \cdot 5H_2O$ and a 254 nm UV detector. The L(+) enantiomer was detected at 13.615 min, and the D(−) enantiomer peak was detected at 15.525 min.

3. Results

3.1. Bioreactor Fermentations

The *L. pentosus* ATCC 8041 strain successfully fermented synthetic galactose and glucose in a MRS media and 30% (*w*/*v*) *K. alvarezii* hydrolysates, as shown in Figures 1–4. Applying a 0.6 g/L inoculum in hydrolysates, the depletion of fermentable sugars was observed between 12 and 16 h after inoculation of acclimated *L. pentosus* in hydrolysate. After pulse injection of sugars, a two-day period was necessary for total consumption of galactose, reaching up to 90.9 g/L of lactic acid. In the extended continuous fed-batch fermentation assay, the completion of the constant continuous injection of substrate took about 100 h, with the final lactic acid titers reaching 132.6 g/L.

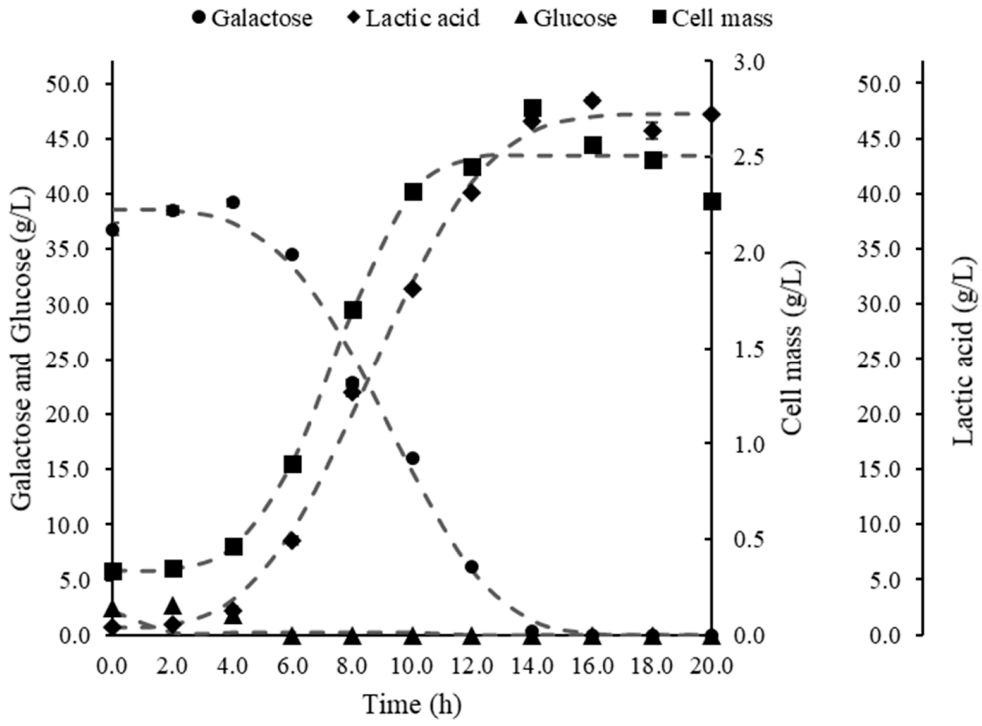

Figure 1. Batch fermentation kinetics of lactic acid by *Lactobacillus pentosus* from the Man-Rugosa-Sharpe medium containing initial galactose and glucose concentrations similar to *Kappaphycus alvarezii* hydrolysates. Operating conditions: pH 6, 37 °C, and 150 rpm. Standard errors in sampling were ≤1.

Productivity for simple batch fermentation with a 0.6 g/L inoculum was 3.57 g/(L.h). When executing feeding strategies, pulse and extended continuous fed-batch total production rates were 1.37 and 1.10 g/(L.h), respectively. Yields for fermentation with a pulse injection were 1.17 g/g, while continuous batch yields reached 1.04 g/g. Simple batch yield was 1.37 g/g. The maximum cell mass in fed-batch application was a 2.3 g/L cell concentration.

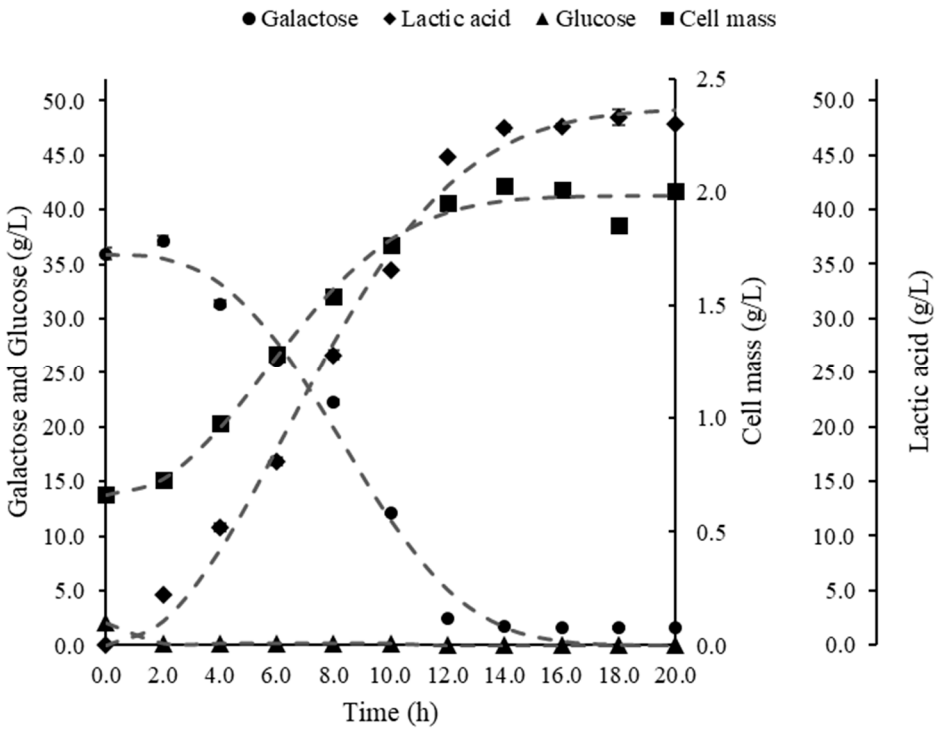

Figure 2. Batch fermentation kinetics of lactic acid by acclimatized *Lactobacillus pentosus* from detoxified *Kappaphycus alvarezii* hydrolysates in a 2L bioreactor with a 1L working volume. Inoculum size of 0.6 g/L. Operating conditions: pH 6, 37 °C, and 150 rpm. Standard errors in sampling were ≤1.

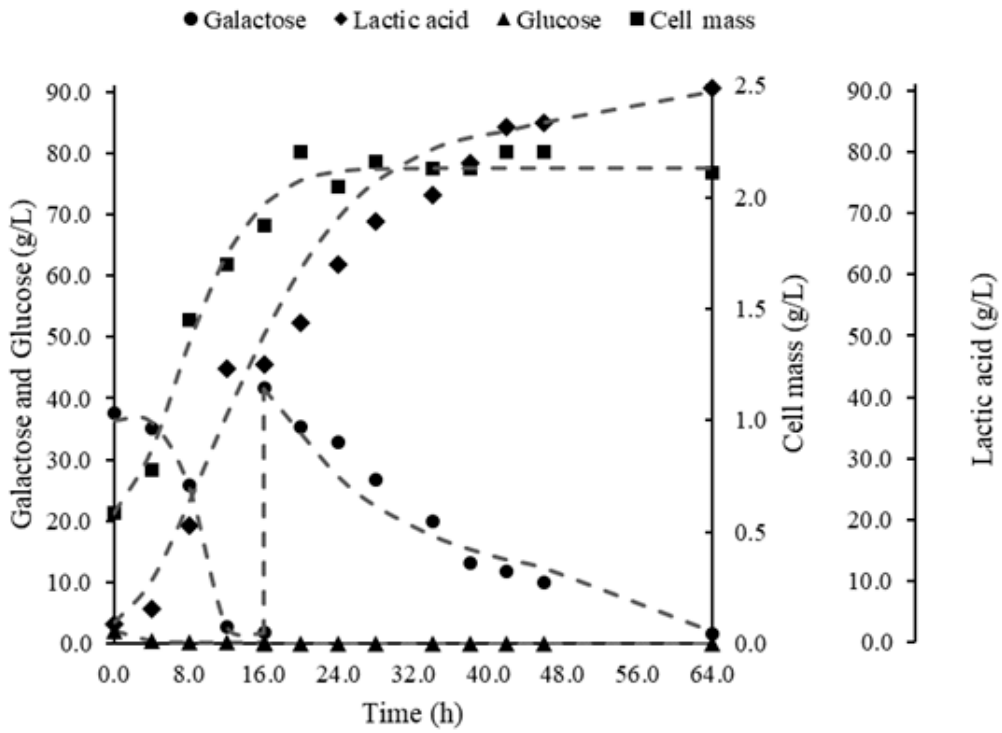

Figure 3. Pulse fed-batch fermentation kinetics of lactic acid by acclimatized *Lactobacillus pentosus* from detoxified *Kappaphycus alvarezii* hydrolysates in a 2L bioreactor with a 1L working volume. After initial simple batch operation, a 400 g/L galactose solution was injected at 16 h elevating concentration close to initial values. Operating conditions: pH 6, 37 °C, and 150 rpm. Standard error in sampling were ≤1.

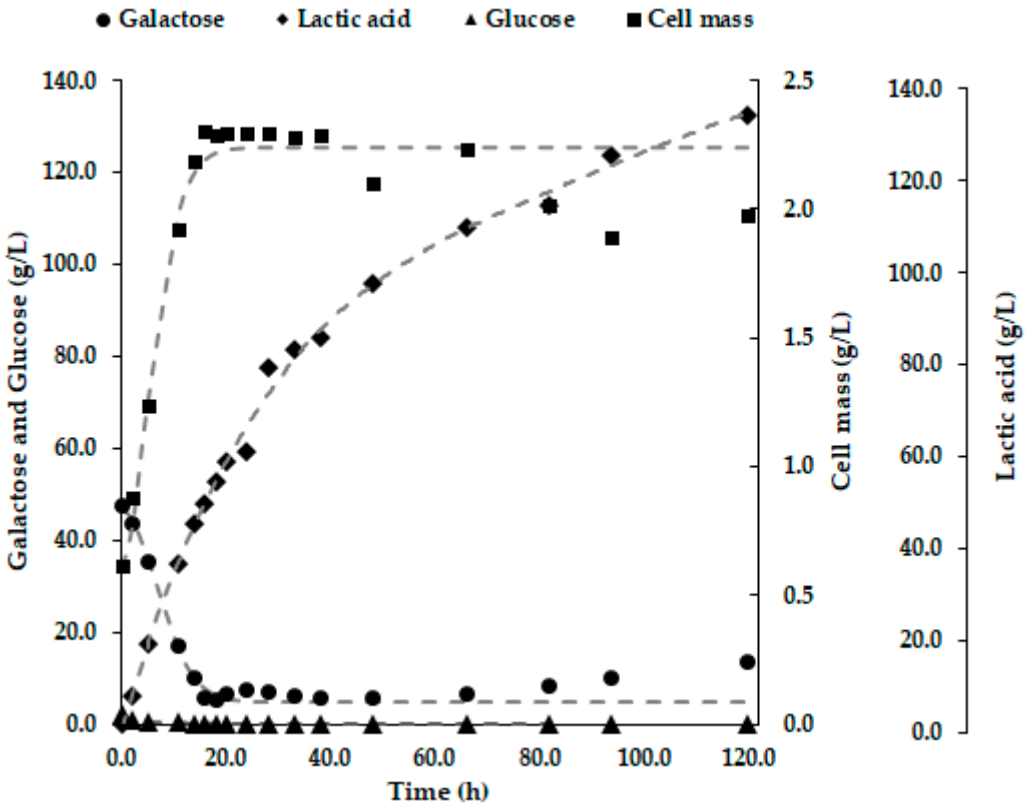

Figure 4. Extended continuous fed-batch fermentation kinetics of lactic acid by acclimatized *Lactobacillus pentosus* from detoxified *Kappaphycus alvarezii* hydrolysates in a 2L bioreactor with a 1L working volume. The reactor operated as a simple batch up to 14 h of fermentation, when a 400 g/L galactose solution was injected at a constant 2 mL/h flow rate. Operating conditions: pH 6, 37 °C, and 150 rpm. Standard errors in sampling were ≤1.

Synthetic MRS batch fermentation had a global production rate of 3.37 g/(L.h) and a 1.28 g/g yield of lactic acid. Total consumption of galactose occurred only in the synthetic MRS medium, leading to a final 47 g/L lactic acid solution after 14 h; there was a similar timeframe for the fermentations taking place in algae biomass hydrolysates. In all cases, glucose was entirely consumed during the first 2 to 4 h after inoculation, and the percentage of substrate reduction was 90% or higher.

The chiral HPLC column analysis showed that *L. pentosus* produced a near-racemic mixture of roughly 49.5% L(−) lactic acid and 50.5% D(+) lactic acid in all bioreactor fermentations.

3.2. Regenerability of Activated Charcoal

The activated charcoal powder efficiently removed HMF from hydrolyzed *K. alvarezii* solutions even after being regenerated three times over (Table 1), and HMF concentrations registered by HPLC were less than 0.70 g/L for hydrolysates detoxified with all regenerated charcoal. After each regeneration, a slightly lower amount of galactose and glucose was present after detoxification, starting with around 41 g/L for newly purchased charcoal, and 38 g/L for charcoal regenerated for the third time. The fermentable sugar loss for using regenerated charcoal was 8, 9, and 18% for the first, second and third regeneration, respectively. The little glucose present in the hydrolysate was totally absorbed by regenerated charcoal starting from the second regeneration.

Table 1. Galactose, glucose, and 5-hydroxymethylfurfural (HMF) concentrations in hydrolysates after detoxification with subsequently regenerated activated charcoal.

Activated Charcoal	Galactose (g/L) *	Glucose (g/L) *	HMF (g/L) *
Newly purchased activated charcoal	41.00 ± 0.44	3.60 ± 0.10	0.70 ± 0.01
After 1st regeneration	38.83 ± 0.12	1.00 ± 0.15	0.50 ± 0.01
After 2nd regeneration	37.40 ± 0.70	Not detected	0.21 ± 0.01
After 3rd regeneration	33.93 ± 0.06	Not detected	Not detected

* Concentration of overlimed hydrolysates before charcoal treatment were as follows: Galactose 43.5 g/L, glucose 6 g/L, and 5-hydroxymethylfurfural 9.3 g/L [14].

4. Discussion

The results of this study demonstrate the benefits of using larger inoculum and fed-batch strategies for lactic acid production of *K. alvarezii* hydrolysates with *L. pentosus* when compared to previously reported data. Fed-batch operation can lead to a high titer of lactic acid in the final solution, with high production rates.

4.1. Comparison of Bioreactor Operation Systems

The acclimation of *L. pentosus* to *K. alvarezii* hydrolysates has already been proven to be essential for the efficiency of fermentation processes and for reducing lag phases of bacterial growth [14]. Cubas-Cano et al. [39] demonstrated how xylose consumption increased two-fold with long-term evolutionary engineered *L. pentosus*. The cells acclimated to *K. alvarezii* hydrolysates performed in a similar efficiency to non-acclimated cells in synthetic media, as can be seen in Table 2. The combination of a continuous carbon source feeding strategy with a large inoculum of acclimated cells resulted in a 9% better yield than in batch fermentation of galactose-rich MRS media.

Table 2. Lactic acid production resultsfor different bioreactor strategies.

Fermentation Parameter	Batch [a]	Batch [b] [14]	Batch [c]	Pulse Fed-Batch [c]	Extended Fed-Batch [c]
Final lactic acid solution (g/L)	47.17 ± 0.12	29.39 ± 0.32	49.96 ± 0.15	90.96 ± 0.32	132.59 ± 0.40
Percentage reduction of substrate %	100	94.4	95.7	95.7	89.4
Productivity(g/(L.h))	3.37	1.05	3.57	1.37	1.10
Yield (g/g)	1.28	1.07	1.37	1.17	1.04
Inoculum size (g/L)	0.4	0.3	0.6	0.6	0.6
Maximum cell concentration (g/L)	2.76 ± 0.14	1.65 ± 0.04	2.00 ± 0.01	2.20 ± 0.04	2.29 ± 0.03
Overall fermentation time (h)	14	28	14	64	120
Final media volume (L)	1	1	1	1.1	1.2

[a] Non-acclimated *Lactobacillus pentosus* in synthetic MRS media with 40 g/L galactose and 4 g/L glucose. [b] Acclimated *L. pentosus* in 25% (*w*/*v*) *Kappaphycus alvarezii* hydrolysate. [c] Acclimated *L. pentosus* in 30% (*w*/*v*) *K. alvarezii* hydrolysate.

As can be observed by curves in Figures 3 and 4, the end of cellular growth and high lactic acid concentrations have a retardant effect on product formation rate. After pulse injection upon galactose depletion, the total substrate consumption took 48 h, which is a three times larger period than the first carbohydrate consumption period. Maintaining galactose concentrations at a fixed level of about 5 g/L (Figure 4) was achieved with an addition of less than 1 g/h of galactose to fermentation media while operating at a continuous feeding rate, leading to an extended fermentation of more than four days. Fermenting hydrolyzed food waste with *L. pentosus*, Lobeda et al. [35] used three additional pulse injections of 100 mL of 400 g/L glucose and fructose concentrated food waste hydrolysates, reaching 157 g/L of lactic acid in about 80 h of fermentation. After the third injection, an amount of 125 g/L of lactic acid remained in the bioreactor, but the microorganism was unable to further consume substrate and continue the production of lactic acid. Similar bacterial behavior was observed in this current study, as production rates were reduced as lactic acid concentration reached 70 g/L. Substrate availability can also play a role in productivity

dynamics. Lobeda et al. [35] replenished the substrate level as it reached around 25 g/L while the pulse in this study was applied upon depletion.

As expected, operating with a continuous substrate feed resulted in the superior yield and productivity when compared to pulse injection. Higher concentrations were reached in the same time period. Upon 64 h of fermentation, the extended fed-batch bioreactor contained 108 g/L of lactic acid with a 1.67 g/(L.h) production rate, whereas a 91 g/L concentration of lactic acid in a pulse injection bioreactor was observed, thus showing that high substrate titers can reduce production rates. A similar titer of lactic acid as the highest reported in bioreactor fermentation using *L. pentosus* [35] was achieved, reaching up to 132.6 g/L in 120 h. The >1 yields in all fermentation assays indicate the use of more complex molecules not detected by HPLC for lactic acid production by *L. pentosus*.

Similar behavior during lactic acid bacteria fermentations is reported in the literature. Bai et al. [30] showed a more than three-day decrease in fermentation time with a continuous flow of glucose to *L. lactis* instead of having all the glucose injected at the beginning, thereby demonstrating the delay caused by substrate inhibition. Producing lactic acid with *L. casei*, Ding et al. [33] maximized production rates using exponential fed-batch strategies, with a 2.14 g/(L.h) rate compared to 1.82, 1.55, and 1.34 g/(L.h) for constant fed-batch, pulse fed-batch, and batch operations, respectively. In the fermentation of lactic acid utilizing *Enterococcus munditii*, continuously fed-batch bioreactors reached double lactic acid concentrations in comparison to batch fermentation [32]. Oliveira et al. [31] reported less productivity in the continuous fed-batch compared to batch operation. Nevertheless, yields for fed-batch strategies were higher, and continuous feeding was significantly more productive than pulse injection of substrate in that case. Machado et al. [34] achieved double the productivity and around 36% higher lactic acid concentrations using fed-batch strategies when compared to ideal conditions of batch fermentation with *L. plantarum.*

Regarding most comparisons present in the recent literature, it stands clear that continuous fed-batch operation has significant advantages when applying lactic acid bacteria for lactic acid production. Exponential strategies for bioreactor feeding show more promise than constant feeding [31].

Inoculum size also seems to play a significant role in the production rates and fermentation time. In comparison to previous batch fermentation [14], which started with 0.3 g/L of *L. pentosus* cells, a decrease of 12 h was observed for reaching minimal galactose concentrations, and three times the production rates were recorded when a double-sized inoculum was injected to hydrolyzed media. Although some reports of *Lactobacillus* strains such as *L. rhamnosus*, *L. delbrueckii*, *L. bulgaricus*, and *L. casei* showed no significant effect regarding inoculum size [40,41], Warandi et al. [42] reported a decrease in pH and increase in viable cells of *L. plantarum* with the increase of inoculum size. The effects of the toxicity of algal biomass on certain bacteria species are not fully understood, thus a larger inoculum can ensure the survival rate of inoculated cells and make for significantly shorter lag phases, as shown by the results of this study.

The fermentation of a solution containing solely hexoses resulted in the production of lactic acid without ethanol and acetic acid coproduction; this is due to the activation of only the EMP metabolic pathway. Similar to the findings of Wischral et al. [13], where the same strain was applied, *L. pentosus* produced a mixture of lactic acid enantiomers. In this study, the D to L lactic acid ratio was lower, indicating that more L-lactate is produced when hexoses are available to microorganisms.

Although a racemic mixture is a less desired product in bio-produced lactic acid, recent studies show that manipulation of *L. plantarum* strains can yield pure D(−) lactic acid. The production of only one of the enantiomers is carried out using L-dehydrogenase or D-dehydrogenase deficient strains [43]. Okano et al. [44] showed that introducing lactate oxidase from *Enterococcus* sp. can lead to L-lactate removal and production of pure D(−) lactic acid. If pentoses are available in fermentation media, specific genes can be cloned into L-dehydrogenase deficient *L. plantarum* strains [45,46].

As shown in Tabacof et al. [14], production of lactic acid from macroalgae has been intensifying in the last decade. Reports of lactic acid production using brown and green macroalgae such as *Laminaria japonica* and *Ulva* sp., reaching lactic acid concentrations of 37.7 and 36.8 g/L, can be found in the literature. As a red seaweed consisting of polysaccharides containing mainly hexose fermentable sugars, *K. alvarezii* hydrolysate fermentation can be used with a wider range of microorganisms, and is thus a sound choice as a raw material for lactic acid production.

4.2. Regeneration of Activated Charcoal

When regenerating activated carbon saturated with benzene and toluene, Carratalá-Abril et al. [38] reported a close to 100% efficiency of activated charcoal after regeneration processes. The authors cite the choice of temperature and purge gas as important factors for efficient regeneration, and that regenerability can be related to the size and format of certain molecules.

While a reasonable recuperation is possible when charcoal is used to treat solutions containing a wide array of organic compounds, total regeneration is not always achievable due to the many larger unknown molecules present in such solutions. *K. alvarezii* biomass hydrolysates can contain some larger complex molecules that could have not sublimated during the thermal treatment. Additionally, no neutral purge gas was pumped into the muffle during the activated charcoal regeneration. Thermal treatment at elevated temperatures can alter the pore morphology of activated charcoal [47]. Glucose has been shown to oxidate at elevated temperatures [48], and similarly, HMF, galactose, and glucose could have been oxidated during the thermal regeneration process that took place with ambient air circulation for gas removal, and these oxidation reactions may have altered the pore morphology in the charcoal, allowing it to absorb the sugars alongside the HMF content, explaining the reduction in selectivity after consecutive regenerations. Further investigation of charcoal morphology, utilizing methods such as scanning electron microscopy [49], should be conducted for a better understanding of the activated charcoal regeneration process.

Ahuja et al. [50] showed that the solution from digestion of corn cobs in dilute acid can be regenerated successfully three times over, and the authors declared that about 38% of operational costs can be reduced due to said regeneration. The active carbon used for detoxification of the *K. alvarezii* hydrolysate, when regenerated, successfully removed the remaining HMF from solutions, and selectivity reduction was noted as consecutive regenerations took place. After the first and second regeneration, 7–9% of fermentable sugars were removed alongside the HMF content. At the third regeneration process, the galactose losses were close to 20%, an amount that can make regeneration not cost worthy. The downside of the thermal treatment applied to activated carbon is the reduction of the selectivity of active carbon, resulting in absorption of more carbohydrates jointly with furfural compounds.

The process used in this study left cellulosic residue. In some cases, part of the biomass itself can be used for activated carbon production, as in the case of sugar beet pulp when used for ethanol production [51]. As described by Fazal-ur-Rehman [52], temperature ranges and manufacturing procedures for the production of activated charcoal are specific to each type of plant matter used as a raw material, making it difficult to verify the cost benefit for producing activated charcoal. The carrageenan content present in the *K. alvarezii* biomass used in this study was estimated to be of 41% [14], leaving 59% of potential biomass for other applications, and due economic viability tests should be studied for each raw material to make sure it is viable for activated charcoal production.

Other methods for promoting the reuse of activated charcoal are available, such as advance oxidative processes [53], and various desorption and decomposition methods [54]. The best method of activated carbon regeneration after *K. alvarezii* hydrolysate treatment should be further researched to lower the operational costs of a future industrial endeavor.

4.3. K. alvarezii Biorefinery for Lactic Acid Production Proposal

From data obtained in previous studies [14] and bioreactor strategies investigated, a scheme for the production of lactic acid from *K. alvarezii* biomass can be proposed (Figure 5). On a laboratory scale, 1 ton of washed dry seaweed can yield about 115 kg of lactic acid. On an industrial scale, applying more efficient liquid sequestration in filtration phases, and washing and extracting machinery, the yield could become significantly higher.

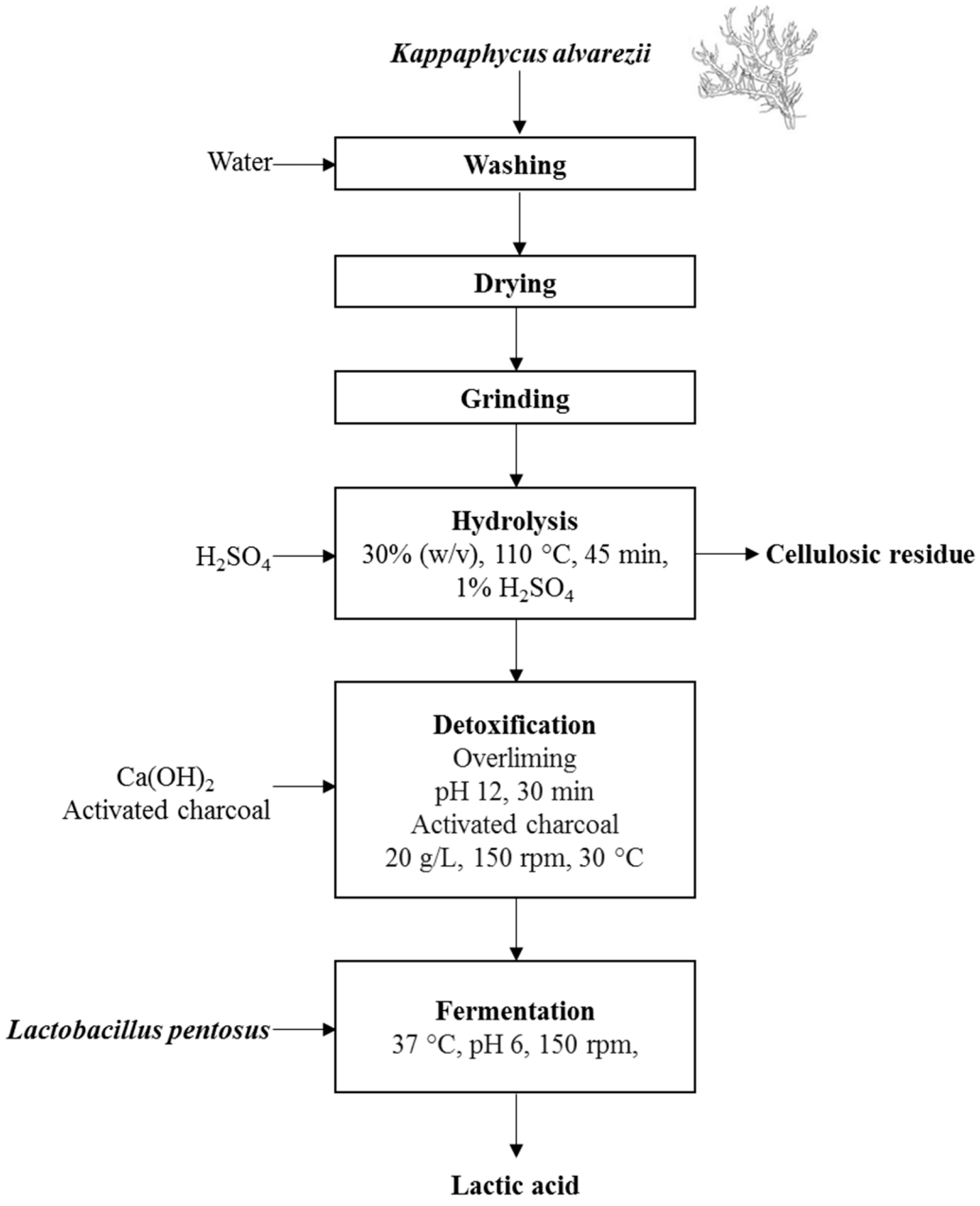

Figure 5. *Kappaphycus alvarezii* biorefinery scheme for lactic acid production. Filtration steps for hydrolysis and detoxification are omitted.

The cellulosic residue left after hydrolysis can be utilized for further lactic acid production, either by enzymatic scarification and fermentation [27], or by thermal treatment at high temperatures assisted by alkaline catalyzers [55]. Alternatively, the cellulose could be submitted to other useful industrial operations such as thermal combustion for energy production [24]. Additionally, the enzymatically obtained monomers were already shown

to be a potential substrate for ethanol production [22,24,56–58], which could be another alternative for a versatile biorefinery operation.

Since the formation of lactic acid during the fermentation of galactose by *L. pentosus* is growth-associated, higher production rates could be achieved with sequential batch fermentations. As acclimation of cells is essential to the process [14], the use of cell mass recycling or discharge leftovers as inoculum for further fermentations should take place to ensure the use of *L. pentosus* cells that are already adapted to *K. alvarezii* hydrolysates, and to avoid the need of the acclimation process before each new batch.

If high titers are desired in final fermentation solutions, an extended fed-batch approach could be chosen, depending on the cost efficiencies of separation operations for product processing. In any case, further investigation and fine tuning of bioreactor fermentation should be carried out to make the production of lactic acid from *K. alvarezii* biomass viable.

The demand for lactic acid is on the rise due to PLA, which is a high-quality biodegradable plastic. Cheap, fast growing, and environmentally beneficial industrial crops as feedstock are needed to make biorefineries for lactic acid production viable. Seaweeds, and especially *K. alvarezii*, for its high carbohydrate content are one of the most interesting choices available for farming. Process fine-tuning and optimization are key for large-scale production.

Finding a sustainable renewable raw material for bioproducts that can be upscaled to mass production is of importance for creating more environmentally friendly industrial processes. This study endeavored to amplify production of lactic acid from *K. alvarezii* hydrolysate, spotlighting the biomass of this alga as a future raw material for ample production of this important and widely used molecule.

5. Conclusions

The processing of macroalgae via fermentation is still at an early and academic stage, and technological maturity for both cultivation and chemical processing has yet to be achieved. *K. alvarezii* is a widely farmed seaweed, which can prove to be a sustainable raw material for lactic acid production. This study shows the potential of applying feeding strategies for augmenting yield and final concentrations in the lactic acid fermentation of *K. alvarezii* hydrolysates with *L. pentosus*. A simple batch with high initial cell concentrations rendered better production rates. Fed-batch strategies made for high titers and yields. In comparison to the latest study, more than triple the productivity can be achieved with a larger inoculum size, with a 30% higher yield. Extended continuous fed-batch and pulse fed-batch operations reached high lactic acid concentrations. Extended fed-batch fermentation reached 18% more lactic acid content in similar fermentation times when compared to pulse fed-batch operations. Activated charcoal used for detoxification of *K. alvarezii* hydrolysates can be regenerated using a simple thermal treatment, thus potentially reducing operation costs, adding yet another point in favor of a future industrial venture. In order to achieve even better results, more modes of fermentation conduction should be explored, such as continuous operations and exponential feeding. Immobilization of *L. pentosus* cells, and the use of an even larger bacterial presence in the fermentation medium can elevate rates of volumetric production as well. For future applications of lactic acid production from *K. alvarezii* biomass, research should strive to improve the liberation of fermentable sugars from the polysaccharide carrageenan, and in addition to the thermochemical treatment and detoxification processes, new technological areas such as enzymatic hydrolysis should be considered. A search for microorganisms capable of processing hydrolysates should also take place to widen the variety of options for large scale use. Alongside technological aspects, agricultural, social, logistic, economic, and government policy fields should be studied for algal biomass to gain a firm footing in the bioprocess industry. Even though more fine-tuning and investigation on the subject are needed, the results of this work reveal the promise in a scheme of a large-scale 3G lactic acid biorefinery.

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