REVISTA MATÉRIA V.24 N.02
ISSN 1517-7076 articles e-12363, 2019

Enzymatically and/or thermally treated Macroalgae biomass as feedstock for fermentative H_2 production

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ABSTRACT

Due to its high carbohydrate content, algae biomass can be employed as feedstock to produce hydrogen (H2) by fermentation. However, to make the carbohydrates entrapped within the cell wall more bioavailable, algae biomass must be treated before fermentation. We submitted Kappaphyccus alvarezzi macroalgae biomass to autoclave (at 120 °C and 1 atm for 6 h) treatment and/or enzymatic (Celluclast® and/or a recombinant β-glucosidase) hydrolysis, to break down complex carbohydrates into available sugars that were used to produce H2 by fermentation. Macroalgae biomass treated with Celluclast®+β-glucosidase and with combined thermal treatment and enzymatic hydrolysis reached very similar TRS productivities, 0.24 and 0.22 g of TRS/L.h, respectively. The enzymatically treated biomass was employed as feedstock to produce H2 by Clostridium beijerinckii Br21, which afforded high yield: 21.3 mmol of H2/g of dry algae biomass. Hence, treatment with Celluclast® and recombinant β-glucosidase provided macroalgae biomass for enhanced bioconversion to H2 by C. beijerinckii Br21.

Keywords: Kappaphyccus alvarezzi, Clostridium beijerinckii, Biohydrogen, Cellulase, β-glucosidase.

1. INTRODUCTION

Nowadays, one of the most important environmental issues is to replace finite, polluting fossil fuels with sustainable fuels [1,2]. In this context, hydrogen (H_2) is an alternative fuel to the traditional ones: H_2 combustion produces water only, and this fuel has three times higher energy potential than gasoline (142 $kJ.g^{-1}$) [1,3,4,5]. However, physical-chemical methods, which also depend on fossil fuels or require a large amount of energy, are mainly employed to obtain H_2 [4]. Alternatively, biological routes, such as fermentation, can be used to obtain H_2 [1,3]. The fermentative route is promising in terms of cost and sustainability because it employs renewable raw materials, including carbohydrate-rich biomasses, at ambient temperature and pressure [6,7].

Due to its high carbohydrate content, macroalgae biomass is a suitable carbohydrate source for biotechnological processes, like fermentative H_2 production [7,8]. Compared to higher plant biomass, algae offer a number of potential advantages: 1) they convert sunlight to biochemical energy more efficiently than terrestrial plants; 2) they grow on vast tracts of sea by action of sunlight only, without the need for fertilizers; 3) their production does not depend on arable land availability, so their cultivation does not compete with food production; 4) they consume CO_2, thereby helping to reduce greenhouse gas emissions; and 5) they do not contain lignin, which simplifies biomass saccharification processes for further use in fermentation [6-16].

Most carbohydrates are entrapped within the algae cell wall, so H_2 production by algae biomass fermentation requires cell wall disruption. Different methods - chemical, physical, biological, or a combination of them - can be applied to algae biomass to make oligo- and monosaccharides available [10, 12, 17]. Some methods can decompose sugars and generate fermentation inhibitors, like 5-hydroxymethylfurfural (HMF) and organic acids [18].

Thermal methods are cost-effective because they do not require that any chemicals other than water be added or recovered. For example, hydrothermal hydrolysis uses water at high temperatures (above 150°C) and controlled atmosphere to break polysaccharides into smaller molecules. In the specific case of algae bio-

Autor Responsável: Emelin Leandro Rodrigues Data de envio: 23/04/2018 Data de aceite: 12/12/2018

10.1590/S1517-707620190002.0678

mass, which does not contain lignin, the hydrothermal method is very promising [12, 13]. Compared to other methods, such as acid treatment, hydrothermal hydrolysis provides high sugar recovery and produces inhibitors at low concentration [13, 18]. However, hydrothermal biomass treatment demands special reactors that can support high temperature and pressure [12,13]. For this reason, here we employed a modified hydrothermal method based on autoclave, an easily available lab instrument, to treat algae biomass.

In this study, we use the red seaweed *Kappaphyccus alvarezzi* as macroalgae biomass because of its high carbohydrate content (about 50%). The carbohydrates present in this algae consist of heterogeneous polysaccharides called carrageenans, which are composed mainly of D-galactose and 3,6-anhydro-D-galactose sulfated at the β-1,4- and α-1,3 bonds [19]. We applied autoclave treatment and/or enzymatic hydrolysis to the *K. alvarezzi* biomass and determined sugar and inhibitor concentrations in the hydrolysates aiming at their further use in H_2 production via fermentation.

2. MATERIALS AND METHODS

2.1 Macroalgae biomass

The *K. alvarezzi* biomass, grown in the Atlantic, was provided by the Fisheries Institute, Ubatuba, São Paulo (SP) (GPS coordinates 23°27′5,8″S; 45°02′49,3″W), after natural air-drying. Approximately 25 g of the dried seaweed biomass was washed five times with 1 L of deionized water to remove impurities. The sample was dried in an air circulation oven at 50 °C for 24 h, milled (SL31, Solab - Brazil), and screened through a 45-mesh sieve (0.355 mm) (Bertel, Brazil) to standardize the biomass for the treatments.

2.2 Algae biomass treatments

Autoclave treatment and enzymatic hydrolysis were carried out alone or in combination. The biomass was treated in 125-mL erlenmeyers containing 0.5 g of dry algae biomass and 50 mL of deionized water or buffer (in the case of enzymatic hydrolysis), to give 10 g/L algae suspensions. All the experiments were conducted in triplicate, with a control. The total reducing sugars (TRS) concentration of the control was subtracted from the TRS concentration of the sample.

Autoclave treatment

Autoclave treatment was accomplished in a bench vertical autoclave (Phoenix Luferco) at 121 °C and 1 atm. After treatment, the suspension was filtered through a 0.45-µm porous acetate-cellulose membrane to determine the TRS concentration and the concentration of potential fermentation inhibitors (acetic acid and HMF). As for the control, an algae suspension was left at ambient temperature for the same duration of the thermal treatment.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out with 0.5 g of *K. Alvarezzi* biomass in 50 mL of 0.1 moL/L citrate buffer, pH 4.8. Next, 60 enzymatic units per gram of dry algae biomass (U/g) was added. The following enzymes were employed: Celluclast® (provided by Novozymes, São José dos Pinhais, Brazil) and a recombinant β-glucosidase (Bglhi) from *Humicola insolens* [20]. The enzymes were added alone or together after the temperature had stabilized at 50 ºC, which was maintained during the assay. In the end, 1-mL samples were collected to quantify the TRS and HMF concentrations. As for the control, algae biomass was suspended in buffer and incubated at the same temperature used during enzymatic hydrolysis but without addition of enzymes.

Combined treatment

The combined treatment consisted in autoclave treatment followed by enzymatic hydrolysis. The erlenmeyer containing the algae suspension (10 g/L) in deionized water was autoclaved at 121 °C and 1 atm for 6 h. After that, a sample of the treated suspension was collected and filtered, and its TRS content was measured. The algae (in suspension) treated by autoclave was added with 30 U of each enzyme (Celluclast® and recombinant β-glucosidase) per gram of algae, and the algae suspension was kept at 50 °C. Finally, 1-mL samples

were taken to determine TRS and inhibitors. The control was not thermally treated in the first step, but it was incubated at 50 °C without enzymes in the second step.

2.3 Hydrolysis yield

To calculate the amount of sugar released after the autoclave, enzymatic, and combined treatments, the *K. alvarezzi* total carbohydrate concentration of 51.33% measured by DALBELO [21] was considered, as described in Equation 1.

$$Y_{TRS} = \frac{[TRS]*V}{MA*\%C}*100 \qquad \text{Equation 1}$$

Y_{TRS} = hydrolysis yield as percent of TRS (%).

[TRS] = TRS concentration in the treated suspension (g/L).

V = algae suspension volume (0.05 L).

MA = algae mass in the assay (g).

%C = 0.513 percent of total carbohydrate content in the algae biomass.

2.4 Recombinant β-glucosidase expression and purification for enzymatic hydrolysis

Bglhi was overexpressed in *Escherichia coli* BL21 (DE3) after transformation with the previously constructed plasmid pET28_*bglhi* containing the Bglhi coding sequences [20]. The cells were grown in HDM medium (25 g/L yeast extract, 15 g/L tryptone, and 10 mmol/L $MgSO_4$) supplemented with specific antibiotics at 37 °C to an OD_{600} of approximately 0.6. Protein expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside to the culture medium at a final concentration of 1 mmol/L. After 5-h induction at 37 °C, the cells were collected by centrifugation at 5000 x *g* and 4 °C for 20 min. The pellet was re-suspended in lysis buffer (50 mmol/L HEPES buffer, pH 8.0, containing 500 mmol/L NaCl and 1% Triton X-100 (v/v)) and disrupted by sonication. Cell debris was removed by centrifugation, and the recombinant protein was purified from the supernatant by nickel affinity chromatography (HisLink™, Promega), according to the manufacturer's instructions.

2.5 Cellulase and β-glucosidase enzymatic activity assays

Celluclast® activity was determined by using filter paper as substrate. After 60 min, the DNS method was used to quantify TRS release, as described in GHOOSE [22].

The β-glucosidase activity was determined by using the synthetic substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) at 2 mmol/L. The yellow *p*-nitrophenol product was quantified at 410 nm, according to the methodology described by SOUZA *et al*. [20]. In both cases, one enzyme unit (U) was defined as the amount of enzyme that released 1 μmol of product per min.

2.6 Fermentative H_2 production assay

Fermentation assays were conducted by using the filtered algae suspension obtained by enzymatic hydrolysis, which was the one with the highest TRS productivity. The H_2-producing *C. beijerinckii* Br21, which can produce H_2 from D-galactose, the main algae biomass monosaccharide, was employed. This microorganism was cultivated as described by FONSECA *et al.* [23]. The culture was prepared 24 h before the inoculum was cultivated, and the culture with 0.1 optical density at 600 nm was added to the hydrolysate.

The fermentation assays were performed in 50-mL vials containing 14 mL of the algae sample in 0.22-μm membrane and 1 mL of inoculum, added to previously sterilized vials. Macro- and micronutrients were added to the hydrolysate, as described by FONSECA *et al.* [23]. After inoculation, argon gas was bubbled into the vials, which were sealed with rubber stoppers or metal stoppers. After 72 h at 35°C, the gas from the vial headspace was analyzed by gas chromatography. The assay was accomplished in duplicate. Culture medium samples were taken at the beginning and at the end of the fermentation assays to measure ART and pH.

The volume of produced H_2 was calculated by considering the headspace volume and the gas composition according to the ideal gas equation, (P.V = n.R.T), where P is pressure (1 atm), V is the H_2 volume, n

is the number of moles of H_2, R is the universal constant of an ideal gas (0.082 atm L/K·mol), and T is the absolute temperature (K), as described by FONSECA *et al.* [23].

The H_2 yield from the hydrolysate ($Y_{H2/S}$) was determined according to Equation 2.

$$Y\frac{H_2}{S} = \frac{\Delta H_2}{\Delta S} \qquad \text{Equation 2}$$

$Y_{H2/S}$ = H_2 yield calculated from the sample ART concentration.

ΔH_2 = difference between H_2 concentration, in moles (considering H_2 molecular weight of 2 g/mol), at the beginning and at the end of the fermentative assay.

ΔS = difference between ART concentration in moles (considering sugar as glucose molecular weight, 180 g/mol) at the beginning and at the end of the fermentative assay.

The H_2 yield as a function of the initial algal biomass (Y) was also calculated as the ratio between the number of moles of produced H_2 and the initial dry algae biomass.

2.7 Analytical Methods

Samples of the algae suspensions collected after autoclave treatment and enzymatic hydrolysis were filtered through 0.45-µm acetate cellulose membrane before analytical determinations were carried out. The TRS concentration was obtained by using the 3,5-dinitrosalicyclic acid (DNS) method, as described by Miller [24].

The length of the carbohydrate products formed after autoclave treatment and after enzymatic hydrolysis with the two enzymes was analyzed by thin layer chromatography (TLC). Aliquots of the filtered samples were treated with 10% trichloroacetic acid (w/v), to precipitate proteins and long-chain carbohydrates. The samples were kept at room temperature for 30 min and centrifuged at 10,000 x *g* for 15 min. The supernatants (10 µL) were analyzed by TLC on silica gel G-60 plates (10×15 cm, DC-Alufolien Kiesel gel 60, Merck, Darmstadt, Germany), as described by CARLI *et al*. [25].

HMF in the hydrolysates was quantified by high performance liquid chromatography (HPLC). To this end, 500 µL of the sample was injected into a high performance liquid chromatograph (Shimadzu, Japan) under the following conditions: ion exclusion column Aminex HPX-87H 300 x 7.8 mm) from Bio Rad, mobile phase = 5 mM sulfuric acid in milli Q water, flow = 0.6 mL/min (Pressure = 68 Kgf/cm^2), detector operating at 190 nm, 205 nm, and 276 nm = diode array (model SPDM10A-VP), and Refractive Index (Model: RID-10A).

Following the procedures described in a previous work by our group [23], the composition of the gas originating from the fermentative assays was identified by gas chromatography (GC). A 100-µL aliquot collected from the bioreactor headspace was injected into the chromatograph GC 2014 Shimadzu (Japan) chromatograph equipped with a thermal conductivity detector (TCD) with a gas-tight syringe. The chromatographic column (2 m x 4.7 mm) consisted of 5-Å molecular sieves. Argon at a flow rate of 30 mL/min was employed as the carrier gas.

2.8 Statistical analysis

To compare the kinetic parameters obtained in treatment tests, analysis of variance (ANOVA) and Tukey test at a 5% significance level were accomplished by using the software Statistic 7.0.

3. RESULTS AND DISCUSSION

3.1. Macroalgae treatment

Figure 1 presents the algae suspension TRS concentration after autoclave treatment (0–6 h). The TRS concentration did not increase significantly in the first two hours of treatment. Thereafter, the TRS concentration increased gradually until it reached a maximum of 0.95 g/L after 6 h of autoclave treatment at 120 °C and 1 atm. This concentration corresponded to a hydrolysis yield (Y_{TRS}) of ca. 18.5 % (Figure 1) and was similar to

the yield obtained by BARREIRO *et al*. [26], who recovered 19.5, 38, and 21.9% of total organic carbon (TOC) in hydrolysates after hydrothermal treatment of the macroalgae *Fucus vesiculosus*, *Laminaria saccharina*, and *Alaria esculenta*, respectively. However, the aforementioned authors employed a temperature of 350 °C for only 15 min of hydrothermal treatment [26].

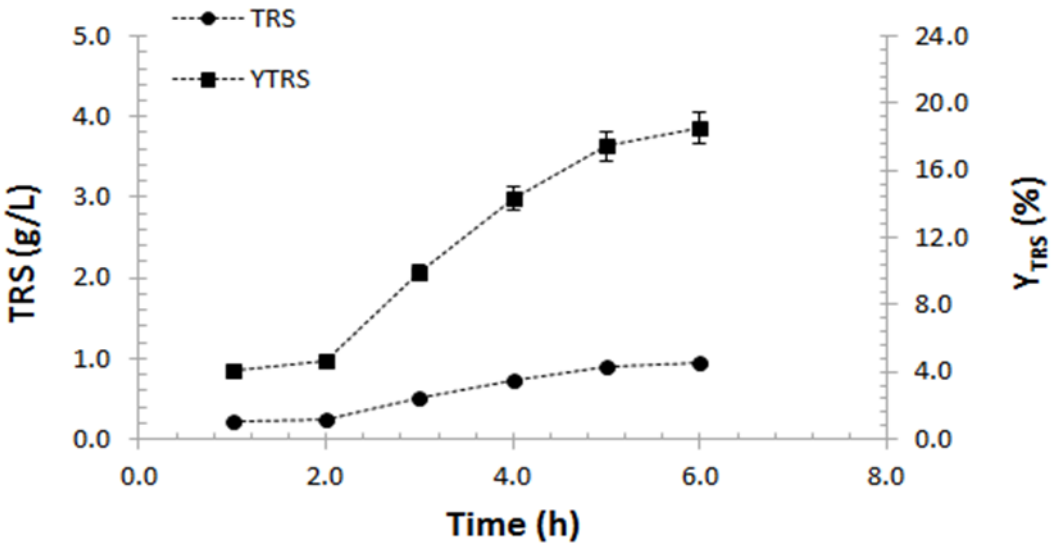

Figure 1: TRS concentration during the autoclave treatment at 120°C. The percent TRS yield was calculated by considering 51.3% total carbohydrate content in *K. alvarezii*.

Figures 2a, 2b, and 2c represent the TRS concentration during enzymatic hydrolysis of algae biomass by Celluclast® alone, recombinant β-glucosidase alone, and a mixture of both enzymes (Celluclast® + β-glucosidase), respectively. Celluclast® alone afforded hydrolysis yield of 23.4 and 31.2 % after 6 and 12 h, respectively (Figure 2a). Macroalgae cell wall contains cellulose as well as carrageenan. Both polysaccharides bear β-1,4-glucosidic bonds, so endocellulases such as Celluclast® can potentially depolymerize them. Endoglucanases act within the polysaccharide chains, to release oligosaccharides with reducing ends. These oligosaccharides can be detected by the DNS method used to quantify TRS herein and, depending on their molecular mass, they can be further fermented [24].

On the other hand, β-glucosidases are more active on small oligosaccharides, mainly the glucose disaccharide linked by β-1,4 bonds; i.e., cellobiose [27]. The yield of macroalgae hydrolysis by β-glucosidase alone was very low, ca. 4 %, even after 6 h (Figure 2b). Nevertheless, we expected that β-glucosidase could complement the Celluclast® action by hydrolyzing the released oligosaccharides. In fact, β-glucosidase together with cellulase increased the TRS concentration in the hydrolysate (Figure 2c). The hydrolysis yield obtained with Celluclast® alone and with the mixture Celluclast® + β-glucosidase was 31 (after 12 h) and 37% (after 8 h), respectively. Brown seaweed *Ecklonia radiata* saccharification by Celluclast® together with other hydrolytic enzymes also improved the total sugar yield slightly as compared to Celluclast® alone [28]. According to a literature review [8] of carrageenan enzymatic hydrolysis, only endotype carrageenases that act on carrageenan β-1,4-linkages (to produce oligosaccharides and carrabiose) have been reported. For complete carrageenan enzymatic monomerization, currently unavailable enzymes like exotype carrageenases and carrabiose hydrolases must be identified [8]. On the basis of our results, even though Celluclast® is not specific for carrageenan, it is a potential biocatalyst for carrageenan-containing macroalgae biomass, such as the red *K. alvarezzi* macroalgae. In addition, recombinant β-glucosidase increases TRS concentration in the algae hydrolysate even more. Hence, the enzyme mixture employed here is a good alternative for algae hydrolysis.

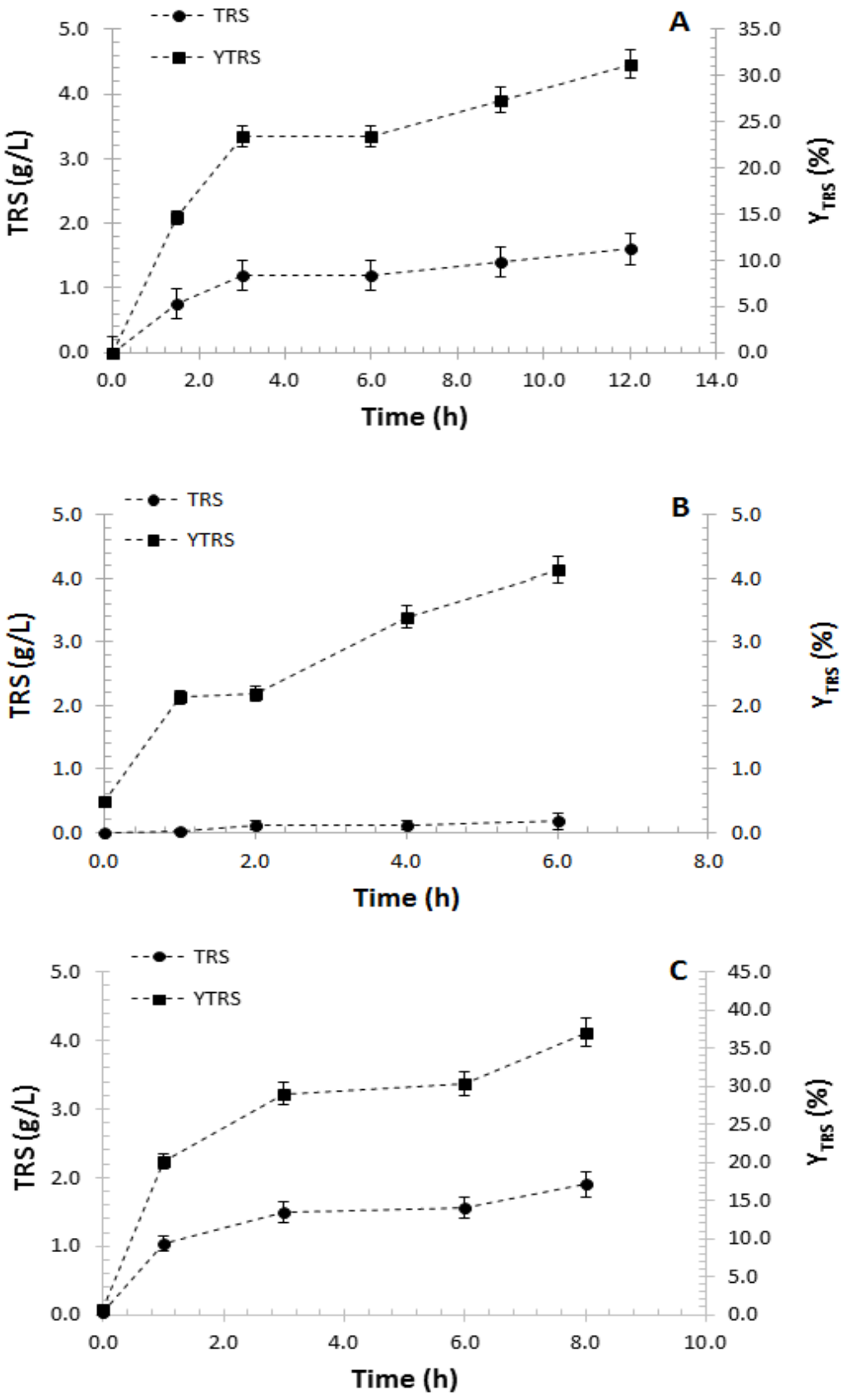

Figure 2: TRS concentration and hydrolysis yield after enzymatic treatments. A) Celluclast ®, B) β-glucosidase, and C) Celluclast® + β-glucosidase. All the treatments used total enzyme concentration of 60 U/g of algae biomass. The percent TRS yield was calculated by considering that *K. alvarezii* presented 51.3% of total carbohydrate content.

Finally, we combined autoclave treatment (6 h) and enzymatic hydrolysis (Celluclast® + β-glucosidase) (Figure 3). The TRS concentration in the hydrolysate at the beginning of the assay was ca. 1 g/L (time zero), which was the TRS concentration in the hydrolysate after 6 h of thermal treatment in the autoclave. Enzymatic hydrolysis with Celluclast® and β-glucosidase increased the TRS concentration in the thermal hydrolysate from 1 to 3.2 g/L, which corresponded to a total saccharification yield of 61.5%. This represented an increase of over 100% in the yield obtained after thermal hydrolysis yield alone (Figure 1) and an increase of 24% in the yield obtained after enzymatic hydrolysis with both enzymes, which was 37% (Figure 2c). LEE, *et al.* [29] recovered 44.8% of D-galactose and 3,6-anhydro L-galactose, the main red macroalgae cell wall monosaccharides, when they used acid-base buffer (20 mM Tris–HCl) and agar hydrothermal pretreatment at 170 °C, for 10 min, followed by enzymatic hydrolysis. Our saccharification yields were higher than the values obtained by LEE et al. [29] even though they used specific *Saccharophagus degradans* 2-40T agarolytic recombinant enzymes.

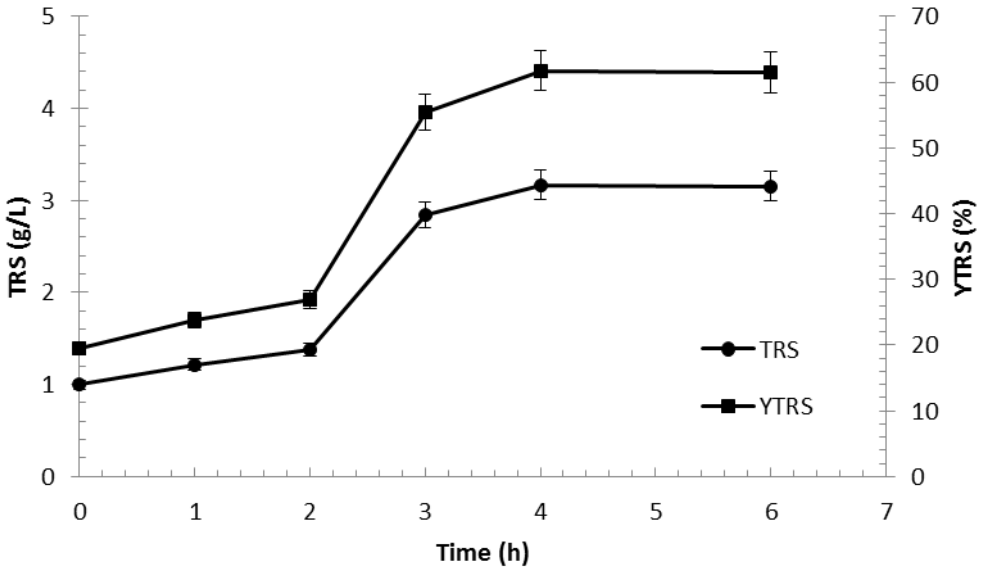

Figure 3: TRS concentration and hydrolysis yield obtained after thermal treatment at 120 °C and 1 atm for 6 h and enzymatic (Celluclast® and β-glucosidase) hydrolysis. The percent TRS yield was calculated by considering that the *K. alvarezzi* total carbohydrate content was 51.3%.

Table 1 summarizes the algae biomass hydrolysis productivity by taking the time employed in the different assays into account. According to the results, the combined treatment (autoclave treatment + enzymatic hydrolysis) reached superior TRS productivity (0.32 g of TRS/L.h) as compared to Celluclast®+β-glucosidase treatment, 0.24 g of TRS/L.h.

Table 1: Hydrolysis yield, TRS concentration, and TRS productivity obtained after the different treatments applied to the *K. alvarezzi* biomass.

TREATMENT	Y_{ART} (%)	TRS IN HYDROLYSATE (g/L)	TREATMENT TIME (h)	TRS PRODUCTIVITY (g of TRS/L.h)
Autoclave	18.5	1.0 (± 0.1)^{c}	6	0.17
Celluclast®	31.0	1.6 (± 0.2)^{b}	12	0.13
β-glucosidase	4.0	0.2 (± 0.1)^{d}	8	0.02
Celluclast® + β-glucosidase	37.0	1.9 (± 0.2)^{b}	8	0.24
Autoclave + enzymatic hydrolysis*	61.5	3.2 (± 0.2)^{a}	6 + 4 (10)	0.32

*Celluclast® + β-glucosidase. a, b, c, d : Different small caps in the same column indicate significant difference between different treatments, as revealed by Tukey test, $p < 0.05$.

3.2 Macroalgae hydrolysate chemical characterization

The hydrolysis yield was based on the TRS concentration in the hydrolysates only, but oligosaccharide size and fermentation inhibitors can further affect fermentation. Figure 4 illustrates the TLC of the macroalgae hydrolysates as compared to the TLC of standard monosaccharides (Glu – glucose; Gal – galactose) and disaccharide (Cel – cellobiose).

Besides the distinct TRS concentrations in the hydrolysates from autoclave treatment and from enzymatic hydrolysis (Table 1), the carbohydrate polymerization degree was also different. The autoclave treatment (Figure 4- t_0-t_4) provided hydrolysates containing high-molecular-mass TRS because the carbohydrates remained at the bottom of the TLC plate. On the other hand, enzymatic treatment (Fig 4, lane 1) promoted higher polysaccharide polymerization: TLC revealed the monosaccharides glucose and galactose as well as the disaccharide cellobiose.

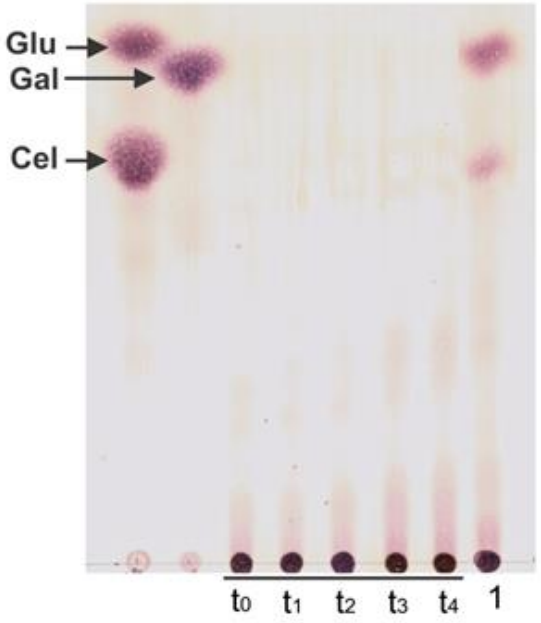

Figure 4: Time-course analysis of macroalgae autoclave treatment: the times were 0 (lanes t_0), 1 h (lanes t_1), 3 h (lanes t_2), 4 h (lanes t_3), and 6 h (lanes t_4), and enzymatic treatment (Celluclast® + β-glucosidase) (lane 1). Standards: Glu, glucose; Gal, galactose; Cel, cellobiose.

Previous studies have shown that *C. beijerinckii* Br21 can produce H_2 from glucose, galactose, and cellobiose, but not from crystalline cellulose [23]. H_2-producing *C. beijerinckii* Br21 genome contains eleven β-glucosidase genes (GenBank access no. KT626859). This should aid oligosaccharide fermentation and enable cellobiose fermentation.

Apart from different carbohydrate types, algae biomass hydrolysates may contain significant levels of non-sugar components, such as 5-HMF, a product of hexose degradation that is toxic to microbes used to convert biomass to biofuels [18, 30, 31]. Therefore, it is desirable that hydrolysates contain fermentable sugars but low inhibitor concentration. We analyzed all the samples treated in this study, to detect HMF at 0.1, 0.5, 1.1, 1.9, 2.8, and 7.6 mg/L after autoclave treatment of the sample for 1, 2, 3, 4, 5, and 6 h, respectively. Such concentrations were very low as compared to fermentation inhibitory concentrations described for H_2-producing *Clostridium* in the literature: above 1.0 g/L [18, 30, 31].

3.3 Fermentative hydrogen production from macroalgae hydrolysate

Because the hydrolysate obtained after algae biomass enzymatic hydrolysis presented the highest TRS productivity but no inhibitors, we employed it as substrate for H_2 production in fermentative assays. During the fermentative assay, the H_2-producing *C. beijerinckii* Br21 consumed ca. 1 g/L TRS from the hydrolysate (5.5 mmol/L, considering TRS as glucose) and produced 11.9 mmol of H_2 after 48 h. The yield ($Y_{H2/S}$) was 2 mmol of H_2/ mmol of glucose equivalent and represented 50% of the stoichiometric yield of H_2 production from glucose by *Clostridium* sp, which is 4 mols of H_2 per mol of glucose [1]. The hydrogen yield from algae biomass, 23.8 mmol of H_2/g of dry algae biomass, was much higher than the yields of between 1.5 and 7.6 mmol of H_2/g of algae biomass reported in the literature (Table 2).

Table 2: Macroalgae hydrolysate fermentation yield for H_2-production by *C. beijerinckii* Br21 as compared to the literature.

MACROALGAE	TREATMENT	H_2-PRODUCING CULTURE	Y (mmol of H_2/g of dry algae biomass)	REFERENCE
K. alvarezzi	Enzymatic	*C. beijerinckii* Br21	23.8	This work
Saccharinasculpera	Pulverization	*Vibrio tritonius*	2.5**	[32]
L. japônica	Thermal 180 °C/40 min.	Anaerobic sludge	4.4	[33]
L. japônica	Acid + thermal (12% HCl, 140 °C/60 min)	Anaerobic sludge	6.7	[33]
G. amansii	Acid + thermal (1.5% H_2SO_4, 180 °C/15 min)	Anaerobic sludge	1.5	[34]
Arthrospiraplatensis + *Laminaria digitata*	Acid + thermal (2.5% H_2SO_4, 135 °C /15 min)	Anaerobic sludge	3.5*	[35]
G. amansii	Acid + thermal (1% H_2SO_4, 121 °C/30 min)	Anaerobic sludge	2.1**	[36]

*mmol of H_2/g of volatile solids**Calculated from manuscript data.

Few literature works have addressed the use of macroalgae as substrate for H_2 production (see Table 2). Most research on macroalgae as substrate for H_2 production has employed acid and thermal treatments for biomass hydrolysis [18]. These treatments could produce much higher inhibitor concentration as compared to enzymatic hydrolysis. In addition, most of the literature works have used mixed cultures such as anaerobic sludges from wastewater treatment plants as H_2 producer, but these cultures are not as specific as pure H_2-producing microorganisms. Only one paper has employed a pure *Vibrio tritonius* culture as biocatalyst, but the yield was 10 times lower than ours [32]. Here, we used a H_2-producing *Clostridium* strain to ferment macroalgae hydrolysate for the first time. We chose to employ the recently isolated *C. beijerinckii* Br21 strain because it can produce H_2 from galactose as substrate at high conversion ratio (2.02 mmol of H_2/mmol of galactose) [23]. Moreover, the Br21 strain can produce hydrolytic enzymes, which could improve pretreated biomass hydrolysis. These *C. beijerinckii* Br21 strain characteristics help to explain its high performance in H_2 production from macroalgae biomass as compared to other works.

4. CONCLUSION

This is the first report that has employed macroalgae biomass (*K. alvarezzi*) as feedstock for H_2 production by a *Clostridium* strain. Both the autoclave treatment and enzymatic hydrolysis broke down *K. alvarezzi* biomass to a greater or to a lesser degree, to produce hydrolysates with low fermentation inhibitor concentration and available sugars for fermentative H_2 production by *Clostridium beijerinckii* Br21. Autoclave treatment followed by enzymatic hydrolysis (combined treatment) provided a hydrolysate with higher TRS concentration. Taking the total hydrolysis time into account, the TRS productivity achieved during the combined treatment (thermal treatment + enzymatic hydrolysis) was about 30% higher than the TRS productivity achieved with enzymatic hydrolysis (Celluclast® + β glucosidase). The use of the hydrolysate from enzymatic hydrolysis as feedstock to produce H_2 gave high yield as compared to literature data on the use of mixed microorganism cultures to produce H_2. In conclusion, both treatments were efficient methods to prepare macroalgae biomass for enhanced bioconversion to H_2 by *C. beijerinckii* Br21.

5. ACKNOWLEDGMENTS

This work was supported by FAPESP 2015/06074-1; ELR received a grant from Programa Unificado de Bolsas; BCF received a grant from CAPES; LPM received a post-doctoral scholarship from FAPESP; and SC received a master scholarship from CAPES.

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