



Use of Algae Biomass Obtained by Single-Step Mild Acid Hydrolysis in Hydrogen Production by the β -Glucosidase-Producing *Clostridium beijerinckii* Br21

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Abstract

Macroalgae biomass is a potential feedstock for fermentative H₂ production: it has high carbohydrate concentration and is lignin-free. Here, we optimize a mild acid treatment of *Kappaphycus alvarezii* biomass by an experimental design 2⁴. The optimal acid treatment conditions were 90 °C, HCl concentration of 55.9 mmol/L, 0.375 g of algae mass, and 8 h of treatment. Under these conditions, the hydrolysate presented mono-, but also di- and oligosaccharides. We used this hydrolysate as substrate for fermentative hydrogen (H₂) production by the *Clostridium beijerinckii* Br21 grown in two different conditions: (1) in medium containing galactose and no β -glucosidase activity; (2) in medium with cellobiose to stimulate β -glucosidase activity, which was 0.33U/mL. The fermentative assay conducted in the presence of the *C. beijerinckii* Br21 with β -glucosidase activity provided higher H₂ concentration and yield as compared to the assay accomplished in the presence of the inoculum with no β -glucosidase activity—171.76 ± 2.10 and 140.95 ± 10.92 mL of H₂/L, and 70.3 ± 0.9 and 62.7 ± 4.9 mL of H₂/g of dry algae, respectively. Therefore, a fermentative β -glucosidase-producing organism such as *C. beijerinckii* Br21 can potentially complete biomass saccharification for later renewable H₂ production.

Keywords *Kappaphycus alvarezii* · Acid treatment · Factorial design · Biohydrogen

Statement of Novelty

Kappaphycus alvarezii biomass has never been explored as feedstock for H₂ production. A biomass hydrolysate containing mostly di- and oligosaccharides and only small monosaccharide concentrations was used as substrate for fermentative H₂ production by *Clostridium beijerinckii* Br21. The fermentation inoculum grown in cellobiose presented β -glucosidase activity of a *C. beijerinckii* strain, which has been described for the first time in this work.

β -glucosidase activity stimulation in the inoculum helped *C. beijerinckii* Br21 to produce higher H₂ concentration per unit of algae biomass as compared to the inoculum grown in galactose with no β -glucosidase activity. Biomass hydrolysis to the monosaccharide level was not necessary because the H₂-producing microorganism can produce hydrolases that help to consume higher-molecular-weight saccharides.

Introduction

Hydrogen (H₂) is a clean fuel; its burning produces water only [1–3]. However, current strategies to produce H₂ demand high amounts of energy or rely on fossil fuel-based methods [4]. H₂ can also originate from renewable fermentation of carbohydrate-rich materials, such as wastewater, wastes, and biomasses, which is a more sustainable and economically viable process [3]. Various renewable biomasses can be used as low-cost substrate for fermentative H₂ production, which significantly reduces production costs [5, 6].

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In the last years, algae biomass has become a promising feedstock to produce biofuels, including biohydrogen, the so-called third-generation H₂ [5–10]. As a substrate for fermentative biofuel production, algae biomass offers many advantages: it sequesters high CO₂ amounts, grows at high rate, and does not require arable land. In addition, algae biomass lacks hemicellulose and lignin, which facilitates access to its carbohydrates [5, 8]. Consequently, algae biomass pretreatment to convert polymeric carbohydrates into monomeric sugars for further fermentation calls for milder conditions as compared to lignocellulose pretreatment [5–8].

According to the literature, brown macroalgae like *Laminaria japonica* [7] and red microalgae like *Gelidium amansii* [9] have high carbohydrate content and therefore display the greatest potential for application as feedstock for biohydrogen production [10]. The red macroalgae *Kappaphycus alvarezii* also has high carbohydrate content (ca. 50% d.w) and has already been employed as substrate for bioethanol production [11]. Nevertheless, there are no studies on the use of this macroalgae as substrate for fermentative H₂ production. *K. alvarezii* biomass contains mainly heterogeneous polysaccharides known as carrageenans, which consist mostly of D-galactose and 3,6-anhydro-D-galactose (AHG) with different degrees of sulfate groups, linked by β-1,4 and α-1,3 bonds [10, 12].

Although algae biomass does not contain lignin, it still has a complex structure that requires a pretreatment step necessary [6]. Additionally, fermentative bacteria usually have low hydrolytic activity, so pretreatment releases organic substances from the algal cells and makes them readily biodegraded. Several pretreatment technologies that have been developed to treat lignocellulosic biomasses, such as milling, heating, and ultrasonic, acid, basic, ozone, and enzymatic treatments, can also be employed to treat algae biomass [6].

Because acid pretreatment is easy to accomplish, is inexpensive, and can be applied on an industrial scale, it has been the most commonly used technology to pretreat biomasses for fermentable sugar production from carrageenan-rich biomasses [13, 14]. The hydrolysis reaction conditions, including acid concentration, temperature, and reaction time, vary depending on the carrageenan type [10, 13]. The acid and temperature conditions employed during algae biomass hydrolysis may result not only in monosaccharides, but also in sugar degradation products. Indeed, dehydrated galactose, like AHG from carrageenan, is more susceptible to degradation as compared to the D-galactose hydrated form. AHG can be promptly converted into 5-hydroxymethylfurfural (HMF), a well-known fermentation inhibitor [10, 15]. Therefore, mild acid hydrolysis conditions is more suitable to saccharify algae biomass for further fermentation [9, 10].

Here, we optimize acid hydrolysis conditions for *K. alvarezii* biomass for later application of this biomass in fermentative H₂ production, and we employ *Clostridium beijerinckii*

Br21 (GenBank accession number KT626859), isolated in our laboratory, as H₂-producing organism to ferment the algae acid hydrolysate. This strain can consume a wide scope of substrates and has a large number of genes encoding for glycosyl hydrolases [16]. Hence, we hypothesize that the *C. beijerinckii* Br21 enzymes can help to complete the hydrolysis of the mild acid pretreated algae biomass.

Materials and Methods

Macroalgae Biomass

The *K. alvarezii* macroalgae biomass was obtained from the Fisheries Institute, Ubatuba, São Paulo, Brazil (GPS coordinates 23°27'5.8"S; 45°02'49.3"W). This seaweed is cultivated in an open raft system. The macroalgae biomass was collected and dried under solar light before being sent to the lab. In the lab, the seaweed biomass was washed three times in distilled water, to remove salt and impurities, and dried in an oven with air re-circulation at 60 °C for 14 h. After drying, the seaweed was milled in a knife mill (Solab model SL 31, Brazil) and sieved at #35 mesh, to standardize it for the acid hydrolysis assays.

Seaweed Biomass Chemical Composition Analysis

The total solids, moisture, and ash contents in the macroalgae biomass were analyzed according to the methodology proposed by the National Laboratory of Renewable Energies—NREL, from the US Department of Energy [17]. The total carbohydrate content in the algae was determined according to the NREL methodology [18], modified by addition of concentrated H₂SO₄ to hydrolyze the biomass before the total sugars content was quantified. This methodology has also been described for analysis of the carbohydrate content in lignocellulosic materials [19]. The protein content was determined by the Lowry method modified by Hartree [20]. Sulfate was quantified by the methodology described by Hayashi [21]. First, the biomass was digested by addition of 0.1 mL of ethanol 92% and 1.0 mL of HCl 0.5 mol/L to 0.05 g of algae (dry mass basis), which was followed by autoclaving at 120 °C for 15 min. After that, a reaction with the gelatin reagent + BaCl₂ was performed, and the absorbance was read at 550 nm [21].

All the analytical quantifications were conducted at least in triplicate.

Surface Response Methodology (SRM) for Algae Biomass Acid Hydrolysis Optimization

Surface-response methodology (SRM) and multi-response analysis [22] helped to optimize the experimental conditions [temperature (X1), time (X2), algae mass (X3), and

acid concentration (X_4) that led to the highest total reducing sugars (TRS) concentration possible. A 2^4 full factorial central composite design (star configuration) aided in this purpose (Table 2). The experimental intervals of the independent variables analyzed in the SMR experiments (Table 2) were chosen on the basis of literature [13, 14, 23] and of previous experiments performed in the laboratory with the one factor at a time (OFAT) methodology, which provided the highest TRS concentration and the lowest HMF concentration possible. The preliminary tests showed the optimum hydrolysis conditions were temperature between 50 and 90 °C, algae mass between 0.075 and 0.375 g, HCl concentration between 11.2 and 55.9 mmol/L, and treatment time between 7 and 11 h. The effect of temperature, time, algae mass, and acid concentration on TRS concentration was determined with the aid of STATISTICA® software 7.0. The data were fit to a second-order equation (Eq. 1) as a function of the independent variables at 90% confidence level, and an analysis of variance (ANOVA) was carried out to evaluate the significance of this mathematical model ($p < 0.05$).

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{123}X_1X_2X_3 + b_{124}X_1X_2X_4 + b_{134}X_1X_3X_4 + b_{134}X_1X_3X_4 + b_{1234}X_1X_2X_3X_4, \quad (1)$$

where b_n corresponds to constant regression coefficients; Y_i refers to the dependent variables TRS; and X_1 , X_2 , X_3 , and X_4 are the coded independent variables temperature, HCl concentration, time, and algae mass, respectively.

After the surface-response results were obtained, the process conditions were optimized by using the software Excel® 2010, with the algorithm generalized reduced gradient (GRG).

Finally, hydrolysates prepared in the optimal conditions helped to validate the optimized conditions provided by response surface methodology (RSM). The validation experiments were accomplished in triplicate, and the resulting hydrolysates were characterized with respect to TRS. Comparison of the values predicted by the model with the actual values was made through the relative error generated by the complete model obtained by the coded values (Eq. 2).

$$\hat{\epsilon}_{RMC} = \left(\frac{Y - Y_{MC}}{Y} \right) \times 100 \quad (2)$$

where $\hat{\epsilon}_{RMC}$ = relative error of the complete model, Y = experimental value; Y_{MC} = value predicted by the complete model.

Acid Hydrolysis Assays

All the hydrolysis assays were conducted in 30 mL of HCl solution in a 125-mL Erlenmeyer; the temperature was controlled with a water bath. The acid treatment conditions were tested according to a 2^4 full factorial central composite design (star configuration, Table 2). The acid treatment was interrupted by cooling the mixture in an ice bath, which was followed by filtration through Whatmann® filter paper number 1 under vacuum. The residual solid algae retained in the filter paper was washed with distilled water, and the liquid was collected in the same volumetric flask (50 mL) as the hydrolysate, to ensure that all the solubilized sugars were removed from the residual algae and to standardize the final volume of the liquid hydrolysate for the chemical analyses (TRS, monosaccharides, and HMF). Before the flask volume reached 50 mL, pH was adjusted to neutral with sodium hydroxide 5 mol/L. The acid hydrolysis assays were carried out at least in duplicate. Hydrolysis under the optimal conditions was accomplished in triplicate.

Hydrolysate Chemical Analysis

The TRS concentration in the algae hydrolysate was determined by the 3,5-dinitrosalicylic acid (DNS) method described by Miller [24]. The length of the carbohydrate products arising after algae acid treatment optimization was analyzed by thin layer chromatography (TLC). Aliquots of the filtered samples were treated with 10% trichloroacetic acid for 30 min, to precipitate proteins and long-chain carbohydrates. The samples were centrifuged at 10,000×g for 15 min, and the supernatants (10 µL) were analyzed by TLC on silica-gel G-60 plates (10 × 15 cm, DC-Alufolien Kieselgel 60, Merck, Darmstadt, Germany) [25]. The monosaccharides (D-glucose and D-galactose) and HMF in the hydrolysates were 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) equipped with an Aminex HPX-87H Ion Exclusion Column (300 × 7.8 mm) from Bio Rad. The mobile phase consisted of sulfuric acid 5 mM in milli Q water at a flow rate of 0.6 mL/min (Pressure = 68 Kg/cm²). The detector was a diode array spectrophotometer (Model: SPD10A-VP) operating at 190, 205, and 276 nm with Refractive Index (Model: RID-10A). The carbohydrates were measured with a refraction index detector (RID), and 5-HMF was analyzed by the diode array spectrophotometer at 280 nm.

Fermentative Assays for H₂ Production

Culture Medium and Inoculum

Clostridium beijerinckii Br21, which was isolated in our laboratory and can consume the main monosaccharides from algae biomass (galactose, glucose, and mannose) was employed to produce H₂ by fermentation [22]. The microorganism was cultivated in a culture medium designated CH medium [26] at pH 7.0 and 35 °C for 48 h, under anaerobiosis. The CH medium consisted of glucose (5.0 g/L), yeast extract (1.0 g/L), Na₂HPO₄ (5.0 g/L), KH₂PO₄ (1.0 g/L), NaCl (1.0 g/L), MgSO₄·7H₂O (0.1 g/L), FeSO₄ (0.025 g/L), and a trace elements solution (2.0 mL/L) containing H₃BO₃ (2.86 g/L), MnSO₄·4H₂O (2.03 g/L), and FeCl₃ (0.1 g/L). All the chemicals were analytical grade.

The *C. beijerinckii* Br21 inoculum used in the H₂ fermentative assays was prepared by two different approaches. In the first approach, *C. beijerinckii* Br21 was cultivated in CH medium containing galactose instead of glucose as carbon source. In the second approach, *C. beijerinckii* Br21 was grown in cellobiose as carbon source to stimulate intracellular β-glucosidase production.

β-Glucosidase Activity Assays

The *C. beijerinckii* Br21 cultures harvested in galactose or in cellobiose were employed as crude extract that might contain β-glucosidases. The *Clostridium* cells were disrupted by sonication; the cell debris was removed by centrifugation; and the supernatant was used in the enzymatic assays.

The β-glucosidase activity was assayed by using *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc; Sigma-Aldrich Chem. Co, St. Louis, MO, USA) as substrate. *p*NP-Glc hydrolysis was estimated discontinuously, at 35 °C [27]. The standard assay conditions were bis-tris buffer 50 mmol/L pH 6.0 containing 2 mmol/L *p*NP-Glc in a final volume of 0.6 mL. All the enzymatic assays were performed in duplicate. One enzyme unit was defined as the amount of enzyme that released 1 μmol of product/min.

Fermentation Assay

The hydrolysate obtained after algae biomass acid treatment under optimal conditions was employed as the only carbon source in the fermentative assays. The algae hydrolysate (14 mL) was supplemented with the CH medium nutrients described above (item 2.4.1), filtered under sterile conditions through a cellulose acetate filter (0.22-μm), and added to pre-sterilized 50-mL flasks. One milliliter of

the *C. beijerinckii* Br21 inoculum prepared by one of the two different approaches described in “Culture Medium and Inoculum” section was added to the fermentation flask. The inoculum had optical density of 0.1 at 600 nm, which corresponded to 6 × 10⁷ cells/mL according to a previously established correlation [22]. After this procedure, the tubes were bubbled with argon gas for 3 min (to establish anaerobiosis), closed with a rubber cap and an aluminum seal, and incubated at 35 °C and 200 rpm. All the fermentative assays were conducted in triplicate.

Sugar consumption during fermentation was determined by collecting liquid samples at the beginning and at the end of the fermentative assays to measure TRS.

The gas composition was also analyzed by periodically withdrawing 100 μL of gas from the headspace of the fermentation vials with a gas-tight syringe. The gas composition was determined on a Shimadzu GC (Japan) GC 2014 gas chromatograph equipped with a thermal conductivity detector (TCD). The column consisted of molecular sieves 5A (2 m × 4.7 mm); argon at a flow rate of 30 mL/min was used as the carrier gas. The injector, column, and detector temperatures were 80, 50, and 100 °C, respectively. The H₂ volume was determined by the total volume (mL) of the headspace multiplied by the percentage of H₂ analyzed in the headspace gas.

To calculate the product (H₂) conversion factor in relation to the substrate (TRS) consumed in the fermentation assays, the final H₂ volume was transformed (in mmol) and divided by the substrate (in mmol) consumed during the test (Eq. 3). The TRS concentration was converted into millimoles of glucose by dividing it by the glucose molecular weight (MW = 180 g/mol):

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad (3)$$

where $Y_{P/S}$ = yield or factor of substrate (TRS as glucose) conversion into product (H₂); ΔS = Difference between the number of mmol of substrate at the beginning and at the end of the test; ΔP = mmol of H₂ formed during the fermentative assay.

The H₂ yield relative to the amount of seaweed biomass was also calculated. The H₂ volume (in mL) was divided by the seaweed initial dry mass (in grams) (Eq. 4).

$$Y_{P/M} = \frac{\Delta P}{M}, \quad (4)$$

where, $Y_{P/M}$ = product yield in relation to the algae mass; ΔP = milliliter of H₂ formed in the fermentative assay; M = dry algae biomass employed in the fermentative assays.

The fermentation assay kinetic parameters were statistically compared by Tukey's test with 95% confidence with STATISTICA® software 7.0.

Table 1 *Kappaphycus alvarezii* biomass chemical composition

Compound	(%)
Total solids	97.8 ± 2.1
Ash ^a	11.1 ± 0.6
Carbohydrates ^a	51.3 ± 2.1
Protein ^a	4.1 ± 0.03
Sulphate ^a	13.6 ± 2.2
Other ^a	24

^aPercentage in dry mass basis

Results and Discussion

Algae Biomass Chemical Composition

The *K. alvarezii* biomass chemical composition, depicted in Table 1, was similar to the composition of different *K. alvarezii* strains; i.e. between 54.6 and 55.8% carbohydrates, 11% ash, and 10.1% mean sulfate concentration [28]. Abd-Rahim et al. [13] described the *K. alvarezii* chemical composition as 19.70%, 59.58%, and 5.74% ash, total carbohydrates, and proteins, respectively. The sulfate amount in *K. alvarezii* varies depending on the strain and on the carrageenan type and content. Commercial carrageenans contain between 22 and 38% sulfate [29]. However, the sulfate content in

Table 2 Central composite design matrix with the variables temperature, HCl concentration, algae mass, and, time, as well as the response for total reducing sugars (TRS) in the algae hydrolysates

Assay	X ₁ [temperature (°C)]	X ₂ [HCl (mmol/L)]	X ₃ [algae mass (g)]	X ₄ [time (h)]	TRS (g/L)	HMF ^a (mg/L)	Glucose ^a (mg/L)	Galactose ^a (mg/L)
1	- 1(60)	- 1(22.4)	- 1(0.15)	- 1(8)	0.86	0	0	0
2	- 1(60)	- 1(22.4)	- 1(0.15)	+ 1(10)	0.94	0	0	0
3	- 1(60)	- 1(22.4)	+ 1(0.3)	- 1(8)	1.52	0	0	0
4	- 1(60)	- 1(22.4)	+ 1(0.3)	+ 1(10)	1.49	0	0	0
5	- 1(60)	+ 1(44.7)	- 1(0.15)	- 1(8)	1.01	0	0	0
6	- 1(60)	+ 1(44.7)	- 1(0.15)	+ 1(10)	1.00	0	0	0
7	- 1(60)	+ 1(44.7)	+ 1(0.3)	- 1(8)	1.93	0	0	0
8	- 1(60)	+ 1(44.7)	+ 1(0.3)	+ 1(10)	1.71	0	0	0
9	+ 1(80)	- 1(22.4)	- 1(0.15)	- 1(8)	1.24	0	0	0
10	+ 1(80)	- 1(22.4)	- 1(0.15)	+ 1(10)	1.30	56.32	26.02	20.08
11	+ 1(80)	- 1(22.4)	+ 1(0.3)	- 1(8)	2.44	58.35	6.04	54.23
12	+ 1(80)	- 1(22.4)	+ 1(0.3)	+ 1(10)	2.49	54.09	26.95	21.51
13	+ 1(80)	+ 1(44.7)	- 1(0.15)	- 1(8)	1.38	33.66	20.38	28.47
14	+ 1(80)	+ 1(44.7)	- 1(0.15)	+ 1(10)	1.38	28.95	20.32	17.44
15	+ 1(80)	+ 1(44.7)	+ 1(0.3)	- 1(8)	2.72	55.02	20.99	35.76
16	+ 1(80)	+ 1(44.7)	+ 1(0.3)	- 1(10)	2.52	82.64	17.83	8.91
17	- 2(50)	0(33.5)	0(0.225)	0(9)	1.01	0	23.82	0
18	+ 2(90)	0(33.5)	0(0.225)	0(9)	2.03	307.5	6.61	8.32
19	0(70)	- 2(11.2)	0(0.225)	0(9)	1.49	8.19	0	5.87
20	0(70)	+ 2(55.9)	0(0.225)	0(9)	1.77	13.42	0	0
21	0(70)	0(33.5)	- 2(0.075)	0(9)	0.61	2.47	0	0
22	0(70)	0(33.5)	+ 2(0.375)	0(9)	2.18	10.99	20.47	9.56
23	0(70)	0(33.5)	0(0.225)	- 2(7)	1.38	3.60	18.27	9.49
24	0(70)	0(33.5)	0(0.225)	+ 2(11)	1.80	9.60	19.29	11.69
25 (C)	0(70)	0(33.5)	0(0.225)	0(9)	1.58	8.84	21.00	8.73
26 (C)	0(70)	0(33.5)	0(0.225)	0(9)	1.64	12.14	0	0
27 (C)	0(70)	0(33.5)	0(0.225)	0(9)	1.59	11.38	0	0
28 (C)	0(70)	0(33.5)	0(0.225)	0(9)	1.69	8.58	0	0

^aVariables not considered in the optimization

X1, X2, X3, and X4 are the of coded variables

Zero(0) means that the concentration is under the method detection limit

the whole macroalgae biomass has been described to range between 8 and 12% [28].

Regarding the use of this macroalgae biomass for fermentative purposes, its total carbohydrate content is higher than 50% and is in the same order of magnitude of the total carbohydrate content of other macroalgae biomasses that have been studied as substrates for H₂ production; e.g., *G. amansii* [9] and *L. japonica* [7], 67.3 and 59.7% total carbohydrate content, respectively. Therefore, the algae we investigate here is a potential feedstock for H₂ production.

Acid Hydrolysis Optimization

Table 2 lists the TRS, HMF, D-glucose, and D-galactose concentrations in the algae submitted to acid treatment carried out according to the conditions (temperature, HCl concentration, algae mass, and time) established by the 2⁴ experimental design matrix.

On the basis of the results displayed in Table 2, acid treatment of 0.3 g of algae biomass at 80 °C in HCl 44.7 mmol/L for 8 h (assay 15) affords the highest TRS concentration.

In all the assays, the glucose and galactose concentrations range from 6.04 to 26.95 mg/L and from 5.87 to 54.23 mg/L, respectively. Hence, the mild acid treatment conditions applied here generate monosaccharides at very low concentrations, so that the TRS concentration is probably represented by di-, tri-, or oligosaccharides. Thin layer chromatography of the hydrolysate (TLC; Fig. 2) confirms this effect. Because HMF originates from hexoses dehydration, it also emerges at low concentration (between 0 and 307.5 mg/L) albeit its concentration is sometimes higher than the monosaccharide concentrations. Tests accomplished at the highest temperatures, 80 and 90 °C, give the highest HMF concentrations, 82.64 and 307.5 mg/L, respectively (Table 2). According to Yun et al. [30], acid treatment is the method that is the most commonly employed to hydrolyze carrageenan-containing algae biomass, but it requires high temperature, large acid concentration, and long time to yield monosaccharides. Nevertheless, these severe acid hydrolysis conditions degrade D-galactose and 3,6-anhydro-D-galactose (AHG), which are carrageenan monosaccharides, to HMF [30]. Park et al. [9] reported that *G. amansii* hydrolysis with 2% sulfuric acid at 121 °C for 59 min produces D-galactose and AHG. However, AHG rapidly degrades to HMF, levulinic acid, and formic acid, which are fermentation inhibitors. We did not target AHG in our chromatographic analyses, but it may have been readily converted into HMF, which could be the reason why HMF concentration is sometimes as high as or even higher than the monosaccharide concentrations.

We used the TRS concentration and not the monosaccharide concentrations to optimize the algae acid treatment because the H₂-producing *C. beijerinckii* Br21 employed

here can ferment different types of sugars [22]. Equation 5 refers to the mathematical model we used to determine the TRS concentration in the hydrolysates after statistical analyses of the results. The linear parameters of temperature (X₁) and algae mass (X₃) and the interaction between these variables (X₁X₃) influence the TRS values more than the other variables (p < 0.1).

$$\begin{aligned} \text{TRS} = & 1.620 + 0.296X_1 - 0.025X_1^2 + 0.077X_2 \\ & + 0.490X_3 + 0.030X_3^2 + 0.130X_1X_3 \\ & - 0.031935X_2X_4 - 0.038X_3X_4 \end{aligned} \quad (5)$$

Analysis of variance (ANOVA) shows that the model we used to calculate the TRS concentration in the hydrolysates (Eq. 5) is statistically significant and predictive at a confidence level of 90% (p < 0.1) because F_{calculated} > F_{listed} and their F ratio (F_{calculated}/F_{listed}) is higher than the corresponding F_{listed}. We generated the response-surface curves for TRS for further fermentation (Fig. 1).

According to the surface-response curves, the TRS concentration increases with increasing HCl concentration and temperature (Fig. 1a). Simultaneous rise in temperature and algae mass also increases TRS concentration (Fig. 1b). The algae mass limits ART concentration. Concerning the variables time and temperature, the maximum ART concentration is reached at the highest temperatures (Fig. 1c). At higher temperatures, longer times do not boost the ART concentration in the algal hydrolysate. Therefore, the shorter times proposed in the experimental design are sufficient to reach high TRS concentrations (Fig. 1c).

On the basis of Eq. 5, the optimal conditions to reach the highest TRS concentration are 90 °C, 55.9 mmol of HCl/L, 0.375 g of algae, and 8 h, which theoretically afford 3.94 g of TRS/L. Algae acid hydrolysis conducted in the optimal conditions, in triplicate, helped to validate the optimization methodology. The relative deviation value revealed that the predicted and experimental values correlate well (Table 3).

The algae hydrolysate TRS concentration (3.5 g/L) is slightly lower than the predicted TRS concentration. The hydrolysate also contains HMF at 0.28 g/L. We performed the assays under low algae biomass load (0.375 g in 50 mL), to obtain enough sugar concentration and small amounts of inhibitors in the hydrolysate for further fermentation. Low inhibitor concentrations eliminate the need for a detoxification step. Higher algae biomass loading (about 10%) leads to higher TRS concentration in the hydrolysate, but also to higher concentration of fermentation inhibitors, which are beyond the lower TRS extraction yield. Meinita et al. [31] found optimal conditions for acid *K. alvarezii* hydrolysis when they used HCl as catalyst and optimized the temperature, reaction time, algae concentration, and acid concentration as 130 °C, 15 min,

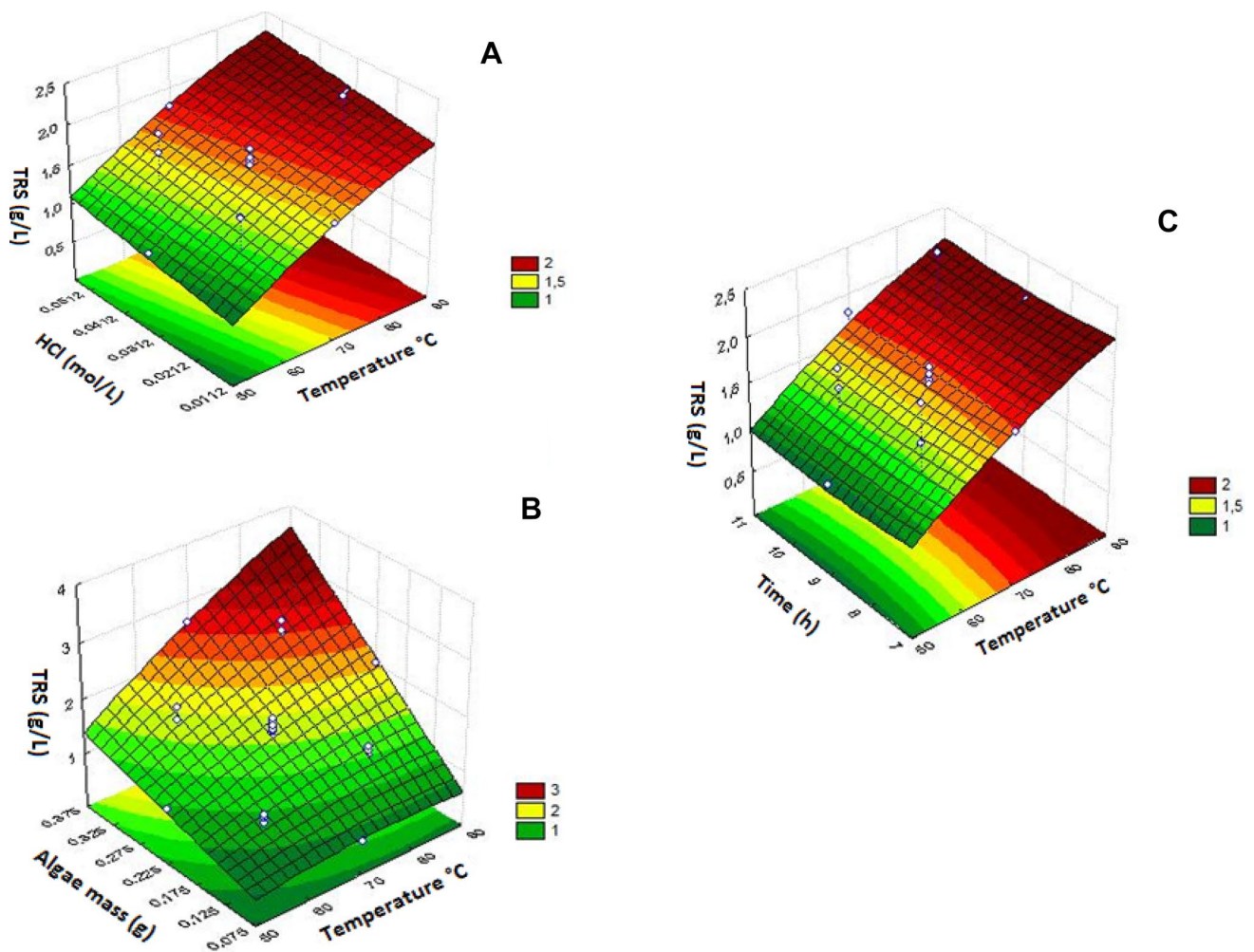


Fig. 1 TRS concentration surface-response as a function of acid concentration versus temperature (a), algae mass versus temperature (b), time versus temperature (c)

Table 3 Predicted and experimental TRS concentration responses in the optimal pretreatment conditions (90 °C, HCl at 55.9 mmol/L, 0.375 g of algae, and 8 h)

Response	Predicted	Experimental	RD ^a (%)
TRS (g/L)	3.94	3.50	- 7.0

^aRelative deviation (RD): [(experimental value - predicted value)/experimental value] × 100

10 g of substrate/100 mL, and HCl 0.2 M, respectively. This optimal condition produced 22.68 g/L of TRS in the hydrolysate, but fermentation inhibitors also arose: HMF and levulinic acid at 0.8 and 2.8 g/L, respectively. HMF and levulinic acid concentrations of about 0.5 and 1.5 g/L, respectively, inhibit 50% of H₂ production by a mixed culture [15, 32]. Here, we carried out algae hydrolysis under the optimized conditions at 0.7% (w/v) algae loading, which yielded a TRS concentration of 3.5 g/L and an

HMF concentration of 0.28 g/L in the hydrolysate. These values do not represent potential inhibition. Interestingly, low HMF concentrations, ca 0.25 g/L, have been considered to stimulate fermentation [32].

The presence of low concentrations of monosaccharides and of their degradation derivative (HMF) in the liquid does not mean that the structure of the algae polysaccharide remained intact, but that the hydrolysis conditions probably sufficed to release oligosaccharides only, as demonstrated by TLC (Fig. 2). Controls C1 and C2 in Fig. 2 correspond to algae and water only and showed that untreated algae biomass has high- molecular-weight polysaccharides that are retained at the bottom of the TLC (Fig. 2). On the other hand, D-glucose and D-galactose, which have the lowest molecular weight, migrate to the top of the TLC plate, followed by the disaccharide cellobiose, which stops just below the monosaccharides. The algae hydrolysate (TRS; Fig. 2) displays stains mainly at the same height as the disaccharide

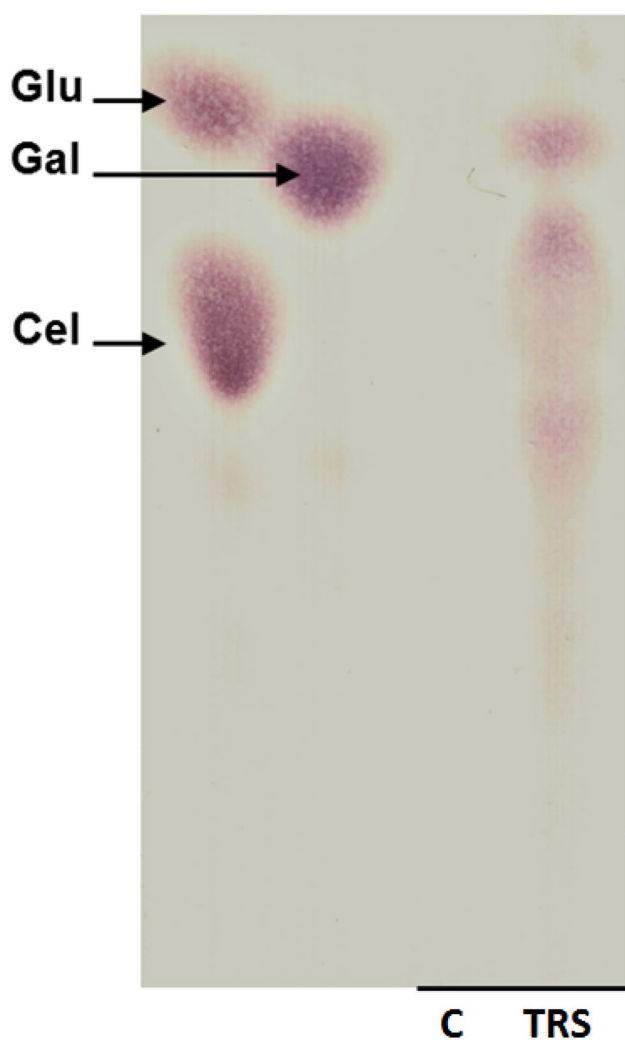


Fig. 2 TLC of the assayed samples. *Glu* glucose, *Cel* cellobiose, *Gal* galactose, *C* control (algae and water), *TRS*-hydrolysate attained at the optimized conditions (90 °C, HCl at 55.9 mmol/L, algae mass of 0.375 g, and 8 h)

as well as below, which indicates that TRS also refer to oligosaccharides.

The low degree of hydrolysis we achieved for the *K. alvarezii* biomass hydrolysate could avoid prompt dehydrated galactose degradation under acid conditions. Nevertheless, successful employment of such hydrolysate as substrate will depend on the fermentative microorganism ability to convert higher sugars into biofuels.

Fermentative Assays for H₂ Production with Algae Hydrolysate

We employed the hydrolysate obtained from the algae subjected to acid treatment under optimal conditions, which yielded the highest TRS concentration, and the TRS were the only carbon source in the fermentative assays for H₂

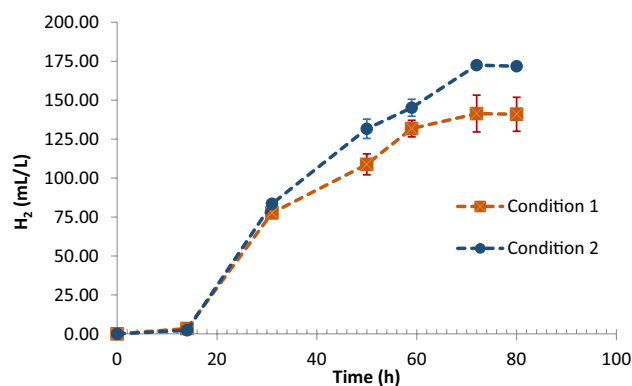


Fig. 3 H₂ concentration as a function of time in the fermentative assays conducted with inoculum prepared under different conditions: condition 1—inoculum grown in galactose and presenting no β -glucosidase activity; condition 2—inoculum grown in cellobiose and presenting 0.33 U/mL of initial β -glucosidase activity

production. Although the sugars present in the hydrolysate consist mostly of oligosaccharides, *C. beijerinckii* Br21 bears a high amount of genes encoding for glycosyl hydrolases [16], which could help in the final algae biomass hydrolysis steps. Here, the *C. beijerinckii* Br21 cell extract cultivated in galactose or in cellobiose exhibits no or β -glucosidase 0.33 U/mL activity, respectively. Albeit low, we have succeeded in detecting β -glucosidase activity by a *C. beijerinckii* strain for the first time.

Figure 3 presents the H₂ concentration measured during the fermentative assays that employed the algae hydrolysate as substrate and the inoculum cultivated in galactose or in cellobiose, named conditions 1 and 2, respectively. The H₂ concentration obtained with *C. beijerinckii* displaying β -glucosidase activity (condition 2, 171.76 ± 2.10 mL of H₂/L) is significantly higher ($p < 0.05$) as compared to the inoculum without initial β -glucosidase activity (condition 1, 140.95 ± 10.92 mL of H₂/L). β -Glucosidases hydrolyze short-chain oligosaccharides and cellobiose, to yield glucose, which is fermentable. Therefore, β -glucosidase may help to increase the content of fermentable sugars, such as glucose, in biomass hydrolysates, and consequently raise the amount of generated biohydrogen [33]. Glucose assimilation is usually preferred over assimilation of other carbohydrates and, depending on the glucose concentration, the catabolic repression effect may emerge; i.e., the presence of glucose inhibits assimilation of other sugars [34]. Galactose and glucose (the main red macroalgae cell wall monosaccharides) are hexoses, but the bacterial cell takes them up differently. Galactose first needs to be converted into glucose by the Leloir pathway. In this process, galactose is converted into glucose-1-phosphate, which consumes more ATP than glucose conversion into this same molecule. For this reason, galactose as substrate leads to lower fermentation yields

Table 4 Maximum H₂ concentration, Y_{P/S} and Y_{P/M} from the fermentative assays conducted in the presence of *C. beijerinckii* Br21 cultivated in galactose (1) or in cellobiose (2)

Inoculum condition	H ₂ max (mL of H ₂ /L)	Y _{P/S} (mmol of H ₂ /mmol of ART as glucose) ^a	Y _{P/M} (mL of H ₂ /g of dry algae)
1	140.95 ± 10.92	0.76 ± 0.05	62.7 ± 4.9
2	171.76 ± 2.10	0.87 ± 0.09	70.3 ± 0.9

^aConverted considering TRS as glucose; molecular weight = 180 g/mol

[22]. Moreover, disaccharides such as cellobiose as well as small oligosaccharides are only used if the fermentation microorganism can hydrolyze these molecules to their respective monosaccharides [33].

Apart from higher H₂ concentration, the assay accomplished with *C. beijerinckii* presenting β-glucosidase activity (Fig. 3, condition 2) gives significantly higher H₂ yield relative to the amount of seaweed biomass employed in the fermentation as compared to the assay performed with the inoculum grown in galactose (Table 4). This result confirms that the β-glucosidase activity in the *Clostridium* grown on cellobiose improves the use of the algae hydrolysate.

As for the ratios of conversion of TRS as glucose to H₂ (Y_{P/S}), they were 0.76 and 0.87 mmol of H₂/mmol of TRS as glucose for conditions 1 and 2, respectively, which represents 19 and 22% if we consider the maximum H₂ stoichiometric yield from glucose as 4 mol of H₂/mol of glucose, respectively.

When it comes to pretreated red algae as feedstock, the yield 70.3 mL of H₂/g of dry algae is very promising as compared to other literature results. Only one paper has reported a much higher yield, 159.6 mL of H₂/g of dry weight of *L. japonica* pretreated at 4.8% HCl and 93 °C for 23 min and loaded onto an anaerobic sludge [35]. Our values are higher than the data obtained by Liu and Wang [36], which was 43.65 mL of H₂/g of dry algae when they employed *L. japonica* treated with HCl 1 mol/L for 30 min loaded at 1% onto an anaerobic sludge. Our values were also higher than the 53.5 mL of H₂/g of dry algae obtained from a *G. amansi* hydrolysate in the presence of a mixed culture at 150 °C for 15 min [9]. In the latter article, fermentation was only possible after a detoxification step with activated carbon [9].

Finally, our results show that, although the algae hydrolysate we obtained from a mild acid treatment presents low monosaccharide content, it can be employed as substrate for H₂ production by *C. beijerinckii* Br21. Indeed, stimulating β-glucosidase production by *C. beijerinckii* Br21 improves H₂ production and yield. This enzyme can hydrolyze cellobiose and other di- and oligosaccharides present in biomass hydrolysates [33] and could thus generate more

fermentable sugars as compared to the assay where there was no β-glucosidase activity stimulation.

Conclusions

The factorial design we employed here showed that the variables temperature and algae mass are the most significant when it comes to obtaining high TRS concentrations from *K. alvarezii* biomass. Mild acid treatment conditions mainly provide di- and oligosaccharides as well as low monosaccharide and HMF concentrations. Here, we have described β-glucosidase activity for the *C. beijerinckii* strain cultivated in cellobiose for the first time. This activity helped to increase fermentative H₂ production from the *K. alvarezii* hydrolysate.

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References

1. Veras, S., Mozer, T.S., dos Santos, D.C.R.M., César, A.S.: Hydrogen: trends, production and characterization of the main process worldwide. *Int. J. Hydrog. Energy* **42**, 2018–2033 (2017)
2. Xia, A., Cheng, J., Song, W., Su, H., Ding, L., Lin, R.: Fermentative hydrogen production using algal biomass as feedstock. *Renew. Sustain. Energy Rev.* **51**, 209–223 (2015)
3. Shobana, S., Kumar, G., Bakonyi, P., Saratale, G.D., Al-Muhtaseb, A.H., Nemestóthy, N., Bélafi-Bakó, K., Xia, A., Chang, J.S.: A review on the biomass pretreatment and inhibitor removal methods as key-steps towards efficient macroalgae-based biohydrogen production. *Bioresour. Technol.* **244**(2), 1341–1348 (2017)
4. Dincer, A., Acar, C.: Review and evaluation of hydrogen production methods for better sustainability. *Int. J. Hydrog. Energy* **40**, 11094–11111 (2015)
5. Wang, J., Yin, Y.: Fermentative hydrogen production using various biomass-based materials as feedstock. *Renew. Sustain. Energy Rev.* **92**, 284–306 (2018)
6. Wang, J., Yin, Y.: Fermentative hydrogen production using pretreated microalgal biomass as feedstock. *Microb. Cell Factories* **17**, 22 (2018)
7. Jung, K.W., Kim, D.H., Shin, H.S.: Fermentative hydrogen production from *Laminaria japonica* and optimization of thermal pretreatment conditions. *Bioresour. Technol.* **102**(3), 2745–2750 (2011)
8. Vassilev, S.V., Vassileva, C.G.: Composition, properties and challenges of algae biomass for biofuel application: an overview. *Fuel* **181**, 1–33 (2016)
9. Park, J.H., Yoon, J.J., Park, H.D., Kim, Y.J., Lim, D.J., Kim, S.H.: Feasibility of biohydrogen production from *Gelidium amansii*. *Int. J. Hydrog. Energy* **36**(21), 13997–14003 (2011)
10. Yun, E.J., Kim, H.T., Cho, K.M., Yu, S., Kim, S., Choi, I.G., Kim, K.H.: Pretreatment and saccharification of red macroalgae to produce fermentable sugars. *Bioresour. Technol.* **199**, 311–318 (2016)

11. Hargreaves, P.I., Barcelos, C.A., da Costa, A.C., Jr. Pereira, N.: Production of ethanol 3G from *Kappaphycus alvarezii*: evaluation of different process strategies. *Bioresour. Technol.* **134**, 257–263 (2013)
12. Teh, Y.Y., Lee, K.T., Chen, W.H., Lin, S.C., Sheen, H.K., Tan, I.S.: Dilute sulfuric acid hydrolysis of red macroalgae *Eucheuma denticulatum* with microwave-assisted heating for biochar production and sugar recovery. *Bioresour. Technol.* **246**, 20–27 (2017)
13. Abd-Rahim, F., Wasoh, H., Zakaria, M.R., Ariff, A., Kapri, R., Ramli, N., Siew-Ling, L.: Production of high yield sugars from *Kappaphycus alvarezii* using combined methods of chemical and enzymatic hydrolysis. *Food Hydrocoll.* **42**(2), 309–315 (2014)
14. Meinita, M.D.N., Marhaeni, B., Winanto, T., Setyaningsih, D., Hong, Y.K.: Catalytic efficiency of sulfuric and hydrochloric acids for the hydrolysis of *Gelidium latifolium* (Gelidiales, Rhodophyta) in bioethanol production. *J. Ind. Eng. Chem.* **27**, 108–114 (2015)
15. Siqueira, M.R., Reginatto, V.: Inhibition of fermentative H₂ production by hydrolysis byproducts of lignocellulosic substrates. *Renew. Energy* **80**, 109–116 (2015)
16. Fonseca, B.C., Riaño-Pachón, D.M., Guazzaroni, M.E., Reginatto, V.: Genome sequence of the H₂-producing *Clostridium beijerinckii* strain Br21 isolated from a sugarcane vinasse treatment plant. *Genet. Mol. Bio.* (2018) (in print)
17. Wychen, S.V., Laurens, L.M.L.: Determination of Total Solids and Ash in Algal Biomass: Laboratory Analytical Procedure (LAP). National Renewable Energy Laboratory, Golden (2013)
18. Wychen, S.V., Laurens, L.M.L.: Determination of Total Carbohydrates in Algal Biomass: Laboratory Analytical Procedure (LAP). National Renewable Energy Laboratory, Golden (2013)
19. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D.: Laboratory Analytical Procedure (LAP). National Renewable Energy Laboratory, Golden (2008)
20. Hartree, E.F.: Determination of protein: a modification of the lowry method that gives a linear photometric response. *Anal. Biochem.* **48**(2), 422–427 (1972)
21. Hayashi, L., Paula, E.J., Chow, F.: Growth rate and carrageenan analyses in four strains of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales) farmed in the subtropical waters of São Paulo State, Brazil. *J. Appl. Phycol.* **19**(5), 393–399 (2007)
22. Fonseca, B.C., Guazzaroni, M.E., Reginatto, V.: Fermentative production of H₂ from different concentrations of galactose by the new isolate *Clostridium beijerinckii* Br21. *Int. J. Hydrog. Energy* **41**(46), 21109–21120 (2016)
23. Park, J.-H., Cheon, H.-C., Yoon, J.-J., Park, H.-D., Kim, S.-H.: Optimization of batch dilute-acid hydrolysis for biohydrogen production from red algal biomass. *Int. J. Hydrog. Energy* **38**, 66130–66136 (2013)
24. Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**(3), 426–428 (1959)
25. Carli, S., Meleiro, L.P., Rosa, J.C., Moraes, L.A.B., Jorge, J.A., Masui, D.C., Furriel, R.P.M.: A novel thermostable and halotolerant xylanase from *Colletotrichum graminicola*. *J. Mol. Catal. B Enzymatic* **133**, S508–S517 (2016)
26. Chen, W.H., Chen, S.Y., Khanal, S.K., Sung, S.: Kinetic study of biological hydrogen production by anaerobic fermentation. *Int. J. Hydrog. Energy* **31**(15), 2170–2178 (2006)
27. Souza, F.H.M., Nascimento, C.V., Rosa, J.C., Masui, D.C., Leone, F.A., Jorge, J.A., Furriel, R.P.M.: Purification and biochemical characterization of a mycelial glucose- and xylose-stimulated β-glucosidase from the thermophilic fungus *Humicola insolens*. *Process Biochem.* **45**(2), 272–278 (2010)
28. Masarin, F., Cedeno, F.R., Chavez, E.G., de Oliveira, L.E., Gelli, V.C., Monti, R.: Chemical analysis and biorefinery of red algae *Kappaphycus alvarezii* for efficient production of glucose from residue of carrageenan extraction process. *Biotechnol. Biofuels* **9**, 122 (2016)
29. Van de Velde, F., Knutsen, S.H., Usov, A.I., Rollema, H.S., A.S. Cerezo: ¹H and ¹³C high resolution NMR spectroscopy of carrageenans: application in research and industry. *Trends Food Sci. Technol.* **13**(3), 73–92 (2002)
30. Yun, E.J., Lee, S., Kim, H.T., Pelton, J.G., Kim, S., Ko, H.J., Choi, I.G., Kim, K.H.: The novel catabolic pathway of 3,6-anhydro-L-galactose, the main component of red macroalgae, in a marine bacterium. *Environ. Microbiol.* **17**(5), 1677–1688 (2015)
31. Meinita, M.D., Hong, Y.K., Jeong, G.T.: Comparison of sulfuric and hydrochloric acids as catalysts in hydrolysis of *Kappaphycus alvarezii* (cottonii). *Bioprocess. Biosyst. Eng.* **35**, 123–128 (2012)
32. Kumar, G., Cheon, H.C., Kim, S.H.: Effects of 5-hydroxymethylfurfural, levulinic acid and formic acid, pretreatment byproducts of biomass, on fermentative H₂ production from glucose and galactose. *Int. J. Hydrog. Energy* **39**(30), 16885–16890 (2014)
33. Singh, G., Verma, A.K., Kumar, V.: Catalytic properties, functional attributes and industrial applications of β-glucosidases. *3 Biotech* **6**(1) 3 (2016)
34. Bruder, M., Moo-Young, M., Chung, D.A., Chou, C.P.: Elimination of carbon catabolite repression in *Clostridium acetobutylicum*—a journey toward simultaneous use of xylose and glucose. *Appl. Microbiol. Biotechnol.* **99**, 7579–7588 (2015)
35. Jung, K.W., Kim, D.H., Kim, H.W., Shin, H.S.: Optimization of combined (acid + thermal) pretreatment for fermentative hydrogen production from *Laminaria japonica* using response surface methodology (RSM). *Int. J. Hydrog. Energy* **36**(16), 9626–9631 (2011)
36. Liu, H., Wang, G.: Fermentative hydrogen production from macro-algae *Laminaria japonica* using anaerobic mixed bacteria. *Int. J. Hydrog. Energy* **39**(17), 9012–9017 (2014)