AIMS Bioengineering, 3 (3): 400-411.
DOI: 10.3934/bioeng.2016.3.400
Received: 19 June 2016
Accepted: 21 August 2016
Published: 25 August 2016

http://www.aimspress.com/journal/Bioengineering

Research article

Optimization of enzymatic saccharification of *Chaetomorpha linum* biomass for the production of macroalgae-based third generation bioethanol

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Abstract: To evaluate the efficacy of marine macro-algae *Chaetomorpha linum* as a potential biofuel resource, the effects of the enzymatic treatment conditions on sugar yield were evaluated using a three factor three level Box-Behnken design. The hydrothermally pretreated *C. linum* biomass was treated with *Aspergillus niger* cellulase at various liquid to solid ratios (50–100 mL/g), enzyme concentrations (10–60 U/g) and incubations times (4–44 h). Data obtained from the response surface methodology were subjected to the analysis of variance and analyzed using a second order polynomial equation. The fitted model was found to be robust and was used to optimize the sugar yield (%) during enzymatic hydrolysis. The optimum saccharification conditions were: L/S ratio 100 mL/g; enzyme concentration 52 U/g; and time 44 h. Their application led to a maximum sugar yield of 30.2 g/100g dry matter. *Saccharomyces cerevisiae* fermentation of the algal hydrolysate provided 8.6 g ethanol/100g dry matter. These results showed a promising future of applying *C. linum* biomass as potential feedstock for third generation bioethanol production.

Keywords: enzyme technology; cellulase; bioconversion; modelling; optimization

1. Introduction

There is increasing interest in algae as one of the alternative renewable sources of biomass for production of bioethanol, which is considered as third-generation biofuel [1,2]. Unlike terrestrial crops cultivated for biofuel production, algae do not require agricultural land for cultivation and various species are able to grow in brackish or salt water avoiding competition for land and fresh water required for food production [2]. Furthermore, bioethanol from marine-based biomass holds significant potential because of their low percentage of lignin and hemicellulose in comparison with other lignocellulosic biomasses [3].

Recently, marine macroalgae, an abundant and carbon-neutral renewable resource, have gained considerable global attention as a third-generation biomass that can be used in bioenergy production [4,5]. Different macroalgae groups belonging to brown seaweed such as *Laminaria japonica*, *Undaria pinnatifida*, and *Sargassum horneri*, and red algal species such as *Gelidium amansii*, *Kappaphycus alvarezii*, and *Gracilaria salicornia* have been considered as potential sources for bioconversion to ethanol [5,6–10]. Trivedi et al. [11] used the green macroalgae, *Ulva fasciata* Delile as a bioethanol feedstock. The feasibility of producing bioethanol from the green macroalgae *Chaetomorpha linum* was also investigated by Schultz-Jensen et al. [12]. *C. linum* was chosen as target, since it contains more cellulose (35–40 g/100g dry matter, DM) than other algae. Furthermore, the cellulose content of *C. linum* is similar to that of land-based biomass. However, any future successes of macroalgal-derived biofuel will be dependent on achieving optimised, energy efficient processes in cultivation, harvesting, post-harvest treatments and fuel production [13,14].

Similar to other cellulosic biomasses, glucan from seaweed can be converted by enzymes into sugars suitable for ethanol fermentation. To convert glucan into the fermentable sugars, either acid hydrolysis or enzymatic hydrolysis needs to be performed. The limitations of acid hydrolysis can be by-products inhibition on yeast growth, neutralization before fermentation and expensive constructional material due to corrosion risks. Furthermore, high enzymes prices play a crucial role when feasibility is of concern. Enzyme saccharification is chosen even though high cost of biocatalysts because of high conversion yield of glucose [11,15].

Optimization of enzymatic hydrolysis process is one of the most important stages in the development of an efficient and cost effective saccharification strategy. The process efficiency depends on several parameters such as enzyme, substrate loading, pH, temperature and incubation time [11,15]. The optimal enzymatic process conditions vary also depending on the composition of carbohydrates between the green, brown and red algae [15]. Optimization of multifactorial system by conventional techniques is generally done with one-factor at a time. However, this type of methodology is time consuming and does not reveal the interactive effects between the factors [16]. Response Surface Methodology (RSM) is mainly used for statistical model building and location of maxima [17–20]. Statistical designs such as RSM have proved efficient for optimization of enzymatic hydrolysis and fermentation of macroalgae feedstocks [5,15,21].

The objectives of this research are performed according to Box-Behnken experimental design and response surface methodology (RSM) to understand the relationship between the critical factor involved in enzymatic degradation of *C. linum* biomass and to optimize the condition for saccharification prior to ethanol fermentation. All the results obtained in this work would provide a sound basis for assessing the valorization of *C. linum* biomass into third-generation biofuel.

2. Material and Methods

2.1. Harvest and preparation of macroalgae biomass

The green seaweed *C. linum* was harvested in February 2014 from the coastal region of Monastir, Tunisia. The Seaweed samples were washed thoroughly with tap water to remove salts, epiphytes and debris and dried to a constant weight at temperature of 50 °C for 48 h. After drying, the seaweed samples were powdered using grinder for chemical characterization and enzymatic cellulose hydrolysis.

2.2. Chemical characterization of C. linum biomass

The seaweed samples were analysed for the total protein, carbohydrates, lipid, ash and crude fibre (dietary fibre) contents. Moisture and ash contents were determined according to the A.O.A.C. method [22] by drying the macroalgae at 105 °C for 24 h followed by incineration at 550 °C for 12 h. Total carbohydrates content was measured using the phenol-sulfuric acid method of Dubois et al. [23]. Crude protein determination involved the use of routine Kjeldhal nitrogen assay (N × 5) [24]. Total fiber content was analyzed according to the AOAC enzymatic-gravimetric method of Prosky et al. [25]. Lipid content was determined by exhaustively extracting samples in a Soxhlet apparatus as described by Chirapart et al. [26]. All the analyses were done in triplicates with results expressed with standard deviations (n = 3) against dry weight.

2.3. Enzymatic hydrolysis of C. linum biomass

Commercial *Aspergillus niger* cellulase (0.8 enzyme Units/mg solid, Sigma C1184-25KU, Sigma-Aldrich, USA; 1 U corresponds to the amount of enzymes which liberates 1 μmol glucose from carboxymethylcellulose per minute at pH 5.0 and 37 °C) was employed for *C. linum* cellulose hydrolysis. Dried algal powder (1 g) moistened with different volume of sodium acetate buffer (pH 5.0) was pretreated by autoclaving at 121 °C and 1.5 bars for 20 min, cooled and then hydrolyzed with different concentration of cellulase and incubated for different time intervals on an orbital shaker with a speed of 100 rpm at 37 °C. Each reaction system was fortified with 1% sodium azide to prevent contamination. Control of each reaction mixture was performed by replacing the active crude enzymes with heat inactivated (100 °C, 10 min) enzymes. Samples were taken out periodically and centrifuged. The reducing sugar of each reaction mixture was measured spectrophotometrically using 3,5-dinitrosalisylic acid (DNS) method [27]. Sugar yield was calculated on *C. linum* biomass, using the following equation [28]: Sugar Yield (%) = 100 (sugar produced during hydrolysis/gram of *C. linum* biomass).

2.4. Statistical optimization of enzymatic saccharification by RSM

The optimization of enzymatic hydrolysis of biomass was carried out for enzyme concentration, incubation period and liquid to solid ratio following the Box-Behnken experimental design given by tables 1 and 2. The Box-Behnken experimental design was set up to look for the best experimental conditions of three independent factors affecting the efficiency of the saccharification.

Table 1. Experimental domain of the Box–Behnken design.

Variable	Factor	Unit	Low level	High level
X_1	L/S ratio	mL/g	50.0	100.0
X_2	Enzyme conc.	U/g	10.0	60.0
X_3	Time	h	4.0	44.0

Table 2. Experimental conditions of the Box-Behnken design in coded and natural variables and the corresponding experimental and predicted responses.

N°Exp	X1	X2	X3	L/S (ml/g)	Enzyme (U/g)	Time (h)	Measured sugar yiel (%)	Estimated sugar yield (%)
1	–1.0	–1.0	0.0	50.0	10.0	24.0	6.91	5.63
2	1.0	–1.0	0.0	100.0	10.0	24.0	6.07	4.47
3	–1.0	1.0	0.0	50.0	60.0	24.0	8.53	10.13
4	1.0	1.0	0.0	100.0	60.0	24.0	11.65	12.93
5	–1.0	0.0	–1.0	50.0	35.0	4.0	9.51	8.74
6	1.0	0.0	–1.0	100.0	35.0	4.0	4.51	4.06
7	–1.0	0.0	1.0	50.0	35.0	44.0	20.01	20.46
8	1.0	0.0	1.0	100.0	35.0	44.0	26.00	26.77
9	0.0	–1.0	–1.0	75.0	10.0	4.0	2.25	4.30
10	0.0	1.0	–1.0	75.0	60.0	4.0	8.85	8.02
11	0.0	–1.0	1.0	75.0	10.0	44.0	17.92	18.75
12	0.0	1.0	1.0	75.0	60.0	44.0	30.04	27.99
13	0.0	0.0	0.0	75.0	35.0	24.0	11.26	10.21
14	0.0	0.0	0.0	75.0	35.0	24.0	8.83	10.21
15	0.0	0.0	0.0	75.0	35.0	24.0	9.61	10.21
16	0.0	0.0	0.0	75.0	35.0	24.0	13.06	10.21
17	0.0	0.0	0.0	75.0	35.0	24.0	8.27	10.21

The sugar yield response can be described by the following second-order model adequate for predicting the responses in the experimental region:

$$\eta = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X^2 X^3 \quad (1)$$

where, η: the theoretical response function, X_j: coded variables of the system, β_0, β_j , β_{jk}, and β_{jj}: true model coefficients.

The observed response y_i for the ith experiment is

$$y_i = \eta_i + e_i \ (e_i : \text{error}) \quad (2)$$

The model coefficients β_0, β_j, . . ., and β_{jj} are estimated by a least squares fitting of the model to the experimental results obtained in the 12 design points of the three-variable Box-Behnken design (Table 2). For the estimated values of these coefficients, the symbols b_0, bj , . . . , and bjj will be used.

The computed values of the responses are designated by $\hat{y}_i$

$$\hat{y} = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 \quad (3)$$

The four replicates at the center point (runs n 13 to 17) are carried out in order to estimate the pure error variance. The fitted polynomial model was then expressed as two and three dimensional surface plots to illustrate the relationship between the responses and interaction effects of the variables and to look for the optimal experimental conditions [29–30]. Statistical analysis of the data and surface plots were performed using the experimental design software NemrodW [31].

2.5. Fermentation of algal hydrolysate

To prepare a starter, the baker's yeast *Saccharomyces cerevisiae* was cultured in 250 mL cap flasks containing 100 mL Yeast extract Peptone (YP) medium and incubated in an orbital shaker at 150 rpm for 24 h at 30 °C. Ethanol production was studied in triplicates using optimized enzymatic hydrolysate of *C. linum*. The saccharification broth was sterilized by filtration (0.2 µm filter membrane) and added to YP medium with the proportion (9/1). The prepared medium was inoculated with 10% v/v of starter culture of *S. cerevisiae* and incubated at 30 °C with a shaking speed of 150 rpm [32]. After 48 h of incubation, the fermentation broth was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was then analyzed for ethanol content using ethanol FS kit marketed by Diagnostic System International [33]. Ethanol yield was calculated as total amount of g ethanol obtained per 100 g DM of *C. linum* before pretreatment.

3. Results and Discussion

The chemical composition of *C. linum* was summarized in Table 3. It has a similar trend to that reported by Ben Yahmed et al. [32]. Generally, *C. linum* biomass is characterized by relatively high content of carbohydrates and fibers compared to other macroalgae such as *G. salicornia* [34] and *S. japonica* [35], which made it a good source for bioenergy production. Previous investigations of the detailed chemical composition of *C. linum* also showed high content in polysaccharides (20–40 g cellulose/100g DM) [12,32]. Schultz-Jensen et al. [12] reported that *C. linum* consisted of 34–38 g glucan, 9–10 g arabinan, 6 g xylan, 14 g pectin, 7 g non hydrolysable organic components, 21–23 g ash, and 6 g wax per 100 g DM. Other seaweeds used for biofuel production e.g. *Ulva lactuca* and *Gracilaria longissima* contained less glucan (6 g and 20 g/100g DM, respectively) [12, 36].

In this study, an attempt was made to optimize enzymatic hydrolysis conditions of hydrothermally pretreated *C. linum* biomass using a three levels three factors Box-Behnken design. This experimental design reduced the number of experiments in comparison to others, so it is more efficient and easier to arrange and to interpret [37]. Furthermore, it has the advantage that it does not contain combinations for which all process variabes are simultaneously at their highest or lowest levels, thereby, it is useful in avoiding experiments performed under extreme experimental conditions, for which unsatisfactory results might occur [38].

The experimental saccharification conditions of pretreated *C. linum* biomass were shown in Table 2. The observed values of sugar yields were used to compute the model coefficients using the least square method [16]. The final empirical model describing the relationship between the variables and the sugar yield from enzymatic hydrolysis of pretreated *C. linum* biomass in terms of coded values can be written as follows:

$$\hat{y} = 10.21 + 0.41\,X_1 + 3.24\,X_2 + 8.61\,X_3 - 0.84\,X_1^2 - 1.08\,X_2^2 + 5.64\,X_3^2 + 0.99\,X_1X_2 + 2.75\,X_1X_3 + 1.38\,X_2X_3 \quad (4)$$

Where $\hat{y}$: the sugar yield (%); X_1, X_2 and X_3: the coded values of test variables L/S ratio, enzyme concentration and hydrolysis time, respectively. X_1X_2, X_1X_3 and X_2X_3: the interaction effects of L/S ratio and enzyme concentration, L/S ratio and hydrolysis time, enzyme concentration and hydrolysis time, respectively.

Table 3. Proximate chemical composition (mean ± SD, n = 3) of *C. linum* biomass. Ash, carbohydrates, protein, lipid, and crude fiber are relative to total dry weight.

Components	Contents (%)
Moisture	13.96 ± 1.32
Ash	14.83 ± 0.36
Protein	10.56 ± 0.22
Lipid	1.89 ± 0.04
Carbohydrates	42.45 ± 2.94
Crude fiber	29.76 ± 1.54

The quality of the fitted model for enzyme hydrolysis of *C. linum* biomass was evaluated based on the correlation coefficient R^2. The R^2 for the obtained equation was found to be 0.961. This indicated that 96.1% of total variation in enzyme saccharification was attributed to the experimental factors studied. A regression model with $R^2 > 0.90$ is considered to have a very high correlation [16] and then the R^2 of 0.961 was considered as a good fit of the model. The value of the adjusted determination coefficient was also high (adjusted $R^2 = 0.910$), supporting for high significance of this model.

The adequacy of the model was further justified through analysis of variance (ANOVA) of the quadratic model for the sugar yield reponse (Table 4). The Fisher's test (F-test) carried out on experimental data make it possible to estimate the statistical significance of the proposed model [16]. The high F-test value of the model with *p*-value less than 0.01 and non-significant lack of fit suggested that the model was adequate to predict the percentage of sugar yield during enzyme hydrolysis within the range of the studied three factors.

The parameter estimates and the corresponding *P*-values suggest that, among the independent variables, X_2 (enzyme concentration) and X_3 (hydrolysis time) have significant effects on sugar yield (%) and maximum effect was shown by hydrolysis time (*p*-value less than 0.001), followed by enzyme concentration (*p*-value less than 0.01). The quadratic terms of X_3 and interactions between X_1 and X_3 also have significant effects on the sugar yield (Table 5).

To examine the interaction of the variables and to determine the optimum level of each variable for maximum response, three-dimensional response surface plots and two dimensional isoresponse curves were plotted against two experimental factors while maintaining the other factor constant at its central value (Figure 1).

Table 4. ANOVA for response surface quadratic model for enzymatic saccharification of *C. linum* biomass.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio	*P*-value (Significance)
Regression	857.4700	9	95.2744	19.0471	< .0001(***)
Residuals	35.0143	7	5.0020		
Lack of fit	19.7614	3	6.5871	1.7274	29.9% (NS)
Error	15.2529	4	3.8132		
Total	892.4843	16			

***Significant at the level 99.9% ; N.S.: non significant.

Table 5. Results of regression analysis of enzymatic saccharification of *C. linum* biomass.

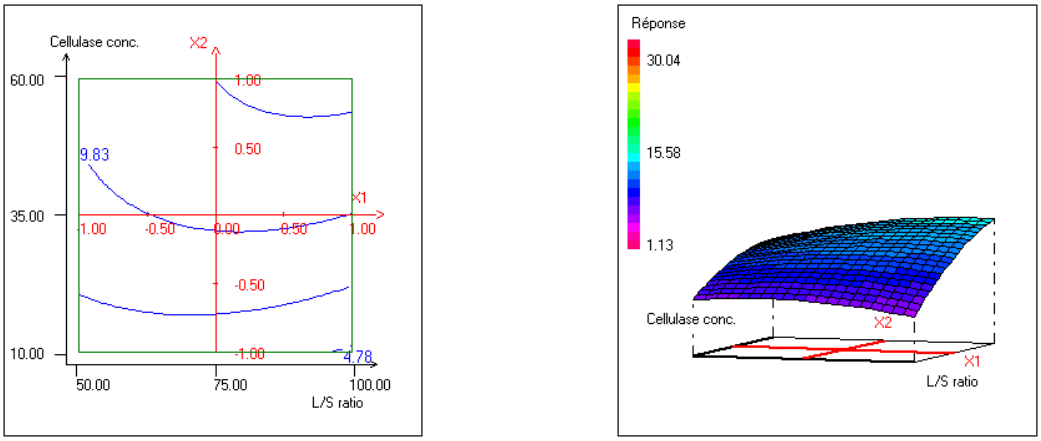
Source	Coefficient	Ecart-Type	t.exp.	Significance %
b0	10.206	1.000	10.20	*
b1	0.409	0.791	0.52	62.5% (N.S.)
b2	3.240	0.791	4.10	*
b3	8.606	0.791	10.88	*
b11	–0.837	1.090	–0.77	47.3% (N.S.)
b22	–1.079	1.090	–0.99	35.7% (N.S.)
b33	5.638	1.090	5.17	*
b12	0.990	1.118	0.89	40.9% (N.S.)
b13	2.747	1.118	2.46	*
b23	1.380	1.118	1.23	25.7% (N.S.)

*Significant at the confidence level 95.0%; NS not significant at the confidence level 95.0%.

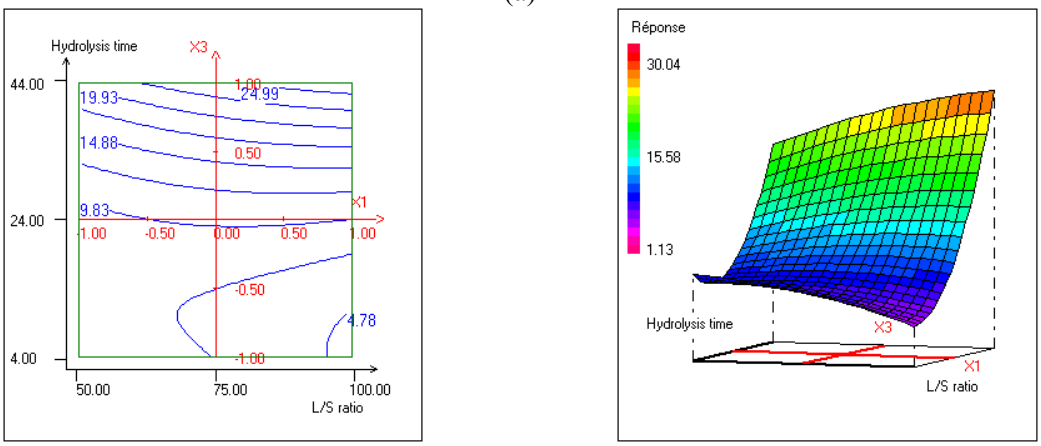
The effects of L/S ratio and enzyme concentration on the sugar yield (%), when the hydrolysis time was at its center point, are shown in Figure 1a. The reducing sugar yields were estimated to increase linearly with increase in enzyme concentration from 10 to 60 U/g and ranged between 5 and 12%. When enzyme concentration was at low levels, non significant improvement in the sugar yield was observed by increasing the L/S ratio. The reducing sugar yield was maximized at high levels of enzyme concentration and medium to high levels of L/S ratio.

Figure 1b shows the effects of L/S ratio and hydrolysis time on sugar yield (%), when the enzyme concentration was at its center level. A positive interaction between L/S ratio and hydrolysis time was observed. Indeed, it was observed that at middle to high level of L/S ratio (80–100 mL/g) and high level of hydrolysis time (> 40 h) the reducing sugar yield was high (≈ 25%).

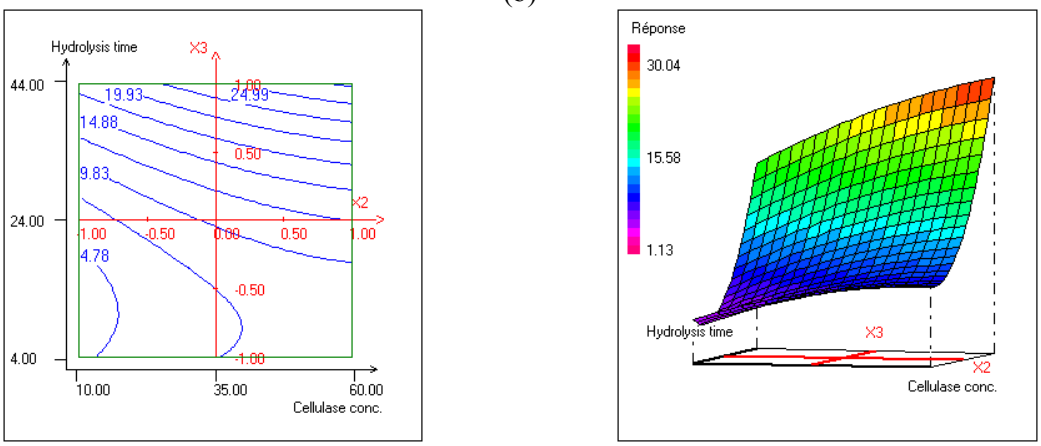
The effects of enzyme concentration and hydrolysis time on the sugar yield (%), when the time was at its center level, are shown in Figure 1c. This figure revealed the low yields of reducing sugar at low levels of enzyme concentration and low levels of incubation time. Reducing sugar yield was found to be consistently increased with raise in hydrolysis time and enzyme concentration. It was also found that the effect of reaction time was more significant than that of the enzyme concentration. High reducing sugar yield (25%) was observed with high levels of enzyme concentration (40–60 U/g) and high level of incubation time (44 h).



(a)



(b)



(c)

Figure 1. Contour plots and three-dimensional response surface and showing interactive effects of (a) L/S ratio and enzyme concentration, (b) L/S ratio and hydrolysis time and (c) enzyme concentration and hydrolysis time on the enzymatic saccharification yield of *C. linum* biomass.

As the sugar yield can be maximized especially with a long treatment duration (Figures 1b and c), we fixed the incubation time at its high level (44.0 h), and we ploted enzyme concentration versus

redox L/S ratio (Figure 2) to look for the highest sugar yield. Response analysis using NemrodW software predicted the maximum sugar yield of 30 ± 3% during enzyme hydrolysis of *C. linum* biomass under the optimum process conditions i.e. when L/S ratio was 100 mL/g, hydrolysis time was 44 h and enzyme concentration was 52 U/g. To validate the predicted sugar yield, an experiment was conducted with the above mentioned optimum conditions of each variable as developed by the model. The optimal experimental sugar yield response for pretreated *C. linum* biomass was 29.6 g sugars/100g DM and it was in good agreement with predicated value.

The conversion efficiency of polymeric carbohydrates from *C. linum* into simpler sugars by *Aspergillus niger* enzymes under optimal conditions was 71%. It was about 18–fold higher than the yield obtained before optimization (hydrothermally pretreated *C. linum* biomass). Our results revealed the superiority of *C. linum* biomass for achieving high sugar yield under enzymatic optimized conditions in comparison to other green seaweed biomasses. For examples, pre-heat treatment of biomass from the green seaweed *Ulva fasciata* Delile in aqueous medium at 120 °C for 1 h followed by incubation in 2% (v/v) enzyme (cellulase 22119) for 36 h at 45 °C gave a maximum yield of 20.6 g sugars/100g DM [11]. The green seaweed *Monostroma nitidum*, has also been used as the raw material for the production of reducing sugars [38]. Glucan in this seaweed was hydrolyzed using a commercial enzyme (Cellulosin T2) at 37 °C for 48 h. Although the conversion rate of glucans reached 79.9%, as a result of applying hydrothermal pretreatment at 100 °C for 30 min prior to enzymatic saccharification, the yield of glucose was still only 11 g glucose/100g biomass [39].

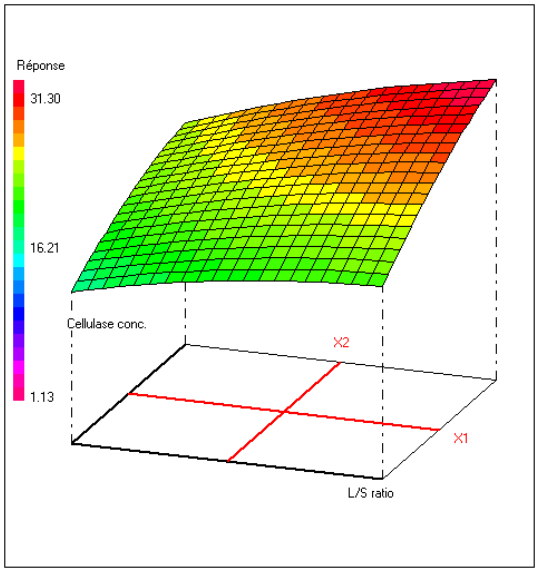


Figure 2. Contour plots for the effect of L/S ratio and cellulase concentration at constant incubation time (44 h) on the enzymatic hydrolysis yield.

The ethanol yield after fermentation of optimized enzymatic hydrolysate from *C. linum* with *S. cerevisiae* reached an average of 8.6 g ethanol/100g DM. This yield was similar to those reported in other studies [12,32]. Indeed, the hydrolysis of *C. linum* feedstock with a crude enzyme preparation, produced from *Aspergillus awamori*, at 45 °C, pH 5 for 30 h gave the maximum yield of fermentable sugar of 22 g/100g DM. An ethanol yield of about 9.3 g/100g pretreated algae was obtained after alcoholic fermentation by *S. cerevisiae* [32]. The ethanol yields produced from

C. linum pretreated with hydrothermal treatment, wet oxidation, steam explosion, plasma assisted pretreatment and ball milling were varied between 11 to 18 g ethanol/100g DM [12]. The seasonal variability and environmental constraints which affect the chemical composition of *C. linum* as well as the type of saccharification treatments were the most significant factors affecting the ethanol yield from *C. linum* biomass [12,32].

4. Conclusion

In the present study, a three factors three levels Box-Behnken design and the response surface methodology provide the development of a polynomial model for optimization of enzymatic saccharification of *Chaetomorpha linum* biomass for the production of macroalgae-based third generation bioethanol. Response surface plot in three-dimension obtained from the empirical quadratic model can show the interaction effect of two variables on the studied response and the optimum values of the selected variables are obtained from response surface plot, too. Based on the statistical optimization, optimal sugar and ethanol yields were 30.2 g sugars/100g DM and 8.6 g ethanol/100g DM, respectively. These results will provide basic information applicable to further studies on the use of the green seaweed *C. linum* as a renewable feedstock for bioenergy production.

Acknowledgments

The authors thank the South African/Tunisia-research partnership programme bilateral agreement 2014 and the Tunisian Ministry of Higher Education and Scientific Research for financial support.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Demirbas MF (2011) Biofuels from Algae for sustainable Development. *Appl Energ* 88: 3473–3480.
2. Milledge JJ, Smith B, Dyer PW, et al. (2014) Macroalgae-Derived Biofuel: A Review of Methods of Energy Extraction from Seaweed Biomass. *Energies* 7: 7194–7222.
3. Tsuji A, Tominaga K, Nishiyama N, et al. (2013) Comprehensive Enzymatic Analysis of the Cellulolytic System in Digestive Fluid of the Sea Hare *Aplysia kurodai*. Efficient Glucose Release from Sea Lettuce by Synergistic Action of 45 kDa Endoglucanase and 210 kDa ß-Glucosidase. *PLoS ONE* 8: e65418.
4. Meinita MDN, Kang JY, Jeong GT, et al. (2012) Bioethanol production from the acid hydrolysate of the carrageenophyte *Kappaphycus alvarezii* (cottonii). *J App Phy* 24: 857–862.
5. Borines MG, Leon RL, Cuello JL (2013) Bioethanol production from the macroalgae *Sargassum spp*. *Bioresource Technol* 138: 22–29.
6. Khambhaty Y, Mody K, Gandhi MR, et al. (2012) *Kappaphycus alvarezii* as a source of bioethanol. *Bioresource Technol* 103: 180–185.

7. Kim HM, Wi SG, Jung S, et al. (2015) Efficient approach for bioethanol production from red seaweed *Gelidium amansii*. *Bioresource Technol* 175: 128–134.
8. Cho YK, Kim H, Kim SK (2013) Bioethanol production from brown seaweed, *Undaria pinnatifida*, using NaCl acclimated yeast. *Bioproc Biosyst Eng* 36: 713–719.
9. Lee JY, Kim YS, Um BH, et al. (2013) Pretreatment of *Laminaria japonica* for bioethanol production with extremely low acid concentration. *Renew Energ* 54: 196–200.
10. Li K, Liu S, Liu X (2014) An overview of algae bioethanol production. *Int J Energ Res* 38: 965–977.
11. Trivedi N, Gupta V, Reddy CRK, et al. (2013) Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga *Ulva fasciata* Delile. *Bioresource Technol* 150: 106–112.
12. Schultz-Jensen N, Thygesen A, Leipold F, et al. (2013) Pretreatment of the macroalgae *Chaetomorpha linum* for the production of bioethanol-Comparison of five pretreatment technologies. *Bioresource Technol* 140: 36–42.
13. Milledge JJ, Harvey PJ (2016) Potential process 'hurdles' in the use of macroalgae as feedstock for biofuel production in the British Isles. *J Chem Technol Biot* 91:2221–2234.
14. Yanagisawa M, Kawai S, Murata K (2013) Strategies for the production of high concentrations of bioethanol from seaweeds. *Bioengineered* 4: 224–235.
15. Jang JS, Cho YK, Jeong GT, et al. (2012) Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed *Saccharina japonica*. *Bioproc Biosyst Eng* 35: 11–18.
16. Myers RH, Montgomery DC, Anderson-Cook CM (2009) Response Surface Methodology: Process and Product Optimization Using Designed Experiments, Wiley, New York, USA.
17. Myers RH, Montgomery DC (1995) Response Surface Methodology: Process and Product Optimization Using Designed Experiments, Wiley, New York, USA.
18. Mathieu, Phan-Tan-Luu R (1997) Approche méthodologique des surfaces de réponse. In *Plans d'expériences: Application à l'entreprise*, Technip, Paris, France, 211–278.
19. Goupy J (1999) Plans d'Expériences Pour Surfaces de Réponse, Dunod, Paris, France.
20. Tye YY, Lee KT, Abdullah WNW, et al. (2015) Effects of process parameters of various pretreatments on enzymatic hydrolysability of *Ceiba pentandra* (L.) Gaertn. (Kapok) fibre: A response surface methodology study. *Biomass Bioenerg* 75: 301–313.
21. Ra CH, Kim SK (2013) Optimization of pretreatment conditions and use of a two-stage fermentation process for the production of ethanol from seaweed, *Saccharina japonica*. *Biotech Bioproc Eng* 18: 715–720.
22. AOAC International (2005) Official methods of analysis of AOAC International, 18th edn., Revision I, Gaithersburg, MD.
23. Dubois M, Gilles KA, Hamilton JK, et al. (1956) Colorimetric Method for Determination of Sugars and Related Substances. *Anal Chem* 28: 350–356.
24. Angell AR, Mata L, Nys RD, et al. (2016) The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. *J App Phycol* 28: 511–524.
25. Prosky L, Asp NG, Schweizer TF, et al. (1988) Determination of insoluble, soluble, and total dietary fiber in foods and food products: interlaboratory study. *J Assoc Anal Chem* 71: 1017–1023.

26. Chirapart A, Praiboon J, Puangsombat P, et al. (2014) Chemical composition and ethanol production potential of Thai seaweed species. *J Appl Phycol* 26: 979–986.
27. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem* 31: 426–428.
28. Idrees M, Adnan A, Sheikh S, et al. (2013) Optimization of dilute acid pretreatment of water Hyacinth biomass for enzymatic hydrolysis and ethanol production. *J Excli* 12: 30–40.
29. Neifar M, Jaouani A, Kamoun A, et al. (2011) Decolorization of Solophenyl red 3BL polyazo dye by laccase-mediator system: Optimization through response surface methodology. *Enzyme Res* doi:10.4061/2011/179050.
30. Neifar M, Kamoun A, Jaouani A, et al. (2011) Application of Asymetrical and Hoke designs for optimization of laccase production by the white-rot fungus *Fomes fomentarius* in solid-state fermentation. *Enzyme Res* doi:10.4061/2011/368525.
31. Mathieu D, Nony J, Phan-Tan-Luu R (2000) NEMROD-W Software, LPRAI, Marseille, France.
32. Yahmed NB, Jmel MA, Alaya MB, et al. (2016) A biorefinery concept using the green macroalgae *Chaetomorpha linum* for the coproduction of bioethanol and biogas. *Ener Convers Manage* 119: 257–265.
33. Neifar M, Chouchane H, Maktouf S, et al. (2016) Improved sugar yield for bioethanol production by modelling enzymatic hydrolysis of *Peganum Harmala* biomass through response surface methodology. *Ijes* 5: 22–28.
34. Wang X, Liu X, Wang G (2011) Two-stage hydrolysis of invasive algal feedstock for ethanol fermentation. *J Integr Plant Biol* 53: 246–252.
35. Lee JY, Li P, Lee J, et al. (2013) Ethanol production from *Saccharina japonica* using an extremely low acid pretreatment followed by simultaneous saccharification and fermentation. *Bioresource Technol* 127: 119–125.
36. Coppalo F, Simoncini E, Pulselli RM (2009) Bioethanol potentials from marine residual biomass: an energy evaluation, In: Brebbia, Tiezzi, Eds., *Fifth International Conference on Ecosystems and Sustainable Development*, Italy.
37. Majumder A, Singh A, Goyal A (2009) Application of response surface methodology for glucan production from *Leuconostoc dextranicum* and its structural characterization. *Carbohyd Polym* 75: 150–156.
38. Ferreira SLC, Bruns RE, Ferreira HS, et al. (2007) Box-Behnken design: An alternative for the optimization of analytical methods. *Anal Chim Acta* 597: 179–186.
39. Okuda K, Oka K, Onda A, et al. (2008) Hydrothermal fractional pretreatment of sea algae and its enhanced enzymatic hydrolysis. *Chem J Technol Biotechnol* 83: 836–41.

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