Scientific Research Publishing

Advances in Microbiology, 2018, 8, 378-389
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Fermentative Bioethanol Production Using Enzymatically Hydrolysed *Saccharina latissima*

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How to cite this paper: Lamb, J.J., Sarker, S., Hjelme, D.R. and Lien, K.M. (2018) Fermentative Bioethanol Production Using Enzymatically Hydrolysed *Saccharina latissima*. *Advances in Microbiology*, **8**, 378-389.
https://doi.org/10.4236/aim.2018.85025

Received: March 5, 2018
Accepted: May 27, 2018
Published: May 30, 2018

Abstract

The increased demand for machinery and transport has led to an overwhelming increase in the use of fossil fuels in the last century. Concerning the economic and environmental concern, macroalgae with high fermentable polysaccharide content (mainly mannitol, cellulose and laminarin), can serve as an excellent alternative to food crops for bioethanol production, a renewable liquid fuel. In this study, *Saccharina latissima*, a brown macroalgae readily available on the Norwegian coast was used as the carbohydrate source for the fermentative production of bioethanol. The macroalgae harvested was found to contain 31.31 ± 1.73 g of reducing sugars per 100 g of dry *Saccharina latissima* upon enzymatic hydrolysis. The subsequent fermentation with *Saccharomyces cerevisiae* produced an ethanol yield of 0.42 g of ethanol per g of reducing sugar, resulting in a fermentation efficiency of 84% as compared to the theoretical maximum. Using these results, an evaluation of the fermentation process has demonstrated that the brown macroalgae *Saccharina latissima* could become a viable bioethanol source in the future.

Keywords

Macroalgae, *Saccharina latissimi*, Fermentation, Hydrolysis, Bioethanol

1. Introduction

Recent increased environmental concerns globally have resulted in an increased interest in developing economically viable methods for producing alternative renewable fuels for transportation. Biofuels like biodiesel, bioethanol, and biogas are considered to be promising fuel sources due to their sustainability, adaptability and low environmental impact [1] [2] [3] [4], and can be used in conventional internal combustion engines when blended with fossil fuels [5]. Bioetha-

nol is one such renewable example that has already gained acceptance. Currently, the majority of bioethanol is produced using the first and second-generation substrates. These substrates require a land area that competes with current food crops [6] [7].

With the excessive use of pesticides and fertilizer in such terrestrial substrate production, concerns for the environment are rising. The production of ethanol from such substrates also has many hurdles such as high cost of production, structural characteristics, geographic latitude and limited yield [10].

An alternative to terrestrial substrates is the use of third generation marine substrates like seaweeds (photosynthetic organisms) for bioethanol production [11] [12]. Seaweeds can be considered the ocean's version of terrestrial plants, as they are also composed of rigid polysaccharide-based structures, and collect vast quantities of polysaccharides, which many upon hydrolysis can be fermented to produce ethanol [13]-[18]. Macroalgae use as a bioethanol substrate has several advantages over terrestrial plants, where they have significantly larger area productivity (**Table 1**), do not compete with conventional food-based agriculture, do not require irrigation, recycle ocean bicarbonate, and are compatible with existing production streams and biorefineries [19] [20] [21]. Despite this, saccharification of biomass into fermentable sugars for bioethanol production still remains to be one of the main challenges [22].

In Nordic countries, where significant levels of terrestrial agriculture are not possible due to the winter climate, macroalgae offers a feasible alternative. For example, Norway has an extensive coastline of relatively warm water (considering the latitude) due to the Gulf stream, providing perfect growing conditions for the largely abundant carbohydrate-rich (laminarin, mannitol and alginate) sugar kelp *Saccharina latissima* [23] [24]. Laminarin and mannitol serve as storage carbohydrates in *S. latissima* that accumulate in the summer, while alginate is a structural carbohydrate. Laminarin and mannitol are substrates that can be fermented to produce ethanol by many various microbes [25] [26]. This is not the case for alginate, which is challenging without the use of specific genetically modified organisms [27] [28].

In this study, *S. latissima* from Trondheimsfjord, Norway, was used for the production of bioethanol from their glucose, laminarin and mannitol (fermentable)

Table 1. Major bioethanol crops and macroalgae comparison.

Substrate	Average yield (kg/ha/year)	Dry weight of hydrolysable carbohydrates (kg/ha/year)
Wheat (grain)	2800	1560
Maize (kernal)	4815	3100
Sugar beet	47,070	8825
Sugar cane	68,260	11,600
Macroalgae [8]	75,000	4500

Data was obtained from [9], unless otherwise stated.

carbohydrates. Additionally, a straight-forward carbohydrate extraction method was used to lower potential process costs. An evaluation of the process was developed to demonstrate that the brown macroalgae *Saccharina latissima* could become an economically viable bioethanol source in Nordic countries.

2. Materials and Methods

2.1. Macroalgae Collection

S. latissimi, an abundant macroalgae found in large numbers along the Norwegian west coast, was collected from Trondheimsfjord (N 63˚26'56", E 10˚10'48") near Trondheim, Norway in August of 2017. The macroalgae were subsequently washed using tap water to remove particulates from the surface. *S. latissima* was then milled using a tabletop blender with 10 mL of deionized water per 1 kg of macroalgae to produce a dense macroalgae pulp. The pulp was dried for 48 h at 30˚C and then stored in airtight plastic bags in a dry location for further use.

2.2. Enzymatic Hydrolysis

Commercial *β*-glucanase (G4423) from *Trichoderma longibrachiatum* (Sigma Aldrich, Germany) was used during enzymatic hydrolysis. This was an enzymatic mixture of *β*-1-3/1-4-glucanase, xylanase, cellulase, *β*-glucosidase, *β*-xylosidase, *α*-L-arabinofuranosidase and amylase activities. The macroalgae pulp (10% w/v) was suspended in 0.15 M sodium carbonate (Na_2CO_3) solution to a volume of 25 L at a starting pH of 9.0 in a stirred tank (CE640, Gunt, Germany) for 2 h at 50˚C. After 2 h, the pH was adjusted to 6 using HCl acid solution. Then 5 mg of enzyme mix per g of macroalgae dry weight was added to the solution, and left in the stirred tank for 48 h. Samples were taken every 12 h and analyzed for glucose concentration using a hexokinase glucose assay kit (GAHK20-1KT, Sigma, Germany).

2.3. Carbohydrate Characterization

Total carbohydrates, reducing sugar and glucose content were both determined by acidic treatment of pre-hydrolysis dry biomass. Biomass (0.5 g) was treated with 5 mL of 72% (v/v) H_2SO_4 at room temperature for 30 minutes with constant stirring via a magnetic stirrer. The sample was then diluted to a volume of 50 mL with deionized water, then autoclaved at 121˚C for 30 minutes. Once cooled, NaOH was added to the sample to reach a pH of 7.5. The total carbohydrates in the sample were then determined by using a phenol-sulfuric acid method [29]. Reducing sugars in the sample were determined using a dinitrosalicylic acid method [30]. Glucose content of the hydrolysate was determined using a hexokinase glucose assay kit (GAHK20-1KT, Sigma, Germany).

2.4. Ethanol Characterization

Ethanol concentration was determined using spectrophotometric measurements at 267 nm in a potassium dichromate and perchloric acid solution [31].

2.5. Macroalgae Fermentation

Saccharomyces cerevisiae was chosen for the fermentation experiments as it is a well understood fermentative organism. The organism was supplied with the fermentation equipment (Gunt, Germany). The 25 L of hydrolysate was used in the fermentation tank as the substrate (CE640, Gunt, Germany). 1 g of yeast per L of hydrolysate was added, along with 0.3% (w/v) of yeast extract and 1% (w/v) peptone and adjusted to a pH of 6.8 to support yeast growth. Fermentation was performed at 30˚C for 48 hours. The reducing sugar and ethanol content of the fermented hydrolysate was measured at 12 h intervals throughout the fermentation. Samples were centrifuged at 10,000 × g for 15 minutes at 4˚C, and the supernatant removed for analysis. Reducing sugars in the supernatant were determined using a dinitrosalicylic acid method [30].

2.6. Statistical Data Analysis

All experiments within this study were conducted in triplicate with the results displayed as mean values ± the standard deviation.

3. Results

The sugar kelp *S. latissima* (Figure 1) is naturally occurring and fast-growing macroalgae that can be found on the extensive coastlines of Nordic countries like Norway, which contains large amounts of carbohydrates and proteins. With the demand for biofuels increasing in recent years, it seems imperative that enzymatically treated macroalgae be identified as a potential source of bioethanol to achieve a biorefinery approach.

3.1. Carbohydrate Yield of *S. latissima*

The total carbohydrate, reducing sugar and post-hydrolysis glucose yield was determined using samples collected in August of 2017 (Table 2). Before enzymatic hydrolysis, the total carbohydrates were 58% ± 2.6% of the dry weight of *S.*

Figure 1. *Saccharina latissima*. Image of *Saccharina latissima* obtained from Trondheimsfjord (N 63˚26'56", E 10˚10'48") near Trondheim, Norway in August of 2017.

Table 2. Carbohydrate composition of *S. latissima.*

Carbohydrate group	Relative percentage of dry weight
Total carbohydrates	58 ± 2.6
Total reducing sugars	37 ± 1.1
Glucose (pre-hydrolysis	11 ± 1.2

latissima. The reducing sugar content was 37% ± 1.1% of the dry weight of *S. latissima*, whereas content was 11% ± 1.2% of the dry weight of *S. latissima.* This is within the expected range of carbohydrate composition based on similar research of *S. latissima* [32], including recent research undertaken using *S. latissimi* from Trondheimsfjorden [33] [34].

3.2. Enzymatic Hydrolysis of *S. latissima*

The enzymatic hydrolysis of *S. latissima* was undertaken to hydrolyze the storage carbohydrates into reducing sugars during a two h macroalgae lysis step, followed by a 48 h enzymatic hydrolysis step for later use in fermentation. An enzymatic mixture of β-1-3/1-4-glucanase, xylanase, cellulase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, and amylases was used. The macroalgae pulp was suspended in 0.15 M sodium carbonate (Na_2CO_3) solution at a starting pH of 9.0 in a stirred tank. Measurements of the reducing sugar concentration were obtained every 12 h throughout the hydrolysis process.

The initial macroalgae lysis step liberated 9.18 ± 1.21 g/L of reducing sugar. The following enzymatic saccharification during the hydrolysis process yielded a further 22.13 ± 1.43 g/L after 48 h (**Figure 2**; **Table 3**). This resulted in a total reducing sugar concentration of 31.31 ± 1.73 g/L after the hydrolysis was complete. With the total reducing sugar content observed to be 37 ± 1.1 using the dinitrosalicylic acid method [30] before hydrolysis, the calculated efficiency of reducing sugar release from the macroalgae lysis and enzymatic hydrolysis process was calculated to be 85%.

The maximum rate of enzymatic saccharification of the macroalgae was observed after 2 h of incubation (**Figure 3**; **Table 4**), with a gradual decline after that, a typical saccharification efficiency relationship observed in other similar studies [19] [35] [36]. It has been speculated that this decline in hydrolysis rate could be the result of inhibition of the enzymes by the products glucose and cellobiose [19] [36] [37].

3.3. Fermentation of *S. latissima* Hydrolysate

The fermentation of *S. latissima* was performed at 30˚C for a period of 48 h. The hydrolysate was used as the bioethanol fermentation substrate. The yeast *Saccharomyces cerevisiae* was used for the fermentation process, with the maximum theoretical ethanol yield of 0.51 g per g of reducing sugar. Measurements of the reducing sugar and ethanol (**Figure 4**) concentration were obtained every 12 h throughout the fermentation process (**Table 3**). The maximal ethanol concentration

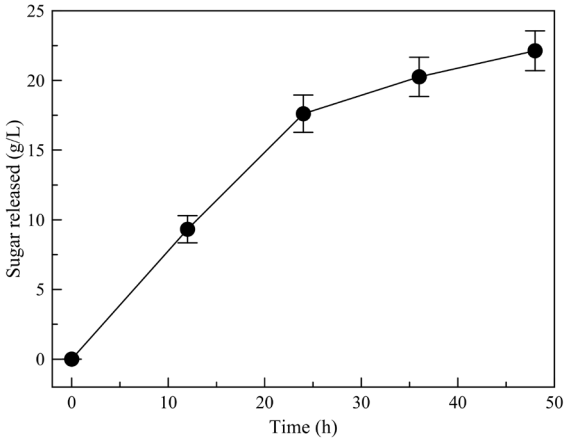

Figure 2. Carbohydrate enzymatic hydrolysis. The concentration of reducing sugars released in grams per liter from the enzymatic hydrolysis of *Saccharina latissima* over a period of 48 hours. The results displayed are mean values of triplicate experiments with the standard deviation displayed as error bars.

Table 3. Hydrolysis yields from *S. latissima*.

Time (h)	Total sugar concentration (g/L)	Sugar released via hydrolysis (g/L)	Saccharification rate (g/L)
0	9.18 ± 1.21	0	0
12	18.50 ± 1.41	9.32 ± 0.98	0.78 ± 0.06
24	26.19 ± 1.78	17.61 ± 1.34	0.69 ± 0.02
36	28.44 ± 1.65	20.26 ± 1.41	0.22 ± 0.03
48	31.31 ± 1.73	22.13 ± 1.43	0.16 ± 0.02

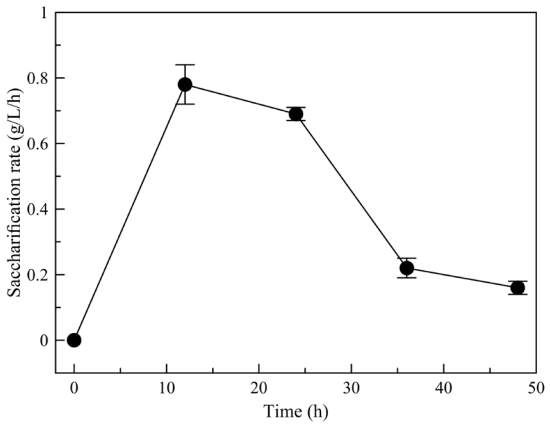

Figure 3. The rate of saccharification during enzymatic hydrolysis. The rate in grams of sugar per liter, per hour of saccharification as a result of the enzymatic hydrolysis of *Saccharina latissima* over a period of 48 hours. The results displayed are mean values of triplicate experiments with the standard deviation displayed as error bars.

was reached after 36 h, at 13.02 ± 0.61 g/L. With the initial glucose concentration of 31.31 ± 1.73 g/L, the maximum ethanol yield from *S. latissima* was 0.42 g ethanol per 1 g of reducing sugar, 84% of the theoretical yield.

Table 4. Fermentation yields from *S. latissima*.

Time (h)	Total sugar concentration (g/L)	Ethanol concentration (g/L)	Theoretical yield (g/L)	Efficiency (%)
0	31.31 ± 1.73	0	0	0
12	15.83 ± 1.21	6.16 ± 0.32	7.74	40
24	6.59 ± 0.78	10.98 ± 0.56	12.36	71
36	1.56 ± 0.62	13.02 ± 0.61	14.88	84
48	0.2 ± 0.17	12.83 ± 0.55	15.56	82

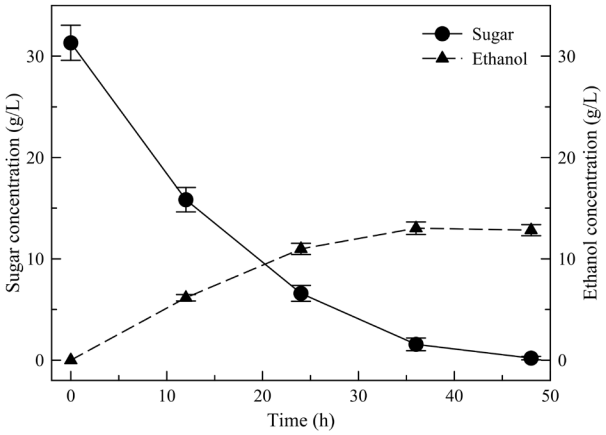

Figure 4. Sugar and ethanol concentration during fermentation. The concentration of reducing sugars and ethanol in grams per litre during the fermentation of the enzymatic hydrolysate of *Saccharina latissima* over a period of 48 hours. The results displayed are mean values of triplicate experiments with the standard deviation displayed as error bars.

The yield observed in this study is amongst the higher known yields as compared to other observations made when using macroalgae as the bioethanol substrate (**Table 5**). Furthermore, since the experiments were undertaken at a large volume (not batch tests), these results display the potential for such bioethanol production up-scaling to industrial levels. After the 36 h point, the ethanol yield was observed to decline very slightly, and this may be due to the metabolism of the yeast strain, which can consume ethanol [35] [36] [37] [48].

The ethanol yield observed in this study is comparable with the ethanol yields observed in lignocellulosic materials. Maize was observed to produce 0.48 g ethanol per 1 g of glucose [49], *Prosopis juliflora* 0.49 g ethanol per 1 g of glucose [35], *Lantana camara* 0.48 g ethanol per 1 g of glucose [36], and newspaper waste 0.39 g ethanol per 1 g of glucose [37].

3.4. Future Biorefinery Prospects of *S. latissima*

Our results from *S. latissima* fermentation observed when extracting with sodium carbonate that by using 1 kg of wet *S. latissima* as the initial biomass, the amount of reducing sugars available was 31.3 g, which can then be fermented into ~ 13 g of ethanol. This carbohydrate-rich macroalgae could be used as the

Table 5. Comparison of glucose and ethanol yields from other macroalgae (modified from [19] [38]).

Macroalgae	Ethanol yield (g/g sugar)	Reference
Saccharina japonica	0.41	[28]
Sargassum sagamianum	0.38	[39]
Saccharina japonica	0.17	[40]
Kappaphycus alverzii	0.39	[41]
Laminaria japonica	0.41	[42]
Gracilaria verrucosa	0.43	[19]
Kappaphycus alverzii	0.37	[43]
Gelidium amansii	0.38	[44]
Ulva fasciata	0.45	[38]
Gracilaria salicornia	0.08	[45]
Saccharina japonica	0.41	[28]
Ulva pertusa	0.38	[46]
Alaria crassifolia	0.28	[46]
Gelidium elegans	0.38	[46]
Sargassum sagamianum	0.13 - 0.23	[47]

raw material for bioethanol production, opening up further economic opportunities in aquaculture. By using macroalgae for the production of bioethanol, the requirement for fresh water, fertilizers and agricultural land for bioethanol production will significantly reduce. Furthermore, Nordic countries will be able to produce bioethanol locally, previously unfavorable due to the limited amount of agricultural land available, from naturally occurring *S. latissima*.

The cost of processing macroalgae for ethanol production can be kept low by employing cost-effective processing methods as used in this study; however, the cost of harvesting large quantities of macroalgae, as well as its delivery to the fermentation plant, are still significant barriers that require attention for the implementation of this technology at an industrial scale.

4. Conclusion

This study has demonstrated the potential for *S. latissima* as biomass for the production of bioethanol. This could also be linked to current alginate extraction industries to form an *S. latissima*-based biorefinery in Nordic countries. The ethanol yield observed was among the higher ethanol yields reported in the literature, suggesting *S. latissima* could be significant biomass for bioethanol production in Norway. The vast coastlines in Nordic countries like Norway provide an extensive area for macroalgae production, in a natural, sustainable manner. Not only does the use of macroalgae from the ocean help reduce ocean acidification and mitigate climate change, but it also separated bioethanol biomass pro-

duction from terrestrial agriculture that is essential for food production.

Acknowledgements

Jacob Lamb acknowledges the support from the ENERSENSE research initiative, and his research was supported by a post-doctoral fellowship from the Norwegian University of Science and Technology—NTNU.

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