

Carbohydrate Yield and Biomethane Potential from Enzymatically Hydrolysed *Saccharina latissima* and Its Industrial Potential

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Abstract

The demand for fuel for utilisation of machinery and transport has culminated in large amounts of fossil fuel usage in the last century. The environmental dangers attached with the usage of fossil fuels have created a large demand for alternative sources of fuels. There is an array of polysaccharides contained within macroalgae, such as mannitol, cellulose and laminarin. These polysaccharides have potential for production of alternative biofuels; however, they are not easily accessible for biological digestion. By pretreatment of macroalgae with enzymes, these polysaccharides may be easier to access by microbes, allowing effective utilization in anaerobic digestion. *Saccharina latissima*, available in abundance on the Norwegian coast line, is a brown macroalgae with a high level of carbohydrates. This study assesses the ability for utilisation of enzymatically pre-treated *Saccharina latissima* for production of biogas through anaerobic digestion. The harvested *Saccharina latissima* was analysed to contain 30.11 ± 2.30 g of reducing sugars per 100 g of dry sample upon enzymatic hydrolysis. This was able to yield 459 ± 30 mL per gVS of biogas through anaerobic digestion, with a methane content of 56%. This suggests a biomethane potential of 1760 m³ per ha of productive sea floor growing *Saccharina latissima*. An evaluation of this process has been performed to demonstrate the industrial potential of *Saccharina latissima* in biogas production.

Keywords

Macroalgae, Hydrolysis, Biogas, Biomethane, Enzymatic Hydrolysis

1. Introduction

The development of alternative fuels to reduce the requirement of fossil fuel us-

age have become of increased interest in recent decades; however, the production of alternative fuels like biofuels is economically unfavourable compared to the fossil fuel market. Despite this, many biofuels (e.g., bioethanol, biogas and biodiesel), are promising alternatives due to their potential sustainability and low environmental impact [1] [2] [3] [4]. Furthermore, many of these biofuels can be utilised by conventional means (e.g., internal combustion engines) when supplemented with fossil fuels [5]. Biogas is a renewable fuel that is already utilised globally as an alternative to fossil fuels, being able to be injected directly into pre-existing natural gas networks. Currently, the majority of biogas production relies on the utilisation of biological waste products as the substrate (e.g., agricultural waste & food industry waste), meaning for many years it has been considered a waste-to-energy route. Although there is still opportunity for significant increases in production from these waste products, other areas of unutilised biomass are of increased interest to extend from the waste-to-energy capacity, and towards a more global and high capacity model. Furthermore, to avoid utilisation of substrates that compete with food crops and require large amounts of land area [6] [7], unused biomass is of great interest, avoiding obstacles such as limited yields, geographic latitude, structural characteristics and high production costs [8].

An alternative is to utilise marine biomass as a substrate, avoiding many of the problems observed in terrestrial substrates. Macroalgae are photosynthetic organisms that utilise the solar irradiation to fix carbon for their metabolism forming rigid polysaccharide-based structures. They grow wild throughout the oceans on coastal areas, collecting significant quantities of polysaccharides that can be hydrolysed into simple polysaccharides for straight-forward utilisation in anaerobic digestion. Additionally, macroalgae tend to have a high content of solids ranging between 8.3% - 22% [9] [10] [11], reducing the requirement of thickening, dewatering and drying of the substrate. Macroalgae have a large area of productivity globally, high production rates per area (**Table 1**), do not compete with conventional food-based agriculture, do not require fertilization or irrigation, recycle ocean bicarbonate and are compatible with existing production streams and biorefineries [12] [13] [14]. The main drawback currently is the saccharification of microalgal biomass into anaerobically digestible polysaccharides for significant biogas production.

Table 1. Major bioethanol crops and macroalgae comparison (modified from (17, 36)).

Substrate	Average yield (kg ha year)	Dry weight of hydrolysable carbohydrates (kg ha year)
Wheat (grain)	2800	1560
Maize (kernel)	4815	3100
Sugar beet	47,070	8825
Sugar cane	68,260	11,600
Macroalgae (17, 31)	75,000	4500

There is a significant lack of terrestrial agriculture production in Nordic countries due to the winter climate (low temperatures and light levels), where the production of biomass on land is a slow process. Here, the potential of food production from terrestrial agriculture cannot be reduced for production of biomass for biofuels. In these cases, macroalgae provide a significant alternative as a biomass source. The extensive coastline of Norway is a prime example, where the warm waters from the gulf stream provide the perfect growing conditions for macroalgae like *Saccharina latissima*, a carbohydrate-rich macroalgae [15] [16]. *S. latissima* contains the carbohydrates laminarin, mannitol and alginate in significant quantities, where laminarin and mannitol serve as energy storage carbohydrates, and alginate as a rigid structural carbohydrate. Although laminarin and mannitol can be anaerobically digested by various microbes to produce biogas, alginate is rather challenging to hydrolyse, reducing its digestibility during biogas production.

Here, samples of *S. latissima* that were obtained from Trondheimsfjord in Norway have been assessed for their biogas production potential from their digestible carbohydrates. Using a carbohydrate extraction procedure previously described [17], further easily digestible carbohydrates were obtained from the macroalgae. This allowed the biogas potential of *S. latissima* to be significantly increased, providing the foundation for its utilisation in the biogas industry in the Nordic countries.

2. Materials and Methods

2.1. Macroalgae Collection

S. latissima was collected from Trondheimsfjord (N63°26'56", E10°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 table-top 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. Algal Pulp Lysis

The dry macroalgae pulp (10% w/v) was suspended in 0.15 M sodium carbonate (Na_2CO_3) solution to a volume of 1 L at a starting pH of 9.0 in a stirred beaker for 2 h at 50°C.

2.3. 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 lysed algal pulp was adjusted to pH 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 flask for

48 h at room temperature. Samples were taken every 12 h and analyzed for glucose concentration using a hexokinase glucose assay kit (GAHK20-1KT, Sigma, Germany).

2.4. 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) H₂SO₄ 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 [18]. Reducing sugars in the sample were determined using a dinitrosalicylic acid method [19]. Glucose content of the hydrolysate was determined using a hexokinase glucose assay kit (GAHK20-1KT, Sigma, Germany).

2.5. Inoculum

The microbial inoculum for the biomethane potential experiments was obtained from the Biokraft biogas plant (Skogn, Norway), from a large-scale continuous mesophilic multifuel anaerobic digester. Prior to the biomethane potential test, the inoculum was incubated at 39 °C to reduce endogenous biogas production. The inoculum was diluted to 9 g volatile solids (VS) per liter. The diluted inoculum had a pH of 7.5.

2.6. Biomethane Potential

The processed samples were digested anaerobically in sealed batch bottles. These were performed in triplicate with inoculum and processed samples, with inoculum alone as a control batch. The following was added as trace minerals and nutrients: 10 mM NH₄Cl, 8 mM NaCl, 1 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O and 0.3 mM Na₂S·3H₂O, as previously described [20]. The final liquid volume of each experiment was 250 mL. The bottles were flushed with nitrogen gas and closed with rubber septums and sealed as described by Ekstrand *et al.* [21]. These were incubated at 39 °C indefinitely with regular agitation.

2.7. Composition Analysis

Analysis of the dry matter (DM) and volatile solids (VS) we performed on the steam exploded birch wood and inoculum before further experimentation using standard methods [22]. The dry matter was determined by incubation of the birch wood and inoculum at 105 °C for 24 h. The mass of the samples after the incubation was compared to the mass pre incubation to acquire a percentage of DM. New samples were then incubated at 550 °C for 4 hours in a muffle furnace. The weight of the samples after incubation in the muffle furnace were compared to the weight of the sample before incubation to acquire a percentage of VS. The DM was determined to be 14.63%; whereas, the VS was determined to be 9.13%.

2.8. Gas Production Volume

Measurement of the produced gas volume was achieved using an optimised liquid displacement method. The volume of gas was then adjusted for temperature using Charles's law.

2.9. Gas Composition and Calculation

Regular 10 mL samples of gas from each experiment were extracted for compositional analysis. The composition was analysed using gas chromatography (SRI 8610C, SRI Instruments, USA), equipped with a thermal conductivity detector using hydrogen as the carrier gas. A standard mixture of CO₂, CH₄, H₂ and N₂ was used as a calibrating gas. Using the volume of gas calculated with the liquid displacement method, and the percentage of methane present in the samples, methane production was calculated for the experiments. All methane production levels are the average of 3 separate experiments and have been corrected by subtracting the endogenous methane production levels for the inoculum only experiments.

2.10. Statistical Data Analysis

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

3. Results

S. latissima is a naturally occurring macroalgae that has a fast growth rate and is found in many locations on the extensive Nordic coastline. With increasing demand for substrates for biomethane production, it seems logical to utilise this macroalgae due to the considerable amounts of carbohydrates it produces. By exposing the microalgae to an enzymatic hydrolysis pre-treatment, the increases in straight forwardly digestible carbohydrates is of great interest to the biogas industry.

3.1. *S. latissima*'s Carbohydrate Content

Using the collected samples of *S. latissima*, the total carbohydrates, reducing sugars and the glucose yield were determined (**Table 2**). These observations are similar to those previously obtained using the same methodology [17]. Before the enzymatic hydrolysis, the total carbohydrates consisted of $55\% \pm 3.1\%$ of the dry weight of *S. latissima*. Furthermore, the reducing sugar content was $36\% \pm 1.9\%$ of the dry weight, whereas the glucose content was $10\% \pm 1.8\%$ of the dry weight of *S. latissima*. These observations of carbohydrate composition are in agreement with previously observed compositions of *S. latissima*, including studies of *S. latissima* obtained from Trondheimsfjorden [17] [23] [24] [25].

3.2. Enzymatic Hydrolysis of Polysaccharides from *S. latissima*

During a 2 h basic (pH 9) lysis method at 50°C in a 0.15 M Na₂CO₃ solution, the

polysaccharides from *S. latissima* were extracted. The lysate was then altered to a neutral pH (pH 6) and exposed to an enzyme mixture of β -1-3/1-4-glucanase, xylanase, cellulase, β -glucosidase, β -xylosidase, α -L-arabinofuranosidase, and amylases for a period of 48 h at room temperature. The reducing sugar concentration of the sample was measured every 12 h during the enzymatic hydrolysis process.

From the initial basic lysis of *S. latissima*, 8.37 ± 0.53 g/L of reducing sugars were released. Further enzymatic hydrolysis yielded a further 21.74 ± 1.77 g/L of reducing sugars after the 48 h incubation period (**Figure 1**; **Table 3**). The total reducing sugars obtained from the basic lysis and enzymatic hydrolysis was observed to be 30.11 ± 2.30 g/L of reducing sugars. When using the dinitrosalicylic acid method for determining total reduced sugars [19], the maximum amount of reducing sugars obtainable from the sample of *S. latissima* was observed to be $36\% \pm 1.9\%$ of the dry weight of reducing sugars before the lysis and enzymatic hydrolysis. Therefore, the efficiency of this process was determined to be 83.6% of the maximum obtainable reducing sugars, which is in agreement with previously observed results [17].

With regard to the rate of the enzymatic hydrolysis of the polysaccharides from *S. latissima*, the maximum rate was observed at 12 h of incubation with the enzyme mixture (**Figure 2**; **Table 3**). The rate gradually declined after this period, as typically observed in similar studies [12] [17] [26] [27]. Although it has been speculated that the decline in rate of the enzymatic hydrolysis may be due to the inhibition of the enzymes by the liberated glucose and cellobiose [12] [27] [28], it may also be due to the reduction of functional enzyme units in relation to the reduced amount of complex polysaccharides left.

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

Carbohydrate group	Relative % of dry weight
Total carbohydrate	55 ± 3.1
Total reducing sugar	36 ± 1.9
Glucose (pre hydrolysis)	10 ± 1.8

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

Time (h)	Total sugar concentration (g/L)	Sugar released via hydrolysis (g/L)	Saccharification rate (g/L/h)
0	8.37 ± 0.53	0	0
12	17.20 ± 1.17	8.83 ± 0.64	0.74 ± 0.05
24	25.25 ± 1.54	16.88 ± 1.01	0.67 ± 0.03
36	28.32 ± 2.17	20.95 ± 1.63	0.34 ± 0.05
48	30.11 ± 2.30	21.74 ± 1.77	0.07 ± 0.01

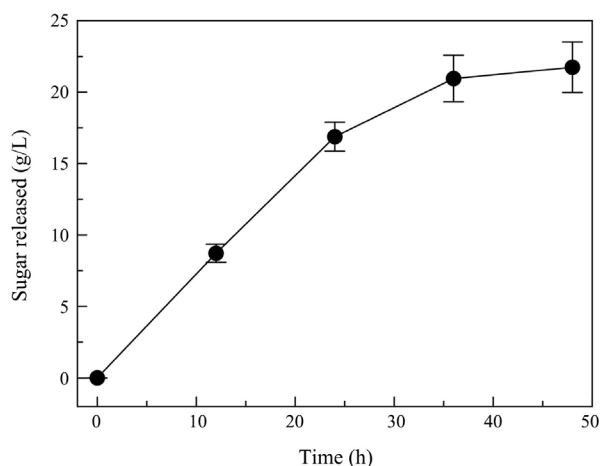


Figure 1. Enzymatic hydrolysis of carbohydrates. Reducing sugars released from the enzymatically hydrolysed *S. latissima* over a 48 h incubation period in g/L. The experiment was produced in triplicate, and the results displayed are the mean values of the 3 replicates with their associated standard deviations as error bars.

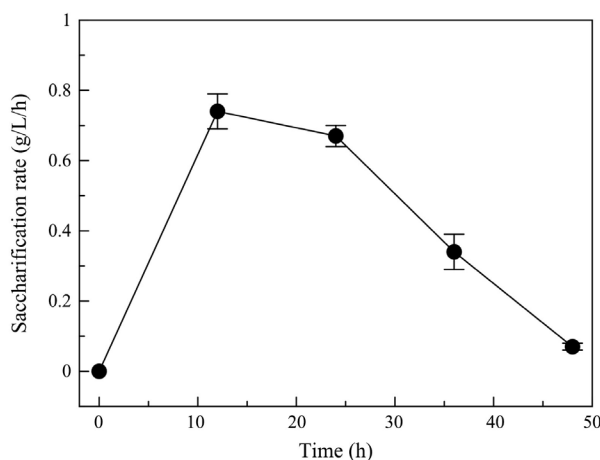


Figure 2. Saccharification rate during enzymatic hydrolysis of *S. latissima*. The amount of reducing sugars per hour were measured to show the relative rate of saccharification. The rate is shown in g/L/h of reducing sugars yielded during the 48 h incubation period. The experiment was produced in triplicate, and the results displayed are the mean values of the 3 replicates with their associated standard deviations as error bars.

Anaerobic Digestion of the Hydrolysate from S. latissima

The anaerobic digestion of the hydrolysate from *S. latissima* was performed using inoculum from a biogas plant near Trondheim, Biokraft (Skogn, Norway). The sample was added in a 2:1 (VS/VS) ratio with the inoculum in 500 mL flasks sealed with rubber septums and flushed with nitrogen gas for 5 minutes to obtain an anaerobic environment. The bottles were then incubated at 39°C indefinitely, with regular mixing. The biogas production was measured regularly using a liquid displacement method, and gas samples were assessed for composition using a GC machine (SRI 8610C, SRI Instruments, USA).

Two experiments were prepared for anaerobic digestion to determine the effect of the enzymatic hydrolysis on the amount of biogas produced; one with the

lysed algal pulp without the enzyme hydrolysis step, and one with the lysed algal pulp with the enzyme hydrolysis step. The total biogas obtained from the anaerobic digestion of *S. latissima* algal pulp without enzyme hydrolysis was 372 ± 22 mL per gVS. This is a similar biogas volume to previously observed values [20] [29] [30], but variation is expected due to seasonal and locational differences. Comparatively, the total biogas obtained from the anaerobic digestion of *S. latissima* algal pulp with enzyme hydrolysis was 459 ± 30 mL per gVS (Figure 3; Table 4). The gas contained 56% CH₄ as determined by GC, resulting in a biomethane production of 208 ± 12 and 257 ± 17 mL per gVS for the algal pulp without and with enzyme hydrolysis, respectively (Figure 3; Table 4). Furthermore, the enzyme hydrolysis step was observed to increase the rate at which biogas was produced, reaching a near full digestion earlier than the algal pulp without the enzyme hydrolysis step.

3.3. Future Industrial Biogas Production from *S. latissima*

The results observed in this study of the anaerobic digestion of *S. latissima* show that the extraction of reducing sugars from 1 kg (w/w) of the macroalgae yields 30.11 ± 2.30 g of reducing sugars. The subsequent enzymatic hydrolysis allows the straight-forward anaerobic digestion of these carbohydrates into 459 ± 30 mL per gVS of biogas, resulting in 257 ± 17 mL per gVS of biomethane from *S. latissima*. With respect to area required, as shown in Table 1, the amount of wet macroalgal biomass obtainable from 1 ha of sea floor is as high as 75,000 kg [31]. The VS of *S. latissima* was determined to be 9.13%, resulting in 6847.5 kgVS per ha. Therefore, 1 ha of *S. latissima* could potentially yield 3142 m³ of biogas, composed of 1760 m³ of biomethane. Therefore, the carbohydrate-rich macroalgae *S. latissima* has a large potential for utilisation as a substrate for biogas production. Furthermore, the utilisation of fresh water sources, fertilisers and terrestrial land is not required due to their ability to grow wildy in the Ocean.

Table 4. Biogas and biomethane yields from *S. latissima*.

Time (days)	Biogas (mL/gVS)	Biomethane (mL/gVS)	Biogas (mL/gVS)	Biomethane (mL/gVS)
0	0	0	0	0
4	22 ± 4	12 ± 2	11 ± 4	6 ± 2
7	78 ± 6	44 ± 3	44 ± 4	25 ± 2
10	167 ± 9	93 ± 5	83 ± 6	47 ± 3
14	257 ± 17	144 ± 10	141 ± 13	79 ± 7
17	326 ± 22	182 ± 13	191 ± 15	107 ± 8
20	385 ± 31	215 ± 18	213 ± 26	119 ± 15
24	405 ± 26	227 ± 15	268 ± 22	150 ± 12
27	420 ± 28	235 ± 16	292 ± 22	164 ± 12
30	433 ± 26	242 ± 15	313 ± 20	175 ± 11
34	440 ± 33	247 ± 19	335 ± 24	188 ± 13
37	453 ± 31	254 ± 18	357 ± 22	200 ± 12
40	459 ± 30	257 ± 17	372 ± 22	208 ± 12

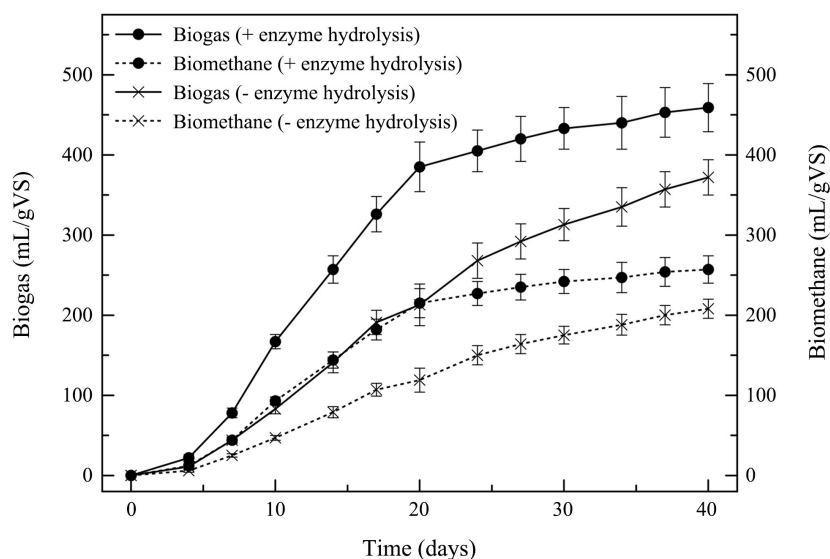


Figure 3. Biogas and Biomethane Potential. The potential of biogas and biomethane production was measured over a period of 40 days. The gas produced was then calculated for production per gVS. The solid lines represent the accumulative biogas production throughout the incubation in mL/gVS, whereas the dashed lines represent the biomethane content of this produced biogas during the incubation in mL/gVS. The experiment was produced in triplicate, and the results displayed are the mean values of the 3 replicates with their associated standard deviations as error bars.

The advantages of CO₂ sequestration associated with the cultivation of macroalgae are also significant. Typically, the utilisation of 1 tonne of macroalgae sequesters 6 times the amount of CO₂ emitted during the processing, maintenance and transport of the feedstock [32], and the close proximity of macroalgae farms to sources of pollution can improve their growth rate, while cleaning the water of excess nutrients [30]. Macroalgae also have a high level of macro nutrients, improving the digestate as a biofertilizer [30] [33]. Finally, the C:N ratio desired for optimal anaerobic digestion is between 20:1 - 30:1 [34], where a ratio below 15:1 can result in excess levels of ammonia in the anaerobic digestion process, leading to unstable biogas production [35]. The C:N ratio of *S. latissima* has been observed to be 22:1, suggesting that it is an ideal substrate for anaerobic digestion [11].

Despite this, it should be noted that the impact of the extensive harvesting of wild macroalgae in these regions is unknown and may cause local ecological issues. The turnover of macroalgae in Nordic regions is relatively high, but excessive removal of macroalgae from the seabed in a specific area could result in the collapse of the community, which may take several years to recover. Alternative methods of industrial-scale growth could avoid these drawbacks, by the growth of macroalgae on suspended systems in deeper waters. If the waters are deep enough, there would be a limited amount of wild macroalgae present in the area; however, microalgae would then be competing for light with these artificial systems, and their growth could be reduced.

4. Conclusion

The research undertaken in this study has presented the potential for utilisation of a common macroalgae (*S. latissima*) present in the Nordic coastal regions for production of biomethane through a straight-forward pre-treatment, followed by anaerobic digestion. The biomethane yield obtained with the enzymatically hydrolysed algae suggests a potentially of 1760 m³ of biomethane per ha of productive sea floor could be obtained. This implies that *S. latissima* could provide a significant biomass source from the production of biomethane in the Nordic regions. Furthermore, the rate increase observed in biogas production as a result of the enzymatic hydrolysis allows for a shorter retention time in continuous feed anaerobic digesters, increasing the potential biogas yield over time. The Nordic coastlines provide a natural, untapped area for biomass production that could be maintained in a sustainable manner. Not only would the continual harvesting and natural cultivation of macroalgae from the sea floor help mitigate the rising levels of dissolved carbon in the oceans, but it would also increase the production of the non-fossil fuel biogas due to the larger variety of excessable biomass sources.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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