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Deconstruction of Macroalgae:

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Final Report of Macroalgae as a Biomass Collaboration between JBEI and StatOil 2010-2014

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Introduction

Abstract

The goal of this collaborative project was to explore the feasibility of using macroalgae as a feedstock for biofuel. Specifically to focus on the conversion of macroalgae to biofuels or other valuable co-products which are low carbon, cost-effective, and sustainable. The main objectives were to develop a library of the composition of macroalgae, develop optimal pretreatments, investigate and determine and optimize enzymatic cocktails capable of efficiently hydrolyzing the resultant polysaccharides into monomeric sugars. The initial technical objective was to identify, verify, and develop new modes of pre-treatment and evaluate enzymes for hydrolysis of macroalgae to fermentable sugars.

Goals

I. Discuss methods that provided numbers for compositional analysis.

II. Test pretreatment, and compare to no pretreatment.

III. The direct use of enzyme on the biomass was able to produce monomeric sugars. The commercial enzymes resulted in more than 99% sugar in less than 5 hrs. This was better than the previously published enzymes.

IV. JBEI is interested in halo-tolerant enzymes for sugar degradation. Initial studies of enzymatic hydrolysis from a library of GH1s starting with those that have high activity on ß1-3 and ß1-6 glucose bonds and a high salt sensitivity.

V. Verify the ability to ferment the produced hydrolysates.

VI. Discuss future research directions for understanding the potential commercialization of algal biomass to monomeric sugars. In particular high biomass loading resulted in lower yields possibly due to an increase in viscosity or increase in salt content. Also, the GH1 library has a large number of halo-tolerant enzymes so investigating the exact sensitivity will be important. Further screening the ability of other enzyme families and their ability to break down the different components. Developing a screen and investigating other enzyme sources will help to find more halo-tolerant and thermophilic enzymes.
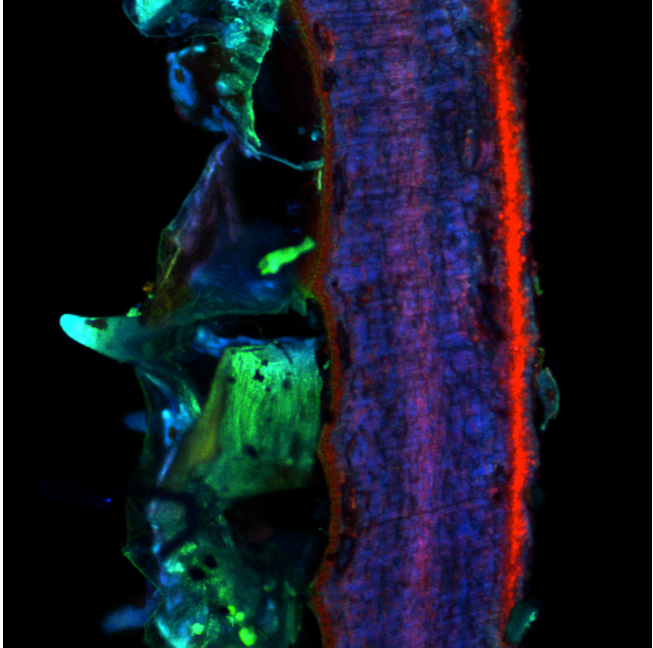
Introduction

In the race to reduce our dependence on petroleum and other non-renewable carbon sources, advanced biosynthesized chemical production has become a global focus. One extremely promising biomass as a sugar source for renewable biochemical synthesis production is algae. Both microalgae and macroalgae are thought to minimally compete with established and projected food supplies and require significantly less land than terrestrial biomass sources to generate significant amounts of renewable chemicals. Macroalgae has been commercially collected for many years in weights of kilotons in China, Philippines, Indonesia, Chile and countries in Europe [1-4], and there are large areas of unused shorelines that have potential for algae cultivation. It has been estimated that using 0.09% of the potential United States offshore 'Exclusive Economic Zone' to grow macroalgae could replace 1% of the current United States fuel consumption [4]. Further, many countries with current algae farms are developing low impact renewable cultivation methods providing guidance for sustainable farming [2, 5, 6]. Components of the biomass such as ash, complex sugars or phenols not easily used for biochemical production have shown the potential for commercial value to help reduce costs, one of the key hurdles for implementing renewable biosynthetic replacements [7-10].

Multiple evaluations of the feasibility of Macroalgae as a sugar source for biofuel production have been preformed. Most of these studies make over reaching estimations and struggle to meet production. The United States had a government supported macroalgae industry during World War I that produced both potash and acetone. The industry struggled then and closed shortly after the war due to the lack of understanding of the product and the need for technological advances to reduce labor costs [11]. Further, the industrial importance and use of alginate had not taken off. Since the 1910's, multiple other countries have figured out how to harvest and improve the efficiency to grow algae. The commercial importance of alginate and market size has grown and seems to sustain a business platform growing and harvesting algae.

Feasibility studies of the use of algae as a sugar source for biofuel production have made assumptions that may not be realistic due to currently published technology. Important considerations are the varying compositional analysis, high biomass loading complications, viscosity and salinity challenges.

While macroalgae has been shown to have promise for biofuel or renewable chemical production, and multiple studies have measured sugar content, both the conversion of algal polysaccharides into sugar and the use of the liberated sugars for synthesis of a renewable chemical product has not been optimized [12, 13].

Section I – Composition and Methods

Introduction

To obtain a compositional profile for the seasonal and geographical variants, we first did a literature review, see Appendix A.

The brown macroalgae, *S. latissima* has been shown to have some of the highest sugar content in the form of laminarin and mannitol. Multiple species of brown algae, including *S. latissima* harvested off the coast of United Kingdom, have shown potential for production of ethanol and biogas [14, 15]. In the present work, we have sought to both characterize the biomass composition and maximize sugar production from *S. latissima* harvested of the Norwegian Coast. In addition since fermentation can be affected by other materials in the supernatant (salt etc.), all of the harvests have been used to produce pinene with an *E. coli*-based advanced biosynthesis pathway [16-18] to demonstrate suitability of hydrolysate resulting from macroalgae.
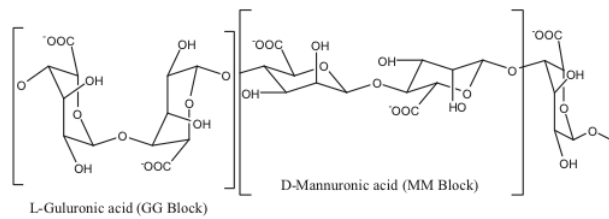
Alginate   Fucoidans
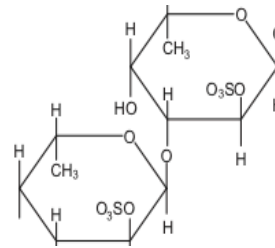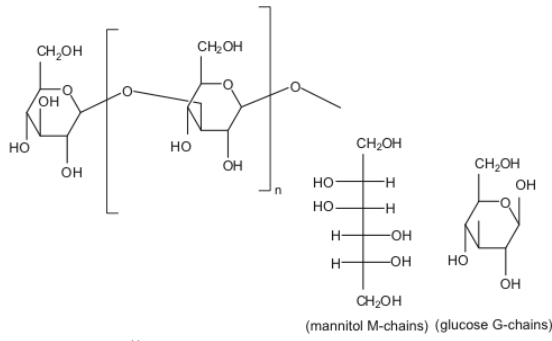
Laminarin   Mannitol
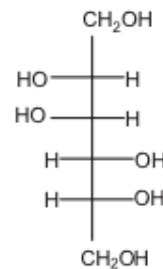
Figure 1: Carbohydrates found in brown macroalgae
Carbohydrates found in brown macroalgae, adapted from Anastasakis et al. 2011 [19]

Lifecycle modeling has shown seasonal harvesting and compositional determination to be important for maximizing fermentation. Higher harvesting yields do not equate to higher fermentation yields, rather macroalgal composition plays an important role [20]. Thus, the regional growth differences even within the same strain can vastly change the best method to optimize sugar production for use in biosynthesis. It is important to understand these parameters because results, either under or over estimations of potential yield, can greatly impact overall feasibility.

The recoverable concentration of fermentable sugars has been shown to vary with harvest time [20-23]. The polysaccharide profile of macroalgae includes alginate, fucoidan, mannitol and laminarin (Figure 1-2). Alginic acid is the copolymer of (1-4)-linked α-L-guluronic acid and (1-4)-linked β-D-mannuronic acid, arranged in blocks of polymannuronic acids or polyguluronic acids, and sequences of alternating mannuronic and guluronic acids (Figure 3). Alginate, the salt form of alginic acid has numerous usages such as for food additives or for medical purposes. World-wide commercial production of alginate was valued at 213 million in 2003 [3], with potential for co-

production of an easily fermentable sugar source of both mannitol and laminarin. Mannitol is synthesized in algae as one of the major photosynthetic products [24, 25] and is found in quantities as high as 20-30% in brown algae (*Phaeophyta*) and has been shown to function as a mean to control cell turgor in response to osmotic stress, as an antioxidant, and as a free radical scavenger [24]. Mannitol has been shown to be fermentable into ethanol, however mannitol to ethanol conversion results in a negative redox balance and requires oxygen for biofuel production [26]. Laminarin is polysaccharide of (1,3)-β-D-glucan with β (1,6) branching ending with either a mannitol or a glucose residue about 5000 kDa in size [27]. It is the primary storage of glucose in the algae, and the specific target of previous bioconversion attempts with use of a laminarinase [12, 13].

Compositional Characterization

S. latissima was collected off the coast of Norway in December 2010, July 2011 and August 2011. Analysis of *S. latissima* harvested off the coast of Norway showed that as expected there are compositional differences between harvests. The July and August 2011 harvests contained 11-18% glucan (laminarin), 17-25% alginate, 20-23% mannitol, 30-37% ash, and <1% protein (Figure 2, Table 1). The July and August compositional profiles were similar to the profiles of brown macroalgae harvested at a similar seasonal harvests off the coast of Ireland [23]. Interestingly, the December 2010 harvest has similar amounts of glucose as the August harvest, namely 10% and 13% respectively. The mannitol content of the December harvest was 6%, which is lower than expected, but similar to that previously reported for January. The other compositional components were as expected: 43% alginate, 25% ash, and <1% protein. While the composition is slightly different from previously reported batches, none of the previous compositional analyses are of macroalgae harvested off the coast of Norway and composition is expected to change with seasonal variation and harvesting methods. Alginate was extracted using an adaptation of multi-step process to get a relative quantifiable amount and was burned to adjust for the ash content of the material [27, 28], then characterized by Fourier transform infrared microscopy (FTIR) [29-32]. The extracted alginate showed that the mannuronic acid to guluronic acid ratio as measured by the ratio of FTIR peaks at 1030 to 1090 cm^{-1}, were 0.64 ± 0.016, 0.64 ± 0.016 to 0.68 ± 0.005 for July, December and August respectively (Figure 4), NMR confirms the extracted material was alginate

(Figure 5). X-ray diffraction analysis of the ground algae was done to evaluate the presence of cellulose and crystallinity [33-35]. Multiple sharp peaks in the XRD pattern of the microalgae suggests a high level of sea salt that matches with the compositional analysis (Figure 6). The broad feature around 16-25° 2θ is likely due to the presence of amorphous alginate or cellulose. The crystalline peaks present in the XRD pattern were indexed to the following phases: NaCl (Fm-3m), KCl (Fm-3m), $CaCO_3$ (Pmcn) and D-mannitol (P2$_1$) (Figure 6).
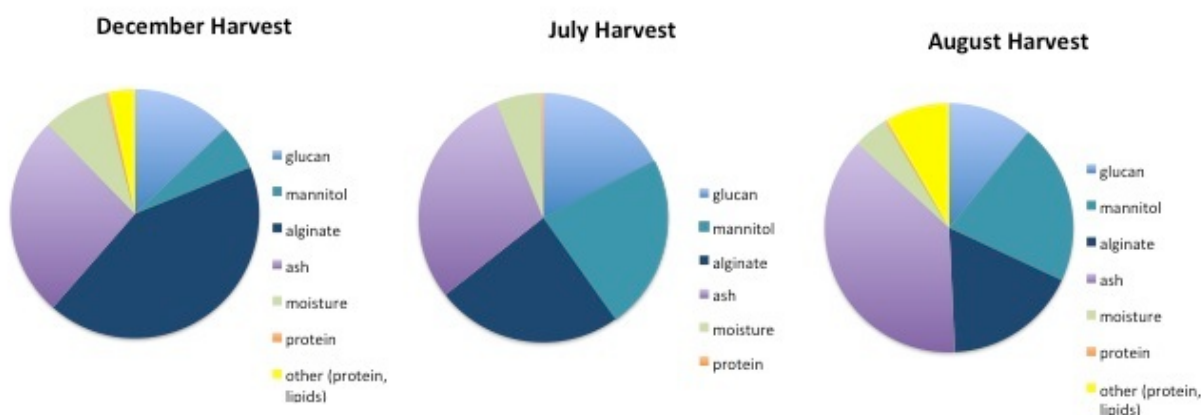
Figure 2: Compositional profile of *S. latissima*
Compositional provile of S.latissima as a function of three different seasonal harvests.

Table 1: Composition of macroalgae
as a function of seasonal harvest, values shown ± SD.

			Composition			
Harvest Time	Glucan	Mannitol	Ash	Moisture	Alginate	Protein
December 2010	13.0% ± 0.4 %	5.9% ± 0.4%	24.99% ± 0.01%	8.4% ± 0.3%	43% ± 4%	0.4%± 0.1%
July 2011	17.9% ± 0.3%	25% ± 1%	30.31% ± 0.01%	6.0% ± 0.3%	25% ± 4%	0.18% ± 0.08%
August 2011	10.2% ± 0.9%	18% ± 2%	37.6% ± 0.4%	4.5% ± 0.1%	17.3% ± 0.8%	0.2% ± 0.1%

Alginate

Is a polymer of (β-D-mannuronic acid (M), α-L-guluronic acid (G)), see review [36], and air dried macroalgae are composed of 12-24 wt% alginate [37]. Lowering the pH increases the viscosity [37]. Alginate absorbs a large amount of divalent cations, and is particularly sensitive to Ca^{2+}

concentration calcium is chelated in the GG blocks in an "egg-box" structure, selectivity $Mg^{2+} <$ $Ca^{2+} < Sr^{2+} < Ba^{2+}$ [38]. The G rich segments regulate Ca^{2+} gel formation and have stronger gel strength while MG rich segments regulate solubility in dilute acid [37]. An important factor in using alginate as a carbon source for fermentation is that alginate has a positive redox balance and thus can balance out mannitol digestion (in theory 2:1, but in experiments ~2.5:1) [39]

The composition not only impacts industrial relevant factors such as viscosity, gel strength, fermentation balances and ion absorption. The guluronate content increases in zoospore culturing comparitive to gemephyte,
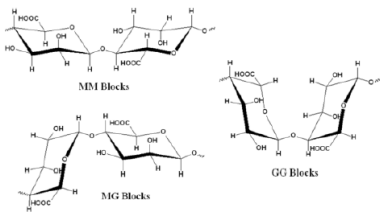
Figure 3: Structure of Polymeric Blocks of Alginate
Structure of polymeric blocks of alginate [40]

Laminaria japonica, Japan NMR [41]

Production method of high purity l-guluronic acid metal salt or d-mannuronic acid metal salt. [42]

Alginate Extraction:

Notes:

This method is involved with many steps. Skipping a step will still result in a reduced recovery of alginate and can effect the yield.

Alginate was extracted using a multistep process as described in [28] with Na_2CO_3 and $CaCl_2$. The recovered alginate was then ashed at 575 °C for 3 h to account for the mineral content, the difference between the 105 °C dried weight and the ashed values were used to calculated the alginate concentration.

Technique adapted from

1. "Characterization of Polysaccharides extracted from brown seaweeds" L.-E. Rionx, S.L.Turgeon, M Beaulieu [27]

2. "Changes in alginate molecular mass distributions, broth viscosity and morphology of Azotobacter vinelandii cultered in shake flasks" C. Pena, N Campos, E Galindo [28]

Method:

1. 85% EtOH wash at room temperature, shake once every 30 min for 12 hours
2. Centrifuge 15,000g for 10 min (2 x 12 hrs), decant (keep solids)
3. 70% EtOH, room temperature, shake once every 30 min for 5 hours
4. Centrifuge 15,000g 10 min (2x5hrs), deacant (keep solids)
5. 2% $CaCl_2$ (w/v) 70°C 3hrs decant (liquid, laminarin, fucodian)
6. Centrifuge 15,000g for 10 min, decant (keep solids)
7. 0.01M HCl ~pH2, 70°C for 3hrs, decant (liquid, fucodian)
8. 3% Na_2CO_3 70°C for 3hrs decant (liquid, alginate)

Suggested extraction:

*precipitate alginate using IPA(2) or Acetone(1)

*precipitate laminarin using 50 mM imidazole (Sigma, USA)

Method Notes: I use isopropyl alcohol to precipitate, then filter the sample with Whitman Filter Paper 1- alginate will clog if using too much alginate. (~50-100 mg alginate for ~2 inch filter). Let it dry then, store in tube remove from filter paper before rewetting. I also then ash ~ 100 mg the sample without the filter paper at 575°C and subtract that % ash to get an alginate yield. I found that if I let it sit in either the $CaCl_2$ or the Na_2CO_3 longer than I get a "higher yield" however, when I ash the samples I just get an increase in the ash.

Solutions:

40mL - 85% EtOH = 35.789mL 95% EtOH + 4.210 H_2O

3% w/v Na_2CO_3 sodium carbonate, white crystal powder 1.5g / 50mL

2% $CaCl_2$ (w/v) MSDS 2 health, 2 reactive, use cold water to mix ($6H_2O$ 0.6mg / 12mL)

Alginate Characterization:

Notes: These two methods provide a measurement of the composition of the alginate

FT-IR (Fourier Transform Infrared) the peaks at 1090 cm^{-1} mannuronic and at 1030 cm^{-1} guluronic acid are used to measure relative ratio of guluronic acid and mannuronic acid components in the recovered biomass. It needs to be done on relatively pure extracts, and not as accurate from the raw algae samples. ([29, 31, 32] in [30])

It should be noted that polymannuronate has a weaker interaction with cations, where the polyguluronates form an egg-box structure and can effect the IR spectrum [29].
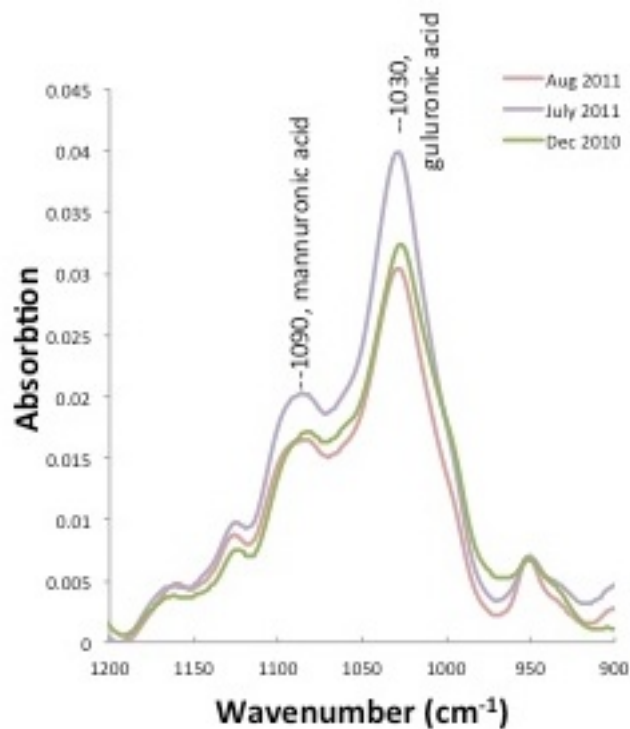
Figure 4: FTIR extracted alginate spectra
FTIR extracted alginate spectra used to calculate mannuronic acid to guluronic acid ratio using peaks at 1090 cm^{-1} (mannuronic acid) and 1030 cm^{-1} (guluronic acid) Shown is an average of 3 aliquots per sample and 3 extractions per harvest. ANOVA with a Tukey's HSD posthoc test comparing the difference in the ratio between harvests $F_{(2,11)} = 19.1$ P < 0.001, Dec versus July p = 0.989, Dec versus Aug p < 0.005, Aug versus July p < 0.001.

NMR

alginate- hydrothermal conditions, depolymerization [43]

monomeric concentration Grasdalen 1983 in [44]
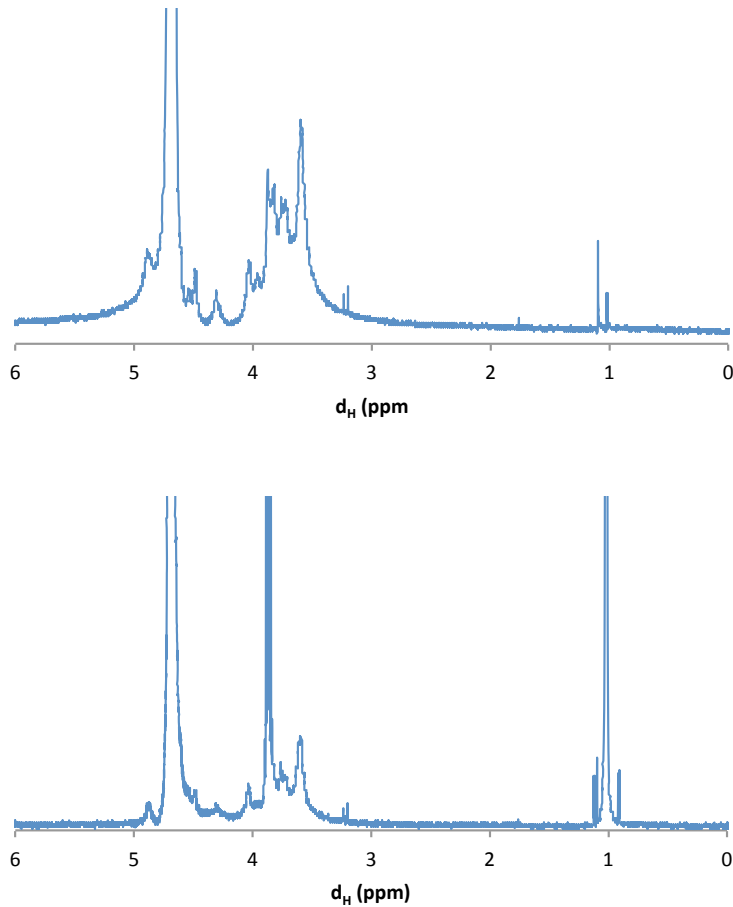
Figure 5: NMR of Alginate
NMR of alginate, room temperature Top: of purchased alginate (Sigma-Aldrich, Sodium Alginate from *L. digitata*) Bottom: Alginate extracted from the July 2012 batch of *S. lattisima*

Acid Hydrolysis Analysis

The National Renewable Energy Laboratory (NREL) protocol for determination of structural carbohydrates and lignin in biomass is the standard protocol for total sugar analysis of lignocellulosic (NREL, [45]). This protocol has been adapted and is used with the following procedure for the analysis of the macroalgae. 100-300 mg of biomass is placed in 2 mL of 96%

sulfuric acid. This is then stirred for 1 hour at 30 °C, 56 mL of DI water is then added, the container sealed and autoclaved on liquid cycle for 1 hour. The resultant liquid is then filtered and run on either HPAEC or HPLC. Samples were filtered with a 0.45 µm filter before being analyzed.

HPAEC Analysis

High pressure anion exchange chromatography (HPAEC) analysis can be used for the general composition and composition of the following structural carbohydrates of algae: mannitol, glucose, xylose, fucose. Carbohydrates were analyzed by HPAEC on an ICS-3000 system (Dionex, Sunnyvale, CA) equipped with an electrochemical detector and a 4 x 250mm CarboPac SA10 analytical column. A injection volume of 10 µL of the sample was injected into the column and was eluted with 1 mM KOH for 14 min. Samples were typically diluted 100 fold for acid hydrolysis or 1000 fold for saccharification hydrolysate. The flow rate of the eluent was maintained at 1.2 mL/ min. Standards were made for mannitol, glucose, xylose, fucose at 6, 10, 25, 50, and 100 µM.

HPLC analysis

High performance liquid chromatography (HPLC) analysis was also used to calculate the general composition and composition of the following structural carbohydrates of algae: glucose, xylose, cellubiose. Carbohydrates were analyzed on an Agilent system equipped with a DAD and an H analytical column. An injection volume of 10 µL of the sample was injected into the column and was eluted with 4% sulfuric acid for 14 min. The flow rate of the eluent was maintained at 0.6 mL/min. Standards were made for glucose, xylose and cellubiose at 2, 1, 0.5, 0.25, and 0.125 g/L.

Mannitol

Can be quantitated with either the HPAEC and HPLC, HPAEC with the CarboPac SA10 analytical column (µM levels), and by HPLC with the H analytical column (mM levels).

Ash

Ash content was measured using procedure of NREL [46]. Briefly, a know amount of algae was placed in a pre-dried (575 °C for 4 hours, stored in a desiccator) and weighed crucible. Samples were then dried at 105 °C until a stable weight (generally overnight). These were then put into muffle furnace with the following program:

Ramp from room temperature to 105 °C

Hold at 105°C for 12 minutes

Ramp to 250 °C at 10°C / minute

Hold at 250 °C for 30 minutes

Ramp to 575 °C at 20 °C / minute

Hold at 575 °C for 180 minutes

Allow temperature to drop to 105 °C

Hold at 105 °C until samples are removed

Reweigh Crucible and remaining sample, using same timing as measuring the 105 °C dry weight (ie immediately out of the oven or cool for 1 hr in desiccator).

Protein

Protein was extracted from algae using glass bead homogenation, Coomassie Blue (BioRad) with BSA standards. Adapted from Weis, V. M., E. A. Verde and W. S. Reynolds. 2002 [47].

Solutions:

100 mM Tris 10 mM EDTA 100 mM NaCl

The day of use, add 1 aliquot of Protease inhibitor cocktail (PIC) to 10 ml of buffer Acid washed glass beads (400-600 um).

B. Extraction of algal protein homogenate

1. Work with 1.5 ml of above algal pellet. Freeze the remainder

2. To this 1.5 ml, add 10 ml of FSW with 2% triton. Resuspend algae.

3. Spin at 2,500 g for 6 min. Supernatant should have greenish-yellow tint. Pour off supernatant

4.   Rinse pellet once in FSW and respin.

5.   Pour off supernatant and add about 3.75 ml of extraction buffer (with PIC). Resuspend algae and place suspension in a **glass** culture tube.

6.   Add 1-2 ml of glass beads (acid washed).

7.   V ortex suspension for 30sec and then place on ice for 30 sec.

8.   Repeat vortex and icing a total of 20 times

9.   Pipette out suspension, away from glass beads and place in microfuge tubes.

10. Spin at 15,000 rpm in microfuge for 5 min. Resulting supernatant should be a deep, clear orange.

11. Determine protein concentration with Bradford assay.

Conductivity

Conductivity was measured using a Horiba pocket conductivity meter, samples were diluted 10 and 100 fold and confirmed for linearity of measurement.

X-ray powder diffraction measurements

Powder X-ray diffraction (XRD) patterns of biomass samples were obtained using a PANalytical Empyrean diffractometer equipped with a PIXcel3D detector operated in 1D scanning mode. Samples from three replicates were mixed for XRD analysis. Scans were collected at 45 kV and 40 mA with a wavelength of 1.5418 Å (CuKα radiation). A reflection-transmission spinner was used as a sample holder and the spinning rate was set at 4 rpm. Scattering intensities were measured using the Bragg–Brentano (θ-2θ) geometry over an angular range of $5° < 2\theta < 55°$ with a step size of 0.026° and a step time of 300 seconds.
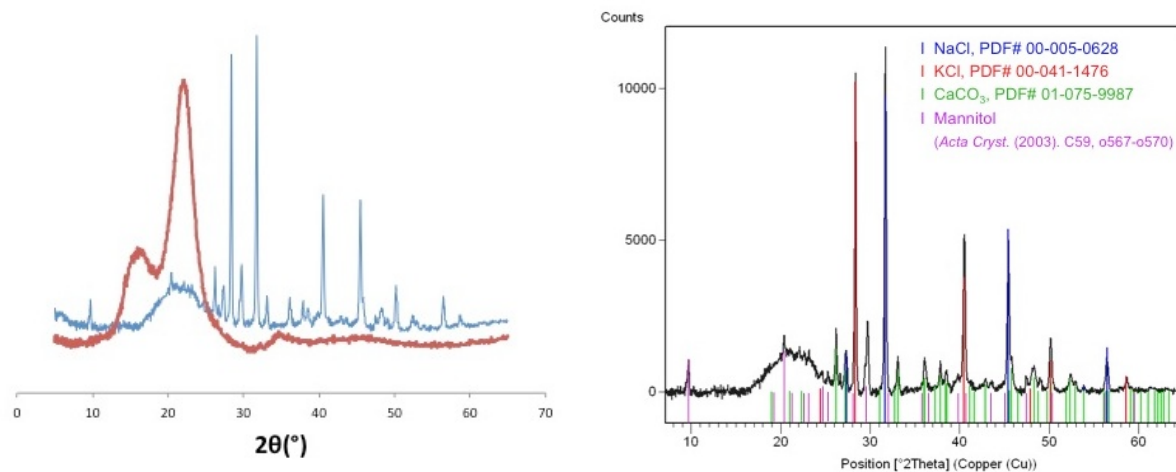
Figure 6: XRD of Macroalgae
XRD of biomass left: Comparison of XRD of cornstover (red), and December 2010 batch macroalgae (blue). Right: X-ray diffraction of algae with marked peaks.

TGA

 Thermo-gravitational Analysis

A thermogravimetric analyzer (TGA/DSC 1, STAR^e system, Mettler-Toledo Inc., Colombus, OH) equipped with a high-throughput auto sampler. approximately 5 – 10 mg of sample was used. Multiple heating programs were used. The samples were heated in the presence of Argon at a flow rate of 25 ml/min. The data was collected using STAR^e Excellence software (Figure 7).
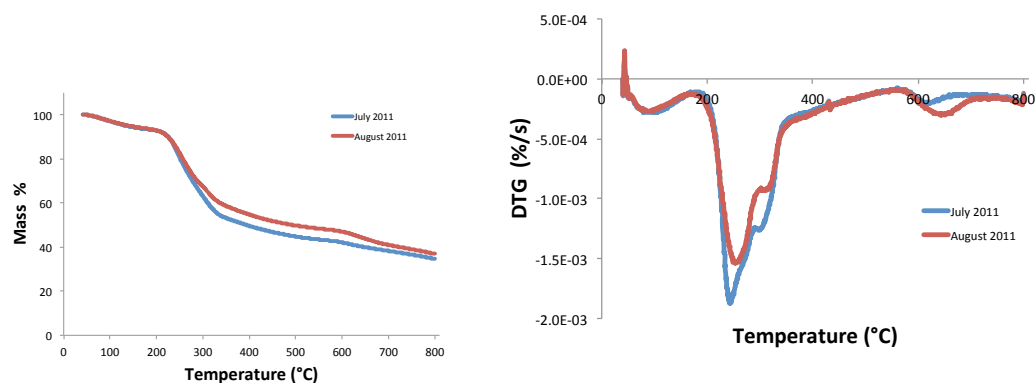
Figure 7: TGA of Macroalgae

TGA of two harvests of July and August batch of *S. lattisima*. The temperature of the sample was increased at a rate of 10°C/min from 35 to 800°C (left) show mass as a function of temperature increase and (right) show the rate of mass loss as a function of temperature.

Section II - Pretreatments

In a study by Adams et al., the use of pretreatment as typically used in lignocellulosic biomass sources for monomeric glucose production and resulting ethanol yield was evaluated. However, the highest recovery was reported for enzymatic hydrolysis without any additional pretreatment which had a yield of 0.45% (v/v) of ethanol [14]. They hypothesized that their initial attempts at pretreatment resulted in poor yields due to high salt content [14]. Attempting a treatment in a PARR reactor where temperature and pressure was controlled resulted in little to no sugar release, and reduced the overall saccharification yield (Figure 8-10) possibly due to release of components like glycolic acid, lactic acid, or other additional unknown compounds (Figure 10).

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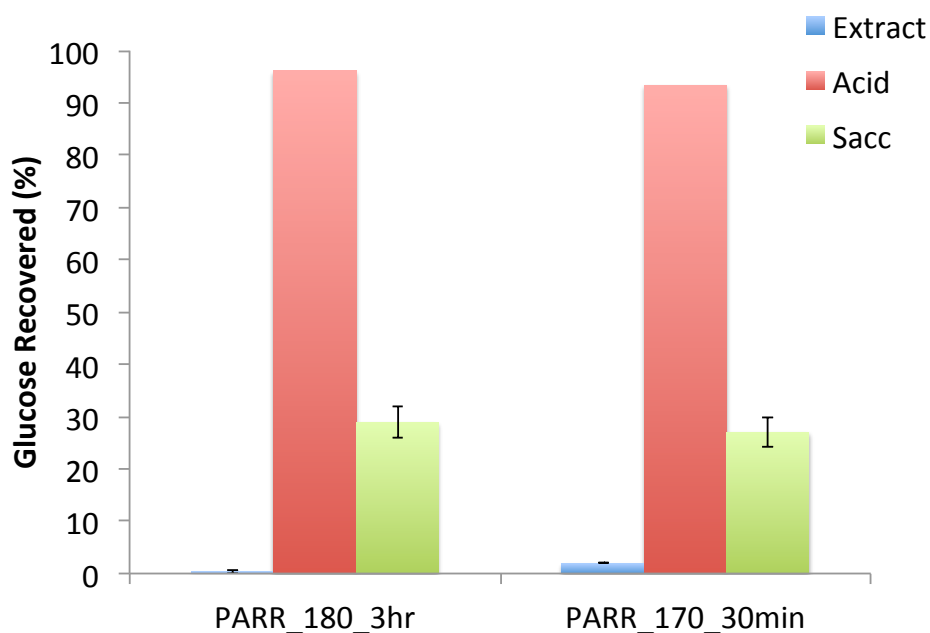
Figure 8: PARR hydrolysis of Macroalgae
Evaluation of high temperature and pressure pretreatment of macroalgae in the PARR reactor for either 180 °C for 3 hours or 170 °C for 30 min at 2200 psi 3% wt dried algae/ DI water.
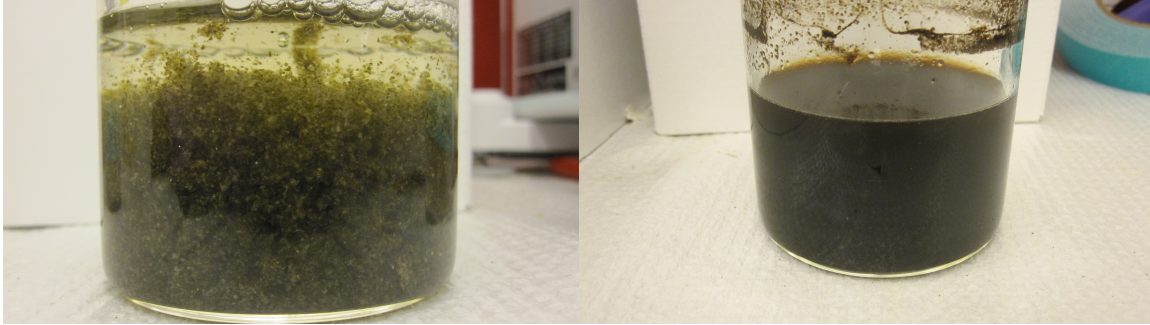
Figure 9: Photograph of PARR treatment
PARR pretreated algae before (left) and after (right).
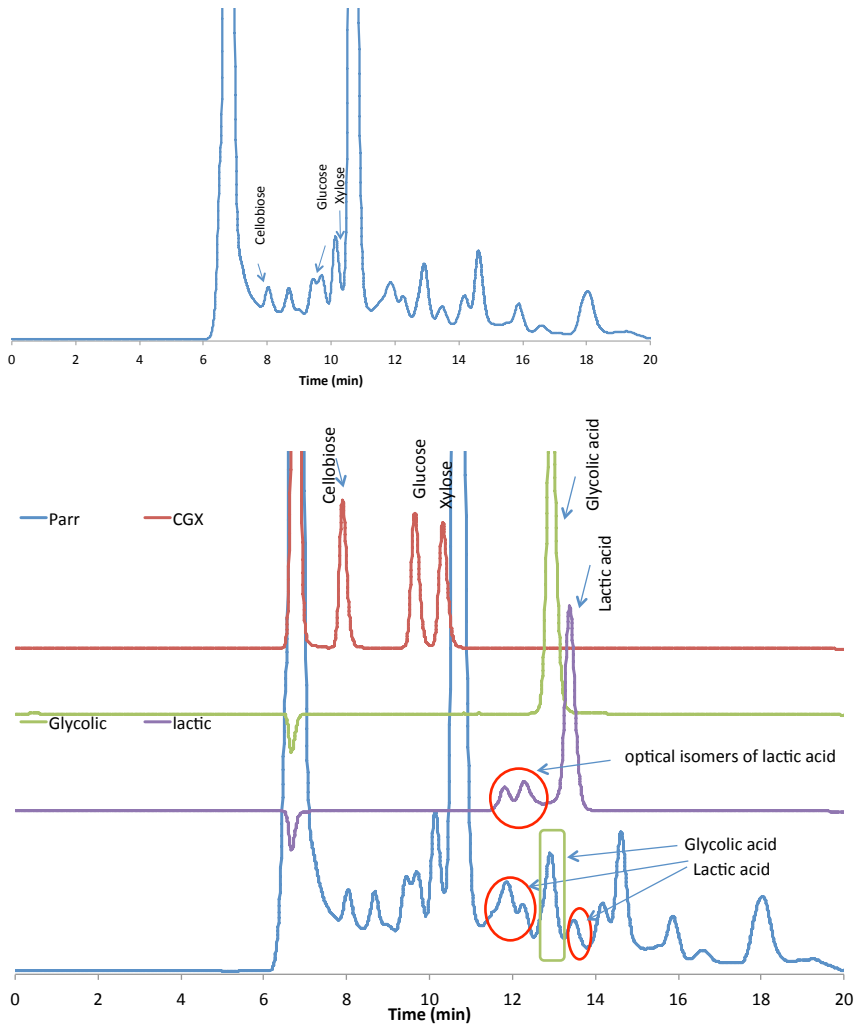
Figure 10: HPLC of PARR results
Resulting pretreated mixture from PARR reactor

Ionic Liquid pretreatment

Ionic liquid pretreatment has been used successfully to reduce the recalcitrance of lignocellulosic biomass. Typical treatment involves heating the biomass in ionic liquid. We attempted this with the S. latissima with a treatment of 3 hrs 140 °C. Only 9 ± 2% of the starting sugar was recovered (Figure 11), while, the IR spectrum shows and increase in the peaks around 1050 suggesting increased sugar concentration in the recovered biomass (Figure 12).
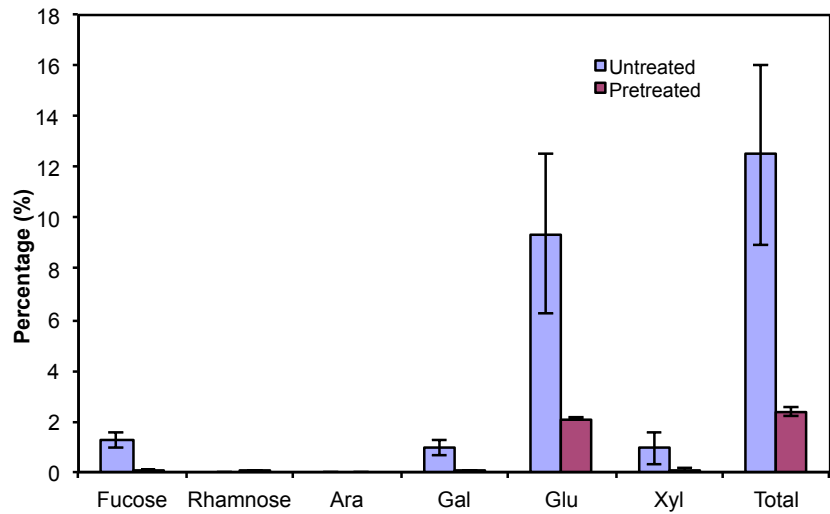
Figure 11: Sugar composition of ionic liquid pretreated biomass
Analysis done by HPAEC.
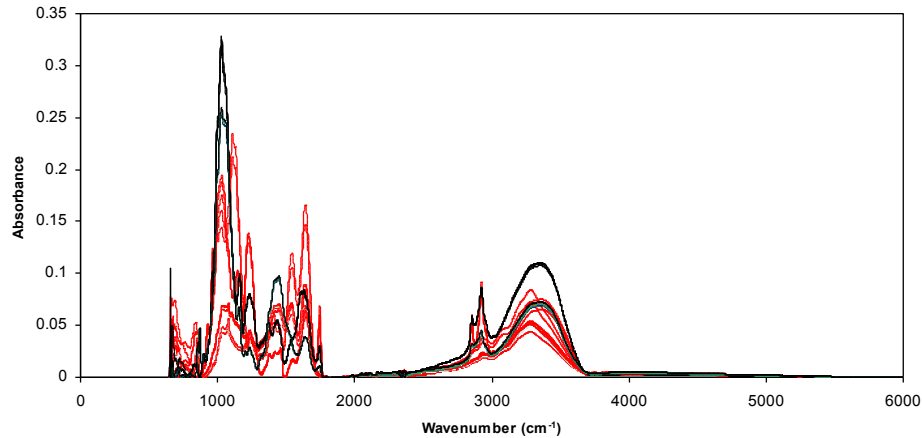
Figure 12: FTIR of macroalgae before and after IL pretreatment
(red untreated, black IL pretreated)

Section III – Initial Enzymes for Biomass
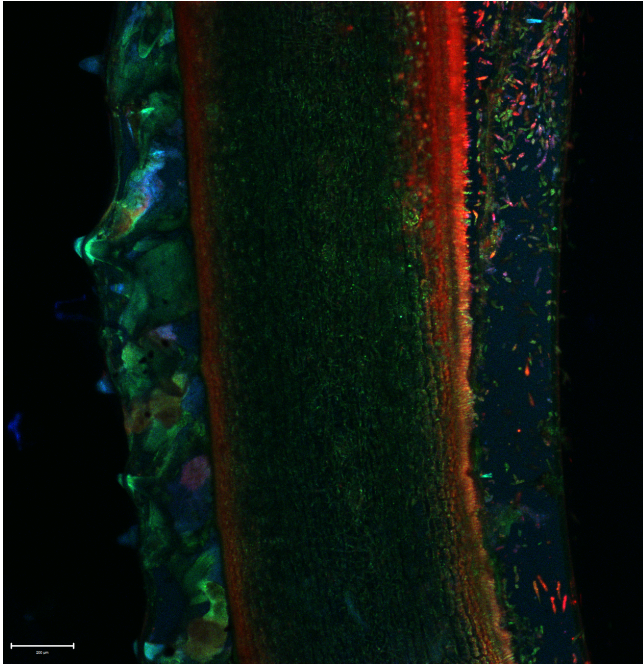
Abstract

Previously, enzymatic saccharification of *S. latissima* using laminarinase has produced moderate yields of sugars that are fermented to ethanol, but there remains no optimized enzyme mixture for this feedstock. Enzymatic hydrolysis with a cellulase cocktail augmented with laminarinase released 60% more glucose than when using laminarinase alone.

The combination of a cellulase cocktail with laminarinase resulted in enhanced glucose release from macroalgae. Increasing biomass loading is not straightforward due to increases in alginate and salt concentration; further research into specific design parameters will play an important role in reducing the process intensity and overall feasibility.

Background

One technique to monitor IL pretreatment is imaging the autofluoresence of biomass at increasing durations of IL pretreatment. These imaging studies have shown a key step in [C$_2$mim][OAc] pretreatment is cell wall swelling [35, 48]. The composition of the biomass [49-

51] and extent of lignification further affect biomass stability and saccharification kinetics [52-55].

Results and Discussion:
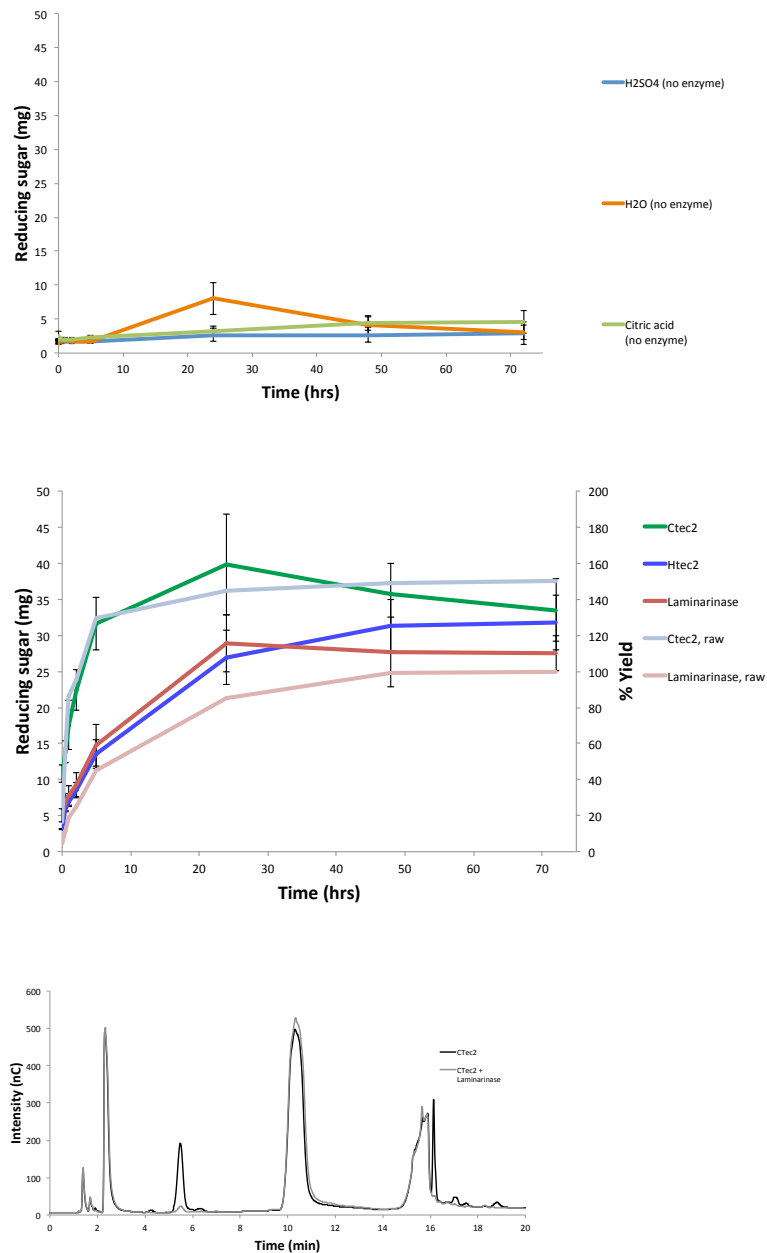
Figure 13: Sugar yields as a function of enzyme cocktail
Analysis done by DNS (top) or HPAEC (bottom)

Previous studies of macroalgae were done using harvested frozen samples with the enzyme laminarinase. We evaluated sugar yields using the same enzyme laminarinase at similar loading of 0.1 U laminarinase per 25 g, using freeze-dried samples to compare our results with the published reports. It was possible to recover ~100% of the glucose as measured from enzymatic hydrolysis using the laminarinase as shown by Adams et al. [14]. To test the possibility of hydrolyzing β (1-4) glucan bonds of cellulose, we digested the algae using a combination of enzymes optimized for digestion of cellulosic biomass (Cellic® CTec2 and HTec2 from Novozymes at a loading of 20 mg protein per g glucan and 2 mg protein per g glucan respectively, Figure 13-15). Interestingly, higher amounts of monomeric glucose were released using the CTec2 than with the laminarinase (Figure 13-15). Furthermore, we observed enhanced glucose release when using the combination of CTec2 with either laminarinase or HTec2 (Figure 13-15). In addition, we found a 2-fold increase in hydrolysis kinetics using CTec2 and further enhancement with the combination of CTec2 with either HTec2 or Laminarinase than either enzyme alone (Table 2). HPAEC analysis confirmed that the CTec2 alone was not hydrolyzing all of the potential monomeric sugars (). We then tested the combination of reduced enzyme loading of CTec2 and HTec2, and found that the enzyme loading could be easily reduced from the recommended 20 mg/g glucan to 5 mg/g glucan with a 5 g glucan per liter loading without a change in yield of final released glucose.

As there was equivalent sugar released from ground as unground (~ 1- 2 cm^2) pieces, we attempted confocal fluorescent imaging during enzymatic saccharification to investigate a potential mechanism. As expected from the enzymatic hydrolysis, there is immediate break down of the biomass during the first 5 hours (Figure 15). We expect that the breakdown of the algae occurs even faster during the batch enzymatic hydrolysis due to mixing that does not occur in the well on the microscope. Interestingly, the outer peripheral layer as seen on the left side of the slice seems to mechanistically inhibit biomass degradation. This is expected, as the outer layer has been shown to contain inhibitory chemicals, phenols and tannins, and provides mechanical support [44, 56].
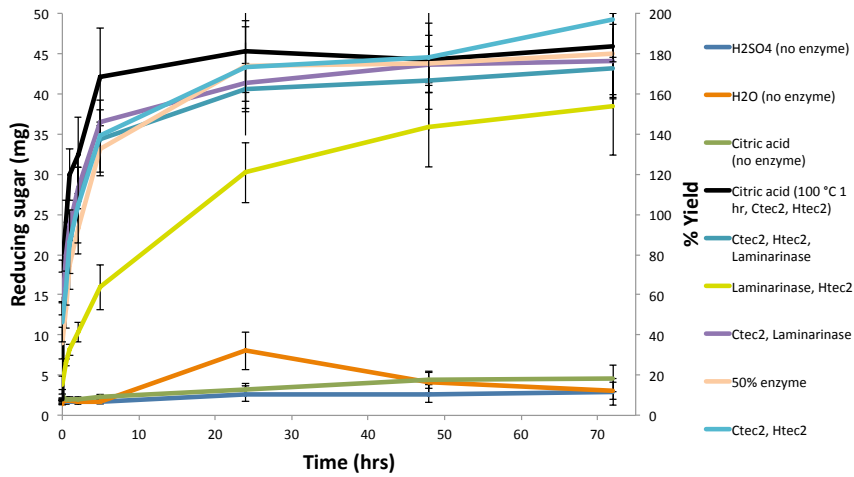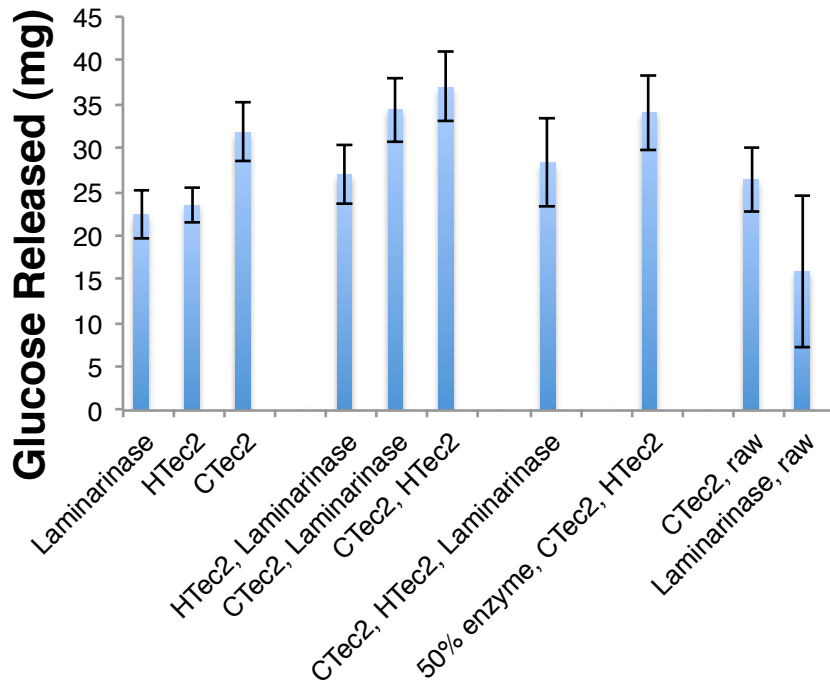
Figure 14: Sugar yields versus enzyme
Timecourse of sugar released during enzymatic saccharification of macroalgae, 5 mL at 5 mg glucose per mL, 50 °C 72 hours glucose measured by HPAEC. Values show with ± 1 SD.

Impact of Salt on Sugar Yields

Higher biomass loading during saccharification can help to reduce operating costs. Increased biomass loading from 2, 4, and 8 wt % during saccharification resulted in sugar yields ranging from 0.103, 0.100, to 0.03 gram glucose per gram biomass per liter for the July harvest, respectively. Previous studies have hypothesized a salt sensitivity on saccharification/ethanol yields above 90

mM [14, 15]. Additionally, high ash content suggested that high ionic strength could play a role in the inhibition (Figure 2, Table 1).

To reduce the soluble salts recovered in our biomass, the algae samples were rinsed using DI water. This was done quickly as to minimize the absorption into the dried algae samples. We analyzed the recovered wash for conductivity and sugars. The lower wash volumes had high concentrations of both salt and sugars. Increasing the wash volume did not proportionally increase the amount of sugar and salt lost in the wash. As expected because of its solubility, a large amount of the sugars lost was in the form of mannitol (Table 3). We quantitated the loss of high molecular weight sugars by acid hydrolysis of the wash and alginate extraction of the solids and found an increase in alginate content in the recovered solids (Table 3).

After the wash the samples were lyophilized to obtain a quantitative comparison of the composition before and after washing. To further confirm the loss of salt and other insoluble material we measured the ash content of the recovered solids (Table 3). Washing increased the alginate percentage of biomass, but reduced the total mass of ash in the samples. This resulted in 71 wt% recovery of the initial biomass (dry weight). We then attempted similar saccharification studies using the same glucose loadings: 0.5 wt% to 2 wt% glucan loadings of both the washed and unwashed samples. The monomeric sugar recovery of the washed sample at the same glucan loading was 94% of the unwashed sample at 0.5 wt% glucan loading, and 50% glucose recovery of that of the unwashed samples at 1 wt% glucan loading. Washing reduced the glucose recovery to an undetectable amount at loadings above 1 wt% glucan. The lower ratio of glucose to alginate in the total biomass required higher total alginate loading to achieve similar loadings of glucan for the washed material versus the raw biomass. Increased alginate concentration is known to significantly increase viscosity [28, 37, 57]. Our washed biomass had a higher alginate to glucan ratio than the unwashed samples and therefore increased viscosity mixtures. The increased viscosity could be acting as inhibitor due to uneven mixing. A more intense washing could help to reduce the viscosity of the alginate by removing ions such as calcium which is known to help stabilize the alginate and increase the viscosity, however, the more vigorous the wash the more sugar is dissolved into the wash solution.

While we obtain increased yields of sugars from the macroalgae using a combination of enzymes, we have found that we get decreased sugar yields after washing the algae. Rinsing the algae results in loss of mannitol and small easily dissolvable sugars, which if not recovered can reduce the potential chemical yield. However, rinsing also reduces the overall phenol and high salt content, which can increase the sugar yield [14, 15].
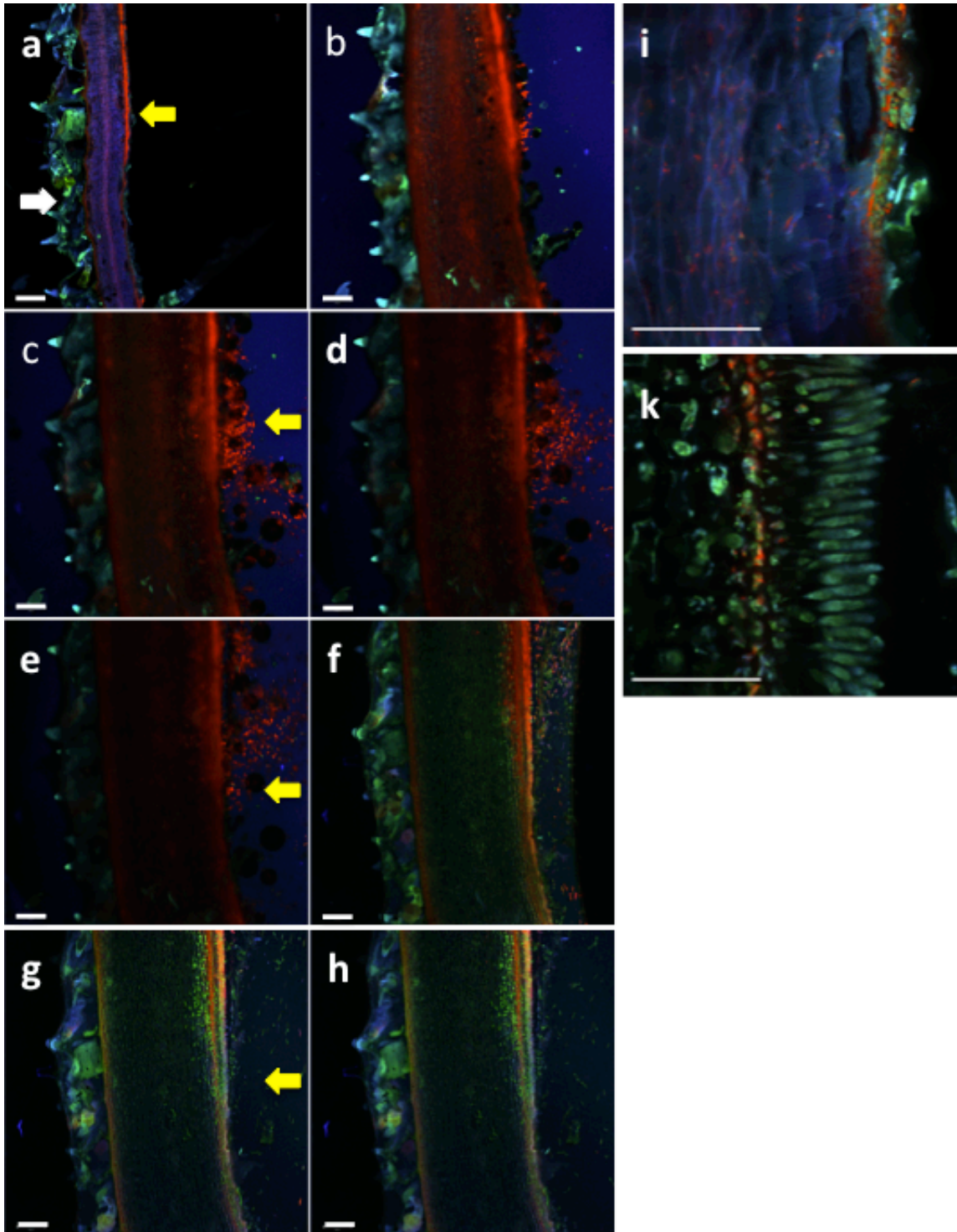
Figure 15: Confocal imaging of enzymatic saccharification
Autofluorescence generated by samples of *S. latissima* during enzymatic digestion after application of CTec2 and HTec2 in 50mM citrate buffer at 50 °C (a) 5x, before solution, (b)5x, 0 min (t = 0 at application of solution) (c) 5x, 15 min, (d) 5x, 30 min, (e) 5x, 1 hr, (f) 5x, 3 hr 30 min, (g) 5x, 5hr, (h) 5x, 5 hr 30 min, (i) 40x, before solution, (k) 40x, 5 hr 30 min scale bars show 100 μm. White

arrows point to outer peripheral layer. Yellow arrows point to side without outer layer and show release of cellular bodies.

Conclusions

Macroalgae shows promise as a potential biomass source for production of fermentable sugars and for advanced biochemical production. A mixture of CTec2 and HTec2 releases the maximum amount of glucose from *S. latissima*. The hydrolysates generated from *S. latissima* are as effective as the glucose control for fermentation with *E. coli* to make the renewable chemical pinene. Increasing the solids loading to a more relevant industrial concentration is not straightforward due to inhibitory effects of increasing alginate and salt concentration. The specific characteristics of the hydrolysate such as viscosity and salt concentration are important for increasing product yields.

Methods

Samples of *Saccharina latissima* were collected off the coast of Norway in Dec 2010, July 2011 and Aug 2011. The samples were then freeze-dried for 1 to 2 days. These samples were then stored in 4°C before and after shipping. Shipping of the freeze-dried algae to Joint BioEnergy Institiute was done at room temperature. Moisture content was continually checked and biomass used was adjusted appropriately. All measurements are calculated from the dried weight.

Enzymatic Saccharification

Enzymatic saccharification of algae samples were carried out at 50 °C and 150 rpm in a reciprocating shaker (Enviro-Genie, Scientific Industries, Inc.) in 50 mM citrate buffer (pH of 4.8). The glucan content in the solution was maintained at 5 g glucan per liter, unless otherwise noted. 20 mg protein/ g glucan of Cellic® CTec2 (Novozymes) and 2 mg protein/g glucan of Cellic® HTec2 (Novozymes) and/or 0.1 U/ 20 g glucan Laminarinase (1 U/ 31 mg Sigma, Adams 2009), were used for hydrolysis reactions unless otherwise noted. 60 µL of the supernatant was taken at specific time intervals (0,0.5, 1, 2, 5, 24, 48, 72 h) to monitor the hydrolysis reaction. The reducing sugars in the supernatant were measured using the dinitrosalicylic acid assay, (DNS), Amplex® Red Glucose/Glucose Oxidase Assay Kit (Molecular Probes, Invitrogen) or HPAEC.

Solutions of D-glucose were used as standards in the DNS and Amplex assays. All assays were performed in triplicates. Error bars show the standard error of triplicate measurements. Amplex® Red Assay was done following the manufacturer's instructions and absorbance was measured at 560 nm.

The aliquots of the supernatants were centrifuged at 10,000g for 5 min, and the reducing sugars in the supernatant were measured using the DNS assay. Each saccharification comparison was run concurrently with all samples in the same comparison to eliminate potential differences in temperature history or other parameters. The rate of hydrolysis was calculated based on the sugar released in the first 30 min of hydrolysis. The supernatant collected after 72 h of hydrolysis was analyzed with HPAEC for the monosaccharide composition. All assays were performed in triplicates, unless noted. It should be noted that the DNS assay does not account for the hydrolysis reaction stoichiometry, of cellulose and hemicellulose upon complete hydrolysis. However, there is minimal hemicellulose released.

Confocal Imaging
Confocal Fluorescence Imaging
S. latissima samples were cut with a razor blade from freeze-dried samples from the December 2010 harvest. These sections were stored at 4°C until imaged. Slices were placed between a coverslip and slide with enough buffered enzyme to wet each sample about 150uL. Autofluorescence images during heating were collected with a Zeiss LSM 710 confocal system mounted on a Zeiss inverted microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY). A 405 nm diode laser and a 488 nm argon laser were used for sequential excitation over a 410-759 nm range with 5x, 10x or 40x objectives. The resulting images were analyzed using the Zen software and reproduced in pseudo color (Zeiss).

Table 2: Hydrolysis rate obtained as a function of enzyme used

					CTec2 Laminarinase	HTec2 Laminarinase	CTec2, unground
	Laminarinase	CTec2, ground	HTec2	CTec2 HTec2			
Rate (mg/L/min)	46 ± 11	92 ± 15	39 ± 3	117 ± 8	132 ± 14	44 ± 4	95 ± 3

Biomass Loading (2% w/w)

*Data based on DNS assay reducing sugar release at 30 min

Table 3: Composition of washed macroalgae samples as a function of season

Composition

Harvest Time	Glucan	Mannitol	Ash	Moisture	Alginate	Solid Recovery
July Washed 2011	17.1% ± 0.6%	20% ± 2%	28.0% ± 0.2%	3.2% ± 0.5%	39% ± 7%	78% ± 7%
August Washed 2011	12.6% ± 0.5%	16% ± 1%	33.5% ± 0.6%	2.0% ± 0.1%	36% ± 2%	72% ± 2%

Section IV – Enzymes

Introduction

Background

From the previous work, the Novozymes cocktails Celliac CTec2 and HTec2 show increased release of glucose from algae than using laminarinase alone. The laminarinase from Trichoderma sp. (L5272) has been discontinued from Sigma-Aldrich.

Richard Heins, Samuel Deutsch, Edward Rubin, Sangeeta Nath, Kenneth Sale, Blake Simmons at JGI and JBEI have been working on sequencing, expressing and characterizing GH1 and endocellulase activity see Table 4. In the process of characterization of the GH1 activity they looked at various dimers of glucose. Some of the GH1s have activity on β 1-3 and β 1-6 bonds that are prevalent in algae (Table 4,5).

Enzyme Families

http://www.cazy.org/ ([1] Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. **Nucleic Acids Res** 37:D233-238 [PMID: 18838391].)

Preliminary Halo and Thermo Tolerant Enzyme Screen

Table 4: GH1 substrate specificity

Substrate specificities of selected GH1s towards various carbohydrate substrates

Enzyme	Reaction temp (°C)[b]	Specific activity (U/mg)[a] Laminaribiose (β1,3)-Glc-Glc	Cellobiose (β1,4)-Glc-Glc	Gentibiose (β1,6)-Glc-Glc	Glucomannan (β1,4)-Glc-Man	Galactobiose (β1,4)-Gal-Gal	Mannobiose (β1,4)-Man-Man
204 Cow_Rumen	30	54.57	8.62	0.16	1.80	0.07	0
4 Streptomyces-sp.	40	8.51	6.99	0.95	2.57	0.09	0
202 Cow_Rumen	40	20.03	3.12	0.69	2.49	0	0
22 Thermobifida-fusca	50	42.58	34.81	0.62	22.21	0.07	0.42
37 Alicyclobacillus-acidocaldarius	55	95.09	70.77	6.96	34.02	0.91	0
72 Caldivirga-maquilingensis	60	53.24	28.04	13.34	17.61	2.00	0
83 Anoxybacillus-flavithermus	60	71.59	4.16	0.58	3.29	0.02	0
89 Halothermothrix-orenii	60	62.15	29.36	7.12	18.22	1.94	0
125 Meiothermus-ruber	60	4.63	2.81	1.79	0.53	0	0
157 Clostridium-thermocellum	65	127.66	3.22	0.98	5.15	0.92	0.24
84 Thermosipho-africanus	70	94.00	59.1	2.65	37.20	0.41	0.32
85 Thermosipho-africanus	70	100.57	38.17	13.70	27.73	2.44	0
116 Thermobaculum-terrenum	75	75.89	49.80	15.99	46.11	10.17	0
8 Sulfolobus-solfataricus	80	19.22	16.90	30.90	11.89	5.07	0
36 Sulfolobus-acidocaldarius	80	48.39	8.36	3.57	4.51	1.90	0

[a] Determined with 10 mM (final concentration) of each substrate in 50 mM acetate buffer pH 5 for 10 minutes. A unit is defined as 1 μmol of monomer produced in one minute per mg enzyme as measured by HPLC for all substrates except. Values represent the average of three measurements with a standard deviation of 10% or less.

[b] Highest temperature at which enzyme retained >= 90% activity after a 1 hr preincubation.

Study 1:

In order to test the sensitivity and evaluate the process, we started with a small collection of the above in-house enzymes at JBEI (Table 5).

Saccharification parameters: 24 hours, 3 mg laminarin or algae/mL, 40 μL per 100 μL reaction

We also tried Kevin Chen's mutlisubstrate Cel5C2 enzyme (endocellulase activity). Rich selected for high β 1-3 and β 1-6 activity.

Table 5: Selected GH1 enzymes of interest for algae deconstruction

Enzyme			% activity IL 70C 1 day	uL Enz	uL Buffer	Stability Temp (Rich 1 hr)	Actual rxn temp (24 hrs)
8	Sulfolobus solfataricus	Archaea	4	12.07	67.93	80	70
116	Thermobaculum terrenum	Bacteria	0	6.91	73.09	75	70
21	Thermus nonproteolyticus	Bacteria	18	8.67	71.33	85	70
89	Halothermothrix orenii	Bacteria	0	12.93	67.07	60	45
37	Alicyclobacillus acidocaldarius	Bacteria	0	19.25	60.75	55	45
k	Cel5C2			2.59	200		

It seems the Enzyme-8 from Sulfolobus solfataricus P2, is the highest activity at 24 hours producing about 55% of laminarin and 70% from algae (Figure 16). Working on calibrating other peaks to have a better understanding of Kevin's Cel5C2 enzyme activity (but the slight increase in glucose is promising).
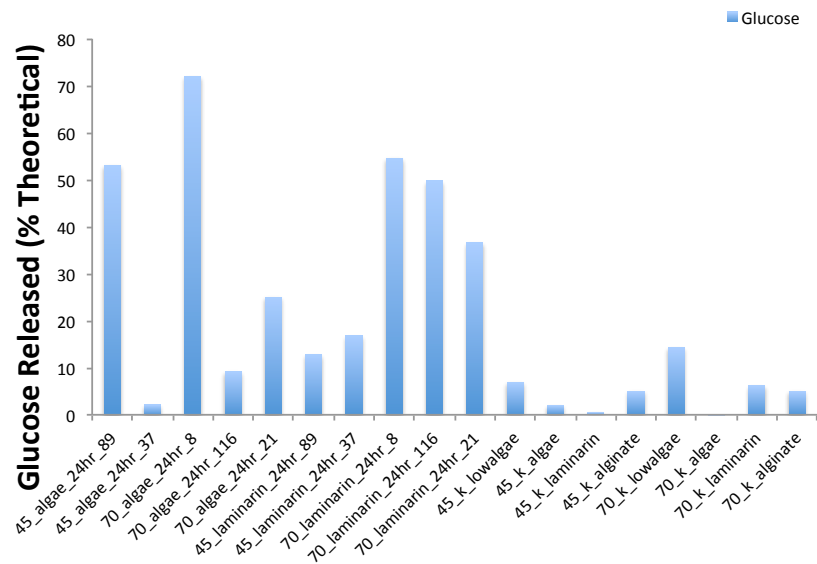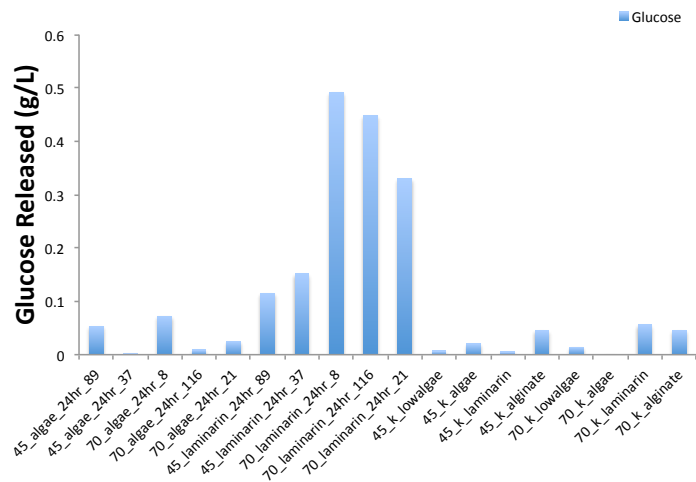
Figure 16: GH1 algae screen
Absolute glucose (top) release versus theoretical glucose released (bottom)
(relative to control)

Preliminary Experiment 2:

1 hour, 3 mg laminarin or 6 mg algae/mL final concentration, 50uL reaction volume in triplicate, NaOAc pH 4.9, used to calculate the specific activity

Evaluated a larger collection of GH1 enzymes. As expected from the first experiments 116 and 8 worked well on the laminarin. Unexpectedly, 116 released the most glucose from the algae samples.

Table 6: GH1 algae specific substrates

Substrate specificities of selected GH1s towards various carbohydrate substrates

| | | Specific activity (U/mg)[a] | |
| | | Laminarin | Algae |
Enzyme	Reaction temp (°C)[b]	(β1,3) & (β1,6)	(β1,3) & (β1,6)
204 Cow_Rumen	30	0.01	0.04
4 Streptomyces-sp.	40	0.08	0.05
202 Cow_Rumen	40	0.42	0
22 Thermobifida-fusca	50	0.01	0.11
37 Alicyclobacillus-acidocaldarius	55	1.10	0.21
72 Caldivirga-maquilingensis	60	0.27	0.21
83 Anoxybacillus-flavithermus	60	0.70	0.09
89 Halothermothrix-orenii	60	1.00	0.09
125 Meiothermus-ruber	60	3.72	0
157 Clostridium-thermocellum	65	0.36	0.07
84 Thermosipho-africanus	70	0.15	0.04
85 Thermosipho-africanus	70	0.76	0
116 Thermobaculum-terrenum	75	11.57	0.49
8 Sulfolobus-solfataricus	80	11.56	0.13
36 Sulfolobus-acidocaldarius	80	0.08	0

[a] Determined with 10 mM (final concentration) of each substrate in 50 mM acetate buffer pH 5 for 10 minutes. A unit is defined as 1 μmol of monomer produced in one minute per mg enzyme as measured by HPLC for all substrates. Values represent the average of three measurements with a standard deviation of 10% or less.

[b] Highest temperature at which enzyme retained >= 90% activity after a 1 hr preincubation.

Section V – Fermentation
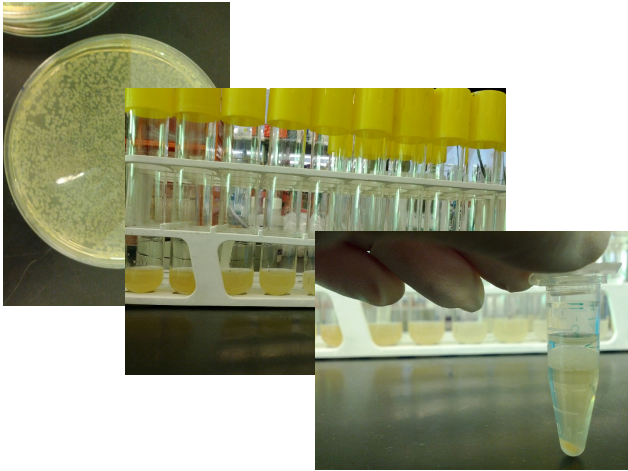
While macroalgae hydrolysate has typically been used for ethanol or methanol production, a more novel complex chemical production has not been demonstrated. The macroalgae hydrolysates were used as a carbon source for the production of pinene, a relatively high value chemical precursor, making use of a novel two plasmid *Escherichia coli* system. The yields obtained from the macroalgae hydrolysates resulted in equivalent pinene production from the *E. coli* as compared to *E. coli* grown on glucose. Further, the enzymatic saccharification hydrolysates were useful for the novel microbial production of pinene with no further treatment and/or purification. These results indicate that macroalgae represent an attractive feedstock for the production of advanced microbial synthesized high-value renewable chemicals.

Advances in understanding microorganism metabolism has allowed molecular engineering to produce novel and complex biofuels and chemicals, including butanol, fatty acid esters, methyl ethyl ketones and terpenes [16-18]. The microbiological production of these chemicals helps to reduce our dependence on fossil fuels. Testing and use of developed microorganisms with biomass-generated hydrolysates is necessary to begin to understand the biomass specific process hurdles that could prevent commercialization [16-18]. Components found in the hydrolysate of biomass such as salts, phenols, or other materials can inhibit microbial growth and can have an impact on the efficiency of conversion. This is particularly important for the microbes and pathways being manipulated for production of advanced chemicals, such as pinene, which have low

yields even under ideal conditions. Previous efforts to produce pinene have resulted in production around 1 mg/L for 2% switch grass loading, equivalent to a ~1% glucose hydrolysate loading. As scaffolding has been shown to increase flux through metabolic pathways [58], here we have made a protein fusion of pinene synthase and geranyl diphosphate synthase to increase the pinene titers (see Methods).

Pinene Production from Macroalgal Hydrolysate

Pinene is a terpene that is a high value chemical precursor for multiple commercial components, such as aromatic chemicals in cosmetics, and is currently extracted from plants such as pine or eucalyptus trees [59]. Using an *E. coli*-based production system [16-18] pinene was produced from both the hydrolysates obtained from macroalgae and a glucose control. To increase the flux through the pinene biosynthetic pathway, we made a protein fusion of pinene synthase to geranyl diphosphate synthase increase pinene production [58]. We found that the hydrolysate made using either the (1) CTec2 and HTec2 or (2) CTec2 and Laminarinase contained the largest amount of sugars but produced similar amounts of pinene. The unground but crushed samples produced the same amount of sugar after saccharification as the ground samples, and the hydrolysates resulted in similar pinene production (Figure 17). Interestingly, a 0.6 wt% glucose loading which resulted from 60% of the medium being replaced with a 1 wt% glucose hydrolysate resulted in similar pinene production. Doubling the solids loading for the saccharification step resulted in the expected increase in sugar present in the hydrolysate 2 wt% glucose hydrolysate and a 1.2 wt% final concentration, but the pinene production was significantly reduced (Figure 18). The reduction in yield could be due to the increased viscosity from alginate of salts similar to the inhibition of the enzymatic saccharification of the washed samples. This suggests that the parameters for optimizing hydrolysate concentration are not the only parameters necessary to optimize for biosynthetic chemical production. Increased salt and alginate concentrations of higher loadings could also be critical parameters for commercialization or even large scale processing during bioconversion (Figure 19).
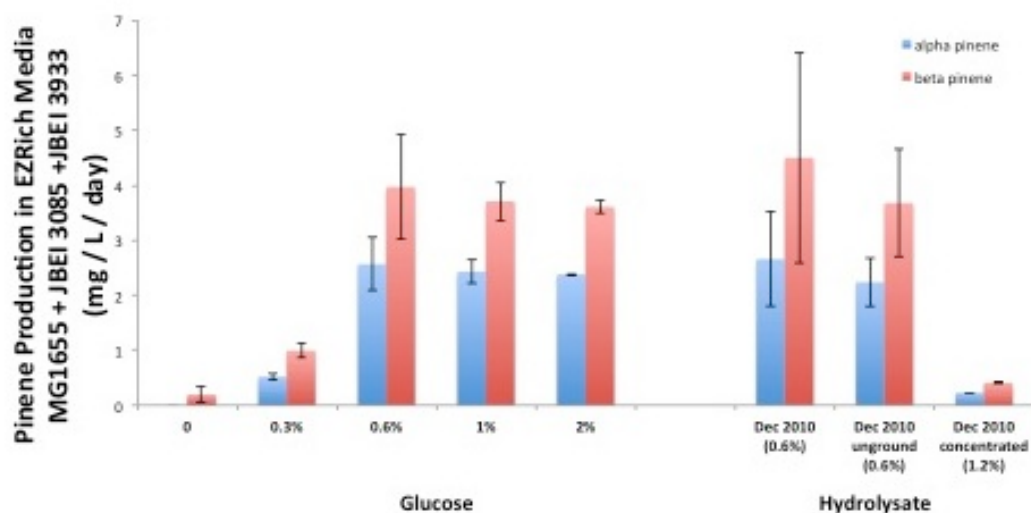
Figure 17: Pinene production in E. coli
Pinene production from glucose control (0, 0.3%, 0.6%, 1%, 2% (w/v) glucose) and
enzymatic saccharification hydrolysates derived from macroalgae enzymatic
hydrolysis with CTec2 and HTec2 for December 2010 harvest at 0.6% glucose
loading, and a twice as concentrated hydrolysate (1.2% glucose loading equal
volume replaced). Values shown ± 1 SD.

Pinene production in E. coli.

E. coli MG1655 was co-transformed with pJBEI-3085 carrying the mevalonate pathway

from aceoacetyl-CoA transferase to isopentenyl diphosphate isomerase [18] and JBEI -

3933 carrying a protein fusions of *Abies grandis* pinene synthase linked to *A. grandis*

geranyl diphospahte synthase using a (Gly-Ser-Gly)$_2$ linker. Fusion of proteins have been

shown to increase the flux through metabolic pathways [58]. Pre-cultures of *E. coli*

MG1655 harbouring the appropriate plasmids were used to inoculate at a 1:25 dilution

pinene production medium (Teknova, EZ-Rich, 1% (v/v) glucose, 100mg/L ampicillin,

30 mg/L chloramphenicol, 5 ml total volume). The cultures were grown at 37 °C for 3 h

(200 r.p.m., OD$_{600}$=0.6–0.8) before induction with 1 mM IPTG and overlayed with 20%

dodecane. After growth for 72hrs at 30 °C (200 r.p.m.), 10 µL of the dodecane overlay

were sampled and diluted into 90 µl of ethyl acetate spiked with terpinene as an internal

standard. The samples were analysed by GC/MS (Agilent 6890 with Agilent 5973 Mass

selective detector) with a DB5 column (30 m×0.25 mm ID ×0.25 μm film) using either an alpha or beta pinene standard curve using the following conditions: inlet at 230 °C, 2 ml min^{-1} constant flow, transfer line at 300 °C, ion source at 230 °C, scan *m/z* 50–300. Oven: 60 °C for 1 min, ramp at 20 °C min^{-1} to 120°C, ramp at 50°C to 250 °C.
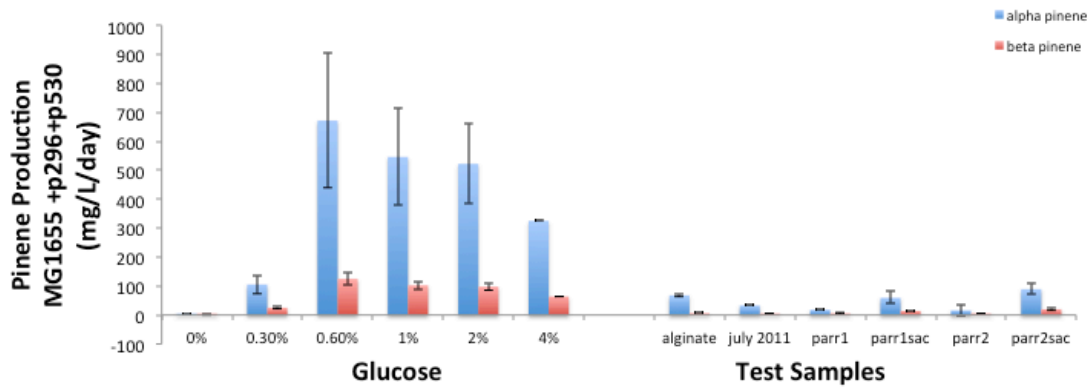
Figure 18: Pinene production of additional enzymatic saccharifications
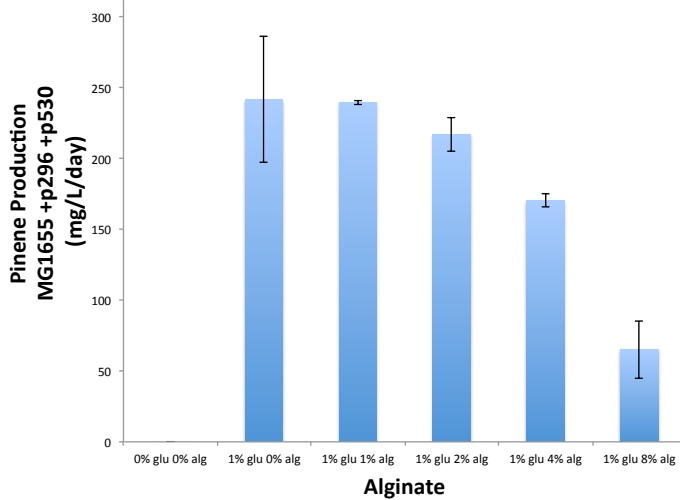
Figure 19: Pinene production versus alginate concentration

Section VI - Future Directions and Initial Results

High-throughput screening

The NX^P robot has been tuned to aliquot into a 96 well plate ground Algae (dried 40

mesh), laminarin, alginate (both high and low viscosity) distribution, evaluated with CTec2 and DNS. Following optimization, 15 g/L ground Algae was dispensed with precision of 3% STD across the plate (Figure 20). This variance has since been reduced by the addition of magnets to the paddle wheel stirring reservoir.
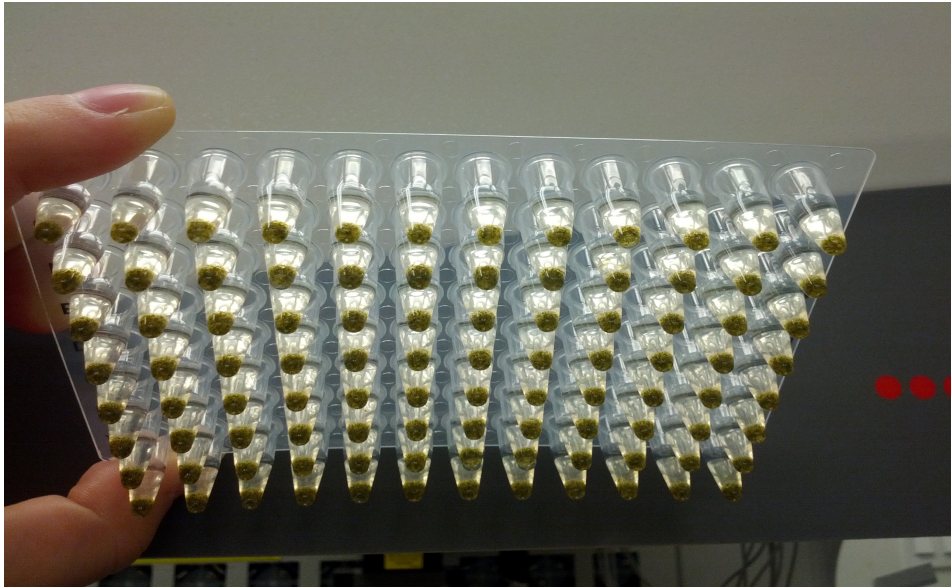
Figure 20: Dispensed macroalgae
Algae from the Aug 2012 harvests at 15 g/L diluted ~2 fold.

2013-11-25_October_Ctec2_no-oil 4.5% STD across plate
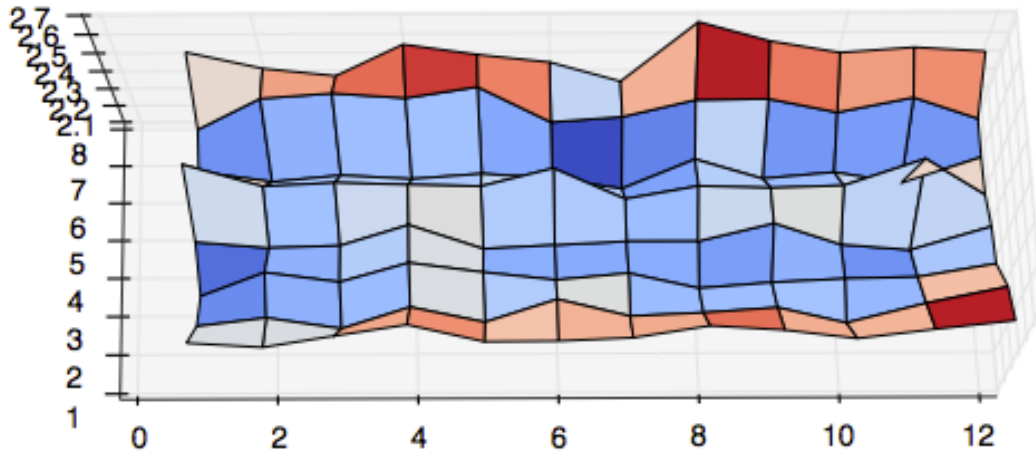
2013-11-25_August_Ctec_no-oil 3% STD across plate
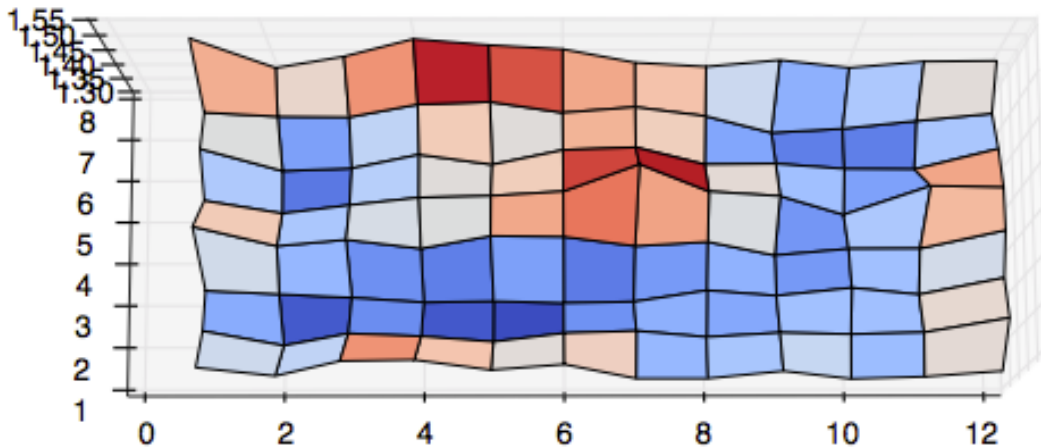
Figure 21: Saccharification profile across a 96 well plate
Dispensed algae from the October 2013 (top) and Aug 2012 (bottom) harvests at 15 g/L
diluted ~2 fold. Saccharification was done at 50 °C for 24 hrs, CTec2 20 mg/g glucan,
HTec2 2 mg/g glucan, in pH 5 citrate buffer (heat map shows relative values reflecting
subtle changes across the plate red high – blue low).

To check that our DNS screen is a useful proxy for glucose release, dose-response
experiments were carried out in 96 well plates with a serial dilution of the CTec2/HTec2
cocktail. Plates were incubated 24 hours and assayed by DNS and HPLC (Figures 21-23).

Sugar Release (Abs) vs. Enzyme Loading (mg/g glucan CTec2/HTec2) with model
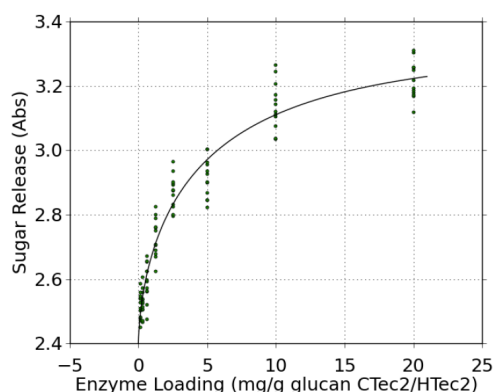
Figure 22: Enzyme loading for dispensed plate analysis
Dispensed algae from the October 2013 and Aug 2012 harvests at 15 g/L diluted ~2 fold.
Saccharification was done at 50 °C for 24 hrs, with a two-fold serial dilution of
CTec2/HTec2 from 0 mg/g glucan to CTec2 20 mg/g glucan, HTec2 2 mg/g glucan in pH
5 citrate buffer. Data fit using a Hocket-Sherby 2D exponential function.
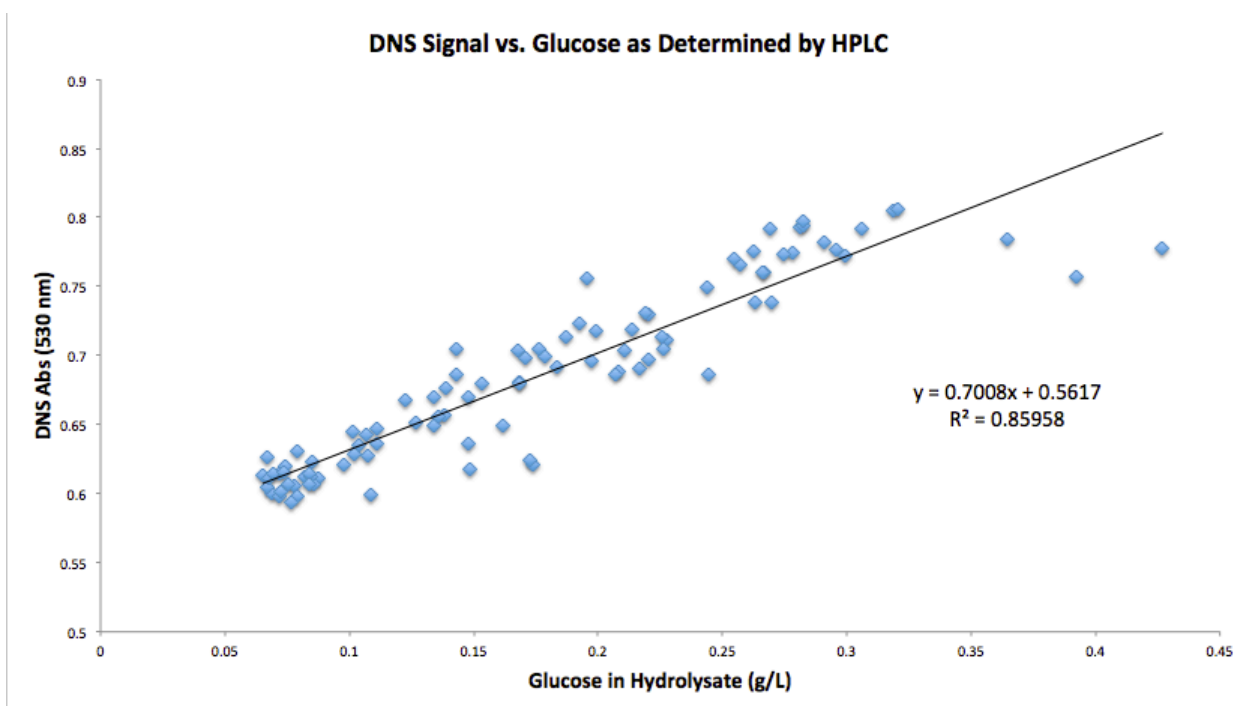
Figure 23: DNS versus HPLC results
DNS Absorbance at 530 nm vs. glucose in hydrolysate as determined by HPLC for the
same plate as shown in Figure 3. Data fit with a linear regression.

Enzyme profiling

There are currently 3 sets of enzymes to screen via robotics:

1. Follow up on initial screen with Rich's GH1 enzymes. Expand to get a pH and temp profile for larger scale trials on algae, starting with enzyme 8 and 116, possibly followed with profile across other enzymes of interest found in Table 7.

By screening over a pH gradient (4.2 - 8.0 50mM citrate buffer) and a temperature gradient (60 – 85°C), identified likely T_{opt}/pH_{opt} for GH1 #8 (pH 5.7 / 78°C). DNS absorbance data were measured in duplicate and analyzed by Design-Expert Version 8 software with a quartic model, and lambda = -1. Will continue using this pipeline for other GH candidates, (Figure 24).
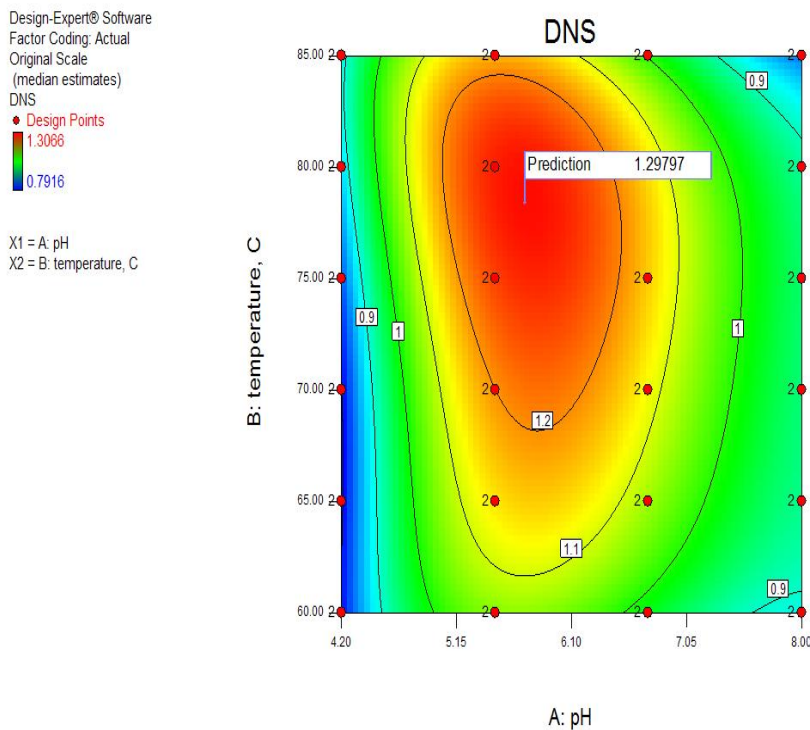
Figure 24: Contour plot of GH1 activity
Quartic fit with prediction of optimum DNS release parameters for GH1 #8, 24 hrs run across a pH gradient between 4.2 and 8, and temperature gradient between 60 and 85 °C.

2. Screening potential candidates from different thermophilic and halotolerant pools of enzymes. Created cell pellets (Protocol for 96 well plate celllysate.docx) from Taya's HTP screen that expressed. They will be lysed and tested at pH 5 over a temperature gradient.

Table 8: List of enzymes from JBEI thermotolerant and halo tolerant enzymes
Taya's screen expressed in cell pellets (2012_10_Screening List_short.docx, 2013_10_Taya_Plate contents.xlsx – antibiotic resistance listed)
Mesophilic fungal

TIL-1	Cel7A_Cth_CBH_1	Chaetomium thermophilum ALKO4265
TIL-3	Cel7_Cth_CBH_2	Chaetomium thermophilum CT2
TIL-4	Cel7A_Tau_CBH	Thermoascus aurantiacus ALKO4242
TIL-5	Cel7A_Ath_CBH	Acremonium thermophilum
TIL-6	Cel7A_Tem_CBH_IB	Talaromyces emersonii (structure 1Q9H)
TIL-7	Cel6A_Tem_CBH_2	Talaromyces emersonii
TIL-8	Cel45A_Hin_EG_1	Humicola insolens
TIL-9	Cel5A_Tem_EGI	Talaromyces emersonii
TIL-10	Cel5A_Tau_EGI	Thermoascus aurantiacus var. levisporus
TIL-12	Cel7A_Hgr_EGI	Humicola grisea var. thermoidea
TIL-14	Cel45_Hgr_EGIII	Humicola grisea var. thermoidea
TIL-15	Cel45_Hgr_EGIV	Humicola grisea var. thermoidea
	Thermophilic Fungal	
TIL-17	Cel6A_Hje_CBHII	Hypocrea jecorina (aka, T. reseii)
TIL-18	Cel7B_Hje_EGI	Hypocrea jecorina (aka, T. reseii)
TIL-20	Cel12A_Hje_EGIII	Hypocrea jecorina (aka, T. reseii)
TIL-21	Cel5B_Hje_EGVIII	Hypocrea jecorina (aka, T. reseii)
	Thermophilic Bacterial	
TIL-57	J24	Paenibacillus provencensis
TIL-58	J26	Rhodothermus marinus

TIL-59	J28	Thermobaculum terrenum
TIL-60	J29	Bacillus halodurans
TIL-61	J30	Mitsuokella multacida
TIL-62	J36	Rhodothermus marinus
TIL-63	J37	Prevotella ruminicola
TIL-64	J40	Caldicellulosiruptor saccharolyticus
TIL-65	J41	Pyrococcus horikoshii
TIL-66	Cel9A	Alicyclobacillus Acidocaldarius (pRAH0008)
TIL-67	Cel5A	Thermotoga Maritima (pRAH0010)
TIL-68	J5	Gemmatimonas aurantiaca
TIL-69	J16	Rhodothermus marinus
TIL-77	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-78	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-81	Cellobiohydrolase A (1,4-beta-cellobiosidase A) (EC:3.2.1.91)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-82	Cellobiohydrolase A (1,4-beta-cellobiosidase A) (EC:3.2.1.91)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-83	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-85	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-86	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-89	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)

TIL-91	Cellobiohydrolase A (1,4-beta-cellobiosidase A) (EC:3.2.1.4)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-94	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-95	Cellobiohydrolase A (1,4-beta-cellobiosidase A) (EC:3.2.1.8)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-96	Cellobiohydrolase A (1,4-beta-cellobiosidase A) (EC:3.2.1.8)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-98	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	Rice-straw enriched compost microbial community from Berkeley (Thermophilic 454/Illumina Combined June 2011 assem)
TIL-99	family 48 glycoside hydrolase	Thermobispora bispora
TIL-100	family 12 glycoside hydrolase	Thermobispora bispora
TIL-101	cellulose-binding family II protein	Thermobispora bispora
TIL-102	beta-glucosidase	Thermobispora bispora
TIL-103	family 3 glycoside hydrolase domain-containing protein	Thermobispora bispora
	Mesophilic fungal	
TIL-105	GH5 (CAZ67882)	Thermoascus aurantiacus
TIL-106	GH61(PDB:3ZUD_A)	Thermoascus aurantiacus
TIL-107	GH3_C (ABX79552)	Thermoascus aurantiacus
	Mesophilic Bacterial	
TIL-112	GH9_meso_650950961	Clostridium acetobutylicum DSM 1731
TIL-114	GH9_meso_640946212	Bacillus pumilus SAFR-032
TIL-115	GH9_meso_643607670	Clostridium cellulolyticum H10
TIL-116	GH9_meso_646370686	Fibrobacter succinogenes succinogenes S85, ATCC 19169
TIL-117	GH9_meso_647681987	Micromonospora carbonacea var. africana. ATCC 39149
TIL-120	GH5_meso_2507042280	Brenneria salicis Dye EX2, ATCC 15712

TIL-122	GH5_meso_2511533243	Bacillus subtilis spizizenii TU-B-10, DSM 15029
TIL-123	GH5_meso_638925493	Dokdonia donghaensis MED134
TIL-124	GH5_meso_641415155	Coprococcus eutactus ATCC 27759
TIL-130	GH5_meso_645959419	Butyrivibrio crossotus DSM 2876
TIL-132	GH5_meso_2509839911	Cytophaga fermentans IAM 14302, DSM 9555
TIL-137	GH9_meso_643607676	Clostridium cellulolyticum H10
TIL-139	GH9_meso_645657384	Clostridium papyrosolvens DSM 2782
TIL-141	GH9_meso_650460979	Vibrio furnissii 2510/74, NCTC 11218
TIL-143	GH9_meso_638072236	Cytophaga hutchinsonii ATCC 33406
TIL-144	GH9_meso_646185055	Vibrio mimicus VM573
TIL-145	GH9_meso_648715300	Acetivibrio cellulolyticus CD2, DSM 1870
TIL-147	GH9_meso_651514356	Paenibacillus sp. HGF5
TIL-153	GH9_meso_651110860	Clostridium acetobutylicum EA 2018
	Halophilic Bacterial	
TIL-166	GH3_halo_2510061272	Clostridium alkalicellulosi Z-7026, DSM 17461
TIL-172	GH3_halo_646747158	Zunongwangia profunda SM-A87
TIL-173	GH3_halo_643612427_BlpI	Halothermothrix orenii H 168
TIL-175	GH3_halo_643610841	Halothermothrix orenii H 168
TIL-176	GH3_halo_646385968	Haliangium ochraceum SMP-2, DSM 14365
	Thermophilic Bacterial	
TIL-177	GH1	R. Heins GH1_8
TIL-178	GH1	R. Heins GH1_21
TIL-179	GH1	R. Heins GH1_26
TIL-180	GH1	R. Heins GH1_30
TIL-181	GH1	R. Heins GH1_36
TIL-182	GH1	R. Heins GH1_37
TIL-183	GH1	R. Heins GH1_67
TIL-185	GH1	R. Heins GH1_72
TIL-186	GH1	R. Heins GH1_216
TIL-187	GH1	R. Heins GH1_76

TIL-188	GH1	R. Heins GH1_84
TIL-189	GH1	R. Heins GH1_85
TIL-190	GH1	R. Heins GH1_92
TIL-191	GH1	R. Heins GH1_93
TIL-192	GH1	R. Heins GH1_116
TIL-193	GH1	R. Heins GH1_125
TIL-194	GH1	R. Heins GH1_139
TIL-195	GH1	R. Heins GH1_147
TIL-196	GH1	R. Heins GH1_156
TIL-197	GH1	R. Heins GH1_160

3. Testing of different known laminarinases that have known structures.

Through Genscript, synthesized 11 previously crystallized laminarinases cloned into a pET28a vector. Laminarinases were expressed, purified, and are currently being profiled on CM-Curdlan as a first pass for ß-1,3 activity at relevant conditions (Table 9). Promising candidates will be profiled on macroalgae to determine optimal conditions (Figure 25).

Table 9: Potential Laminarinases

Name	PDB ID	source organism	Host	Assay Temp (C)	aa sequence	Length (aa)
Lam1	3AZX	Thermotoga maritima MSB8	E. coli	40	MEDEDKVEDWQLVWSQEFDDGVIDPNIWNFEIGNGHAKGIPGWGNGELEYYTDENAFVENGCLVIEARKEQVSDEYGTYDYTSARMTTEGKFEIKYGKIEIRAKLPK GKGIWPALWMLGNNIGEVGWPTCGEIDIMEMLGHDTRTVYGTAHGPGYSGGASIGVAYHLPEGVPDFSEDFHIFSIEWDEDEVEWYVDGQLYHVLSKDELAELGLEW VFDHPFFLILNVAVGGYWPGYPDETTQFPQRMYIDYIRVYKDMNPETITGVEHHHHHH	272
Lam2	3ILN	Rhodothermus marinus	E. coli	80	MRLPHWELVWSDEFDYNGLPDPAKWDYDVGGHGWGNQELQYYTRARIENARVGGGVLIIEARRESYEGREYTSARLVTRGKASWTYGRFEIRARLPSGRGTWPAIW MLPDRQTYGSAYWPDNGEIDIAEHVGFNPDVVHGTVHTKAYNHLLGTQRGGSIRVPTARTDFHVYAIEWTPEEIRWFVDDSLYYRFPNERLTNPEADWRHWPFDQPF HLIMNIAVGGTWGGQQGVDPEAFPAQLVVDYVRVYRWVE	252
Lam3	2VY0	Pyrococcus furiosus	E. coli	90	MVPEVIEIDGKQWRLIWHDEFEGSEVNKEYWTFEKGNGIAYGIPGWGNGELEYYTENNTYIVNGTLVIEARKEIITDPNEGTFLYTSSRLKTEGKVEFSPPVVVEARIKL PKGKGLWPAFWMLGSNIREVGWPNCGEIDIMEFLGHEPRTIHGTVHGPGYSGSGKGITRAYTLPEGVPDFTEDFHVFGIVWYPDKIKWYVDGTFYHEVTKEQVEAMGY EWVFDKPFYIILNLAVGGYWPGNPDATTPFPAKMVVDYVRVYSFVSG	264
Lam4	1MAC	Bacillis macerans	E. coli		MGSVFWEPLSYFNPSTWEKADGYSNGGVFNCTWRANNVNFTNDGKLKLGLTSSAYNKFDCAEYRSTNIYGYGLYEVSMKPAKNTGIVSSFFTYTGPAHGTQWDEIDI EFLGKDTTKVQFNYYTNGVGGHEKVISLGFDASKGFHTYAFDWQPGYIKWYVDGVLKHTATANIPSTPGKIMMNLWNGTGVDDWLGSYNGANPLYAEYDWVKYTS N	213

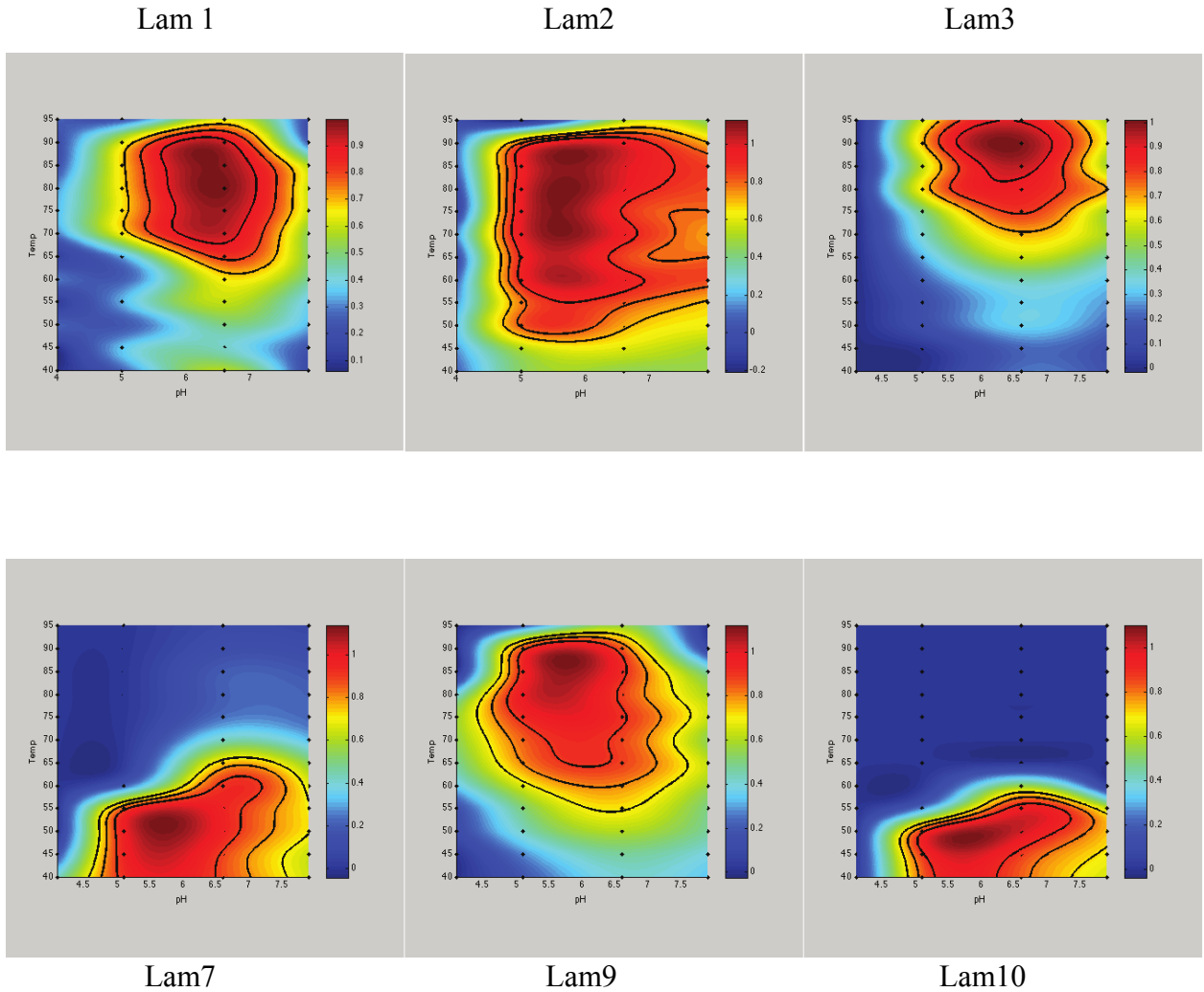|						
Lam5	1GBG	Bacillus licheniformis			MQTGGSFYEPFNNYNTGLWQKADGYSNGNMFNCTWRANNVSMTSLGEMRLSLTSPSYNKFDCGENRSVQTYGYGLYEVNMKPAKNVGIVSSFFTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVNLGFDAANSYHTYAFDWQPNSIKWYYVDGQLKHTATTQIPQTPGKIMMNLWNGAGVDEWLGSYNGVTPLYAHYNWVRYTKR	215
Lam6	3DGT	Streptomyces sioyaensis	E. coli	70	MSAPAPPSGWSQVFLDDFDGAAGSSVNTANWQFDTGTSYPGGAGNWGTGEVESMTSSTSNVSLDGNGDLLITPRRDASGNWTSGRIETTRTDFQPPAGGKLRVEARLQMPNVTGDAAAGYWPAFWMLGAPFRGNYQNWPGVGELDIMENVQLNKTWATMHCGTSPGGPCNETSGIGNSTACPNTTCQSGFHTYTMEWDRSVSPEAIRFSVDGVTYQTVTANQMDAATWTNATNHGFFVILNVAMGGGFPGAFGGGPTGATEPGHPMVVDYYQVTSLSPGL	281
Lam7	2HYK	Nocardiopsis sp.strain F96	E. coli	Thermophillic	MTESDMRATLVWSDEFDGPAGSAPDPANWNHETGDHGWGNNELQNYTDSRANSALDGNGNLVITARQEADGGYTSARLTTQNKVQPQYGRVEASIQIPRGQGIWPAFWMLGADFPNTPWPDSGEIDIMENIGREPHLVHGSLHGPGYFGGEPLTGSYMHPQGWSFADTFHTFAVDWRPGSITWSVDGVAYQTYTSADTRGNPWVFDQPFFMILNVAVGGDWPGYPDGSTQFPQEMRVDYVRVYELG	246
Lam8	1MVE	Fibrobacter succinogenes	E. coli	45	MVSAKDFSGAELYTLEEVQYGKFEARMKMAAASGTVSSMFLYQNGSEIADGRPWVEVDIEVLGKNPGSFQSNIITGKAGAQKTSEKHHAVSPAADQAFHTYGLEWTPNYVRWTVDGQEVRKTEGGQVSNLTGTQGLRFNLWSSESAAWVGQFDESKLPLFQFINWVKVYKYTPGQGEGGSDFTLDWTDNFDTFDGSRWGKGDWTFDGNRVDLTDKNIYSRDGMLILALTRKGQESFNGQVPRD	243
Lam9	4DFS	Thermotoga petrophila RKU-1			MGNSIHMYTEEDEDKVEDWQLVWSQEFDDGVIDPNIWNFEIGNGHAKGIPGWGNGELEYYTDENAFVENGCLVIEARKEQVSDEYGTYDYTSARMTTEGKFEIKYGKIEIRAKLPKGKGIWPALWMLGNNIGEVGWPTCGEIDIMEMLGHDTRTVYGTAHGPGYSGGASIGVAYHLPEGVPDFSEDFHIFSIEWDEDEVEWYVDGQLYHVLSKDELAELGLEWVFDHPFFLILNVAVGGYWPGYPDETTQFPQRMYIDYIRVYEDKNP	268
Lam10	3ATG	Cellulosimicrobium cellulans			MAPGDLLWSDEFDGAAGSAPNPAVWNHETGAHGWGNAELQNYTASRANSALDGQGNLVITARREGDGSYTSARMTTQGKYQPQYGRIEARIQIPRGQGIWPAFWMLGGSFPGTPWPSSGEIDIMENVGFEPHRVHGTVHGPGYSGGSGITGMYQHPQGWSFADTFHTFAVDWKPGEITWFVDGQQFHRVTRASVGANAWVFDQPFFLILNVAVGGQWPGYPDGTTQLPQQMKVDYVRVYDNGSGSSNPGNPGTGLPT	257
Lam11	1UPS	Clostridium perfringens	E. coli		MKDFPANPIEKAGYKLDFSDEFNGPTLDREKWTDYYLPHWCKDPESAKANYRFENGSLVEYITEDQKPWCPEHDGTVRSSAIMSFDKSWIHNFSGTTDNHERNEWRGYTTKYGYFEIRAKLSNTGGGGHQAWWMVGMQDDTNDWFNSKQTGEIDILETFFSKKDTWRIAAYGWNDPNFQTSWTISEDKVPSGDPTSEYHIYAMEWTPTALKFYYDNELFKVIYGSPDYEMGTILNIYTDAGSGAHNDVWPKEWAIDYMRVWKPVDGYKESESLNNYLIRNRQTGKFLYIEENNDKVSYGDITLKNEKNAKWSKEYRDGYTLLKNNETGEYLNIENQTGYIEHGKVPKTWWSAQWSEVPVDGYTRFVNRWKPNMSIHTESYEGVLQYGNVPNTYWTSQWQLIPVE	404

Figure 25: Laminarinase profiles versus pH and Temp
Lam1, Lam2, Lam3, Lam7, Lam9 and Lam10 profiles (top to bottom, left from right).
All laminarinase screening was done at 0.5% CM-Curdlan 20 min preincubation at temp
range with enzyme & buffer premixed then substrate addition and incubated for 10 min at
temp grad. DNS assay with 2 mM glucose, read at 520 nm.

Alginate Assay

Environmental DNA has been extracted from several environments of interest, including salt ponds and aquatic sediments. After purification of high molecular weight DNA (>20kb fragments) using a Boreal Aurora SCODA machine, DNA was end repaired and ligated into a fosmid vector from Epicentre. Glycerol stocks of *E. coli* fosmid libraries were prepared for the following experiments:

1. Solid media tolerance assays: library will be plated on minimal media agar plate with alginate as sole carbon source. Inserts from any surviving colonies will likely contain genes of interest.

2. Liquid media DNS assay: single colonies will be picked to 96 well plates and grown in LB/alginate media for 24 hours. Culture will be spun down and an aliquot will be taken and sugar release will be measured using the DNS assay. The inserts from any hits will likely contain genes of interest.

Fosmid libraries will also be prepared from strain CAIM 615, which is currently being isolated. Initial screening did not result in possible colonies.

Hydrolysate Production

We attempted producing 100 mL of hydrolysate from the October 2013 batch of algae. Initial trials of small volume saccharification resulted in reduction of sugar after 24 hrs. The hydrolysate was plated on LB and algae plates at 50°C and then picked for colony PCR, and found to be from the genus *Bacillus*.

Attempts to work out a low key pretreatment resulted in wet autoclaving at 121°C for 30 min to release and maintain the most amount of sugar (Figure 26).

We have since run many 10 mL-scale saccharifications of CTec2 (20 mg/g glucan) and HTec2 (2 mg/g glucan) in 50 mM Citrate Buffer, pH 4.95 on 40 mg/mL and 80 mg/mL *S. latissima* from the October and August harvests. Despite promising early data, we have continued to see relatively low yields of glucose released (<40% from the 40 mg/mL algae loading in the most recent trial) most likely due to contamination.
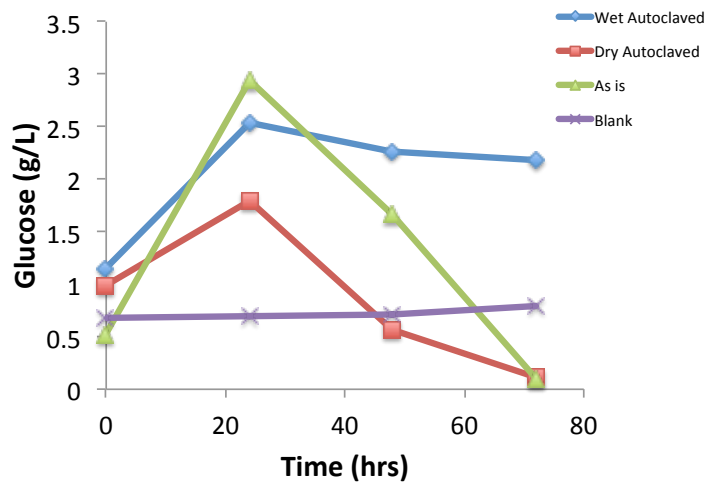

Figure 26: Time course of saccharification of Oct 2013 harvest
 S. latissima with CTec2 (20 mg/g glucan) and HTec2 (2 mg/g glucan) in 50 mM Citrate Buffer, pH 4.95. Shows that wet autoclaving keeps the Bacillus from eating the released glucose.

Halo and Thermo Tolerant Enzymes

Using the results of the screening, we were able to pair a GH1 with a Laminarinase to examine potential synergy. The GH1 #8/Laminarinase #1 resulted in high sugar release. Increasing the loading we found that it produced higher yields that the CTec2/HTec2 combination (Figure 27).
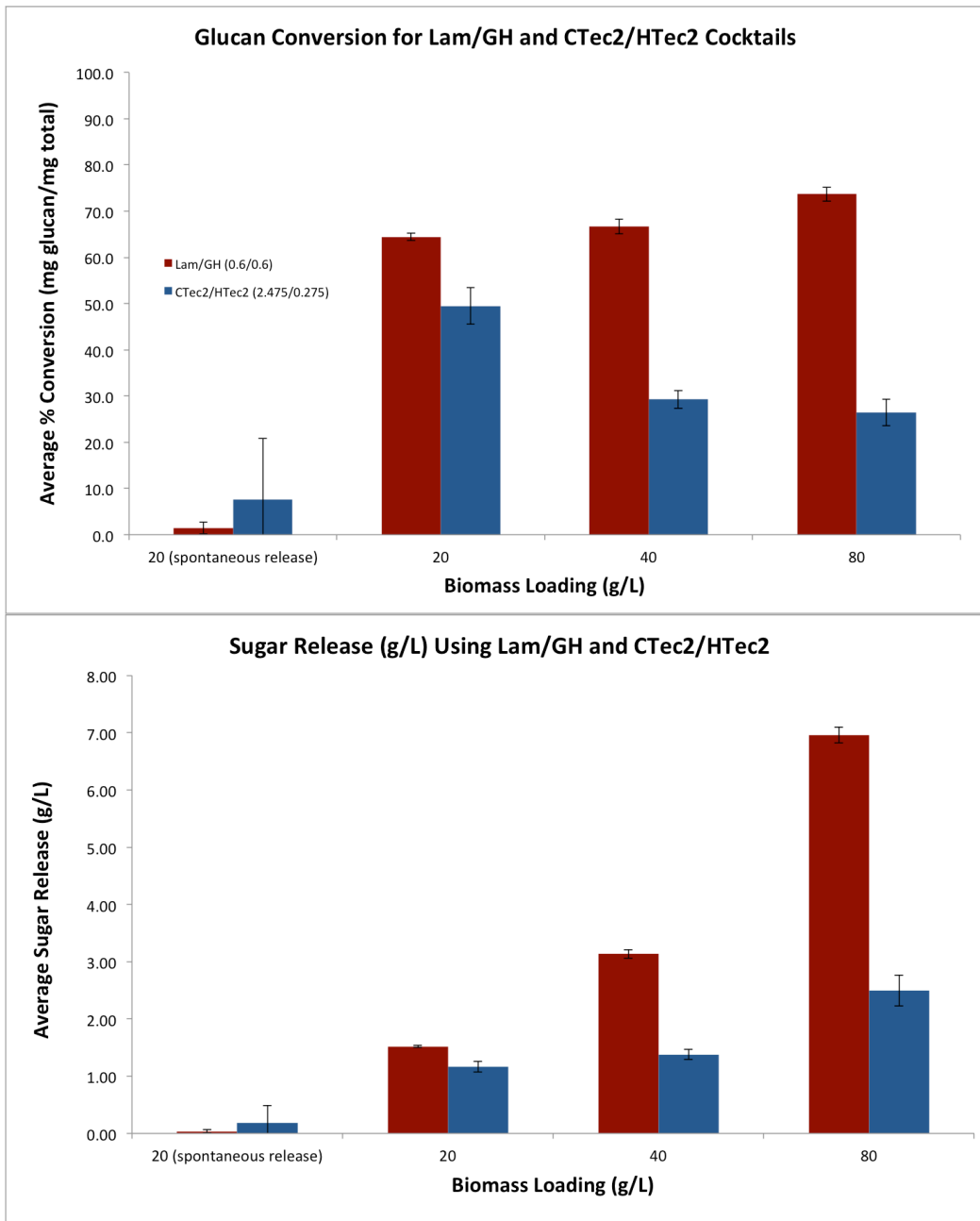

Figure 27: Comparison of CTec2/HTec2 mixture versus Lam 1/GH1 #8 mixture

Economics of a macroalgae biorefinery: (How) can it be viable?

Macroalgal biomass is generally considered as a prospective feedstock for biofuel production as it offers a number of benefits [14, 60-62] including: 1) it is an abundant renewable carbon source, 2) its growth does not require arable land, fertilizer or fresh water, 3) it is not a primary food crop, 4) it grows rapidly (e.g., 2,960 dry MT/km^2/yr [61]). The presence of significant quantities of polysaccharides (i.e., laminarin, alginate, and small amounts of other sugars) and sugar alcohols (i.e., mannitol), the absence of a secondary cell wall (i.e., no lignin) and the low levels of hemicellulose make certain species of brown macroalgae (e.g., *Saccharina Latissima*) particularly attractive for the production of biofuels [63]. Successful deployment of macroalgae-based biorefineries, however, depends on their economic viability at industrial scale. With that in mind, the key objective of this study was to carry out a detailed technoeoconomic analysis (TEA) of this process to understand the economic potential and cost drivers of macroalgal biorefineries.

In this work, a detailed process model for a macroalage-to-ethanol biorefinery was built. Given the current status and possible technological advances, several scenarios were constructed by varying key process and economic parameters. These include: 1) macroalgae price ($50 to 200/dry MT), 2) overall yield (50 to 80%), 3) solids loading in hydrolysis (5 to 20%), and 4) enzyme loading (10 to 20mg protein/g polysaccharide). With a delivered macroalge price of $100/MT, depending on the maturity of the other process parameters (i.e., yield, solids, and enzyme loading), the Minimum Ethanol Selling Price (MESP) was observed to be in the range of $3.6-$8.9/gal. Overall, the price of macroalgae and the yield remain key parameters in determining the economic feasibility of the biorefinery. For instance, the feedstock price needs to be less than $50/MT to ensure MESP is lower than $3/gal (at 80% yield) and with every $50/MT increase in the price of macroalgae, the MESP would increase by $0.6-1/gal (for the yield range studied). Solids loading

was observed to be as significant as the yield as it affects both the capital and operating expenses. In addition to the macroalgae-to-ethanol biorefinery configuration, we have studied other configurations that could possibly improve the economics of biorefinery – these additional configurations include the possibility to co-produce alginate and ethanol.

Summary

Macroalgae, particularly *S. latissima*, shows promise as a biomass source for renewable chemical production. Hurdles for use at commercial scale are increasing biomass loading, reducing contamination problems, and having more reliable methods for characterization. All pretreatment methods resulted in reduced yields compared to straight enzymatic saccharification. While the commercially available enzyme mixtures CTec2 and HTec2 produce high yields and fast kinetics, they have significantly reduced yields at more industrially relevant loadings (>20 g/L). Using high throughput screening methods has allowed us to find 2 potential thermotolerant enzymes that work on algal substrates, providing a mixture that overcomes potential viscosity and salt restrictions of higher biomass loading. Using a mixture of GH1 #8and laminarinase #1 picked from the screening results in a mixture that has retained efficiency at higher biomass loadings (>80 g/L) – providing a more relevant mixture for industrial use.

Acknowledgements

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Appendix A

Papers

Chessa Scullin, Vitalie Stavila, Anita Skarstad, Jay D. Keasling, Blake A. Simmons, and Seema Singh**, Optimization of Renewable Pinene Production from the Conversion of Macroalgae Saccharina latissima**, Bioresource Technology

Conferences

International Conference on Algal Biomass, Biofuels and Bioproducts

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Investigation of Bacterial Isolates for Algal Polysaccharide Alginase and Laminarinase Activity

Chessa Scullin, Anita Skarstad, Børre Tore Børresen, Evy Mellemsæther, Blake Simmons and Seema Singh

2012

Optimization of Sugar Production from the Brown Algae *Saccharina latissima*

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Records of Invention

Thermotolerant mixture of Glucoside hydrolase family 1, beta-glucosidases and laminarinase for algal biomass deconstruction

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