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ORIGINAL PAPER

Application of ensilage as a green approach for simultaneous preservation and pretreatment of macroalgae *Ulva lactuca* for fermentable sugar production

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Abstract
Green macroalgae *Ulva lactuca* could be a potential marine biomass feedstock for the production of biofuel and biochemicals. However, the high moisture content makes long-term preservation of fresh *Ulva* biomass a challenge. Ensilage has been suggested as a green approach to preserving and pretreating fresh biomass without intensive energy input. In this study, silage additives including cellulase complex and inoculum of *Lactobacillus plantarum* were tested and applied to circumvent the difficulties associated with ensilage of the *Ulva* species, such as insufficient water-soluble carbohydrate and low lactic acid bacteria (LAB) count. The experimental results with statistical analysis indicated that the addition of both cellulase complex at 10 carboxymethyl cellulose unit (CMCU)/g dry biomass and inoculum of *Lactobacillus plantarum* at 10^6 cfu/g dry biomass was necessary to drop silage pH value to lower than 4 in 15 days. The successful preparation of *Ulva* silage could retain around 92% solid and most of the carbohydrates and the ensiled *Ulva* biomass could produce more reducing sugar than fresh biomass by dilute acid hydrolysis at high solid content and moderate temperature. Moreover, with further enzymatic hydrolysis, the *Ulva* silage proved to be fermentable by LAB for lactic acid production. The results suggested that ensilage could be a useful process for simultaneous preservation and pretreatment of *Ulva* biomass for fermentable sugar production.

Keywords Ensilage · Biomass preservation · Pretreatment · Fermentable sugar · *Ulva lactuca*

Introduction

During the last decade, global climate change has raised public awareness about human beings' excessive reliance on the usage of petroleum for energy and chemical production. Biorefinery of plant biomass to bio-based chemical and energy, such as lactic acid and bioethanol, has been suggested as part of the solutions to achieving sustainable developments. (Srirangan et al. 2012; del Castillo-Romo et al. 2018). Nevertheless, despite the renewable characteristics, the increasing demands of the food crops and terrestrial plant biomass for biorefinery directly compete with the arable lands and agricultural resources (Havlík et al. 2011; Valentine et al. 2012). These kinds of conflicts will only be elevated over the time with the growth of global population. In order to avoid this dilemma, aquatic biomass such as algae has been defined as the third-generation feedstock for biorefinery (Hoevers 2011). Among all kinds of aquatic biomass, algae are commonly acknowledged for their potential as a renewable resource to generate a variety of chemicals and fuels (Jones and Mayfield 2012; Bikker et al.2016; Resdi et al. 2016).

Sea lettuce belongs to the family of green macroalgae *Ulvaceae* which commonly exist in the littoral zone of the coast (Zhu et al. 2016). The *Ulva species* have been traditionally used as animal feed, herbal medicine, and food ingredient in many human civilizations (Vázquez-Rodríguez and Amaya-Guerra 2016). Additionally, the *Ulva* species have been extensively evaluated for different types of applications related to environmental biotechnology such as bioremediation of wastewater, bioabsorption of toxic metal ions from water bodies (Henriques et al. 2017; Shaaban et al. 2017), and biological indicator of the aqueous environment (Farias et al. 2017). Because of their rapid nutrient uptake capabilities, promotion of biomass reproduction and

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wide ranging salinity tolerance, many investigations have suggested that *Ulva lactuca* could also be used as a new feedstock for bioenergy production (Bruhn et al. 2011; van der Wal et al. 2013; Chen et al. 2015a, b). Yet, research related to storage and supply logistics of the *Ulva* species or other macroalgal biomass is relatively limited in comparison with studies of biomass conversion technology. Storage of macroalgal biomass is a challenge because of its high water content, and freshly harvested macroalgal biomass can spoil rapidly if the storage is not properly implemented.

Conventional storage of macroalgal biomass is usually done by oven dry or sun dry (Foscarini and Prakash 1990; Mabeau and Fleurence 1993); in addition, storage at low temperature may also work in preserving fresh macroalgae biomass (Onodera et al. 2011). However, these storage approaches require either high energy consumption or permissible weather conditions, which might not suitable in terms of low cost and a consistent supply of feedstock for bio-based chemicals and fuels (Kadam et al. 2015; Franco et al. 2017). Ensilage is a wet storage approach for biomass preservation, which had been traditionally employed in the industrial animal agriculture. The main objective of ensilage is to prevent the loss of carbohydrates by creating a low-pH environment where the microbial activity is inhibited or decreased. Storage of biomass through the ensilage process has many advantages over dry storage including low risk of fire and less loss of dry matter (Oleskowicz-Popiel et al. 2011). Nevertheless, in comparison with the ensiling of lignocellulosic biomass, there is only some information available on the ensiling of macroalgae. An early study done by Black (1955) examined ensiling of brown seaweeds for animal feed and chemical processing purposes and concluded that the macroalgae supports lactic acid fermentation; however, pH values of the seaweed silages did not decline below the desired level. A similar investigation reported by Herrmann et al. (2015) also indicated that high buffering capacities, insufficient fermentable sugars and low initial counts of lactic acid bacteria (LAB) are the main challenges for ensiling macroalgae. Many technical reports and research studies recommend that LAB counts should be higher than 10^5 CFU/g biomass for silage preparation (Weinberg and Muck 1996; Muck 2008; Basso et al. 2012; Abdul Rahman et al. 2017). There is no strict required value of initial fermentable sugar content for silage preparation, because the sugar profile varies with different sources of biomass. For ensilage, the amount of fermentable sugar is usually measured as water-soluble carbohydrates (WSC). A report suggests that at least 7% WSC is sufficient for preparing wheat straw silage (Yang et al. 2006). Another report suggests that at least 2.5% WSC is required to obtain an acceptable silage quality of fresh material, while 2% WSC is adequate for inoculated silage (Pettersson and Lindgren 1990). In order to accelerate anaerobic fermentation and drop the pH level, LAB inoculum, cell degrading enzymes, and chemical additives are often added at the beginning of ensilage (Kung and Shaver 2001). A successful preparation of macroalgae silage as fish hatchery feeds was done by adding LAB and yeasts at the beginning of ensilage (Uchida et al. 2004). Ensilage can also be employed as a biological approach to pretreat biomass for further processing. For example, enzymatic digestibility of grape pomace and sugar beet pulp was improved by ensilage (Zheng et al. 2011a, b; 2012). Thus, the aim of this study was to apply ensilage as a green approach for simultaneous preservation and pretreatment of *Ulva lactuca* and to test the fermentability of *Ulva* silage for biorefinery purposes by lactic acid fermentation. In order to overcome the problems associated with low initial LAB and low soluble carbohydrates in the *Ulva* biomass, the effects of factors including addition of cell wall degrading enzyme and inoculation of LAB at the beginning of ensilage on the carbohydrate preservation and subsequent dilute acid hydrolysis were investigated and reported. Additionally, lactic acid fermentation was performed to evaluate the fermentability of *Ulva* silage.

Materials and methods

Biomass preparation

Biomass of *U. lactuca* was purchased from local residents who collected the fresh biomass from the coast near the Hoping Island (Keelung, Taiwan). The fresh biomass was used directly or stored in Ziploc® bags at − 20 °C until use.

Chemicals and enzymes

All chemicals used in the present study are analytical grade purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) except the sulfuric acid used to prepare the mobile phase for high-performance liquid chromatography (HPLC) analysis is HPLC grade. Cellulase enzyme complex ACCELLERASE®1500 was given by DuPont™ Genencor® Science (Rochester, NY, USA) as a gift.

LAB cultivation and ensilage inoculum preparation

Lactobacillus plantarum purchased from Bioresource Collection and Research Center (BCRC# 10069, Hsinchu, Taiwan) was used to ensile the biomass of *U. lactuca* and to ferment acid hydrolysate of ensiled biomass. *L. plantarum* was maintained and precultured in MRS broth (HiMedia M369-500G, Midland Scientific, Inc. Omaha, NE, USA). The preparation of ensilage inoculum was done according to the reference published by Zheng et al. (2011a, b).

Ensilage of *U. lactuca*

A 2^2 experimental design plus a central point was performed to test the effects of ensilage additives, cellulase (10 CMCU/g dry biomass) and LAB (1×10^6 cfu/g dry biomass), on the carbohydrate recovery after ensilage and the yield of reducing sugar after dilute acid hydrolysis. Triplicates of five base experimental combinations (Table 1) were generated by using a statistical software Design Expert v8 (Stat-Ease, Inc. MN, USA) and carried out in random order. Ensilage of sea lettuce was conducted in 500-ml airtight screw-capped PP jars at 25 °C in an isothermal incubator. Before ensilage, the water content of *Ulva lactuca* was first determined, and then 59 g equivalent dry weight biomass was packed into the jars. The final moisture content was adjusted to 80% by adding distilled water which contains the designed amount of LAB and cellulase. The content in the jar was manually mixed by using a stir rod and then tightly screw caped. The ensilage period was 30 days and the pH of each silage fermentation was monitored using a potable pH meter (CLEAN PH30 Tester, Clean Instrument Co., Ltd, New Taipei City, Taiwan) by opening jar at storage periods of 0, 7, 15, 22 and 30 days in an anaerobic glove box. After ensilage, total solid, WSC, total carbohydrates, and the biomass composition were determined. The organic acids in silage effluent and cold water extracts of silage samples were analyzed by HPLC.

Dilute acid hydrolysis of *Ulva* biomass

Dilute acid hydrolysis was carried out in 250-ml screw-capped laboratory bottles (DURAN®, GL 45, Wertheim/Main, Germany) with 100 ml total reaction volume. Ensiled and raw biomass of sea lettuce was hydrolyzed in the presence of a designated concentration of sulfuric acid at 15% (w/w) solid loading and 80 °C for designated period. Two milliliters of the well-mixed hydrolysate was neutralized with $CaCO_3$, and then the liquid and solid in the hydrolysate were separated by centrifugation at 6000 ×g for 20 min. The amount of reducing sugar in the supernatant was determined by dinitrosalicylic acid (DNS) method. The content of monosaccharides, organic acids, and fermentation inhibitors in the hydrolysate was identified and quantified using HPLC.

Table 1 Coded and actual value of experimental design factors

Factor	Coded		Actual value[a]
Addition of cellulase complex	−1	+1	0 10 CMCU/g dry biomass
Inoculation of LAB			0 10^6 cfu/g dry biomass

[a]*CMCU* Carboxymethyl cellulose unit, *cfu* colony-forming unit

Lactic acid fermentation of acid hydrolysate

The acid hydrolysate (4% H_2SO_4, 120 min) of silage with both additives was neutralized to pH 5 by slow addition of 6 N NaOH. The neutralized acid hydrolysate was further hydrolyzed with ACCELLERASE®1500 (0.2 ml/g silage, dry basis). The reducing sugar concentration was also determined by DNS method. Then, the enzymatic hydrolysate was used as solution to prepare MRS medium without addition of glucose and the final concentration was also adjusted to 2% (w/w) by addition of sterilized DI water. Overnight culture of LAB (BCRC 10069) was inoculated to 25 ml silage hydrolysate MRS medium in a 125-ml screw-capped Erlenmeyer flask at initial optical cell density at 600 nm (OD_{600}) equal to 0.2. Two milliliters of samples was withdrawn at 0, 8, 16, 24, 48, and 72 h of fermentation in an anaerobic glove box. The optical cell density and the concentration of lactic acid, acetic acid, and monosaccharides were determined.

Analytical methods

The pH values were averaged from three measurements of each sample by using a pH meter. The solid content was determined according to the NREL Laboratory Analytical Protocol (LAP) "Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples" (Sluiter et al. 2008). For compositional carbohydrate analysis, silage and raw biomass were dried at 45 °C and powdered by bead beating (Mini-BeadBeater-1, Bio Spec Products Inc., Bartlesville, OK, USA) with 3 stainless beads (diameter 3.2 mm) in 2-ml capped stainless steel microvials (catalog # 2007, Bio Spec). The powdered biomass was hydrolyzed by two-stage sulfuric acid hydrolysis according to a published report (Chen et al. 2015a, b) modified from the NREL Laboratory Analytical Protocol (LAP) "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al. 2012). The acid hydrolysate was first neutralized using $CaCO_3$ followed by static precipitation at 4 °C overnight and centrifugation at a relative centrifugal force of 6000 ×g for 20 min. The content of monosaccharides, organic acids and fermentation inhibitors resulted from ensilage and acid hydrolysis was analyzed by HPLC equipped with a Rezex ROA-organic acid column and guard cartridge (Phenomenex Inc., Torrance, CA, USA), RID and UV detectors ($\lambda = 210$ nm).

Data analysis

All presented values are the average of three replicates, unless specified otherwise. Design Expert V8 was used to perform statistical analyses (Version 8.0; SAS Institute,

Raleigh, NC). The statistical significance of treatment effects and mean comparisons were determined by analysis of variance (ANOVA) with significance level $\alpha = 0.05$.

Results and discussion

Effect of additives on the pH variation over the ensiling period

A quick and effective front-end fermentation during early ensiling is essential to drop the pH to lower than 4. The low-pH environment can prevent microbial growth and reduces the loss of dry matter and nutrients (Zheng et al. 2011a, b). In order to circumvent the problems associated with ensiling macroalgae, including insufficient fermentable sugars and low initial LAB count (Herrmann et al. 2015), commercial cellulase complex and LAB inoculum were applied in this study as silage additives. Because the biomass of *Ulva* sp. contains cellulose as part of its carbohydrate structure (Trivedi et al. 2011), the aim of adding cellulase complex was to release some fermentable sugars from the *Ulva* biomass at the beginning of ensilage and the LAB inoculum could successively promote the lactic acid fermentation. The result (Fig. 1) showed that only the silage treated with both additives could reach the target pH (< 4) in 15 days from the beginning of ensilage. There are a limited number of successful cases that can be found on the ensilage of Ulva species (pH < 4); one report demonstrated that additions of certain types of cellulase and co-inoculation of LAB and yeast could be beneficial for preparation of fermented marine silage feed from *Ulva reticulate* (Felix and Pradeepa 2011). Here, both the addition of cellulase complex (p value < 0.0001) and LAB inoculum (p value < 0.0001) were required to drop the pH value of the silage based on the statistical analysis (Table 2). Among all experimental sets, only the silage received both the cellulase complex (10 CMCU/g dry biomass) and LAB inoculum (10^6 cfu/g dry biomass), and it reached a pH value lower than 4 at the end of ensilage period. The addition of cellulase (coded coefficient = −0.66) seems to be slightly more important than the inoculum of LAB on silage (coded coefficient = −0.27). For silages without the addition of the cellulase complex, the pH remained above 5.5 and high concentrations of volatile fatty acids (VFA) were observed (Table 3), with the sign of spoilage and growth of molds appearing after 30 days of ensiling. These results are similar to reports that examined the ensilage of macroalgae without supplement of silage additives (Herrmann et al. 2015) or with inoculum of LAB only (Cabrita et al. 2017). The formation of VFA could be attributed to the activity of clostridia and other unwanted microbes. Clostridia and mold growth is not favored for ensilage because they can consume lactic acid and produce VFA resulting in a pH rise that opposes the preservation mechanism of ensilage. The growth of unwanted clostridia, mold, and yeast can also degrade carbohydrates and protein into VFA, ammonia, and biogas, leading to substantial loss of nutrients and dry matter (Kung Jr 2001). Based on the pH value (Table 3) and solid recovery (Table 4), a good quality silage of *Ulva lactuca* could be prepared by addition of cellulase complex and LAB inoculum at the beginning of ensilage.

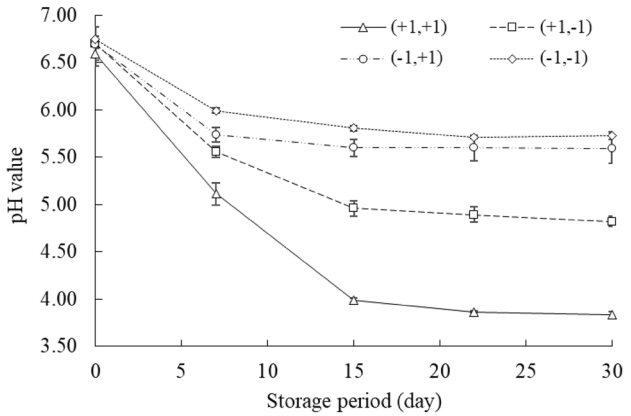

Fig. 1 Changes of silage pH value during the ensiling period. (+, +) with cellulase and LAB inoculum, (+, −) with cellulase only, (−, +) with LAB inoculum only, and (− ,−) without silage additives

Table 2 Summary of analysis of variance (ANOVA)

Source	Sum of squares	*df*	Mean square	*F* value	*p* value
Block	2.2388	1	2.238802		
Model	6.571825	3	2.190608	296.5625	< 0.0001*
A-Cellulase complex	5.1614	1	5.161408	698.7466	< 0.0001*
B-LAB inoculum	0.9020	1	0.902008	122.113	< 0.0001*
AB	0.5084	1	0.508408	68.82784	< 0.0001*
Pure error	0.0739	10	0.007387		
Corrected total	8.8845	14			

Final equation in terms of coded factors: pH = 4.52-0.66A-0.27B-0.21AB

*Statistical significance

Table 3 Final pH, organic acids, and VFA concentration after a 30-day ensilage

Silage preparation		Final pH	Organic compounds (mg/g dry matter)	
Cellulase	LAB		Lactic acid	VFA[a]
−1	−1	5.73 ± 0.04	33.5 ± 3.2	29.2 ± 0.9
−1	+1	5.59 ± 0.16	52.8 ± 3.1	22.8 ± 1.9
+1	−1	4.82 ± 0.05	73.7 ± 7.0	18.4 ± 1.6
+1	+1	3.84 ± 0.03	96.0 ± 8.8	10.4 ± 1.1

Numbers are present as average ± standard deviations of triplicate analyses

[a]VFA is the sum of acetic acid, propionic acid, butyric acid, isobutyric acid, and valeric acid

Effect of ensilage conditions on the biomass composition

Table 4 shows that all preparations of silage without addition of the cellulase complex lost more than thirty percent of solid after 30 days of ensiling. The loss of dry matter is clearly caused by the degradation associated with unwanted microbial activity because the pH value did not drop enough to fulfill the preservation mechanism of ensilage. Since the solid lost during the ensiling period is influenced by many factors including biomass type, moisture content, and environmental conditions (Muck 1988), the data derived from one feedstock might not be totally appropriate for predicting other feedstocks. Nevertheless, the solid recovery could still be an indicator of silage quality (Kung Jr 2001). An early report published by FAO has suggested that ensilage could preserve more than 90% of the energy content from the harvested plant biomass (McCullough 1978). For some types of plant biomass such as sugar beet pulp, the loss of dry matter could be negligible if ensilage is effective (Kreuger et al. 2011; Zheng et al. 2011a, b; Liu et al. 2016). On the contrary, some studies have suggested that dry matter loss could be substantially higher (> 10%). In this study, a good quality of Ulva silage with around 92% solid recovery was obtained from the silage preparation with both cellulase complex (10 CMCU/g dry biomass) and LAB inoculum (10^6 cfu/g dry biomass).

All the silage preparations contained relatively less percentage of sugar composition than the raw biomass, except the fraction of rhamnose and arabinose (Table 4). Since the pH values of silages without both additives did not drop below pH 4, the decrease in sugar content might be attributed to the consumption and degradation caused by microbial activity. The degradation of carbohydrates commonly happens in land plants and might be also applicable to macroalgae, for example, the loss of starch content during the postharvest processing (Finger et al. 1999). The data also

Table 4 Solid recovery and biomass compositions of ensiled and fresh *Ulva lactuca* biomass

Silage preparation		Solid recovery (%)	Water-soluble carbohydrate	Biomass component (% of recovered solid)					
Cellulase	LAB			Glucose	Xylose + mannose + galactose	Rhamnose + arabinose	Uronic acid	Lignin	Ash	Other
−1	−1	62.2 ± 2.9	2.2 ± 0.1	8.7 ± 0.6	5.1 ± 0.6	10.4 ± 1.3	10.6 ± 1.8	3.4 ± 0.4	29.9 ± 0.7	31.9 ± 3.3
−1	+1	65.9 ± 3.1	2.6 ± 0.2	10.71 ± 1.0	5.1 ± 0.5	10.3 ± 0.8	11.3 ± 1.8	3.3 ± 0.3	27.2 ± 1.2	32.6 ± 2.3
+1	−1	82.5 ± 1.6	4.4 ± 0.2	11.7 ± 0.7	6.6 ± 0.6	11.7 ± 0.8	13.7 ± 1.4	2.8 ± 0.2	22.0 ± 0.5	31.6 ± 2.7
+1	+1	91.8 ± 1.3	3.6 ± 0.6	13.8 ± 2.1	9.2 ± 0.5	12.6 ± 0.9	14.0 ± 2.9	2.5 ± 0.3	20.4 ± 0.5	27.4 ± 2.1
Fresh biomass		–	1.1 ± 0.1	16.8 ± 1.4	9.7 ± 0.7	11.9 ± 1.2	17.2 ± 1.2	2.3 ± 0.1	19.1 ± 0.2	21.9 ± 3.5

Numbers are presents as average ± standard deviations of triplicate analyses

show that the content of WSC is relatively low in the fresh biomass. The increased content of WSC in all silage preparations might have resulted from the partial breakdown of the carbohydrate structure during the ensilage period, especially the silage prepared with addition of the cellulase complex. The partial breakdown of cell components could also support better ensilage by increasing the accessibility of the cell walls and cytoplasmic carbohydrates for LAB which has cell-surface enzyme complexes that can degrade and utilize oligo- or poly-saccharides (Siezen et al. 2006).

The lignin and ash content in the recovered solid increased for all prepared silage in comparison with fresh biomass (Table 4). Since the content of each composition is a relative value to the dry matter, the increase in lignin and ash might have resulted from the loss of other dry matters such as carbohydrates and protein.

Effect of ensilage on dilute acid hydrolysis of *Ulva* silage

Normally, pretreatment is employed to improve the efficiency of chemical or enzymatic hydrolysis. Several pretreatment methods, including chemical, physical, and biological approaches, have been developed to increase fermentable sugar yields from lignocellulosic biomass. Ensilage could also be employed as an approach for biological pretreatment of biomass (Richard et al. 1998). For example, reports published by Ambye-Jensen, Morten et al. suggest that ensilage could be used as a biological pretreatment for grass (Ambye-Jensen et al. 2013a, b) and decrease the required temperature in the hydrothermal pretreatment of wheat straw (Ambye-Jensen et al. 2013). Another benefit is that the application of ensilage can improve the enzymatic digestibility of sugar beet pulp (Zheng et al. 2011a, b). Although *Ulva* species do not contain a significant amount of lignin which might hinder the efficiency of hydrolysis, high temperature is usually required to release the fermentable sugars from *Ulva* biomass (Choi et al. 2012; Hamouda et al. 2016). Therefore, diluted acid hydrolysis at high solid loading (15% w/w) and low temperature (80 °C) was performed to examine the pretreatment effect of ensilage on *Ulva lactuca* biomass. Figure 2 shows that under all tested conditions the silage could produce more reducing sugar than fresh biomass after dilute acid hydrolysis. The best reducing sugar yield was around 155 mg/g dry biomass. Because the yield of reducing sugar was comparable to the data published in other reports (Table 5), the result suggests that ensilage could be employed as an integrated green approach to simultaneously preserve and pretreat *Ulva* biomass. The higher reducing sugar yield means that the ensilage did reflect the pretreatment effect and decreased the required temperature and acid concentration of diluted acid hydrolysis.

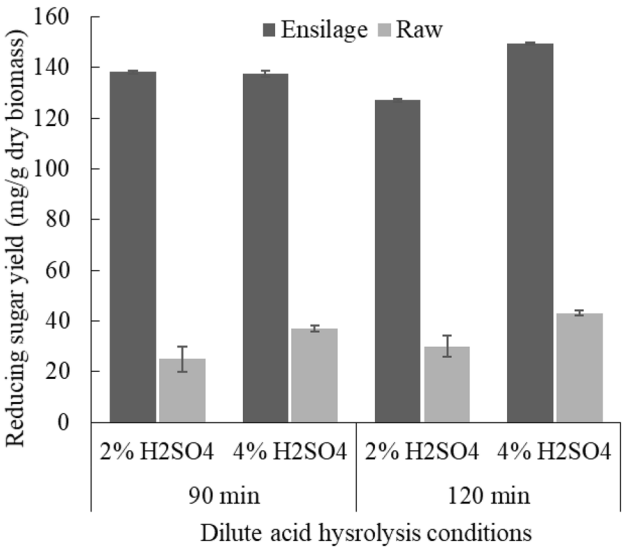

Fig. 2 Reducing sugar yield from dilute acid hydrolysis of *Ulva* biomass at different conditions

Fermentability of *Ulva* silage hydrolysate

In order to test the fermentability of *Ulva* silage, lactic acid fermentation was performed after enzymatic hydrolysis of the neutralized acid hydrolysate. The result indicated that the additional enzymatic hydrolysis could increase the reducing sugar yield from 155 to 198 mg/g dry biomass. The yield of reducing sugar was comparable to previous reports published by Trivedi et al. (2013) and Kim et al. (2011). The results also suggested that the hydrolysate could be used as a substitute of glucose in MRS medium and supported lactic acid fermentation (Fig. 3). During the fermentation period, glucose was completely consumed in 24 h and other sugars were only partially consumed. The yield of lactic acid was around 0.58 g per g of consumed reducing sugars. The results derived from this study suggest that the ensilage could be a useful approach to preserve and pretreat *Ulva* biomass for fermentable sugar production. Additionally, the fermentation by-product including unfermented sugars, lignin, and microbial biomass could also be a good substrate for anaerobic digestion. The integration of fermentation and anaerobic digestion allows for complete utilization of *Ulva* silage with a better energy balance (Alrefai et al. 2017).

Conclusion

In this study, ensilage was successfully employed as a green method to simultaneously preserve and pretreat macroalgae *Ulva lactuca* by adding cellulase complex and LAB inoculum. The silage of *Ulva* lactuca retained more than ninety percent of solid and most of the carbohydrates

Table 5 Comparison of sugar yields reported by different hydrolysis approaches and conditions

Ulva species	Hydrolysis conditions					Sugar yield (mg/g dry biomass)	References
	Solvent or buffer concentration	Temperature (°C)	Pressure (MPa)	Timing (min)	Solid loading (%)		
Ulva pertusa	5% H_2SO_4	120	0.015	30	3	430	Jang et al. (2012)
Ulva lactuca	1N H_2SO_4	121	0.5	30	50	158	El-Sayed et al. (2016)
Ulva pertusa	H_2O	150	15	15	10	41.6	Choi et al. (2012)
Ulva lactuca	7.5% wt H_2SO_4/wt dry matter	150	NA	10	10	~150	van der Wal et al. (2013)
Ulva lactuca	6% wt NaOH/wt dry matter	85	NA	240	10	~120	
Ulva sp.	2% H_2SO_4	121	NA	30	15	225	Jiang et al. (2016)
Ulva meridiomalis	50 mM H_2SO_4	140	NA	10	5	~140	Tsubaki et al. (2014)
	50 mM HCl					~145	
	50 mM PW[a]					~150	
	50 mM H_2SO_4					~74	
	50 mM HCl					~73	
	50 mM PW[a]					~83	
Ulva lactuca	4% H_2SO_4	80	Ambient	120	15	155	This study

[a]*PW* phosphotungstic acid

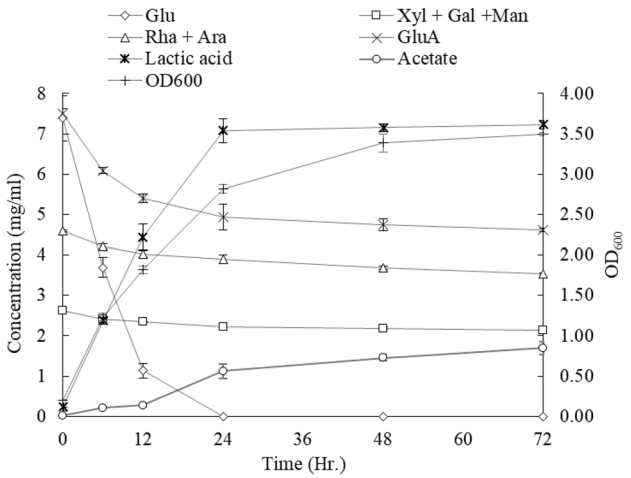

Fig. 3 Lactic acid fermentation profile of *Ulva* silage hydrolysate

at the end of ensiling period. The ensiled *Ulva lactuca* showed higher digestibility than fresh biomass by diluted acid hydrolysis at moderate temperature and high solid loading. Additionally, the hydrolysate of *Ulva* silage was also proven to be fermentable by LAB for lactic acid production. Since the successful preparation of *Ulva* silage is relatively limited, the results derived from this study could be useful for the future development of utilizing *Ulva* biomass as feedstock for bioproductions.

References

Abdul Rahman N, Abd Halim MR, Mahawi N, Hasnudin H, Al-Obaidi JR, Abdullah N (2017) Determination of the use of *Lactobacillus plantarum* and *Propionibacterium freudenreichii* application on fermentation profile and chemical composition of corn silage. Biomed Res Int 2017:8

Alrefai R, Benyounis KY, Stokes J (2017) Integration approach of anaerobic digestion and fermentation process towards producing biogas and bioethanol with zero waste: technical. J Fundam Renew Energy Appl 7(6):243

Ambye-Jensen M, Johansen KS, Didion T, Kádár Z, Schmidt JE, Meyer AS (2013a) Ensiling as biological pretreatment of grass (*Festulolium Hykor*): the effect of composition, dry matter, and inocula on cellulose convertibility. Biomass Bioenerg 58:303–312

Ambye-Jensen M, Thomsen ST, Kádár Z, Meyer AS (2013b) Ensiling of wheat straw decreases the required temperature in hydrothermal pretreatment. Biotechnol Biofuels 6(1):116

Basso FC, Bernardes TF, Roth APDTP, Lodo BN, Berchielli TT, Reis RA (2012) Fermentation and aerobic stability of corn silage inoculated with *Lactobacillus buchneri*. Revista Brasileira de Zootecnia 41:1789–1794

Bikker P, van Krimpen MM, van Wikselaar P, Houweling-Tan B, Scaccia N, van Hal JW, Huijgen WJ, Cone JW, López-Contreras AM (2016) Biorefinery of the green seaweed *Ulva lactuca* to produce animal feed, chemicals and biofuels. J Appl Phycol 28(6):3511–3525

Black W (1955) The preservation of seaweed by ensiling and bactericides. J Sci Food Agric 6(1):14–23

Bruhn A, Dahl J, Nielsen HB, Nikolaisen L, Rasmussen MB, Markager S, Olesen B, Arias C, Jensen PD (2011) Bioenergy potential of Ulva lactuca: biomass yield, methane production and combustion. Biores Technol 102(3):2595–2604

Cabrita ARJ, Maia MRG, Sousa-Pinto I, Fonseca AJM (2017) Ensilage of seaweeds from an integrated multi-trophic aquaculture system. Algal Res 24:290–298

Chen B, Zou D, Jiang H (2015a) Elevated CO_2 exacerbates competition for growth and photosynthesis between *Gracilaria lemaneiformis* and *Ulva lactuca*. Aquaculture 443:49–55

Chen K-Y, Zheng Y, Cheng Y-S (2015b) Integrated alkali pretreatment and preservation of wet lettuce (*Pistia stratiotes*) by lactic acid bacteria for fermentable sugar production. Biomass Bioenergy 81:249–255

Choi W, Han J, Lee C, Song C, Kim J, Seo Y, Lee S, Jung K, Kang D, Heo S (2012) Bioethanol production from *Ulva pertusa Kjellman* by high-temperature liquefaction. Chem Biochem Eng Q 26(1):15–21

del Castillo-Romo AA, Morales-Rodriguez R, Román-Martínez A (2018) Multiobjective optimization for the socio-eco-efficient conversion of lignocellulosic biomass to biofuels and bioproducts. Clean Technol Environ Pol

El-Sayed W, Ibrahim H, Abdul-Raouf U, El-Nagar M (2016) Evaluation of bioethanol production from *Ulva lactuca* by *Saccharomyces cerevisiae*. J Biotechnol Biomater 6(226):2

FAO ANIMAL PRODUCTION AND HEALTH PAPER. Rome, Italy, Food and Agriculture Organization of the United Nations, p 12

Farias D, Hurd C, Eriksen R, Simioni C, Schmidt E, Bouzon Z, Macleod C (2017) In situ assessment of *Ulva australis* as a monitoring and management tool for metal pollution. J Appl Phycol 29(5):2489–2502

Felix S, Pradeepa P (2011) Seaweed (*Ulva reticulata*) based fermented marine silage feed preparation under controlled conditions for *Penaeus monodon* larval development. J Mar Sci Res Dev 1(1):103

Finger FL, Endres L, Mosquim PR, Puiatti M (1999) Physiological changes during postharvest senescence of broccoli. Pesquisa Agropecuária Brasileira 34:1565–1569

Foscarini R, Prakash J (1990). Handbook on *Eucheuma* seaweed cultivation in Fiji. Handbook on *Eucheuma* seaweed cultivation in Fiji

Franco RT, Buffière P, Bayard R (2017) Optimizing storage of a catch crop before biogas production: impact of ensiling and wilting under unsuitable weather conditions. Biomass Bioenergy 100:84–91

Hamouda RA, Sherif SA, Dawoud GTM, Ghareeb MM (2016) Enhancement of bioethanol production from *Ulva fasciata* by biological and chemical saccharification. Rendiconti Lincei 27(4):665–672

Havlík P, Schneider UA, Schmid E, Böttcher H, Fritz S, Skalský R, Aoki K, Cara SD, Kindermann G, Kraxner F, Leduc S, McCallum I, Mosnier A, Sauer T, Obersteiner M (2011) Global land-use implications of first and second generation biofuel targets. Energy Pol 39(10):5690–5702

Henriques B, Rocha LS, Lopes CB, Figueira P, Duarte AC, Vale C, Pardal MA, Pereira E (2017) A macroalgae-based biotechnology for water remediation: simultaneous removal of Cd, Pb and Hg by living *Ulva lactuca*. J Environ Manag 191:275–289

Herrmann C, FitzGerald J, O'Shea R, Xia A, O'Kiely P, Murphy JD (2015) Ensiling of seaweed for a seaweed biofuel industry. Biores Technol 196:301–313

Hoevers R (2011) Aquatic biofuels for local development. FACT Foundation, Amsterdam

Jang S-S, Shirai Y, Uchida M, Wakisaka M (2012) Production of mono sugar from acid hydrolysis of seaweed. Afr J Biotechnol 11(8):1953–1963

Jiang R, Linzon Y, Vitkin E, Yakhini Z, Chudnovsky A, Golberg A (2016) Thermochemical hydrolysis of macroalgae *Ulva* for biorefinery: Taguchi robust design method. Sci Rep 6:27761

Jones CS, Mayfield SP (2012) Algae biofuels: versatility for the future of bioenergy. Curr Opin Biotechnol 23(3):346–351

Kadam SU, Álvarez C, Tiwari BK, O'Donnell CP (2015) Processing of seaweeds. Seaweed Sustainability. Elsevier, Amsterdam, pp 61–78

Kim N-J, Li H, Jung K, Chang HN, Lee PC (2011) Ethanol production from marine algal hydrolysates using *Escherichia coli* KO11. Biores Technol 102(16):7466–7469

Kreuger E, Nges IA, Björnsson L (2011) Ensiling of crops for biogas production: effects on methane yield and total solids determination. Biotechnol Biofuels 4(1):44

Kung L Jr (2001) Silage fermentation and additives. Sci Technol Feed Ind 17:145–159

Kung L, Shaver R (2001) Interpretation and use of silage fermentation analysis reports. Focus Forage 3(13):1–5

Liu S, Ge X, Xu F, Li Y (2016) Effect of total solids content on giant reed ensilage and subsequent anaerobic digestion. Process Biochem 51(1):73–79

Mabeau S, Fleurence J (1993) Seaweed in food products: biochemical and nutritional aspects. Trends Food Sci Technol 4(4):103–107

McCullough ME (1978) New trends in ensiling forages, Ruminant nutrition: selected articles from the world animal review

Muck R (1988) Factors influencing silage quality and their implications for management. J Dairy Sci 71(11):2992–3002

Muck R (2008) Improving alfalfa silage quality with inoculants and silo management. In: Proceedings of the 70th annual cornell nutrition conference for feed manufacturers, Cornell University Syracuse, NY

Oleskowicz-Popiel P, Thomsen AB, Schmidt JE (2011) Ensiling—wet-storage method for lignocellulosic biomass for bioethanol production. Biomass Bioenergy 35(5):2087–2092

Onodera M, Yoshie-Stark Y, Suzuki T (2011) Changes of the components of fresh seaweed, *Undaria pinnatifida*, by different strage conditions. Trans Jpn Soc Refrig Air Condit Eng 25:35–46

Pettersson KL, Lindgren S (1990) The influence of the carbohydrate fraction and additives on silage quality. Grass Forage Sci 45(2):223–233

Resdi R, Lim JS, Kamyab H, Lee CT, Hashim H, Mohamad N, Ho WS (2016) Review of microalgae growth in palm oil mill effluent for lipid production. Clean Technol Environ Pol 18(8):2347–2361

Richard TL, Proulx S, Moore KJ, Shouse S (1998) Ensilage technology for biomass pre-treatment and storage. In: 2001 ASAE annual meeting, american society of agricultural and biological engineers

Shaaban AE-SM, Badawy RK, Mansour HA, Abdel-Rahman ME, Aboulsoud YI (2017) Competitive algal biosorption of Al^{3+}, Fe^{3+}, and Zn- and treatment application of some industrial effluents from Borg El-Arab region, Egypt. J Appl Phycol 29(6):3221–3234

Siezen R, Boekhorst J, Muscariello L, Molenaar D, Renckens B, Kleerebezem M (2006) *Lactobacillus plantarum* gene clusters encoding putative cell-surface protein complexes for carbohydrate utilization are conserved in specific gram-positive bacteria. BMC Genom 7(1):126

Sluiter A, Hames B, Hyman D, Payne C, Ruiz R, Scarlata C, Sluiter J, Templeton D, Wolfe J (2008) Determination of total solids in biomass and total dissolved solids in liquid process samples National Renewable Energy Laboratory, Golden, CO, NREL Technical Report No. NREL/TP-510-42621, pp 1–6

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2012) Determination of structural carbohydrates and lignin in biomass (revision). Laboratory analytical procedure. National Renewable Energy Laboratory, Golden, pp 1–15

Srirangan K, Akawi L, Moo-Young M, Chou CP (2012) Towards sustainable production of clean energy carriers from biomass resources. Appl Energy 100:172–186

Trivedi N, Gupta V, Kumar M, Kumari P, Reddy C, Jha B (2011) An alkali-halotolerant cellulase from Bacillus flexus isolated from green seaweed *Ulva lactuca*. Carbohyd Polym 83(2):891–897

Trivedi N, Gupta V, Reddy CRK, Jha B (2013) Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga *Ulva fasciata* Delile. Biores Technol 150:106–112

Tsubaki S, Oono K, Hiraoka M, Ueda T, Onda A, Yanagisawa K, Azuma J-I (2014) Hydrolysis of green-tide forming *Ulva* spp. by microwave irradiation with polyoxometalate clusters. Green Chem 16(4):2227–2233

Uchida M, Amakasu H, Satoh Y, Murata M (2004) Combinations of lactic acid bacteria and yeast suitable for preparation of marine silage. Fish Sci 70(3):507–517

Valentine J, Clifton-Brown J, Hastings A, Robson P, Allison G, Smith P (2012) Food vs. fuel: the use of land for lignocellulosic 'next generation' energy crops that minimize competition with primary food production. GCB Bioenergy 4(1):1–19

van der Wal H, Sperber BLHM, Houweling-Tan B, Bakker RRC, Brandenburg W, López-Contreras AM (2013) Production of acetone, butanol, and ethanol from biomass of the green seaweed Ulva lactuca. Biores Technol 128:431–437

Vázquez-Rodríguez JA, Amaya-Guerra CA (2016) *Ulva* genus as alternative crop: nutritional and functional properties. Alternative crops and cropping systems. InTech

Weinberg ZG, Muck R (1996) New trends and opportunities in the development and use of inoculants for silage. FEMS Microbiol Rev 19(1):53–68

Yang HY, Wang XF, Liu JB, Gao LJ, Ishii M, Igarashi Y, Cui ZJ (2006) Effects of water-soluble carbohydrate content on silage fermentation of wheat straw. J Biosci Bioeng 101(3):232–237

Zheng Y, Yates M, Aung H, Cheng Y-S, Yu C, Guo H, Zhang R, VanderGheynst J, Jenkins BM (2011a) Influence of moisture content on microbial activity and silage quality during ensilage of food processing residues. Bioprocess Biosyst Eng 34(8):987–995

Zheng Y, Yu C, Cheng Y-S, Zhang R, Jenkins B, VanderGheynst JS (2011b) Effects of ensilage on storage and enzymatic degradability of sugar beet pulp. Biores Technol 102(2):1489–1495

Zheng Y, Lee C, Yu C, Cheng Y-S, Simmons CW, Zhang R, Jenkins BM, VanderGheynst JS (2012) Ensilage and bioconversion of grape pomace into fuel ethanol. J Agric Food Chem 60(44):11128–11134

Zhu D, Wen X, Xuan X, Li S, Li Y (2016) The green alga *Ulva lactuca* as a potential ingredient in diets for juvenile white spotted snapper *Lutjanus stellatus* Akazaki. J Appl Phycol 28(1):703–711