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Pretreatment and fermentation of salt-water grown algal biomass as a feedstock for biofuels and high-value biochemicals

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ABSTRACT

Utilization of halotolerant algal strains and saline water resources is necessary to increase the areas available for algal cultivation and to mitigate fresh water use issues. However, the added salt content from salt-water algae cultivation may impact downstream biomass pretreatment and conversion processes especially when combined with the salt that is generated as part of our Combined Algal Processing (CAP) scheme which includes pretreatment with sulfuric acid for cell disruption and carbohydrate hydrolysis. Here we compared the pretreatment, fermentation, and lipid extraction processes on salt-water versus fresh-water grown algal biomass. Response surface pretreatment plots showed that a broader range of conditions for the salt-water grown algae species yield > 90% sugar yield compared to a narrower range for fresh-water species. Despite this, we anticipated that high salt content would inhibit fermentation of algal sugars, a key element the CAP scheme and sought to reduce the formation of additional salt from pretreatment and neutralization by substituting oxalic acid for sulfuric acid. The sugar release response surface for oxalic acid was of a different shape than that of sulfuric acid but achieved > 90% sugar release at 2% acid. Fermentation results showed that for *Saccharomyces cerevisiae*, the added salts had minimal impact on sugar utilization rates or ethanol production. For *Actinobacillus succinogenes* however, a significant delay was observed due to the additional salts and fermentation was further delayed or inhibited by pretreatment with oxalic acid. This was remedied by removal of the oxalic acid either by the addition of $CaCO_3$ or trioctylamine whereupon fermentation rates recovered. Differences in lipid extraction yields were observed from the fermentation broth between the salt-water grown and fresh-water grown algal biomass and was species dependent.

1. Introduction

The combined algal process (CAP) and parallel algal process (PAP) represent pathways for upgrading algal biomass into fuels and chemicals [1,2]. Both pathways begin with acid pretreatment of algal biomass followed by carbohydrate fermentation (e.g. to ethanol) and lipid extraction in a single vessel (CAP) or a solid-liquid separation prior to fermentation due to non-solids compatible product purification methods (e.g. succinic acid crystallization) (PAP). These approaches are based on a hybrid biochemical/thermochemical processing strategy for selective recovery and conversion of algal biomass components, namely carbohydrates to fuels or chemicals and lipids to renewable diesel blendstock. We have explored various options for these processes to build the concept of a flexible, multi-product algal biorefinery to mitigate the current reality that algal biomass feedstock is expensive and requires near-complete utilization of the cellular components to be competitive with petroleum. To date these processes have been demonstrated using freshwater algal biomass. Water supply is a critical resource consideration in outdoor algal cultivation due to the significant sustainability issues of using water that would otherwise be used for municipal or agricultural uses. Halotolerant algae are receiving greater focus for biofuel production because of their ability to thrive in brackish or saline water which will greatly reduce if not entirely eliminate the use of fresh water for algae cultivation. If the CAP and PAP processes are to remain relevant, it will be necessary to address their compatibility with biomass grown in saltwater, and thus, one of the key challenges is the impact of this increased salt content in the harvested biomass on the pretreatment and fermentation operations.

The dilute sulfuric acid pretreatment process has been successfully developed as a highly effective approach to facilitate the conversion of algal carbohydrates, lipids, and protein into biofuels and bioproducts [3]. In dilute sulfuric acid pretreatment, microalgal carbohydrates are

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hydrolyzed into monomeric sugars, which can then be converted in CAP or PAP platforms. After dilute sulfuric acid pretreatment, the resulting algal slurry needs to be neutralized with alkali, typically sodium hydroxide, prior to fermentation. The resulting sodium sulfate generated by the pretreatment and neutralization can inhibit fermentation and may be exacerbated with the high salt content carried along with algal biomass (harvested at 20% solids) from saltwater cultivation. On the other hand, inorganic salts and metal salts present in the seawater cultivation media may be beneficial during pretreatment [4–7]. To date, reports of fermentation of algal biomass used either biomass that has been grown in low-salinity growth media or employed a wash step with deionized water to reduce the salts carried over from cultivation [1,8,9]. Washing of the harvested biomass, while effective for laboratory research, would be prohibitively costly in a commercial scale biorefinery [10].

The objective of this work was twofold; 1) to understand the impact on fermentation of the additional sodium sulfate and 2) to evaluate the replacement of sulfuric acid with a dicarboxylic acid to avoid creating additional sodium sulfate. Oxalic acid has previously been evaluated as an alternative catalyst in place of sulfuric acid in the pretreatment process for cellulosic biomass and has the additional potential for recovery and reuse from the hydrolysate [11,12]. We present here the results from salt-water grown algae pretreated with sulfuric or oxalic acids.

2. Materials and methods

2.1. Algae biomass

The Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University (ASU) provided *Desmodesmus* sp. C046 [13], *Nannochloris* sp. [14] [Michael Guarnieri and Lukas Dahlin, unpublished, NREL], and *Scenedesmus acutus* LRB-AP 0401 biomass [1]. All algae were grown in 1.5 in. wide flat panel PBR's. The two halotolerant strains were grown in f/2 seawater media [15] at 35 ppt salinity and *S. acutus* was grown as previously described [1]. The algal strains were harvested 11 days post N-depletion using a continuous centrifuge.

2.2. Compositional analysis

Total carbohydrate in whole algal biomass was hydrolyzed by a two-stage acid hydrolysis procedure and quantified by HPLC analysis [16]. Lipid content was determined by using in situ transesterification [17]. Nitrogen content of the samples was determined by combustion using a TruSpec CHN analyzer (LECO, USA) and a protein conversion factor was used to estimate the protein content in microalgal biomass [18]. Ash content was analyzed by a procedure based on NREL standard Laboratory Analysis Procedure [19]. The methods used to determine protein, carbohydrate, FAME, and ash content of the various fractions after pretreatment, fermentation, or extraction have been described [16,19–21].

2.3. Central composite design for oxalic acid pretreatment

A central composite design (CCD) experiment with 3 factors (temperature, acid concentration, and time) and 3 levels was used to evaluate the effectiveness of sulfuric and oxalic acids in pretreating saltwater grown algal biomass. The experimental design (Table 1) consisted of a total of 20 runs with the center point at 155 °C, 10 min, and 1.5% (w/w) acid concentration for oxalic acid pretreatment and a center point at 150 °C, 10 min, and 1.25% (w/w) acid concentration for sulfuric acid. The tests with the center point conditions were performed in 6 replicates. Based on the CCD, deionized water, sulfuric or oxalic acid, and biomass slurry (15%, w/v) were sequentially fed into a 10 mL microwave tube. Each tube was heated to the pretreatment temperature and held for a specified time. After pretreatment, the biomass was allowed to cool to ambient temperature. A 2 mL aliquot of the pretreated sample was filtered through a 0.22 μm membrane and the liquid used for carbohydrate analysis. The pH value of the algal slurry was measured before and after each pretreatment.

2.4. Pretreatment and hydrolysate preparation for fermentation

Pretreatment of algal biomass was performed in a ZipperClave® (Autoclave Engineers, Erie, PA) batch reactor with a 4 L volume (2 L working volume). The Zipperclave® reactor pretreats biomass at high-solids concentrations using direct steam injection for rapid heating. Mixing is achieved using an anchor-type impeller with customized lifting wedges that sweep the reactor bottom to provide mixing and lifting of the biomass under high-solids loading conditions. Wet algal paste (300 g) was loaded into the reaction container and acid and water were added to achieve a final solid loading of 20% (w/w) at an acid concentration of 2% (w/w) based on the total water in the system. Algal biomass was pretreated at 155 °C for 15 min. Pressure in the reactor was typically 70 psi. At the end of the pretreatment reaction, the sample canister was removed and cooled. The pretreated algal hydrolysate slurry (PAHS) was then removed and refrigerated. PAHS was neutralized with NaOH just prior to fermentation and used un-sterilized. For pretreatment with oxalic acid, $CaCO_3$ was used to remove the oxalic acid by precipitation of calcium oxalate and render it non-toxic. Solid $CaCO_3$ powder was added and stirred vigorously until the pH was 5.0. Alternatively, trioctylamine (TOA) was used to remove the remaining oxalic acid. Oxalic acid PAHS (12 mL) at pH 2.0 was mixed with 10 mL of a TOA (20%)-oleyl alcohol solution and stirred on a magnetic stir plate for 2 h. The mixture was then centrifuged at 2000*g* for 5 min for phase separation. The pH of the bottom liquor phase increased to 4.3 indicating that the oxalic acid had been removed from the liquor.

2.5. Fermentation of algal carbohydrates

Ethanol fermentations were conducted in shake flasks. An overnight seed culture of *Saccharomyces cerevisiae* D5A [22] was grown in 200 mL yeast extract-peptone-dexterose media (YPD, 1% yeast extract, 2% peptone, 20 g/L glucose) in a 500 mL baffled flask at 37 °C, 225 rpm from a 1:10 dilution of a fresh culture grown overnight the previous night. From this 2nd overnight seed culture, shake flasks were inoculated at an initial OD600 = 1. For triplicate shake flask experiments, 50 mL of neutralized (pH 5.5) PAHS was added to 125 mL baffled flasks. Flasks were inoculated, capped with a water trap, and incubated at 37 °C and 150 rpm. Samples were taken periodically to track sugar consumption and ethanol formation.

Succinic acid fermentation was conducted in screw-capped bottles using wild-type *A. succinogenes* 130Z (ATCC 55618) [23] acquired from the American Type Culture Collection. Culture stocks were stored at −80 °C in a cryopreservation solution (40% glycerol solution mixed with an equal volume of cells). Cells were revived by transferring a 1 mL frozen seed into 50 mL tryptic soy broth (TSB) media (SigmaAldrich, cat# 22092) in 100 mL bottles incubated at 37° C and 150 rpm overnight. The next day, 10% of the revived culture was transferred to 50 mL TSB in 100 mL bottle incubated at 37 °C at 150 rpm and used as a seed culture for inoculation after overnight growth. In each bottle, 40 mL of neutralized PAHS (pH 6.0) were combined with 2.5 mL of corn steep liquor (200 g/L), 4 mL of yeast extract (60 g/L), 2.5 mL of salts stock (20×), and 1 mL of phosphate salts stock (50×). The 20× stock salts solution contained 1 g/L NaCl and $(NH_4)_2SO_4$, 0.2 g/L $MgCl_2$-6 H_2O, and $CaCl_2$-2 H_2O. The 50× stock phosphate salts solution contained 1.5 g/L of both K_2HPO_4 and KH_2PO_4. Fermentations were incubated as described. Samples were taken periodically to track sugar consumption and succinic acid formation.

Table 1
CCD experimental conditions for microwave pretreatment using sulfuric or oxalic acid.

Experimental run	Temp (°C)	H_2SO_4 (% w/w)	Time min	Experimental run	Temp (°C)	Oxalic acid (% w/w)	Time min
1	175	2.00	5	1	155	1.50	10
2	150	2.50	10	2	170	2.00	5
3	175	0.50	15	3	155	1.50	1.6
4	150	0.00	10	4	155	1.50	10
5	150	1.25	10	5	130	1.50	10
6	150	1.25	10	6	155	1.50	10
7	150	1.25	10	7	155	0.66	10
8	125	2.00	15	8	170	2.00	15
9	150	1.25	10	9	170	1.00	5
10	175	0.50	5	10	170	1.00	15
11	125	0.50	15	11	140	1.00	5
12	150	1.25	1.6	12	155	1.50	10
13	150	1.25	10	13	155	1.50	10
14	108	1.25	10	14	155	2.34	10
15	125	0.50	5	15	180	1.50	10
16	125	2.00	5	16	140	2.00	15
17	150	1.25	10	17	140	2.00	5
18	150	1.25	18.4	18	155	1.50	18.4
19	192	1.25	10	19	140	1.00	15
20	175	2.00	15	20	155	1.05	10

2.6. Carbohydrate and acids analysis

Acids and ethanol were quantified by high-performance liquid chromatography (HPLC) on an Agilent 1100/1200 HPLC system with RID detection. A Bio-Rad Aminex HPX-87H column was used with a flow rate of 0.6 mL/min using 0.01 N H_2SO_4 as the mobile phase. Each sample injection volume was 6 μL and had a run time of 50 min. The column and detector temperatures were both set at 55 °C. The sugars glucose and mannose were quantified by high performance anion exchange (HPAE) on a Thermo Scientific Dionex ICS 5000 system equipped with pulsed amperometric detection (PAD). A Dionex CarboPac PA20 column preceded by a Dionex AminoTrap was run at 0.5 mL/min and 35 °C for both column and detector compartments using the quadruple waveform recommended by Dionex for carbohydrate detection. Samples were injected at 10 μL and an eluent of 27.5 mM sodium hydroxide was used to separate the monosaccharides followed by a gradient from 2 to 17% of 1 M sodium acetate and 100 mM sodium hydroxide. All samples were filtered through a 0.2 μm nylon filter and diluted as necessary prior to analysis.

2.7. Lipid extraction from the fermentation broth

Fermentation broth was mixed with an equal volume of hexane in a 50 mL Erlenmeyer flask. The mixture was stirred overnight on a multi position magnetic stir plate (Velp, Bohemia, NY, US) after which, the stillage and solvent mixture was transferred to centrifuge tubes for phase separation at 2000*g* for 10 min. The hexane phase was collected in a pre-weighed glass tube and evaporated in a TurboVap concentration workstation (Caliper Life Sciences, East Lyme, CT) at 40 °C. Afterwards, the glass tubes containing the crude lipid fractions were placed in a vacuum oven at 40 °C overnight to evaporate the residual solvent. The fermentation broth was extracted using hexane three times. The FAME content of the extracted lipid stream was measured [21] and total recovery was calculated based on the measured FAME content of the starting material. The purity of the FAME in the extracted oil was determined by dividing the amount (g) of FAME in the oil by the total weight of the extracted oil (g) [1].

3. Results and discussion

3.1. Compositional analysis of algal biomass

In this study, two halotolerant strains, *Desmodesmus* sp. C046 and *Nannochloris* sp., were evaluated for susceptibility to acid pretreatment. As shown in Table 2, the combined FAME and carbohydrate contents were higher than 50%, indicating that these two batches of biomass are acceptable CAP feedstocks to provide sufficient content for fermentation. A fresh water strain, *S. acutus* with high FAME and carbohydrate content, was previously used as a feedstock [1,2] and was used here to compare fermentation efficiency with the halotolerant strains. As expected, the ash content in halotolerant strains is higher than that in biomass grown in fresh water. This adds to the challenge of reducing acid loading because the ash provides additional buffering capacity. On the other hand, the salts present in seawater also may help catalyze carbohydrate hydrolysis [4–6].

3.2. Sulfuric acid pretreatment of halotolerant algal biomass

The pretreatment responses appear to be consistent for the two-halotolerant strains, with a different behavior for the fresh water *S. acutus* biomass (Fig. 1). With 2% acid concentration, both of the halotolerant strains exhibit a broader range of pretreatment conditions that achieve high sugar yields, while the optimal pretreatment condition is narrower for the fresh water strain. We speculate that some transition

Table 2
Biomass composition of two salt-water and one fresh water grown algal species.

	FAME (%)	Total carbohydrate (%)	Glucose (%)	Mannose (%)	Protein (%)	Ash (%)	Mass closure (%)
Desmodesmus	30.0 +/- 0.1	37.7 +/- 1.0	24.8 +/- 0.6	12.1 +/- 0.4	10.2 +/- 0.0	7.6 +/- 0.0	85.5
Nannochloris	16.5 +/- 0.2	34.2 +/- 1.7	25.7 +/- 1.1	3.4 +/- 0.2	15.6 +/- 0.0	12.5 +/- 0.1	82.9
Scenedesmus	30.8 +/- 0.4	44.3 +/- 1.4	34.9 +/- 1.2	8.8 +/- 0.2	10.9 +/- 0.0	1.6 +/- 0.1	87.6

Data shown as the mean n = 3.

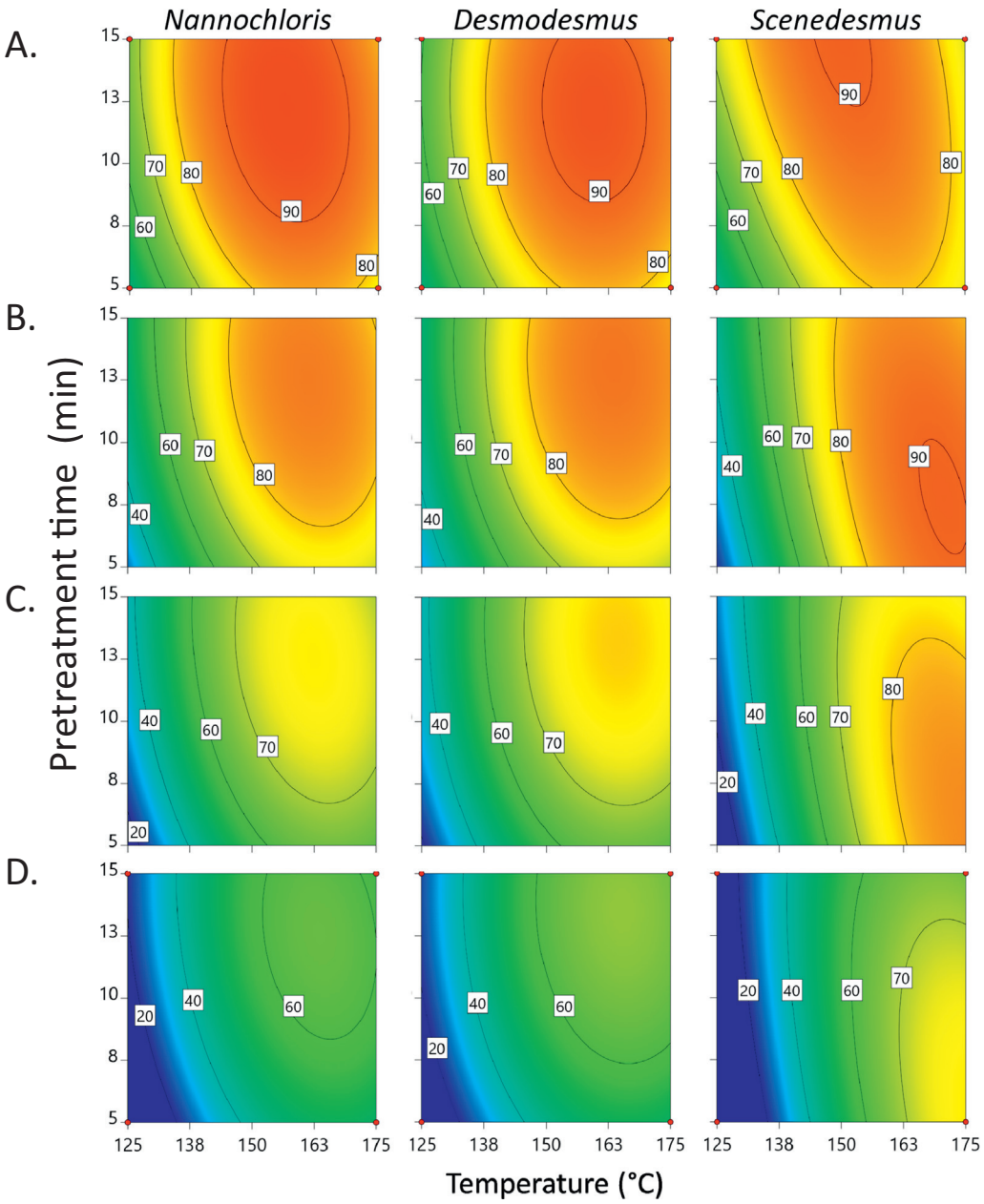

Fig. 1. Combined monomeric glucose and mannose yield in the aqueous phase of a pretreated slurry following a CCD of pretreatment conditions of varying temperature (125 °C–175 °C), time (5–15 min), and sulfuric acid concentrations. A: 2%; B: 1%; C: 0.75%; D: 0.5%. The contours are percent yield as labeled.

metals from seawater in the halotolerant biomass behave as Lewis acids, assisting carbohydrate hydrolysis under these conditions, and therefore lead to a broader range of optimal conditions for obtaining high sugar yields [7]. Monomeric glucose and mannose yields in excess of 80% were achieved for the three strains at 1% acid, but the range of optimal pretreatment conditions (temperature and time) for the halotolerant strains were narrower compared to the fresh water strain. Optimal conditions for *S. acutus* were still quite broad at 1% acid concentration shifting to a higher temperature and shorter time. When the acid concentration was reduced to 0.75%, sugar yields of 80% could still be achieved for the fresh water strain while sugar yields for the halotolerant stains were only > 70%. Finally, when the acid concentration was reduced to 0.5%, the highest sugar yields for halotolerant biomass were above 60%, while the sugar yield for *S. acutus* still reached above 70%. It can be observed from Fig. 1 that when the acid concentration is reduced, the optimal conditions for pretreatment of halotolerant biomass remain relatively unchanged while the optimal pretreatment conditions for *S. acutus* shifts to higher temperatures. The different responses of the salt water and fresh water biomass might be attributed to their differences in polysaccharide structures, compositions, and salt effects. Statistical analysis identifies different critical pretreatment factors between halotolerant biomass and fresh water biomass. Based on ANOVA, for fresh water *S. acutus,* temperature is the most significant factor, followed by acid concentration, and time. However, in *Nannochloris* and *Desmodesmus* biomass, the most significant factor is acid concentration, followed by temperature, and time (results not shown). These results are consistent with the surface response plots (Fig. 1). We speculate that the high ash content in the salt water biomass has a strong buffering effect to neutralize acid, leading to the lower sugar yield, especially with reduced acid concentration.

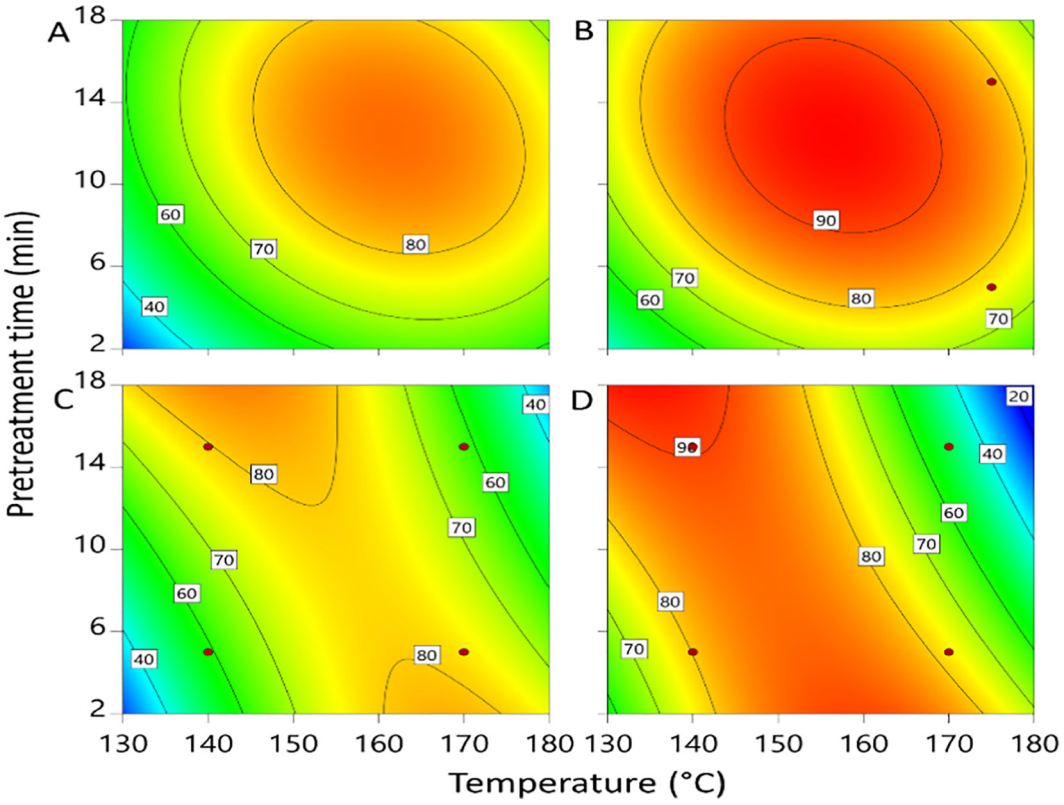

Fig. 2. Response surface of combined monomeric glucose and mannose yield from pretreatment of *Nannochloris* using oxalic acid and sulfuric acid. A: 1% sulfuric acid; B: 2% sulfuric acid; C: 1% oxalic acid; D: 2% oxalic acid. The contours are percent yield as labeled.

Table 3
Monomeric and total (monomeric and oligomeric) glucose and mannose yields from pretreated salt-water and fresh-water grown algal biomass.

		Glucose yield (%)		Mannose yield (%)	
Strain		Sulfuric	Oxalic	Sulfuric	Oxalic
Desmodesmus	Monomeric	83.7 ± 4.4	79.3 ± 3.0	82.7 ± 6.2	77.4 ± 8.3
	Total	94.9 ± 0.1	90.2 ± 0.9	94.3 ± 0.3	89.9 ± 7.8
Nannochloris	Monomeric	57.6 ± 8.9	47.6 ± 10.7	24.3 ± 13.1	9.7 ± 1.7
	Total	67.6 ± 7.6	62.0 ± 15.8	20.5 ± 2.2	18.5 ± 4.8
Scenedesmus	Monomeric	83.0 ± 1.3	65.6 ± 3.3	80.1 ± 0.8	61.4 ± 3.4
	Total	83.5 ± 1.9	85.3 ± 5.7	83.9 ± 2.0	81.8 ± 4.9

Data shown as the mean n = 3.

3.3. Comparison of oxalic acid and sulfuric acid pretreatment of halotolerant algal biomass

The combined glucose and mannose yield from pretreatment with oxalic acid manifests as a saddle shape which is quite different from the responses obtained using sulfuric acid indicating that the mechanism of oxalic acid hydrolysis is different from that of sulfuric acid. It has been reported that dicarboxylic acids may impart more efficient hydrolysis over a range of temperature and pH values due to their two pKa values and reduce sugar degradation [24,25]. As shown in Fig. 2, there are two optimal conditions for sugar release using oxalic acid. A high sugar yield can be achieved either with shorter time (< 2 min) and higher temperature (155 °C) or longer time (16 min) and lower temperature (145 °C). At low severity (low temperature and less time), there is not enough energy to activate the oxalic acid to obtain high sugar yields. On the other hand, the sugar yields dropped dramatically with higher pretreatment severity, probably because oxalic acid is not stable at higher temperatures and is converted to carbon dioxide and water. Indeed, pH increased after the higher temperature treatments (data not shown). The low thermostability of oxalic acid might provide a route to remove the residual acid by converting it into carbon dioxide and water using a heat shock step at a later stage during pretreatment.

3.4. ZipperClave pretreatment

Results from the small-scale response surface experiments were used to scale up the pretreatment into the 2 L ZipperClave reactor to compare sugar yields released from each strain and type of catalyst. Using 2% (w/w) loading for both sulfuric and oxalic acids, pretreatment of *Desmodesmus* gave monomeric glucose yields near 80% while total glucose (oligomeric and monomeric) yields exceeded 90% (Table 3). Monomeric mannose yields were slightly lower yet total mannose yields also exceeded 90%. Monomeric sugar release from the salt-water species *Desmodesmus* compared favorably to that of the fresh-water *Scenedesmus*. Monomeric and total glucose and mannose yields were much lower in the pretreated *Nannochloris* for both catalysts. Mass recovery after pretreatment was lower in *Nannochloris* than *Desmodesmus*, 71% and 105%, respectively. Concentration of hydroxmethylfurfural was similar in all of the pretreatments and ranged from 1.6 to 2.3 g/L.

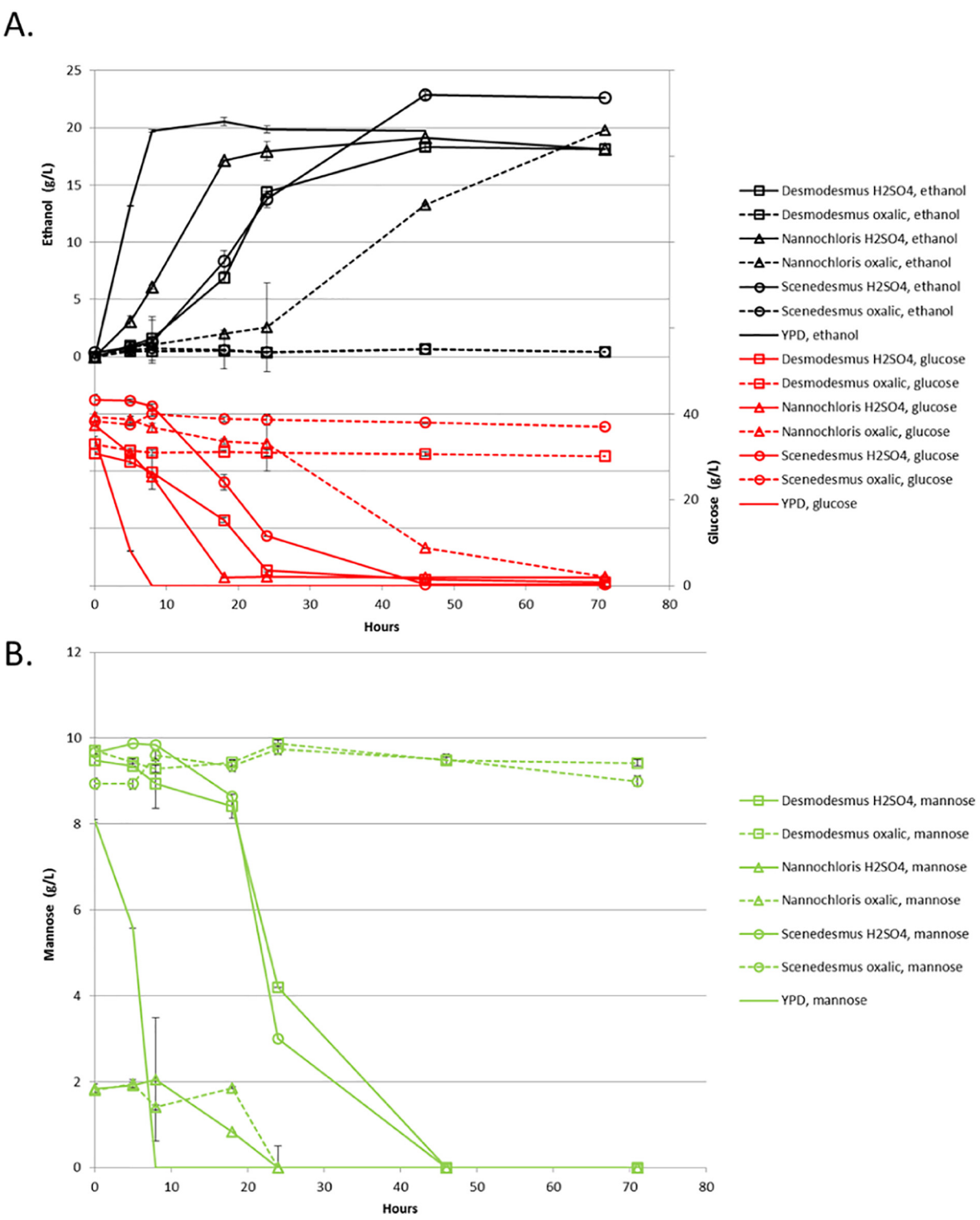

Fig. 3. Ethanolic fermentation of algal hydrolysates by *S. cerevisiae*. A. Ethanol production and glucose utilization. B. Mannose utilization. Data shown as the mean n = 3.

3.5. Pretreated Algal Hydrolysate Slurry fermentation

Previously, fresh water grown, pretreated algal biomass was fermented with *S. cerevisiae* to produce ethanol or *A. succinogenes* to produce succinic acid. Here, we extend those fermentations to two salt water grown algae species, *Desmodesmus* and *Nannochloris*, with the fresh water grown algae species, *S. acutus*, for comparison. Algal biomass was pretreated with either H_2SO_4 or oxalic acid and neutralized with NaOH. Though much work has recently been done on improving the salt tolerance of *S. cerevisiae* and other yeasts [26–29], the added salts present in the algal biomass from the salt water algae cultivation did not affect *S. cerevisiae* as fermentation proceeded as rapidly as with the fresh water *S. acutus* biomass (Fig. 3A, B). A long lag phase was however observed for *A. succinogenes* in the salt water PAHS compared to the fresh water PAHS (Fig. 4A, B). Oxalic acid proved to be inhibitory to yeast at the pretreatment concentration of 2%, though the yeast was

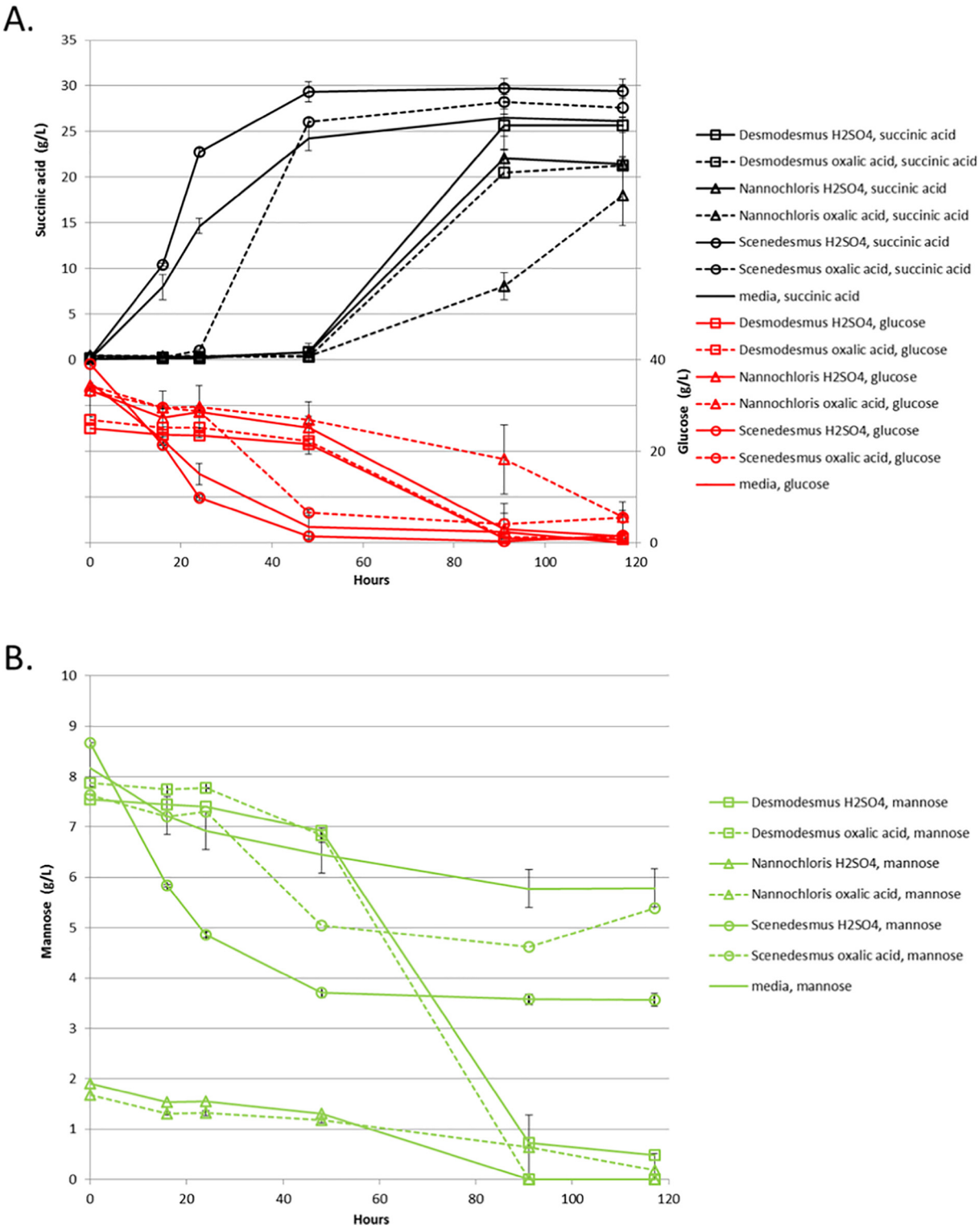

Fig. 4. Succinic acid fermentation of algal hydrolysates by *A. succinogenes*. A. Succinic acid production and glucose utilization. B. Mannose utilization. Data shown as the mean n = 3.

eventually able to overcome this inhibition after 24 h in the pretreated *Nannochloris* but not in the *Desmodesmus* nor *S. acutus* suggesting that the added salt from salt water cultivation does not contribute to oxalic acid inhibition. Similarly, *A. succinogenes* was able to overcome oxalic acid induced inhibition after a lag phase for all three PAHS. However, the lag phase for the fresh water PAHS was significantly shorter than the lag phase for the salt water PAHS. This suggests that the added salts from the salt water cultivation add to the inhibitory impact of the oxalic acid on *A. succinogenes*. These results also show that either adaptation to or metabolism of the oxalic acid can occur given a long lag phase eventually allowing PAHS fermentation.

Since *S. cerevisiae* could not overcome the toxicity of the oxalic acid, we explored two means of remediating this problem. In one, $CaCO_3$ was used to interact with the oxalic acid to form the non-toxic precipitate

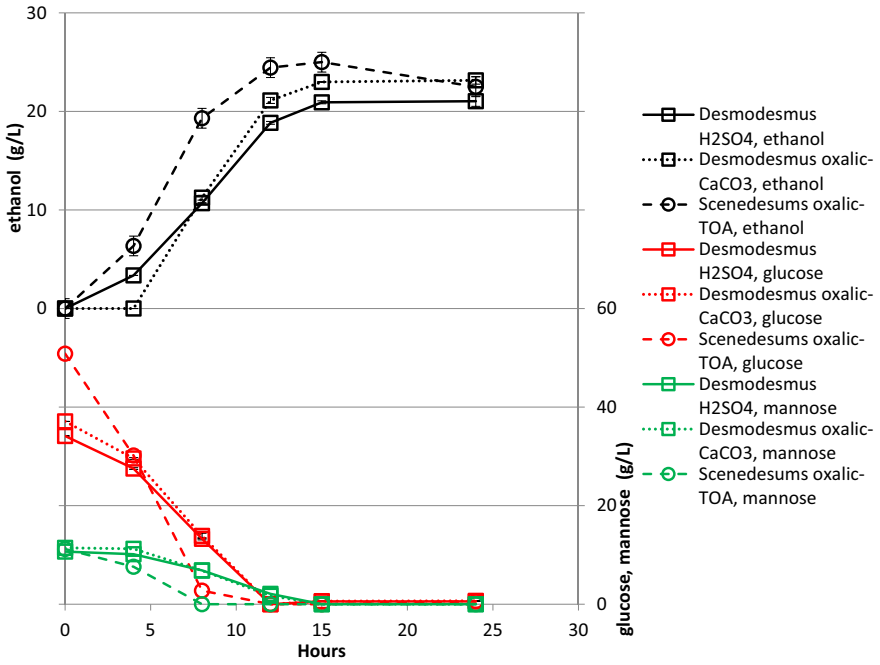

Fig. 5. Fermentation of pretreated algal hydrolysate neutralized with $CaCO_3$ or TOA. Data shown as the mean n = 3.

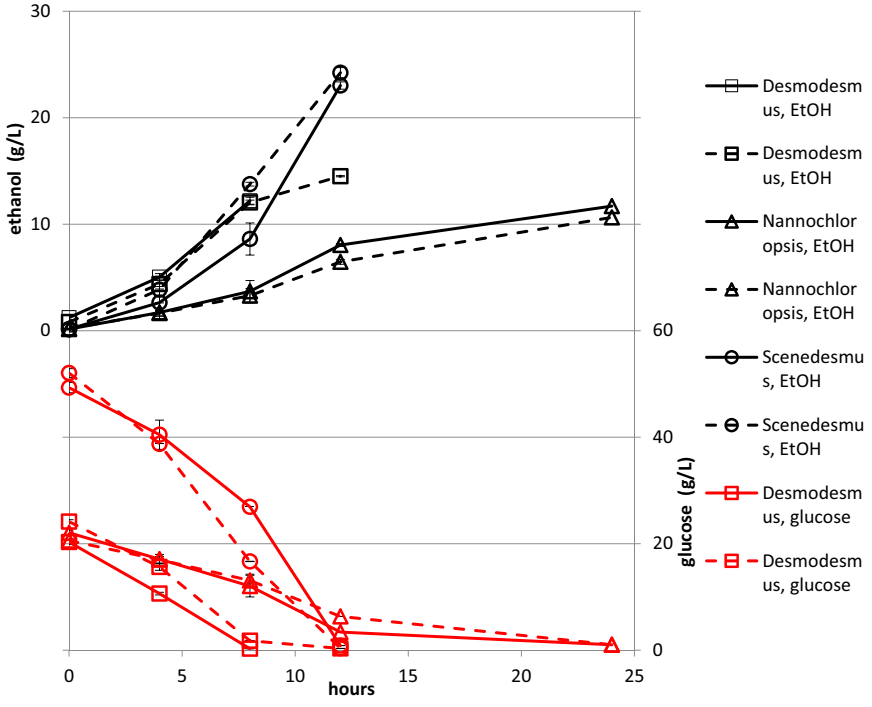

Fig. 6. Ethanol production and glucose utilization in duplicate algae pretreatment runs.

Ca-oxalate. When neutralized with $CaCO_3$, the fermentation proceeded as rapidly as the algal biomass pretreated with H_2SO_4 neutralized with NaOH (Fig. 5). In another test, TOA was used to complex with the oxalic acid which was then removed using oleyl alcohol. The *S. acutus* PAHS de-toxified with TOA fermented even more rapidly suggesting that TOA effectively removes oxalic acid and may also remove other inhibitory factors from PAHS. In addition, there is the potential for recycle of the oxalic acid back to the pretreatment step. Once the TOA-oxalate complex is removed from the liquor by extraction with oleyl alcohol, the oxalic acid can be recovered by reaction with another volatile alkali solution, such as trimethylamine (TMA), to form a trimethylammonium-carboxylic acid salt. This salt is then filtered from the solution and using evaporation, the TMA off-gases leaving crystallized oxalic acid [30]. This method may thus provide a route to fully recycle oxalic acid back to pretreatment while improving fermentation productivity and reducing the overall waste stream from the CAP or PAP platforms.

Though oxalic acid provides satisfactory results for pretreatment and can be detoxified and potentially recycled, it is currently more expensive than H_2SO_4. Thus, to examine the variability between larger scale pretreatment runs in the Zipperclave, H_2SO_4 was used for pretreatment. The fermentation results show that from the perspective of

Table 4
FAME extraction yield from fermentation stillage.

Fermentation product	Algal species	FAME yield (%)			Total FAME yield (%)	FAME purity in extracted oil (%)
		1st	2nd	3rd		
Ethanol	*Desmodesmus*	70.9 ± 5.9	8.6 ± 0.1	3.1 ± 0.9	82.6 ± 6.8	101.6 ± 1.0
	Nannochloris	61.6 ± 17.6	32.0 ± 9.1	10.8 ± 3.7	104.2 ± 4.7	90.5 ± 0.3
	Scenedesmus	87.2 ± 10.3	8.2 ± 3.5	1.3 ± 0.3	96.7 ± 14.1	101.4 ± 0.3
Succinic acid	*Desmodesmus*	46.4 ± 5.4	8.4 ± 0.3	1.8 ± 0.6	56.6 ± 5.7	103.2 ± 0.6
	Nannochloris	40.5 ± 7.7	17.2 ± 3.0	6.2 ± 1.3	63.8 ± 3.3	91.7 ± 0.6
	Scenedesmus	43.1 ± 1.8	3.1 ± 0.1	0.5 ± 0.0	46.7 ± 2.0	102.7 ± 0.1

Data shown as the mean n = 3.

Table 5
Fermentation and extraction product yields.

Fermentation product	Algal species	Fermentation product yield (g product/g glucose + mannose)	Fermentation product yield (g product/g biomass)	FAME yield (g FAME/g biomass)
Ethanol	*Desmodesmus*	0.43 ± 0.01	0.16 ± 0.01	0.25 ± 0.07
	Nannochloris	0.50 ± 0.01	0.19 ± 0.01	0.17 ± 0.05
	Scenedesmus	0.38 ± 0.00	0.17 ± 0.00	0.30 ± 0.10
Succinic acid	*Desmodesmus*	0.79 ± 0.00	0.30 ± 0.00	0.17 ± 0.05
	Nannochloris	0.61 ± 0.01	0.23 ± 0.01	0.11 ± 0.03
	Scenedesmus	0.61 ± 0.00	0.27 ± 0.00	0.14 ± 0.02

Data shown as the mean n = 3.

the generation of fermentation inhibitors during pretreatment and the rates of glucose use and product formation, there was very little difference between the pretreatment runs (Fig. 6). The final titers of ethanol were nearly the same for each PAHS as was the rate of glucose use.

3.6. Lipid extraction

As shown in Table 4, FAME yields from the ethanol fermentation broth are much higher compared to the succinic acid fermentation stillage. Ethanol is known to be effective in lipid extraction [31] and the presence of ethanol in addition to the hexane may have provided a synergistic effect. For the PAHS containing ethanol after fermentation, the FAME extraction yields are in the order of *Nannochloris* > *Scenedesmus* > *Desmodesmus*, with no correlation between extraction yields based on whether the algae were grown in salt or fresh water. Conversely, for the PAHS containing succinic acid after fermentation, the FAME extraction yields are in the order of *Nannochloris* > *Desmodesmus* > *Scenedesmus* showing that the extraction yields from salt-water grown algal biomass were higher than for the fresh-water grown algal biomass. The purity of the extracted FAMEs from the *Desmodesmus* and *Scenedesmus* fermentation broths were much higher than from the *Nannochloris*, possibly due to the lower initial lipid content present in the *Nannochloris* biomass and thus a higher prevalence of non-FAME nonpolar compounds extracted from this strain. The final pH of the fermentation stillage may also impact lipid extraction. The pH of the ethanol fermentation stillage was slightly acidic at pH 5 while the pH for the succinic acid fermentation stillage was near 7. Further research into the detailed extraction kinetics and mechanisms is needed to better understand the effects of pH and presence of additional ethanol or carboxylic acids in order to design better lipid extraction strategies from algal hydrolysates and fermentation stillage. Overall product yields from fermentation and extraction are an important consideration in terms of conversion process performance with a significant proportion of the starting biomass converted into useful products (Table 5).

4. Conclusion

We have demonstrated the compatibility between halotolerant algal biomass and the CAP or PAP platforms. Depending on the organism chosen to produce the fermentation product, the added salt due to cultivation can be inhibitory. To remediate this inhibition, we identified oxalic acid as a potential replacement for sulfuric acid. Though oxalic acid proved even more inhibitory than sulfuric acid to the yeast when left un-treated in the PAHS, neutralization of the residual oxalic acid using $CaCO_3$ or TOA demonstrated fermentation performance on par with pretreated fresh water algal biomass. Thus, in conclusion, dilute acid pretreatment of salt water grown algal biomass yields a suitable media for fermentation, and if overall salt reduction is needed due to salt sensitivity of the fermenting organism, the simplest carboxylic acid, oxalic, is nearly as effective as sulfuric acid at monomeric sugar release. Product yields from fermentation of salt water grown pretreated algal biomass were on par with that from fresh water grown algal pretreated biomass and thus salt water grown algae present a suitable feedstock for conversion to renewable biofuels and high-value chemicals.

Authors contributions

All authors contributed in the design, execution, and analysis of their respective experimental contributions as well as critical editing of the final manuscript. Specifically; E. P. Knoshaug performed the ethanol fermentations and wrote the publication. T. Dong performed the CCD experiments, lipid extractions, and co-wrote the publication. R. Spiller performed the succinic acid fermentations. N. Nagle performed the large-scale pretreatment. P. T. Pienkos led the overall effort providing guidance for experimental design.

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Competing interests

The authors declare that they have no competing interests.

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