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Enriching Duckweed As an Energy Crop for Producing Biobutanol Using Enzyme Hydrolysis Pretreatments and Strengthening Fermentation Process Using pH-stat

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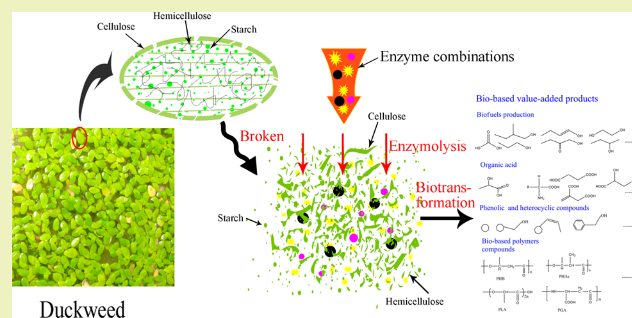
Supporting Information

ABSTRACT: Large scale production of biobutanol from a lignocellulosic feedstock for alternative fossil fuels consumption has garnered much interest by researchers in renewable energy. However, making biobutanol from lignocellulose requires the development of novel, renewable, nonfood sources for biofuel production and sustainable biorefining technology that maximizes the utilization of feedstock is indispensable. Duckweed (*Lemnaceae*) is a family of aquatic plants that in early trials has demonstrated great potential as an alternative nonfood energy feedstock for ethanol production. However, research on methods to obtain higher biobutanol yield from this plant is thus far insufficient. In this study, we tested several hydrolysis procedures with different enzyme combinations for duckweed pretreatment in detail. We then assessed the efficiency of these treatments for biobutanol production via fermentation with *Clostridium acetobutylicum*, using separate hydrolysis and fermentation (SHF) and simultaneous saccharification fermentation (SSF) and modulation of pH with pH-stat. The highest concentration of butanol and total solvent produced via SHF were 11.63 g/L and 24.06 g/L, respectively, using an enzyme hydrolysis method 4 (EHM4) with pH control. With SSF and controlled pH, butanol and total solvent concentrations achieved by EHM4 were 13.56 and 26.78 g/L, respectively, which was 14% and 10% higher than with SHF. Our results also show that duckweed is a promising feedstock for biobutanol production via comparison experiments. This study shows an additional advantage of using duckweed as a fermentation substrate is the potential to use simple enzyme hydrolysis instead of complex pretreatment. Having demonstrated the greatest butanol yield thus far, this study indicates that duckweed is a very promising bioenergy crop for industrial biobutanol development.



KEYWORDS: *Butanol, Fermentation, Duckweed, Pretreatment, Energy crop*

INTRODUCTION

Renewable sources of bioenergy currently under development include bioethanol and biobutanol from sugar cane, corn, and cassava and biodiesel from various plant oils such as coconut, palm, and rapeseed. Some of these sources are also used as food sources for humans and livestock; thus, a contradictory "food vs. fuel" issue has propelled researchers to develop new, renewable, nonfood sources for biofuel production. Duckweed, a family of the fastest growing aquatic angiosperms[1] that represent the world's smallest flowering plants, has received increasing attention as a potentially inexpensive and sustainable source of nonfood plant biomass for producing biofuels.[2] Additionally, duckweed has several advantages over alternative starch-rich biomass sources such as the feedstocks corn and cassava.[3−5] First, the growth characteristics of duckweed guaranteed that it has few requirements: its rapid growth guarantees a maximum biomass yield in a short time, at least amount of cost;[6] it can thrive in polluted water and, thus, does not compete with agriculture for cultivated land; it can be more easily harvested with a handy net compared with some microalgae rich in bio-oil. Second, duckweed can be simply pretreated because it is easily ground with low-level power required. Third, the biofuel produced from duckweed is considered a "green fuel" because the plant consumes carbon dioxide as it grows, which is the same byproduct produced when the fuel is burned. Additionally, duckweed is a potential bioenergy source with high-starch and low-lignin that might be suitable for bioconversion into biofuels via fermentation.[7]

Previous studies have reported the use of duckweed as a raw material for biofuel production, specifically for the production of biogas and bio-oil via pyrolysis[8,9] or thermolysis.[10] While duckweed tissues have been gasified in a thermochemical-based

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superstructure to produce diesel, kerosene, and gasoline,[11] in general duckweed is used to produce traditional ethanol[2,12] and biogas[13,14] by fermentation. These studies show that duckweed can produce significant quantities of starch, which is readily available for ethanol production via bioconversion; however, the ethanol is a low energy value biofuel. Therefore, existing biofuel production approaches are not yet sufficient to meet the requirements for industrial application. Thus, there is a need for more research to develop the methods and microbial resources required for duckweed utilization.

Renewable biobutanol, obtained by fermentation of organic material by microorganisms, has been proposed as one solution to the energy crisis,[15] in particular as a replacement for ethanol.[16] However, there has only been an isolated case report on using duckweed to produce butanol or higher alcohols by bioconversion, and it demonstrated a low yield.[17,18]

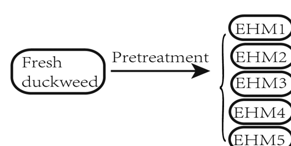
Therefore, here we enriched the potential of duckweed as an energy crop for producing high biobutanol from a species of wild duckweed, *Landoltia punctata*, via fermentation by a strain of *Clostridium acetobutylicum*. Specifically, with the aim of controlling the enzymatic hydrolytic process in order to improve the growth of microorganisms and obtain a higher sugar yield, we tested various enzymatic hydrolysis procedures involving different combinations of enzymes. Additionally, we assessed the effectiveness of using the resulting hydrolysates for further fermentation to produce biobutanol via two methods: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

MATERIALS AND METHODS

Experimental Design. Experiments were conducted following the methodology illustrated (Figure 1). Specifically, duckweed was pretreated using five different methods of enzyme hydrolysis. *C. acetobutylicum* ATCC 4259 was then used to ferment the products of hydrolysis.

Basic Assessment of Duckweed Composition. Duckweed was dehydrated to a lower moisture content (~75%) with a spin dryer before hydrolysis. Duckweed was hydrolyzed with 1% H_2SO_4, and then its starch content was calculated based on total sugar content (starch content = glucose content × 0.91).[19,20] Crude protein (CP) content was measured as CP = Kj·*N* × 6.25 (Kj indicates the Kjeldahl method).[21,22] Cellulose content was measured using spectrophotometry. Specifically, 10 g of duckweed was placed in 1 L of water with 60 mL of 60% H_2SO_4, and the plants were left to decompose for 30 min. Subsequently, 2% anthrone reagent (v/v) was added to the hydrolyzed mixture, and after 2 min the absorbance at 620 nm was measured.[23,24] The cellulose content of samples was then calculated according to a standard curve: cellulose content *Y* (%) of duckweed = *X* (cellulose content of standard sample) × *a* (diluted multiples) × 100/*W* (total weight of samples). Lignin content was determined using acetyl bromide according to standard methods.[25,26] Trace elements in duckweed were determined as follows: samples were washed with deionized water, dried at 80 °C, milled to powder, and finally digested using a wet digestion method.[27,28] The elemental composition in the digested solution was analyzed by using atomic absorption spectrometry (Z-2300, Hitachi, Japan). After analysis, compositions of duckweed were presented in Table 1.

Pretreatment



Fermentation

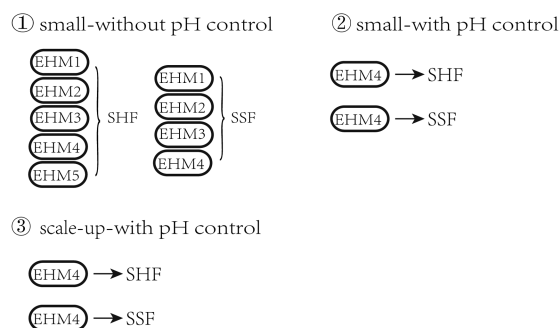


Figure 1. Methodology illustrated for the experimental process. EHM1, EHM2, EHM3, EHM4, and EHM5 indicate duckweed was pretreated using five different methods of enzyme hydrolysis.

Enzymolysis Pretreatment of Duckweed. *L. punctata* was harvested with nets from a wild pond in Xinjin County, China, rather than cultured artificially indoors. This was left to dry naturally until the surface of the duckweed contained no water and then dried at 60 °C to remove water until the final moisture content was ~55%. Each sample of dried duckweed (100 g per sample) was ground into a slurry, suspended in 400 mL of water, and then stirred. The samples were then pretreated using five different methods of enzymatic hydrolysis combinations as described in the following subsections. A pretreatment process similar to liquid hot water pretreatment (LHW) with relatively low temperature and pressure was conducted.[29,30] The duckweed samples were pretreated at a constant temperature of 150 °C and constant pressure of 0.5 MPa for 2 h prior to enzymatic hydrolysis with autoclave sterilizer.

Enzymatic Hydrolysis Method 1 (EHM1). In EHM1, the pH value of the sample was adjusted to 6.2 using Na_2CO_3 (5%, w/v). Next, 10 mL $CaCl_2$ (5%, w/v) was added, and the mixture was heated in a water bath at 90−95 °C for 20 min. Subsequently, 0.2 mg/g of α-amylase (120 KUN/g, equivalent to 8000 U/g; Novozymes, Tianjin) was added to the substrate, and the mixture was liquefied at 70−80 °C for 2 h. The resulting solution was heated at 95 °C for 10 min and cooled to 55 °C. The pH of the solution was again adjusted to 4.5 using 1% (v/v) H_3PO_4, and then 0.1 (mg/g) glucoamylase (Novozymes) was added (300 AGU/mL, where 1 AGU is the quantity of enzyme required to hydrolyze 1 μg of maltose in 1 min) to the liquefied solution and maintained at 60−65 °C for 4 h. Finally, the hydrolysate was cooled to room temperature and was used in the fermentation experiment.

Enzymatic Hydrolysis Method 2 (EHM2). In EHM2, the pH value of the sample was adjusted to 5.5 with 1% H_3PO_4. Next, 0.2 mg/g of α-amylase (120 KUN/g) was added to the slurry, and hydrolyzed at 60 °C for 6 h.

Enzymatic Hydrolysis Method 3 (EHM3). In EHM3, 10 mL of $CaCl_2$ (1%, w/v) was added to the sample, followed by 0.2 mg/g α-amylase (120 KUN/g), and hydrolyzed at 90−95 °C for 2 h. The pH value was adjusted to 5.2 with 1% H_3PO_4, and then 0.1 mg/g of β-amylase (50 000 U/g; Anked Bioengineering, Wuhan) and 0.1 mg/g of pullulanase (400 PUN/ml, where 1 PUN is the quantity of enzyme required to hydrolyze 1 μg maltose in 1 min; Novozymes) were added to the solution and saccharified at 50 °C for 20 h.

Enzymatic Hydrolysis Method 4 (EHM4). In EHM4, the pH value of the slurry was adjusted to 6.0 with 1% H_3PO_4. Next 0.2 mg/g of α-amylase (120 KUN/g) was added and then hydrolyzed at 60 °C for 6 h. Subsequently, 0.2 mg/g of β-amylase was added and then saccharified at 50 °C for 20 h. Following saccharification, 0.2 mg/g cellulase (500 000 U/g, where enzyme activity (U/g) is defined as follows: CMCA = 1 g enzyme powder decomposes the substrate CMC-Na to produce 1 mg glucose with treatment at 50 °C and pH 4.8 for 1 h; Thinkly, China) and 0.2(mg/g) Optimash BG (containing 5.4 U β-glucosidase activity and 1.9 U β-xylosidase activity; Genencor, USA) were added to the solution. The reaction mixture was then buffered with 50 mM phosphate buffer at pH 5.0 and incubated in a

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Table 1. Main Components of Wild Duckweed *Landoltia punctata* (before fermentation: 0 h)[a]

main composition of duckweed (%)					
sample	water	cellulose	protein	starch	lignin
dried duckweed		44.08 ± 0.57	20.25 ± 0.43	24.89 ± 0.31	2.98 ± 0.24
fresh duckweed	80 ± 5.0	5.44 ± 0.21	2.47 ± 0.17	3.04 ± 0.13	0.33 ± 0.078
metallic element composition of duckweed (μg/g)					
composition	concentration	composition	concentration	composition	concentration
Mg	6.04 ± 0.67	Cr	3.67 ± 0.81	Zn	272.01 ± 3.59
P	313.03 ± 2.63	Mn	473.91 ± 3.71	Pb	27.26 ± 1.42
K	1261.71 ± 4.28	Cd	7.91 ± 0.69	Al	451.46 ± 2.53
Ca	131.45 ± 2.86	Fe	775.41 ± 5.52		

[a]Content (g) of various main composition of duckweed measured in 10 g pretreatment samples.

rotary shaker (HZQ-X500; Yiheng, Shanghai, China) at 55 °C and 220 rpm for 72 h.

Enzymatic Hydrolysis Method 5 (EHM5). In EHM5, the sample was pretreated at 121 °C for 1 h, which is similar to the thermal hydrolysis process with a relatively low temperature.

Microorganism and Inoculum Preparation. The *C. acetobutylicum* ATCC 4259 was obtained from the American Type Culture Collection (ATCC, Manassas, USA) and stored in sterile distilled water at 4 °C. A TGYM medium (10 g Tryptone, 10 g glucose, 5 g glucose, 2 g NaCl, and 1 L purified water) was prepared for the rejuvenation process as follows: 5 g of meat medium (Sigma-Aldrich, Beijing) was dissolved in 50 mL of distilled water, and 2 g of glucose was added to the solution. The mixture was then autoclaved at 121 °C for 20 min and subsequently cooled to 80 °C. Preserved strains were added to the TGYM medium, which was heat shocked at 80 °C for 2 min and then allowed to cool in ice cold water for 1 min. The heat shocked spores were incubated in an anaerobic jar at 35 °C for 48 h.

To prepare the fermentation inoculum for strain cultivation, a 100 mL TGYM medium containing 20 g/L glucose, 5 g/L yeast extract, 3 g/L ammonium acetate, 1 g/L sodium chloride, 1 g/L KH_2PO4, 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, 0.02 g/L $MnSO_4 \cdot 7H_2O$, and 0.02 g/L $FeSO_4 \cdot 7H_2O$ was prepared and autoclaved at 115 °C for 20 min. Once the medium had cooled to 35 °C, 0.5 g/L cysteine was filtered (using a 0.22-μm Millipore filter; GEMA Medical SL, Chengdu) into the medium. Approximately 5 mL of the prepared rejuvenation bacterial culture was then added to the medium and left to grow at 37 °C until the optical density reached 1.5.

Acetone, Butanol, Ethanol Fermentation Experiments in Small Anaerobic Bottles. In order to evaluate the effect of different procedures on fermentation, our experiments were divided into two sets: one set conducted using SHF after enzymatic hydrolysis was complete and another set conducted using SSF. All fermentation procedures were conducted using sterile conditions (following sterilization at 121 °C and 0.5 MPa for 20 min) unless stated otherwise.

SHF Procedure. In SHF, batch cultures were carried out statically. Fermentation bottles were sparged with filtered oxygen-free nitrogen gas to maintain strictly anaerobic conditions. For batch cultures without pH control, the hydrolysates were collected for fermentation after the pretreatment, and their sugar compositions were determined prior to fermentation. For batch cultures with pH control, pH-stat was performed on the enzymatic hydrolysates of duckweed. The fermentation cultures were maintained anaerobically, and the pH was automatically maintained at 5.5 by a pH controller (PHC-2201; Able, Tokyo, Japan). Each hydrolysate was concentrated to 150 mL, its sugar composition was determined, and it was fermented in a 500 mL glass anaerobic bottle (Haimen Huakai, Haimen, China) sealed with butyl rubber. After the addition of 1 g of yeast extract and 2 g of peptone to each bottle, the pH of the fermentation substrates was adjusted to 6.8 with 1% NaOH. These solutions were sterilized at 115 °C for 20 min and cooled to room temperature. Subsequently, 5 mL of a mixture solution containing a combination of P2 trace elements (50 g/L KH_2PO_4, 50 g/L K_2HPO_4, and 220 g/L CH_3COONH_4), vitamins (0.1 g/L para-aminobenzoic acid, 0.1 g/L thiamin, and 0.001 g/L biotin), and minerals (20 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $MnSO_4 \cdot H_2O$, 1 g/L $FeSO_4 \cdot 7H_2O$, and 1 g/L NaCl) was filtered for sterilization (0.22-μm Millipore filter) and added to each bottle. The bottles were then inoculated with ~3 mL of fermentation inoculum (OD_{600} = 1.57).

SSF Procedure. In SSF, the fermentation procedure was simultaneously conducted with and without pH control alongside enzymatic hydrolysis. Supplementary trace elements and fermentation conditions were the same as those used in SHF. Pure nitrogen was injected into the fermentation bottles with a latex tube to maintain anaerobic conditions via expelling oxygen. Fermentation was conducted at 37 °C for 96 h, during which time samples were periodically withdrawn with a sterile syringe through the rubber stopper in order to analyze sugars, organic acid, pH, and ABE production.

Fermentation Experiments with Pure Glucose and Sugar Mixture. In an additional experiment used for comparison with our pretreatment designs, we assessed the use of absolute glucose and a mixture of sugars (47.69 g/L glucose, 8.21 g/L xylose, 2.54 g/L arabinose, 1.37 g/L cellobiose, 2.11 g/L galactose, 1.68 g/L mannose, and 1.51 g/L maltose), mimicking the sugar composition of hydrolysates from EHM4, as fermentation substrates for producing ABE under the same fermentation conditions previously described with pH control.

Bioreactor Scale-up of Butanol Fermentation. In order to assess the fermentation effect on a larger scale, a 20-L stirred-tank bioreactor (Holves Company, China) with a mode of sterilization (high temperature steam sterilization), mechanical stirring system (straight stirring of fermentation, special standard turbine agitator propeller with two layers of six flat inclined-blade and curved blades for increasing the contact interface), and speed control (50−1000 rpm) was used instead of small bottles. The fermentation procedure was simultaneously conducted with controlled pH alongside enzymatic hydrolysis. Fresh duckweed (7 kg) was pretreated in the bioreactor using EHM4 but with an enzyme dosage 50 times higher than that used in the small anaerobic bottles for fermentation. *C. acetobutylicum* ATCC 4259 (500 mL inoculum size, OD_{600} = 2.3) was then inoculated into the fermentation substrate. N_2 gas was injected into the reactor to remove oxygen and then sealed the reactor. To ensure that the duckweed slurry could be fully utilized by *C. acetobutylicum* ATCC 4259, the solution was stirred for 5 min every 5 h during fermentation time for 12 h. Generated gases were also released every 5 h. The ABE products were determined after fermentation had completed.

■ ANALYTICAL PROCEDURES

The ABE production (acetone, butanol, and ethanol) was measured with a gas chromatograph (GC) equipped with a flame ionization detector (model 6890 GC [Agilent Technologies, CA, USA] with a model 7673A automatic injector, sampler, and controller [Hewlett-Packard]). Alcohols were separated using a ZB-WAX capillary column (30 m, 0.25 mm inside diameter, 0.25 μm film thickness; Phenomenex, PA, USA). The GC oven temperature was held initially at 40 °C for

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Table 2. Compositions from Hydrolysate of Duckweed for Different Enzyme Hydrolysis Methods (Fermantation Substrates of SHF at 0 h)[a]

	sugars components (g/L) using different pretreatment methods				
sugars	EHM1	EHM2	EHM3	EHM4	EHM5
glucose	30.77 ± 2.39	25.41 ± 1.35	36.41 ± 1.16	47.69 ± 1.84	
xylose	7.27 ± 1.79	3.22 ± 1.48	5.17 ± 1.29	8.21 ± 1.42	6.17 ± 0.12
arabinose	1.54 ± 0.49	2.44 ± 0.16	1.16 ± 0.21	2.54 ± 0.17	
cellobiose	4.16 ± 1.14	2.18 ± 0.48	3.37 ± 0.19	1.37 ± 0.19	1.35 ± 0.13
galactose	1.79 ± 0.11	1.47 ± 0.24	2.31 ± 0.11	2.11 ± 0.31	
mannose	1.12 ± 0.41	1.05 ± 0.12	1.89 ± 0.22	1.68 ± 0.30	
maltose	2.35 ± 0.32	2.18 ± 0.14	2.22 ± 0.11	1.51 ± 0.06	1.17 ± 0.17
total sugars	49.00	37.95	52.53	64.11	9.69

[a]EHM1, EHM2, EHM3, EHM4, EHM5: indicate the different pretreatment methods.

5 min and was then raised stepwise by 15 °C/min until it reached 150 °C, and then by 50 °C/min to 250 °C where it was held for 4 min. Helium was used as the carrier gas, with an inlet pressure of 9.3 lb/in.[2] The injector and detector were maintained at 220 °C. A 1-μL volume of supernatant from the culture broth was injected in split-injection mode at a 1:30 split ratio. Isobutanol was used as the internal standard.

The phenol-sulfuric acid method[31−34] was used to determine total residual sugars. The constituent sugars were detected with a Dionex UltiMate3000 liquid chromatograph in a column packed with Aminex HPX-87H (Hercules, CA, USA: carbohydrate analysis column Aminex HPX-87P Column 300 × 7.8 mm, catalog 125−0098 serial 426070, 5 mM H_2SO_4, 0.6 mL/min; column temperature at 65 °C). The RI detector, used to detect the liquid refractive index, was run at 45 °C. The concentrations of the sugars were determined by extrapolation from standard curves. Butyric and acetic acids were also determined with the Dionex UltiMate3000 liquid chromatograph but in a column packed with Aminex HPX-87H and 0.05 mM H_2SO_4 on Chromosorb WAW. Chromatography was conducted at an injector temperature of 175 °C, detector temperature of 180 °C, and oven temperature of 125 °C.

Glucuronic acid, p-coumaric acid, syringic acid, and ferulic acid were determined with a DIONEX UltiMate 3000 liquid chromatograph in a column packed with Aminex HPX-87H and 0.05 mM H_2SO_4 on a Chromosorb WAW. The chromatography was conducted at an injector temperature of 175 °C, detector temperature of 180 °C, and oven temperature of 125 °C. Determination of furfural and 5-hydroxymethylfurfural were determined with HPLC according to the methods.[35,36]

■ STATISTICAL ANALYSIS

For each treatment, we calculated the mean response and SD of variables. Variation was analyzed using ANOVA, and mean separation was performed using Fisher's Least Significant Difference ($P \leq 0.05$), in SPSS 21.0. Unless otherwise indicated, three replicates of each experiment and assay were conducted.

■ RESULTS AND DISCUSSION

Selection of Enzymatic Hydrolysis and Fermentation Processes. Acid hydrolysis (before fermentation, 0 h) was used to determine duckweed composition. The main compositions and nutritional microelements of duckweed were measured and used as baseline data (Table 1). LHW, a common method, is used for lignocellulose pretreatment before enzyme hydrolysis. Treatment of cellulose with LHW resulted in destruction of the cell wall of the solid interface and thus facilitated enzyme hydrolysis.[37−39] Therefore, a pretreatment process similar to LHW was conducted in order to facilitate enzymatic hydrolysis and obtain maximum sugars. Research on basic information is a necessary prerequisite for subsequent enzymatic hydrolysis optimizing processes, in order to determine optimal fermentation substrates.

Generally, acid hydrolysis of lignocellulosic biomass would require detoxifying the toxic compounds such as furfural and hydroxymethylfurfural in hydrolysate product in order to enhance fermentation rates and obtain high yields.[40,41] However, the detoxification process is not generally necessary when enzyme hydrolysis pretreatment is used because barely or trace amounts of inhibitors are produced in the enzymolysis process. Different sets of specific enzymes can produce different kinds of sugars that adapt to the uptake of different microorganisms. This is an important factor for the fermentation effect to obtain a high yield in the bioconversion process. For example, glucose is generally produced by enzyme combination: α-amylase and glucoamylase hydrolysis. Malt oligosaccharides are also produced by α-amylase, and maltose is generally produced by the combination of α-amylase, pullulanase, and β-amylase. The structural characteristic of duckweed is significantly different from other crops rich in intricate lignocellulose. We therefore researched in depth several hydrolysis methods and tested the effects of different enzyme combinations, instead of single enzymes as in previous studies.

After preprocessing, the sugar composition of the hydrolysates was determined before starting the fermentation experiment (Table 2). The pretreatment groups EHM3 and EHM4 produced more total sugar, as compared with EHM1, EHM2, and EHM5. The results showed that different amounts and varieties of sugars can be obtained by using the appropriate enzyme combination. We thus concluded that enzyme combination hydrolysis is an appropriate pretreatment method, which confers a great advantage in production of sufficient quantities and variety of monosaccharides from duckweed.

SHF and SSF are the most commonly used methods in the production of biofuel via fermentation. SSF uses simultaneous conversion of sugars to overcome the slow sugar uptake rate in SHF, which is caused by sugar accumulation after enzymatic hydrolysis. Here, we further investigate and detail the effect of producing butanol from duckweed using enzymatic hydrolysates. In butanol fermentation, pH is an important factor for improving butanol yield, making it necessary to investigate the effect of pH control. We therefore first investigated ABE

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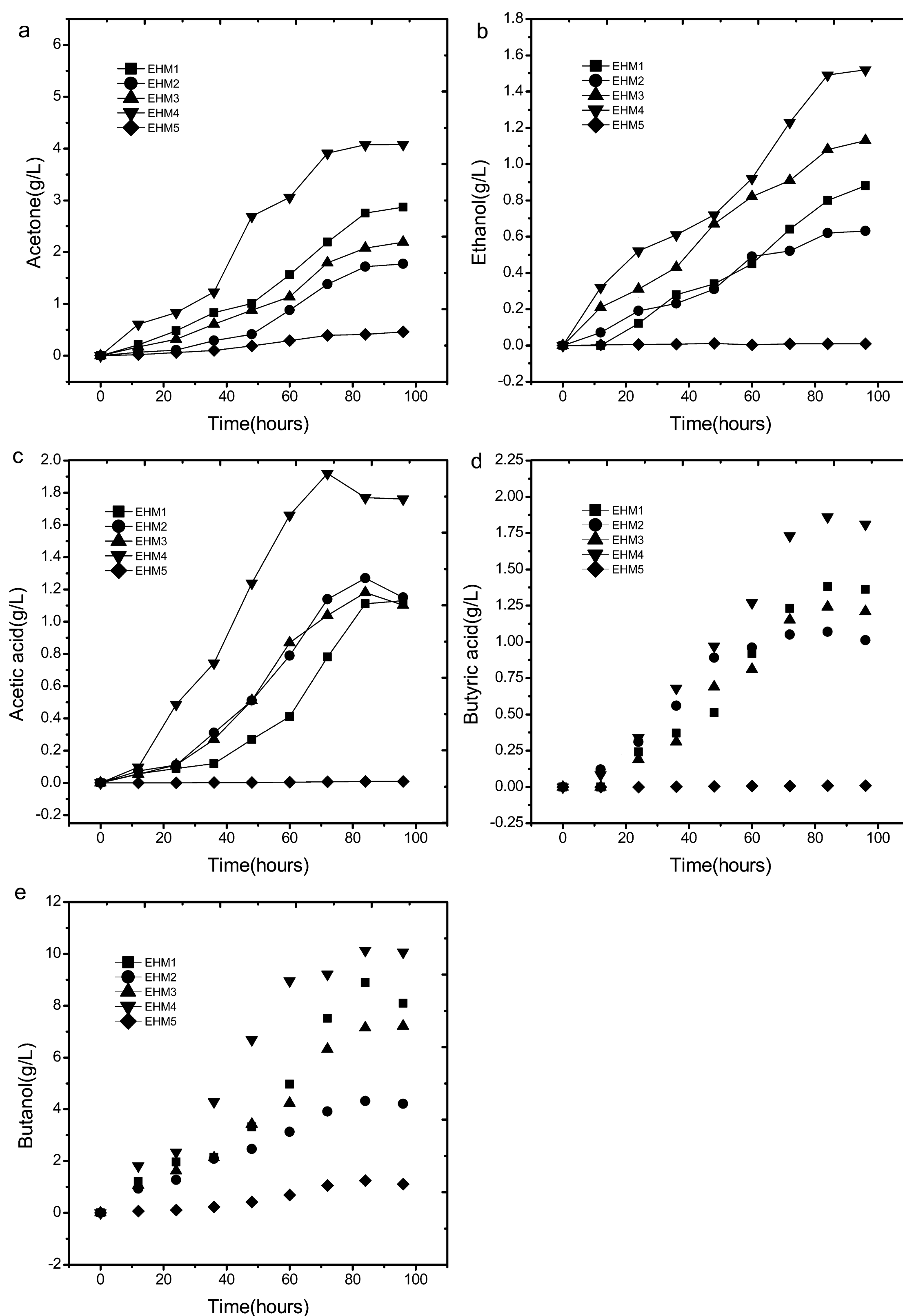

Figure 2. Products of fermentation over time given different enzyme hydrolysis methods conducted via SHF in small anaerobic bottles without pH control. EHM1, -2, -3, -4, and -5 denote enzyme hydrolysis methods 1, 2, 3, 4, and 5, respectively. Error bars indicate SD ($n = 3$).

fermentation by comparing the ability of *C. acetobutylicum* ATCC 4259 to produce butanol from different hydrolysates of duckweed. This was carried out during the decomposition of pretreated duckweed using SHF and SSF in small anaerobic bottles without pH control, so as to determine the optimal substrate and fermentation method to obtain the highest quantity of butanol. Second, we further investigated ABE production in small anaerobic bottles with pH control using the optimum fermentation substrate of duckweed. Third, we further conducted scaled-up butanol fermentation reactions in

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Table 3. Highest ABE Yield Using Hydrolysate of Duckweed, Pure Glucose, and Mixture Sugars As Substrate by SHF, SSF[a]

parameters	SHF			SSF				
	EHM4^{smH1}	EHM4^{smH2}	EHM4^{scH}	EHM4^{smS1}	EHM4^{smS2}	EHM4^{scS}	Glu.	MixSug.
butanol (g/L)	10.1 ± 1.15	11.6 ± 1.38	11.3 ± 0.69	11.8 ± 1.47	13.5 ± 1.18	13.2 ± 2.30	13.9 ± 1.72	12.2 ± 1.69
acetone (g/L)	4.07 ± 0.36	5.41 ± 0.29	4.41 ± 0.42	5.42 ± 0.26	5.45 ± 0.37	5.15 ± 0.41	6.32 ± 0.27	5.43 ± 0.33
ethanol (g/L)	1.49 ± 0.07	2.79 ± 0.23	2.43 ± 0.18	2.49 ± 0.47	3.19 ± 0.39	3.07 ± 0.75	2.41 ± 0.34	2.87 ± 0.48
total ABE (g/L)	15.69 ± 1.37	19.83 ± 1.51	18.1 ± 2.37	19.77 ± 1.44	22.2 ± 1.62	21.48 ± 1.87	22.64 ± 2.0	20.58 ± 1.34
total acids (g/L)	3.73 ± 0.73	4.23 ± 0.81	3.61 ± 0.79	4.21 ± 1.02	4.58 ± 0.52	4.2 ± 0.83	5.02 ± 0.92	4.65 ± 0.58
total solvents (g/L)	19.42 ± 2.34	24.06 ± 2.78	21.77 ± 0.73	23.98 ± 3.63	26.78 ± 3.21	26.68 ± 2.76	27.66 ± 2.8	25.23 ± 2.57
total residual sugars (g/L)	8.92 ± 1.57	8.7 ± 1.34		5.92 ± 1.87	5.3 ± 1.63		6.67 ± 1.79	12.26 ± 3.52
ABE yield (g/g)	0.16	0.20		0.20	0.22			
productivity (g/L·h)	0.19	0.25	0.23	0.25	0.28	0.28	0.29	0.26
butanol/total solvents (%)	0.52	0.48	0.52	0.49	0.51	0.50	0.50	0.49
fermentation time (h)	96	96	96	96	96	96	96	96

[a]ABE yield (g/g): total ABE production divided by gross weight of duckweed. Productivity (g/L·h): Total solvents divided by fermentation time. Different letters in table indicate significant differences based on multiple comparisons ($P < 0.05$). EHM4^{smH1}: fermentation in small anaerobic bottles via SHF without pH control. EHM4^{smH32}: fermentation in small anaerobic bottles via SHF with pH control. EHM4^{scH}: fermentation for scale-up in bioreactor via SHF with pH control. EHM4^{smS31}: fermentation in small anaerobic bottles via SSF without pH control. EHM4^{smS32}: fermentation in small anaerobic bottles via SSF with pH control. EHM4^{scS}: fermentation for scale-up in bioreactor via SSF with pH control.

a bioreactor, to assess scalability of the laboratory results. After continuous fermentation for 96 h, the change in various parameters was measured and the target products of fermentation were determined.

ABE Production in Small Anaerobic Bottles. First, butanol production during SSF peaked at 84 h in fermentation without pH control; the highest level of butanol (11.86 g/L), acetone (5.42 g/L), ethanol (2.49 g/L), and total solvent of ABE (19.77 g/L, compared with 15.69 g/L for SHF) were produced using the EHM4 substrate. Similarly, during SHF, the highest butanol yield (10.13 g/L), acetone and ethanol were obtained from the EHM4 substrate (Figure 2; Table 3). The butanol yields from SHF were 14.6% and 6.6% lower than those from SSF for EHM4 and EHM3, respectively (Figure 3; Table 3). However, the ethanol yield from SSF was not always higher than from SHF (Figures 3 and 4; Table 3). The highest yields of acetic acid (1.92 and 2.22 g/L for SHF and SSF, respectively), butyric acid (1.86 and 2.01 g/L for SHF and SSF, respectively), and total acid (4.21 g/L, compared with 3.73 g/L for SHF) were achieved from EHM4 substrates (Figures 2 and 3). Additionally, ABE production rates were higher with SSF (mean = 0.2 g/g, compared with 0.16 g/g for SHF), as was productivity from the total duckweed substrate (0.25 g/L·h, compared to 0.19 g/L·h for SHF). Additionally, glucose was almost completely consumed when using EHM4, and the level of glucose and xylose consumption was higher than that of all other substrates such as cellobiose (Supporting Information Figure S1). Total residual carbohydrates were higher in SHF than in SSF, regardless of substrates (Supporting Information Figures S2b and c). The level of consumption for total carbohydrates was also higher for all substrates when using SSF compared to SHF, which explained why more butanol was produced from the EHM4 substrate with SSF.

Second, having determined that EHM4 was the optimum fermentation substrate without pH control, we investigated the efficiency of butanol production with pH control. Figure 3 shows the temporal yield profiles of butanol and other solvent products in small EHM4 anaerobic batch cultures where pH was controlled. Butanol concentration increased with pH control for both SHF and SSF (see Figures 2e and 3e vs Figure 4a and b). In both methods, the consumption of total sugars stalled after 72 h, but total residual sugars were lower with pH control than without (see Figure S2b and c vs Figure 4c). The highest butanol concentrations in the batch fermentations were 11.63 g/L for SHF and 13.56 g/L for SSF, while total ABE production was 19.83 g/L for SHF and 22.2 g/L for SSF (Figure 4a and b; Table 3), all of which were higher than the corresponding values obtained without pH control (Table 3).

Experimental results showed that the butanol yield from SSF was higher than that from SHF (Figures 2 and 3 and Table 3), because SSF uses simultaneous conversion of sugars to avoid the slow sugar uptake rate in SHF. Additionally, we saw that the optimum substrate for obtaining the highest butanol yield was from the EHM4 substrate without pH control regardless of using SHF or SSF (Figure 4). The productivity data in Table 3 also indicate that controlling the pH can increase the final butanol yield from the EMH4 duckweed substrate.

The pH would have been measured and observed during the time course of the fermentation. The pH is apparently a factor in maximizing yield; total butanol yield has a close relationship to pH value, as the concentration of acids in the fermented product leads to pH change and also indirectly reflects the efficiency of fermentation. In this study, final pH values are different for different substrates, after SSF fermentation. Total acid production was lower when using SHF as compared to SSF when pH was not controlled (Figure S2a); however, when pH was controlled, total acids were almost equal between the two methods (Table 3). In the SHF cultures, the high pH of EHM1, -2, -3, and -5 compared with that of EHM4 suggests that there was an untimely end to fermentation (Figure S2a). Therefore, the change of pH also clarified the data for the high butanol titer that was presented in Figure 4.

In addition, residual carbohydrates were higher when using SHF, as compared with SSF, regardless of pH control (Figure S2b and c), which demonstrates an advantage of simultaneously conducting enzymatic hydrolysis and fermentation. This result suggests that the final ABE concentration may have been higher, and the ABE production faster, with SSF, because of sugar removal during the saccharification process, which eliminated product inhibition.

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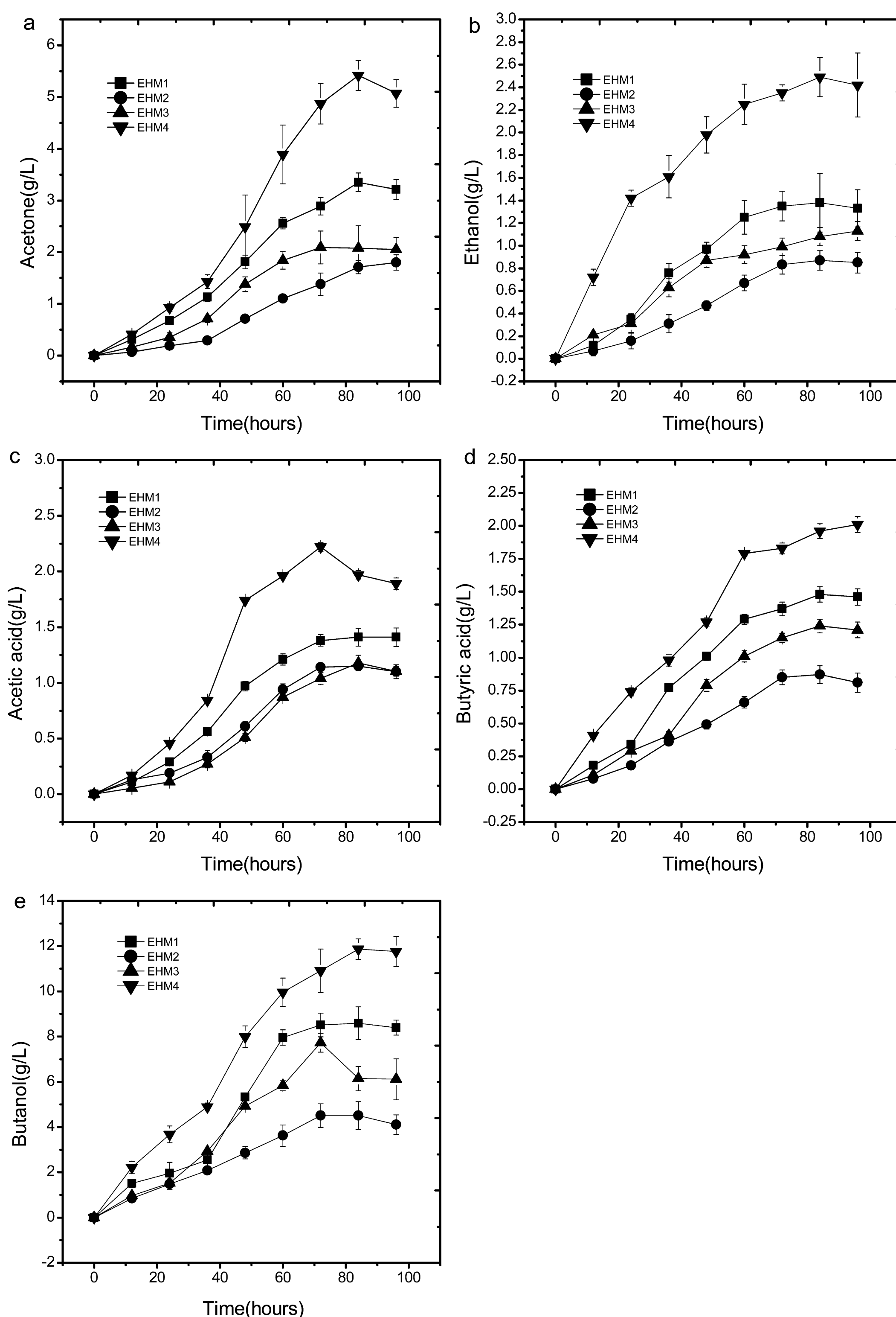

Figure 3. Products of fermentation over time given different enzyme hydrolysis methods conducted via SSF in small anaerobic bottles without pH control. EHM1, -2, -3, and -4 denote enzyme hydrolysis methods 1, 2, 3, and 4, respectively. Error bars indicate SD (n = 3).

Taken together, the data from the above analyses suggest that the best fermentation substrate is a product of EMH4 and that the optimal fermentation process for obtaining high butanol yield is using SSF with pH control.

Scaled-up Butanol Fermentation in a Bioreactor. To investigate the potential value of using duckweed on an industrial scale, the fermentation of duckweed was scaled up for a 20-L bioreactor. Using EHM4 substrate, the highest yields of butanol were 11.32 and 13.26 g/L for SHF and SSF, respectively, whereas the yields for acetone were 4.41 and 5.15 g/L and those for total solvents were 21.77 and 26.68 g/L, for SHF and SSF, respectively (Figure 5a and b; Table 3). The yield of butanol obtained from the scaled up fermentation was similar to that of the small-scale flask experiments with

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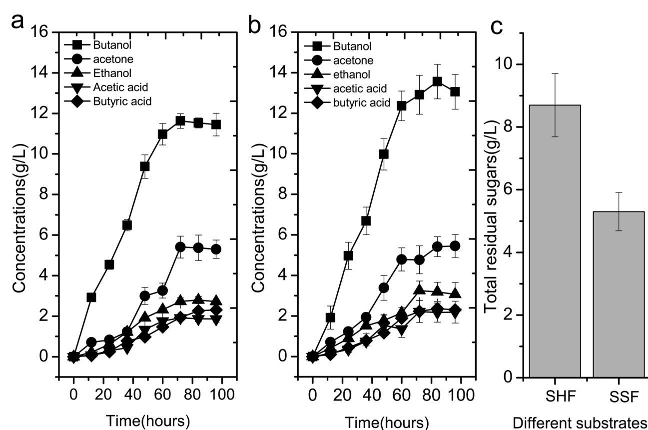

Figure 4. Temporal change in fermentation products and total residual sugars when using enzyme hydrolysis method 4 (EHM4) via SHF and SSF in small anaerobic bottles with pH control. Fermentation products resulting from (a) SHF and (b) SSF methods. (c) Total residual sugars after SHF and SSF. Error bars indicate SD (n = 3).

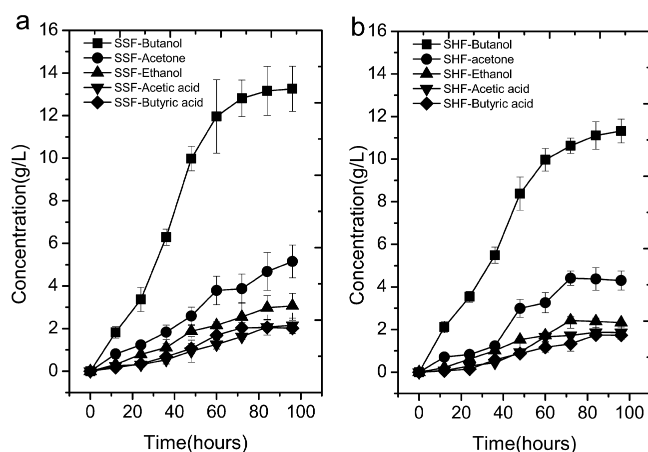

Figure 5. Temporal change in fermentation products when using enzyme hydrolysis method 4 (EHM4) with SHF and SSF, pH control, and scaled-up butanol fermentation in the bioreactor. Change of fermentation products during (a) SSF and (b) SHF. Error bars indicate SD (n = 3).

controlled pH (e.g., 13.56 vs 13.26 g/L for SSF; 11.63 vs 11.32 g/L for SHF). The yield of total solvents was also similar using these methods.

These results suggest that butanol yield does not decrease as compared to prior yield from fermentation in small anaerobic bottles (Figure 5). At the moment, the use of agricultural crops rich in lignocellulose to produce butanol is far from industrial feasibility, regardless of acid hydrolysis or enzyme hydrolysis applications. In contrast, we obtained a relatively high butanol yield from substrates of duckweed that underwent enzymatic hydrolysis, which showed scalability of the results from a small-scale laboratory model to industrial large-scale application. These results show the potential for duckweed development in the butanol industry and strongly suggest that butanol production from duckweed may be feasible on an industrial scale.

Comparison Experiments with Pure Glucose and Sugar Mixtures. In order to adequately confirm the potential of duckweed use for butanol production, a comparison experiment was necessary with pure glucose and artificial sugar mixtures, to mimic the sugar compositions of hydrolysate from duckweed, after butanol production from the three substrates. Butanol production was highest when using pure glucose (13.91 g/L), and there was no significant difference in yield (13.56 g/L) from the EHM4 substrate using SSF with pH control, but a significant difference ($P < 0.05$) resulted from the use of EHM4 substrate SHF. The highest yield of total ABE was obtained when using pure glucose (22.64 g/L), and this was significantly different ($P < 0.05$) from the yield obtained with the EHM4 substrate of SHF (Figure S3; Table 3). When using the sugar mixture as a substrate, the highest butanol yield was 12.28 g/L; however, the highest total ABE yield with sugar mixture was 20.58 g/L, which was lower than the yield from SSF with pH control (22.2 g/L; Figure S4; Table 3). In terms of fermentation productivity, the rate of sugar consumption and ABE productivity appeared to produce results similar to duckweed hydrolysates as substrates for fermentation (Table 3). Interestingly, the ability of *C. acetobutylicum* ATCC 4259 to produce butanol from duckweed when using EHM4 pretreatment, SSF, and pH control was similar to the production of butanol from pure glucose (Figure S3, Table 3). The comparison experiments further validated the potential of duckweed as an energy crop for butanol production by the same fermentation strain. The consistent performance by the bacterial strain clearly indicated the feasibility of duckweed to be a fermentation substrate. Additionally, the almost identical ABE yields obtained from duckweed processed with SSF VS fermentation of mixed sugars (Figure S4, Table 3) showed that inhibitors in the pretreated substrate, such as furfural and hydroxymethyl furfural, may not reach levels which suppress bacterial growth (Table 4). The combination of different enzymatic hydrolysis procedures is an effective method that prevents the inhibiting effect of inhibitory compounds for growth of microorganisms.[42−46] Therefore, the conditions used in the EHM4 pretreatment procedure may be advantageous for fermentation and, thus, yield more butanol.

Table 4. Inhibitor Determination from Acid Hydrolysates of Duckweed[a]

compounds	concentration (mg/L)
furfural	39.15 ± 0.92
hydroxymethylfurfural (HMF)	6.19 ± 0.32
glucuronic acid	ND
p-coumaric acid	ND
syringic acid	ND
ferulic acid	ND

[a]ND: Not detected.

Overall, the analyses above showed that pretreatment using SSF was more effective than that using SHF when duckweed is used as a feedstock to produce butanol. EHM4 is apparently a suitable pretreatment method for increasing ABE production from duckweed. Therefore, we suggest that duckweed, as a new feedstock energy plant, has the potential to be exploited for efficient ABE production in industry application.

■ CONCLUSION

In conclusion, the potential of ABE production from a duckweed substrate was investigated by using different enzyme hydrolysis combinations, strengthening the fermentation process with pH-stat, and comparison fermentation experiments. Our research results further show the potential for duckweed to be an energy crop for biobutanol production, as

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well as the promise of enzyme hydrolysis producing. Duckweed is an energy-efficient substrate for butanol production, and we suggest that it could be used to produce biobutanol as a future biofuel. Our study also provides evidence for further development of duckweed as a feedstock for industrialized biofuel production.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.5b00538.

Figures S1−S5, including the data of changing of sugar compositions, pH value and residual carbohydrates (PDF)

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Author Contributions

Haifeng Su participated in the conception, design, data collection and analysis, and drafted the manuscript. Guihua Xu assisted the laboratory work, results interpretation and the manuscript revision. Yuanjian Xu provided financial support and participated in the partial discussion of the results and revised the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation; LHW, liquid hot water pretreatment; EHM1, Enzymatic hydrolysis method 1; EHM2, Enzymatic hydrolysis method 2; EHM3, Enzymatic hydrolysis method 3; EHM4, Enzymatic hydrolysis method 4; EHM5, Enzymatic hydrolysis method 5

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