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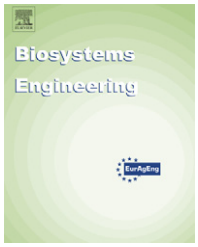


Research Paper

# Production of high-starch duckweed and its conversion to bioethanol

*Jiele Xu* [a], *Weihua Cui* [b], *Jay J. Cheng* [a,*], *Anne-M. Stomp* [c]

[a] *Department of Biological and Agricultural Engineering, Campus Box 7625, North Carolina State University, Raleigh, NC 27695-7625, USA*
[b] *China University of Geosciences (Beijing), School of Water Resources and Environment, Beijing 100083, China*
[c] *Department of Forestry and Environmental Resources, Campus Box 8008, North Carolina State University, Raleigh, NC 27695-8008, USA*

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Growing high-starch duckweed for its conversion to bioethanol was investigated as a novel technology to supplement maize-based ethanol production. Under the fall (autumn) climate conditions of North Carolina, the biomass accumulation rate of *Spirodela polyrrhiza* grown in a pilot-scale culture pond using diluted pig effluent was 12.4 g dry weight $m^{-2}$ $day^{-1}$. Through simple transfer of duckweed plants into well water for 10 days, the duckweed starch content increased by 64.9%, resulting in a high annual starch yield of $9.42 \times 10^3$ kg $ha^{-1}$. After enzymatic hydrolysis and yeast fermentation of high-starch duckweed biomass in a 14-l fermentor, 94.7% of the theoretical starch conversion was achieved. The ethanol yield of duckweed reached $6.42 \times 10^3$ l $ha^{-1}$, about 50% higher than that of maize-based ethanol production, which makes duckweed a competitive starch source for fuel ethanol production.



## 1. Introduction

Maize (corn) grain is currently the dominant feedstock for bioethanol production in the United States. In 2009, the US ethanol industry produced a record 40 billion litres of ethanol from maize starch, at an increase of 18% over the previous year (RFA, 2010). However, since maize is also an important food/feed source, its conversion for energy purposes would put much stress on food/feed supplies (Sun & Cheng, 2002). Moreover, intensive maize production has raised environmental concerns. Maize has high requirements for agricultural inputs, which results in substantial environmental pollution, and its cultivation causes more total soil erosion than that of any other crop (Pimentel, 2003). Therefore, it is necessary to explore novel starch sources to supplement maize starch to make the development of ethanol industry more sustainable and environmentally friendly.

Duckweed (*Lemnaceae*) is a free-floating aquatic plant that not only has a longer growing period but also grows faster than most other plants including field crops. It can grow all seasons in areas with warm climates and doubles its biomass within two days under the optimal conditions (Landesman, Parker, Fedler, & Konikoff, 2005). Duckweed has been used in the treatment of domestic effluent for more than two decades (Oron, 1994; Oron, De-Vegt, & Porath, 1988; Van der Steen, Brenner, & Oron, 1998). Because of its tolerance to high nutrient levels and preferred absorption of ammonium, duckweed has also been successfully used to recover the nutrients from pig effluents (Bergmann, Cheng, Classen, & Stomp, 2000; Shen, Xu, Hu, Zhao, & Liu, 2006; Xu & Shen, 2011).

* *Corresponding author*. Tel.: +1 919 515 6733; fax: +1 919 515 7760.
E-mail address: jay_cheng@ncsu.edu (J.J. Cheng).
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Research has shown that duckweed is a potential starch source for ethanol production (Cheng & Stomp, 2009). Depending on the duckweed species and the growing conditions applied, starch contents ranging from 3 to 75% have been reported (Landolt & Kandeler, 1987; Reid & Bieleski, 1970). Reports also show that the starch content of duckweed can be considerably increased by manipulating growing conditions, e.g., pH, phosphate concentration, and nutrient starvation (Cui, Xu, Cheng, & Stomp, 2010; McLaren & Smith, 1976; Tasseron-De-Jong & Veldstra, 1971). A previous laboratory study has shown that, at a temperature of 5 °C and a photoperiod of 12 h, the duckweed starch content increased by 59.3% through simple transfer of fresh duckweed fronds of *Spirodela polyrrhiza* from a nutrient-rich solution to well water for two days (Cui et al., 2010). Although duckweed-to-ethanol conversion appears to be a promising technology to supplement maize-based ethanol production and some laboratory-scale experiments have already shown encouraging results, a comprehensive investigation based on a larger scale operation is required to better evaluate the technical and economic viability of this technology.

In this study, *S. polyrrhiza*, a local duckweed strain, from North Carolina, that was found to have a great potential for starch accumulation, was used for starch production. A pilot-scale duckweed culture pond was operated for the production of biomass using pig effluent lagoon water. Nutrient starvation was imposed on duckweed plants by growing postharvest duckweed in well water for starch accumulation. Some studies show that salinity stress favours the starch synthesis in some plants (Rathert, 1983; Yin et al., 2010). To evaluate its effect on duckweed, starch accumulation at different loadings of sodium hydroxide (NaCl) were investigated. A 14-l fermentor was used for the enzymatic hydrolysis and yeast fermentation of high-starch duckweed for ethanol production. A preliminary comparison was conducted between maize- and duckweed-based ethanol productions to better assess the promise of duckweed-to-ethanol conversion. In addition, the nutrient removal from wastewater caused by duckweed growth was also examined because it provides a remarkable environmental benefit and a potential economic opportunity in the future commercialisation of this technology.

## 2. Materials and methods

### 2.1. Experimental layout

Fig. 1 is a schematic diagram of the experimental layout. Due to the different growing conditions required, two sequential steps are involved in the production of high-starch duckweed: biomass production and starch accumulation. Duckweed plants from each step were sampled for starch analysis. Since protein is the other important component of duckweed and a valued ingredient of the residual duckweed meal that can be used to produce valuable by-products such as animal feed supplements or organic fertiliser (Ahmad, 1990; Islam et al., 2004), its change was also examined. The postharvest high-starch duckweed was subjected to enzymatic hydrolysis for fermentable sugar production, and then the hydrolysate was fermented anaerobically by yeast to produce ethanol. The total reducing sugars in the hydrolysate and the ethanol in the fermentation liquor were measured, with the reaction efficiencies determined to evaluate the performance of the overall conversion.

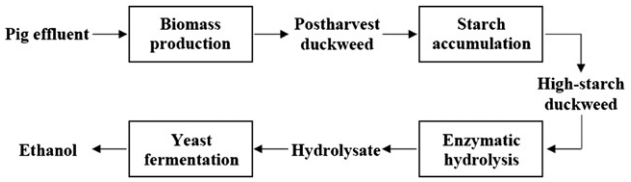


**Fig. 1 – Schematic diagram of the experimental layout.**

### 2.2. Biomass production

A pilot-scale duckweed culture pond of 300 $m^2$ in surface area and 0.6 m in depth was operated for biomass production at Barham Farm, Zebulon, NC, USA (Fig. 2a). Pig effluent from the pig rearing buildings on the farm flows into an ambient temperature anaerobic digester for primary treatment. The treated effluent was stored in a lagoon from which the high nutrient liquid was pumped regularly into the duckweed culture pond to maintain the ammonium ($NH_4$–N) concentration of pond water at about 20 mg $l^{-1}$. The newly produced

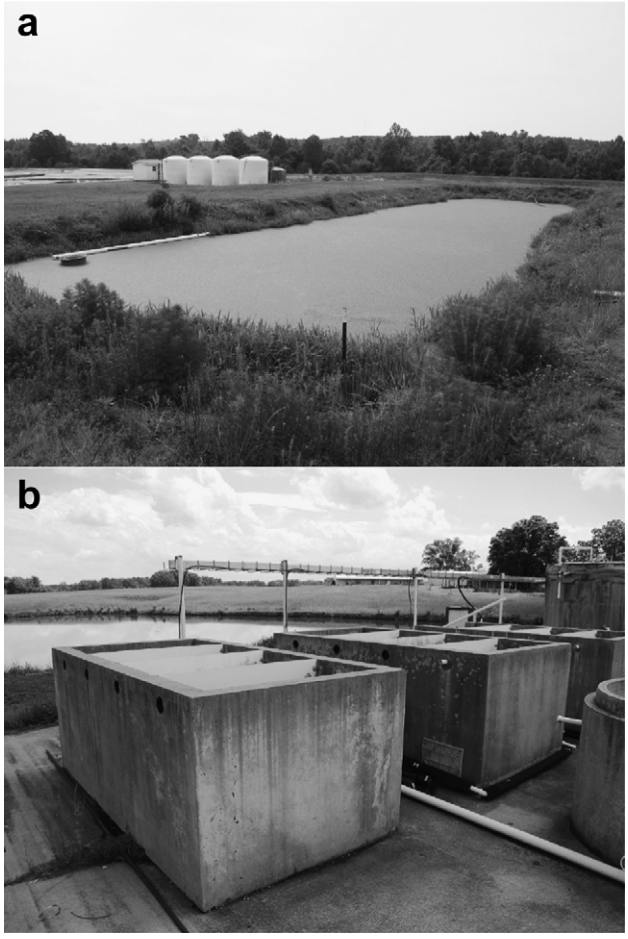


**Fig. 2 – Experimental setups for (a) biomass production and (b) starch accumulation.**

biomass from duckweed growth was harvested from the pond three times a week, keeping the pond covered by approximately two layers of duckweed fronds. The biomass yields were recorded and the compositions of duckweed plants were analysed for four consecutive weeks from August 30 to September 26, 2010 to evaluate the performance of the duckweed culture system. The nutrient removal ability of the duckweed system was evaluated by carrying out a mass balance of $NH_4$–N and o-$PO_4$–P to the culture pond.

### 2.3. Starch accumulation

Fresh harvested duckweed was transferred into 1.5 × 3.2 × 1.3 m (W × L × D) concrete tanks filled with 1 m of well water for starch accumulation under nutrient starvation stress (Fig. 2b). In each tank, 3 kg of fresh duckweed covered the water surface with approximately two layers of fronds. The effect of salinity stress was investigated by conducting another batch of experiment in 0.5 × 1.0 × 0.5 m (W × L × D) water tubs filled with 0.4 m of well water. In each tub, 300 g of fresh duckweed was added and a desired amount of NaCl was dissolved into the well water to obtain a NaCl concentration of 0, 10, 20, or 30 mmol $l^{-1}$. The range of NaCl concentration investigated was determined based on a preliminary study which showed that NaCl concentrations higher than 37.5 mmol $l^{-1}$ were detrimental to duckweed plants. The duckweed was sampled every other day to monitor its compositional change until the starch content became stabilised. The water lost to evaporation was replaced with well water every other day throughout the experimental period. When the starch content of the duckweed grown in well water levelled off, all the duckweed biomass (from both batch experiments) was harvested using a strainer. After the fresh weight of duckweed was recorded, the duckweed was scattered onto a concrete board and sun dried for three days. The dried biomass was collected in plastic bags, sealed, and delivered to the Bioproducts Development Lab in the Department of Biological & Agricultural Engineering at NCSU for enzymatic hydrolysis and yeast fermentation.

### 2.4. Enzymatic hydrolysis and yeast fermentation

The enzymatic hydrolysis and yeast fermentation were conducted in a 14 l (10 l working volume) continuous stirred tank reactor (CSTR) fermentor (Bioflo 110, New Brunswick, NJ, USA). The CSTR fermentor test setup is shown in Fig. 3. The method for enzymatic hydrolysis of starch was adapted from Megazyme Total Starch Assay Procedure (2008) and Sluiter and Sluiter (2005). To improve enzyme accessibility, the dry duckweed was ground using a Cyclotec™ 1093 Sample Mill (Foss in North America, Eden Prairie, MN, USA) with a 2 mm screen. One thousand gram of duckweed biomass (dry basis), 4 l of MOPS buffer (pH 7), and 1.5 × $10^6$ units of α-amylase (Sigma A3404, Sigma–Aldrich, Inc., St. Louis, MO, USA) were mixed in the fermentor. The slurry was incubated at 90 °C for 45 min and an agitator rotation speed of 150 rpm was applied throughout the hydrolysis. After the reaction time elapsed, glacial acetic acid was added to adjust the pH of the hydrolysate to 4.5 and 2 × $10^5$ units of pullulanase (Sigma P2986, Sigma–Aldrich, Inc., St. Louis, MO, USA) was added for further hydrolysis. The hydrolysate was incubated at 60 °C for 30 min.

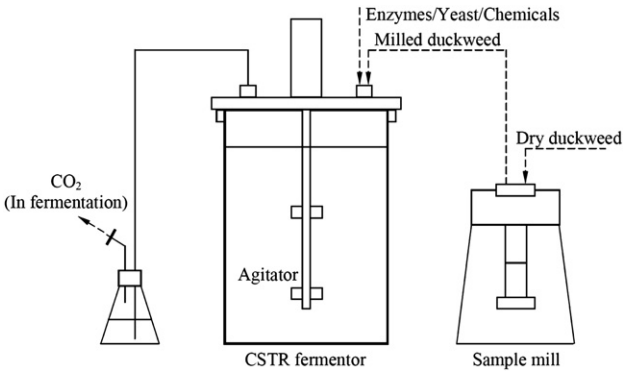


**Fig. 3 – CSTR fermentor test setup.**

After that, heating was stopped for a while to let the temperature decrease. When the temperature dropped to 50 °C, $10^5$ units of amyloglucosidase was added and the hydrolysate was incubated at 50 °C for 4 h. After enzymatic hydrolysis, deionised water was added to bring the total volume to 5 l. A small amount of liquid sample was collected for sugar analysis.

Enough *Saccharomyces cerevisiae* (ATCC 24859) cells for ethanol fermentation were grown according to Chen, Sharma-Shivappa, Keshwani, and Chen (2007). Desired volume of inoculum was used to inoculate the hydrolysate to reach a cell concentration of 6.2 g dry matter $l^{-1}$, and the hydrolysate was adjusted to pH 7 by adding 2 N NaOH. Yeast fermentation was conducted under an anaerobic condition at 30 ± 1 °C for 72 h and the liquid sample was collected for ethanol analysis after fermentation.

### 2.5. Analytical methods

The water temperature and light intensity were recorded respectively using UA-001-64 HOBO Temperature/Alarm Pendant Data Logger and UA-002-64 HOBO Temperature/Light Pendant Data Logger (Onset Computer Corporation, Bourne, MA, USA). The fresh and dry weights of duckweed were measured according to Xu and Shen (2011). The starch analysis method was adapted from Sluiter and Sluiter (2005) and the protein analysis method was adapted from Casal, Vermaat, and Wiegman (2000). The characteristics of pig effluent lagoon water and well water were determined by analysing $NH_4$–N, nitrate nitrogen ($NO_3$–N), nitrite nitrogen ($NO_2$–N), total phosphorous (TP), K, Na, Ca, Mg, Zn, Cu, and pH. Water samples from duckweed culture pond were analysed for $NH_4$–N and o-$PO_4$–P since they are the major nutrients in pig effluents that can be readily utilised by duckweed. Standard Methods for the Examination of Water and Wastewater (1995), and Methods for Chemical Analysis of Water and Wastes (1983) were used for water sample analysis. Total reducing sugars in the hydrolysate were measured using 3,5-dinitrosalisylic acid (DNS) method adapted from Miller (1959) and Ghose (1987). The ethanol in the fermentation liquor was measured using a Shimadzu high performance liquid chromatography (HPLC).

## 3. Results and discussion

### 3.1. Biomass production

During the four-week biomass production period, the temperature of duckweed pond water generally fluctuated between 20 °C and 30 °C, a temperature range suitable for the fast growth of *S. polyrrhiza* (Docauer, 1983, pp.223), and the average maximum daytime light intensity was 2.89 mmol $m^{-2}$ $s^{-1}$, which was substantially higher than that applied in the previous laboratory-scale studies (Cheng et al., 2002; Cui et al., 2010) and favourable for the improved biomass production. The characteristic of pig effluent lagoon water was quite stable during the biomass production period, and the typical composition of lagoon water is shown in Table 1. The lagoon water was fed to the duckweed culture pond three times a week to maintain a desired $NH_4$–N concentration of 20 mg $l^{-1}$. Under a harvest frequency of three times a week, a total of 1008 kg fresh duckweed was harvested during the four-week period. Since the average moisture content of the harvested duckweed was 89.7%, a dry biomass yield of 12.4 g $m^{-2}$ $day^{-1}$ was achieved, which was comparable to the yield of 15 g dry biomass $m^{-2}$ $day^{-1}$ obtained by growing duckweed (*Lemna gibba*) in domestic wastewater (Oron, 1994; Oron et al., 1988). The starch and protein contents of duckweed were stable, respectively averaging 18.8 and 25.4% of dry biomass. Based on the total volume of lagoon water added to the duckweed pond and the nutrient levels of lagoon and pond water, a mass balance of $NH_4$–N and o-$PO_4$–P was conducted to evaluate the nutrient removal ability of duckweed pond. Since the lagoon water added to the pond was sufficient to supplement water loss to evaporation and no obvious changes in the water level was observed, it is assumed that the total volume of pond water was constant. Over the four-week period, the total amount of $NH_4$–N and o-$PO_4$–P removed by the duckweed pond were respectively 9.07 and 0.85 kg. The corresponding $NH_4$–N and o-$PO_4$–P removal rates achieved were 1.08 g $m^{-2}$ $day^{-1}$ and 0.10 g $m^{-2}$ $day^{-1}$, respectively. The $NH_4$–N removal rate was comparable with that reported by Cheng et al. (2002). In their field study, growing *Lemna minor* 8627 on diluted pig effluent in batch experiments under the natural autumn climate conditions of North Carolina resulted in peak $NH_4$–N and TP removal rates of 1.29 g $m^{-2}$ $day^{-1}$ and 0.59 g $m^{-2}$ $day^{-1}$, respectively. The lower o-$PO_4$–P removal rate obtained in this study was probably due to the reduced phosphorus concentration of the swine lagoon liquid used.

**Table 1 – Characteristics of pig effluent lagoon water for duckweed cultivation and well water for starch accumulation.**

| Ion | Pig effluent lagoon water [a] (mg/L) | Well water (mg/L) |
|---|---|---|
| $NH_4$–N | 290 | 0.01 |
| $NO_3$–N | 5.47 | 0.01 |
| $NO_2$–N | 4.70 | 0.00 |
| TP | 12.1 | 0.06 |
| K | 926 | 2.80 |
| Na | 384 | 6.63 |
| Ca | 22.2 | 4.04 |
| Mg | 41.4 | 2.03 |
| Zn | 0.28 | 0.01 |
| Cu | 0.09 | 0.00 |
| pH | 8.4 | 7.2 |

a Sampled on September 8, 2010.

### 3.2. Starch accumulation

The starch accumulation test was conducted in early October, right after the biomass production test. Although the average maximum daytime light intensity of 2.91 mmol $m^{-2}$ $s^{-1}$ was comparable with that during biomass production period, the water temperature during starch accumulation period significantly dropped. The average highest and lowest water temperatures were, respectively, 24.2 °C and 14.8 °C, decreased by 3.6 °C and 7.4 °C compared with those during biomass production period, respectively. Although the lower water temperatures would negatively affect biomass production, they would probably favour the accumulation of starch due to the reduced respiration of duckweed plants at night when starch consumption is substantial. The characteristic of well water used is shown in Table 1. Since there were almost no nutrients in well water, the duckweed roots were found remarkably elongated to 5–6 cm over the starch accumulation period. This normally indicates a high photosynthesis rate of duckweed and a low nitrogen supply from the medium (Landolt & Kandeler, 1987). Under nutrient starvation stress, the starch accumulation in duckweed plants was triggered and the starch content reached 29.8% after the duckweed was grown in well water for eight days (Fig. 4a). Salinity stress significantly ($P < 0.05$) accelerated starch accumulation at the

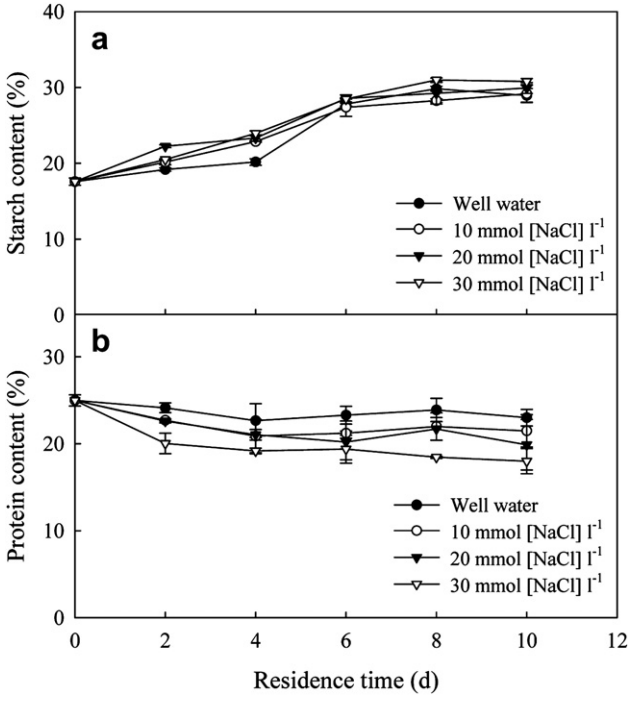


**Fig. 4 – Changes of duckweed (a) starch and (b) protein under salinity stress in the starch accumulation experiment.**

initial stage of the experiment. After four days, the starch contents at NaCl loadings of 10, 20, and 30 mmol $l^{-1}$ were, respectively, 13.4, 15.7, and 18.7% higher than that with no NaCl addition. However, the advantage of imposing salinity stress for improved starch accumulation was less apparent later in the experiment. After eight days when the starch contents generally levelled off, only applying the highest NaCl loading of 30 mmol $l^{-1}$ led to a statistically significant ($P < 0.05$) increase in duckweed starch, while the starch content achieved was only 4% higher than that at no NaCl addition. Therefore, primary cost-benefit analysis is necessary prior to determining whether to apply salinity stress in practical application. The protein content of duckweed significantly ($P < 0.05$) decreased after the duckweed plants were transferred from nutrient-rich pond water to well water (Fig. 4b). This is probably because of the rise of the relative proportion of starch in duckweed biomass. It is also reported that the reduced nitrogen availability in the medium can also result in a reduction in duckweed protein (Chaiprapat, Cheng, Classen, & Liehr, 2005; Leng, Stambolie, & Bell, 1995). With the increase of NaCl loading, the protein content of duckweed significantly ($P < 0.05$) decreased. The protein contents of duckweed stressed by NaCl at 10, 20, and 30 mmol $l^{-1}$ were respectively 6.57, 13.5, and 21.7% lower than that of duckweed without salinity stress. This could be the result of the protein degradation at salt stress (Becker & Fock, 1986; Khan & Gulzar, 2003), and similar trend was found in our previous study at higher NaCl loadings.

Unexpectedly, although without nutrient supply, after 10 days of starch accumulation, the duckweed biomass increased by 81.4, 82.7, 83.0, and 79.8% respectively at NaCl loadings of 0, 10, 20, and 30 mmol $l^{-1}$. Taking the biomass growth into account, the total amount of starch that can be obtained from the duckweed biomass increased by 198.6–214.8% at NaCl loadings from 0 to 30 mmol $l^{-1}$ after the starch accumulation step. The substantial biomass growth probably occurred at the initial stage of the experiment when the duckweed mat was still in proper thickness. The duckweed mat became very thick later in the experiment due to the accumulation of biomass, which would block the light penetration and substantially restricted the growth of duckweed especially the plants trapped lower in the mat. Moreover, the overcrowding itself would inhibit duckweed growth (Xu & Shen, 2011). On the other hand, although the protein content decreased, the total amount of protein increased by 66.9–29.5% at NaCl loadings from 0 to 30 mmol $l^{-1}$ with the biomass growth. Since duckweed was not likely to get access to nitrogen in well water, there must be other nitrogen sources for its protein synthesis. Based on the references, there are two potential nitrogen sources: nitrate stored in the vacuoles of duckweed frond cells and nitrogen provided by $N_2$-fixing microbes. Nitrate, which is the other form of nitrogen that can be readily used by duckweed, was constantly found in the duckweed culture pond. It can be accumulated in inactive 'storage pool' in the duckweed frond when nitrogen is sufficient and brought back to active 'metabolic poor' for reduction under starvation condition (Aslam, Okas, & Huffaker, 1976; Landolt & Kandeler, 1987). $N_2$-fixing heterotrophic bacteria and cyanobacteria can play a significant role in nitrogen supply. It was reported that 15–20% of the nitrogen requirement of the duckweed can be supplied through biological nitrogen fixation by bacteria attached to duckweed mats (Zuberer, 1982). Further studies, however, are required to better understand these or other potential pathways for nitrogen supply.

### 3.3. *Bioethanol production*

The high-starch duckweed harvested from concrete tanks for ethanol production had a final starch content of 31%. After the enzymatic hydrolysis of duckweed biomass using α-amylase, pullulanase, and amyloglucosidase, the reducing sugar recovery reached 96.8%. In the fermentation of hydrolysate (72 h), the yeast loading applied was 6.2 g dry matter $l^{-1}$, which was lower than those recommended in other fermentation researches (Chen et al., 2007; Palmarola-Adrados, Choteborska, Galbe, & Zacchi, 2005). However, at the lower yeast loading, the ethanol production still reached 97.8% of the theoretical yield. The overall starch conversion rate was 94.7%, which indicates that the high-starch duckweed can be very readily converted for bioethanol production.

To better evaluate the promise of using duckweed to produce bioethanol, the ethanol yield from using newly developed duckweed-to-ethanol technology was compared with that from well established maize-to-ethanol conversion. Based on the report of Renewable Fuels Association (2010), the average maize-based ethanol yield was $4.31 \times 10^3$ l $ha^{-1}$ in 2009. The calculation for duckweed-based ethanol yield was conducted based on the data obtained in this study as well as the following assumptions: 1) duckweed can grow nine months under the climate conditions of North Carolina and the growth rate maintains the same as that observed during this study; 2) the moisture and starch contents of high-starch duckweed as well as the starch-to-ethanol conversion rate in a full-scale operation are the same as those obtained in our study; 3) the water surface required for starch accumulation is the same as that required for duckweed biomass production. Based on the above assumptions, a dry biomass yield of $3.35 \times 10^4$ kg $ha^{-1}$ can be achieved using the present growing method. Through the simply transfer of fresh duckweed plants from pig effluent to well water for 10 days, a starch yield of $9.42 \times 10^3$ kg $ha^{-1}$ was achieved, which leads to an ethanol yield of $6.42 \times 10^3$ l $ha^{-1}$, about 50% higher than that obtained using maize.

## 4. Conclusions

Duckweed grew very rapidly in anaerobically treated swine wastewater and the pilot-scale duckweed culture pond can produce 12.4 g dry biomass $m^{-2}$ $day^{-1}$. Under nutrient starvation condition, starch content of duckweed was substantially increased and the total amount of starch tripled after 10 days of starch accumulation. Compared with maize-to-ethanol conversion, a 50% higher ethanol yield can be achieved using the technology developed in this research, making duckweed-to-ethanol conversion a promising technology to supplement maize-based ethanol production. Besides higher ethanol yield, the environmental benefit brought by this technology was remarkable. A total amount of 9.07 kg $NH_4$–N and 0.85 kg o-$PO_4$–P were removed by the 300 $m^2$ duckweed pond over a period of four weeks. A primary cost-benefit

analysis is required before the economic promise of this novel technology can be determined.

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