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Research paper

# Duckweed biomass as a renewable biorefinery feedstock: Ethanol and succinate production from *Wolffia globosa*

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ABSTRACT

For evaluating duckweed biomass as a bioresource, the specific growth rate and the chemical constituents of duckweed of four kinds were investigated. *Spirodela polyrrhiza*, *Lemna minor*, *Wolffia arrhiza*, and *Wolffia globosa* commonly showed high specific growth rates of 0.22–0.30 $d^{-1}$ with initial concentrations of nitrogen >3.0 kg $m^{-3}$ and phosphorus >5.0 kg $m^{-3}$. All duckweeds had high sugar contents greater than 300 g $kg^{-1}$ of dry mass. Especially, vegetative fronds of *W. globosa* showed the highest sugar content of 410 g $kg^{-1}$ of dry mass. The duckweed biomass was pretreated easily by heating at 121 °C for saccharification using α-amylase and amyloglucosidase. The ethanol yield of *W. globosa* biomass in the simultaneous saccharification and fermentation (SSF) using the enzymes and dry yeast was 170 g $kg^{-1}$ of dry mass, whereas the succinate yield in the SSF using the enzymes and *Actinobacillus succinogenes* was 200 g $kg^{-1}$ of dry mass. The production rates of ethanol and succinate from the *W. globosa* biomass were estimated as 0.58 kg $m^{-2}$ $y^{-1}$ and 0.68 kg $m^{-2}$ $y^{-1}$, respectively. The biomass of duckweed, with its high growth rate and high starch content, can be an excellent renewable feedstock for the production of ethanol and succinate as building block chemicals for the replacement of petrochemicals.



## 1. Introduction

Floating aquatic plants such as duckweed grow quickly and absorb large amounts of nutrients, making them useful for purifying eutrophicated wastewater including that of paddy fields and wetlands [1–4]. The aquatic plant biomass used for water purification should then be harvested for final removal of nutrients from the water bodies. Although the harvested biomass is usually discarded as agricultural waste, the aquatic plant biomass is regarded as a promising biorefinery material for producing low-value biofuels and high-value chemicals for the replacement of petrochemicals. A co-beneficial system using constructed wetlands planted with floating aquatic plants is proposed for biorefinery material production and nutrient removal from wastewater [2,3,5].

Ethanol has been produced by fermentation of glucose obtained by saccharification of agricultural biomass such as corn cobs and sugar cane bagasse. For use instead of terrestrial plants, floating aquatic plants are widely regarded as the next promising biorefinery material. Aquatic plants have many benefits such as growing on and in bodies of water without competition against most grains and vegetables for arable land. Especially, the vegetation form of floating aquatic plants will facilitate their movement and harvest. Moreover, duckweed species have high starch contents which can be saccharified easily to glucose [2,3,6,7].

Despite those advantages, data on ethanol production from floating aquatic plants are limited except for water hyacinth *Eichhornia crassipes*, water lettuce *Pistia stratiotes* L. [8], and duckweed *Lemna minor* [3], *Spirodela polyrrhiza* [2], and *Wolffia arrhiza* [7]. As far as we know, no reports in the relevant literature describing succinate production from aquatic plants are available. Succinate is a C-4 linear saturated dicarboxylic acid and a top value-added chemical produced from agricultural biomass as a key building block for the production of various chemicals [9,10].

To evaluate duckweed biomass as a bioresource, the specific growth rates and chemical constituents of duckweed of four kinds were investigated in this study: *L. minor* [11], *S. polyrrhiza* [12], *W. arrhiza* [1], and *W. globosa*. The biomass of *W. globosa* as a model duckweed species was thermally pretreated for saccharification of starch to obtain glucose as the substrate for ethanol and succinate fermentation. Finally, ethanol and succinate were produced from

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the *W. globosa* biomass to demonstrate its potential as a renewable feedstock for use in biorefineries.

## 2. Materials and methods

### 2.1. Plant materials

*L. minor*, *S. polyrrhiza*, and *W. globosa* used in this study were sourced from Hokkaido University Botanical Gardens [11], the campus of University of Yamanashi [12], and a pond in Nara Park, Japan, respectively. *W. arrhiza* was given by Prof. E. Landolt of the Swiss Federal Institute for Technology [1].

### 2.2. Measurement of specific growth rates of duckweed

Duckweed was routinely cultivated in 100-fold diluted solution of Hyponex 6-10-5, a commercial liquid fertilizer (Hyponex Japan Corp. Ltd., Japan) in a plant growth chamber (LH-200-RDH, Nippon Medical & Chemical Instruments Corp., Ltd, Japan) at 28 °C under fluorescent lamps with a 16 h $d^{-1}$ photoperiod. The 100-fold diluted Hyponex solution contains total nitrogen 6.0 kg $m^{-3}$, nitrate-N 2.9 kg $m^{-3}$, ammonium-N 1.05 kg $m^{-3}$, phosphorus 10.0 kg $m^{-3}$, potassium 5.0 kg $m^{-3}$, magnesium 50 g $m^{-3}$, manganese 1 g $m^{-3}$, boron 5 g $m^{-3}$, and other minerals.

To measure the specific growth rate (μ), each duckweed was precultivated in 100 $cm^3$ of 100-, 200-, or 1000-fold diluted Hyponex solution in a 300 $cm^3$ flask at 28 °C under fluorescent lamps with a 16 h $d^{-1}$ photoperiod at 7500 lx for 7 d. Subsequently, 2 stocks of *S. polyrrhiza* [11], 10 stocks of *L. minor*, 50 stocks of *W. arrhiza* [1], and 100 stocks of *W. globosa* were cultivated in the 100 $cm^3$ solutions at 28 °C under fluorescent lamps with a 16 h $d^{-1}$ photoperiod at 7500 lx for 7 d. The μ value was defined by eq. (1).

$$\mu = \frac{1}{T}\ln\frac{X_f}{X_i} \tag{1}$$

where $T$ is the cultivation period (d), $X_i$ is the initial biomass (g-wet), and $X_f$ is the final biomass (g-wet).

### 2.3. Measurement of chemical constituents of duckweed

*L. minor*, *S. polyrrhiza*, *W. arrhiza*, and *W. globosa* were cultivated in 200 $cm^3$ of 100-, 200-, or 1000-fold diluted Hyponex solution in a 500 $cm^3$ flask at 28 °C under mercury lamps with a 16 h $d^{-1}$ photoperiod at 7500 lx for 7 d in a greenhouse. Fresh vegetative fronds of each duckweed were washed manually using tap water, dried at 60 °C, and powdered to pass a 0.8 mm-mesh sieve. The powdered samples were immediately used for chemical analysis of monosugars, starch, crude protein, lignin, and ash.

For turion formation, *S. polyrrhiza* and *W. globosa* were cultivated in 200 $cm^3$ of A&H solution [13] in a 500 $cm^3$ flask at 28 °C under mercury lamps with a 16 h $d^{-1}$ photoperiod at 7500 lx for 14–28 d. Turions that sank to the bottom were collected and used for chemical analysis.

The monosugars and lignin contents were measured according to the laboratory analytical procedures of the National Renewable Energy Laboratory (NREL) for standard biomass analysis [14]. The starch contents were measured using a total starch assay kit (Megazyme International Ireland, Ltd., Ireland). The ash content was measured using the laboratory analytical procedures of NREL [15]. The crude protein content and the total nitrogen content were measured according to the method set out in Japan Industrial Standard K0102 [16].

### 2.4. Pretreatment and saccharification of duckweed biomass

As pretreatment for saccharification, 50 g-wet of the *W. globosa* biomass (mainly vegetative fronds) was washed gently by water, drained, and autoclaved at 121 °C for 20 min. For the glucose recovery test, the pretreated biomass was used for enzymatic saccharification using $2.9 \times 10^3$ units of α-amylase (Wako Pure Chemical Industries Ltd., Japan) and 75 units of amyloglucosidase (Wako Pure Chemical Industries Ltd.) with 50 $cm^3$ of $50 \times 10^{-3}$ mol $m^{-3}$ citrate buffer solution (pH 5.0) for 12 h at 37 °C.

### 2.5. Ethanol fermentation of duckweed biomass

For ethanol production, the pretreated *W. globosa* biomass (mainly vegetative fronds) was used in the simultaneous saccharification and fermentation mode (SSF). In a 300 $cm^3$ flask, 50 g-wet of the pretreated *W. globosa* biomass was mixed with $1.5 \times 10^3$ units of α-amylase, 38 units of amyloglucosidase, and 50 mg of a dried yeast (*Saccharomyces cerevisiae*, Super camellia; Nisshin Seifun Group Inc., Japan) with 50 $cm^3$ of $50 \times 10^{-3}$ mol $m^{-3}$ citrate buffer. The flasks were kept at 37 °C on a rotary shaker (1.33 Hz) for 12 h.

### 2.6. Succinate fermentation of duckweed biomass

The pretreated biomass of *W. globosa* (mainly vegetative fronds) was also used for succinate production in the SSF using the enzymes and a bacterial strain, *Actinobacillus succinogenes* ATCC55618 with $CO_2$ supply. A colony of *A. succinogenes* on a trypticase soy broth (TSB; Becton Dickinson, NJ, USA) agar plate was cultivated and then inoculated into 1 $cm^3$ of TSB in a 10 $cm^3$ bottle and cultivated on a reciprocal shaker (2.00 Hz) at 28 °C for 24 h. Subsequently, 100 $mm^3$ of the culture was transferred into 1 $cm^3$ of TSB in a 10 $cm^3$ bottle and cultivated on the rotary shaker for 18 h for precultivation. In a 100 $cm^3$ bottle, 50 g-wet of the autoclaved biomass was added to 50 $cm^3$ of a medium (yeast extract 5.0 kg $m^{-3}$, $NaHCO_3$ 10.0 kg $m^{-3}$, $NaH_2PO_4 \cdot H_2O$ 8.5 kg $m^{-3}$, $K_2HPO_4$ 15.5 kg $m^{-3}$, pH 7.0) with $2.9 \times 10^3$ units of α-amylase, 75 units of amyloglucosidase, and the bacterial culture at 0.1 optical density at 600 nm. The SSF mixture was stirred at 5.00 Hz and 37 °C with $CO_2$ (>99.5%) supply at 50 $cm^3$ $min^{-1}$.

### 2.7. Chemical analysis in SSF

Samples were collected periodically from the SSF bottles for chemical analysis. Before determination of chemical concentrations, samples were centrifuged (15,000 × *g*, 10 min, 4 °C) and filtrated by 0.45 mm filter units to separate the supernatant and precipitates. Glucose, ethanol, and succinate concentrations were measured using HPLC (liquid chromatograph LC-10AT; Shimadzu Corp., Japan) with a refractive index detector (RID-10A; Shimadzu Corp.) using an exclusion column (300 × 7.8 mm, Bio-Rad Aminexion HPX-87H; Bio-Rad Laboratories Inc., USA) maintained at 65 °C. The mobile phase was $5 \times 10^{-3}$ mol $m^{-3}$ sulfuric acid at a flow rate of 10 $mm^3$ $s^{-1}$.

## 3. Results and discussion

### 3.1. Specific growth rate and chemical constituents of duckweed

Fig. 1 shows the specific growth rate of duckweed in batch cultivation using the Hyponex solutions for 7 d. *S. polyrrhiza*, *L. minor*, *W. arrhiza*, and *W. globosa* commonly showed high specific growth rates of 0.22–0.30 $d^{-1}$ with initial concentrations of nitrogen >3.0 kg $m^{-3}$ and phosphorus >5.0 kg $m^{-3}$. Reportedly, the

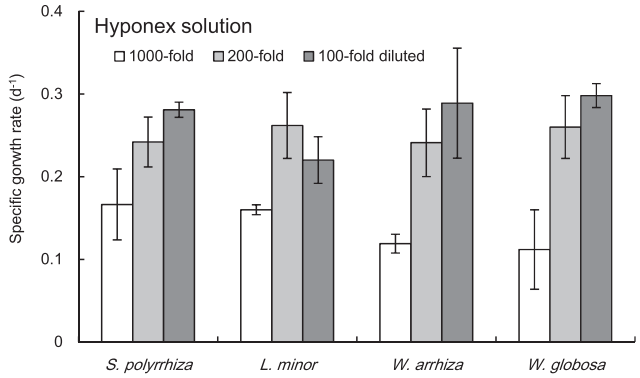


**Fig. 1.** Specific growth rate of duckweed grown in 100-, 200-, and 1000-fold diluted Hyponex solutions at 28 °C under 16 h $d^{-1}$ photoperiod at 7500 lx for 7 d (average ± standard deviation, $n = 3$).

maximum specific growth rates of *L. minor* and *W. arrhiza* were, respectively, 0.40–0.47 $d^{-1}$ [17,18] and 0.35–0.38 $d^{-1}$ [4]. Generally, the specific growth rate of floating aquatic plants is limited by the nutrient concentrations and light intensity [4,17,18].

The water contents (mass fraction) in the fresh biomass of *S. polyrrhiza*, *L. minor*, *W. arrhiza*, and *W. globosa* were 940–950, 920–960, 950–970, and 920–950 g $kg^{-1}$, respectively. Fig. 2 shows average chemical constituents of vegetative fronds of duckweed grown in 100-, 200-, and 1000-fold diluted Hyponex solutions. They had high sugar contents with greater than 300 g $kg^{-1}$ of dry mass. Especially, *W. globosa* showed the highest sugar content of 410 g $kg^{-1}$ of dry mass, of which starch-glucose as the sugar occupied 65%. Based on the experimentally obtained results with assumption of no limitation of water surface and nutrients, the dry biomass and starch yields of *W. globosa* are estimated as 3.39 kg $m^{-2}$ $y^{-1}$ and 1.15 kg $m^{-2}$ $y^{-1}$, respectively, which are comparable to those of typical corn production (1.20 kg $m^{-2}$ $y^{-1}$) [19]. Reportedly, *S. polyrrhiza* and *Lemna gibba* also have high biomass yields of 4.50 kg $m^{-2}$ $y^{-1}$ [2] and 5.5 kg $m^{-2}$ $y^{-1}$ [20,21], respectively.

The low contents of lignin in duckweed might reduce the pretreatment and enzyme dosages. In addition, the crude protein contents of duckweed were 200–250 g $kg^{-1}$ of dry mass, suggesting that it is a nutritious food for fish and animals [22]. Unknown constituents and loss/error in the measurement were 150–250 g $kg^{-1}$ of dry mass in the duckweed biomass. Possible chemicals in the unknown constituents were lipids [23], apiose [3], and pectin [24].

Table 1 presents starch and water contents of the vegetative fronds and turions of *S. polyrrhiza* and *W. globosa* grown in 1000-fold diluted Hyponex solution. The vegetative fronds change to turions as a dormant form in undesirable circumstances such as cold seasons and starvation conditions. The turions contain large amounts of starch and sink to the bottom because of a change in density for survival in unfavorable circumstances [25]. Especially, the *W. globosa* turions showed extremely high starch contents of 490 g $kg^{-1}$ of dry mass. The duckweed biomass is more valuable as a starch resource that can be a feedstock of biorefinery materials if formation of turions from the vegetative fronds can be controlled. In the case of *W. arrhiza* used for wastewater treatment, the turion formation was induced when the specific growth rate decreased below 0.085–0.12 $d^{-1}$ in low-nitrogen and low-phosphorus conditions [4,26].

### 3.2. Ethanol production from duckweed

Because of difficulty with mass production of turions, the *W. globosa* biomass consisting mainly of the vegetative fronds was pretreated at 121 °C for 20 min for glucose production followed by enzymatic saccharification. Results show that 92.3% of starch in the biomass was converted to glucose. The soft tissue of duckweed is pretreated easily and by heating and saccharification by amylase, which presents economical advantages over cellulase, without complicated treatments such as alkali/oxidative treatment for saccharification using cellulase [7] and enzyme cocktails of cellulase and β-glucosidase [6]. Although the pretreatment condition might be harsh for the soft biomass, fermentation media used for lab-scale experiments are usually sterilized by autoclaving.

Fig. 3 presents the occurrence and consumption of glucose and production of ethanol from the pretreated *W. globosa* biomass in the SSF mode. The concentrations of starch-glucose and other glucose in the pretreated biomass were inferred to be 15.1 kg $m^{-3}$ and 5.3 kg $m^{-3}$ in the SSF medium, respectively. Ethanol production proceeded smoothly; the biomass was finally converted to

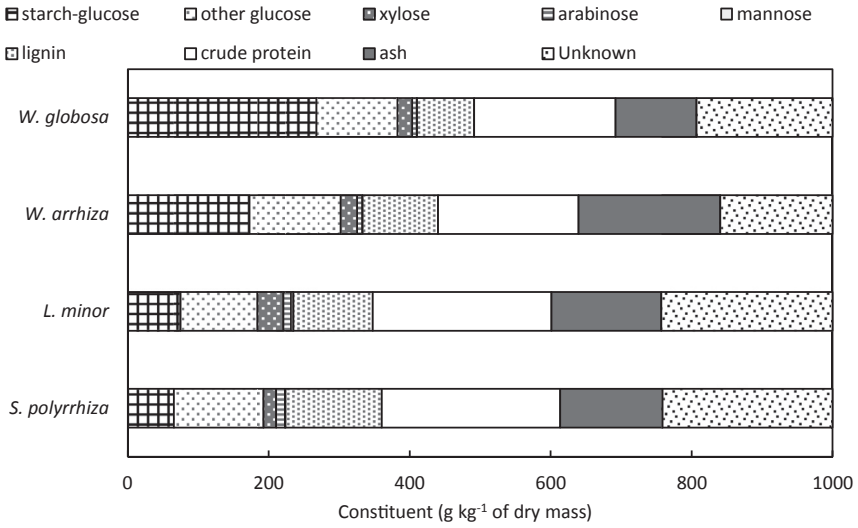


**Fig. 2.** Chemical constituents of duckweed (vegetative fronds), *S. polyrrhiza*, *L. minor*, *W. arrhiza*, and *W. globosa* (average of biomass grown in 100-, 200-, and 1000-fold diluted Hyponex solutions).

**Table 1**
Starch and water contents in vegetative fronds and turions of *S. polyrrhiza* and *W. globosa* grown in 1000-fold diluted Hyponex solution.

| | | Water content (g $kg^{-1}$ of fresh biomass) | Starch content (g $kg^{-1}$ of dry biomass) |
|---|---|---|---|
| *S. polyrrhiza* | Vegetative frond | 940 | 110 |
| | Turion | 590 | 370 |
| *W. globosa* | Vegetative frond | 940 | 220 |
| | Turion | 920 | 490 |

10.8 kg $m^{-3}$ of ethanol in 12 h with a transient accumulation of glucose at 8 kg $m^{-3}$. The ethanol yield in the SSF mode was 170 g $kg^{-1}$ of dry mass, which is comparable to those of water hyacinth (160 g $kg^{-1}$ of dry mass) and water lettuce (170 g $kg^{-1}$ of dry mass) [8] and slightly lower than that of *S. polyrrhiza* (260 g $kg^{-1}$ of dry mass) [27]. Glycerol and acetate were also accumulated slightly as byproducts in the SSF mode. Therefore, the ethanol production can be estimated as 0.58 kg $m^{-2}$ $y^{-1}$.

Reportedly, the ethanol yield of starch-rich turions of *W. arrhiza* in the SSF using an amylase mixture was 250 g $kg^{-1}$ of dry mass [7]. That of the vegetative fronds was 160 g $kg^{-1}$ of dry mass in the SSF mode using cellulase, although that with the amylase mixture was only 70 g $kg^{-1}$ of dry mass [7]. The main form of the *W. globosa* biomass used for ethanol production was not turions but vegetative fronds. These results demonstrate that the vegetative fronds of *W. globosa* can be converted efficiently to ethanol in the SSF using amylase. A higher ethanol yield will be obtained if starch-rich turions of *W. globosa* are used.

### 3.3. Succinate production from duckweed

Fig. 4 presents succinate production from the pretreated *W. globosa* biomass in the SSF mode. After 48 h, the *W. globosa* biomass was ultimately converted to 12.0 kg $m^{-3}$ of succinate and 5.4 kg $m^{-3}$ of acetate as a byproduct. The yield of succinate and acetate was inferred as only 200 g $kg^{-1}$ of dry mass and 90 g $kg^{-1}$ of dry mass, respectively. Therefore, the succinate production can be estimated as 0.68 kg $m^{-2}$ $y^{-1}$.

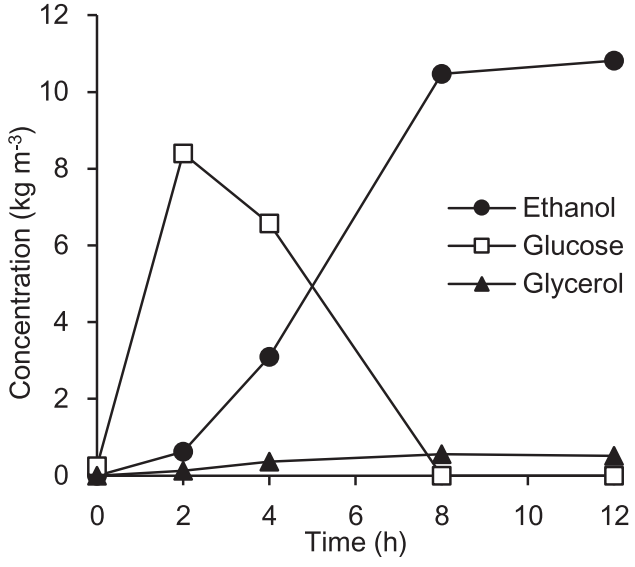


**Fig. 3.** Ethanol production from the *W. globosa* biomass in SSF using α-amylase, amyloglucosidase, and dry yeast (*Saccharomyces cerevisiae*).

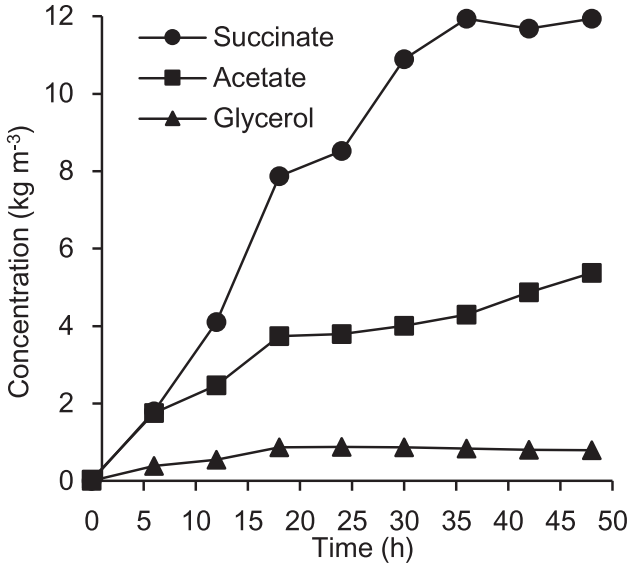


**Fig. 4.** Succinate production from the *W. globosa* biomass in the SSF using α-amylase, amyloglucosidase, and *A. succinogenes*.

A typical reaction for succinate production is expressed as eq. (2).

$$C_6H_{12}O_6 + CO_2 \rightarrow HOOC - CH_2 - CH_2 - COOH + CH_3COOH + HCOOH \quad (2)$$

Theoretically, the succinate yield can be enhanced with a bacterial strain that consumes hydrogen as shown eq. (3) [9].

$$C_6H_{12}O_6 + 2CO_2 + 2H_2 \rightarrow 2HOOC - CH_2 - CH_2 - COOH + 2H_2O \quad (3)$$

This report is the first of a trial of succinate production from floating aquatic plants. Optimization of the fermentation process will bring higher yields of succinate. The $CO_2$ concentration in the fermentation culture of *A. succinogenes* is an important factor for enhancing the succinate yield [28]. Succinate as a fermentation product presents distinct advantages over ethanol. During ethanol fermentation, 2 mol of $CO_2$ is emitted from 1 mol of glucose ($C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$), whereas succinate fermentation consumes $CO_2$. Furthermore, integrating succinate and ethanol fermentation is expected to decrease carbon loss as waste $CO_2$ and is expected to produce three commercial products: succinate, ethanol, and diethyl succinate [9].

## 4. Conclusions

Duckweed biomass, with its high growth rate and high starch content, can be an excellent feedstock for the production of ethanol and succinate as building block chemicals for the replacement of petrochemicals.

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