Original article

Production of bioethanol from four species of duckweeds (*Landoltia punctata*, *Lemna aequinoctialis*, *Spirodela polyrrhiza*, and *Wolffia arrhiza*) through optimization of saccharification process and fermentation with *Saccharomyces cerevisiae*

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ABSTRACT

Duckweeds are promising potential sources for bioethanol production due to their high starch content and fast growth rate. We assessed the potential for four species, *Landoltia punctata*, *Lemna aequinoctialis*, *Spirodela polyrrhiza*, and *Wolffia arrhiza*, for bioethanol production. We also optimized a possible production procedure, which must include saccharification to convert starch to soluble sugars that can serve as a substrate for fermentation. Duckweeds were cultivated on 10% Hoagland solution for 12 days, harvested, dried, homogenized, and dissolved in solutions that were tested as substrates for bioethanol production by the yeast *Saccharomyces cerevisiae*. First, we optimized the saccharification process, including the ideal ratio of the enzyme used to convert starch into simple sugars. The greatest starch-to-sugar conversion was obtained when the α-amylase and amyloglucosidase was 2:1 (v/v) and with a 24 h incubation period at 50 °C. After saccharification, the solutions were incubated with the yeast, *S. cerevisiae*. The fermentation process was carried out for 48 h with 10% (v/v) yeast inoculum. The ethanol content was maximal approximately 24 h after the start of incubation, and the sugars and protein were minimal, with little change over the next 24 h. The final ethanol concentration obtained were 0.19, 0.17, 0.19, and 0.16 g ethanol/g dry biomass for *L. punctata*, *L. aequinoctialis*, *S. polyrrhiza*, and *W. arrhiza* respectively. We suggest that these four species of duckweed have the potential to serve sources of bioethanol and hope that the procedure we have optimized proves useful in the endeavour.

1. Introduction

The world's energy demands from all energy sources are increasing, and this includes an increased demand for the energy from fossil fuels. At the same time, the supply of fossil fuels is limited, particularly as they are a non-renewable resource. Alternative energy sources from renewable materials, therefore, will likely be an important component in meeting the world's energy needs in the future (Joshi et al., 2017, Owusu & Asumadu-Sarkodie, 2016, Real et al., 2016). The U.S. Energy Information Administration, in its 2019 International Energy Outlook (IEO) reference case, predicted that global energy consumption would increase by more than 30% from 2018 to 2050 and that the consumption of bioenergy would increase during that time by 3% per year (https://www.eia.gov/outlooks/ieo/).

Bioethanol is an important biofuel. It is an appealing energy source as it is clean and produced from renewable sources. It is produced through fermentation by microorganisms by one of two methods, distinguished by the raw materials (Baeyens et al., 2015, Naghshbandi et al., 2019). In the first method, bioethanol is generated from raw materials that are rich in starches and sugars such as corn, cassava, and sugar cane (Zabed et al., 2017). This method is not considered ideal as these materials are also food sources for humans or animals. In the second method, bioethanol is produced by the fermentation of lignocellulose acquired from agricultural waste and forestry products (Rastogi & Shrivastava,

2017). The fermentation of these waste products overcomes the problem of competition with useful products, but these materials must be pre-treated to break down the structure of the lignin-carbohydrate complex (Nielsen et al., 2019, Soccol et al., 2019).

It would be ideal to locate a source for bioethanol that would not compete for valuable resources and not require this pre-treatment. Here, we consider species of duckweeds as candidates for bioethanol production. Duckweeds are the smallest aquatic plants and have growth rates that can be as much as 10 times faster than corn or rice, requiring only 2–4 days to double their biomass (Xu et al., 2011, Ziegler et al., 2015). Also, duckweeds are not food crops.

An additional consideration in bioethanol production is the conversion efficiency of starch into sugars (Zabed et al., 2017). Starch is a glucose polymer consisting of amylose and amylopectin. The ratio of these two varies with species and is a factor in conversion efficiency of starch to sugar (Lin et al., 2018, Sun et al., 2017). Duckweeds have 35.7% amylose and 64.3% amylopectin (Lee et al., 2016). Therefore, enzymatic saccharification is a necessary step to hydrolyze these type of starch into simple sugars before it can be efficiently fermented. Efficient saccharification requires identifying the optimal enzyme concentration, pH, temperature, and incubation time (Rattanaporn et al., 2018, Bala & Singh, 2019). In this study, we optimize the saccharification process and the bioethanol production process of four duckweeds species.

2. Methods

2.1. Cultivation of duckweeds

Four species of duckweeds (*Landoltia punctata*, *Lemna aequinoctialis*, *Spirodela polyrrhiza*, and *Wolffia arrhiza*) were cultivated for 12 days on 10% Hoagland solution. Plants were placed in $40.5 \times 31.5 \times 15.5$ cm trays in a screenhouse illuminated with direct sunlight. Plant were weighed daily on a digital balance after blotting them on a filter paper to remove excess solution. The growth curve was determined from these daily measurements of fresh weight (Sembada & Faizal, 2019, Faizal & Putra, 2019). The specific growth rate showed the slope of a growth curve and was defined as the growth rate of biomass per unit of time. The specific growth rate in this study was calculated using the following formula (Shuler & Kargi, 2011):

$$\frac{dx}{dt} = \mu.x$$

$$\int_{t_0}^{t} \frac{dx}{x} = \int \mu dt$$

$$x = x_0 t = t_0$$

$$\ln x - \ln x_0 = \mu.t$$

$$\mu = \frac{\ln x_t - \ln x_0}{\Delta t}$$

In this equation above, x is the weight of the plant (g), t is the time of logarithmic phase (days), and μ is the rate of plant growth (days^{-1}). Doubling time is the time required for plants to double their biomass. Doubling time (dt) was calculated from the following formula (Shuler & Kargi, 2011):

$$dt = \frac{\ln 2}{\mu}$$

In this equation, dt is the doubling time and μ is the plant growth rate (days^{-1}), calculated during the exponential growth phase.

2.2. Harvesting, drying, and dissolving duckweed biomass

Plants were harvested 12 days after the start cultivation and placed in a plastic container coated with aluminum foil. Plants were dried to constant weight in an oven at 65 °C for 48 h, ground in a blender to a powder, and sieved to uniform consistency. Five grams of the powdered biomass were dissolved in 4 mL of 25 mM sodium acetate at pH 5.5, then brought up to a volume of 100 mL with distilled water (Yu et al., 2014). The resulting solution was incubated at 90 °C for 30 min and filtered through Whatmann filter paper no. 1 to remove solids.

2.3. Enzymatic saccharification

Three solutions of the enzymes, α-amylase and amyloglucosidase (Novozymes™) were prepared in different ratios: 1:1, 1:2, and 2:1, respectively. The enzyme solutions were added to 90 mL of duckweed solutions proportional to the respective starch content in dry biomass of each species (1 mL enzyme solution/1 mg starch) in a 125 mL Erlenmeyer flask and incubated for 6, 12, 18, 24, 30, or 36 h in a shaker incubator (125 rpm, 50 °C).

2.4. Culture of Saccharomyces cerevisiae

The yeast *S. cerevisiae* was used in the fermentation process. Before the process, it requires multiplication and then adaptation with three activations. Media were sterilized by autoclaving at 121 °C and 1.5 atm.

Multiplication. Potato Dextrose Agar (PDA) media (39 g/L) was completely dissolved in hot water. Then, 10 mL of sterilized media was added to a tube in an oblique position to form a slanted agar, and 1 loop of yeast was rubbed onto the surface.

Activation. Potato Dextrose Broth (PDB) media (26.5 g/L) was completely dissolved in hot water, and 100 mL aliquots were placed in 125 mL flasks. Next, 1–2 loop of the yeast from the multiplication culture were added to the flasks, and the flasks were then placed in a shaker incubator (37 °C, 125 rpm) for 24 h; this completed Activation 1. After this time, 90 mL of the yeast cultivation media were placed in a 125 mL flask, sterilized, and cooled. 10 mL of the culture from Activation 1 was added, and the flasks were placed in a shaker incubator (37 °C, 125 rpm) for 24 h, completing Activation II. Activation III was carried out by repeating Activation II, except that the flasks were incubated for 24 h and then subcultured.

2.5. Fermentation

Yeast from the final subcultured of Activation III was used in the fermentation. 10 mL of the yeast was added to a 125 mL flask containing 90 mL of duckweeds solution. The flasks were incubated at 37 °C without agitation. The flasks were sampled every 4 h to measure the reducing sugars, protein, ethanol, number of yeast cell, and pH. The starch and ammonium content were measured at the beginning and end of the fermentation process.

2.6. Measurement of starch content

Eight hundred microliters of the duckweed solution were added to 200 μL Lugol reagent, and the absorbance of the solution was measured at a wavelength of 580 nm in a spectrophotometer. The starch content was interpolated with a standard curve.

2.7. Measurement of glucose content

Glucose was measured with the Dinitrosalicylic (DNS) method (Miller, 1959). Three mL of duckweed solution were mixed with

3 mL of DNS reagent, heated at 90 °C for 5–15 min, and 1 mL of Rochelle's salt (40%) was added. The solution was cooled to room temperature and its absorbance was measured at 575 nm.

2.8. Measurement of protein content

Five milliliters of Bradford reagent were added to 100 μL of duckweed solution. The absorbance was measured at 595 nm. The protein content was interpolated from a standard curve using bovine serum albumin.

2.9. Measurement of ammonium content

Ammonium concentration was assessed with the Nessler method (Zhou & Boyd, 2016). Seignette reagent and Nessler reagent (0.1 mL each) were added to 5 mL of duckweed solution, and the solution was agitated with a vortex. Ten minutes after agitation, the absorbance was measured at a 420 nm. Ammonium content was interpolated from a standard curve.

2.10. Measurement of ethanol concentration

The ethanol concentration of the duckweed solutions was carried out by a method developed by Crowell and Ough (1979). Ten milliliters of dichromate reagent were added to 2 mL of duckweed solution, which was then incubated in a water bath at 60 °C for 20 min and left undisturbed until it reached room temperature. Absorbance of the solution was measured at 600 nm.

2.11. Yeast cell count

Yeast cells were counted with a hemocytometer. One to two drops of solution from fermentation process were introduce into the counting chamber of the hemocytometer, which was placed under a light microscope. Yeast cells in the chamber were counted.

3. Results and discussions

3.1. Growth kinetics of duckweeds

The growth curve for all four species of duckweed during the 12 days cultivation is presented in Fig. 1, based on fresh weight accumulation. The fresh weights of all plants were similar at the start of the cultivation. The curves for all species were sigmoid, with a lag phase, an exponential (logarithmic) phase, and a stationary phase. The lag phase lasted approximately from days 0–1, immediately after the plants had been transferred from the acclimatization medium to the cultivation medium. The acclimatization period was imposed to allow the plants to adjust to the cultivation condition so that the lag phase would be short.

The growth curve was dominated by an exponential phase from days 1–11, at which point there was little or no increase in fresh weight. A long exponential phase is expected because duckweed fronds continue to grow as long as they have adequate space and nutrients (Sembada & Faizal, 2019). S. polyrrhiza is the largest species in the duckweed family, consistent with our finding that this species had the greatest fresh weight of the four species by the end of cultivation period. The specific growth rates and doubling times are listed in Table 1.

The doubling time of duckweeds measured in this study is within the range (3–6 days) reported by Yu et al. (Yu et al., 2014). S. polyrrhiza had the highest specific growth rate of the four species, which is consistent with its greater biomass at the end of the study (Fig. 1). Li et al. (Li et al., 2016) measured a specific
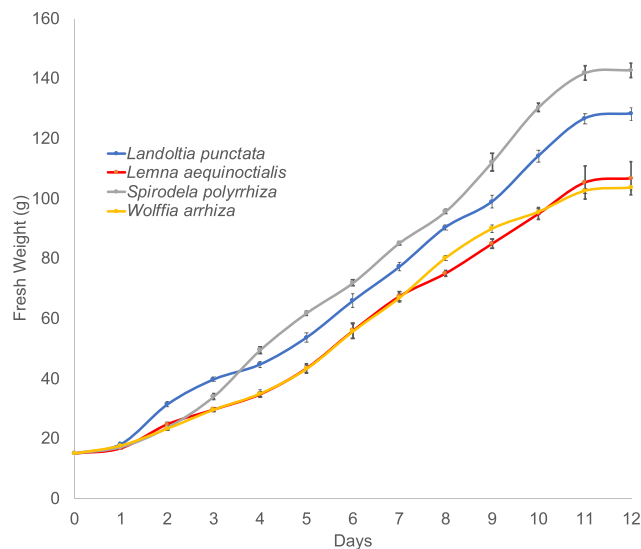

Fig. 1. Duckweeds growth curve. Data represent mean ± SE of the mean.

Table 1
Specific growth rate and doubling time of duckweeds during cultivation.

Species	Specific growth rate (days^{-1})	Doubling time (days)
L. punctata	0,19 ± 0,001	3,57 ± 0,02
L. aequinoctialis	0,18 ± 0,003	3,77 ± 0,07
S. polyrrhiza	0,21 ± 0,002	3,28 ± 0,03
W. arrhiza	0,18 ± 0,002	3,94 ± 0,04

Data represent mean ± SE of the mean.

growth rate of 0.19 days^{-1} for both L. punctata and L. aequinoctialis on the same medium used here; these values agree with ours.

3.2. Optimization of the saccharification process

Saccharification is performed to break down starch into simple sugar that can be fermented. This is often done enzymatically, with α-amylase and amyloglucosidase. Before saccharification process, we measured the initial starch and sugar content of the four duckweed species as depicted in Table 2.

In this study, our goal was to optimize the saccharification process. Specifically, we wished to identify the optimal period of incubation of the plant material with the enzymes, and the ideal ratio of the enzymes.

The time course for the percentage conversion of starch to sugar for different enzyme ratios is shown in Fig. 2. The time courses for the conversion of starch to sugar have similar patterns for the four duckweed species. Conversion increased with incubation time for approximately 24 h, with little or no increase after that. Of the three ratios of enzymes tested, a ratio of α-amylase to amyloglucosidase of 2:1 was most effective in all four species. This enzyme ratio yielded approximately 80% conversion of starch to sugar in all four species.

Table 2
Starch and reducing sugar content of duckweed solutions before saccharification.

Species	Starch content (g/L)	Sugar content (g/L)
L. punctata	13,64 ± 0,15	1,10 ± 0,03
L. aequinoctialis	12,48 ± 0,05	1,10 ± 0,01
S. polyrrhiza	14,91 ± 0,16	1,12 ± 0,01
W. arrhiza	11,50 ± 0,26	1,13 ± 0,01

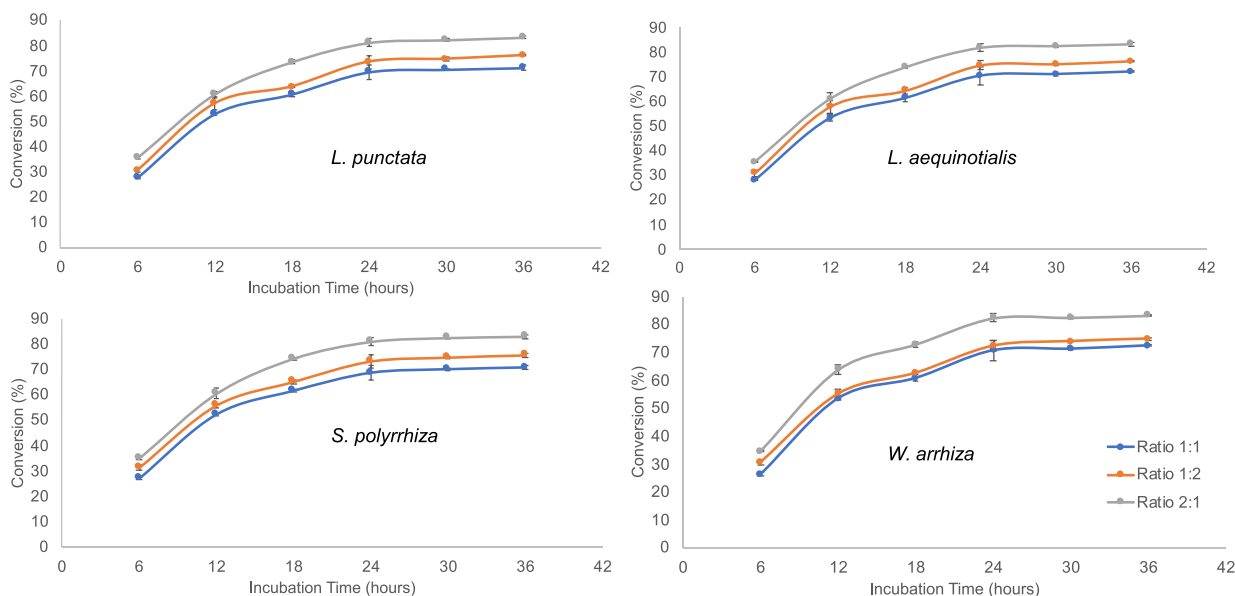Data represent mean ± SE of the mean.

Fig. 2. Time course of enzymatic conversion of starch to sugar by solutions from four duckweed species. Solutions were incubated in the presence of different ratios α-amylase to amyloglucosidase and the disappearance of starch was measured. Data represent mean ± SE of the mean.

The percentage of starch converted was significantly different than the control ($p < 0.05$) for all species at 6, 12, 18, and 24 h. The differences between the 2:1 enzyme ratio and the other enzymes ratios were also significant ($p < 0.05$) at the later time points. We conclude that the optimal incubation time is 24 h and the optimal enzyme ratio is 2:1 (α-amylase to amyloglucosidase).

The α-amylase enzyme breaks the glycosidic α-1,4 bond in both amylose and amylopectin (Hall, 2009), while bypassing α-1,6 glycosidic bonds. Alpha-amylase hydrolyzes amylose to maltotriose and maltose, while it releases the oligosaccharide dextrin from amylopectin. Dextrin is then broken down by amyloglucosidase, which can break both α-1,4 and α-1,6 bonds, into glucose monomers (Hall, 2009). Duckweeds starch has a high content of amylopectin compared to amylose, so the result of the action of α-amylase is mostly dextrin. The products of this reaction are then cut into glucose monomers by amyloglucosidase.

The final products of enzymatic starch breakdown are sugars, including glucose. Glucose is a feedback inhibitor of α-amylase (Alrumman, 2016). After 24 h of incubation with the enzyme, we observed that starch conversion was approximately 80% in all species, and this did not increase with time. We suggest that glucose, which was at a high concentration in the incubation medium at 24 h, inhibited α-amylase.

3.3. Composition of duckweed solutions after saccharification

The starch and sugar content of the duckweed solutions were measured after saccharification is shown in Table 3.

Table 3
Starch and reducing sugar content of duckweed solutions after saccharification.

Species	Starch Content (g/L)	Sugar Content (g/L)
L. punctata	2,40 ± 0,06[b]	12,17 ± 0,14[b]
L. aequinoctialis	2,08 ± 0,07[c]	11,29 ± 0,05[c]
S. polyrrhiza	2,61 ± 0,06[a]	13,18 ± 0,19[a]
W. arrhiza	1,82 ± 0,05[d]	10,61 ± 0,18[d]

Data represent mean ± SE of the mean; Different letters indicate significant differences ($P < 0.05$) according to Duncan test.

A comparison of Tables 2 and 3 reveals that saccharification dramatically decrease the starch contents and increased the sugar content of the duckweed solutions, for all four species. This is consistent with the results in Fig. 2 and the expected action of the enzymes.

Fermentation for the production of bioethanol requires a sugar concentration of around 10 g/L to 20 g/L (Talebnia et al., 2010). Sugar concentrations below 10 g/L are not acceptable because small amounts of bioethanol they will yield. All four species of duckweed solutions had more than 10 g/L sugars. The solution containing *S. polyrrhiza* had the greatest concentration.

The amount of protein and ammonium in the duckweed solutions is presented in Table 4.

In batch bioethanol fermentation, nitrogen must be available to support fermentation by the yeast. The minimum nitrogen source is considered to be 10% of the available carbon source. This minimum requirement is met by all four species. Our results are consistent with those of Chen et al. (Chen et al., 2012), who found that the production of bioethanol from duckweed plants did not require nitrogen supplementation. This is an advantage of duckweed as a source of bioethanol, since nitrogen would not need to be supplied and costs will be lower.

3.4. Growth kinetics of S. Cerevisiae in the fermentation process

The growth of *S. cerevisiae* was measured during the fermentation process, based on the number of cells counted in a hemocytometer at any given time. The growth curves for *S. cerevisiae*

Table 4
Protein and ammonium content (as a measure of N) of duckweed solutions after saccharification.

Spesies	Protein Content (g/L)	Ammonium Content (g/L)
L. punctata	1,31 ± 0,06[a]	0,18 ± 0,003[b]
L. aequinoctialis	1,20 ± 0,07[b]	0,16 ± 0,004[c]
S. polyrrhiza	1,35 ± 0,03[a]	0,20 ± 0,003[a]
W. arrhiza	1,13 ± 0,02[b]	0,14 ± 0,004[d]

Data represent mean ± SE of the mean; Different letters indicate significant differences ($P < 0.05$) according to Duncan test.

during the fermentation process for the four duckweed species are presented in Fig. 3.

The biomass of the yeast began to increase immediately after the start of fermentation process and was maximum at about 20 h for all species, after which there was no or little continued yeast growth. We conclude that the yeast was in an exponential or logarithmic phase from 0 to 20 h after the start of the process, and then entered into a stationary phase from 20 to 48 h. There are two reasons that the growth of the yeast would decline after 20 h. The sugars in the medium serve as the anaerobic substrate for the yeast, but as the yeast uses the sugars, there is less substrate available. The second consideration is that the ethanol content has increased with fermentation, and as the ethanol accumulates, it becomes toxic or even lethal to the yeast, and inhibits its growth. We conclude that the yeast grew vigorously in the medium for the first 20 h. There is also evidence from the growth curves that there is a relationship between bioethanol and yeast growth.

The specific growth rate of *S. cerevisiae* grown in the *L. punctata* was $0.21 \pm 0.02 \ h^{-1}$ with a doubling time of $3.33 \pm 0.31 \ h$; for *L. aequinoctialis* these values were $0.21 \pm 0.02 \ h^{-1}$ and $3.37 \pm 0.33 \ h$, respectively; for *S. polyrrhiza* they were $0.22 \pm 0.02 \ h^{-1}$ and $3.16 \pm 0.27 \ h$, and for *W. arrhiza,* they were $0.20 \pm 0.02 \ h^{-1}$ and $3.50 \pm 0.33 \ h$. No significant difference was observed in the specific growth rate or doubling time of *S. cerevisiae* in the medium derived from any of duckweed species.

3.5. Levels of glucose and ethanol during the fermentation process

The concentration of glucose and ethanol in the fermentation medium were measured every 4 h for the 48 h fermentation process (Fig. 4). During the first 24 h of fermentation, the glucose declined steadily with a concomitant increase in ethanol. Neither the sugar, nor the ethanol concentration changed much after 24 h. These results are consistent with the idea that the yeast used the sugars in fermentation and produced ethanol. We conclude

that the duckweed solutions were promising to support bioethanol fermentation.

Glucose is the main carbon source for fermentation by *S. cerevisiae*. The percentage of sugar to bioethanol conversion was 73.87% in *L. punctata,* 73.61% in *L. aequinoctialis,* 71.52% in *S. polyrrhiza* was, and 71.04% in *W. arrhiza*. When the ethanol production is expressed relative to plant dry biomass (g ethanol/g dry biomass; $[g_e/g_{db}]$), the values are 0.1 g_e/g_{db} for *L. punctata*, 0.17 g_e/g_{db} for *L. aequinoctialis*, 0.19 g_e/g_{db} for *S. polyrrhiza*, and 0.16 g_e/g_{db} for *W. arrhiza*. These values agree with those of Yu et al. (2014) who observed values for duckweed in the range of 0.17–0.19 g/g.

3.6. Protein and ethanol levels during the fermentation process

The concentration of protein and ethanol were measured in the fermentation media every 4 h for the 48 h of the fermentation process. The results are shown in Fig. 5. During the first 24 h of fermentation, the protein concentration declined steadily with a concomitant increase in ethanol. Neither the protein nor the ethanol concentration changed much after 24 h. This time course is consistent with the conclusion that the protein was utilized by the yeast as a N source during fermentation, and we suggest that the duckweed protein was a good N source for *S. cerevisiae*.

3.7. pH profile during the fermentation process

The fermentation process for the production of bioethanol from *S. cerevisiae* has an optimum pH of 4–5. The time course of the pH during the fermentation process is shown in Fig. 6 for the four duckweed species. The pH for all species during the fermentation was initially adjust at 6, but by 6–10 h after the start, had decreased to approximately 4–5, and remained in that range. Azhar et al. (Mohd Azhar et al., 2017) stated that the optimum pH for the fermentation process by *S. cerevisiae* for bioethanol production is in the range of 4–5.
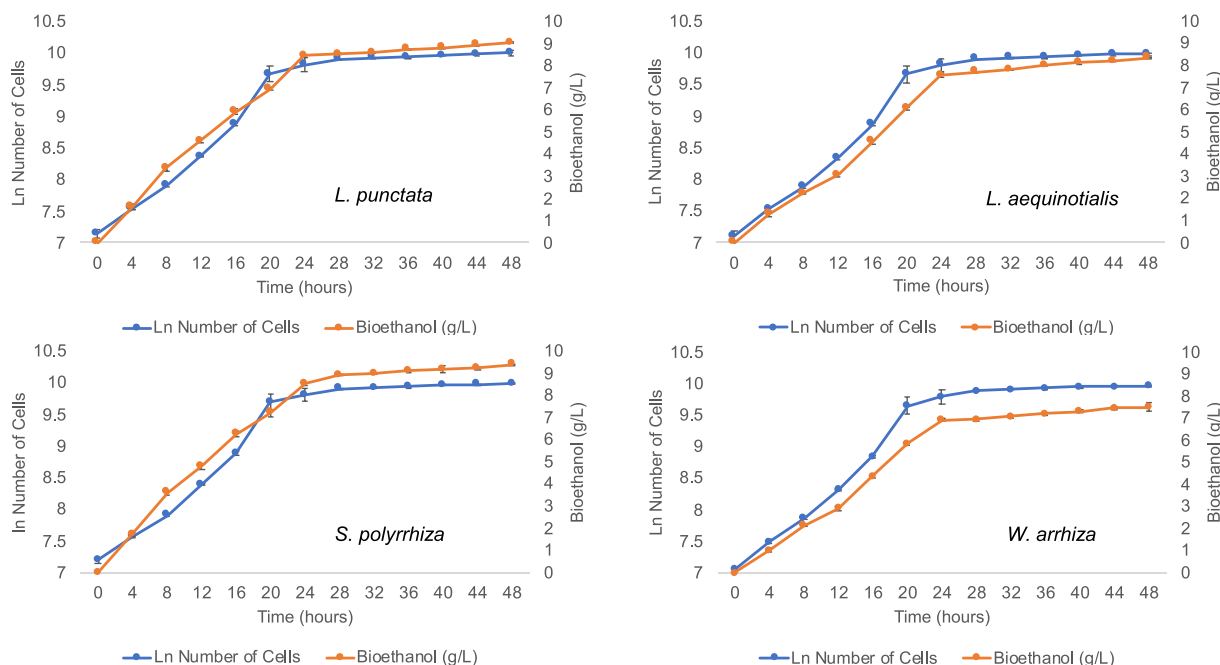

Fig. 3. Time course of the growth of *S. cerevisiae* and its production of ethanol during the fermentation process in solutions from four species of duckweed, after enzymatic hydrolysis of the starch in the solutions. Data represent mean ± SE of the mean.
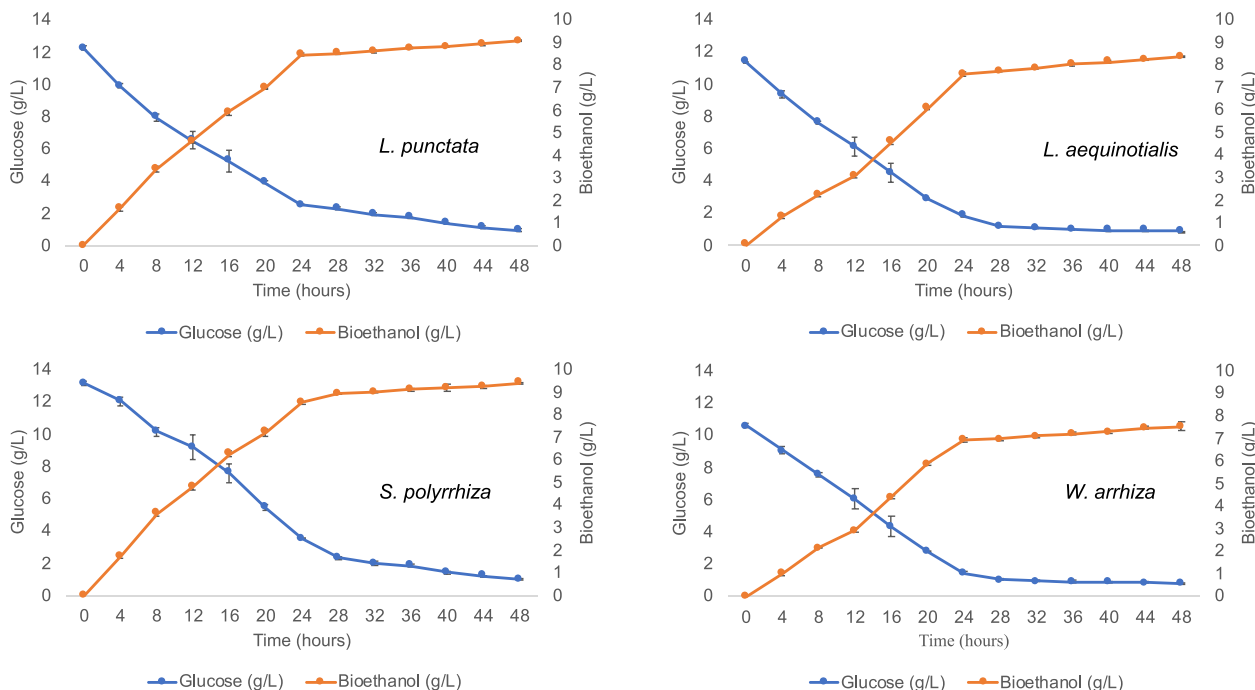
Fig. 4. The time course of the concentration of glucose and ethanol in the fermentation process of solutions from four species of duckweed after enzymatic hydrolysis of the starch in the solutions. Fermentation was performed by *S. cerevisiae*. Data represent mean ± SE of the mean.
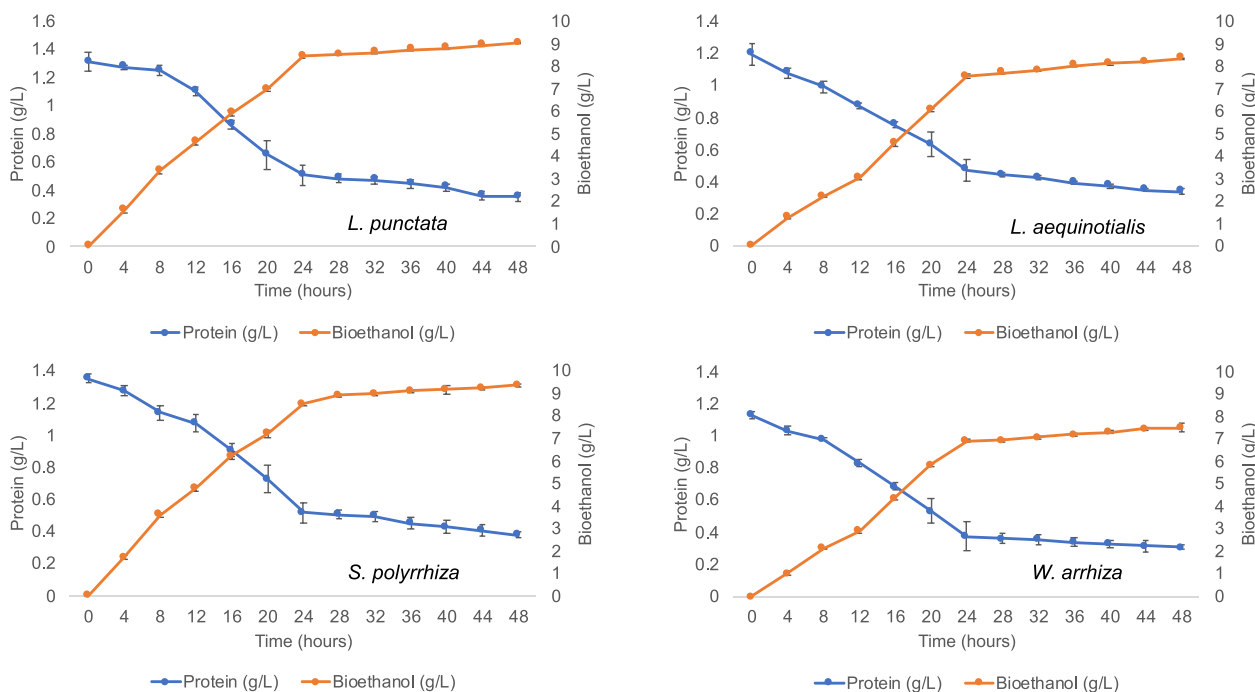

Fig. 5. Time course of the concentration of protein and ethanol in the fermentation process of solutions from four species of duckweed after enzymatic hydrolysis of the starch in the solutions. Fermentation was performed by *S. cerevisiae*. Data represent mean ± SE of the mean.

pH is an important parameter in the fermentation process and affects many biological processes in fermentation, including enzymes activity. One example is alcohol dehydrogenase which converts glucose into ethanol in *S. cerevisiae*. Alcohol dehydrogenase has a pH optimum of 4–5 (Hall, 2009). It also regulates protein conformation, and this would affect the enzymes for bioethanol production and growth.

4. Conclusion

Four duckweed species have been cultivated in a controlled environment. We have identified the saccharification and fermentation processes for optimal bioethanol production by these plants. Specifically, the optimal starch-to-sugar conversion required the enzyme α-amylase and amyloglucosidase in a ratio of 2:1 (v/v)
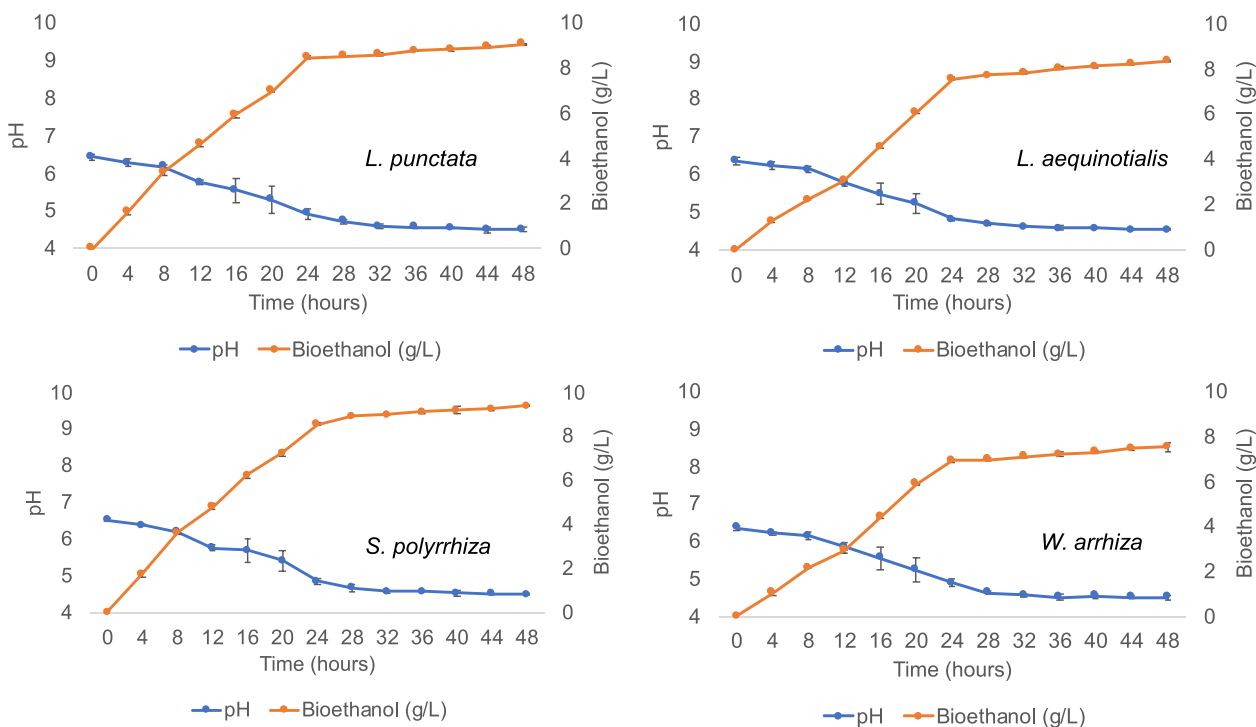
Fig. 6. Time course of the pH in the fermentation process of solutions from four species of duckweed after enzymatic hydrolysis of the starch in the solutions. Fermentation was performed by *S. cerevisiae*. Data represent mean ± SE of the mean.

and fermentation for 24 h at 50 °C. We hope this study will aid future large-scale industrial application for production of bioethanol.

Author contributions

A.F., A.A.S, and N.P. designed the study, developed the methodology, performed the experiment, analyzed the data, and wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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