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Research Article
Characterization of Starch from Duckweeds and Its Conversion Into Reducing Sugars Via Enzymatic Saccharification

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Abstract

Background and Objective: Bioethanol derived from plants is a renewable energy source and promising alternative to fossil fuels. It can be produced from plant starch by fermentation, but the starch must first be broken down to sugars, a process known as saccharification. Duckweeds are small, fast-growing plants that are easy to cultivate and accumulate high levels of starch and hold promise as a bioethanol source. Here, the growth parameters and starch content of three species of duckweeds were examined and assessed the efficiency of enzymatic saccharification. **Materials and Methods:** The duckweeds used in this study were *Landoltia punctata*, *Lemna aequinoctialis* and *Wolffia arrhiza*. The saccharification process was performed at 50°C for 24 hrs. Three starch-degrading enzymes were assessed in the saccharification process, specifically, α-amylase, β-amylase and glucoamylase, in equal amounts (1 mL per mg starch) but four different combinations. **Results:** The measured doubling times were, respectively, 3.57±0.02, 3.77±0.07 and 3.94±0.04 days and the initial starch contents were 0.28±0.02, 0.26±0.01 and 0.24±0.02 g/g. The greatest percentage of starch conversion to sugar was observed when all three enzymes were used in saccharification. The conversion percentages were 82.0±1.3%, 80.8±1.9% and 81.4±1%, for *L. punctata*, *L. aequinoctialis* and *W. arrhiza*, respectively. **Conclusion:** Results concluded that duckweeds have the potential to serve as a substrate in the fermentation process to produce bioethanol and other products.

Key words: Duckweeds, starch, reducing sugar, saccharification, α-amylase, β-amylase, glucoamylase

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Biofuels provide an energy source derived from living materials. They are a promising alternative to fossil fuels for several reasons, including their sustainability and potential generation of fewer greenhouse gases than fossil fuels. Duckweeds have generated interest recently as a source of biomass for biofuels, particularly bioethanol, which is derived from the fermentation of sugars and starches in biomass[1,2]. Duckweeds are fast-growing, small aquatic plants that produce large amounts of starch[3,4]. They have short doubling times and multiply easily in the right conditions[5,6]. Notably, duckweed produces an estimated 28 tons of starch per hectare per year, while corn, a major source of bioethanol, produces only six tons per hectare per year[7]. For these reasons, duckweeds are considered a promising potential source of biomass for the generation of bioethanol.

Bioethanol production requires fermentation by microorganisms such as yeast, which uses sugar as the main substrate. Therefore, an initial step in bioethanol production is to convert the plant starch to sugar. This is done enzymatically and is known as saccharification[8,9]. The relative success of this process depends on the variety of enzymes used, the length of incubation time, incubation temperature and the pH of the mixture. There are different starch-degrading enzymes: α-amylase, β-amylase, glucoamylase (α-glucosidase), isoamylase, pullulanase I, pullulanase II and cyclodextrin glycosyl transferase[10,11]. Alpha-amylase cleaves glucose residues at the α-1,4 linkages, β-amylase cleaves glucose residues at the end of the starch chain and glucoamylase cleaves branch chains bound by α-1,6 linkage[10].

Using duckweeds in bioethanol production requires the characterization of the starch produced by the plants. Initial characterization was undertaken by de Souza Moretti *et al.*[12], who focused on amylose and amylopectin, as these affect starch processing at later stages. Here, in this study the starch from duckweed was characterized and its conversion into sugars via the saccharification process, to maximize the yield of sugars for subsequent fermentation was done. Three types of saccharification enzymes: α-amylase, β-amylase and glucoamylase were assessed. Growth parameters of the plants were also measured.

MATERIALS AND METHODS

Study area: This study was conducted at the Laboratory of Biomass Production and Laboratory of Microbiology, School of Life Sciences and Technology, Institut Teknologi Bandung from September, 2020 to October, 2019.

Cultivation of duckweeds: Three species of duckweed, *Landoltia punctata*, *Lemna aequinoctialis* and *Wolffia arrhiza*, were used in this study. They were obtained from commercial duckweed farming in Purwodadi, Central Java. A collection of plants that weighed 20 g (fresh weight) was placed in trays (40.5×31.5×15.5 cm) containing 4 L of 10% Hoagland's solution. The trays were placed in a cultivation chamber equipped with cool-white tubular lamps for 10 days. The plants were weighed daily to generate fresh-weight growth curves. The doubling times and specific growth rates were obtained from the following formula[1], where x was the fresh weight of plants (g), t was the number of days in cultivation, μ was the specific growth rate ($days^{-1}$) and dt was doubling time (days):

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

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

Starch extraction: After 10 days of cultivation, plants were harvested for starch extraction according to the method of Chen *et al.*[13]. First, the plants were soaked in 90% C_2H_5OH for 24 hrs, with solvent changes every 6 hrs. At the end of this step, the plant material has lost its green colour. The plant material was rinsed with distilled water three times and crushed thoroughly to extract the starches. The extract containing the starch was mixed with 0.1% NaOH, rinsed with distilled water three times and dried in an oven for 24 hrs at 45°C.

Measurement of amylose and amylopectin content of the extracted starch: One hundred milligrams of the extracted starch was placed in a glass beaker and 1 mL of 95% C_2H_5OH and 9 mL of 1 M NaOH were added. The suspension was heated in boiling water for up to 10 min, cooled to room temperature and diluted to 100 mL with distilled water. Then, 2.5 mL of the diluted suspension was mixed with 7.5 mL of distilled water and 0.75 mL of chromogenic solution (CH COOH:2% KI/I2, 1:1). The mixture was left for approximately 20 min for the colour to develop and the absorbance was measured at the 625 nm with a spectrophotometer (Shimadzu UV-Vis Spectrophotometer

UV-1280, Japan). The absorbance was used to estimate the amylose concentration based on the equation generated from a standard curve. The amylopectin content in the sample was calculated from the total starch content minus the amylose content[14].

Preparation of biomass suspensions for subsequent experiments: Plants were harvested after 10 days of cultivation and dried in an oven at 60°C for 48 hrs. The dried biomass was ground into powder and sieved to uniform particle size. Approximately 5 g of powdered biomass were dissolved in 4 mL of 25 mM $C_2H_3NaO_2$ at pH 5.5, then diluted to 100 mL with distilled water[14]. The resulting suspension was incubated at 90°C for 30 min, filtered through filter paper (Whatman® qualitative filter paper Grade 1, Sigma-Aldrich Corporation USA) to remove solids and used in subsequent experiments.

Analysis of three starch-degrading enzymes in the saccharification process: The effect of three starch-degrading enzymes on their effectiveness in the saccharification of the duckweed suspension was evaluated. These were: α-amylase, β-amylase and glucoamylase (Novozymes A/S, Bagsværd, Denmark). The enzymes were added to 90 mL of duckweed suspension in an amount determined by the starch content of each species: 1 mL of enzyme solution for each mg of starch[1]. Assays were done with all possible combinations of two enzymes or with all three, in equal ratios. The saccharification process was performed in a shaker incubator (125 rpm, 50°C) for 12 hrs.

Measurement of starch content: Suspensions of duckweed (800 µL), prepared as described above, were mixed with 200 µL of Lugol's reagent (I_3K) and left undisturbed for 10 min. Then, the absorbance was measured at 580 nm with a spectrophotometer (Shimadzu UV-Vis Spectrophotometer UV-1280, Japan). Starch content was determined from a standard curve[1].

Measurement of sugar content: The reducing sugars were measured in the biomass suspensions. 3 mL of suspension were mixed with 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent, heated for 15 min at 90°C and left undisturbed at room temperature. Next, 1 mL of 40% potassium sodium tartrate tetrahydrate ($NaKC_4H_4O_6{\cdot}4H_2O$) was added and the absorbance was measured at 575 nm with a spectrophotometer (Shimadzu UV-Vis Spectrophotometer UV-1280, Japan). The glucose concentration was determined with the equation generated from a glucose standard curve[1].

Statistical analysis: Data obtained in this study was evaluated by using descriptive statistics (mean and population standard deviation) in Microsoft Excel and using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) in IBM SPSS Statistics 26.

RESULTS AND DISCUSSIONS

Growth characteristics of duckweeds: The growth curves of each species of duckweed in our conditions were prepared and are shown in Fig. 1. These were used to calculate the specific growth rates and doubling times (Table 1) according to the equations presented above. The differences between specific growth rates and doubling times of the three species were not significant ($p<0.05$). The doubling times agreed closely with those of Faizal *et al.*[1] for this same species. Both studies also agreed with Iwano *et al.*[15] who concluded that the doubling time for duckweeds is 3.1-4 days. The fast growth of duckweeds made them very powerful to be a candidate substrate for saccharification and bioethanol production. When compared to other bioenergy crops, duckweeds had a relatively shorter growing time. The growing period of sugar beet, sugar cane, maize and cassava were 175-200 days[16], 12 months[17], 141-180 days[18], 8-12 months[19], respectively.

Amount of starch, amylose, amylopectin and reducing sugars in duckweeds: Amylose and amylopectin are the main components of plant starch. These were measured for all three duckweed species after 10 days in culture. The percentages of amylose and amylopectin in the starch were, respectively: 18.19±0.2% and 81.81±0.2% for *L. punctata*, 16.81±0.86% and 83.19±0.86% for *L. aequinoctialis* and 15.07±0.87% and 84.93±0.87% for *W. arrhiza*. The ratios of amylose and amylopectin contents for *L. punctata*, *L. aequinoctialis* and *W. arrhiza* were 0.22±0.01, 0.20±0.02 and 0.18±0.02, respectively. The differences between percentages of amylose and amylopectin contents and the ratios between them were significant ($p<0.05$) in three species. Fig. 2 showed the amylose and amylopectin contents and Fig. 3 showed the ratio of amylose and amylopectin for the three species.

Liu *et al.*[20] observed that starch in *L. aequinoctialis* was made up of 16.94-20.55% amylose and 79.45-83.06% amylopectin, with an amylose to amylopectin ratio of 0.2-0.26.

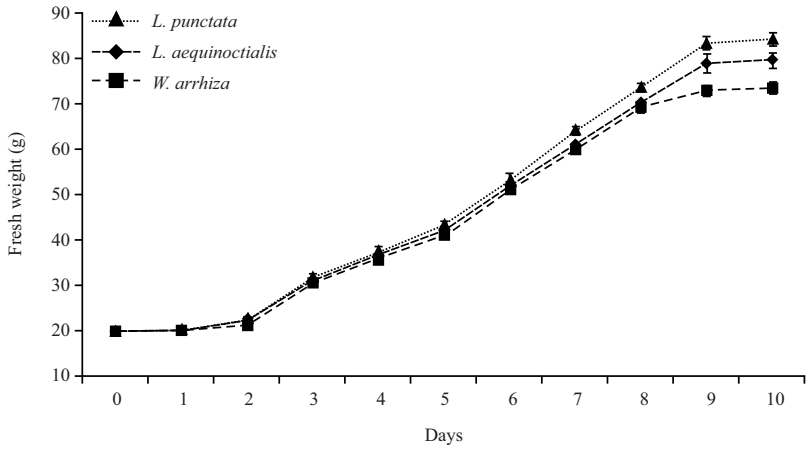

Fig. 1: Fresh weight of the duckweeds *L. punctata*, *L. aequinoctialis* and *W. arrhiza* in culture

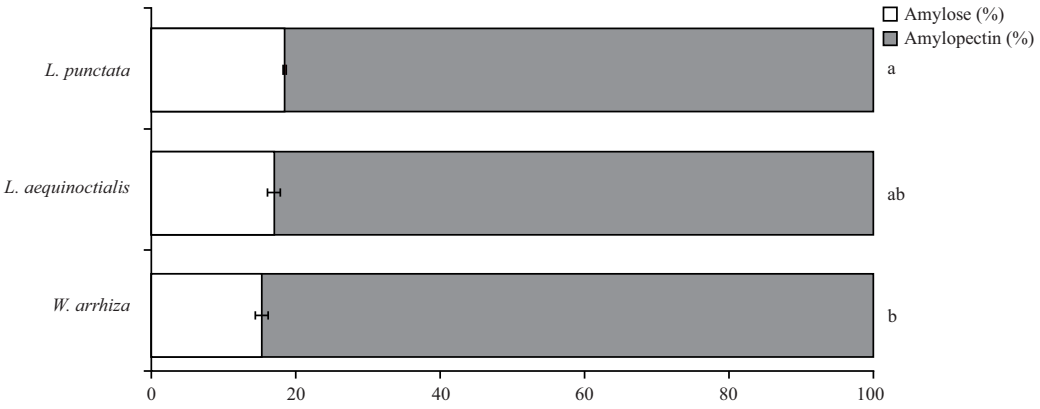

Fig. 2: Percentage of total starch comprised of amylose and amylopectin in duckweeds *L. punctata*, *L. aequinoctialis* and *W. arrhiza*

Different letters indicate significant differences ($p<0.05$) according to the Duncan's test

Table 1: Growth kinetics of duckweeds *L. punctata*, *L. aequinoctialis* and *W. arrhiza* grown in our conditions

Species	Specific growth rates (days^{-1})	Doubling time (days)
L. punctata	0.19±0.01	3.70±0.13
L. aequinoctialis	0.18±0.01	3.83±0.13
W. arrhiza	0.17±0.01	3.96±0.18

Values represent Mean±standard deviation

These values agreed well with current results. Yu *et al.*[14] also measured amylose and amylopectin in *L. aequinoctialis*. They reported that amylose content was 13.4-20.12% of the total starch and the amylopectin was 79.88-86.6%, the ratio of amylose to amylopectin was 0.15-0.25. These values were also similar to current measurements. The amount of starch and sugar in duckweeds is important in the evaluation of saccharification, as the percentage of conversion of the starches into sugars by enzymes must be determined. The reducing sugars and total starch content for all three species of duckweed were shown in Table 2 (before saccharification). The differences between starch contents in the three species were significant ($p<0.05$) but no significant differences in reducing sugar contents.

Starches and sugars contents of duckweeds after saccharification: During saccharification, starches are broken down into simple sugars, which is necessary for the next stage, fermentation, to proceed efficiently. We tested three starch-degrading enzymes (α-amylase, β-amylase and glucoamylase)

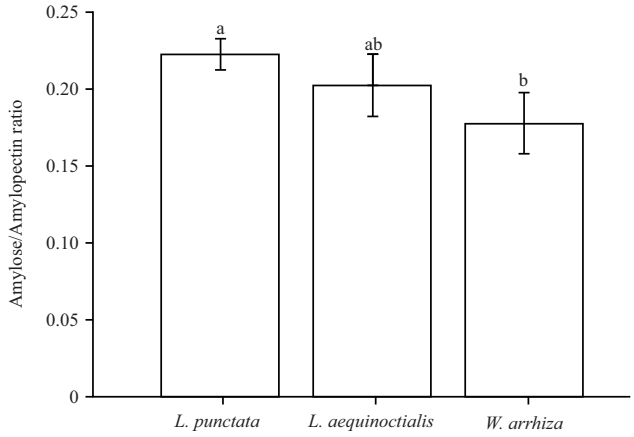

Fig. 3: Ratios of amylose and amylopectin in the duckweeds *L. punctata*, *L. aequinoctialis* and *W. arrhiza*
Different letters indicate significant differences (p<0.05) according to the Duncan's test

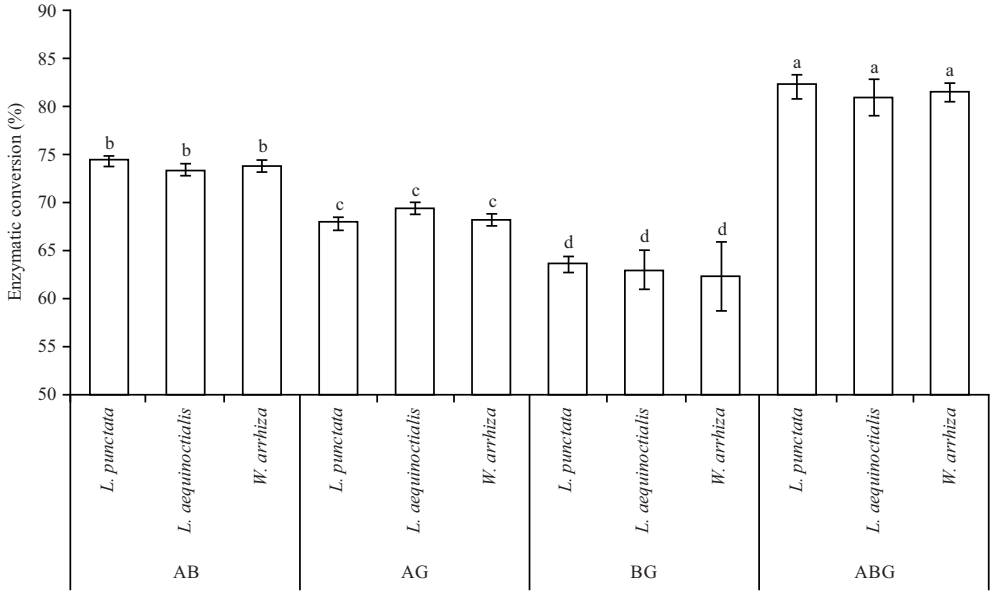

Fig. 4: Enzymatic conversion percentage of starch to sugar in saccharification of the duckweeds *L. punctata*, *L. aequinoctialis* and *W. arrhiza*
Saccharification was carried out in the presence of different combinations of starch-degrading enzymes, (A, α-amylase, B, β-amylase: G, glucoamylase) and Different letters indicate significant differences (p<0.05) according to the Duncan's test

in equal quantities and different combinations for their effectiveness in saccharification. The four combinations were: AB (α-amylase, β-amylase), AG (α-amylase, glucoamylase), BG (β-amylase, glucoamylase) and ABG (α-amylase, β-amylase, glucoamylase). The differences between starch and reducing sugar contents after saccharification were significant (p<0.05) among the three species as shown in Table 3. The conversion percentages from starch into reducing sugar during saccharification were depicted in Fig. 4. The differences between conversion percentages during saccharification were also significant (p<0.05).

The combination of three starch-degrading enzymes gave the highest conversion of starch into reducing sugar. β-amylase attacked starch from the non-reducing ends of both amylopectin and amylose. It attacked and broke the α-1,4 glycosidic bond to release maltose, but could not break the α-1,6 bond, producing beta-limit dextrins[21]. α-amylase cleaved starch by breaking the α-1,4 glycosidic bond of reducing and non-reducing ends in beta-limit dextrins molecule, thereby causing the release of alpha-limit dextrins[22]. The rest of the α-1,6 glycosidic bond would be broken by glucoamylase. This enzyme could hydrolyze both α-1,4 and

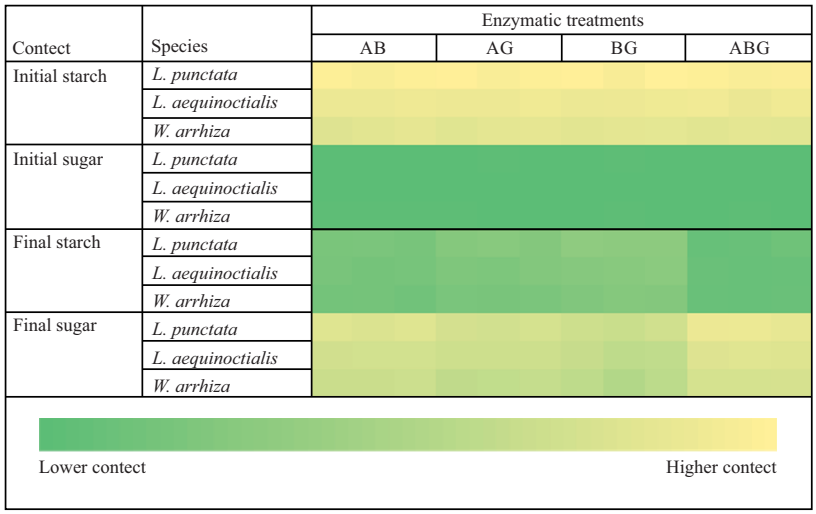

Fig. 5: Heat map visualization in the changes of starch and sugar during saccharification in the duckweeds *L. punctata*, *L. aequinoctialis* and *W. arrhiza*

Saccharification was carried out in the presence of different combinations of starch-degrading enzymes and (A, α-amylase, B, β-amylase: G, glucoamylase)

Table 2: Starch and reducing sugar content of the duckweeds *L. punctata*, *L. aequinoctialis* and *W. arrhiza*

Species	Starch (g/g)	Reducing sugar (g/g)
L. punctata	0.28±0.02^{a}	0.02±0.002^{a}
L. aequinoctialis	0.26±0.01^{b}	0.02±0.001^{a}
W. arrhiza	0.24±0.02^{c}	0.02±0.002^{a}

Different letters indicate significant differences (p<0.05) according to the Duncan's test

Table 3: Amount of starch and reducing sugar after saccharification in the duckweeds *L. punctata*, *L. aequinoctialis* and *W. arrhiza*

	Starch (g/g)				Reducing sugar (g/g)			
Species	AB	AG	BG	ABG	AB	AG	BG	ABG
L. punctata	0.07±0.002^{e}	0.087±0.003^{bc}	0.102±0.003^{a}	0.045±0.005^{g}	0.233±0.003^{b}	0.216±0.002^{c}	0.204±0.005^{d}	0.255±0.004^{a}
L. aequinoctialis	0.065±0.003^{e}	0.078±0.003^{d}	0.092±0.004^{b}	0.044±0.001^{g}	0.214±0.001^{c}	0.205±0.005^{d}	0.188±0.005^{e}	0.234±0.002^{b}
W. arrhiza	0.059±0.002^{f}	0.071±0.002^{e}	0.084±0.002^{c}	0.043±0.002^{g}	0.201±0.002^{d}	0.188±0.003^{e}	0.175±0.008^{f}	0.22±0.001^{c}

Saccharification was carried out in the presence of different combinations of starch-degrading enzymes, (A, α-amylase, B, β-amylase: G, glucoamylase) and different letters indicate significant differences (p<0.05) for each treatment according to the Duncan's test

α-1,6 glycosidic bonds in the starch molecule[23]. The combination of α- amylase and β-amylase enzymes would degrade starch more rapidly than alone, but both depend on the glucoamylase to facilitate the complete production of reducing sugars.

In a study of saccharification in *L. aequinoctialis*, Yu *et al.*[14], used three starch-degrading enzymes, specifically α-amylase (Sigma A4582), α-amyloglucosidase (Sigma A7095) and pullulanase (Sigma P1067) and a 30-h incubation at 50°C. The percentage conversion of starch to sugars was 94.14% under these conditions. In saccharification of *L. minor*, Zhao *et al.*[24], used cellulase, β-glucosidase, a cell-wall degrading enzyme cocktail and a 24 hrs incubation at 50°C. They observed a percentage of enzymatic conversion of approximately 80%. Another study revealed the possibility of using a simultaneous process of both saccharification and fermentation for bioethanol production in duckweeds[25].

Current results were visualized with a heat map, shown in Fig. 5. The initial sugar content of all three duckweed species was low initially, which is shown as dark green on the heat map, but after the saccharification process, sugar levels were high, indicated by yellow on the heat map. The opposite pattern was apparent for starch content and they appear in the heat map as initially yellow but changing to green. Results concluded that the saccharification process was efficient in the conversion of starch to sugar for the various enzyme treatments, as is apparent in the visualization.

CONCLUSION

The duckweeds studied here, *L. punctata*, *L. aequinoctialis* and *W. arrhiza* had high starch content, 0.28±0.02, 0.26±0.01 and 0.24±0.02 g/g, respectively. A successful saccharification process was identified for converting these starches to simple

sugars, which can then be used as raw materials for various products, including bioethanol, through the fermentation process. The highest conversion of starches into sugars was obtained when α-amylase, β-amylase and glucoamylase were all three used in saccharification. The final percentages of enzymatic conversion for the three species were 82±1.3%, 80.8±1.9% and 81.4±1 % for *L. punctata*, *L. aequinoctialis* and *W. arrhiza*, respectively.

SIGNIFICANCE STATEMENT

This study discovered that starch derived from duckweed has the potential to be developed into various types of bioproducts. One of the processes that must be passed is through fermentation which must be preceded by saccharification. We found the optimal combination of enzymes to convert duckweed starch to readily fermentable sugar. This study will help the researcher to uncover the critical areas of the upstream process of bioproduct production and also provide some information for the scale-up process.

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