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Improving Production of Bioethanol from Duckweed (*Landoltia punctata*) by Pectinase Pretreatment

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Abstract: *Landoltia punctata*, a widely distributed duckweed strain with the ability to accumulate starch, was used as a novel feedstock for bioethanol production by *Saccharomyces cerevisiae*. To improve ethanol production, pectinase pretreatment was used to release much more glucose from *L. punctata* mash and the pretreatment conditions (enzyme loading, temperature and pretreatment time) for the duckweed were optimized by using a surface response design. The results showed that maximum glucose yield was 218.64 ± 3.10 mg/g dry matter, which is a 142% increase compared to the untreated mash, with a pectinase dose of 26.54 pectin transeliminase unit/g mash at 45 °C for 300 min. Pectinase pretreatment apparently changed the ultrastructure of *L. punctata*, as evidenced by scanning electron microscopy analysis. Further fermentation experiments were performed and 30.8 ± 0.8 g/L of ethanol concentration, 90.04% of fermentation efficiency and 2.20 g/L/h of productivity rate were achieved. This is the highest ethanol concentration reported to date using duckweed as the feedstock.

Keywords: duckweed; *Landoltia punctata*; pectinase; ethanol fermentation; surface response design; *Saccharomyces cerevisiae*

1. Introduction

Liquid biofuels, such as bioethanol, obtained from biomass are considered a promising alternative to fossil fuels in the effort to reduce greenhouse gas emissions and meet the strong global demand for energy. Currently, bioethanol is mainly produced on an industrial scale from feedstocks containing starch and sugar, such as corn in the USA and sugarcane in Brazil [1]. However, these ethanol production modes have some inherent problems, including food security and agricultural land insufficiency [2]. The same dilemma also exists in the ethanol production using other feedstocks containing abundant carbohydrates, such as sweet potato and cassava, which have the potential to be converted into ethanol [3,4]. Although lignocellulosic biomass is regarded as a promising feedstock for ethanol production, there are still several obstacles (for example, the lack of an efficient, economical and environmentally friendly pretreatment process) to be overcome for economically feasible ethanol production [5]. Therefore, exploring new alternative feedstocks for ethanol production is an urgent topic.

Duckweed is a small, green floating aquatic plant belonging to the Lemnaceae family that can be easily found in quiescent or slowly flowing waters and also in relatively polluted waters worldwide [6]. It has a longer production period than most other plants, even growing year-round in some areas with a warm climate [7]. It accumulates its biomass at more rapid rates than other higher plants, including agricultural crops [8]. Duckweed has a doubling time of 2–7 days [6,9]. Under ideal conditions, a doubling time of 20–24 h was observed in *Wolffia microscopica* (Griffith) Kurz [10]. Besides, duckweed displays a high ability to remove nutrients from wastewater, and it has been widely applied for the treatment of municipal and industrial wastewaters in many countries, including Bangladesh, Israel and the USA [11–15].

The annual yields of the duckweed *Spirodela polyrrhiza* and *Lemna gibba* were 20.4 and 54.8 t/ha in dry matter (DM) [16,17], whereas the yields of corn and corn stover are 5.22 and 7.66 t/ha, respectively [18,19]. Therefore, duckweed could produce a large quantity of biomass. *Landoltia punctata* is a duckweed strain widely distributed in China and successfully employed for wastewater treatment [20]. Although previous studies indicated that *L. punctata* possessed the ability to accumulate a high content of starch (3%–75%) [21], the potential of duckweed as a novel bioenergy biomass was not recognized until recently [22]. However, there are only a few published reports on ethanol fermentation from duckweed [22–24], the ethanol concentration and fermentation efficiency of the duckweed fermentation remains unanswered at laboratory scale and no report focuses on processes for improving the ethanol yield from it.

The limitation of ethanol fermentation from duckweed is attributed to the low ethanol concentration and fermentation efficiency. Fortunately, early studies indicated that pectinase pretreatment has been used for improving ethanol production from various feedstocks [25,26]. Compared with physical or thermo-chemical processes, pectinase treatment requires less energy and produces no inhibitory factors, and the treatment conditions are environmentally friendly, making the process much economical and easy to perform [27].

The aims of this study were to improve the ethanol yield from *L. punctata* by increasing sugar release using pectinase pretreatment. The enzyme pretreatment variables (enzyme loading, temperature and pretreatment time) were optimized using a response surface methodology (RSM) and a

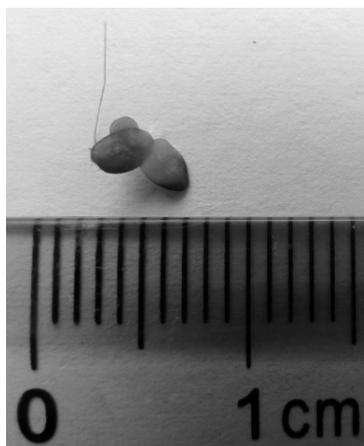
Box-Behnken design for maximum glucose release. Finally, ethanol production from pretreated *L. punctata* mash by *Saccharomyces cerevisiae* was also investigated.

2. Experimental Section

2.1. Duckweed Collection and Preparation

The duckweed *Landoltia punctata* (G. Meyer) Les & Crawford was grown under natural conditions and collected from a fish pond in Qionglai, Chengdu, China (Figure 1). The pond is about 50 m in length, 35 m in width and 40–80 cm in depth. The pond is almost fully covered by *L. punctata*. The duckweed was collected in February, washed with tap water and then dried at 60 °C for 2 days. The dried sample was milled and screened through 80 mesh. The powder was stored in desiccators at room temperature until being further processed.

Figure 1. The duckweed *L. punctata*.



2.2. Microorganism and Media

The *Saccharomyces cerevisiae* strain CCTCC M206111 was isolated from wine lees and used for ethanol fermentation in this study. The strain was maintained on YPD agar plates (1% yeast extract, 2% peptone, 2% glucose and 1.5% agar) at 4 °C subcultured every 4 weeks. The inoculum was developed by growing the cells at 30 °C and 150 rpm for 16 h in a 250 mL Erlenmeyer flask containing 100 mL of sterile culture medium consisting of (g/L): glucose, 100; yeast extract, 8.5; (NH₄)₂SO₄, 1.3; MgSO₄·7H₂O, 0.1; CaCl₂, 0.06. The initial pH of the medium was adjusted to 6.0 using 2 M HCl or 2 M NaOH. The *L. punctata* mashes at different solid-liquid ratios (SLRs) were used in both pretreatment and fermentation experiments. All the media were autoclaved at 115 °C for 20 min before use.

2.3. Enzymatic Pretreatment

Liquefaction enzyme (Liquozyme Supra at 90 kilo Novo α -amylase unit (KNU)/mL, Novozymes, Beijing, China) and Glucoamylase (Suhong GA II at 500 Novo glucoamylase unit (AGU)/mL, Novozymes, Beijing, China) were used for biomass mash liquefaction and saccharification. The enzyme

used for the *L. punctata* pretreatment was pectinase (Pectinex Ultra color at 11173 pectin transeliminase unit (PECTU)/mL, Novozymes, Beijing, China). The liquefaction of the mash was performed at 95 °C (0.15 KNU/g mash) for 10 min prior to pectinase pretreatment. The liquefied mash was autoclaved at 115 °C for 20 min and then cooled to room temperature. Pectinase pretreatment and starch saccharification were carried out simultaneously in 250 mL flasks containing 100 g of the mash at pH 6.0 and 50 °C for 2 h. Glucoamylase was added with 1.5 AGU/g mash in pretreatment experiments. Each pretreatment was performed in triplicate.

2.4. Experimental Design and Statistical Analysis

The optimal pectinase pretreatment conditions of the *L. punctata* mash for a maximum sugar yield were determined using a Box-Behnken design (BBD) to study the independent variables that have significant effects on the fermentable sugar yield. Such variables as the pretreatment temperature, pretreatment time and pectinase dose were selected and designated as X_1 , X_2 and X_3 , respectively. The range of variables and their levels are given in Table 1, which were based on our preliminary experiments. The complete design consisted of 15 experimental points containing three replications at the center points for estimating the purely experimental variance. The experimental data from the BBD were analyzed by multiple regressions to fit second-degree polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y represents the response (glucose yield, mg/g DM); X_1 , X_2 and X_3 are the coded variables; β_0 is a constant; β_1 , β_2 and β_3 are the linear coefficients; β_{12} , β_{23} and β_{13} are the cross product coefficients; β_{11} , β_{22} and β_{33} are the quadratic coefficients. The Design Expert software 7.1.3 (Stat-Ease Inc., Minneapolis, MN, USA) was used for the experimental design and the subsequent multiple regression analysis. The adequacy of the fitted quadratic model of the glucose yield was tested using variance analysis (ANOVA). The quality of the obtained polynomial equation was evaluated statistically by the coefficient of determination R^2 , and its significance was determined by an F -test.

Table 1. The levels of the variables used in the Box-Behnken design.

Independent variables	Symbols	Coded levels		
		-1	0	1
Pretreatment temperature (°C)	X_1	45	50	55
Pretreatment time (min)	X_2	60	180	300
Pectinase dose (PECTU/g mash)	X_3	5	17.5	30

2.5. Ethanol Fermentation

The fermentation experiments of pretreated *L. punctata* mash were carried out in 250 mL flask containing 100 g mash in batch model. The flask was inoculated with 10% v/w yeast inoculum and the reaction mixture was incubated at 30 °C and 220 rpm. The flasks were sealed with rubber stoppers equipped with hypodermic needles for CO₂ venting. Samples were withdrawn periodically after inoculation for the analysis of the concentrations of ethanol and residual glucose. The ethanol fermentation efficiency and ethanol productivity were calculated as follows:

$$\text{Fermentation efficiency (\%)} = \frac{\text{Ethanol produced in fermentation}}{\text{ethanol produced in theoretical}} \times 100\% \quad (2)$$

$$\text{Ethanol productivity rate (g/L/h)} = \frac{\text{Final ethanol concentration (g/L)}}{\text{fermentation time (h)}} \quad (3)$$

2.6. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed to analyze the ultrastructural changes of the enzyme-treated samples of *L. punctata*. The samples were dehydrated in a graded ethanol series and lyophilized to dryness. The samples were then coated with gold prior to imaging using an S3400+ EDX SEM (Hitachi, Tokyo, Japan) with an accelerating voltage of 10 kV [28].

2.7. Analytical Methods

The starch content of *L. punctata* was measured using a total starch kit (Megazyme International Ireland Co., Ltd., Wicklow, Ireland) according to the manufacturer's instructions. The chemical composition (extractives, cellulose, xylan, galactan, arabinan, acid insoluble lignin and ash) of the duckweed was determined following the method referring to the National Renewable Energy Laboratory (NREL) analytical methods [29]. The crude protein in the biomass was determined as Kjeldahl nitrogen $\times 6.25$. The viscosity of the duckweed mash was determined according to the previous report [4]. The fermentation mash mixed with a certain amount of water was centrifuged at 12,000 rpm for 5 min. The supernatant was determined for reducing sugar and ethanol. The amounts of monosaccharide in the liquid samples were determined using high-performance liquid chromatography (HPLC) (Waters 2795, Milford, MA, USA) with Evaporative Light-scattering Detector (ELSD) (All-Tech ELSD 2000, Deerfield, IL, USA). The samples were filtered through a 0.22 μm filter before the HPLC analysis. The monosaccharides were separated on an Aminex HPX-87-Pb column (Bio-Rad, Hercules, CA, USA) at 79 $^{\circ}\text{C}$ using deionized water as the eluent at a flow rate of 0.6 mL/min. Nitrogen was carrier gas at the pressure of 2.8 Bar and the draft temperature was 95 $^{\circ}\text{C}$ for the ELSD detector. The total sugar of *L. punctata* mash was quantitatively assayed by the same method after hydrolyzed with 0.7 M H_2SO_4 at 100 $^{\circ}\text{C}$ for 120 min [30]. The ethanol concentration was determined using gas chromatography (FULI 9790, FULI Corp., Hangzhou, China) with flame ionization detector and a stainless steel column (3.2 mm \times 2 m). Nitrogen was carrier gas at a flow rate of 30 mL/min. The temperature of the column, detector and injector were 95, 150 and 150 $^{\circ}\text{C}$, respectively. Prior to ethanol determination, the sample was mixed with an inner standard *n*-propanol (98:2, v/v). All of the experiments were conducted in triplicate.

3. Results and Discussion

3.1. Raw Material Composition

The chemical composition of *L. punctata* was determined and the results are presented in Table 2. The cellulose content was in agreement with data for *Lemna minor* in previous reports [31]. The galactose and arabinose suggested that the duckweed *L. punctata* could contain certain amount of pectin. Starch was the main polysaccharide in the raw material. The high content of polysaccharides, together with the low lignin content ($5.55 \pm 0.36\%$), makes this feedstock a promising resource for

ethanol production. Moreover, the high protein content in *L. punctata* indicated that no other nitrogen source was necessary for the fermentation, which could help to reduce the total cost of ethanol production.

Table 2. Composition of *L. punctata*.

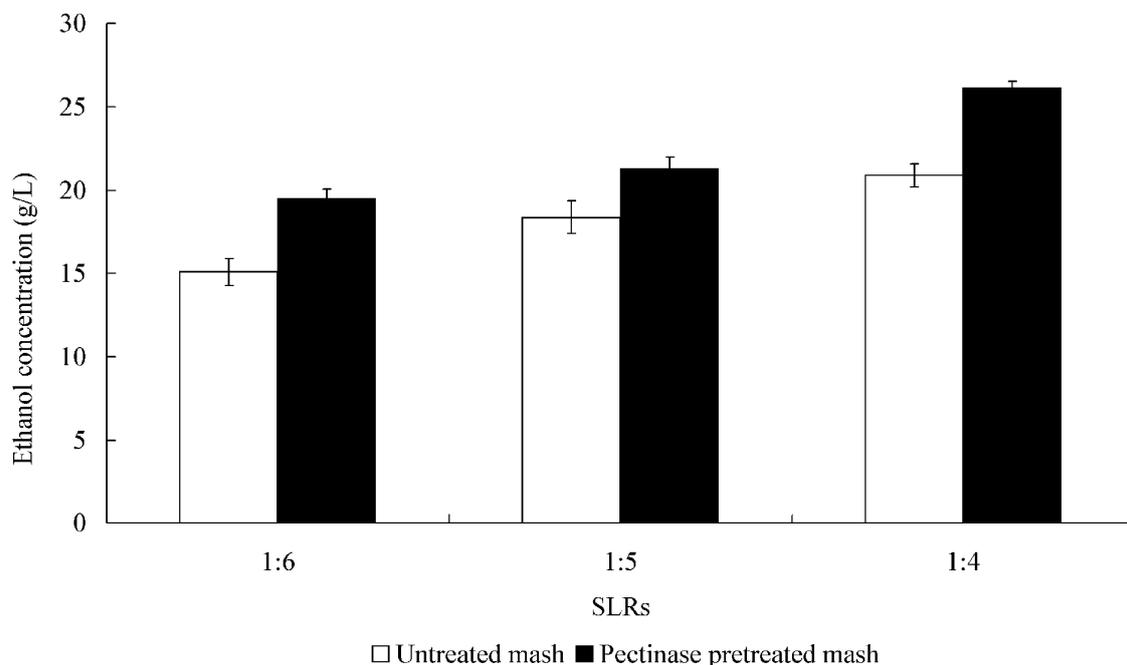
Component	% w/w DM
Extractives	13.04 ± 1.98
Crude protein	16.27 ± 0.12
Starch	24.59 ± 0.67
Cellulose	13.31 ± 0.41
Xylose	1.61 ± 0.01
Galactose	3.46 ± 0.32
Arabinose	1.32 ± 0.02
Acid insoluble lignin	5.55 ± 0.36
Ash	3.48 ± 1.0

3.2. Ethanol Fermentation of the Pectinase Pretreated Duckweed Mash

Ethanol fermentation was carried out after liquefaction and 2 h of saccharification at SLRs from 1:6 to 1:4. Although ethanol concentrations of 15.07 ± 0.8 g/L, 18.38 ± 1 g/L and 20.91 ± 0.7 g/L were obtained at SLRs of 1:6, 1:5 and 1:4, respectively, their fermentation efficiencies were low (below 75%). Furthermore, the high total residual sugar (over 20 g/L mash, based on the glucose content) and low residual reducing sugar (7 g/L) in the fermented mash suggested that the yeast cells could efficiently utilize the released glucose but that it was not fully released. Thus, proper processes should be used for improving glucose release of *L. punctata* mash to improve its ethanol production.

According to recent studies, pectinase has been applied in biomass pretreatment and has positive effects on the ethanol fermentation process due to the hydrolysis of pectin and the breakdown the complex structure of the feedstock [32]. In addition, compared with the physical or thermo-chemical pretreatment applied to lignocellulosic biomass, the enzyme pretreatment was performed under mild conditions and was more environmentally friendly [33,34]. Therefore, pectinase was used in this study to improve the ethanol production from *L. punctata*. The duckweed was pretreated by pectinase with the dose of 7 PECTU/g mash at 50 °C for 2 h at the SLRs from 1:6 to 1:4. The fermentation was then performed for approximately 13 h. The results are shown in Figure 2. The highest ethanol concentration of 26.12 ± 0.4 g/L was obtained from the pectinase pretreated mash at the SLR of 1:4. The fermentation efficiency was over 90% and the residual sugar was below (7 g/L) after pectinase pretreatment, which indicated that the pectinase pretreatment facilitated the saccharification of starch in the mash. In addition, viscosity reduction of the mash was observed after the enzyme pretreatment. The viscosity of the mash were 926 ± 35 , 4610 ± 90 and 16504 ± 176 mPa·S and 471 ± 43 , 3362 ± 104 and 7155 ± 127 mPa·S before and after pectinase pretreatment at the SLRs of 1:6, 1:5 and 1:4, respectively. The results suggested that ethanol concentration and fermentation efficiency could be significantly improved by pectinase pretreatment. Therefore, further study would focus on optimization of pectinase pretreatment conditions to achieve higher glucose yield, and eventually higher ethanol concentration, which was crucial for its practical application on ethanol production.

Figure 2. Ethanol production from *L. punctata* mash pretreated by pectinase at different solid-liquid ratios (SLRs). (□): Ethanol production from untreated mash; (■): Ethanol production from pectinase pretreated mash (7 PECTU/g mash). Error bars represent standard error.



3.3. Optimization of Pectinase Pretreatment

To improve the glucose yield and ethanol concentration from duckweed, three variables involved in the pectinase pretreatment, including pretreatment time, pectinase dose and pretreatment temperature, were optimized by using BBD at the SLR of 1:3. The experimental conditions and the results are shown in Table 3.

To estimate the maximum glucose yield corresponding to the three variables, a second-order polynomial equation was used to calculate the optimal levels of these variables. By applying a multiple regression analysis to the glucose yield data, the values of the coefficients were calculated and the following equation was obtained to express the role of the variables and their second-order interactions on the glucose yield:

$$Y = 189.05 - 2.71X_1 + 10.86X_2 + 7.74X_3 + 1.25X_1X_2 - 4.77X_1X_3 - 4.59X_2X_3 - 0.88X_1^2 + 7.32X_2^2 - 5.47X_3^2 \quad (4)$$

where Y was the predicted glucose yield, X_1 was the pectinase pretreatment temperature, X_2 was the pretreatment time and X_3 was the pectinase dose. The coefficient values of Equation (4) were tested for their significance using the Design-Expert software 7.1.3. As shown in Table 4, the linear coefficients (X_2 and X_3), quadratic term coefficients (X_2X_2 and X_3X_3) and cross product coefficients (X_1X_3 and X_2X_3) were significant on the basis of $p < 0.05$. The linear coefficient X_1 was insignificant, indicating that the pectinase activity was stable within the selected temperature range (from 45 °C to 55 °C), whereas the pretreatment time (X_2) and enzyme dose (X_3) expressed strong linear effects on the glucose yield.

Table 3. Box-Behnken design for the three variables showing observed and predicted results for glucose yield of *L. punctata* under pectinase pretreatment.

Run No.	Coded variables			Glucose yield (mg/g DM)	
	X_1	X_2	X_3	Observed	Predicted
1	-1	-1	0	188.53	186.82
2	1	-1	0	181.99	178.39
3	-1	1	0	206.48	210.08
4	1	1	0	204.94	206.66
5	-1	0	-1	171.67	174.11
6	1	0	-1	174.41	177.73
7	-1	0	1	200.52	197.20
8	1	0	1	184.17	181.73
9	0	-1	-1	168.97	168.25
10	0	1	-1	201.15	201.57
11	0	-1	1	189.82	189.36
12	0	1	1	203.63	207.55
13	0	0	0	192.93	188.05
14	0	0	0	187.67	188.05
15	0	0	0	186.54	188.05

Table 4. Regression coefficients of the predicted quadratic polynomial model.

Factor	Coefficient estimate	Standard error	F value	p value
Intercept	189.05	1.991729427	-	-
X_1	-2.71125	1.219680201	4.9413678	0.0768
X_2	10.86125	1.219680201	79.298993	0.0003
X_3	7.7425	1.219680201	40.296793	0.0014
X_1X_2	1.2500	1.724888281	0.5251678	0.5011
X_1X_3	-4.7725	1.724888281	7.655436	0.0395
X_2X_3	-4.5925	1.724888281	7.0888598	0.0447
X_1X_1	-0.880833333	1.795320644	0.2407152	0.6445
X_2X_2	7.319166667	1.795320644	16.620314	0.0096
X_3X_3	-5.473333333	1.795320644	9.2943655	0.0285

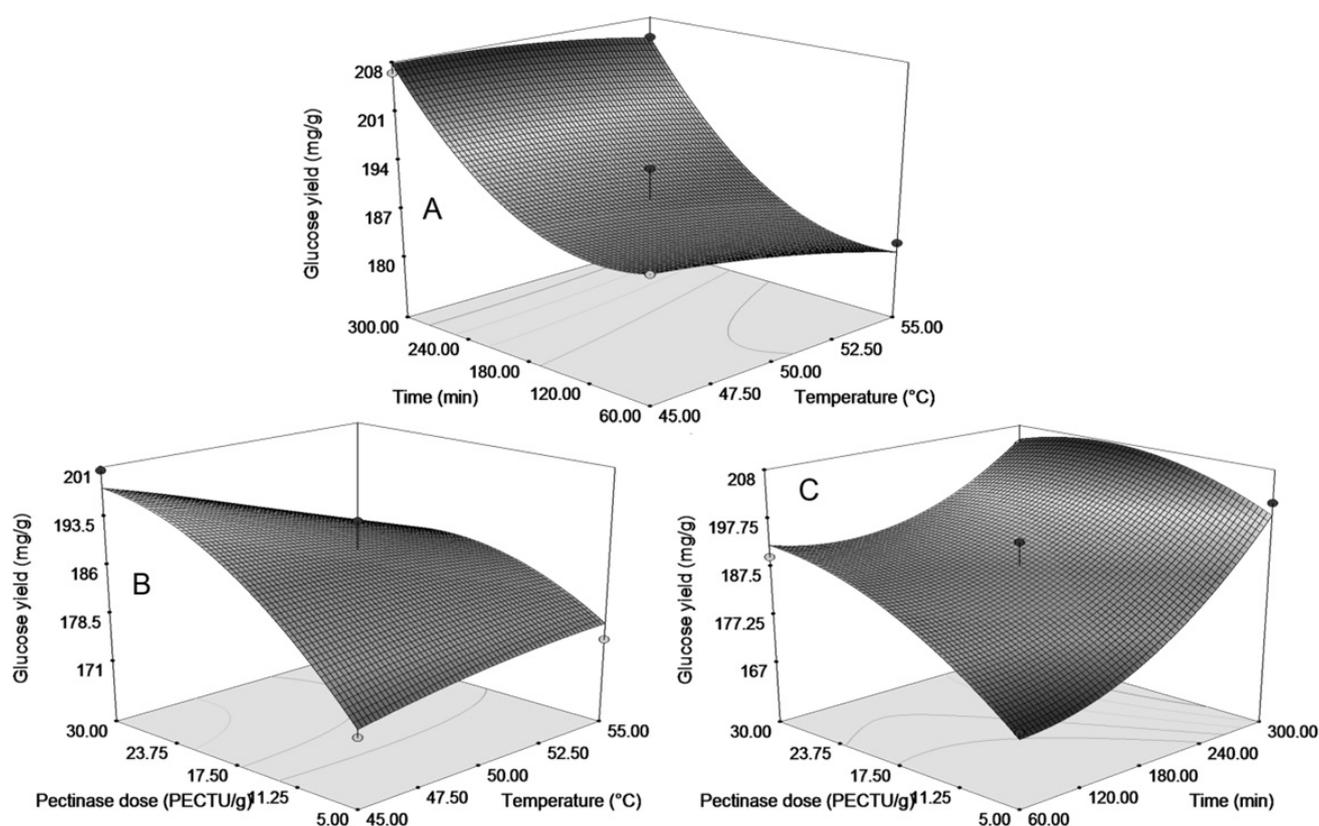
An analysis of variance (ANOVA) for the quadratic polynomial model is presented in Table 5. We found that the model was highly significant, as evidenced from the calculated F value (18.68) and low probability (0.0025). The fitness of the model was examined by determination coefficient R^2 , which was 0.9711, indicating that the sample variation of more than 97% was attributed to the variables and that only less than 3% of the total variance could not be explained by the model. A regression model with an R^2 value higher than 0.9 could be considered as having a high correlation [35]. The adjusted determination coefficient (Adjusted $R^2 = 0.9191$) indicated a high degree of correlation between the observed and predicted values. The model also showed statistically insignificant lack of fit ($p > F = 0.5246$). Therefore, the model was supposed to be adequate for prediction of optimal conditions within the range of variables employed.

Table 5. ANOVA for the regression model.

Source	Sum of squares	Degree of freedom	Mean square	F value	$p > F$
Model	2000.8	9	222.31	18.68	0.0025
Residual	59.5	5	11.9	-	-
Lack of Fit	36.25	3	12.08	1.04	0.5246
Pure Error	23.26	2	11.63	-	-

A 3D response surface curve was generated from the predicted model to better understand the effects of the variables on glucose yield. Statistical analysis indicated that the glucose yield was significantly affected by the pectinase dose and pretreatment time, and a significant effect of their interaction was also observed (Figure 3 and Table 4). Although the temperature was not significant for the glucose yield, it decreased with increasing temperature, which could be due to inactivation of pectinase under high temperature. The optimal pretreatment conditions were calculated from the results using the Design-Expert software. The optimal variables were as follows: 45 °C, 300 min pretreatment time and 26.54 PECTU/g mash of pectinase. The model predicted that the maximum glucose yield would be 210.67 mg/g DM of *L. punctata* under the above optimal conditions.

Figure 3. Response surface plots of glucose yield by pectinase pretreatment as a function of (A) time and temperature; (B) temperature and enzyme dose; (C) time and enzyme dose under the pretreatment conditions.

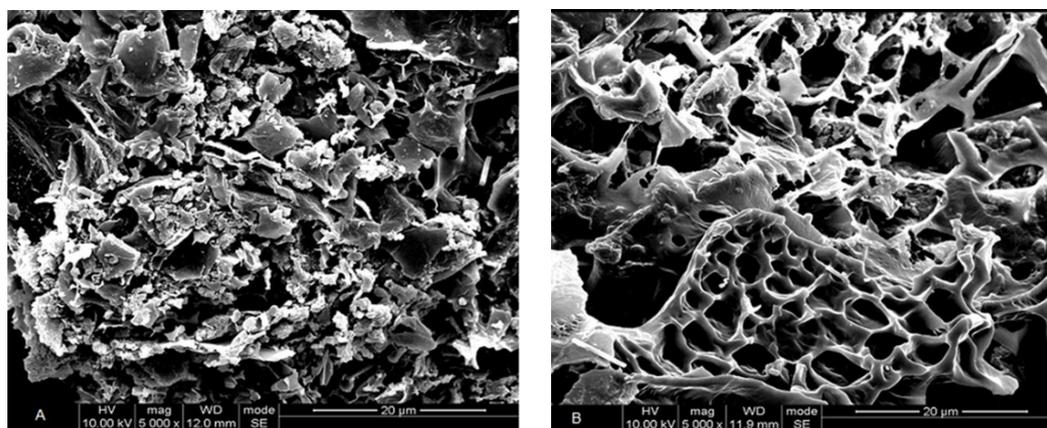


3.4. Validation of the Experimental Design

A validation experiment was conducted using the optimal conditions, resulting in a glucose yield of 218.64 ± 3.10 mg/g DM, which was in good agreement with the predicted value. The glucose yield was 90.00 ± 4.19 mg/g DM of the untreated mash, thus a 142% increase in the glucose yield had been obtained. This result corroborated the predicted value and indicated that the quadratic model could be used to predict the interactions between the three variables and the glucose yield. The results also suggested that pectinase pretreatment was essential for increasing the glucose yield of *L. punctata*.

The ultrastructural changes in the pectinase pretreated *L. punctata* mash under the optimal conditions were revealed by SEM. As shown in Figure 4A,B, the porosity of the pretreated mash was obviously increased and breakdown of irregular materials that tightly wrapped the biomass was also observed after the pectinase pretreatment (Figure 4B). According to the obtained results, it was likely that the increase of glucose yield in the raw material was mainly caused by pectin hydrolyzed after pectinase pretreatment, which would help the starch saccharification. The pectinase pretreatment could also help to reduce the viscosity of *L. punctata* mash [36,37]. However, the mechanism requires further investigation.

Figure 4. Scanning electron microscopy of *L. punctata* mash before and after pectinase pretreatment: (A) untreated *L. punctata* mash; (B) *L. punctata* mash pretreated by pectinase under optimal conditions.

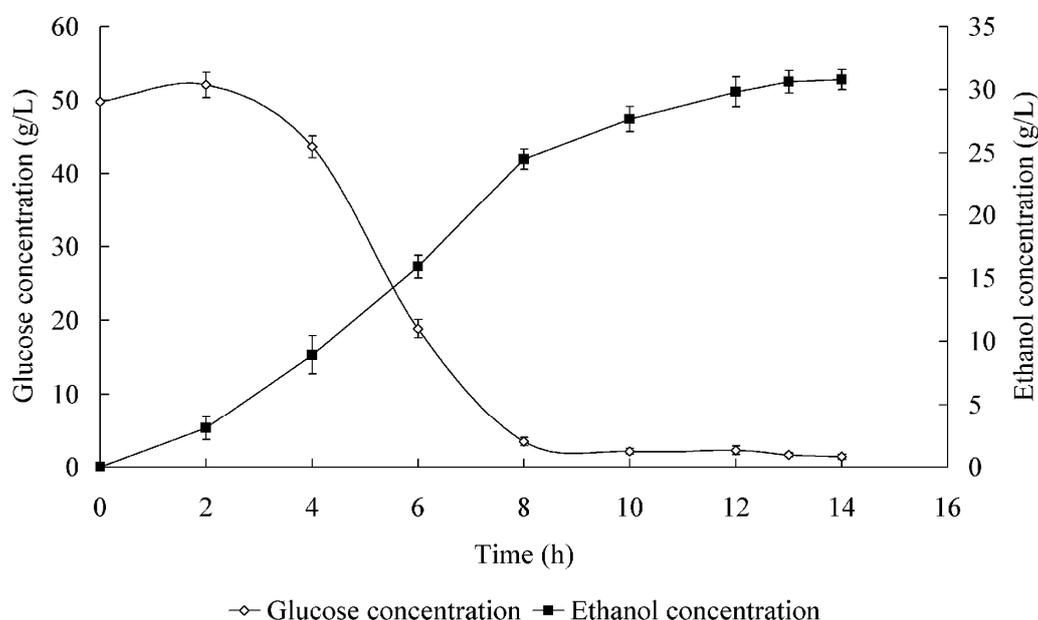


3.5. Fermentation of the Mash with the Optimal Pretreatment Conditions

The *L. punctata* mash pretreated by pectinase under optimal conditions was fermented by *S. cerevisiae* in 250 mL flask at 30 °C and 220 rpm. The initial total sugar of the duckweed mash (based on glucose) was 69.53 g/L. The time course of the fermentation is shown in Figure 5. The fermentation time, final ethanol concentration, ethanol productivity rate, fermentation efficiency and residual glucose were 14 h, 30.8 ± 0.8 g/L, 2.20 g/L/h, 90.04% and 1.4 g/L, respectively. The ethanol concentration of 30.8 ± 0.8 g/L was 29.41% higher than that obtained from the untreated mash. Pectinase pretreatment could significantly improve the fermentation performance of *L. punctata* due to its effort of increasing the glucose yield. The potential annual ethanol yield was 1299.38 gallons/ha based on a common annual yield of 30 t/ha, which is much higher than that from corn stover of 865.59 gallons/ha [19,34] and corn of 641.90 gallons/ha [38]. When compared with lignocellulosic

biomass, *L. punctata* had the advantages of a short fermentation time, high ethanol productivity and environmentally friendly pretreatment conditions in fermentation process [2], which are benefits for the commercial success of ethanol production from this feedstock [23]. However, the starch content of corn ranged from 65% to 75%, which is more stable and higher than that of duckweed (3%–75% DM) in most cases [22]. Due to its high moisture content (over 90% in fresh biomass) and variable starch content (3%–75% DM), further studies should focus on efficient harvesting and processing technologies for duckweed biomass and improving its starch content to obtain even higher ethanol concentration for economical feasibility of ethanol production [39].

Figure 5. Time course of ethanol fermentation of *L. punctata* mash pretreated by pectinase under optimal conditions. (■): Ethanol concentration (g/L); (◇): Glucose concentration (g/L). Error bars represent standard error.



4. Conclusions

As shown in this research, duckweed *L. punctata*, an abundant raw material, is a potential and novel resource for ethanol production. In order to attain higher ethanol concentration and productivity, pectinase was used to improve its glucose yield and eventually increase its ethanol yield. The pretreatment conditions were optimized by Box-Behnken design. Under the optimal conditions (45 °C, 300 min pretreatment time and 26.54 PECTU/g mash of pectinase), it was possible to obtain approximate 30.8 g/L of ethanol in 14 h from the pretreated *L. punctata* mash. This work would pave a way for utilizing a novel renewable feedstock for ethanol production. Nevertheless, the ethanol concentration obtained with the optimized pretreatment conditions is still low for an industrial ethanol process, and further studies are necessary to achieve an economical process.

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