Applied Biochemistry and Biotechnology
https://doi.org/10.1007/s12010-020-03437-0

Bioethanol Production from *Azolla filiculoides* by *Saccharomyces cerevisiae, Pichia stipitis, Candida lusitaniae,* and *Kluyveromyces marxianus*

Mariam H. Chupaza[1,2,3] · Yu-Rim Park[1] · So Hee Kim[1] · Ji Won Yang[1] · Gwi-Teak Jeong[1] · Sung-Koo Kim[1]

Received: 30 July 2020 / Accepted: 29 September 2020/Published online: 07 October 2020

Abstract

Ethanol was produced by separate hydrolysis and fermentation using *Azolla filiculoides* as a biomass. Thermal acid hydrolysis and enzymatic saccharification were used as pretreatment methods to produce monosaccharides from Azolla. The optimal content for thermal acid hydrolysis of 14% (w/v) Azolla weed slurry produced 16.7-g/L monosaccharides by using 200 mM H_2SO_4 at 121 °C for 60 min. Enzymatic saccharification using 16 U/mL Viscozyme produced 61.6 g/L monosaccharide at 48 h. Ethanol productions with ethanol yield coefficients from Azolla weed hydrolysate using *Kluyveromyces marxianus*, *Candida lusitaniae Saccharomyces cerevisiae*, and *Pichia stipitis* were 26.8 g/L (Y_{EtOH} = 0.43), 23.2 g/L (Y_{EtOH} = 0.37), 18.2 g/L (Y_{EtOH} = 0.29), and 13.7 g/L (Y_{EtOH} = 0.22), respectively. *Saccharomyces cerevisiae* produces the lowest yield as it utilized only glucose. Bioethanol from Azolla weed hydrolysate can be successfully produced by using *Kluyveromyces marxianus* because it consumed the mixture of glucose and xylose completely within 60 h.

Keywords Bioethanol · *Azolla filiculoides* · *Kluyveromyces marxianus* · *Candida lusitaniae* · *Pichia stipitis* · *Saccharomyces cerevisiae*

Mariam H. Chupaza and Yu-Rim Park are co-first authors.

✉ Sung-Koo Kim
skkim@pknu.ac.kr

[1] School of Marine Fisheries, and Life Science (Major in Biotechnology), Pukyong National University, 48513 Busan, Republic of Korea

[2] KOICA-PKNU International Graduate Program of Fisheries Science, Pukyong National University, Busan 48513, Republic of Korea

[3] Department of Fishing and Fish Processing, Fisheries Education and Training Agency, P.O. Box 83, Bagamoyo, Costal Region, Tanzania

Introduction

In the twenty-first century, the society is facing global challenges on how and where to get sufficient and sustainable energy for transportation, heating and industrial processes, and raw material for different industries to run the world in a sustainable way [1]. Currently, the goals of study are to reduce our dependency on fossil fuels and prevent further deforestation and competition with foods. This has triggered an extensive search for domestication of new bioenergy feedstock which can generate substantial renewable biomass over a short period with rich bioenergy molecules which can be converted into biofuels using a set of well-established technologies [2]. Since fossil energy are limited and their large-scale use as fuels has a negative impact on global climate, thus fuel alternative resources for energy productions are required [3]. Zhang et al. [4] have shown that renewable energy is recognized as the next generation that will replace human dependency on fossil fuels.

Nowadays, bioethanol has been produced from agricultural feedstock and lignocellulosic biomass in many countries [5]. Biomass based on sugar and starch, such as sugarcane or corn, is regarded as food and feedstock [6]. In this respect, research has now shifted to using nonedible feedstock like lignocellulosic biomass or algal biomass rather than the first-generation carbohydrates rich food crops due to human food security risks associated with the first-generation biofuels [7]. Availability of lignocellulosic materials is opening the potential for different geographic regions to take advantage of locally abundant cellulosic feedstock for ethanol production [8].

The use of wastewater as a source of reclaimed water and key nutrients for growing energy crops would significantly reduce the cost and energy requirement for biofuel production [2]. Aquatic plants represented by submerged, emerged, and free-floating species colonized contaminated wetlands have attracted significant attention as a potential feedstock for second and third generation biofuels because of their ability to produce a large amount of biomass as well as cheap and easy maintenance and harvesting [9].

In the current study, Azolla plant was sampled for ethanol production with the aim of ethanol production from *Azolla filiculoides* by using four different types of yeasts for fermentation. Azolla is a genus of floating aquatic ferns with seven species, distributed throughout tropical and temperate regions [10]. The most remarkable characteristic of Azolla is its symbiotic relationship with the nitrogen-fixing blue-green alga (cyanobacterium), *Anabaena azollae* [11]. The ability of Azolla to fix atmospheric nitrogen allows this fern to grow successfully in aquatic habitats lacking or having low levels of nitrogen. Azolla is one of the fastest growing plants, and it has ability to adapt with wide range of environmental conditions [12]. Azolla is a good example of a plant being a crop in the right place when cultivated for a certain purposes, and it is considered as nuisance weed in some part of the world [13]. In tropical and temperate regions, Azolla species may form thick mats on the relatively placid surface of freshwater ponds, drainage ditches, and rice paddies [14]. Dense Azolla mats prevent light penetration in open water areas, which causes oxygen deficiency in the waters and poor life conditions for fish [15]. Hossain et al. [16] show that the aquatic species like water hyacinth and Azolla can be good cellulosic biomass and can be efficient in releasing sugars in their hydrolysate by cost-efficient treatments for ethanol production .

Material and Methods

Raw Materials

Azolla filiculoides was harvested from Magadu fish farm at Mororgoro region in Tanzania. The weed was dried with sunlight for 1 week and powdered to particle size of less than 100 μm using a roller mill and then sieved with 200-mesh and used for analysis.

The biomass composition was determined by using AOAC method [17] by the Agriculture Science Research Institute under Feed Certification Center at Chungnam National University in Daejeon 34134, Republic of Korea.

Thermal Acid Hydrolysis

Three types of acids (HCl, HNO_3, and H_2SO_4) at different concentrations from 50 to 250 mM were used for slurry content ranging 6 to 16% (w/*v*) using 100-mL working volume in a 250-mL flask. The mixture autoclaved for different time intervals between 15 and 90 min at 121 °C then neutralized by NaOH to pH 5.0. The efficiency of thermal acid hydrolysis was calculated using Eq. (1) as follows:

$$E_P(\%) = \frac{\Delta S_{\text{mono}}(\text{g/L})}{\text{TC (g/L)}} \times 100 \quad (1)$$

where E_p is the efficiency of the pretreatment (%), ΔS_{mono} is the increase in xylose and glucose (g/L) during the thermal acid hydrolysis, and TC is the total carbohydrate concentration (g/L) of the Azolla weed [18].

Optimization of Enzymatic Saccharification

Three types of enzymes Celluclast, Viscozyme, and Cellic C-Tec2 were used to determine the optimal enzyme for saccharification. The Viscozyme (Novozymes, Bagsvaerd, Denmark) was added at different level from 4 to 20 U/mL in 100-mL working volume in a 250-mL flask, then the reaction was performed at 50 °C on a shaking incubator at 150 rpm. Samples of 1 mL were taken periodically from 0 to 72 h and analyzed to assess the degree of enzymatic saccharification. The concentrations of monosaccharides (xylose and glucose) were analyzed using high-performance liquid chromatography (HPLC).

The enzymatic saccharification efficiency was calculated using Eq. (2)

$$E_s(\%) = \frac{\Delta S_g(\text{g/L})}{\text{TF (g/L)}} \times 100 \quad (2)$$

where E_s is efficiency of enzymatic saccharification (%), ΔS_g is increase in monosaccharide concentration (g/L) during enzymatic saccharification after the pretreatment, TF is cellulose content (g/L) in pretreated *A. filiculoides* [19].

Fermentation

Yeast Culture and Adaptation

Saccharomyces cerevisiae KCCM 1129 was obtained from the Korean Culture Centre of Microorganisms (KCCM, Seoul, Korea), *Kluyveromyces marxianus* KCTC 7150, *Candida lusitaniae*, and *Pichia stipitis* KCTC 17574 were obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). These yeasts were grown in YPD medium containing 10-g/L yeast extract, 20-g/L peptone, and 20-g/L glucose as a seed culture. The culture was incubated with agitation at 150 rpm for 24 h at 30 °C. Each cultured yeast strains were sampled to determine the dry cell weight through the optical density at 600 nm (OD_{600}) using the standard curves of dry cell weight and OD_{600}.

Ethanol Fermentation

Four yeasts (*S. cerevisiae, K. marxianus, K. lusitaniae*, and *P. stipitis*) were used for ethanol production during fermentation process. Ethanol fermentation was performed with 100 mL of Azolla weed hydrolysate in a 250-mL Erlenmeyer flask under semianaerobic conditions. The Azolla weed hydrolysates were fermented at 30 °C and 150 rpm and then samples were collected periodically from 0 to 72 h for determination of ethanol and residual sugars stored at − 20 °C prior to analysis. The ethanol concentration was determined by high-performance liquid chromatography (HPLC). The ethanol yield coefficient (Y_{EtOH}) was calculated using Eq. (3) as follows:

$$Y_{EtOH} = \frac{[EtOH]_{max}}{[MS]_{ini}} \tag{3}$$

where Y_{EtOH} represents the ethanol yield coefficient (g/g), $[EtOH]_{max}$ is the maximum ethanol concentration achieved during fermentation (g/L), and $[MS]_{ini}$ is the total initial monosaccharides (xylose + glucose) concentration at the onset of fermentation (g/L) [20].

Analytical Methods

The composition of Azolla weed residue was analyzed using the AOAC method.

Cell growth was analyzed by measuring the OD_{600} via spectrophotometry and was converted to dry cell weight using a standard curve. The samples were centrifuged at 12,000 rpm for 15 min, and the supernatants were used to determine the concentrations of glucose, xylose, and ethanol after filtration through a 0.22-μm syringe filter. The concentrations of glucose, xylose, and ethanol were analyzed by HPLC (Agilent 110 series; Agilent Inc., Santa Clara, CA, USA) with a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad) using filtered and degassed 5 mM H_2SO_4 as an eluent at a flow rate of 0.6 mL/min and a temperature of 65 °C.

Statistical Analysis

Each experiment was carried out in triplicate. The statistical significances of differences in pretreatment, saccharification, and monosaccharide contents were evaluated using a one-way

analysis of variance (ANOVA) and Duncan's multiple range test ($P < 0.05$) in SPSS version 23 (SPSS, Cary, NC, USA).

Results and Discussion

Composition of *Azolla filiculoides*

The biomass composition of Azolla weed was determined by using the AOAC method. It is known that these species contain different levels and compositions of essential metabolites, including proteins, starch, and crude fat, as well as cell-wall components including cellulose, hemicellulose, and lignin [21]. Zouhair et al. [22] also reported that carbohydrates were the major substrates for bioethanol production. The total carbohydrate content of the *Azolla filiculoides* used in this research was 47.7% obtained by adding carbohydrate and fiber contents as shown in Table 1. Cellulose, hemicellulose, and lignin contents are composed of 11.3%, 14.0%, and 28.9%, respectively. The contents of fiber and carbohydrate are higher than those of Miranda et al. [2]. The difference is due to maturity level which is influenced by seasonal changes in temperature and solar radiation [23].

Optimization of Pretreatment Methods of Azolla Weed

Thermal Acid Hydrolysis of *Azolla filiculoides*

Saha et al. [24] confirmed that pretreatment of any lignocellulosic biomass is crucial before enzymatic saccharification. Figure 1 shows monosaccharides concentration increased with increase in slurry content from 6 to 14% (w/v). Lu et al. [25] also stated that high solid contents of slurry produce high monosaccharide contents by the conversion of polysaccharide to monosaccharide. The efficiency of pretreatment (Ep) of 5.1% was obtained using 14% (w/v) Azolla weed and 100 mM H_2SO_4 for 30 min. Beyond 14% (w/v), the efficiency dropped to 4.9% were there was not any significant change in monosaccharide contents, thus, 14% (w/v) was selected as the optimum slurry content. Beyond 14% (w/v), the viscosity was too high to work with the medium. Rosgaard et al. [26] confirms that proper solid contents should be applied during pretreatment.

Figure 2a shows the increase in monosaccharide concentration after thermal acid hydrolysis for 30 min at 121 °C using HCl with concentrations ranging from 50 to 250 mM. Previous research shows high acid concentrations produced high monosaccharide content. The lowest monosaccharide concentration was 0.7 g/L with efficiency of 1% obtained using 50 mM HCl, and the highest monosaccharide concentrations was 4.2 g/L with efficiency of 6.3% reached using 200 mM HCl. Beyond 200 mM, there was no significant difference in monosaccharide concentration; hence, 200 mM was selected as optimal HCl concentration.

Table 1 Composition analysis of *Azolla filiculoides*

Weed form	Moisture	Ash	Fat	Protein	Unit: (%) Carbohydrate
Dry Azolla weed	0.85	26.20	2.21	23.02	47.72

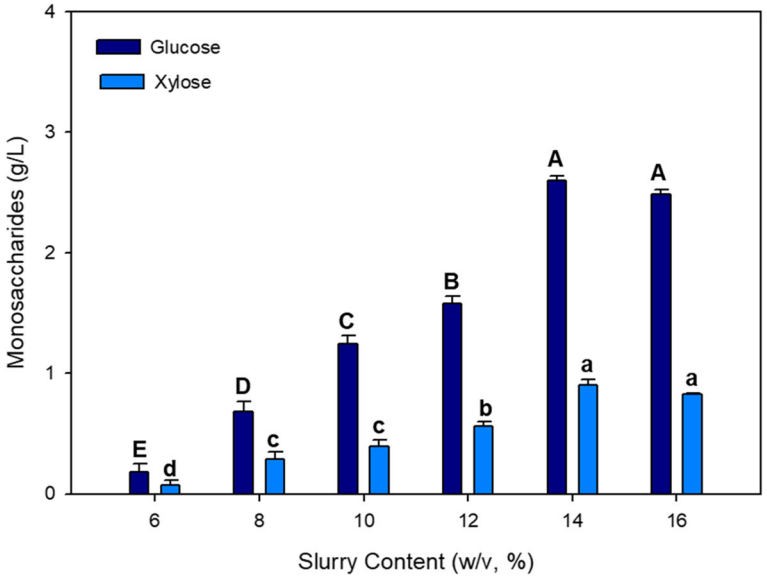

Fig. 1 Optimization of slurry contents of *Azolla filiculoides* for thermal acid hydrolysis (100 mM H_2SO_4 at 121 °C for 30 min) were evaluated using a one-way analysis of variance (ANOVA)

Figure 2b shows the increase in monosaccharide concentration after thermal acid hydrolysis for 30 min at 121 °C using HNO_3 with concentrations ranging from 50 to 250 mM. The lowest monosaccharide concentration was 1.3 g/L with efficiency of 2% obtained using 50 mM HNO_3 and the highest monosaccharide concentrations was 8.9 g/L with efficiency of 13.3% reached using 200 mM HNO_3. Beyond 200 mM, there was no significant difference in monosaccharide concentration; hence, 200 mM was selected as optimal HNO_3 concentration. Dziekońska-Kubczak et al. [27] showed the same result to this study. The pretreatment efficiency of HNO_3 is higher compared with that of HCl.

Figure 2c shows the increase in monosaccharide concentration after thermal acid hydrolysis for 30 min at 121 °C using H_2SO_4 with concentrations ranging from 50 to 250 mM. High hydrolysis yields have been reported when pretreating lignocellulosic materials with H_2SO_4 [28]. The chemical hydrolysis of biomass with dilute sulfuric acid has been recognized by other study [29] as a critical step for removing hemicelluloses from lignocellulosic substrates to facilitate the biological conversion of cellulosic biomass to ethanol. The lowest monosaccharide concentration obtained was 1.4 g/L with efficiency of 2% using 50 mM H_2SO_4 and highest concentration was 11.8 g/L with efficiency of 17.7% using 200 mM H_2SO_4. High acid concentrations showed high monosaccharide content as reported by Chiaramonti et al. [30].

Figure 3 shows thermal acid hydrolysis using different acid hydrolysis time. Monosaccharides concentrations increased with increasing acid hydrolysis time from 15 to 60 min. Beyond 60 min, there was no significant change in monosaccharides concentration. Kityo et al. [31] reported 60 min as optimal hydrolysis time and no significant difference of monosaccharides beyond 60 min. Monosaccharides was increased to 6.9 g/L with efficiency of 10.3% from 15 min to 16.9 g/L with efficiency of 25.3% from 60 min and beyond 60 min monosaccharides dropped to 16.2 g/L monosaccharides with efficiency of 24.3%. Therefore, 60 min was selected as optimal acid hydrolysis time. If the reaction time is longer than 60 min, xylose concentration decreases due to the degradation of xylose according to Chiaramonti et al. [30].

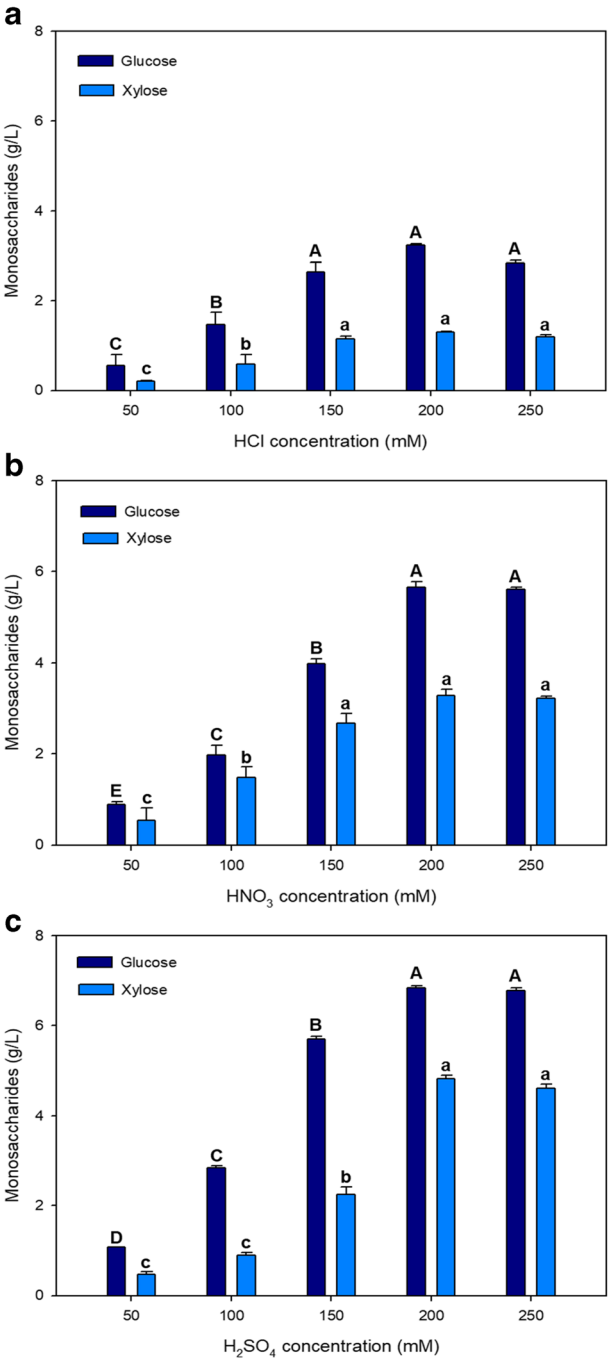

Fig. 2 Thermal acid hydrolysis of *Azolla filiculoides* at 121 °C for 30 min with different Concentrations of **a** HCl, **b** HNO_3, and **c** H_2SO_4 were evaluated using a one-way analysis of variance (ANOVA)

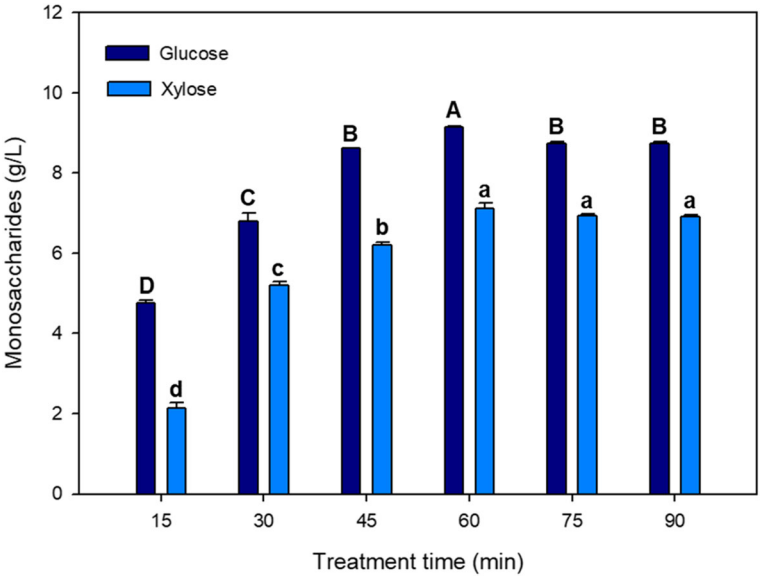

Fig. 3 Thermal acid hydrolysis of *Azolla filiculoides* using different hydrolysis times (200 mM H_2SO_4 at 121 °C for 15 min to 90 min) were evaluated using a one-way analysis of variance (ANOVA)

Figure 4 shows the increase in monosaccharides concentration using optimal hydrolysis time (60 min) at 121 °C and H_2SO_4 acid with concentrations ranging from 50 to 250 mM. Monosaccharides were increased with increasing acid concentrations from 50 to 200 mM. Beyond 200 mM of acid concentration, there was no significant difference in monosaccharides

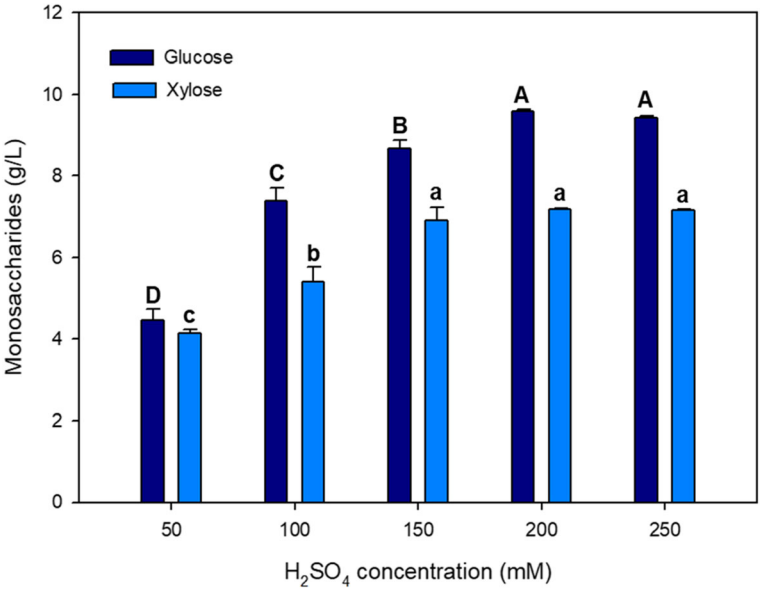

Fig. 4 Thermal acid hydrolysis of *Azolla filiculoides* using different concentrations of H_2SO_4 from 50 to 250 mM at 121 °C for 60 min were evaluated using a one-way analysis of variance (ANOVA)

concentration. Lu et al. [25] reported that an increase in acid concentration produce a high yield of monosaccharides. Difference was observed when Azolla slurry was treated at 121 °C for 30 min as shown in Fig. 1d and 60 min as shown in Fig. 2b. There was an increase of monosaccharides concentration of 4.9 g/L from 11.8 g/L when slurry contents autoclaved for 30 min to 16.7 g/L when slurry contents autoclaved for 60 min. Therefore, 200 mM H_2SO_4 was selected as the optimum acid concentration producing high monosaccharides concentration of 16.7 g/L with 25% efficiency. Kityo et al. [31] reported 200 mM of HNO_3 as optimal acid concentration which produced high monosaccharides concentration.

Enzymatic Saccharification

Figure 5a shows monosaccharide concentrations using 8 U/mL of Viscozyme, Celluclast, and Cellic C-Tec2 enzymes for 72 h. Viscozyme produces high monosaccharides of 35.8 g/L with efficiency of 53.6% followed by Celluclast 32.9 g/L with efficiency of 49.3% and last Cellic

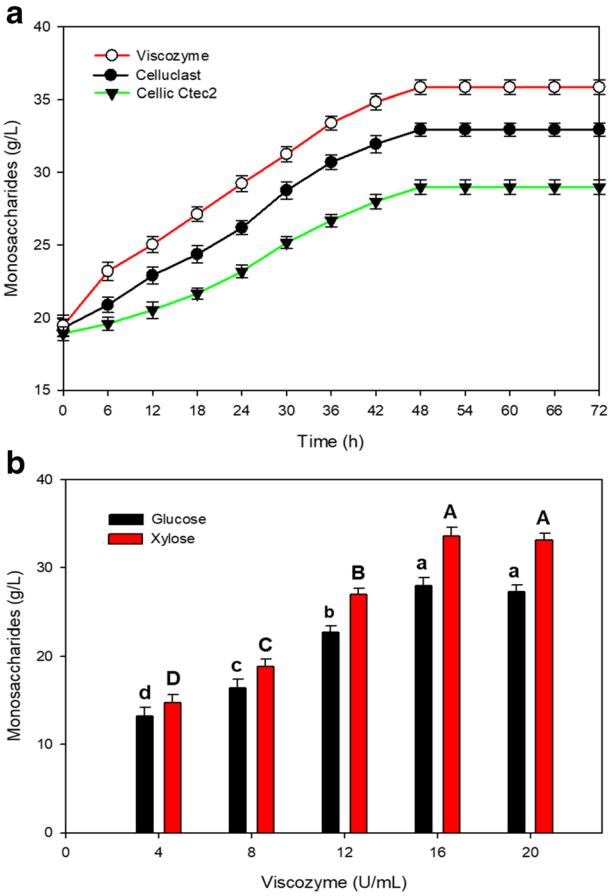

Fig. 5 Enzymatic saccharification of acid hydrolyzed *Azolla filiculoides*. **a** Monosaccharides production using 8 U/mL of Celluclast, Viscozyme and Cellic C-Tec2 with treatment time using *Azolla filiculoides*. **b** Monosaccharides production from *Azolla filiculoides* using various units of Viscozyme were evaluated using a one-way analysis of variance (ANOVA)

C-Tec2 28.9 g/L with efficiency of 43.3%. The enzymes show high concentrations of monosaccharides at 48 h after that no further increase of monosaccharides. In this study, Viscozyme showed high monosaccharide content compared with Celluclast and Cellic C-Tec2, thus, Viscozyme was selected as the optimum enzyme for enzymatic saccharification. Deba et al. [32] showed that Viscozyme contains various enzymatic activities including cellulase and hemicellulase.

Figure 5b monosaccharide concentrations using various units of Viscozyme enzyme from 4 to 20 U/mL. In this study 16 U/mL, was selected as the optimum unit of Viscozyme for enzymatic saccharification because it produces high monosaccharides contents of 61.6 g/L with efficiency of 92.2% while 4 U/mL produces the lowest monosaccharides contents of 13.2 g/L with efficiency of 19.8%. Beyond 16 U/mL, there was not a significant increase in monosaccharides concentration. Saha et al. [33] shows that various pretreatment options can be used to hydrolyze a fiber to monosaccharides increasing amount of fermentable sugars to produce more ethanol.

Fermentation with *K. marxianus*, *C. lusitaniae*, *P. stipitis*, and *S. cerevisiae*

Fermentation with 4 yeasts; *S. cerevisiae*, *K. marxianus*, *K. lusitaniae*, and *P. stipitis* after thermal acid hydrolysis and enzymatic saccharification produces ethanol as shown in Fig. 6.

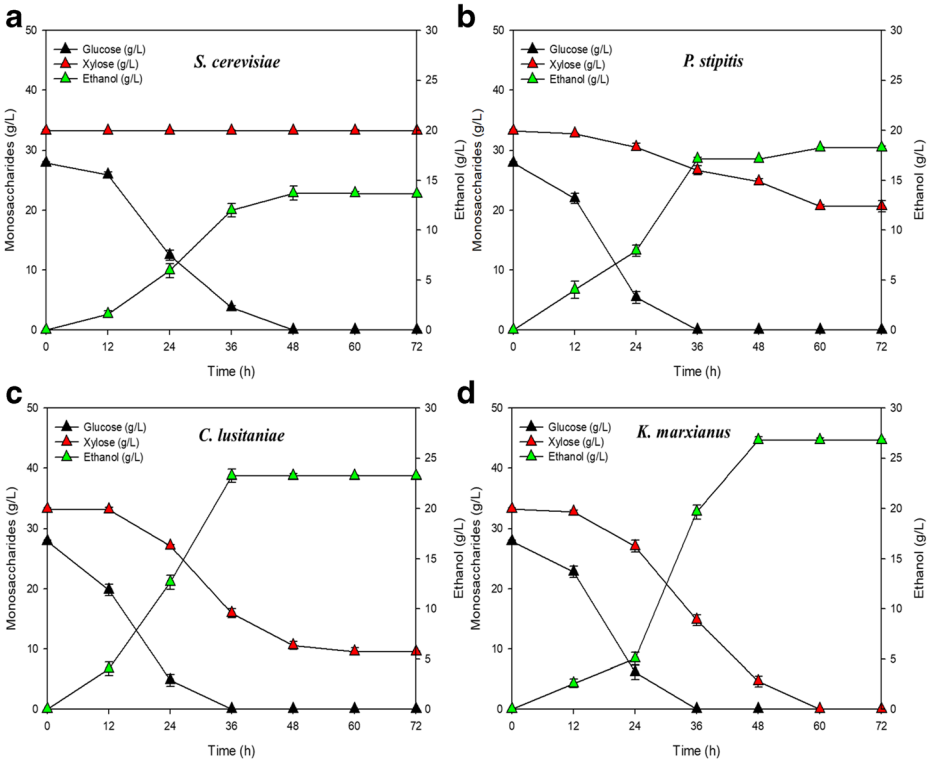

Fig. 6 Bioethanol production from *Azolla filiculoides* hydrolyzate using four yeasts. **a** *S. cerevisiae*, **b** *P. stipitis*, **c** *C. lusitaniae*, and **d** *K. marxianus*

Figure 6a shows ethanol production by using *S. cerevisiae.* In this study, xylose could not be utilized by *S. cerevisiae.* Same results reported by Limtong et al. [34] mentioning pentose sugar cannot be fermented by *S. cerevisiae.* Glucose of 27.8 g/L was fully utilized after 48 h to produce ethanol while 33.2 g/L of xylose remained unused. It has been reported by several other researches that ethanol can be produced from xylose by genetically engineered *S. cerevisiae* [33–36]. *S. cerevisiae* had the lowest yield of 13.7 g/L ethanol with $Y_{EtOH} = 0.22$ consumed glucose only but not xylose.

Figure 6b shows that glucose was fully utilized within 36 h and xylose was utilized 12.6 g/L in 60 h producing 18.2 g/L ethanol with $Y_{EtOH} = 0.29$. It is known that *P. stipitis* is able to consume xylose to produce ethanol despite of preference of glucose to xylose [37]. Glucose inhibits xylose transport by noncompetitive inhibition in the low-affinity proton symport system, where the low-affinity transport is used when sugar concentrations are high and the high affinity systems are used when sugar concentrations are low [38]. Maleszka et al. [39] reported that *C. lusitaniae* produces more ethanol than *P. stipitis* simply because it able to ferment xylose better.

Figure 6c shows how glucose was fully utilized by *C. lusitaniae* in 36 h, however xylose was not completely utilized after 60 h. Previous study indicates that xylose can be consumed completely in 48 h by adaptive evolution process [36]. *C. lusitaniae* was the second yeast to produce 23.2 g/L ethanol with $Y_{EtOH} = 0.37$ as it consumed glucose completely and 23.7 g/L xylose.

Figure 6d shows high utilization of monosaccharides by *K. marxianus* from 27.8 g/L of glucose and 33.2 g/L of xylose yielding high ethanol of 26.8 g/L with $Y_{EtOH} = 0.43$ compared with other three yeasts. It shows that glucose was fully utilized after 36 h while xylose was completely utilized after 60 h. Sunwoo et al. [17] reported that *K. marxianus* can consume both glucose and xylose although observed to utilize xylose after glucose is depleted. It has been reported that *K. marxianus* can grow at 47 °C, 49 °C, and even 52 °C and can produce ethanol even at higher temperatures above 40 °C [35, 36]. Also, *K. marxianus* offers additional benefits including a high growth rate and the ability to utilize a wide variety of industrially relevant substrates such as sugarcane, corn silage juice, molasses, and whey powder [35].

Conclusion

Azolla weed is one of the fastest growing plants on the earth, and it is considered as nuisance in some part of the world. This makes Azolla as a perfect biomass for bioethanol production. Thermal acid hydrolysis and enzymatic saccharification enhanced ethanol production from Azolla hydrolysate by converting cellulose to more fermentable sugar. This study confirms the production of ethanol from *Azolla filiculoides* by using four different types of yeasts, and all of them utilize the mixture of glucose and xylose except for *S. cerevisiae* which utilize only glucose. The optimal pretreatment conditions for the production of monosaccharides from Azolla weed were thermal acid hydrolysis with 200 mM H_2SO_4 with 14% (w/v) slurry at 121 °C for 60 min. The optimal condition for enzymatic saccharification was the treatment of 16 U/mL Viscozyme. The maximum ethanol concentration produced from *Azolla filiculoides* hydrolysate was 26.8 g/L with $Y_{EtOH} = 0.43$ by using *K. marxianus* followed by *C. lusitaniae* with 23.2 g/L with $Y_{EtOH} = 0.37$ then *P. stipitis* and *S. cerevisiae* having the lowest ethanol production of 18.2 g/L with $Y_{EtOH} = 0.29$ and 13.7 g/L with $Y_{EtOH} = 0.22$.

Funding This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2019R1F1A1041288), Republic of Korea.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Lidén, G., & Zacchi, G. (2006). Bio-ethanol-the fuel of tomorrow from the residues of today. *Trends in Biotechnology, 24*(12), 549–556.
2. Miranda, A. F., Biswas, B., Ramkumar, N., Singh, R., Kumar, J., James, A., Roddick, F., Lal, B., Subudhi, S., Bhaskar, T., & Mouradov, A. (2016). Aquatic plant Azolla as the universal feedstock for biofuel production. *Biotechnology for Biofuels, 9*(1), 221.
3. Brouwer, P., van der Werf, A., Schluepmann, H., Reichart, G. J., & Nierop, K. G. J. (2016). Lipid yield and composition of *Azolla filiculoides* and the implications for biodiesel production. *Bioenergy Research, 9*(1), 369–377.
4. Zhang, Q., Wei, Y., Han, H., & Weng, C. (2018). Enhancing bioethanol production from water hyacinth by new combined pretreatment methods. *Bioresource Technology, 251*(11), 358–363.
5. Cho, Y., Kim, H., & Kim, S. K. (2013). Bioethanol production from brown seaweed, *Undaria pinnatifida*, using NaCl acclimated yeast. *Bioprocess and Biosystems Engineering, 36*(6), 713–719.
6. Kim, H., Ra, C. H., & Kim, S. K. (2013). Ethanol production from seaweed (*Undaria pinnatifida*) using yeast acclimated to specific sugars. *Biotechnology and Bioprocess Engineering, 18*(3), 533–537.
7. Kaur, M., Kumar, M., Sachdeva, S., & Puri, S. K. (2018). Aquatic weeds as the next generation feedstock for sustainable bioenergy production. *Bioresource Technology, 251*(10), 390–402.
8. Redding, A. P., Wang, Z., Keshwani, D. R., & Cheng, J. J. (2011). High temperature dilute acid pretreatment of coastal Bermuda grass for enzymatic hydrolysis. *Bioresource Technology, 102*(2), 1415–1424.
9. Miranda, A. F., Liu, Z., Rochfort, S., & Mouradov, A. (2018). Lipid production in aquatic plant Azolla at vegetative and reproductive stages and in response to abiotic stress. *Plant Physiology and Biochemistry, 124*(11), 117–125.
10. Speelman, E. N., Van Kempen, M. M. L., Barke, J., Brinkhuis, H., Reichart, G. J., Smolders, A. J. P., et al. (2009). The Eocene Arctic Azolla bloom: environmental conditions, productivity and carbon drawdown. *Geobiology, 7*(2), 155–170.
11. Lumpkin, T. A., & Plucknett, D. L. (1980). Azolla: botany, physiology, and use as a green manure. *Economic Botany, 34*(2), 111–153.
12. Sood, A., Uniyal, P. L., Prasanna, R., & Ahluwalia, A. S. (2012). Phytoremediation potential of aquatic macrophyte, Azolla. *Ambio, 41*(2), 122–137.
13. Moore, A. W. (1969). Azolla: Biology and agronomic significance. *The Botanical Review, 35*(1), 17–34.
14. Peters, G. A., & Meeks, J. C. (1989). The Azolla-Anabaena symbiosis: basic biology. *Annual Review of Plant Biology, 40*(1), 193–210.
15. Salehzadeh, A., Naeemi, A. S., & Arasteh, A. (2014). Biodiesel production from *Azolla filiculoides* (water fern). *Tropical Journal of Pharmaceutical Research, 13*(6), 957–960.
16. Hossain, R., Chowdhury, M. K., Yeasmin, S., & Hoq, M. M. (2010). Production of ethanol using yeast isolates on water hyacinth and *Azolla*. *Bangladesh Journal of Microbiology, 27*(2), 56–60.
17. Sunwoo, I. Y., Nguyen, T. H., Sukwong, P., Jeong, G. T., & Kim, S. K. (2018). Enhancement of ethanol production via hyper thermal acid hydrolysis and co-fermentation using waste seaweed from Gwangalli beach, Busan, Korea. *Journal of Microbiology and Biotechnology, 28*(3), 401–408.
18. Sunwoo, I. Y., Hau, N. T., Ra, C. H., Jeong, G. T., & Kim, S. K. (2018). Acetone–butanol–ethanol production from waste seaweed collected from Gwangalli beach, Busan, Korea, based on pH-controlled and sequential fermentation using two strains. *Applied Biochemistry and Biotechnology, 185*(4), 1075–1087.
19. Ra, C. H., Jeong, G. T., Shin, M. K., & Kim, S. K. (2013). Biotransformation of 5-hydroxymethylfurfural (HMF) by *Scheffersomyces stipitis* during ethanol fermentation of hydrolysate of the seaweed *Gelidium amansii*. *Bioresource Technology, 140*, 421–425.
20. Ra, C. H., Kim, Y. J., Lee, S. Y., Jeong, G. T., & Kim, S. K. (2015). Effects of galactose adaptation in yeast for ethanol fermentation from red seaweed, *Gracilaria verrucosa*. *Bioprocess and Biosystems Engineering, 38*(9), 1715–1722.

21. Muradov, N., Taha, M., Miranda, A. F., Kadali, K., Gujar, A., Rochfort, S., Stevenson, T., Ball, A. S., & Mouradov, A. (2014). Dual application of duckweed and azolla plants for wastewater treatment and renewable fuels and petrochemicals production. *Biotechnology for Biofuels, 7*(1), 30–47.
22. Zouhair, F. Z., Benali, A., Kabbour, M. R., EL Kabous, K., El Maadoudi, E. h. e., Bouksaim, M., & Essamri, A. (2020). Typical characterization of argane pulp of various Moroccan areas: a new biomass for the second generation bioethanol production. *Journal of the Saudi Society of Agricultural Sciences, 19*(3), 192–198.
23. Reddy, K. R., & DeBusk, W. F. (1985). Growth characteristics of aquatic macrophytes cultured in nutrient-enriched water: II. Azolla, duckweed, and Salvinia. *Economic Botany, 39*(2), 200–208.
24. Saha, B. C., Iten, L. B., Cotta, M. A., & Wu, Y. V. (2005). Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. *Biotechnology Progress, 21*(3), 816–822.
25. Lu, X., Zhang, Y., & Angelidaki, I. (2009). Optimization of H_2SO_4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. *Bioresource Technology, 100*(12), 3048–3053.
26. Rosgaard, L., Andric, P., Dam-Johansen, K., Pedersen, S., & Meyer, A. S. (2007). Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw. *Applied Biochemistry and Biotechnology, 143*(1), 27–40.
27. Dziekońska-Kubczak, U., Berłowska, J., Dziugan, P., Patelski, P., Pielech-Przybylska, K., & Balcerek, M. (2018). Nitric acid pretreatment of *Jerusalem Artichoke* stalks for enzymatic saccharification and bioethanol production. *Energies, 11*(8), 2153–2169.
28. Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresource Technology, 101*(13), 4851–4861.
29. Jang, J. S., Cho, Y. K., Jeong, G. T., & Kim, S. K. (2012). Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed, *Saccharina japonica*. *Bioprocess and Biosystems Engineering, 35*(1–2), 11–18.
30. Chiaramonti, D., Prussi, M., Ferrero, S., Oriani, L., Ottonello, P., Torre, P., & Cherchi, F. (2012). Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method. *Biomass and Bioenergy, 46*, 25–35.
31. Kityo, M. K., Sunwoo, I., Kim, S. H., Park, Y. R., Jeong, G. T., & Kim, S. K. (2020). Enhanced bioethanol fermentation by sonication using three yeasts species and Kariba weed (*Salvinia molesta*) as biomass collected from Lake Victoria, Uganda. *Applied Biochemistry and Biotechnology, 192*(1), 180–195.
32. Deba, A., Azimah, N., Zain, M., & Salleh, M. (2017). Biosugar production from oil palm mesocarp fiber (OPMF) using Viscozyme. *ARPN Journal of Engineering and Applied Sciences, 12*(21), 6225–6237.
33. Saha, B. C., Dien, B. S., & Bothast, R. J. (1998). *Fuel ethanol production from corn fiber current status and technical prospects. Biotechnology for fuels and chemicals*. Humana Press, Totowa, 1998, 115–125.
34. Limtong, S., Sringiew, C., & Yongmanitchai, W. (2007). Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresource Technology, 98*(17), 3367–3374.
35. Nonklang, S., Abdel-Banat, B. M. A., Cha-aim, K., Moonjai, N., Hoshida, H., Limtong, S., Yamada, M., & Akada, R. (2008). High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. *Applied and Environmental Microbiology, 74*(24), 7514–7521.
36. Sunwoo, I. Y., Kwon, J. E., Nguyen, T. H., Jeong, G. T., & Kim, S. K. (2019). Ethanol production from water hyacinth (*Eichhornia crassipes*) hydrolysate by hyper-thermal acid hydrolysis, enzymatic saccharification and yeasts adapted to high concentration of xylose. *Bioprocess and Biosystems Engineering, 42*(8), 1367–1374.
37. Hanly, T. J., & Henson, M. A. (2013). Dynamic metabolic modeling of a microaerobic yeast co-culture: predicting and optimizing ethanol production from glucose/xylose mixtures. *Biotechnology for Biofuels, 6*(1), 44–60.
38. Agbogbo, F. K., & Coward-Kelly, G. (2008). Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. *Biotechnology Letters, 30*(9), 1515–1524.
39. Maleszka, R., Wang, P. Y., & Schneider, H. (1982). Yeasts that ferment D-cellobiose as well as D-xylose. *Biotechnology Letters, 4*(2), 133–136.