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Enhancing Bioethanol Production from *Azolla filiculoides* through Optimization of Pretreatment and Culture Conditions with *Saccharomyces cerevisiae*

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ABSTRACT

Purpose: *The increasing human population and extreme consumption of fossil fuels create the potential to generate alternative energy sources. Bioethanol is a renewable energy resource for fossil fuels and it can be produced from low-cost raw material. This study aimed to convert the low-value freshwater flora into high-value bioethanol using Saccharomyces cerevisiae and to optimize the conditions for yield enhancement with the Azolla filiculoides substrate.*

Research Method: *The freshwater flora were collected, cleaned, dried and then pre-treated with 1 M H_2SO_4 solution at 121 ^{C} for 15 min. The flora with significantly higher yields of reducing sugar and alcohol was chosen for further research. Three pre-treatment techniques, including acid (1 M H_2SO_4), enzymatic (1% α-amylase), and a combination of both (1 M H_2SO_4 and 1% α-amylase) were applied to the selected substrate. The technique that resulted in a significantly higher reducing sugar and alcohol yield was chosen.*

Findings: *The study revealed that the Azolla filiculoides substrate produced significantly higher alcohol yield with Saccharomyces cerevisiae using a combination of chemical and enzymatic pre-treatment techniques. When fermentation was done at varying H_2SO_4 concentrations (0.50-1.75 M), fermentation time (12-60 h), temperature (20-45 ^oC), rotation speed (50- 250 rpm) and inoculum concentration (25-150 g/L), and significantly higher alcohol yield (19 times than the non-optimized) was obtained after 36 h, at 40 ^oC and 0.75 M H_2SO_4 concentration with an inoculum concentration of 75 g/L at 200 rpm.*

Originality/value: *The study concluded that Azolla filiculoides can be used as an efficient raw material for alcohol production.*

Keywords: *Azolla filiculoides*, *Bioethanol, Fermentation, Pre-treatment*, *Saccharomyces cerevisiae*

INTRODUCTION

The world population has been steadily on the rise, and fossil fuels have remained the primary source for fulfilling the majority of the world's energy needs (Sarkar *et al*., 2012). The global economy is currently facing a critical energy crisis as a result of the continuous increase in petroleum-based fuel costs, the adverse environmental effects of fossil fuel combustion, and the depletion of fossil oil resources. Currently, used energy resources are non-renewable fossil fuels. Using fossil fuels is considered unsustainable due to depleting fossil fuel sources and the increased emission of greenhouse gases which have caused acid rain, melting glaciers, and air pollution during the last few decades (Schenk *et al*., 2008). Global warming increases the earth's temperature which

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would be dangerous to the earth's inhabitants such as animals, plants and human beings (Zein and Chehayeb, 2015). It has created importance to develop reduction processes and adopt policies to promote sustainable energy sources to minimize the reliance on petroleum-based fossil fuels and to maintain the sustainability of the environment and economy (Brennan and Owende, 2010; Nguyen and Vu, 2012). Biofuels are alternative and renewable sources to current petroleum-based fuels and are expected to minimize the dependence on petroleum-based fuels (Brennan and Owende, 2010). Production of bioethanol from biomass sources is one of the best alternatives for petroleum-based fuels (Hill *et al*., 2006). Bioethanol is produced from 1st, 2nd, 3rd, and 4th generation feedstocks. First-generation bioethanol is obtained from food crops with high levels of starch and sugar content materials. The main advantages of the 1st generation feedstocks are high sugar production and less conversion cost (Sarkar *et al*., 2012). The usage of this 1st generation of biomass for the production of bioethanol has led to various discussions about rising food prices and the occupation of agricultural land. The problems related to the use of 1st generation feedstocks are partially resolved with the use of the 2nd generation feedstocks. Second-generation bioethanol was obtained from lignocellulosic materials such as municipal waste or forest residues including grass, non-food crops, wood chips and straw (Nigam and Singh, 2011). Second-generation feedstocks are readily available as well as inexpensive and minimize the competition on arable land (Singh *et al*., 2014). However, complications in harvesting, purification, and different pre-treatment procedures have made their products more difficult and uneconomical (Daroch *et al*., 2013). Third-generation feedstocks for biofuels are algal biomass (Brennan and Owende, 2010). Algae produce a higher amount of carbohydrates, proteins and lipids in a short period (Yen and Brune, 2007). Algae can live everywhere such as in salty water, freshwater and even sewage Marine vegetation does not occupy any arable lands and has high efficiency of photosynthesis and a short growth period (Demirbas, 2010; Harun *et al*., 2010). Microalgae, macroalgae marine and freshwater gymnosperms could be promising sources for natural ethanol production as they show an excessive multiplication trend and are generally underutilized. Microalgae include *Spirulina, Chlorella* and green algae and macroalgae include red seaweeds, green seaweeds, and brown seaweeds (Demirbas, 2010).

COVID-19 is a pandemic disease (Zhu *et al*., 2020) and many millions of people have contracted the disease globally (Berardi *et al*., 2020). People who are infected with COVID-19 experience serious respiratory illness. The best way to protect ourselves from the virus is by washing our hands using alcohol-based sanitizers or hand washes frequently and avoiding the face. COVID-19 increases ethanol demand due to infections (Schmitz *et al*., 2020). Frequent hand washing and sanitizing are being suggested by public health organizations around the world. Hand hygiene practices include hand washing, antiseptic hand washing, and antiseptic hand sanitization hand hygiene is considered the basic principle of infection prevention and is vital to minimize the transmission and colonization of infection among healthcare workers and the general public (Mahmood *et al*., 2020). Alcohol-based hand sanitizing can effectively remove 99.9% of pathogens (Hadaway, 2020). The Centers established for Disease Control and Prevention for Fighting COVID-19 suggests the usage of alcohol-based sanitizers for preventive measures. The exact alcoholic content is the key factor in determining the effectiveness of a hand sanitizer (Kampf and Kramer, 2004). Therefore, there is a need to produce large-scale, cost-effective alcohol-based sanitizers constantly so that they would be affordable to the public living all around the world.

Aquatic biomass is recognized as a highly efficient and sustainable source of biomass for the production of bioethanol, due to its remarkable photosynthetic efficiency and area-specific yields. Aquatic biomass does not occupy any arable lands and has a short growth period (Arefin *et al*., 2021). Aquatic biomass such as *Azolla* (Chupaza *et al*., 2021; Christy *et al*., 2020), *Spirodela*

polyrrhiza (Cui and Cheng, 2015), *Landoltia punctate* (Chen *et al*., 2012), and *Lemna minor* (Faizal *et al*., 2021) have been previously used in bioethanol production. *Azolla filiculoides* is a small freshwater fern with a floating leaf that reproduces both asexually by fragmentation and sexually by spores. *Azolla filiculoides* is a rapidly growing aquatic plant that can double its mass every 5–6 days (Kollah *et al*., 2016), facilitated by its association with nitrogen-fixing cyanobacteria. As a result, this aquatic plant can achieve high growth rates without the need for inorganic nitrogen (Brouwer *et al*., 2016). In both tropical and temperate areas, *Azolla* species have been recognized to create dense mats on the calm surfaces of freshwater bodies (Chupaza *et al*., 2021) which can become so dense that they obstruct light penetration into the water. This can result in oxygen depletion and adverse living conditions for aquatic life, particularly fish (Salehzadeh *et al.,* 2014). As a result, *Azolla* is considered a nuisance weed in some parts of the world (Chupaza *et al*., 2021). *Azolla,* which has a high content of cellulose and hemicellulose (35 % dw), can be effectively converted into sugars through inexpensive hydrolysis techniques with high productivity and the ability to grow abundantly in various aquatic environments (Hossain *et al.,* 2010; Miranda *et al*., 2016).

Pre-treatment using dilute acids and alkaline are widely used techniques for treating biomass. Dilute acid pretreatments are favored due to their mild operating conditions, simple procedures, and use of inexpensive chemicals. On the other hand, alkaline pretreatments are known to enhance enzyme accessibility to cellulose by eliminating lignin and certain hemicellulose components during saccharification (Tutt *et al.,* 2012). This step is necessary to modify the structure of the biomass and facilitate the ability of enzymes to break down carbohydrate polymers into fermentable sugars (Mosier *et al*., 2005). Christy *et al*. (2023) studied the utilization of *Chara globularis* as a feedstock for bioethanol production. They found that a combination of diluted sulfuric acid (0.75 M) and 1 % alpha-amylase enzyme pre-treatment resulted in an ethanol yield of 0.8 percent.

Fermentation is a biological process in which microorganisms, such as fungi and bacteria, break down complex organic molecules into simpler ones (Sharma *et al.,* 2020). In the production of bioethanol, these microorganisms play a crucial role by fermenting sugars into ethanol. Various microorganisms have been utilized as biocatalysts for bioethanol production from biomass (Yu *et al*., 2009). The widely used microorganism for household and industrial bioethanol production is *Saccharomyces cerevisiae* (Fernando and Kapilan, 2020). Various environmental factors affect the growth of *Saccharomyces cerevisiae* cells and the enzymatic chemical reactions within them, such as fermentation time, temperature, agitation rate, and inoculum concentration (Kapilan, 2015). In the study conducted by Khambhaty *et al*. (2012), process temperatures of 30 °C were utilized, with incubation times of up to 48 h (2 days) at 150 rpm using a 5% (v/v) concentration of *Saccharomyces cerevisiae*. The study reported bioethanol production of 0.390 g/g using algal feedstock.

Aquatic plants have the potential to contribute to bioethanol production, the use of these resources for this purpose is currently limited. Furthermore, there have been several studies conducted on bioethanol production from *Azolla filiculoides* in the literature, its potential to yield significant amounts of bioethanol remains underexplored. In Sri Lanka, there are abundant and widely distributed under-utilized inland aquatic plant resources that could potentially be used for bioethanol production through a continuous multiplication process in the future. The objective of the study was to convert the low-value *Azolla filiculoides* into high-value bioethanol using *Saccharomyces cerevisiae* and to optimize the conditions for yield enhancement.

MATERIALS AND METHODS

Raw Materials

Freshwater flora such as *Azolla filiculoides*, *Spirodela polyrhiza, Wolffia globosa, Salvinia*

minima, Salvinia natans, Wolffia arrhiza and *Cabomba caroliniana* were collected from various freshwater bodies of the Northern province of Sri Lanka.

Fermentation Medium

The fermentation medium used for alcohol production consists of substrates (after liquefaction and saccharification) 8% (w/v), yeast extract 4 g/L, $MgSO_4.7H_2O$ 4 g/L, KH_2PO_4 8 g/L, ammonium sulfate 4 g/L, respectively in Erlenmeyer flask sterilized using autoclave at 121 ^oC for 15 min at 0.15 MPa pressure.

Inoculum Preparation

The cells of *Saccharomyces cerevisiae* were bought from the local store. *Saccharomyces cerevisiae* was cultured in 100 ml of sterile sucrose solution (50 g/L) by inoculating yeast grains (5 g) and incubated at room temperature for 18 h with shaking at 100 rpm (Inparuban *et al*., 2009).

Determination of Reducing Sugar

The reducing sugar content was determined by using the 3, 5 Dinitrosalicylic acid (DNS) method (Christy *et al*., 2021).

Determination of Alcohol

The alcohol content in the fermented sample was determined by using Dujardin-Salleron ebulliometer and expressed in terms of percentage (v/v) (Christy *et al*., 2023).

Biomass Pre-treatment-

The freshwater floras such as *Azolla filiculoides*, *Spirodela polyrhiza, Wolffia globosa, Salvinia minima, Salvinia natans, Wolffia arrhiza* and *Cabomba caroliniana* were collected from various freshwater bodies in the Northern province of Sri Lanka. Then the collected floras were washed and dried to reduce the moisture content. Subsequently, the substrates were milled, which resulted in the reduction of particle size, and increased the surface area of biomass (Ravindran and Jaiswal, 2016).

Chemical Pre-treatment

The substrates dissolved in distilled water were autoclaved at 121 C for 15 min. Then 1 M H_2SO_4 was added to the substrate solution for acid hydrolysis. Then the mixture was cooled down to room temperature and centrifuged at 8000 rpm for 15 min and neutralized using 4 M NaOH. The supernatant was inoculated aseptically with 10% of 18 h old culture of *Saccharomyces cerevisiae* inoculum. The mixture was incubated at room temperature at 100 rpm and allowed to ferment for 5 days in the fermentation medium. The samples were collected at regular time intervals and reducing sugar and alcohol contents were determined (Christy *et al*., 2021). Flora that produced significantly higher amounts of reduced sugar and alcohol content were chosen for further studies.

Enzymatic Pre-treatment

Azolla filiculoides substrate was taken into a sterile conical flask, and distilled water was added. The flask was autoclaved at 121 ^{C} for 15 min. The substrate was added with 0.1 M phosphate buffer and autoclaved at 121 ^{C} for 15 min. Then the mixture was cooled down to room temperature and centrifuged at 8000 rpm for 15 min. Next 1% of the enzyme α-amylase, diluted with 0.1 M phosphate buffer was added to the mixture and kept at 60 °C for 2 h. The mixture was allowed to ferment with *Saccharomyces*

cerevisiae in the fermentation medium. The samples were collected at regular time intervals and the reducing sugar and alcohol contents were determined (Christ *et al*., 2023).

Combination of Chemical and Enzymatic Pre-treatment

The chemically pre-treated *Azolla filiculoides* supernatant was subsequently used for enzymatic hydrolysis. The supernatant was taken and 1% of the enzyme alpha-amylase, diluted with 0.1 M phosphate buffer was added. The mixture was maintained at a temperature of 60 °C for two hours and then subjected to centrifugation. The mixture was allowed to ferment with *Saccharomyces cerevisiae* in the fermentation medium. At regular intervals, samples were taken and the amounts of reduced sugar and alcohol content were analyzed (Ghazali *et al*., 2016).

Optimization of Sulfuric Acid Concentration in the Pre-treatment

The combined chemical and enzymatically pretreated *Azolla filiculoides* supernatant was treated with different acid concentrations (0.50, 0.75, 1.00, 1.25, 1.50, and 1.75 M). The resulting mixture was added to a fermentation media with *Saccharomyces cerevisiae* and incubated at room temperature at 100 rpm. Then the reduced sugar and alcohol contents were determined (Ghazali *et al*., 2016).

Optimization of Culture Conditions for Alcohol Production

Optimization of the fermentation time: Azolla filiculoides was pre-treated using 0.75 M H_2SO_4 and 1% α-amylase combination. The supernatant was added into the fermentation media with *Saccharomyces cerevisiae* inoculum and incubated at room temperature at 100 rpm. The substrate was incubated at different incubation times (12, 24, 36, 48 and 60 h). The samples were collected at regular time intervals and alcohol yield was determined (Manyuchi *et al*., 2018).

Optimization of the temperature: Azolla filiculoides were pre-treated using 0.75 M H_2SO_4 and 1% α-amylase combination. The supernatant was added into the fermentation media with *Saccharomyces cerevisiae* inoculum and incubated at different temperatures (20, 25. 30, 35, 40 and 45 ^{C}) for 36 h. The samples were collected at regular time intervals and alcohol yield was determined (Manyuchi *et al*., 2018).

Optimization of rotation speed: Azolla filiculoides substrate was pre-treated using 0.75M H_2SO_4 and 1% α-amylase combination. The supernatant was added into the fermentation media with *Saccharomyces cerevisiae* inoculum and incubated at 40 ^{C} for 36 h and at different rotation speeds (50, 100, 150, 200 and 250 rpm). The samples were collected at regular time intervals and alcohol yield was determined (Rodmui *et al*., 2008).

Optimization of inoculum concentration: Azolla filiculoides substrate was pre-treated with 0.75 M H_2SO_4 and 1% α amylase combination. The supernatant was added into the fermentation media with different concentrations of *Saccharomyces cerevisiae* inocula such as 25, 50, 75, 100, 125 and 150 g/L and incubated at 40 ^{o}C for 36 h and at 200 rpm. The samples were collected at regular time intervals and alcohol yield was determined (Manyuchi *et al*., 2018).

Statistical Analysis

All the experiments were conducted in triplicates in a randomized design. Minitab 17.0 software was used to analyze the statistical data. A one-way ANOVA was followed by Tukey's multiple comparison tests with a significance level of $p < 0.05$ to determine the significant difference among the mean values (Keerthiga *et al*., 2022)

RESULTS AND DISCUSSION

Biomass Pre-treatment

The amount of reducing sugar produced by the freshwater flora substrates *Cabomba caroliniana* (17.77 ± 1.025 g/L), *Spirodela polyrhiza* (20.2 ± 0.483 g/L), *Salvinia minima* (25.866 ±0.332 g/L), *Azolla filiculoides* (35.978 ±1.184 g/L), *Salvinia natans* (22.915 ±0.746 g/L), *Wolffia arrhiza* (14.589 ±0.465 g/L), and *Wolffia globosa* (8.289 ± 0.758 g/L) fluctuated from 8.289 g/L to 35.978 g/L after the acid hydrolysis using 1 M H_2SO_4. The *Azolla filiculoides* produced a significantly higher amount of reducing sugar than the other species tested. When fermentation was done using *Saccharomyces cerevisiae*, among the chosen flora substrates, alcohol was produced only from the *Azolla filiculoides* substrate (Figure 01). Therefore, *Azolla filiculoides* was selected for further studies. The higher alcohol yield from the Azolla filiculoides substrate is due to having a higher amount of complex carbohydrates, such as cellulose and hemicellulose, than the other substrates (Hossain *et al.,* 2010; Miranda *et al*., 2016). These complex carbohydrates can be broken down into simpler sugars during the acid hydrolysis process. This could result in a higher initial concentration of reducing sugars (Christy *et al*., 2023).

Chemical Pre-treatment

Azolla filiculoides substrate was pre-treated using 1 M H_2SO_4 and fermented with *Saccharomyces cerevisiae*, and the results of reducing sugar and alcohol yield are shown in Figure 02. *Azolla filiculoides* substrate showed a significant increase in alcohol production until the 2nd day of fermentation by *Saccharomyces cerevisiae* and showed 0.1% ($p < 0.05$) of alcohol yield as the highest production. The amount of reducing sugar was significantly decreased from the 1st day towards the 5th day of fermentation (Figure 02). When rice straw was treated with diluted H_2SO_4 at 121 ºC for one hour, the maximum sugar yield of 84 g/L was obtained after the hydrolysis method (Ren *et al*., 2010). Diluted acid pre-treatment is more effective in hydrolyzing biomass than the other chemical pre-treatment processes (Ibrahim, 2012). Sulfuric acid is commonly used as a pre-treatment agent and is relatively cheap and efficient in hydrolyzing cellulose and is more environmentally friendly (Demirbas, 2008). Higher temperatures are used in this procedure to increase sugar decomposition and produce acceptable rates of glucose production from cellulose (Ibrahim, 2012). The reduction of reduced sugar may be due to the rapid consumption of the reduced sugar by *Saccharomyces cerevisiae* during the fermentation process (Agustini *et al*., 2019). The reduction in the quantity of alcohol produced after 2nd day might be due to the evaporation of alcohol produced at moderately high temperatures and the utilization of alcohol by *Saccharomyces cerevisiae* for its metabolic activities (Mitiku and Hatsa, 2020).

Enzymatic Pre-treatment

Production of reducing sugar after the enzymatic pre-treatment and alcohol production by fermentation of *Azolla filiculoides* using *Saccharomyces cerevisiae* is illustrated in Figure 03. After the enzymatic pre-treatment (1% α-amylase) and fermentation by *Saccharomyces cerevisiae* of *Azolla filiculoides* substrate, the amount of alcohol produced significantly increased from the 1st to the 2nd day of fermentation, reaching a higher alcohol yield of 0.2% ($p < 0.05$). Subsequently, there was a significant decrease in alcohol yield until the 5th day of fermentation and, whereas the amount of reducing sugar showed a significantly reducing trend from the 1st day towards the 5th day of fermentation by *Saccharomyces cerevisiae* with *Azolla filiculoides* substrate (Figure 03). Higher bioethanol production of 2.43 g/L was produced from sugarcane bagasse with a mixture of cellulose and hemicellulose enzymes using *Saccharomyces cerevisiae* (Thontowi *et al*., 2018). Enzymatic treatment (α-amylase) is considered an environmentally friendly method due to the reasons of low energy and no chemical

requirements (Sheikh *et al*., 2010). Enzymatic hydrolysis of the substrate is affected by both the structural features of cellulose and the mode of enzyme action (Yang *et al*., 2011). Enzyme α-amylase specifically catalyzes the hydrolysis of α-1, 4 glycosidic bonds of starch to maltose, dextrin, and a small amount of glucose (Zhang and Lynd, 2004). These molecules are converted into ethanol by yeast. The sudden reduction in the quantity of bioethanol was due to the evaporation of ethanol under the conditions used and the active utilization of ethanol by *Saccharomyces cerevisiae* (Mitiku and Hatsa, 2020).

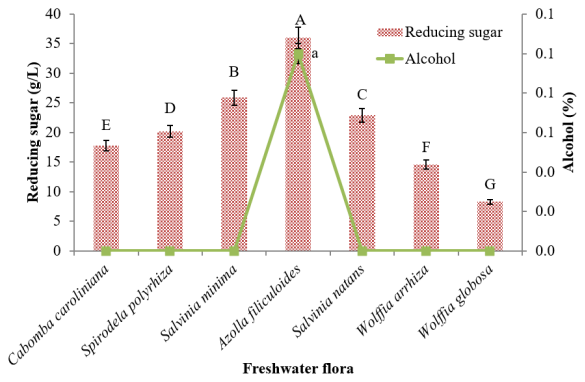

Figure 01: **Changes in different reducing sugar and alcohol yields from diverse freshwater flora on fermentation using *Saccharomyces cerevisiae* after the acid hydrolysis by 1 M H_2SO_4. Different alphabets (A-G), (a) show the significant differences between the mean values.**

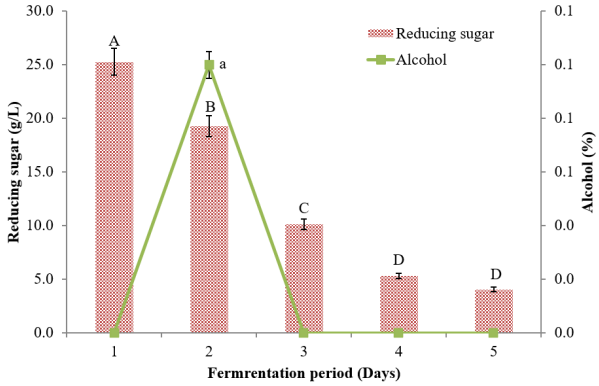

Figure 02: **Quantity of reducing sugar after acid pre-treatment by 1 M H_2SO_4 from Azolla filiculoides and production of alcohol after fermentation using *Saccharomyces cerevisiae*. Different alphabets (A-D), (a) show the significant differences between the mean values.**

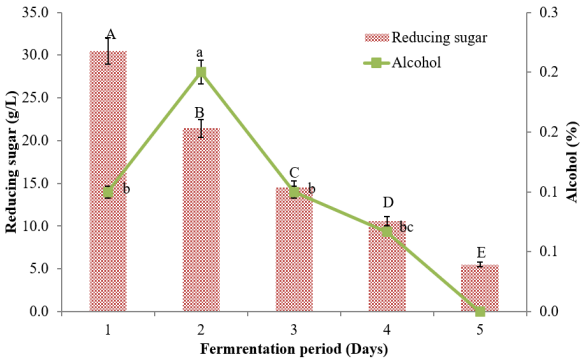

Figure 03: **Quantity of reducing sugar after the enzymatic pre-treatment using 1% α-amylase from *Azolla filiculoides* and production of alcohol after fermentation using *Saccharomyces cerevisiae*. Different alphabets (A-E) (a-c) show the significant differences between the mean values.**

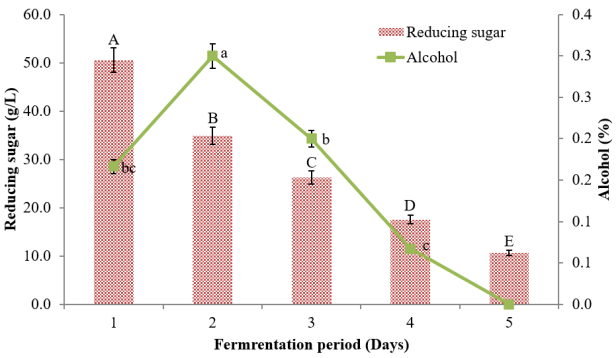

Figure 04: **Quantity of reducing sugar after the chemical (1 M sulfuric acid) and enzymatic (1% α-amylase) pre-treatment from *Azolla filiculoides* substrate and production of alcohol after fermentation using *Saccharomyces cerevisiae*. Different alphabets (A-E), (a-c) show significant differences between the mean values.**

Combination of Chemical and Enzymatic Pre-treatment

Production of reducing sugar after the combination of chemical (1 M H_2SO_4) and enzymatic (1% α-amylase) pre-treatment and alcohol yield by the fermentation of *Azolla filiculoides* substrate using *Saccharomyces cerevisiae* is illustrated in Figure 04. After the combination of chemical and enzymatic treatment, the amount of alcohol produced was significantly increased from the 1st to the 2nd day of fermentation, reaching a higher alcohol yield of 0.3% ($p < 0.05$). Subsequently, there was a significant decrease in alcohol yield until the 5th day of fermentation, whereas the amount of reducing sugar showed a significantly reducing trend from the 1st day towards the 5th day of fermentation by *Saccharomyces cerevisiae* with *Azolla filiculoides* substrate (Figure 04). Bioethanol production of 7.98 % (v/v) was produced from sago starch with 2.5% sulfuric acid concentration using α-amylase and dextrose (Sunaryanto *et al*., 2013). The success of the pre-treatment procedure is generally determined by the acid concentration, temperature, particle size, and reaction time (Chum *et al*., 1990). Acid pre-treatment releases some of the fermentable sugars from the cellulosic biomass and enhances the accessibility of enzymes (α-amylase) for subsequent hydrolysis processes (Pandiyan *et al*., 2019). The main advantages of enzymatic hydrolysis are high process efficiency, no substrate loss and the application of mild and non-corrosive conditions along with the use of non-toxic reagents and biodegradable (Samdhu and Bawa, 1992). Alpha-amylase breaks down the cellulose into dextrin (Puad *et al*., 2018). *Saccharomyces cerevisiae* converts dextrin and fermentable sugars into bioethanol (Gumienna *et al*., 2014).

Among the three pre-treatment techniques, a combination of chemical (H_2SO_4) and enzymatic (α- amylase) treatment yielded significantly higher alcohol after fermentation by *Saccharomyces cerevisiae* than the other techniques used. Therefore, a combination of chemical and enzymatic (α-amylase) pre-treatment was chosen for further studies.

Cultural Conditions for Alcohol Production

Optimization of sulfuric acid concentration: Production of reducing sugar by the combination of chemical and enzymatic (α-amylase) pre-treatment and alcohol yield after fermentation using *Saccharomyces cerevisiae* were determined. Here, instead of acid pretreatment different concentrations of H_2SO_4 were used and the results are shown in Figure 05. When sulfuric acid concentration was increased from 0.5 M to 0.75 M alcohol production increased, reaching a significantly higher alcohol yield of 0.5% ($p < 0.05$). Subsequently, alcohol yield decreased until the 1.25 M sulfuric acid concentrations. Alcohol was not produced when 1.50 M and 1.75 M sulfuric acid concentrations were used in the acid-enzyme combined pretreatment (Figure 05). Therefore, 0.75 M sulfuric acid concentration was chosen as the acid component in the acid α-amylase combined pre-treatment, for further optimization studies for *Azolla filiculoides* substrate (Figure 05). When simultaneous saccharification and fermentation procedures using the commercial cellulase enzyme (Novozyme) were done, a significantly higher ethanol concentration (13.68 g/L) was obtained from rice husk by *Saccharomyces cerevisiae*, when 3% sulfuric acid concentration was used (Novia *et al*., 2017). Extreme acidity may cause sugar degradation during the fermentation process and would lead to an unfavorable effect on sugar conversion (Kefale *et al*., 2012; Nutawan *et al*., 2010).

Optimization of the fermentation time: The effect of fermentation time on the production of alcohol when *Azolla filiculoides* was used as substrate using *Saccharomyces cerevisiae* is shown in Figure 06. Increasing the fermentation time from 12 to 36 hours resulted in a significant increase in alcohol production, reaching its peak (36 hours). However, when the fermentation time was further increased, there was a significant decrease in alcohol production with *Azolla filiculoides* substrate using *Saccharomyces cerevisiae*. Since, a significantly higher alcohol yield (0.7%, $p < 0.05$) was obtained after 36 h of fermentation time of *Azolla filiculoides* substrate using

Saccharomyces cerevisiae, 36 h of fermentation was chosen as the optimum time for further studies (Figure 06). A higher bioethanol yield of 24.8 g/L was obtained at 48 h of incubation with the starch medium (Verma *et al*., 2000). The shorter incubation periods result in insufficient growth of the *Saccharomyces cerevisiae* cells which will decrease the bioethanol production at last. A longer incubation period of fermentation produces a higher concentration of ethanol, which can become toxic to the broth later. Prolonged incubation will result in a decreased ethanol yield because of evaporation (Dash *et al*., 2017).

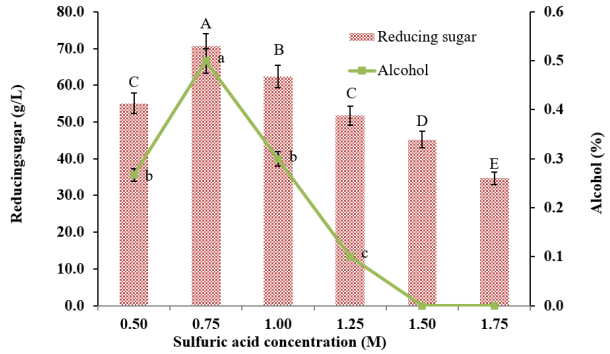

Figure 05: **Production of maximum reducing sugar and maximum alcohol yield after fermentation using *Saccharomyces cerevisiae* when different concentrations of H_2SO_4 in the combined chemical and enzymatic pre-treatment from *Azolla filiculoides* substrate. Different alphabets (A-E) (a-c) show the significant differences between the mean values.**

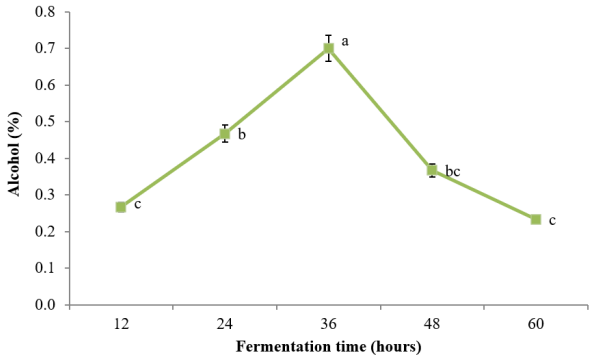

Figure 06: **Effect of fermentation time on alcohol production from *Azolla filiculoides* substrate using *Saccharomyces cerevisiae*. Different alphabets (a-c) show significant differences between the mean values.**

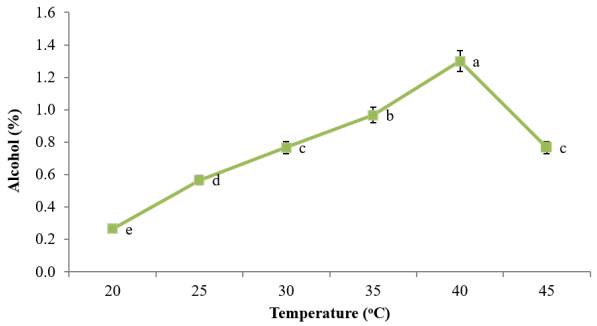

Figure 07: **Effect of different fermentation temperatures on maximum alcohol production from *Azolla filiculoides* substrate using Saccharomyces cerevisiae. Different alphabets (a-e) show the significant differences between the mean values.**

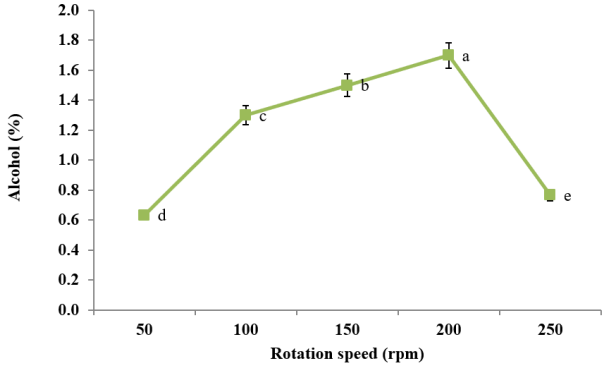

Figure 08: **Effect of different rotation speeds on maximum alcohol production from *Azolla filiculoides*, substrate using *Saccharomyces cerevisiae*. Different alphabets (a-e) show the significant differences between the mean values.**

Optimization of temperature: To optimize the production of alcohol at different temperatures from *Azolla filiculoide,* substrate using *Saccharomyces cerevisiae*, the results of the experiment performed are shown in Figure 07. Increasing the temperature from 20 to 40 ^{o}C resulted in a significant increase in alcohol production, reaching its peak (40 ^{o}C). However, when the temperature was further increased, there was a significant decrease in alcohol production with *Azolla filiculoides* substrate using *Saccharomyces cerevisiae*. Since, a significantly higher alcohol yield (1.3%, $p < 0.05$) was observed at 40 ^{o}C with *Azolla filiculoides* substrate, 40 ^{o}C was chosen as the optimum temperature and used for further studies (Figure 07). The high bioethanol yield of 60 mL/L was achieved from sewage sludge broth at an incubation period of 10 days at 30 °C (Manyuchi *et al*., 2018). The enzymes that control fermentation and microbial activity are susceptible to higher temperatures. At these temperatures, they get denatured and lose their ability to function due to the inactivation of their tertiary structure (Phisalaphong *et al*., 2006; McMeekin *et al*., 2002). Microorganisms that are involved in the fermentation process have an optimum temperature range desirable for their better growth. Therefore, it is required to predetermine an optimum temperature before the fermentation for higher ethanol yield as well as proper microbial growth (Ballesteros *et al*., 2004). Using the too-high or too-low temperature decreases ethanol production as well as inhibits the growth of bacterial cells and *Saccharomyces cerevisiae* and significantly decreases the quantity of fermentation (Manyuchi *et al*., 2018).

Optimization of the rotation speed: Alcohol production at different rotation speeds during fermentation of *Azolla filiculoides* substrate using *Saccharomyces cerevisiae* is shown in Figure 08. Increasing the rotation speed from 50 to 200 rpm resulted in a significant increase in alcohol production, reaching its peak (200 rpm). However, when the rotation speed was further increased, there was a significant decrease in alcohol production with *Azolla filiculoides* substrate using *Saccharomyces cerevisiae*. Since, a significantly higher alcohol yield (1.7%, $p < 0.05$) was obtained at 200 rpm rotation speed, this was selected for further studies (Figure 08). The maximum bioethanol production of 85.73% was produced from stalk juice of sweet sorghum using immobilized *Saccharomyces cerevisiae* at 200 rpm (Liu and Shen, 2008). Agitation influences obtaining higher ethanol production by increasing the permeability of nutrients from the fermentation broth to inner cells as well as by removing bioethanol from interior cells to the fermentation broth. Normally best rotation speed is 150-200 rpm for *Saccharomyces cerevisiae* in the fermentation process (Liu and Shen, 2008). A higher agitation rate affects smooth ethanol production due to restricted metabolic activities (Zabed *et al*., 2014).

Optimization of the inoculum concentration: The production of alcohol varies with different amounts of *Saccharomyces cerevisiae* inoculum when *Azolla filiculoides* substrates were fermented by *Saccharomyces cerevisiae* was studied and the result is shown in Figure 09. Increasing the inoculum concentration from 25 to 75 g/L resulted in a significant increase in alcohol production, reaching its peak (75 g/L). However, when inoculum concentration was further increased, there was a significant decrease in alcohol production with *Azolla filiculoides* substrate using *Saccharomyces cerevisiae*. Since, a significantly higher alcohol yield (1.9%, $p < 0.05$) was obtained at 75 g/L *Saccharomyces cerevisiae* inoculum concentration; this was selected as the optimum inoculum concentration for *Azolla filiculoides* substrate (Figure 09). Higher bioethanol yield was obtained with a concentration of 10% inoculum size in sweet potato flour by co-culture of *Trichoderma* sp. and *Saccharomyces cerevisiae* (Swain *et al*., 2013). *Saccharomyces cerevisiae* was used as the inoculum biocatalyst during the alcohol production from biomass (Manyuchi *et al*., 2018). The inoculum concentration of *Saccharomyces cerevisiae* significantly affects sugar production and ethanol productivity. The biocatalysts will saturate the system once they reach a certain concentration, which will reduce the amount of bioethanol produced (Zabed *et al*., 2014; Laopaiboon *et al*., 2007).

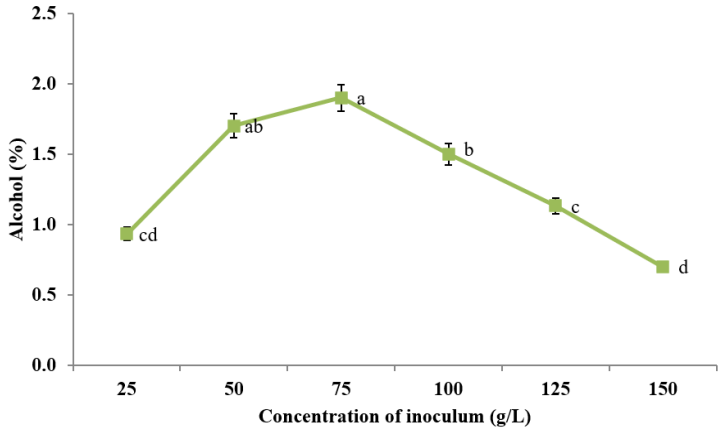

Figure 09: **Effect of different concentrations of *Saccharomyces cerevisiae* inoculum on alcohol production from *Azolla filiculoides* substrate. Different alphabets (a-d) show the significant differences between the mean values.**

In this study, a lower alcohol yield was reported. This could be attributed to several factors that influence the process of bioethanol production. This study used baker's yeast in a liquid media rather than the usual method of employing commercial baker's yeast on solid mediums. This alteration might have potentially reduced the final yield of bioethanol. Christy *et al*. (2023) reported that using *Saccharomyces cerevisiae* (baker's yeast) as an inoculum resulted in lower bioethanol (0.8%) yield when *Chara globularis* was used as a substrate. The composition of *Azolla filiculoides* may vary based on several factors such as environmental conditions of tropical regions and growth stage. This may affect the overall carbohydrate content and accessibility for enzymes.

CONCLUSION

Among the freshwater floral species tested, *Azolla filiculoides* could be used as a good source for bioethanol production using *Saccharomyces cerevisiae.* Bioethanol yield was significantly increased when *Azolla filiculoides* substrate was pretreated with 1 M H_2SO_4 and 1% alpha-amylase combination and fermented by *Saccharomyces cerevisiae*. When the culture conditions were optimized one after another in the order of fermentation time (36 h), temperature (40 °C), agitation rate (200 rpm), and inoculum concentration (75 g/L) after the combined pretreatment with 0.75 M H_2SO_4 and 1% alpha-amylase, alcohol yield was significantly increased (19 times, from 0.1% to 1.9%) than the non-optimized conditions.

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