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Optimization of pre-treatment and culture conditions for bioethanol yield enhancement from *Azolla filiculoides* substrate using *Saccharomyces cerevisiae*

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Abstract

Increasing human population and extreme consumption of fossil fuels create the potential to generate alternative energy sources. Bioethanol is an alternative and renewable energy resource for fossil fuels, which can be generated from low-cost raw materials. The objective of the study is to optimize pretreatment and culture conditions to enhance bioethanol production from *Azolla filiculoides* substrate using *Saccharomyces cerevisiae*. The A. *filiculoides* was collected from fresh-water ponds, cleaned, dried, milled to fine powder and then autoclaved for 15 min at 121 ℃. When A. *filiculoides* substrate was pre-treated with different acids and alkaline separately and alcohol production was monitored, significantly higher alcohol (0.1%) was produced with 1 M H_2SO_4, thus it was selected as the best pre-treatment agent.

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After the pre-treatment with H_2SO_4, the supernatant was subjected to further treatment using 1% alpha-amylase enzyme. This combination resulted in a significantly higher alcohol yield (0.3%) and was selected for further studies. When fermentation was done at varying H_2SO_4 concentrations (0.50 - 1.75 M), fermentation time (12 - 60 h), temperature (20 - 45 ℃), agitation rate (50-250 rpm) and inoculum concentration (25 - 150 g/L) with H_2SO_4 followed by alpha-amylase enzymatic pre-treated A*. filiculoides* supernatant, significantly higher alcohol yield (1.9% - 19 times than non-optimized conditions) was obtained after 36 h, at 40 ℃ and 0.75 M H_2SO_4 concentration with an inoculum concentration of 75 g/L at 200 rpm.

Keywords: Alpha-amylase**;** *Azolla filiculoides*; Bioethanol; Sulfuric acid; Yeast

Introduction

The world population has been increasing considerably and the major part of the global energy demand has been fulfilled by fossil fuel (Sarkar *et al*., 2012). The global economy is experiencing a serious energy crisis due to the continuous increase of petroleum-based fuel prices and the detrimental impacts of fossil fuel burning and the reduction of fossil oil resources. Currently used energy resources are non-renewable and considered unsustainable due to depleting fossil fuel sources and the increased emission of greenhouse gases, which cause acid rain, melting glaciers, and air pollution over the last few decades (Schenk *et al*., 2008). Global warming increases the earth's temperature which 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 fuel and ensure the sustainability of the environment and economy (Brennan and Owende, 2010). Bioethanol is an alternative and renewable source to current petroleum-based fuels and is expected to minimize the dependence on petroleum-based fuels (Brennan and Owende, 2010). When bioethanol is produced from edible feed stocks such as corn and sugar cane, it is called first-generation bioethanol and second-generation bioethanol if the feedstock is lignocellulose. This lignocellulose biomass includes cornstalks,

wood, waste paper products, agricultural and forestry residues, municipal solid waste, and food industry waste. Lignocellulosic biomass comprises cellulose, hemicellulose, lignin, protein, ash, and minor extractives (Toor *et al.*, 2020). Lignocellulosic biomass is being considered a feedstock for bioethanol production due to its relatively low cost of acquisition, availability, and sustainability of supply. Second-generation bioethanol has a greater potential to reduce greenhouse gas emissions compared to first-generation bioethanol. Third-generation bioethanol is obtained when algae are used as the feedstock. Algae bioethanol is gaining traction, possibly due to the high carbohydrate content and absence of lignin in most available algae. With this kind of feedstock, the cost of pre-treatment is expected to reduce as the complex lignin removal process is eliminated (Anyanwu *et al.,* 2018).

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 (Arefin *et al*., 2021). Aquatic biomass does not occupy any arable lands and has a short growth period (Ackman *et al*., 1968). A*. filiculoides*is a small freshwater fern with a floating leaf that reproduces both asexually by fragmentation and sexually by spores. A*. 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 (Peters and Meeks, 1989) 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 (Moore, 1969). *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-treatments 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 (Hsu *et al*., 1980). Ghazalia *et al*. (2016) studied the utilization of durian seed waste as a feedstock for bioethanol production and found that a combination of diluted sulfuric acid (0.1113 M) and alpha-amylase enzyme pre-treatment resulted in an ethanol yield of 0.47 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. The widely used microorganism for household and industrial bioethanol production is S*. cerevisiae* (Fernando and Kapilan, 2020). Various environmental factors affect the growth of S*. cerevisiae* cells and the enzymatic chemical reactions within them, such as fermentation time, temperature, agitation rate, and inoculum concentration (Kapilan, 2015). In a 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 S*. 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 A*. 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 A*. filiculoides* into high-value bioethanol using *S.cerevisiae* and to optimize the conditions for yield enhancement.

Materials and methods

Raw material collection and biomass preparation

The *A. filiculoides* was collected from various freshwater bodies in the Northern Province of Sri Lanka. Then, it was washed, dried and milled. After obtaining a standardized 30 g of milled substrate, it was dissolved in 100 mL of distilled water and subjected to autoclaving at 121 °C for 15 minutes. Following autoclaving, the mixture was allowed to cool down, and 100 mL of H_2SO_4 was added and the substrate solution was then autoclaved for an additional 15 minutes. After cooling to room temperature, the mixture was subjected to centrifugation at 8000 rpm for 15 minutes to separate the components. The supernatant was collected and subsequently neutralized using 4 M NaOH. The neutralized supernatant was then aseptically inoculated with 10% of an 18-hour-old culture of *S. cerevisiae*, which served as the fermentation inoculum. The mixture was placed in an incubator at room temperature, set at 100 rpm, and allowed to ferment for duration of 5 days in the fermentation medium.

Inoculum preparation

The *S. cerevisiae* (yeast strain) was sourced from a nearby market and subsequently incubated in 100 mL of sterile sucrose solution (50 g/L) for 18 hours at 100 rpm at room temperature (Inparuban *et al.,* 2009).

Determination of reducing sugar

The reducing sugar content was determined by using 3, 5 Dinitrosalicylic acid (DNS) method. A series of standard glucose solutions were prepared by diluting different volumes ranging from 0.2 - 1.0 mL of a stock glucose solution (1.0 gL^{-1}) in a series of labelled test tubes. The total volume was made up to 1.0 mL with distilled water. DNS reagent (1.0 mL) was added, and the tubes were heated in a boiling water bath for 5 min. The tubes were cooled, distilled water (10.0 mL) was added, and the absorbance was measured spectrometrically (Spectronic 21D) against a reagent blank at 550 nm. The reagent blank was prepared by taking 1.0 mL of distilled water instead of standard glucose solution. Solutions of unknown concentrations (samples) were treated similarly, and their glucose concentrations were determined using the standard curve for glucose (Christy *et al*., 2021).

Determination of alcohol

Alcohol content of the fermented sample was determined directly for each sample using Dujardin-Salleron ebulliometer and expressed in percentage (v/v) (Christy *et al*., 2023).

Chemical pre-treatment sets

The A*. filiculoides* was hydrolyzed separately using different acids (1 M H_2SO_4, HCl, and HNO_3) and alkaline (1 M NaOH and KOH) and autoclaved. Then the mixture was cooled down and centrifuged, then neutralized. The mixture was allowed to ferment with S*. cerevisiae*. At regular intervals, samples were taken and the amounts of reducing sugar and alcohol content were analyzed.

Combination of chemical and enzymatic treatments

The chemically pre-treated A*. 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 S*. cerevisiae* in the fermentation medium. At regular intervals, samples were taken and the amounts of reducing sugar and alcohol content were analyzed.

Optimization of sulfuric acid concentration in the pre-treatment

The combined chemical and enzymatically pretreated A*. filiculoides* supernatant were 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 S*. cerevisiae* and incubated at room temperature at 100 rpm. Then the reducing sugar and alcohol contents were determined.

Optimization of culture conditions for alcohol production

The optimization process for producing bioethanol from A*. filiculoides* involved a sequential approach where each variable was optimized one after another. After determining the optimized condition, it was maintained constant for subsequent optimization steps. To enhance bioethanol production, a method

combining sulfuric acid and alpha-amylase enzyme hydrolysis was employed, which was then followed by fermentation with S*. cerevisiae*. The optimization process included varying the fermentation time (12 - 60 h), temperature (20 - 45°C), agitation rate (50 - 250 rpm), and S*. cerevisiae* inoculum concentration (25 - 150 gL^{-1}). The reducing sugar and alcohol contents were determined.

Statistical analysis

All the experiments were conducted in triplicates in randomized block 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 $p < 0.05$ was done to determine the significant difference among the mean values.

Results and discussion

Chemical pre-treatment

When A*. filiculoides* were pre-treated with different acids (1 M H_2SO_4, HNO_3 and HCl), and bases (1 M NaOH and KOH) separately, a significantly higher amount of reducing sugar yield was obtained in the acid pre-treatments than the alkaline pre-treatments (Fig.1). Among the three acids used for acidic pre-treatment, the treatment with 1 M sulfuric acid resulted in a significantly higher yield of reducing sugar compared to the other two acids. When acidic pre-treatment was done with 1 M H_2SO_4 only, A*. filiculoides* substrate produced alcohol (0.1%), after fermentation by *S. cerevisiae*. Therefore, acidic pre-treatment with 1 M H_2SO_4 was chosen as the best pre-treatment agent for A*. filiculoides* (Fig.1).

The selection of the appropriate pre-treatment method for the biomass plays a vital role in achieving optimal bioethanol production. The objective of the pre-treatment was to hydrolyze the structure of polysaccharides and facilitate the alkaline or acids to easily break down the polysaccharides into monomers. Monomeric sugars, such as glucose, have the potential to be utilized for the production of various products, including bioethanol and alcohol products (Hill *et al*., 2006). However, the cost of pre-treatment substances can be a significant barrier to the efficient utilization of these sugars (Antunes *et al.,* 2019). The

development of effective and economical pre-treatment methods is crucial to maximizing the output of hydrolysis and downstream processing (Jorgensen *et al.,* 2007). Alkaline conditions, often achieved using sodium hydroxide or potassium hydroxide, are typically used to break down lignocellulosic materials by selectively hydrolyzing lignin. Alkaline hydrolysis primarily targets lignin rather than cellulose and hemicellulose. This process disrupts the lignin structure, making it easier to separate the cellulose and hemicellulose fractions.

HCl is generally considered a weaker acid compared to H_2SO_4 and HNO_3. It has lower reactivity and a lower capacity to break down complex carbohydrates such as cellulose and hemicellulose. As a result, HCl may not be as effective in hydrolyzing the polysaccharides into reducing sugars, leading to lower sugar yields (Christy *et al*., 2023). Pre-treatments that used HNO_3 and H_2SO_4 both yielded significantly higher yields of reducing sugar using A*. filiculoides* substrates compared to other pre-treatment agents. However, A*. filiculoides* produced ethanol after the fermentation using S*. cerevisiae*, if they were pretreated with H_2SO_4 only. Nitric acid pre-treatment may produce toxic substances or inhibitors that can negatively impact downstream processing (Christy *et al.,* 2021) and may inhibit microbial growth and fermentation, resulting in reduced ethanol yield (Esteghlalian *et al.,* 1997).

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 the sugar decomposition and produce acceptable rates of glucose production from cellulose (Xue *et al*., 2009). Similar observations were made in different acids (sulfuric acid and nitric acid) hydrolysis processes where a bioethanol yield of 0.4% was produced only in sulfuric acid treated palmyrah fiber at a temperature of 121 ℃ for 30 min after fermentation using S*. cerevisiae* (Christy *et al.,* 2021).

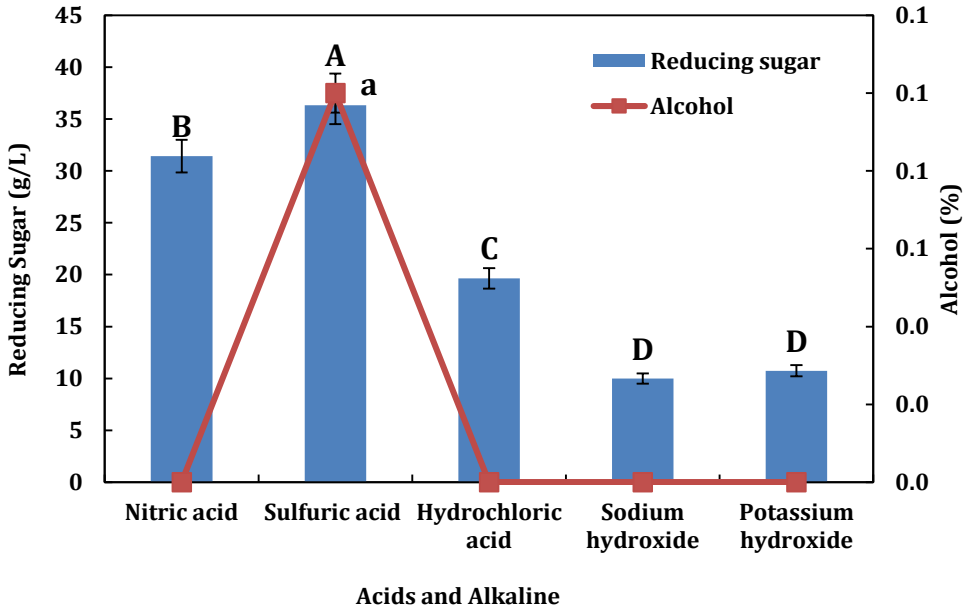

Fig. 1: Quantity of reducing sugar from *A. filiculoides* substrate after the pre-treatment by acids and alkaline and production of alcohol after fermentation using *S.cerevisiae.*

Combination of chemical and enzymatic treatments

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 A. *filiculoides* substrate using S. *cerevisiae* is illustrated in Fig. 2. Ethanol production was significantly increased from the 1st day to the 2nd day of fermentation and after that ethanol production was significantly decreased until the 4th day of fermentation. No ethanol production was observed at 5th day of fermentation. A significantly higher ethanol yield was produced on the 2nd 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 S. *cerevisiae* with A. *filiculoides* substrate (Fig. 2). Acid pre-treatment releases some of the fermentable sugars from the cellulosic biomass and enhances the accessibility of enzymes (α-amylase) for subsequent hydrolysis process (Pandian *et al*., 2019). The 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 S. *cerevisiae.* 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 S. *cerevisiae* (Mitiku and Hatsa, 2020). Bioethanol production of 7.98% (v/v) was produced from sago starch with a sulfuric acid concentration of 2.5% using α-amylase and dextrose (DX) (Sunaryanto *et al*., 2013).

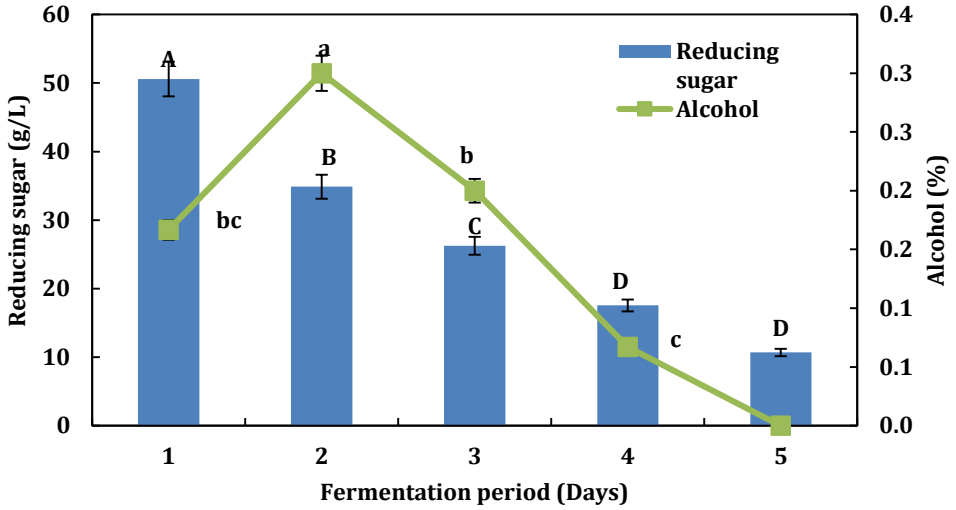

Fig. 2: Quantity of reducing sugar after the chemical (1 M sulfuric acid) and enzymatic (1% α-amylase) pre-treatment from A. *filiculoides* substrate and production of alcohol after fermentation using S. *cerevisiae*.

Optimization of sulfuric acid concentration

When the sulfuric acid concentration was increased from 0.50 M to 0.75 M, the amount of reducing sugar and alcohol content was significantly increased and then reducing sugar and alcohol content were significantly decreased. No alcohol yield was observed at 1.50 M and 1.75 M sulfuric acid concentration. Therefore, the concentration of 0.75 M sulfuric acid was chosen as the acid component in the combined acid α-amylase pre-treatment, for further optimization studies on the A. *filiculoides* substrate (Fig. 3). The effectiveness of the hydrolysis process can be influenced by the concentration of sulfuric acid utilized. If the concentration is too low, the reaction may not proceed quickly enough to be economically viable.

Conversely, if the concentration is too high, side reactions may occur, resulting in the formation of byproducts such as furfural, which can decrease the ethanol yield. It is, therefore, important to carefully control the concentration of sulfuric acid to optimize the hydrolysis process (Kefale *et al*., 2012; Nutawan *et al*., 2010). 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 S*. cerevisiae*, when 3% sulfuric acid concentration was used (Novia *et al*., 2017).

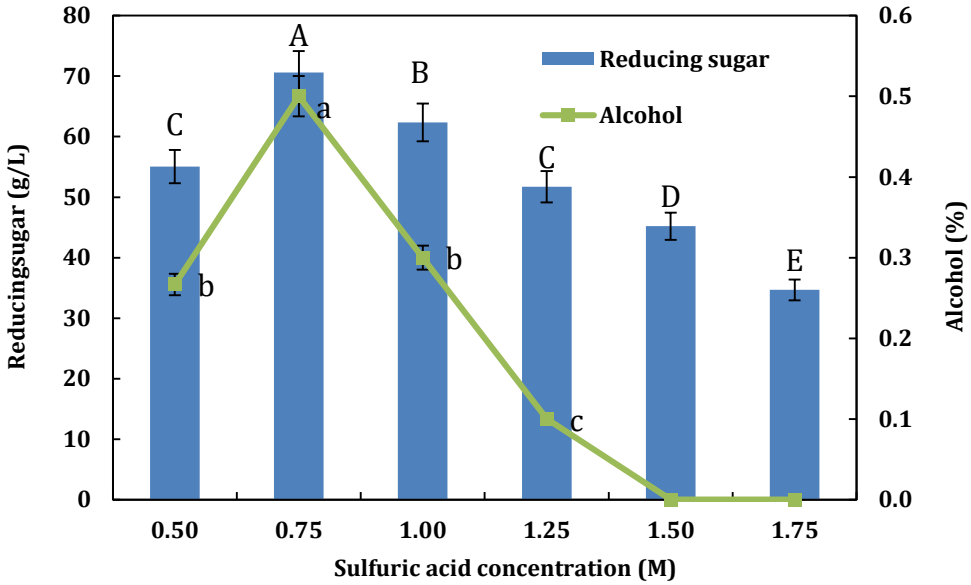

Fig. 3: Production of maximum reducing sugar and alcohol yield after fermentation using S *.cerevisiae* when different concentrations of H_2SO_4 with A*. filiculoides* substrate.

Culture conditions for alcohol production

Optimization of the fermentation time

An increase in fermentation time from 12 to 36 h resulted in a significant increase in alcohol yield, and then the alcohol yield started to decline significantly with A*. filiculoides* substrate using S*. cerevisiae*. Since, a significantly higher alcohol yield was obtained at 36 h of fermentation time using S*. cerevisiae*. The fermentation time of 36 h was chosen as the optimum

time for subsequent studies (Fig. 4). The shorter incubation periods result in insufficient growth of the S*. cerevisiae* cells which will decrease 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). A higher bioethanol yield of 24.8 g/L was obtained at 48 h of incubation with the starch medium (Verma *et al*., 2000).

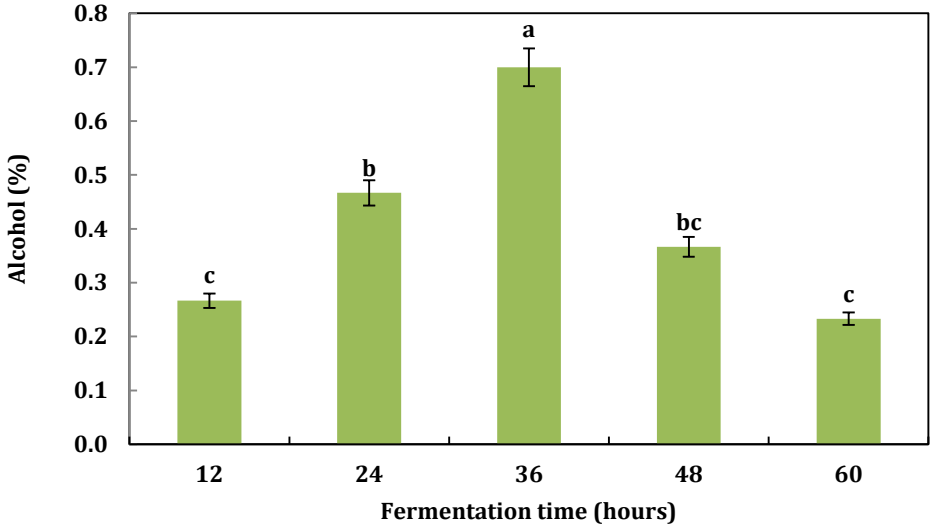

Fig. 4: Changes in fermentation time on alcohol yield from A*. filiculoides* substrate using yeast.

Optimization of temperature

An increase in temperature from 20 °C to 40 °C resulted in a significant increase in alcohol yield and after 40 °C the alcohol yield started to decline significantly with A*. filiculoides* substrate. Since, a significantly higher alcohol yield was observed at 40 °C, this was chosen as the optimum temperature and used for further studies (Fig. 5). Higher temperatures can have a negative impact on the enzymes responsible for controlling fermentation and microbial activity, making them less effective. At these temperatures, they get denatured and lose their ability to function due to the inactivation of their tertiary structure (Phisalaphong *et al*., 2006). The microorganisms involved in the fermentation process have an ideal temperature range for optimal growth, and it is necessary

to determine this range beforehand to achieve a higher ethanol yield and proper microbial growth during fermentation (Ballesteros *et al*., 2004). Using too high or too low temperature decreases ethanol production as well as inhibits the growth of bacterial cells and *S. cerevisiae* and significantly decreases the quantity of fermentation. The high bioethanol yield of 60 mL/L was achieved from sewage sludge broth after an incubation period of 10 days at 30 °C (Manyuchi *et al*., 2018).

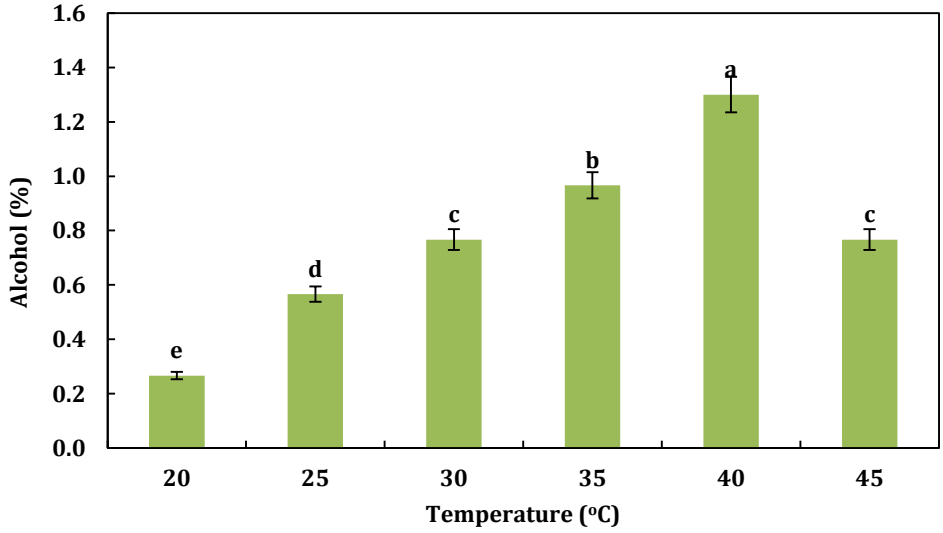

Fig. 5: Changes in different fermentation temperatures on alcohol yield with A. *filiculoides* substrate using yeast.

Optimization of the agitation rate

When the agitation rate increased from 50 to 200 rpm, the alcohol yield was significantly increased with the A. *filiculoides* substrate and then it started to decrease with a higher agitation rate. Since, a significantly higher alcohol yield was obtained at 200 rpm agitation rate, this was selected for subsequent studies (Fig. 6). Agitation can have a positive impact on ethanol production by promoting the movement of nutrients from the fermentation broth into the interior cells and facilitating the release of bioethanol from the cells into the fermentation broth. The optimal rotation speed for the fermentation of yeast is between 150 and 200 rpm (Liu and Shen, 2008). A higher agitation rate can have a negative impact on ethanol production by limiting metabolic activities

and disrupting the smooth production of ethanol (Zabed *et al*., 2014). The maximum bioethanol production of 85.73 % was produced using the stalk juice of sweet sorghum with immobilized yeast at 200 rpm (Liu and Shen, 2008).

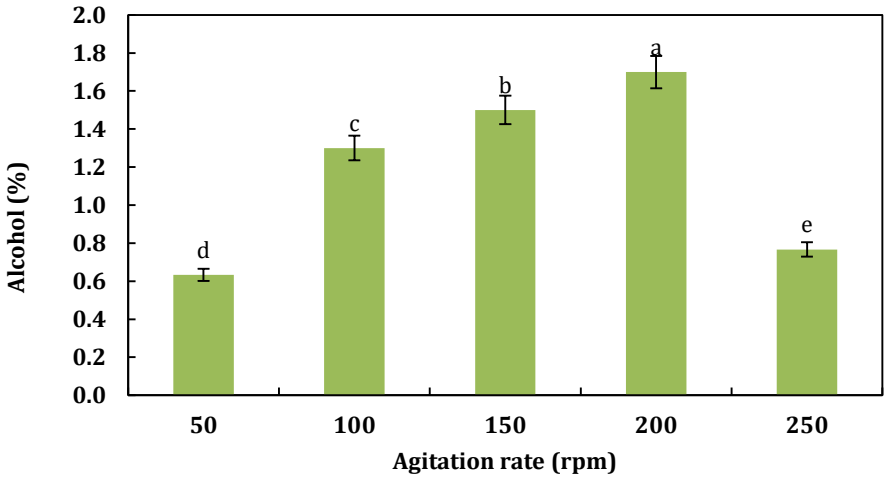

Fig. 6: Changes in different agitation rate on maximum alcohol production with A*. filiculoides,* substrate using yeast.

Optimization of the inoculum concentration

When the inoculum concentration was increased from 25 to 75 g/L, the alcohol production was significantly increased and then it started to decline with increasing inoculum concentration. Since, a significantly higher alcohol yield was obtained at 75 g/L yeast inoculum concentration, this was selected as the optimum inoculum concentration for A. *filiculoides* substrate (Fig. 7). Yeast is commonly employed as the inoculum biocatalyst in the production of alcohol (Manyuchi *et al*., 2018). The inoculum concentration of S*. cerevisiae* significantly affects 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). Swain et al. (2013) reported that a co-culture of *Trichoderma sp*. and S*. cerevisiae* with an inoculum size of 10% in sweet potato flour resulted in a higher yield of bioethanol.

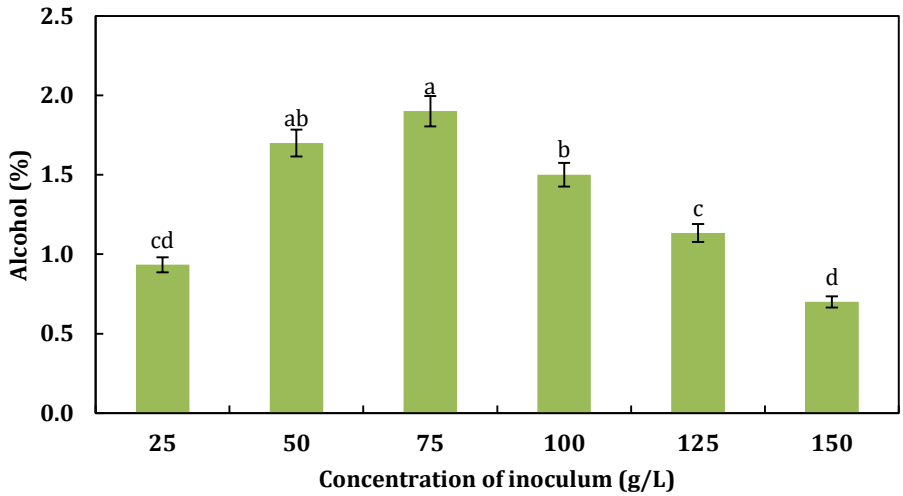

Fig. 7: Changes in different concentration of yeast inoculum on alcohol yield with *A. filiculoides* substrate.

Conclusion

Bioethanol yield was significantly increased when A. *filiculoides* substrate was pretreated with 1 M H_2SO_4 and 1% alpha-amylase combination and fermented by *S. cerevisiae*. When the culture conditions were optimized one after another in the order of fermentation time (36 h), temperature (40 ℃), 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 by 19 times compared to the non-optimized conditions.

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