Bioethanol Production from *Azolla filiculoides* using *Saccharomyces cerevisiae*

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Abstract

Ethanol can be produced from any material that contains sugar, starches and cellulosic materials are used as an ideal and inexpensive raw material in the production of ethanol by fermentation. Cellulosic substrates are easily hydrolyzed into sugars by way of the action of enzymes. Huge amount of diverse under-utilized aquatic sources which are rich in cellulosic substrates are excessively available and widely distributed in Sri Lanka especially in Northern Province. This study was aimed to screen the potential aquatic plants as substrate for ethanol production and to optimize the conditions to obtain higher yield. Fresh water vegetation species such as *Azolla filiculoides, Nelumbo nucifera, Lemna minor, Wolffia globosa, Wolffia arrhiza, Lemna minor and Cabomba caroliniana* were used as substrates for ethanol production by using baker's yeast; *Saccharomyces cerevisiae*, with the liquid fermentation system. Significantly higher amount (0.2%) of bioethanol was produced using *Azolla filiculoides* as substrate. *Azolla filiculoides* was selected for further studies and pretreated with 50 ml of 3% (w/v) acid solutions (H_2SO_4, HNO_3 and HCl) and 3% (v/v) alkaline solutions (NaOH and KOH). Significantly higher amount of yield (0.2%) was obtained with H_2SO_4. Therefore, H_2SO_4 was selected as the best hydrolyzing agent and different concentrations (1-10%) were used for pretreatment.Significantly higher amount of ethanol (0.4%) was measured at 6% of H_2SO_4. When the substrates were hydrolyzed with different incubation periods (15min, 30min and 45min), significantly higher amount of alcohol (0.4%) was obtained at 15 min of incubation period. All the containers were incubated and alcohol was measured at every 12 hours, higher amount of yield (0.5%) was obtained at 54 hours of fermentation time. When Fermentation was carried out with baker's yeast inoculated with peptone, yeast extract and nutrient (PYN) medium at room temperature and pH 7.0 and under the optimized conditions such as 6% of (50ml) H_2SO_4 pretreatment, 15 minutes of incubation period and 54 hours of fermentation time, the ethanol production with *Azolla filiculoides* by *Saccharomyces cerevisiae* was increased by 2.5 times than the non- optimized conditions.

Key Words: *Azolla filiculoides,* bioethanol, fermentation, pretreatment, hydrolysis,*Saccharomyces cerevisiae*

Introduction

The depletion of fossil fuels which meet most of our energy requirements in near future and the pollutants from fossil fuels necessitates the usage of alternative renewable energy sources extensively. Biodiesel and bioethanol produced from biomass sources are one of the best alternatives for petroleum-based fuels and recently, they are commonly used for transportation in many countries (Hill et al, 2006). Bioethanol is basically produced from first or second generation feed stocks. First generation bioethanol is produced from some cereals and legumes such as corn, sugar beet, wheat and barley used for also food sources. Sugars which are obtained from first generation feedstock such as sugar cane, molasses, sugar beet and fruits can be fermented via yeast directly. Advantages of these raw materials are high sugar yields and low conversion cost (Sarkar

et al, 2012). Usage of this first-generation feedstock for bioethanol production leads to various discussions about increasing food prices and occupation of agricultural land. These problems are solved partially by using second generation feedstocks lignocellulosic materials such as waste or forest residues (Nigam and Singh, 2011). Second generation feedstocks have some advantages over first generation feedstocks due to not being used as food source and less land requirement. However, their harvesting, purification and various pre-treatment needs made their production quite challenging and not economical (Daroch et al, 2013).
Azolla filiculoides which are the third-generation feedstock for biofuels are an alternative for the first and second generation feedstocks due to their productivity, easily cultivation methods and convenient harvesting time (John et al, 2011, Daroch et al, 2013). They have cellulosic structure and large amounts of carbohydrates embedded in, so they can also be utilized for bioethanol production. The objective of the study was to convert the low value marine and freshwater plants into high value bioethanol using *Saccharomyces cerevisiae* and optimize the conditions for higher yield.

Materials and Methods

Source of strain and substrate

Baker's yeast (Saccharomyces cerevisiae) was purchased from the local market. Fresh water plants such as *Azolla filiculoides, Nelumbo nucifera, Lemna minor, Wolffia globosa, Wolffia arrhiza, Lemna minor and Cabomba caroliniana* were collected from various fresh water bodies in the Northern Province located in different parts of the Northern Province of Sri Lanka.

Chemicals

All the chemicals were obtained from the standard sources.

Biomass pretreatment

Fresh water plants were washed with distilled water and dried under the direct sunlight for one week to reduce the moisture content and make them more susceptible for milling. The substrates milled with motor and pistil, sieved to pass through a 2.2 mm mesh sieve. Samples (30g) were taken, weighed and added into a 500 ml conical flask and 50 ml of 3% (v/v) H_2SO_4 was added. The flask was plugged with cotton and autoclaved at 121 ℃ for 15 min and the material obtained after treatment was dark in color which was centrifuged in 5000 rpm for 15 minutes then alcohol was determined using an ebulliometer.

Optimization of pretreatment methods

Chemical pretreatment

A. Acid pre-treatment
Thirty grams of *Azolla filiculoides* were taken weighted and then 50 ml of 3% (v/v) H_2SO_4, HCl, HNO_3 were separately added to Azolla *filiculoides* and allowed for 15 minutes for fermentation by the addition of *Saccharomyces cerevisiae*. Ethanol was determined according to section 2.3.
B. Alkaline pre-treatment
Thirty grams of *Azolla filiculoides* were taken and then 50 ml of 3% (v/v) NaOH and KOH were separately added to *Azolla filiculoides* and allowed for 15 minutes for fermentation by the addition of *Saccharomyces cerevisiae*. Ethanol was determined as per section Biomass pretreatment.

Optimization of concentration of H_2SO_4 for acid pretreatment

Thirty grams of *Azolla filiculoides* sample was weighted and pretreated with different concentrations of H_2SO_4 (1-10%) for 15 minutes. Then ethanol was measured as per the section Biomass pretreatment.

Optimization of time for pretreatment
Thirty grams of *Azolla filiculoides* sample was taken and filled into different conical flasks and 50 ml of 6% of H2SO4 was added to each and every conical flask. The conical flasks were autoclaved in different pretreated time intervals ranging from 15 to 45 minutes. Bioethanol production was determined according to the section Biomass pretreatment.

Optimization of fermentation time
Thirty grams of *Azolla filiculoides* were taken into different conical flasks and 50 ml of 6 % of H_2SO_4 was added to each conical flask and pretreated for 30 minutes. And *Saccharomyces cerevisiae* was added into each conical flask and allowed for fermentation. All the containers were incubated and every 12 hours, each sample were taken and alcohol production was measured according to the section Biomass pretreatment. And time used for fermentation measurement were 24h, 36h, 48h, 54h, 60h, 72h and 84hours.

Fermentation under optimized conditions
All the above fermentation procedures were followed with the optimized condition. Ethanol production was measured as per section Biomass pretreatment.

Statistical analysis
All the experiments were conducted in triplicates and the average values were used to plot the graphical representation. Statistical analyses were performed using Minitab 17.0 version. The data were analyzed using one way ANOVA. Tukey's multiple comparison test was used to determine significant difference at $p < 0.05$.

Results and Discussion

Selection of flora
Among the freshwater substrates tested, *Azolla filiculoides* was produced significantly higher amount of alcohol than the other species tested. Thus *Azolla filiculoides* was selected as the best substrate for bioethanol production in further studies

Optimization of pretreatment methods
When 50 ml of 3% H_2SO_4 was used as a pretreatment condition for 15 minutes, *Azolla filiculoides* was produced significantly higher amount of ethanol than any other chemicals used. Thus 3% of H_2SO_4 was selected as a best pretreating agent for further studies.

Optimization of the concentration of H_2SO_4 for acid pretreatment
When 6% of H_2SO_4 (50ml) was used for pretreatment, significantly higher amount of ethanol was produced with *Azolla filiculoides* than other concentrations of H_2SO_4. Thus 6% was selected as optimum concentration for H_2SO_4 for further studies.

Optimization of time for pretreatment
When fermentation was done for 15 minutes incubation period, significantly higher amount of ethanol was produced from *Azolla filiculoides* than other incubation periods. Thus 15 minutes of fermentation was selected as optimized time for further studies.

Optimization of fermentation time
When fermentation was done for 54 hours, a significantly higher amount of ethanol was measured with *Azolla filiculoides* than other fermentation times. Thus 54 hours was selected as optimum fermentation time for further studies.

Fermentation under optimized conditions

After the optimization of fermentation conditions, ethanol production by *Azolla filiculoides* with *Saccharomyces cerevisiae* was increased by 2.5 times than the non-optimized conditions.

Conclusion

Among the fresh water vegetation tested, *Azolla filiculoides* could be used as an efficient source for bioethanol production with *Saccharomyces cerevisiae.* At the room temperature and neutral pH, the optimized conditions such as 6% of (50ml) H_2SO_4 pretreatment, 15 minutes of incubation period and 54 hours of fermentation time, the ethanol production with *Azolla filiculoides* by *Saccharomyces cerevisiae* was increased by 2.5 times than the non- optimized conditions.

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