Journal of the University of Ruhuna
Volume 12 No 1, June 2024. Pp 28-34
DOI: https://doi.org/10.4038/jur.v12i1.8022
ISSN: 2659-2053

RESEARCH ARTICLE

Bioethanol from *Parthenium Hysterophorus* Substrate by *Saccharomyces cerevisiae*

A. Gnanasegaram and R. Kapilan
University of Jaffna, Jaffna, Sri Lanka

Abstract: This study was aimed to determine the efficient part of the *Parthenium hysterophorus* plant as a substrate for bioethanol production and optimize fermentation conditions to enhance the yield. When different parts of *Parthenium hysterophorus* such as stem, leaves, roots, inflorescence, and whole plant were used as the substrate with *Saccharomyces cerevisiae*, significantly higher quantities of bioethanol were produced with whole plant and leaves (1% V/V) than the other plant parts chosen. The entire plant was chosen as a substrate for bioethanol production to address its noxious weed status and simultaneously achieving economically efficient output, making its removal from agricultural land for economic gain. Fermentation conditions were optimized sequentially, changing one factor at a time while keeping other variables constant. The sequence of optimization is time of fermentation, amount of substrate, amount of yeast inoculum, pH of the media and the solution: air space ratio. The optimized fermentation time with the whole plant of *Parthenium hysterophorus* resulted in a 3 times higher ethanol yield on the 4th day of fermentation. When 12.5 g/100 mL amount of whole *Parthenium hysterophorus* plant substrate was used, the ethanol yield was significantly increased by 7.3 times, while using 5 g/100 mL of yeast inoculum resulted in a significantly higher yield. The pH of the media was optimized to 4.8, and the solution: air space ratio was optimized to 1:1.3 bioethanol output was significantly increased by 10 times. After optimizing all culture conditions, the bioethanol yield from the whole plant *Parthenium hysterophorus* substrate was significantly increased (10 times) than the non-optimized conditions.

***Keywords**: Bioethanol, Fermentation, Parthenium hysterophorus, Saccharomyces cerevisiae*

Introduction

Climate change has emerged as a pressing contemporary issue, demanding urgent attention and concerted global efforts. The burning of fossil fuels is the main cause of climate change as it releases greenhouse gases, particularly CO_2. Efforts have been made to capture CO_2 emissions; however, the current techniques are not sufficient to combat the increasing levels of CO_2 in the atmosphere. Additionally, the high demand for fossil fuels is depleting non-renewable energy sources, leading to concerns about future energy supplies (Alalwan et al., 2019).

Biofuels are an alternative to fossil fuels that are produced from renewable sources like plant materials. They offer a way to meet the increasing energy demand while minimizing environmental impact. By transitioning to biofuels, we can reduce greenhouse gas emissions, promote economic development, and enhance energy security (Rodionova et al., 2017).

Bioethanol is simply defined as ethanol produced from biomass (plant material or animal wastes) via biochemical processes using microorganisms (Balat et al., 2008). Bioethanol, which is produced from lignocellulosic biomass resources, has the potential to be a cost-effective alternative to petroleum. When mixed with gasoline, it improves fuel combustion and reduces the emission of CO and unburned hydrocarbons that contribute to smog. Brazil and the USA are the two major ethanol producers accounting for 62% of the world's production (Alvira et al., 2010). *Parthenium hysterophorus* is an annual, noxious, and invasive weed, which belongs to the family Asteraceae. It's biological characteristics such as short life cycle, continuous and profuse flowering until

*Corresponding author: rkapilan@univ.jfn.ac.lk, https://orcid.org/0000-0002-7608-1615

senescence, high seed productivity (up to 15,000 to 100,000 per plant) (Gnanavel and Natarajan, 2013) light seed weight, seed dormancy in adverse environmental conditions, large viable seed bank, and strong regenerative capability make it a highly fecund weed (Tamado et al., 2002). It is commonly known as congress weed and white top weed. The weed is known for its high adaptability over a wide range of environmental conditions and has been successful in naturalizing in various habitats owing to its high reproduction potential and negative allelopathic effect on many other plant species (Gnanavel and Natarajan, 2013).

Parthenium hysterophorus is used as lignocellulosic biomass in bioethanol production. The main importance of lignocellulose biomass is it does not compete with the food industry, and it has become an attractive substance to produce bioethanol (Saha et al., 2013). In this research study, various parts of *Parthenium hysterophorus*, including stems, leaves, whole plants, roots and inflorescences were used as lignocellulosic biomasses. Therefore, the objective of the study was to determine the efficient *Parthenium hysterophorus* plant part as a substrate for bioethanol production and optimize fermentation conditions to enhance the alcohol yield.

Materials and Methods

Chemicals and culture media

All the chemicals were obtained from standard sources. NaOH solution (5%) was used for the pre-treatment, α amylase enzyme (100 g/L) and phosphate buffer (pH 4.8) were used for the hydrolysis step. Basal medium containing 4 g/L yeast extract, 8 g/L KH_2PO_4, 4 g/L $(NH_4)_2SO4$, 2 g/L peptone and 4 g/L $MgSO_4.7H_2O$ was prepared. After autoclaving, a conical flask containing 100 mL media was inoculated with 5 g of *Saccharomyces cerevisiae*. (Commercial yeast, 50 g/L).

Source of strain

Baker's yeast (*Saccharomyces cerevisiae*) was purchased from Cargill's food city, Jaffna, and stored in the refrigerator.

Collection of samples

Leaves, stems, roots, inflorescence, and whole plant of *Parthenium hysterophorus* were collected separately from different regions of the Jaffna peninsula and packed in polythene containers and brought carefully to the laboratory preventing the drop of any parts.

Substrate preparation

Physical pre-treatment

Leaves were collected from the *Parthenium hysterophorus* plants and midribs were removed. Then they were cleaned with running tap water and allowed to air dry. The clean leaves were oven dried at 50 ^oC until a constant weight was obtained. Finally, they were ground, and the resulting fine powder was stored in plastic bottles. Roots, stems, inflorescences, and whole plant which were collected from the *Parthenium hysterophorus* separately, were washed well using clean running tap water and were air dried. Then roots and stems were cut into small pieces using a sharp knife and all those substrates oven dried at 50 ^oC until getting a constant weight. They were ground well using a grinder and stored in plastic bottles separately (Gouveia and Olivera, 2009).

Alkaline pre-treatment

Ten grams of each substrate were taken in 500 mL conical flask separately and 3% NaOH was added into it. Cotton was plugged into each conical flask in airtight manner. Then the substrates were autoclaved at 120 ^oC, 1atm pressure for 40 minutes. Then it was allowed to cool. After that solid phase was separated from the liquid phase. The solid phase was washed with distilled water until neutral pH is obtained and samples were oven dried at 50 ^oC.

Enzymatic hydrolysis

Two hundred milliliters of phosphate buffer, 10 mL α amylase enzyme and 140 mL distilled water were mixed with the neutralized dried sample in 500 mL conical flasks. Conical flasks were shaken by using an orbital shaker at 150 rpm, for 72 hours. The liquid phase was separated by using centrifugation.

Fermentation process

Three hundred milliliters of the enzyme-activated solution was added with 75 mL of fermentation medium (10 g/L yeast extract, 10 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 2 g/L peptone, and 0.5 g/L $MgSO_4.7H_2O$) in a separate conical flask and autoclaved at 121 ^oC, 1 atm pressure for 20 minutes. Then those media were allowed to cool, and 37.5 mL of pre-activated yeast inoculum was added to it. It was kept in an orbital shaker for uniform shaking at 100 rpm, 30 ^oC for 24

hours. Fermentation was in an anaerobic medium achieved upon sealing the conical flask tightly with cotton.

Measuring ethanol percentage

Fifty millilitres of fermented samples were transferred to the falcon tube and centrifuged at full speed. The supernatant was taken carefully, and the pellet was discarded. Then the ethanol percentage of the collected supernatant was measured by using the ebulliometer method. The above procedure was repeated to the five substrates that were obtained from *Parthenium hysterophorus.*

Optimization conditions for bioethanol production

Selection of suitable substrate

Parthenium hysterophorus roots, stems, leaves, inflorescence, and whole plants were used as substrates which underwent the process as mentioned in the above methodology. Then the substrate that produced a high percentage of ethanol was chosen for further optimization conditions.

Optimization of time

Ten grams of whole plant substrate underwent the pre-treatment enzymatic hydrolysis, and it was fermented for 5 days on an orbital shaker at 100 rpm, 30 ^{o}C. Ethanol percentage was measured in 24-hour intervals. Then the time that produced a higher amount of ethanol was chosen for further optimization conditions.

Optimization of the amount of substrate

Different amounts of whole plant samples (2.5 g, 5 g, 7.5 g, 10 g, 12.5 g, and 15 g) were used as substrate to produce ethanol. Those were subjected to pre-treatment, enzymatic hydrolysis, and fermentation for 4 days, separately. After four days, the ethanol percentage was measured by using an ebulliometer. The amount of substrate which produced higher ethanol was chosen for further optimization conditions.

Optimization of the amount of yeast inoculum

The whole plant sample (12.5 g) was processed according to the methodology mentioned previously. Different amount of yeast inoculum (2.50, 3.75, 5.00, 6.25, and 7.50 g/100 mL) was added in different conical flasks which contain fermentation media. It was inoculated at room temperature and allowed for fermentation in an orbital shaker at 100 rpm, 30 ^{o}C for 4 days. After that ethanol percentage was measured by using an ebulliometer. The amount of yeast inoculum that produced a high percentage of ethanol was chosen for further optimization conditions.

Optimization of pH

Alkaline pre-treatment was (3% NaOH) done to the 12.5 g of the whole plant sample. Different pH ranges (2.8, 3.8, 4.8, 5.8, and 6.8) of phosphate buffer were prepared and enzymatic hydrolysis was carried out. It was fermented by using 5 g/100 mL yeast inoculum. Ethanol yield was determined on 4th day to choose the optimized pH of phosphate buffer.

Effect of fermentation solution: air space

Different fermentation solutions: air space (1:10, 1:5, 1:2.5, 1:1.6, 1:1.3, and 1:1) was maintained at different conical flasks. All other conditions were maintained as optimum (whole plant 12.5 g, pH 5.8, yeast inoculum size 5 g/100 mL). Ethanol yield was measured on 4th day.

Statistical analysis

All the experiments were done in triplicates and the average values were used to plot the graphical presentation. Statistical analysis was performed by using the Minitab 20.0 version. The data was analysed using one-way ANOVA. Tukey's multiple comparison test was used to determine significant differences $p < 0.05$.

Results and Discussion

Effect of different Substrates in bioethanol production

Root, stem, inflorescence, leaves, and whole plant samples of *Parthenium hysterophorus* were used as a substrate for the production of bioethanol. Leaves and whole plants produced 0.1% bioethanol after 24 hours of fermentation. On the 4th day of fermentation, leaves and whole plant produced significantly higher ethanol yield (0.4%) than the other *Parthenium hysterophorus* plant parts root (0.17), stem (0.2), and inflorescence (0.15). The nature and structure of cellulose in plants poses challenges in the release of fermentable sugars (Himmel et al., 2010). *Parthenium hysterophorus* parts such as leaves, stems and the whole plant are reasonably satisfactory substrates for bioethanol

production as they perform photosynthesis and contain carbohydrates.

Ethanol production was significantly lower in the roots and inflorescence of *Parthenium hysterophorus* because of a lack of saturated substances. Among the different plant parts of *Parthenium hysterophorus*, the whole plant was chosen as a substrate for further studies because the collection of leaves one by one is a labor-intensive and time-consuming method and the usage of the whole plant involves uprooting the plant from the agricultural land which functions as a productive weed control and management practice.

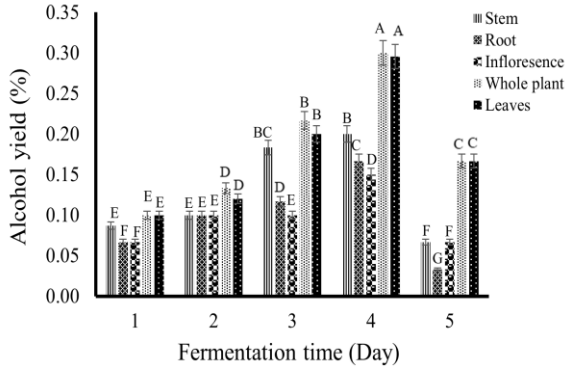

Figure 1. Effect of different substrates on bioethanol production from *Parthenium hysterophorus* using *Saccharomyces cerevisiae*. Alphabets (A, B, C, D, E, F, G) denotes significant differences between the mean values.

Effect of fermentation time

When hydrolysed solution of the whole plant of *Parthenium hysterophorus* was used to ferment at different time intervals (1-5 days), a significantly higher ethanol yield was obtained on the 4th day of fermentation. At this optimization condition ethanol yield was increased by three times (0.1% to 0.3%). On the fourth day of bioethanol production, the yield was significantly higher than the previous three days. This might be due to the time taken by the yeasts to adapt to the media. When suitable conditions for the growth of yeast are eventually achieved on the 4th day the ethanol yield is significantly higher.

Ilangarathna and Kapilan.(2022) reported that 3rd day of fermentation significantly produced higher bioethanol yield from coconut husk fiber using *Saccharomyces cerevisiae*. Short fermentation times decrease ethanol yield efficiency while longer times harm microbial growth due to high ethanol concentration (Nadir et al., 2009, Hossain et al., 2011).

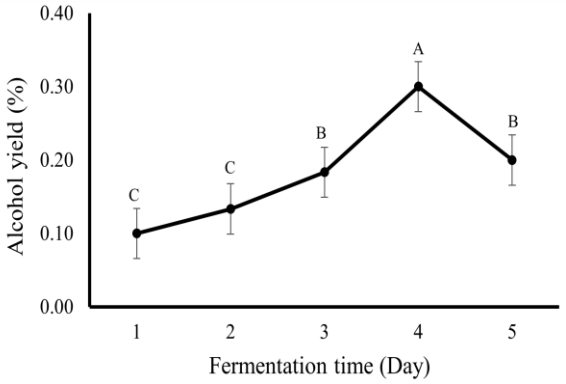

Figure 2. Effect of incubation period on bioethanol production from *Parthenium hysterophorus* using *Saccharomyces cerevisiae*. Different alphabets (A, B, C) denote significant differences between the mean values.

Effect of amount of substrate

When different quantities of whole *Parthenium hysterophorus* plant were used as a substrate, a significantly higher amount of ethanol yield (0.73%) was obtained with 12.5 g/100 mL of whole plant substrate, on the 4th day of fermentation. Therefore, 12.5 g/100 mL whole plant substrate was used for further research studies. At the end of the optimization step bioethanol production from *Parthenium hysterophorus* whole plant substrate was increased by 7.3 times than non-optimized conditions (from 0.1% to 0.73%). When the concentration of the whole *Parthenium* plant sample varies from 2.5 g/100 mL to 12.5 g/100 mL bioethanol production showed an exponential rise, taking more time for adaptation to the medium by yeast cells. The drop in bioethanol produced may be due to the inhibition of ATP synthesis because of the shortage of substrates and, decrease in cell viability due to the metabolically inactiveness of the higher rate of ethanol that produced early and this led to the leakage of intracellular metabolites into the growth medium (Cot et al., 2007).

Effect of yeast inoculum

When yeast inoculum was increased, the amount of bioethanol production from *Parthenium hysterophorus* whole plant was increased by seven times (0.1% to 0.7%) on the 4th day of fermentation. Significantly higher bioethanol yield was obtained when yeast inoculum was 5.0 g/100 mL than the non-optimized conditions (0.1%).

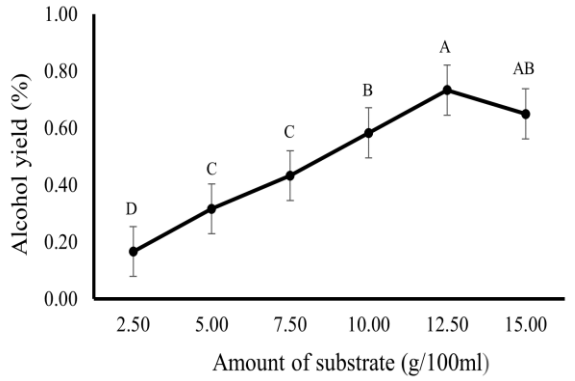

Figure 3. Effect of substrate concentration on bioethanol production from *Parthenium hysterophorus* using *Saccharomyces cerevisiae*. Different alphabets (A, B, C, D) denote significant differences between the mean values.

Therefore, the yeast inoculum concentration was optimized as 5.0 g/100 mL and it was used for further experiments.

Christy et al.,(2023) reported that a yeast inoculum concentration of 75 g/L resulted in a higher yield of bioethanol from *Azolla filiculoides* using *Saccharomyces cerevisiae*. Inoculum concentration does not significantly affect the final ethanol concentration, but it affects the sugar consumption rate and ethanol productivity of *Parthenium hysterophorus* (Laopaiboon et al., 2007). This is because the rapid increase in cell concentration within a certain range reduces fermentation time, as the cells grow quickly and directly consume sugars to produce ethanol (Zabed et al., 2014).

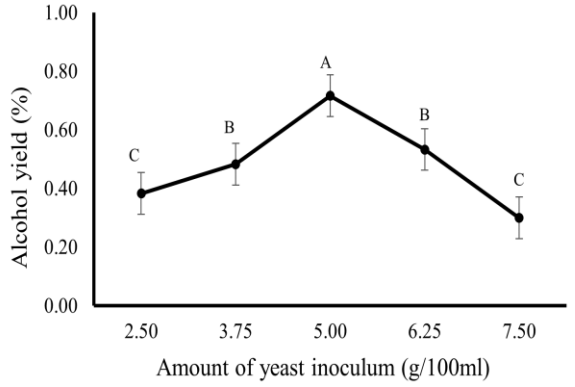

Figure 4. Effect of yeast inoculum size on bioethanol production from *Parthenium hysterophorus* using *Saccharomyces cerevisiae*. Different alphabets (A, B, C) denote significant differences between the mean values.

Effect of pH

When the pH value of the fermentation medium was maintained at 4.8, a significantly higher bioethanol yield was obtained with *Parthenium hysterophorus* whole plant substrate on the 4th day of fermentation. Therefore, this pH was selected as the optimized pH value for further studies. After optimizing the pH value, ethanol production increased by 8.6 times, than the non-optimized condition. Ethanol production is influenced by the pH. It affects yeast growth, fermentation rate, and by-product formation. Additionally, the concentration of H^+ in the fermentation broth can affect the permeability of some essential nutrients into the cells (Zabed et al., 2014). In fermentation for ethanol production, the optimum pH range of *Saccharomyces cerevisiae* is 4.0-5.0 (Lin et al., 2012). When the pH was below 4.0, a longer incubation period is required, but the ethanol concentration is not significantly reduced. However, when the pH was above 5.0, the concentration of ethanol was substantially reduced (Staniszewski et al., 2007). Maintaining the fermentation medium pH at 7, resulted in a higher bioethanol yield (11.20% to 12.60%) from sour banana fruit juice with *Saccharomyces cerevisiae* (Vivekanandaraja and Kapilan, 2021).

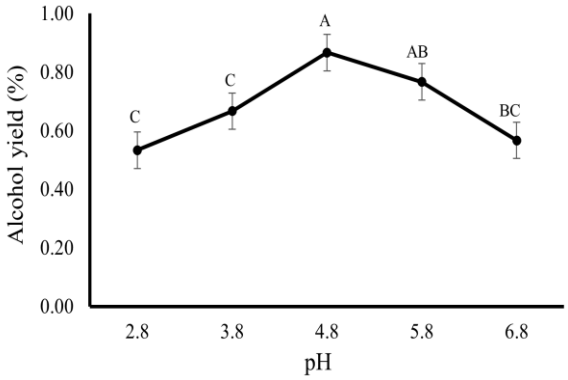

Figure 5. Effect of pH on bioethanol production from *Parthenium hysterophorus* using *Saccharomyces cerevisiae*. Different alphabets (A, B, C, D) denote significant differences between the mean values.

Effect of fermentation solution: air space ratio

When the different fermentation solution (V) air space (v) ratios were maintained, a significantly higher ethanol percentage was obtained with the solution volume was 400 mL (solution (V): airspace (v) = 1:1.3) on the 4th day. Therefore, this was considered as the optimized ratio. At this ratio, the ethanol yield was increased by 10 times than the non-optimized condition. The ratio of air space in the fermentation

solution is determined by the volume of the solution, which has a direct impact on the rate of fermentation and the number of microbial cells. The ratio typically expresses how much oxygen is present in the medium. Anaerobic fermentation occurs when yeast cells convert monomer sugars into ethanol where carbon dioxide released as a by-product at anaerobic conditions (Van der Waarde et al., 1993).

During the fermentation, an anaerobic environment is formed inside the conical flask because of the release of carbon dioxide by-product. However, yeast cells can absorb the oxygen from the substrate they live. If the fermentation solution: air space ratio is high, the presence of additional oxygen in the medium may cause yeast cells to engage in aerobic respiration. As a result, the sugars are completely fermented, producing less ethanol and releasing carbon dioxide. Additionally, if the oxygen concentration is too low this can decrease the viability of the yeast cells (Rosenfeld and Kay, 2003).

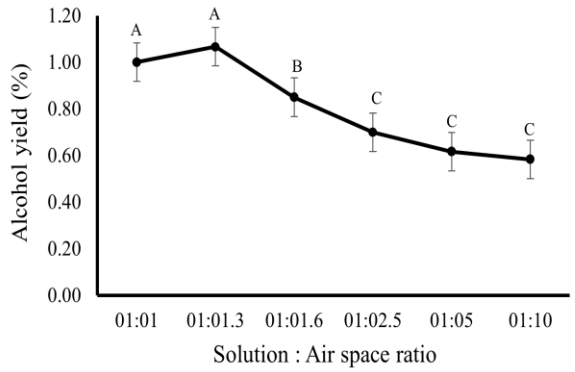

Figure 6. Effect of solution (V): air space (v) ratio on bioethanol production from *Parthenium hysterophorus* using *Saccharomyces cerevisiae*. Different alphabets (A, B, C) denote significant differences between the mean values.

Conclusions

The whole plant of *Parthenium hysterophorus* is an effective substrate for bioethanol production using *Saccharomyces cerevisiae* in the liquid fermentation media among the various parts of the plant chosen for this study. After optimizations of all the culture conditions such as fermentation time, pH, and fermentation solution: air space ratio and media compositions such as amount of *Parthenium hysterophorus* whole plant substrate, and yeast inoculum concentration, the bioethanol yield from *Parthenium* whole plant substrate was significantly increased by ten times. (From 0.1% to 1.0%). To ascertain the commercial viability of this study, it is imperative to conduct an extensive multicenter fermentation study on a large scale.

Acknowledgement

The authors wish to express their sincere gratitude to the National Research Council 2019 for providing the financial support to undertake this project.

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