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ORIGINAL RESEARCH

Bioethanol production from the plant *Impatiens tinctoria* A. Rich. tuber by using *Saccharomyces cerevisiae* fermentation method

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Abstract

The possibility of using bioethanol as an alternative fuel has piqued the interest of biotechnological ethanol production. It's critical to look into the possibilities of using new energy sources that are efficient like oil and that could be utilized instead of or in addition to present fuels and the bioethanol potential from the cellulosic material obtained from the plant *Impatiens tinctoria* A. Rich. tuber was investigated. For the plant *I. tinctoria* tuber, the impacts of several parameters that determine the concentration of bioethanol were examined. Pre-treated lignocellulosic material was treated with 2% diluted H_2SO_4 at 350°C, resulting in a 76.73% w/w carbohydrate concentration. The carbohydrate concentrations were determined by the phenol-sulfuric acid method. The optimized sample was fermented at a pH of 6.0, a reaction temperature of 32.5°C, and a fermentation time of 4 days, yielding a maximum ethanol level of 10.38% v/v as measured by Pycnometer. Under optimum conditions, *I. tinctoria* produced a very promising amount of bioethanol (10.38%), which suggests that it might be used as a lignocellulosic feedstock for bioethanol production instead of food crops.

Keywords: bioethanol, fermentation, lignocellulosic, *Impatiens tinctoria, Saccharomyces cerevisiae*,

Introduction

Many countries, like Ethiopia, currently import a large amount of petroleum products. Benzene is one of the petroleum products that requires a large amount of foreign currency. There is an industry that mixes benzene with ethanol in a plausible proportion to reduce this problem on a national scale. Furthermore, there is a requirement for ethanol at various levels (Benti *et al.,* 2021).

People all over the world are dealing with two major issues: the rapid depletion of fossil fuel reserves and uncontrolled environmental degradation. The possibility of using bioethanol as an alternative fuel has piqued the interest of biotechnological ethanol production. Recent economic and environmental interest has been fueled by price increases and environmental issues caused by fossil fuels. Roots, straws, and fruit peels are among the most abundant lignocellulosic waste materials in the world (Soleimani *et al.,* 2012).

Bioethanol can help us reduce our dependence Bioethanol can help us reduce our dependence on fossil fuels while reducing net emissions of carbon dioxide, the greenhouse gases. Long-term economic and environmental concerns have prompted extensive studies into renewable liquid fuel sources to replace fossil fuels. CO_2 is released when fossil fuels like coal and oil are burned, and this is a key contributor to global warming (Erdei *et al.*, 2010). They burn cleaner than fossil fuels, and the short cycle of growing plants and consuming the fuel they produce does not emit CO_2 (Tekaligne *et al.,* 2015; Kumar et al., 2009). It's critical to look into the possibilities of using new energy sources that are efficient like oil and that could be utilized instead of or in addition to present fuels (Tahir *et al.,* 2010). Bioethanol can be mixed with conventional fuel in amounts as high as 5% without requiring engine modifications (Tekaligne et al., 2015; Talebnia, 2008).

These alternative energy sources should not come from edible sources, as this would increase the cost of these crops, resulting in food insecurity. To address these issues, it is suggested that other agricultural crops such as *I. tinctoria* tuber, which are not used for human or animal use, be researched.

Numerous studies have explored the potential of various plant sources for bioethanol production, contributing to the development of sustainable energy alternatives. The production of bioethanol from a variety of plant materials, including corn (Kumar and Singh, 2019), sugarcane (Cardona *et al.,* 2010), switchgrass (Mariano and Grossmann, 2011), and several types of lignocellulosic biomass sources (Bušić *et al.,* 2018), has been the subject of previous researches.

In order to increase ethanol yield and efficiency, these studies have concentrated on improving fermentation processes, enzymatic hydrolysis methods, and genetic engineering approaches.

However, despite the extensive research in this field, there remains a need to explore novel and underutilized plant sources for bioethanol production. *I. tinctoria* tuber, presents an intriguing potential due to its high carbohydrate content and fast growth characteristics. Moreover, *I. tinctoria* tuber is abundantly available and it is non edible, especially in regions with favorable climatic conditions, making it a promising candidate for bioethanol production (Gizachew, 2023). By focusing on this plant species, we aim to contribute to the diversification of feedstock options for bioethanol production and explore the feasibility of utilizing a previously overlooked resource for sustainable energy generation. This study aims to assess the potential of the species and produce bioethanol on a small scale in the laboratory while studying the effects of different parameters.

Materials and Methods

Materials

Instruments

Alcohol meter (Anton Paar Snap Alcohol Meter, Snap 41), incubator (**Biosan BK-YC80B)**, Drying oven **(Thermo Fisher Scientific Lab Ovens 50-250^{0}C)**, electrical grinder (TOSCANA 1000W), electrical balance (SUN-ACN-320N), UV-Vis spectrometer (NV203 spectrophotometer), fermentation and distillation setup, pycnometer (50 mL, KW 14/23), hydrometer (Araometer nach Dichte fur schwefelsaure Temp. 20^{o}C), digital pH meter **(Adwa Hungary__Jenco 6173)**, heating mantle **(Stuart™ HM2000C)**, and different size Erlenmeyer flask (pyrexed) were the equipment used in this work.

Chemicals

Methylene blue indicator AR grade, Fehling A and Fehling B Fisher schientific, sulfuric acid 98.08%, D (+) – glucose (anhydrous, FUJIFILM Wako), and calcium hydroxide (AR 96%), , sodium hydroxide (1.0N) were the major chemicals used in this study which are mostly obtained from Loba chemicals. Moreover, yeast (*Saccharomyces cerevisiae*) was the biological material that was used in this study to facilitate the fermentation process.

Methods

Collection and Processing of Sample

The plant *I. tinctoria* were collected from the fields of Gayint and Simada towns in South Gondar zone. The plant was collected after identification and authentication of the plant were made by one of the research group members from Biology department. The collected plant material was then transferred to the University of Gondar chemistry department. The sample were freshly washed on running water to remove dust maters.

Feed stock

The primary techniques employed in the production of ethanol from *I. tinctoria* tubers involved several steps to maximize ethanol yield and efficiency. Initially, samples of the tubers were collected, and then they underwent a pre-treatment phase to enhance their susceptibility to hydrolysis. This phase included grinding and milling the tubers to reduce their size, facilitating subsequent processes. Following pre-treatment, the resulting solution containing sugars underwent yeast fermentation to convert the sugars into ethanol. Subsequently, distillation was carried out to separate and concentrate the alcohol. Additionally, hydrolysis was employed to break down the cellulose and hemicellulose molecules into simpler sugars for further characterization. The entire process aimed at optimizing ethanol production from *I. tinctoria* tubers while ensuring efficient utilization of the biomass resource. In the sample preparation phase, 13kg of *I. tinctoria* tuber was manually processed, including size reduction, drying, and grinding. The tubers were meticulously chopped into 3-5 cm pieces with a knife, facilitating drying and grinding procedures conducted in the organic chemistry laboratory at the University of Gondar. These meticulous steps ensured the preparation of high-quality samples for subsequent ethanol production experiments.

The sample was ground with an electronic grinder after drying. The ground mixed sample's maximum particle size is 1-2 mm. The sample with particles bigger than 2 mm was ground repeatedly until all particle sizes were 1-2 mm. Until the following part of the experiment, the sample ware was stored at room temperature. The surface area of the sample was increased by grinding *I. tinctoria* tuber into powder, which improves the contact between hemicellulose and cellulose with dilute acid, reducing cellulose crystallinity (Tekaligne *et al.*, 2015; Onuki, S. et al., 20015).

To increase the yield, a 0.5 % sulfuric acid solution was applied to 50g of each tiny *I. tinctoria* tuber sample in an erlenmeyer flask. For 1 hour, the mixture was heated to a temperature of 125 –130 ^{O}C and a pressure of 25 psi on the heating mantle . The pretreated material was then dried again with at 35°C in an oven before being exposed to further investigation. The pretreatment's goals were to eliminate lignin, reduce cellulose crystallinity, and increase the material's porosity. The Benedict test was used to confirm that the pretreated material was indeed free of lignin. It exhibits a colour change from deep green to red, orange, and yellow (Mishra *et al.,* 2011).

Determination of Moisture Content

The pretreated powder was oven-dried for 48 hours at 60 ^{0}C (to a moisture content of 15%). The oven-drying method was used to determine the moisture content of the samples and weighed. The technique was repeated until the weight remained consistent, indicating that it was free of moisture. After that, the moisture content will be estimated as follows:

$$\% \text{ Moisture content} = \frac{W_1 - W_2}{W_1} X\ 100 \quad (1)$$

Where W_1 was the sample's weight before drying, in grams, and W_2 is the sample's weight after drying, in grams.

The first sample weighed 50 grams before drying; after drying in the oven, the sample weighs 42.5 grams.

The moisture content of the sample is converted to percent using the formula: Moisture content = 50g-42.5g/42.5g/50g X 100 = 15%.

Hydrolysis

Individual sugar molecules can be fermented into alcohol using diluted or concentrated acids to break down the cellulose and hemicellulose polymers in lignocellulosic biomass (Tekaligne *et al.,* 2015; Bušić *et al.,* 2018).

In the triplicated experiment, a quantity of 50g of the pretreated *I. tinctoria* was utilized. The hydrolysis process was carried out for a duration ranging from 2 to 7 days, at various hydrolysis temperatures including room temperature, 27.5, 30, 32.5, 37.5, and 40 ^{O}C. Additionally, acid concentration varied from 0 to 4%, and the biomass concentration was measured in terms of %w/v, with values of 5.30, 6.14, 7.50, 10.00, 13.20, and 15.7.

The dilute acid hydrolysis method was used for the hydrolysis by introducing the substrate to the pretreated material. To prevent acid evaporation owing to heat, the liquid was placed into glass bottles and sealed with corck. The liquid fraction of the hydrolysate samples was filtered collected, and their sugar content was analyzed using the phenol-sulfuric acid method with D (+)-Glucose as a reference.

Determination of carbohydrate concentration by using Phenol-sulfuric acid method

In a hot, acidic medium, glucose is dehydrated to hydroxymethyl furfural. When combined with phenol, this produces a yellow-brown colored product with a maximum absorption wavelength of 490 nm. Among the quantitative assays for carbohydrate measurement, the phenol-sulfuric acid method is the simplest and most dependable. (Krishnaveni *et al.,* 1984). The procedure is straightforward, quick, and practical, and the results are repeatable. The total sugar concentration is measured using spectrophotometry at a wavelength of 490 nm for glucose absorbance, and the quantification is done using a calibration curve with glucose as the standard, and the calculation is done using the equation of linear regression obtained from the calibration curve (Dubois *et al.,* 2008).

Standard and Reagent Solution Preparation

The stock glucose solution was prepared by dissolving 4 grams of glucose in 100 milliliters of distilled water. It's then time to assemble the standard functional solutions. Pipette 1, 2, 3, 4, and 5 milliliter aliquots of stock glucose into a 100 milliliter volumetric flask, then dilute with distilled water to obtain a desired concentration.

The total sugar content was prepared using known concentrations of standard glucose. The standard solutions were made in five concentrations: 0.04, 0.08, 0.12, 0.16, and 0.2 g/ml. These concentrations were made by diluting a stock glucose solution.

In 100 milliliters of water, 5 grams of redistilled (analytical grade) phenol were dissolved. 98.96 mL of 97% sulfuric acid and 1.04 mL of distilled water were combined to make 96% sulfuric acid (Krishnaveni et al., 1984).

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To determine the standard glucose calibration curve, 1 mL of each of the standard solutions was pipetted out and placed into separate test

tube. Subsequently 1 mL of phenol and 5 mL of sulfuric acid were added, the mixture was left for 10 minute at room temperature. After shaking the contents in the test tube for 10 minutes, they were immersed in a water bath at 25–30°C for 20 minutes. Next, the absorbance was measured at 490 nm using a UV-visible spectrophotometer (NV203 Spectrophotometer). The total sugar content of the sample solution was determined using a standard graph.

$$Y=AX+B \quad (2)$$

Y = the sample's absorbance, X = the standard's concentration in g/mL, A is the y-intercept, and B is the slope, and the concentrations of the samples in g/mL are determined from the calibration curve using Equation 3:

$$X = \frac{Y-B}{A} \quad (3)$$

The total sugar content of 50g of samples was determined and reported as gram glucose equivalents (GE) using Equ.4:

$$C = \frac{X * V * D_f}{m} \quad (4)$$

Where C is the amount of sugar content, g/g sample extract, in GE, V is the volume extract, Df is the dilution factor, m is the weight of the sample, X is the concentration of glucose established from the calibration curve (g/mL).

Fermentation

The pH value of the hydrolised solution was altered to a new pH value before introducing the yeast. Baker's yeast (*Saccharomyces cerevisiae*) was used to ferment the hydrolysates obtained from the pretreatment and acid hydrolyzed steam portion of *I. tinctoria*. To accommodate yeast development, the hydrolysates were filtered, and the pH of the fermentation medium was changed to 4, 4.5, 5, 5.5, 6, and 6.5 by adding the needed amount of 4 M NaOH and 2.5 M HCl. Yeast was introduced at a rate of 2, 3, 4, and 5 grams per 100 milliliters.

Fermentation was permitted for 2, 3, 4, and 5 days at room temperature at 27.5, 30, 32.5, 37.5, and 40 degrees Celsius. Using a hydrometer and a pycnometer, the ethanol concentration was calculated based on the density of alcohol distillate at 20°C and expressed in weight percent (w/v) (Igwe, C. *et al.* 2012).

To preserve anaerobic conditions, the flask mouths were firmly sealed with aluminum foil, with an opening to emit CO_2. The opposite end of the outlet was dipped in lime water, which makes the lime water milky, to confirm the release of CO_2. Confirmatory tests with K_2CrO_4 were performed to verify that the distillate was, in fact, ethanol (Mandal and Kathale, 2012).

Distillation

The fractional distillation method was the final step in the *I. tinctoria* tuber trials' ethanol production. It's a technique for separating two liquids with differing boiling points. The temperature of the distillation set up was set at 85^0C for 3 hours. However, numerous distillations are required to attain great purity. It is the process of purification.

Result and discussion

The effect of biomass concentration on hydrolysis

The effect of biomass concentration was investigated at 35^0C for 72 hrs. The highest sugar concentration was obtained at 74.35 % w/w by using the phenol-sulfuric acid method at a 10% biomass concentration. As presented in Table 1 below, the sugar content increased with increasing substrate concentration.

Table 1. Total sugar content (% w/w) determined at various biomass concentrations, 2 % H_2SO_4, 3 days and 35°C

No.	Substrate concentration (%w/v)	Amount of sugar content (%w/w) Phenol sulfuric acid method	Remark
1	5.30	41.41±0.00	
2	6.14	44.54±1.26	
3	7.50	59.33±1.96	
4	10.00	74.35±2.84	Very high
5	13.20	Out of range	Very high
6	15.70	Out of range	Very high

Effect of acid concentration on hydrolysis

Table 2 shows that phenol-sulfuric acid produced the highest sugar content of 76.73 %, utilizing a 2 % acid hydrolysate of *I. tinctoria*, with the lowest yield at 0 % acid concentration (35 %). This demonstrates that 2 % sulfuric acid hydrolysis is more efficient in producing simple sugars than 1, 3, and 4 % sulfuric acid hydrolysis. According to the findings, the amount of sugar gained increases as the acid concentration increases from 0-2 % to 2-4 % and reduces as the acid concentration climbs from 0-2 % to 2-4 %, according to the findings.

Table 2. Total sugar content (%w/w) at different acid concentration (H_2SO_4) hydrolysates of 10 %w/v biomass concentration, three days, and 35°C.

No.	Acid concentration (%v/v)	Amount of sugar content (%w/w) Phenol sulfuric acid method	Remark
1	0	32.22±1.43	Minimum
2	1	68.54±0.97	Average
3	2	76.73±0.07	Maximum
4	3	62.58±1.29	Average
5	4	35.11±2.28	Minimum

Table 3. Ethanol yield at 32.5°C, pH 6.0, 4 g/L yeast extract, and different fermentation time

Day	Specific Gravity		Ethanol yield (%v/v)	
	Hydrometer reading	Pycnometer reading	Hydrometer value	Pycnometer value
2	0.994±0.001	0.9924±0.001	6.42	6.51
3	0.981±0.003	0.9930±0.002	8.47	8.71
4	0.986±0.005	0.9899±0.001	10.16	10.38
5	0.988±0.010	0.9911±0.001	9.83	10.12
6	0.991±0.004	0.9915±0.002	9.24	9.49
7	0.997±0.006	0.994±0.000	8.76	8.92

Fermentation parameters

Numerous variables, including reaction time, temperature, amount of yeast given, and pH, are important during the fermentation process. These variables have a big impact on the bioethanol production process and are necessary to make it work. A pyrometer and a hydrometer were used to measure the density of the experimental data in order to evaluate the influence of these variables. On the basis of these data, the alcoholic content of the bioethanol produced was then computed. This comprehensive approach enables an in-depth understanding of the fermentation process and its results in terms of the production of bioethanol.

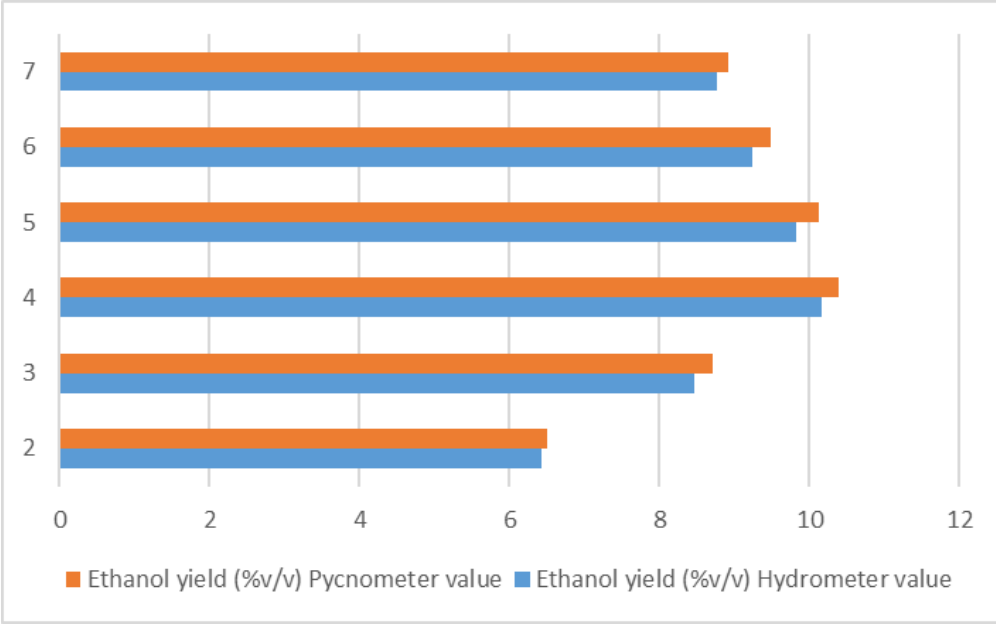

Figure 1. Ethanol yield at 32.5oC, pH 6.0, 4g/L yeast extract and different fermentation time and

The illustration indicates that the pycnometer reading tends to be marginally higher than the hydrometer reading. Despite the observed variance in readings, the disparity between them is not deemed statistically significant.

Discussion

Distillation yields were determined, and the concentration of ethanol (% v/v) was measured using a hydrometer and a pycnometer, taking into account various parameters.

Fermentation time, pH, and temperature are the most important factors in increasing bioethanol output. It was discovered that 35°C and a pH of 6 were the best conditions for obtaining a high bioethanol output. Table 3 and the following chart shows the outcomes of fermentations carried out over different time periods (2, 3, 4, 5, 6, and 7 days).

Fig. 1 and Table 3 showed that after 4 days of fermentation, the highest amounts of ethanol (10.16 and 10.38 %) were obtained from the Hydrometer and Pycnometer measurements, respectively. As the period was extended, the amount of ethanol produced decreased. The

difference between the alcoholic content measured with a Hydrometer and a Pycnometer isn't substantial. The concentration of bioethanol increased as fermentation time increased, then fell as fermentation time increased. According to Table 3 and figure 1 increasing the fermentation days to 6 and 7 days reduces the concentration of bioethanol production to 9.24 and 8.76, respectively, when measured using a hydrometer. After four days of fermentation, the bioethanol yield began to decline. This could be owing to the microorganisms' use of sugar for ethanol generation. It could also be because the hydrolysate contains high quantities of metabolic inhibitors that can prevent fermentation. In comparison to the maximum amount of ethanol obtained from acid hydrolysis of groundnut hulls (6.2 %) and 5.5 percent from rice husks (Ali and Khan, 2014), and the enzymatic fermentation of mango juice (7 - 8.5 %), (Reddy and Reddy, 2015), The amount of ethanol produced by hydrolyzing *I. tinctoria* under optimal conditions (10.16 and 10.38 percent, respectively, according to measurements from a hydrometer and a pyranometer) was extremely good. The results suggest that ethanol produced from *I. tinctoria* may be used in place of other agricultural products such as corn, cassava, and mango juice. *I. tinctoria* can prevent a food disaster by taking the place of food crops in the manufacture of bioethanol because it is not a plant that is edible to humans.

Conclusion

The research indicates that *I. tinctoria* may be a source of sugar or carbohydrates, which is a requirement for the synthesis of bioethanol from plants. The amount of bioethanol that plants can create depends on a number of variables, such as temperature, pH, acidity, and length of fermentation.

The amount of ethanol produced by hydrolyzing *I. tinctoria* under optimal conditions (10.16 and 10.38 percent, respectively, according to measurements from a hydrometer and a pyranometer) was quite satisfactory.

Recommendations

Even though the plant *I. tinctoria* has a higher sugar concentration, the yield of bioethanol is minimal. To achieve a greater yield of bioethanol products there should be optimized conditions related to temperature, pH, acid concentration, and fermentation time. In addition to these, further elemental and impurity analysis should be conducted to determine the quality of bioethanol produced from the plant.

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Conflict of interests

The authors do not have conflict of interest on the publication.

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