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Research Article

PRODUCTION OF BIOETHANOL FROM *IMPATIENS TRINCTORIA* THROUGH MICROBIAL ENZYMATIC HYDROLYSIS

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ABSTRACT

Bioethanol can be produced from starch rich crops and vegetable oils. But these substrates are in limited supply and constitute human food and animal feed. There is growing interest to use cellulosic and lignocellulosic biomass as raw material for bioethanol production. These materials are highly abundant, cheap and ecofriendly compared to other feed stocks. The objective of this study was to produce bioethanol from *Impatiens trinctoria* tuber by hydrolyzing with *Aspergilus niger* and further fermentation with S*accharomyces cerevisiae*. The substrate concentrations of 10, 15 and 20 g; inoculums concentrations of 5, 10 and 15 %; temperature conditions of 25, 30, 35^{o}C; pH conditions of 5, 6 and 7; and incubation periods of 72, 120 and 168 hrs were taken to optimize proper working conditions for *Aspergillus niger*. It was found that the optimum hydrolysis conditions for this fungus were 10 g substrate, 5 % inoculums, pH of 5, incubation period of 72 hrs at 30 ^{0}C. The maximum total sugar, reducing sugar, bioethanol yield and bioethanol concentrations of 5.02 ± 0.07g, 2.28±0.38g, 60.61±.094% and 59.53±2.15% were obtained, respectively in the above conditions. These results indicated that *Impatiens trinctoria* tuber is the potential source for bioethanol production. Further studies are needed to upscale the production of bioethanol from this plant.

Keywords: *Aspergillus niger*, Bioethanol, Fermentation, *Impatiens trinctoria*, Cellulosic, Lignocellulosic, Sugar

INTRODUCTION

Ethanol produced from renewable source and biomass based waste materials are referred as bioethanol. The depletion of fossil fuels, increasing price of petroleum and environmental concerns have initiated people to look for alternative sources of renewable energy (Samsuri *et al*., 2008; Tanaka, 2006). Bioethanol is the cleanest

liquid fuel alternative to fossil fuels (Tanaka, 2006). This product is used as fuel, industrial solvent, cleaning detergent and laboratory solvent. There is an impressive achievement in many parts of the world for the conversion of biomass into bioethanol. Brazil and the United States are the leading bioethanol producers which account for 80% of the total world ethanol production (Entrix, 2010). The Renewable Fuels Association in 2014 had reported that Brazil has produced 16.3 billion liters of bioethanol representing 33.3% of the world's total ethanol production in 2006 and USA had produced 52.6 billion liters of ethanol fuel in 2011 compared with 49.2 billion liters of ethanol produced in 2010 (Baker, 2014). The global ethanol production had increased from 88 billion liters in 2013 to 90.38 billion liters in 2014 (Baker, 2014).

Ethanol is mostly produced from petrochemicals by chemical process. Ethanol can also be produced from starch rich crops, vegetable oils, organic waste and cellulose substrates (Tanaka, 2006). Production of bioethanol from food crops such as sorghum, wheat, maize, sugar cane, cassava and others have raised a serious debate in food security issues especially in developing countries (Ward and Singh, 2002). These substrates are in limited supply and constitute human food and animal feed. There are growing interest to use agricultural by-products of cellulosic and lignocellulosic biomass as raw material for bioethanol production. These materials are highly abundant, cheap, low net CO_2 emission as compared to other feed stocks (Tanaka, 2006; Tomas-Pejo *et al*., 2008). However, these materials have complex structural composition. Therefore, they require physical, chemical and biological pretreatment steps to remove lignin and reduce the crystallinity of cellulose (Sanchez and Cardona, 2008).

Biological pretreatment of cellulosic and lignocellulosic materials involve the use of selective lignin degrading microbes (Itoh *et al*., 2003). These microbes can degrade lignin and hemicelluloses and expose cellulose for hydrolysis (Dashtban *et al.,* 2010). Biological pretreatment is carried out under controlled reaction conditions with few side reactions (Lee, 1997; Samsuri *et al.,* 2008). Biological degradation of cellulosic and lignocellulosic materials is the basic characteristics of some fungal and bacterial species (Lee, 1997; Samsuri *et al*., 2008). These microbes have extracellular oxidative enzymes predominantly lignin peroxidase, manganese peroxidase, laccase and cellulases (Dashtban *et al.,* 2010). Cellulases or glucoside hydrolases (GH) are potential cellulose degrading enzymes which hydrolyze the β - 1, 4 linkages between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Dashtban *et al.*, 2010). The combined action of endoglucanases and exoglucanases enzymes would hydrolyze cellulose to glucose (Zhang *et al*., 2004). A number of fungal species have been reported for their potential to hydrolyze cellulosic and lignocellulosic materials. Among these *Trichoderma species* and *Aspergillus niger* have been most extensively studied for this purpose (Sun and Cheng, 2002). *Aspergillus niger* is known to produce several enzymes for degradation of lignocellulosic material (Jantasila *et al*., 2012).

Impatiens trinctoria is an ornamental plant classified under the family of *Balsaminaceae*. *Impatiens* occurs in tropical and subtropical regions of Africa, Asia, Central America and temperate regions of the Northern hemisphere (Grey-Wilson, 1980; Yuan *et al.*, 2004). This plant is widely found in Eastern and Southern parts of Africa (Yuan *et al.*, 2004). This plant is non-edible, cheap, fast growing plant and doesn't require farming land for its growth. The production of bioethanol from this plant requires liquefaction of the biomass by chemical process, saccharification of liquefied product by hydrolyzing microorganisms and fermentation of glucose to bioethanol using *Saccharomyces cerevisiae*. This yeast is one of the most important microorganisms involved in the fermentation of hydrolyzed substrates to produce bioethanol. *Saccharomyces cerevisiae* is preferred for bioethanol production due to its high ethanol yield, tolerance to ethanol concentration, low accumulation of by-products and good tolerance to substrate concentrations (Lin *et al.*, 2012). The hydrolysis of *Impatiens trinctoria* by *Aspergilus niger* depends on reaction conditions such as substrate concentration, inoculums concentration, temperature, pH and incubation time. Therefore, the objective of this study was to produce bioethanol from *Impatiens trinctoria* tuber by hydrolyzing with *Aspergilus niger* and further fermentation with S*accharomyces cerevisiae.*

MATERIALS AND METHODS

The raw material (*Impatiens trinctoria* L.) was purchased from local Gondar town, Ethiopia. The plant tuber was washed thoroughly to remove dust and debris with tap water and chopped using knife. The chopped sample was dried for 24 hrs at 70^{o}C in incubator in the Department of Biotechnology, University of Gondar in 2013. Then the dried sample was grinded to fine powder using grinder. The fine powder of *Impatiens trinctoria* was sieved by 0.2 mm sieve size, packed in glass beaker and stored in safe area for further use. All experiments were carried out in a completely randomized design in three replications and in triplicates.

Preparation and saccharification of substrate

Preparation of the flour was done according to the method of Ademiluy and Mebpa (2013). An appropriate amount of *Impatiens trinctoria* flour was measured and dissolved in 100 ml of double distilled water in 250 ml screw capped culture bottle. Then all of the culture bottles were supplemented with 0.2 % NH_4Cl as a nitrogen source. These solutions were subjected to saccharification with 3% sulphuric acid at 70 ^{0}C for 90 min by shaking in between to allow uniform reaction and temperature. The solutions were further treated with *Aspergillus niger.* The substrate concentration, temperature, pH and inoculums concentrations for *Aspergillus nige*r were optimized for better hydrolysis of the sample. Saccharification was carried out in stationary methods and the samples were shaken twice for 5 minutes per day.

Fermentation Medium

The hydrolyzed samples were prepared for fermentation process. The samples were autoclaved at 121^{0}C for 30 minutes to facilitate hydrolysis and prevention of contamination. The media

were then cooled and inoculated with 10 % *Saccharomyces cerevisiae* starter culture. The media in screw capped culture bottles were incubated at 30°C and kept at 120 rpm for 168 hrs.

Distillation Process

After fermentation was completed, the distillation process had been carried out using fractional distillation apparatus. The fermented broth was filtered using filter cloth and transferred into round bottom flask and placed on a heating mantle fixed to a distillation column enclosed with running tap water. Another flask was fixed to the other end of distillation column to collect the distillate at 78 ^{0}C and kept for 13 minutes after first drop of the distillate to maintain the uniformity of distillation process.

Optimization of hydrolysis process with *Aspergillus niger*

Amount of the substrate

The optimum amount of substrate was studied by taking a series of screw capped flasks containing 10, 15 and 20 g of *Impatiens trinctoria* flour moistened with an appropriate amount of distilled water in order to contain 80, 85 and 90% (v/w) moisture, respectively. The flasks for each treatment in triplicate were inoculated and incubated at temperature of 30^{o}C for 72 hrs in incubator (Swain *et al.*, 2013; Duhan *et al*., 2013).

Concentrations of the inoculums

The optimum inoculums concentration was studied by taking a series of screwed capped flasks containing 10 g of *Impatiens trinctoria* flour with 5, 10 and 15% *Aspergillus niger* inoculums. The samples were incubated at 30^{o}C for 72 hrs with a pH of 5 for each treatment in triplicate (Duhan *et al*., 2013; Swain *et al.*, 2013).

Optimization of pH

The optimum pH for the hydrolysis of 10 g of *Impatiens trinctoria* flour by 5 % *Aspergillus niger* inoculums were studied at a pH of 5, 6 and 7. Then these solutions for each treatment in triplicates were incubated for 72 hrs at 30^{o}C in shaking incubator (Duhan *et al*., 2013; Swain *et al.*, 2013).

Temperature condition

The optimum temperature for the hydrolysis of 10 g of *Impatiens trinctoria* flour with 5 % *Aspergillus niger* inoculums concentration at a pH of 5 were inoculated and incubated with 25, 30 and 35 °C for 72 hrs for each treatment in triplicates (Duhan *et al*., 2013; Swain *et al.*, 2013).

Time for hydrolysis

The media consisting of 10 g of *Impatiens trinctoria* flour were inoculated with 5 % *Aspergillus niger* inoculums at 30^{o}C, pH of 5 for 72, 120 and 168 hrs for each treatment in triplicates (Duhan *et al*., 2013; Swain *et al.*, 2013).

Determination of different components in the sample

Starch test

This was performed with Iodine test (Colin and Gaultier de Claubry, 1814). The *Impatiens trinctoria* tuber was cut in half at the equator midway and dipped in the iodine potassium iodide solution and soaked for 30 seconds. This dipped sample was taken and rinsed in tap water for 5 seconds. The colour change was noticed for the absence or presence of starch in this sample.

Estimation of Cellulose

The content of the cellulose in the fermenting sample was determined

according to the protocol of Nelson-Somogyi (1952). The anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 ml of concentrated sulfuric acid and stored on ice for 2 hrs before use. After this 0.5 g of the samples were taken in test tubes, mixed well with 3 ml of 80% acetic acid /nitric acid solution (10: 1 ratio) and placed in water bath at 100^{o}C for 30 minutes. Then the samples were centrifuged for 15 minutes while the residue was washed twice with double distilled water and mixed with 10 ml of 67% H_2SO_4 and kept for one hour. From this, 1 ml of the solution was diluted to 100 ml with double distilled water. Then, 1 ml of the diluted solution was taken and mixed with 10 ml of anthrone reagent. The mixture was boiled again for 10 minutes. Finally, the solution was cooled and its absorbance was measured at 630 nm using anthrone and double distilled water as a blank. Cellulose standard curve was used to determine the cellulose content in a given sample. The cellulose content was calculated from 0.5 g sample taken = (mg of cellulose/volume of test sample) X 100.

Estimation of total sugar

The content of the total sugar in the hydrolyzed sample was determined according to the protocol of Nelson-Somogyi (1952). The total sugar was determined by mixing 0.1 g of the samples with 5 ml of 2.5 N HCl in test tubes. The mixture was placed in water bath at 100^{o}C for 3 hrs. Then the boiled samples were cooled to room temperature and neutralized by solid sodium carbonate until it forms foaming. The volume was made up 100 ml with distilled water and centrifuged at 4000 rpm for 15 min. From the supernatant 0.5 ml of aliquots was taken to test tubes and the volume was made 1 ml with distilled water. Then 4 ml of anthrone solution was added and boiled in water bath for 8 min. Finally the boiled samples were cooled and the absorbance was measured at 630 nm. Glucose standard curve was used to determine the total sugar content. The sugar content was calculated from 0.1 g sample taken = (mg of glucose /volume of test sample) X 100.

Estimation of reducing sugar

The content of the reducing sugar in the hydrolyzed sample was determined according to the protocol of Nelson-Somogyi (1952). The alkaline copper tartrate was prepared in the form of solution A and B. Solution A was prepared by dissolving 2.5 g anhydrous sodium carbonate, 2 g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20 g anhydrous sodium sulphate in 80 ml water and finally the volume was made 100 ml. Solution B was prepared by dissolving 15 g copper sulphate in a small volume of distilled water and one drop of sulphuric acid was added and increased the volume to 100 ml. Then 4 ml of solution A and 96 ml of solution B were mixed and stored for use. On the other hand arsenomolybdate reagent was prepared by dissolving 2.5 g ammonium molybdate in 45 ml water and mixed well with 2.5 ml of sulphuric acid. Then 0.3 g of disodium hydrogen arsenate was dissolved in 25 ml of water. Then the solution was mixed well by shaking and incubated at 37°C for 24 hrs. Analysis of reducing sugar in the sample was done by taking 0.1 g of the sample into test tubes and mixed with 5 ml of 80% ethanol. The sample was boiled in water bath for 20 minutes to extract sugar.

This procedure was repeated to extract the whole sugar. Then the supernatants were collected and evaporated at 80 ^{O}C in incubator. Then the dried sugar was dissolved by adding 10 ml of double distilled water. From this, 0.1 ml of dissolved sugar was pipetted out to separate test tubes and the volume was made up 2 ml by adding double distilled water. Additionally 2 ml of double distilled water was pipetted into separated test tube to set a blank. Then 1 ml of alkaline copper tartrate was added to all test tubes and boiled for 10 minutes. The boiled tubes were cooled and 1 ml of arsenomolybdic acid reagent was added to each tube while the volume was increased to 10 ml by adding double distilled water. Finally the absorbance of blue color was read at 620 nm after 10 minutes. The amount of reducing sugar in the sample was determined based on glucose standard curve used in this experiment. The amount of reducing sugar from 0.1 g sample taken = (mg of glucose /volume of test sample) X100.

Determination of bioethanol

The presence of bioethanol was determined following methods of Caputi *et al*. (1959). The test was done by taking 2 ml distillate samples, pinch of potassium dichromate and a few drop of H_2SO_4 into test tubes. The presence of bioethanol was determined based on the colour change to green.

Density of bioethanol

The density of bioethanol was determined according to the protocol of Ademiluy and Mepba (2013). Bioethanol was transferred to pre-weighed measuring cylinder using electronic balance. The weight and volume of bioethanol was recorded carefully. Then density was calculated by using the formula: Density (g/ml) = Mass of bioethanol/Volume of bioethanol.

Determination of bioethanol yield

Bioethanol yield was determined according to the protocol of Otulugbu (2012). It was calculated as follows: Percent bioethanol yield = (Actual value/Theoretical value) X 100. The maximum bioethanol yield of cellulosic material is 34.1 ml from 100ml of the fermentation distillate.

Bioethanol concentration

The concentration of bioethanol was determined following Perry's Chemical Engineers' Handbook (Perry *et al*., 1997). This method uses density of bioethanol after distillation and calculates corresponding bioethanol percentage in each of distillates.

Data analysis

All experiments were carried out in a completely randomized design in triplicates. The results were subjected to analysis of variance (one-way ANOVA) using SPSS Ver. 16.0 software. The mean comparisons were done using Duncan method ($p < 0.05$) when the F-test demonstrated significant.

RESULTS

In the present study, the absence of starch in the *Impatiens trinctoria* flour and piece cutting was exhibited by lack of blue black color with iodine test compared with blue black colour for starch rich potato tuber sample. The cellulose test of the *Impatiens trinctoria* tuber showed a maximum content of 966 mg/g. This covers about 96.6 % of the total content of the *Impatiens trinctoria* tuber plant. This result was calculated based on the cellulose standard curve obtained from an equation: Y= 0.042X - 0.021 with R^2= 0.990, where Y represents the

absorbance and X represents the concentration of unknown cellulose present in the sample.

The cellulosic rich substrate of *Impatiens trinctoria* flour was treated by diluted acid and *A. niger* to produce the fermentable sugar. The total sugar in the flour *of Impatiens trinctoria* tuber before acid hydrolysis was found 43.2 mg/g. This shows that the total sugar found in *Impatiens trinctoria* tuber was about 4.32% w/w. The total and reducing sugars of the samples were calculated based on the glucose standard curve obtained from an equation: $Y= 0.056X + 0.010$ with $R^2=0.989$. It is found that the total and reducing sugar contents increased after treatment with dilute sulphuric acid and *A. niger*.

The mean values of the total sugar produced using *Aspergillus niger* culture for 72 hours days did not show significant difference on the substrate concentration used in this experiment. On the other hand the reducing sugar produced from these substrate concentrations showed significant difference ($p \leq 0.05$). The highest total sugar (5.09 ± 0.38 g) and reducing sugar (2.38 ± 0.07 g) were recorded from the *Aspergillus niger* culture grown in the substrate concentration of 10g. These results were followed by the culture of the *Aspergillus niger* on 20g and 15g of substrate, respectively as shown on Table 1. All the samples incubated for a period of 72 hrs by *Aspergillus niger* showed significant difference for bioethanol concentration. The yield of bioethanol produced from different substrate concentrations was not significant. But the highest bioethanol yield (60.61 ± 0.94 %) and bioethanol concentration (59.53 ± 2.52 %) were recorded from the *Aspergillus niger* inoculums cultured in 10 g substrate as shown on Table 1. These results were followed by the bioethanol yield and concentration obtained from 15g and 20g substrates *hydrolyzed by Aspergillus niger* as shown on Table 1.

Table 1: The mean values of different substances obtained from the hydrolysis of different amounts of *Impatiens trinctoria* by *A. niger* and fermentation with *S. cerevisiae.*

Substances obtained	**Substrate concentration (g)**		
	10	**15**	**20**
Reducing sugar (g)	2.38 ± 0.07^b	2.14 ± 0.9^a	2.08 ± 0.17^a
Total sugar (g)	5.09 ± 0.38^a	4.96 ± 0.47^a	4.98 ± 0.31^a
Bioethanol yield (%)	60.61 ± 0.94^a	60.12 ± 1.47^a	59.63 ± 4.47^a
Bioethanol concentration (%)	59.53 ± 2.52^b	42.65 ± 2.15^a	38.82 ± 2.45^a

***** Values with different letters as superscript are significantly different across the row at $p \leq 0.05$.

The mean values of the total sugar and reducing sugar produced from 10g of substrate hydrolyzed with *A. niger* for 72 hrs of hydrolysis were not significantly different ($p \leq 0.05$). The highest total sugar (5.09 ± 0.38g) and reducing sugar (2.38 ± 0.07) were obtained from the inoculums size of 5% *A. niger* culture grown in the *Impatiens*

trinctoria slurry. These results were followed by the production of total sugar from 10% and 15% *A. niger* inoculums concentration for hydrolysis as shown on Table 2. The reducing sugar and total sugar production were slightly decreased as inoculums concentration increased from 5 to 15%. It was found that different inoculums concentration showed significant difference ($p \leq 0.05$) for bioethanol yield and bioethanol concentration. The highest bioethanol yield (60.61 ± 0.94%) and bioethanol concentrations (59.53 ± 2.52) were recorded from the inoculums concentrations of 5 % with 10 g substrate for all inoculums. These results were followed by bioethanol yield and concentrations obtained from 10 % and 15 % *Aspergillus niger* inoculums used for hydrolysis as shown on Table 2.

Table 2: Shows mean values of different substances obtained from the hydrolysis of *Impatiens trinctoria* by different inoculums concentrations of *A. niger* and fermentation with *S. cerevisiae.*

Substances obtained	**Inoculums concentration (%)**		
	5	**10**	**15**
Reducing sugar (g)	2.38 ± 0.07^{b}	1.17 ± 0.15^{a}	1.32 ± 0.15^{a}
Total sugar (g)	5.09 ± 0.38^{a}	2.42 ± 0.50^{a}	2.17 ± 0.15^{a}
Bioethanol yield (%)	60.61 ± 0.94^{c}	57.62 ± 0.85^{b}	51.32 ± 1.53^{a}
Bioethanol concentration (%)	59.53 ± 2.52^{b}	40.22 ± 0.99^{a}	40.16 ± 0.77^{a}

* Values with different letters as superscript are significantly different across the row at $p \leq 0.05$.

The mean values of the total sugar produced using *A. niger* culture for 72 hours of hydrolysis were significantly different ($p \leq 0.05$) to different temperature conditions. The maximum total sugar (5.09 ± 0.38g) and reducing sugar (2.38 ± 0.07) were obtained from the substrate hydrolyzed by *A. niger* culture grown at 30 ^{o} C. This result was followed by production of total sugar from the substrate hydrolyzed by *A. niger* incubated at 25^{o}C and 35^{o}C as shown on Table 3. The maximum bioethanol yield and bioethanol concentration were obtained at the temperature of 30^{o}C. This was followed by bioethanol yield and bioethanol concentration for the substrate hydrolyzed by *Aspergillus niger g*rown at 35^{o}C. Finally, the lowest result was recorded at 25 ^{o} C for both bioethanol concentration and bioethanol yield as shown on Table 3.

Table 3: Shows mean values of different substances obtained from the hydrolysis of *Impatiens trinctoria* by *A. niger* at various temperature conditions and fermentation with *S. cerevisiae*.

Substances obtained	**Temperature in (^{o}C)**		
	25	**30**	**35**
Reducing sugar (g)	1.75 ± 0.37^{a}	2.38 ± 0.07^{c}	2.13 ± 0.4^{b}
Total sugar (g)	4.81 ± 0.18^{a}	5.09 ± 0.38^{b}	4.74 ± 0.27^{a}
Bioethanol yield (%)	42.35 ± 0.85^{b}	60.61 ± 0.94^{c}	36.71 ± 0.09^{a}
Bioethanol concentration (%)	45.19 ± 0.83^{a}	59.53 ± 2.52^{b}	54.64 ± 1.01^{b}

* Values with different letters as superscript are significantly different across the row at $p \leq 0.05$.

In the present study, maximum reducing and total sugars, bioethanol yield and bioethanol concentrations were obtained from *A. niger* inoculums cultured at the pH of 5. The effect of pH has shown significant difference ($p \leq 0.05$) for all the substances produced. These results were followed by the production of these substances from the substrates hydrolyzed by *Aspergillus niger* inoculums cultured at the pH of 6 and 7 as shown on Table 4.

Table 4: Shows mean values of different substances obtained from the hydrolysis of *Impatiens trinctoria* by *A. niger* at various pH conditions and fermentation with *S. cerevisiae.*

Substances obtained	**pH values**		
	5	**6**	**7**
Reducing sugar (g)	2.38 ± 0.07^{c}	2.08 ± 0.08^{b}	1.60 ± 0.05^{a}
Total sugar (g)	5.09 ± 0.38^{c}	4.68 ± 0.10^{b}	3.75 ± 0.10^{a}
Bioethanol yield (%)	60.61 ± 0.94^{c}	56.94 ± 1.12^{b}	53.28 ± 2.23^{a}
Bioethanol concentration (%)	59.53 ± 2.52^{b}	40.56 ± 0.85^{b}	31.14 ± 0.81^{a}

*Values with different letters as superscript are significantly different across the row at $p \leq 0.05$.

The maximum total sugar, reducing sugar, bioethanol yield and bioethanol concentration were produced by the hydrolysis of 10 g of *Impatiens trinctoria* flour by *Aspergillus niger* for 72 hrs. These results were followed by the hydrolysis of 10 g of *Impatiens trinctoria* flour by *Aspergillus niger* for 120 and 168 hrs as shown on Table 5. These results showed that increasing incubation period from 72 hrs to 168 hrs decreased bioethanol concentration and bioethanol yield.

Table 5: Shows mean values of different substances obtained from the hydrolysis of *Impatiens trinctoria* by *A. niger* at various incubation periods and fermentation with *S. cerevisiae.*

Substances obtained	**Incubation period (hours)**		
	72	**120**	**168**
Reducing sugar (g)	2.38 ± 0.07^{c}	2.12 ± 0.19^{b}	1.96 ± 0.31^{a}
Total sugar (g)	5.09 ± 0.38^{c}	4.86 ± 0.06^{b}	4.25 ± 0.04a
Bioethanol yield (%)	60.61 ± 0.94^{c}	51.56 ± 1.53^{a}	50.34 ± 2.23^{a}
Bioethanol concentration (%)	59.53 ± 2.52^{b}	36.53 ± 0.52^{b}	32.35 ± 0.63^{a}

*The values with different letters as superscript are significantly different across the row at $p \leq 0.05$.

DISCUSSION

The absence of starch in the *Impatiens trinctoria* flour and piece cutting was exhibited by lack of blue black color with iodine test compared with blue black colour for starch rich potato tuber sample. This indicated that the sample taken for this study was rich in cellulosic and lignocellulosis materials. The total sugar and reducing sugars content in the sample of *Impatiens trinctoria* increased after treatment with dilute sulphuric acid and *A. niger*. The dilute sulphuric acid was used to break the complex structure of cellulose and lignocellulose and make it favorable for the hydrolysis by cellulase enzyme released from intact cell of *A. niger*.

The highest total sugar and reducing sugar recorded from the *Aspergillus niger* culture grown in the substrate concentration of 10 g. The decrease in the hydrolysis of the substrate with increase in the concentration of the substrate might be due to the increase in the concentration of the substrate may inhibit the activity of the hydrolytic enzymes in *Aspergillus niger*. The sugars obtained by the hydrolysis of cellulosic materials were used for the production of fermentable sugar by *S. cerevisiae* to produce bioethanol. This was achieved by consuming some fermentable sugars obtained from the hydrolysis of the substrate by *Aspergillus niger*. It was also found that the bioethanol yield and concentrations were directly correlated with the contents of the reducing and total sugars. Previous studies showed that the production of bioethanol from wheat straw (Tutt *et al*., 2012), from rice husks and groundnut hulls (Ali *et al*., 2011) using co-culture of *Aspergillus* and *S.cerevisiae.* Oyeleke and Jubrin (2009) reported the ethanol concentration of 67.7% and 63.8% by using *A. niger* and *Z. mobilis* on guinea corn husk and millet husk, respectively. Ademiluy and Mepba (2013) and Manas *et al.* (2013) obtained 70% ethanol yield from the substrate concentration of 10g cassava using yeast and 58.44% ethanol yield from 50g of sweet potato, respectively.

Determination of optimum inoculums concentration was required for proper hydrolysis of *Impatiens trinctoria*.

The highest total sugar (5.09 ± 0.38g) and reducing sugar (2.38 ± 0.07) were obtained from the inoculums size of 5% *A. niger* culture grown in the *Impatiens trinctoria* slurry. Previous study done on potato starch fermentation by co-culture of *Aspergillus niger* and *S. cerevisiae* reported the maximum sugar production at the inoculums concentration of 10% (Abouzied *et al*., 1986). On the other hand, Ademiluy and Mepba (2013) obtained maximum sugar with 6% inoculums concentration. Mohan *et al*. (2013) obtained the maximum reducing sugar at inoculums concentration of 10 % from agricultural wastes. This indicated that optimum inoculums concentration for the hydrolysis was dependent on the type of substrate.

It was found that different inoculums concentration showed significantly different ($p \leq 0.05$) for bioethanol yield and bioethanol concentration. The highest bioethanol yield (60.61 ± 0.94%) and bioethanol concentrations (59.53 ± 2.52) were recorded from the inoculums concentrations of 5 % with 10 g substrate for all inoculums. It was that indicated that bioethanol yield and bioethanol concentrations were directly correlated with the reducing and total sugars obtained by hydrolysis of the substrate with optimum inoculums concentration. Utulugbu (2012) obtained 73.9% bioethanol from sawdust at 5% inoculums concentration. This was consistent with the present study.

The mean values of the total sugar produced using *A. niger* culture for 72 hours of hydrolysis were significantly different ($p \leq 0.05$) to different temperature conditions. In this study the maximum total sugar and reducing sugar were obtained from the substrate hydrolyzed by *A. niger* culture grown at 30^oC. Ademiluy and Mebpa (2013) reported that 30^oC was an optimum temperature for production of sugar from whole cassava flour. Ado *et al*. (2009) also obtained the maximum reducing sugar at the temperature of 30^oC from cassava using *Aspergillus*. Thus, 30^oC is the optimum temperature for the hydrolysis of *Impatiens trinctoria* tuber. The maximum bioethanol yield and bioethanol concentration were obtained at the temperature 30^o C.

In the present study, maximum reducing and total sugars, bioethanol yield and bioethanol concentrations were obtained from *A. niger* inoculums cultured at the pH 5. Similar result was reported by Mohan *et al*. (2013) for maximum total sugar from agricultural wastes and Magdy *et al*. (2011) obtained maximum reducing sugar from the industrial potato at pH 5. Ado *et al*. (2009) reported for maximum bioethanol yield at pH 5 from corn cobs. It indicated that fungi require lower pH conditions for effective hydrolysis. It was also found that the bioethanol yield and bioethanol concentration were directly correlated with the reducing and total sugars obtained by hydrolysis of the substrate with optimum pH conditions and inoculums concentration.

The maximum total sugar, reducing sugar, bioethanol yield and bioethanol concentration were produced by the hydrolysis of 10 g of *Impatiens trinctoria* flour by *Aspergillus niger* for 72 hrs. Similar results were reported by Ado *et al*. (2009) for maximum bioethanol yield from corn cobs; Utulungbu (2012) from sawdust at incubation period of 72 hrs. Magdy *et al*. (2011) also obtained maximum bioethanol concentration from industrial potato wastes at incubation period of 72 hrs. Thus, these

results are consistent with the present study for incubation period of 72 hrs as optimum period for action of *Aspergillus niger on Impatiens trinctoria.*

CONCLUSION

Impatiens trinctoria is cellulosic, non-edible, cheap, fast growing plant. The production of bioethanol from this plant requires liquefaction of the biomass by dilute sulphuric acid, saccharification of liquefied product by hydrolyzing *Aspergillus niger* and fermentation of glucose to bioethanol using *Saccharomyces cerevisiae*. It was found that the optimum working conditions for this fungus to hydrolyze *Impatiens trinctoria* were 10 g substrate concentration, 5% inoculums concentration, temperature conditions of 30^0C, pH of 5 and incubation period of 72 hrs. In these working conditions the maximum total sugar, reducing sugar, bioethanol yield and bioethanol concentrations of 5.02 ± 0.07g, 2.28±0.38g, 60.61±.094% and 59.53±2.15% were obtained, respectively. These results indicated that *Impatiens trinctoria* tuber can serve as a potential source for bioethanol production.

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