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Evaluation of Bioethanol Production from Enset (*Ensete ventricosum* (Welw.) Cheesman) Processing Waste and Leaf Using *Saccharomyces cerevisiae*

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Abstract: The depletion of fossil fuel reserves and the increasing awareness of greenhouse gas emissions act as the primary driving force for finding alternative renewable energy sources, especially those derived from biomass. This study was conducted with the objective of bio-ethanol production from non- edible plant biomass, Enset [Ensete ventricosum (Welw.) Cheesman] processing waste and leaves in batch fermentation using yeas (S. cerevisiae). Forty grams of acid pre-treated or untreated ensete processing waste and leaves were incubated with 1% of yeast under three different temperature (26°C, 30°C and 40°C) treatments and allowed to ferment over 16 days. Yeast cell density, total reducing sugars and percent of ethanol was measured at 4 days interval spectrophotometrically beginning from the incubation period. Results showed that all measured parameters subsequently decrease with increasing days of fermentation in both acid pre-treated and untreated substrates. Acid pre-treated substrates resulted in higher amounts of cell density, total reducing sugars and bio-ethanol than untreated substrates. Among the different temperatures, 30°C produced more bio-ethanol than others throughout the fermentation periods, and the result was in agreement with cell density and total sugars measured. In conclusion, this study showed that Enset processing waste and leaves can be used as a feedstock for bio-ethanol production and the yield can be increased with acid pre-treatment and incubation under 30°C temperature.

Keywords: Batch Fermentation, Cell Biomass, Bio-ethanol, Enset Processing Waste, Ensete Leaf, Reducing Sugar

1. Introduction

The depletion of fossil fuel reserves and the increasing awareness of greenhouse gas emissions act as the primary driving force for finding alternative renewable energy sources [17]. While solar energy, wind energy and hydroelectricity primarily address the generation of electrical power, the production of biofuels from biomass provides a very promising alternative for fossil derived liquid fuels [10]. The major challenge related to the utilization of biomass for biofuels, however, is the availability of the biomass.

The continued use of fossil fuels to meet the majority of the world's energy demand is threatened by increasing concentrations of CO_2 in the atmosphere and concerns over global warming [28]. The heightened awareness of the global warming issue has increased interest in the development of methods to mitigate GHG emissions [28]. Much of the current effort to control such emissions focuses on advancing technologies that: (i) reduce energy consumption, (ii) increase the efficiency of energy conversion or utilization, (iii) switch to lower carbon content fuels, (iv) enhance natural sinks for CO_2, and (v) capture and store CO_2 Reducing use of fossil fuels would considerably reduce the amount of CO_2 produced, as well as reduce the levels of pollutants [10].

Recently, as to address the uncertain fuel supply, in an effort to reduce carbon dioxide emissions and to provide feasible alternative to fossil transport fuel all over the world, bio-ethanol production has attracted the attention of the world [6]. Bio-ethanol is a volatile and flammable liquid produced through microbial fermentation process, which has a molecular formula of C_2H_5OH [13]. Microscopic yeast cells break down the starch and water, creating ethanol and carbon dioxide as end products. Bio-ethanol can be produced from various sources, like starch crops,

sugar crops, household waste, agricultural waste, fruit juices, fruit wastes etc. Among these sources, non-edible source seems to be the best options [7]. Sufficient use of bio-ethanol as an energy source for transportation or in industries can considerably reduce the nauseating greenhouse gas emissions from transport and industries emission [3]. More job opportunities can be created through bio-ethanol production as well as economic income and energy security [9]. To prevent fuel food crisis lignocelluloses biomass, particularly agricultural residues are converted to useful products such as bio-ethanol [10]. Lignocelluloses biomass consists mainly of lignin, cellulose and hemicelluloses that are present in a different percentage according to the plant type and its parts. It was reported that, the plant leaves contain 15-20% cellulose, 80-85% of hemicelluloses and 0% of lignin [15]. Presently, more researches are focused on non-edible biomass due to their availability and low cost in procurement [16].

Enset [*Ensete ventricosum* (Welw.) Cheesman] is a perennial, herbaceous monocot in the family Musaceae. It resembles the banana plant and is often referred to as 'false banana'. The plant is domesticated only in Ethiopia and is grown in the south and south-western parts of the country serving as a staple/co-staple food for an estimated 10–12 million people [25]. It was reported that Enset starch demonstrated a number of similar physico-chemical properties and comparable binding and disintegrating properties in granulated tablet formulations with potato starch [26]. The purpose of this study was to evaluate the quantity bio-ethanol produced from *Ensete ventricosum* processing waste and leaf in batch fermentation using *S. cerevisiae.*

General objective of the study was:

To evaluate the quantity bio-ethanol produced from *Ensete ventricosum* processing waste and leaf in batch fermentation using *S. cerevisiae.*

Specific objectives of the study were:

1) To evaluate the amount of bio-ethanol production on each period of the fermentation.
2) To evaluate the effects of varying temperatures on the amount of bio-ethanol production, cell density and amount of reducing sugar
3) To determine the effect of acid pre-treatment on the amount of bio-ethanol production, cell density and amount of reducing sugar
4) To determine the effect of fermentation period on the amount of bio-ethanol production, cell density and amount of reducing sugar

2. Materials and Methods

2.1. Description of the Study Area

This study was conducted at Wachemo University, Biotechnology department, plant tissue culture and molecular Biology Laboratory which is found at Hossana Town. Hossana town is located south west of Addis Ababa at a distance of 232 km. The absolute geographic location of Hossana is from 7° North latitude and from 37° East longitudes and altitude of 2722m.a.s.1 [14].

2.2. Sample Collection and Preparation

Enset [*Ensete ventricosum* (Welw.) Cheesman] processing waste and leaf sample was collected from southern nations nationalities and peoples region, Hadiya zone, Hosana and the leaf was cleaned and washed with distilled water vigorously prior to bringing to the laboratory. Enset processing waste and leaf sample was chopped, wiped with cotton dipped in ethanol to clean the surface and dried in oven at 60 ± 3℃ for 24 hrs prior to pretreatment and then was mixed and ground into a fine powder.

2.3. Experimental Design and Fermentation

The experimental design was based on CRD factorial with three replications. Two types of substrates (40gm), i.e., acid pre-treated and untreated Enset processing waste and leaves were evaluated for bio-ethanol production in different levels of temperatures. Acid pre-treatment was done by adding 10ml of 1% H_2SO_4 solution and heated for 15 minutes at temperature of 121°C and pressure of 3 bars. After pre-treatment, the samples were cooled to a temperature below 50°C and pH of the samples was neutralized with K_2CO_3 to pH range of 4.5–5. The pre-treated and untreated substrates were then separately put in 1000ml fermenter together with 1% inoculums (baker's yeast). Inoculums were prepared from dried baker's yeast, (*Saccharomyces cerevisiae*) purchased from market. For this, 1g of yeast was dissolved in 100ml of distilled water [11]. After inoculation, both acid pre-treated and untreated substrates were incubated under different temperature levels (26, 30 and 40°C) for 16 days. The pH of the culture was then adjusted to 4.5—5 with buffer solution. Nutrient supplements prepared by mixing 0.1 g KH_2PO_4, 0.5 g $CaCl_2$, 0.5 g $MgSO_4$, 0.1 g Na_2SO_4 and 0.1 g $(NH_4)_2SO_4$ per liter of distilled water was also added into the sample [1]. The production of bio-ethanol and other parameters were estimated and compared in both pre-treated and untreated substrate at the interval of four days starting from the beginning of the fermentation up to 16^{th} day.

2.4. Determination of Cell Density (Biomass)

Cell density was measured at the 4^{th}, 8^{th}, 12^{th} and 16^{th} day of fermentation using spectrophotometer (Humas Think HS 3300, Korea) at 600 nm absorbance [23]. Dry weight method of cell measurement was used. The cell in the 2ml of the broth sample was separated by centrifugation and the wet weight of the culture was measured immediately and allowed to dry in oven at 100°C for six hour. The difference in weight was calculated and expressed as dry weight in mg /ml. Then the sample was diluted with 20ml distilled water and its absorbance was measured with spectrophotometer at 600 nm. The calibration curve to relate the absorbance with cell dry weight was then generated using baker's yeast.

2.5. Estimation of Total Reducing Sugar

The reducing sugar content of the sample of fermentation

broth was estimated by the method of using D-glucose as standard [19, 22]. Analysis of reducing sugar in untreated & pre-treated samples were determined by using 3, 5-dinitrosalicylic acid (DNS) reagent [18]. A sample (0.05ml) was taken from the filtrate and 0.35ml citrate buffer (pH=6.5) and 0.6ml of Dinitrosalicylic acid (DNS) was added to it. The sample was then boiled for 5 minutes immediately to stop the reaction. The absorbance was measured for reducing sugar at 540nm using spectrophotometer [4]. The amount of reducing sugar in the sample was calculated using standard graph prepared from working standard Glucose [20].

2.6. Qualitative Determination of Bio-ethanol

Presence of alcohol in the distillate was checked by functional group classification test [5]. For this, 1 drop of the unknown sample was added to 1 ml of reagent-grade acetone in a test tube. Then, a drop of the chromic acid/sulfuric acid reagent was directly added into the solution and the mixture was shaken. A reduction of primary or secondary alcohol from orange-red chromic acid/sulfuric acid reagent to an opaque green or blue suspension of Cr (III) salts in 2–5s confirmed the presence of alcohol.

2.7. Quantitative Estimation of Bio-ethanol

One ml of the fermented sample was taken into 500 ml Pyrex distillation flask containing 30 ml of distilled water and then distilled. The distillate was collected in 50 ml flask containing 25 ml of potassium dichromate solution (33.76 g of $K_2Cr_2O_7$ dissolved in 400 ml of distilled water with 325 ml of sulphuric acid and volume raised to 1 liter). About 20 ml of distillate was then collected in each sample and the flasks were kept in a water bath maintained at 60°C for 20 minute. The flasks were cooled to room temperature and the volume was raised to 50 ml. Five ml of this was diluted with 5 ml of distilled water for measuring the optical density at 600 nm using spectrophotometer [8]. A standard curve was prepared under similar set of conditions by using standard solution of ethanol containing 0 to 20% (v/v) ethanol in distilled water and then ethanol content of each sample was estimated [27].

2.8. Data Analysis

The data were analyzed using (SPSS, version 17). Least significant difference (LSD) test was used to identify significant differences among treatment means. P values < 0.05 were considered significant in all cases.

3. Results and Discussion

3.1. Determination of Cell Density (Biomass)

Acid pre-treatment, fermentation time and temperatures had significant effect on cell density (Table 1). In both acid treated and untreated substrates, cell density was found to decrease subsequently with increasing days of fermentation. This may be due to high alcohol content and decrease in fermentable sugar. Compared to acid-untreated substrate, treated substrates showed higher cell density. This shows that acid treatment of substrates facilitates the breakdown of the complex lignocellulosic materials into simple structures that yeasts can consume and reproduce well. As compared to the rest of temperature treatments of this experiment, incubation of the substrate under 30℃ yielded in higher amount of cell density, suggesting this temperature level is optimal for yeast reproduction compared to other temperature levels used (Table 1). The increasing bio-ethanol production with increasing cell biomass indicated that the amount of yeast influenced ethanol production [2].

Table 11. *Cell density (mg/ml) observed at 600nm in different fermentation period. (Values are mean± standard deviation (SD), n=3).*

Temperature	Treatment	Fermentation Period			
		4th day	8th day	12th day	16th day
26℃	Acid Treated	7.44 ± 0.19^{Ba}	7.00 ± 0.12^{Cb}	6.22 ± 0.26^{Ac}	3.31 ± 0.08^{Cd}
	Untreated	6.19 ± 0.12^{Ea}	5.70 ± 0.12^{Eb}	4.30 ± 0.06^{Ec}	3.04 ± 0.18^{Dd}
30℃	Acid Treated	8.66 ± 0.04^{Aa}	7.68 ± 0.03^{Ab}	5.91 ± 0.02^{Bc}	3.79 ± 0.04^{Ad}
	Untreated	7.36 ± 0.15^{Ca}	7.16 ± 0.07^{Bb}	4.89 ± 0.06^{Cc}	3.63 ± 0.07^{Bd}
40℃	Acid Treated	6.34 ± 0.13^{Da}	5.11 ± 0.07^{Fb}	3.84 ± 0.13^{Fc}	2.76 ± 0.07^{Fd}
	Untreated	5.79 ± 0.03^{Fa}	5.73 ± 0.05^{Db}	4.58 ± 0.08^{Dc}	2.81 ± 0.07^{Ed}

Means followed by different small letters in row are significant at P<0.05. Means followed by different capital letter in column are significantly different at P<0.05.

3.2. Quantitative Estimation of Total Reducing Sugar

Similar to cell density results, acid pre-treatment, fermentation time and temperatures had significant effect on the amount of total reducing sugar (Table 2). In both acid treated and untreated substrates, total reducing sugar was found to decrease subsequently with increasing days of fermentation. This may be due to bio-conversion of sugars into ethanol. Compared to acid-untreated substrates, treated substrates showed higher total reducing sugar. This shows that acid treatment of substrates facilitates the breakdown of the complex lignocellulosic materials into sugars that yeasts can consume and convert them into bio-ethanol. As compared to the rest of temperature treatments of this experiment, incubation of the substrate under 30°C resulted in higher amount of reducing sugar, suggesting this temperature level facilitates the conversion of the complex lignocellulosic materials into sugars, and is optimal for yeast to reproduce and consume more sugars for conversion into ethanol (Table 2).

Table 22. *Amount of reducing sugar measured at 540nm (mg/ml) from fermented sample through fermentation period. (Values are Mean ± standard deviation (SD), n=3).*

Temperature	Treatment	Fermentation Period			
		4th day	8th day	12th day	16th day
26℃	Acid Treated	0.16 ± 0.01^{Ca}	0.15 ± 0.01^{Bb}	0.11 ± 0.01^{Cc}	0.10 ± 0.01^{Bd}
	Untreated	0.13 ± 0.20^{Ea}	0.11 ± 0.02^{Eb}	0.10 ± 0.17^{Dc}	0.08 ± 0.01^{Cd}
30℃	Acid Treated	0.38 ± 0.20^{Aa}	0.33 ± 0.02^{Ab}	0.28 ± 0.17^{Ac}	0.16 ± 0.16^{Ad}
	Untreated	0.16 ± 0.01^{Ca}	0.13 ± 0.01^{Cb}	0.10 ± 0.01^{Cc}	0.08 ± 0.01^{Cd}
40℃	Acid Treated	0.20 ± 0.04^{Ba}	0.15 ± 0.03^{Bb}	0.12 ± 0.03^{Bc}	0.10 ± 0.01^{Bd}
	Untreated	0.14 ± 0.02^{Da}	0.13 ± 0.01^{Cb}	0.12 ± 0.01^{Bc}	0.10 ± 0.01^{Bd}

Means followed by different small letters in row are significant at P<0.05. Means followed by different capital letter in column are significantly different at P<0.05.

The trend of reducing sugar content was in line with bio-ethanol production, suggesting that acid pre-treatment facilitated conversion of complex carbohydrates in to simple sugars that would eventually be converted into bio-ethanol. Current result showed the concentration of reducing sugar decreased consistently through fermentation period. This may be due to rapid conversion of reducing sugar into bio-ethanol; the maximum concentration of bio-ethanol was achieved; showing the consumed sugar was converted to bio-ethanol. The current study is similar with that of [24] who reported that maximum bio-ethanol concentration was obtained at 4th day with high concentration of reducing sugar in submerged shake –flask fermentation of Mahula flowers with high reducing sugar concentration.

3.3. Qualitative Determination of Bio-ethanol

The presence of alcohol in the distillate was checked by functional group classification test by using chromic acid in the presence of sulfuric acid. Upon treatment with chromic acid, alcohol functional group is expected to be oxidized while Cr (VI) is being reduced to the blue-green Cr (III) and change its color into blue-green color. In this experiment the same color change occurred, suggesting that ethanol is really produced in the course of incubation [8].

Table 33. *Quantitative estimation of bio-ethanol produced through fermentation period. (Values are mean± standard deviation (SD), n=3).*

Temperature	treatment	Fermentation Period			
		4th day	8th day	12th day	16th day
26℃	Acid Treated	12.45 ± 0.04^{Ea}	9.75 ± 0.04^{Db}	7.94 ± 0.07^{Cc}	5.42 ± 0.24^{Dd}
		10.10 ± 0.09^{Fa}	8.38 ± 0.06^{Eb}	7.72 ± 0.04^{Fc}	2.82 ± 0.07^{Fd}
30℃	Acid Treated	18.92 ± 0.09^{Aa}	15.54 ± 0.04^{Ab}	11.57 ± 0.09^{Ac}	7.38 ± 0.04^{Ad}
		15.81 ± 0.08^{Ba}	10.76 ± 0.04^{Bb}	7.79 ± 0.08^{Dc}	5.86 ± 0.08^{Bd}
40℃	Acid Treated	13.21 ± 0.09^{Ca}	10.02 ± 0.05^{Cb}	7.74 ± 0.07^{Ec}	5.51 ± 0.03^{Cd}
		12.57 ± 0.02^{Da}	10.02 ± 0.03^{Cb}	8.16 ± 0.09^{Bc}	5.15 ± 0.06^{Ed}

Means followed by different small letters in row are significant at P<0.05. Means followed by different capital letter in column are significantly different at P<0.05.

3.4. Quantitative Estimation of Bio-ethanol

Consistent with cell density and total reducing sugars results, the amount of bio-ethanol produced was influenced by acid pre-treatment, fermentation time and temperatures (Table 3). In both acid treated and untreated substrates, the amount of bio-ethanol was found to decrease subsequently with increasing days of fermentation. This may be due to the less amount of cell biomass and total reducing sugars observed with increasing time of fermentation. Compared to acid-untreated substrate, treated substrates showed higher amount of bio-ethanol. This shows that acid treatment of substrates facilitates the breakdown of the complex lignocellulosic materials into sugars that yeasts can consume and convert them into bio-ethanol. [21] Reported that acid pretreatment makes the structure of the substrate less complex, and it becomes more accessible to the enzyme, and hence, more reducing sugars are released. [12] Also reported that dilute sulfuric acid pre-treatment can result in high reaction rates and significantly improve cellulose hydrolysis. As compared to the rest of temperature treatments of this experiment, incubation of the substrate under 30°C resulted in higher amount of bio-ethanol, suggesting this temperature level is optimal for yeast to reproduce and consume more sugars for conversion into ethanol (Table 3). The overall trend in the results of bio-ethanol production was agreed with that of cell density and total reducing sugars measured.

4. Summary, Conclusion and Recommendations

4.1. Summary

Bio-ethanol is a volatile and flammable liquid produced through microbial fermentation process which is derived from cellulosic and lignocellulosic (non- edible) plant biomass. The depletion of fossil fuel reserves and the increasing awareness of greenhouse gas emissions act as the primary driving force for finding alternative renewable energy sources, especially those derived from plant biomass. This study was conducted with the objective of bio-ethanol production from Enset

[*Ensete ventricosum* (Welw.) Cheesman] processing waste and leaves using baker's yeast (*S. cerevisiae*) at different temperatures (26℃, 30℃ and 40℃) for 16 days in batch fermentation.

Acid pre-treatment, fermentation time and temperatures had significant effect on cell density, reducing sugar and bio-ethanol production. The comparison between acid-treated and untreated substrate, acid pre-treated showed the highest cell biomass concentration on 4th day at temperature 30℃. The reducing sugar concentrations were analyzed for optimum temperature in acid-treated and untreated substrates at different temperatures. In both acid treated and untreated substrates, total reducing sugar was found to decrease subsequently with increasing the days of fermentation. As compared to the rest of temperature treatments of this experiment, incubation of the substrate under 30°C resulted in higher amount of reducing sugar on 4th day in acid treated.

The color change occurred during confirmation test of the presence of alcohol in the distillate was blue-green color, positive test, suggesting that ethanol is really produced. Consistent with cell density and total reducing sugars results, the amount of bio-ethanol produced was influenced by acid pre-treatment, fermentation time and temperatures. In both acid treated and untreated substrates, the amount of bio-ethanol was found to decrease subsequently with increasing days of fermentation. Compared to acid-untreated substrate, treated substrates showed higher amount of bio-ethanol. As compared to the rest of temperature treatments of this experiment, incubation of the substrate under 30°C resulted in higher amount of bio-ethanol. Moreover, on 4th day of fermentation period, in consistence with higher cell density and high concentration of reducing sugar, maximum bio-ethanol production was measured in acid-treated substrate at temperature, 30℃. The overall trend in the results of bio-ethanol production was agreed with that of cell density and total reducing sugars measured.

4.2. Conclusion

The study was conducted to evaluate the effects of varying temperatures, acid pre-treatment and fermentation period on the amount of bio-ethanol production, cell density and amount of reducing sugar from Enset processing waste and leaves using *Saccharomyces cerevisiae*. The fermentation was carried out at different temperatures to obtain optimum temperature for maximum cell biomass, higher reducing sugar concentration and maximum bio-ethanol production. Among the different temperatures, 30 ℃ showed the higher cell density, higher reducing sugar concentration and highest percentage of bio-ethanol production in acid-treated substrate throughout fermentation period. Moreover, on 4th day of fermentation period, in consistence with maximum bio-ethanol production, higher cell density and high concentration of reducing sugar were measured in acid-treated substrate at temperature, 30℃. The overall trend in the results of bio-ethanol production was agreed with that of cell density and total reducing sugars measured. In conclusion, this study showed that Enset processing waste and leaves can be used as a feedstock for bio-ethanol production and the yield can be increased with acid pre-treatment and incubation under 30°C temperature.

4.3. Recommendations

1) The study revealed that it is possible to produce bio-ethanol from Enset processing waste and leaves by using *Saccharomyces cerevisiae*, it is recommended to use other microbes those directly degrade cellulosic plant biomass, because the yeast cell is not starch degrading microbe.
2) Further study should be very important to describe how absolute bio-ethanol can be produced from Enset processing waste and leaves by using rotary evaporator, because it is difficult to make pure ethanol since there are other chemicals that can evaporate below the boiling point of ethanol (78℃).
3) Further investigation should be done to analyze the potential of bio-ethanol production from Enset processing waste and leaves using combination of cellulosic enzyme for the purpose of commercialization.

Appendix

Table 44. *Standard curve for determination of cell density.*

Cell density mg/ml	absorbance at 600nm
1.25	0.08
2.5	0.14
5	0.46
10	0.88

Standard stock solution was prepared by taking 1gm of baker's yeast (*S. cerevisiae)* dissolved in 100ml of distilled water with appropriate serial dilution having dilution factor 0.5.

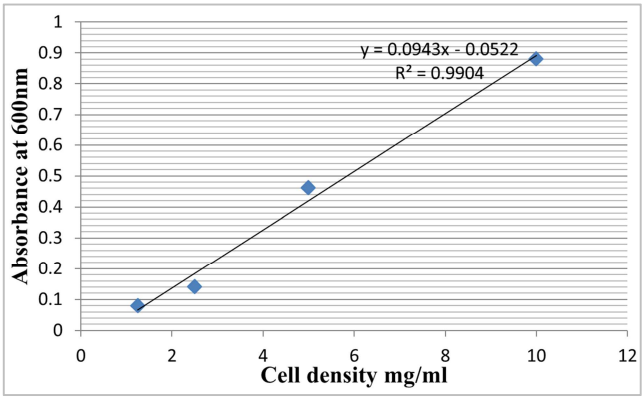

Figure 11. *Standard curve for yeast cell at 600nm in mg/ml.*

Table 55. *Standard curve for determination of glucose concentration.*

Glucose concentration	Absorbance at 540nm
0.2	0.12
0.4	0.23
0.6	0.38
0.8	0.48
1.00	0.66

Standard stock solution was prepared by dissolving 1gm of D-glucose in 100 ml of distilled water and working standard was prepared by diluting 10 ml of stock solution to 100 ml with

distilled water. Standard curve was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard glucose solution and the volume was made up to 3 ml by adding distilled water.

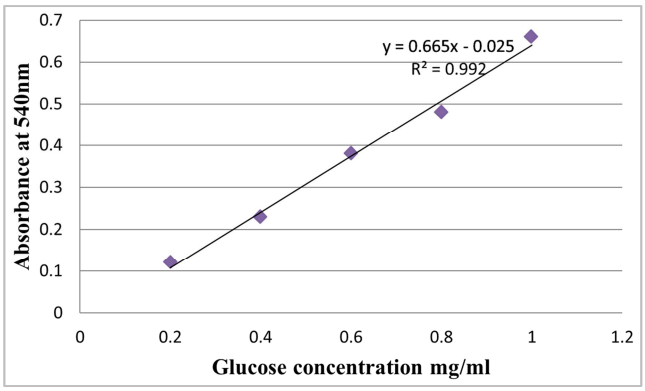

Figure 22. *Standard curve for determination D-glucose concentration at 540nm.*

Table 66. *Standard of ethanol for determination of bio-ethanol content.*

Ethanol concentration (%)	Absorbance at 600nm
0%	0.00
5%	0.36
10%	0.68
15%	0.74
20%	1.02

Ethanol standards were prepared by using ethanol-water solution in the range of 0 – 20% ethanol (v/v). Standard curve was prepared by taking 1 ml of each concentration of the standard solution [0-20% (v/v)] in a 100 ml volumetric flask containing 25 ml of potassium dichromate solution. The samples were heated at 60℃ for 20 minutes in a water bath and then cooled and diluted to 50 ml with distilled water; then the absorbance was recorded at 600 nm.

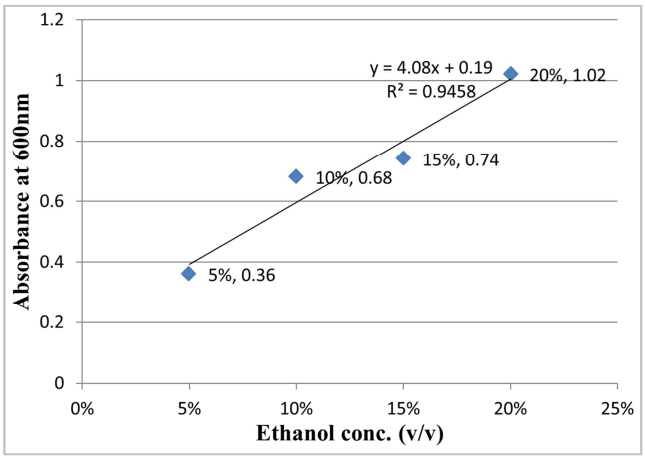

Figure 33. *Standard curve for estimation of ethanol content at 600nm.*

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