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Bioethanol production from sequential acidic-alkaline pretreated sorghum straw hydrolysate and investigation of the effects of fermentation parameters using response surface methodology

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ABSTRACT

The depletion and environmental problems associated with fossil fuels have encouraged us to look for alternative feedstock that do not compromise food security and the environment. Sorghum is a fast-growing crop that can be harvested twice a year and produces both food and straw that can be utilized in the production of bio-based fuels. In this study, the production of bioethanol and the effects of fermentation parameters on ethanol yield are presented. A sequential pretreatment method was employed, using dilute sulfuric acid (1%) at 125 °C in the first stage and dilute sodium hydroxide (1.25 %) at 90 °C in the second stage. The residues left after the sequential pretreatment stage were hydrolyzed using acid hydrolysis. The sugar concentration of the hydrolysates was determined using the phenol sulfuric acid method, and three hydrolysates having sugar levels of 30.42 g/L, 31.79 g/L, and 32.9875 g/L were selected for fermentation. The ethanol yield was determined after 72 hours of fermentation at 30 °C with varying inoculum sizes (5%, 10%, and 15%) and pH (4.5, 5, and 5.5). With a maximum ethanol yield of 0.617 mL/g (48.742%) produced at a sugar content of 32.9875 g/L, pH of 5, and inoculum size of 15%, statistical analysis showed that all three independent parameters affected ethanol yield. According to these findings, while raising sugar content, inoculum amount, and pH all initially result in higher ethanol yields, doing

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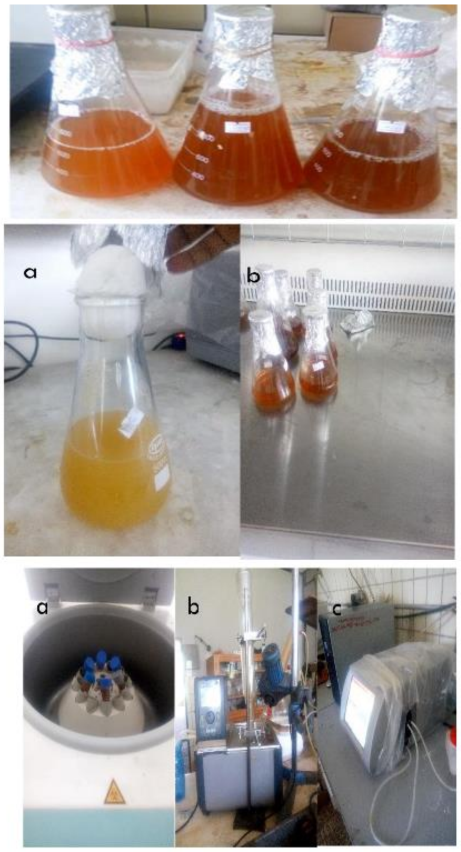
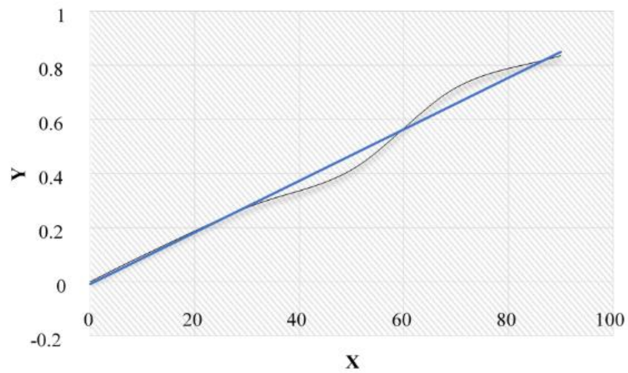
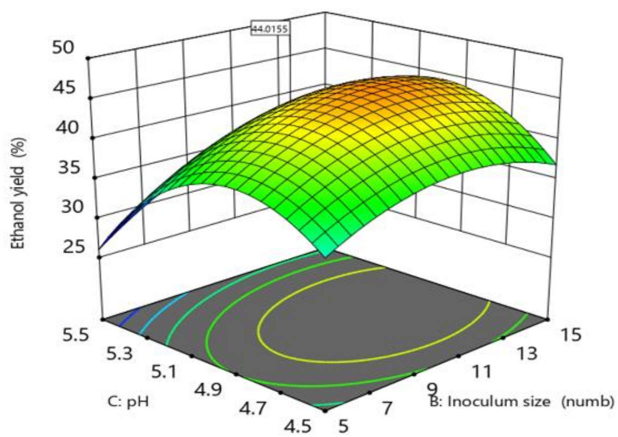
so further reduces yield. So, in order to increase ethanol yield, fermentation conditions must be carefully managed while producing ethanol from sequential acidic-alkaline pretreated sorghum straw. The strategy followed by using sequential acidic-alkaline pretreatment of sorghum straw provides prospects for efficient and effective production of biofuels from alternative feedstock.

Key words: *Yield, Fermentation, Sequential Pretreatment, Sorghum Straw*

GRAPHICAL ABSTRACT







Highlight

- ✓ Bioethanol was produced by using sequential acid-alkali treatment.
- ✓ Hydrolysates were fermented under various circumstances.
- ✓ While raising sugar content, inoculum level, and pH all initially result in higher ethanol yields, doing so further reduces output.

INTRODUCTION

The chemical industry has grown in size and technological maturity through the centuries and has come under pressure to be more sustainable. One of the emerging trends observed in the 21st century is the shift from using non-renewable fossil resources to renewable ones for producing chemicals and materials. Awareness of the level of environmental sustainability has globally spread throughout various industries, and with this paradigm shift, the use of biomass as feedstock for producing fuels and chemicals is perceived as a means of "going green" (Khoo et al., 2016).

Lignocellulose biomasses are considered as a potential sustainable and renewable resource for the production of bio-chemicals due to their global abundance (Khoo et al., 2016). These materials consist of three major components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are structural carbohydrates that can be depolymerized through hydrolysis for bio based chemicals production, while lignin is a complex aromatic polymer which forms a crust surrounding the carbohydrate fraction and performs as a barrier limiting the accessibility of carbohydrates to hydrolytic enzymes or chemicals. There are extensive interactions among these three components, which further improve the stiffness of biomass structure (Xu, 2010). Lignocellulose biomass consists of 35–50 % cellulose, 20–35 % hemicellulose, and 10–25 % lignin. Proteins, oils, and ash make up the remaining fraction (Isikgor & Becer, 2015).

Nowadays, many chemicals are produced from lignocellulosic biomass and ethanol is one of them. Ethanol has wide range of applications due to its renewable nature, ease of storage, the fact that it is free of sulfur, and its less contribution to global warming and air pollution (Rorke & Kana, 2017). Ethanol is commercially produced from edible feedstocks such as corn, maize, sorghum, barley etc. Use of these feedstocks competes with food security. Hence, using non-edible agricultural products can solve both ethanol supply and food security problems.

Huge volumes of cellulosic materials such as straws, stalks, peels etc. are generated as waste from various agricultural activities. These materials can be potential sources for bio based commodities (Adelabu et al., 2018). For example, Sorghum is one of the most cultivated crops in Ethiopia and huge amount of its straw is produced every year. This straw can be a potential source of ethanol as it has high amounts of cellulose and hemicellulose which can be broken down to simple sugars and ultimately fermented to ethanol.

Production of ethanol from lignocellulosic biomass involves four basic steps namely (pretreatment, hydrolysis, fermentation, and purification). Pretreatment is the rate-limiting step for bioethanol production from lignocellulosic biomass, and subsequently intensive studies have been undertaken to improve the pretreatment efficiency. However, so far, most pretreatment methods failed to achieve desirable sugar recovery from both cellulose and hemicellulose in the biomass, which is essential to improve process economics and competitiveness of bioethanol.

Previous researches on bioethanol production employed either single stage acidic or single stage alkaline pretreatment. Acid catalysts, such as sulfuric acid, are less efficient in lignin removal while alkaline catalysts like lime are less effective in hemicellulose degradation. To address the issue, several researches were focused on sequential acidic-alkaline pretreatment. Sequential sulfuric acid-lime pretreatment was used for pretreatment of miscanthus (Guo et al., 2013). But lime pretreatment had its limitations in the two-stage pretreatment. Lime was less soluble in water at high temperatures and lead to inefficient delignification.

Sequential acid alkaline pretreatment of rice straw using sulfuric acid and sodium hydroxide was conducted in 2014. The enzyme Accellerase®1500 was employed for hydrolysis of the pretreated residue. The sequential acid alkaline pretreatment gave a good sugar yield compared to the single acid and base pretreatments, reduces chemical usage, and enzyme dose (Weerasai et al., 2014). Although employing enzyme creates a friendly environment, it is expensive compared to acid hydrolysis which is easy and productive hydrolysis method. Besides, the hydrolysis products (glucose and cellulose chains) inhibit the ability for enzymes to convert cellulose to glucose. As more product is formed, the enzymes become more inhibited by the presence of excess glucose. This ultimately slows down the hydrolysis process yielding low levels of usable hydrolysis product (Maurice & Advisor David DiBiasio Co Advisor Yan Lin at Shanghai Jiao, 2011).

Yield of ethanol is also strongly affected by parameters during fermentation such as temperature, pH, inoculum size, substrate concentration, time, etc. (Darvishi & Moghaddami, 2019). This study presents the production of bioethanol from sequentially acidic-alkaline pretreated sorghum straw and studying the effects of fermentation parameters on ethanol yield. Bioethanol was produced using acidic-alkaline pretreated sorghum straw and acid hydrolysis, and the effects of sugar level, pH, and inoculum size on ethanol yield was studied.

MATERIALS AND METHODS

Chemicals and Reagents

Acetone was employed as a solvent for determining extractives in sorghum straw, and sodium hydroxide (99.8 %, Norbright) was used for neutralization and alkaline pretreatment. Sulfuric acid (98 %) used for pretreatment and hydrolysis was obtained from Loba Chemie. Phenol was used in the determination of the sugar level of hydrolysates, while Urea (> 99.5 %, Unichem), Potato dextrose broth (HiMedia), yeast extract powder (Microexperess), and Hydrous magnesium sulfate were used for medium preparation.

Apparatus and Instrumentation

Samples were ground using a grinder (WRB80), and sieved on AS 200. The physiochemical characterization of sorghum straw powder was conducted using a Soxhlet extractor and an autoclave (LZ-B5O, Shanghai MedEco, China). Pretreatment and hydrolysis experiments were also conducted on LZ-B5O. The absorbance of hydrolysates during phenol sulfuric acid experiments was measured with UV-VIS DOUBLE BEAM UVD-3200 (LABOMED, INC.). Media for the propagation of a culture of Saccharomyces cerevisiae and inoculation were conducted in a laminar air hood, while the fermentation experiments were conducted in a Shaker incubator. A centrifuge and a rotary evaporator were employed to purify ethanol. The composition of ethanol was determined using a digital density meter (DMA 4100 M). The functional groups of the produced product were determined using a Perkin Elmer FTIR Spectrophotometer.

Microorganism

A culture of Saccharomyces Cerevisiae utilized for the fermentation process was brought from the Ethiopian Biotechnology Institute, and further cultivation was done in the Biochemical Engineering lab of Addis Ababa Institute of Technology.

Raw Material Characterization

The study used wet sorghum straw brought from Debrebirhan, Ethiopia; three kilograms of sorghum straw were chopped into pieces using a knife. The chopped pieces were dried in the sun for two days, ground with a grinder, sieved to pass 1 mm mesh according to the NREL laboratory analytical procedure (Hames et al., 2008), and packed in a plastic bag for further processing.

Moisture content, extractive content, cellulose, hemicellulose, and lignin were analyzed according to NREL laboratory analytical procedures (Hames et al., 2012; Sluiter et al., 2007; Sluiter, Hames, et al., 2008; Sluiter, Ruiz, et al., 2008; Wolfrum et al., 2013).

Pretreatment of Sorghum Straw Powder

Two subsequent pretreatment methods: dilute Sulfuric acid and dilute sodium hydroxide treatment were performed aiming to get a better sugar yield. 17 identical experimental runs were performed to handle the prepared sample with the available equipment in Addis Ababa Institute of Technology. Sample was mixed with distilled water in a ratio of 1:10 (g/mL), then pretreatment was carried out sequentially with both methods. During the dilute acid pretreatment, 100 mL dilute sulfuric acid solution of 1 % concentration was added to a solution prepared by mixing 70 grams of powder sorghum straw with 700 mL of distilled water. The solution was treated inside an autoclave and heated at a temperature of 125 °C for 10 minutes. Samples that had been pretreated as stated above were cooled and filtered. Each of the filtrate liquids were held in separate flasks and placed in a refrigerator for preservation while the residues were washed twice to remove the acid, dried in an oven, weighed, and packed in polyethylene bag for alkaline treatment.

During the alkaline pretreatment, 1.25 % sodium hydroxide was mixed with the dried solid residue in a ratio of 10: 1 (mL/g) and pretreated in an autoclave for 10 minutes at 90 °C. The pretreated sample was filtered using vacuum filtration and the filtrates were mixed with previous filtrates and placed in a refrigerator for fermentation and other analysis while the residues were washed twice to remove the base, dried in an oven, weighed, and packed in polyethylene bag for hydrolysis treatment.

Hydrolysis

Dilute sulfuric acid was used for the purpose of hydrolysis. For the purpose of obtaining different sugar concentrations, temperature, time, and acid concentration were varied in the hydrolysis experiment. Diluted H_2SO_4 of concentration 2%, 3% and 4% (v/v), temperature (110 °C, 125 °C and 140 °C), and time (20, 40 and 60) minutes were used.

Determination of Total Carbohydrates

The total carbohydrates of all the hydrolysate was determined using phenol sulfuric acid method. The method here was adopted from (Brummer & Cui, 2005), (Masuko et al., 2005) with little modification.

Preparation of reagents

5 % phenol solution was prepared by weighing 5 grams of phenol and diluting it with 100 mL distilled water while the 96 % sulfuric acid was prepared by mixing 97.96 mL of 98 % sulfuric acid and 2.04 mL distilled water.

Standard curve preparation

The glucose stock solution was prepared in such a way that a 0.03 g of glucose (dextrose) was added in to 500 mL volumetric flask containing distilled water. The mix was stirred and its volume was adjusted to 300 mL by adding distilled water. The final concentration of the stock solution was then adjusted to 100 mg/L. Six dilutions of the stock glucose solutions were made separately by pipetting a known volume of the stock solution (0, 1, 3, 5,7, and 5 mL) in to a 10 mL volumetric flask and filling the volume with distilled water up to the mark. Where the dilute solution prepared by taking no volume of stock was used as a blank solution. The concentrations made for this study were: 0, 10, 30, 50, 70 and 90 mg/L. To determine the standard curve, 4 mL aliquot of each dilutions was taken and pipetted in to separate test tubes. 1 mL of 5 % phenol was added to the test tube containing the sample. After that, 5 mL of 96 % sulfuric acid was added to the mix and vortexed to keep uniformity. The mixture was kept at room temperature for 10 minutes and vortexed again. The mix was then placed in a water bath at 90 °C for 15 minutes. The test tubes were removed from water bath, cooled, and vortexed. Some amount of the mix from each test tube was taken to clear cuvettes and the absorbance was read at 490 nm. The standard curve was then produced by plotting the absorbance versus concentration data. The same procedure used for the standard solutions was followed on the samples, and the concentration was obtained using the standard linear curve equation.

Fermentation

Three hydrolysates having different concentration were prepared. Sugar concentration, pH, and inoculum concentration were varied to study their effect on yield of ethanol.

Figure 1. Selected hydrolysates for fermentation

Media preparation

At the glance, a culture of saccharomyces cerevisiae was brought from Ethiopian Biodiversity institute with a 1.5 mL preservative. A medium was then prepared for further propagation. The medium was prepared by mixing 400 mL distilled water, 40-gram potato dextrose broth, 1-gram yeast extract, 4-gram urea, and 4gram hydrous Magnesium sulphate in 500 mL conical flask. The pH of the medium was adjusted using 4 molar of sodium hydroxide to 5, closed tightly with aluminum foil, and sterilized in an autoclave at 121 ℃ for 15 minutes. Finally, the media was taken out of the autoclave and cooled to room temperature.

Inoculation

The brought culture was added to the cooled medium in a laminar air hood. Then, it was closed using cotton material to allow entry of small amount of air which is used for the propagation of the yeast. Finally, the inoculated medium was incubated in a shaker incubator for 24 hours at 30 ℃.

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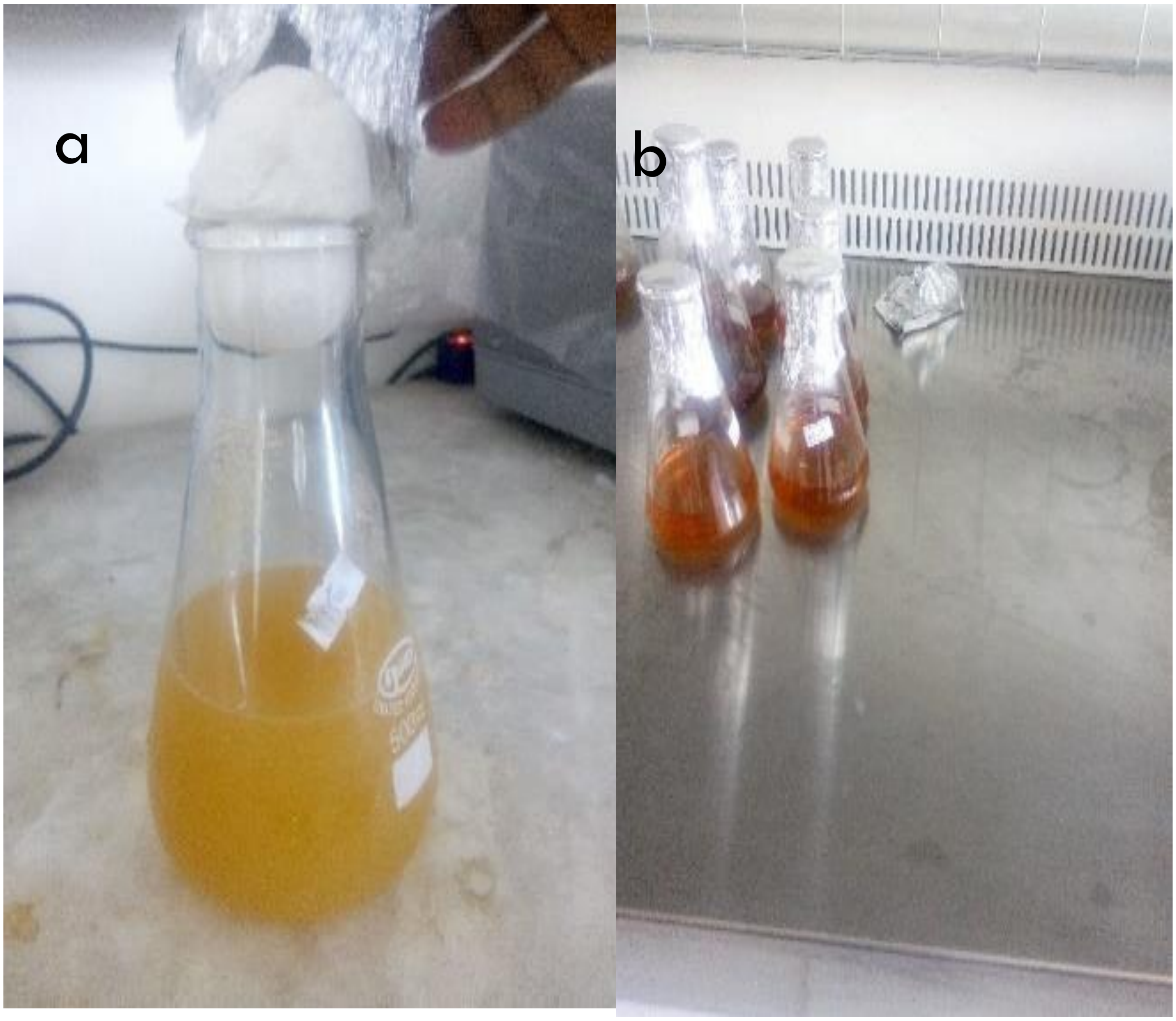

Figure 2. (a) Inoculated medium (b) Samples ready for fermentation

Experimental design

100 mL of sugar solution was taken from three different hydrolysates for fermentation. Sugar level (3.0402 to 3.29875 g/L), inoculum size (5 to 15), and pH (4.5 to 5.5) were investigated. The pH range was made narrow, because previous researches (Adelabu et al., 2018), (Hames et al., 2008) showed that ethanol yield was sensitive to pH variation. The design was done using response surface method, Box Behnken method.

Centrifugation and Evaporation

Fermented samples were centrifuged for 20 minutes with 6000 rpm. Then the centrifuged samples were evaporated in a rotary evaporator at 83 °C for two and half hours.

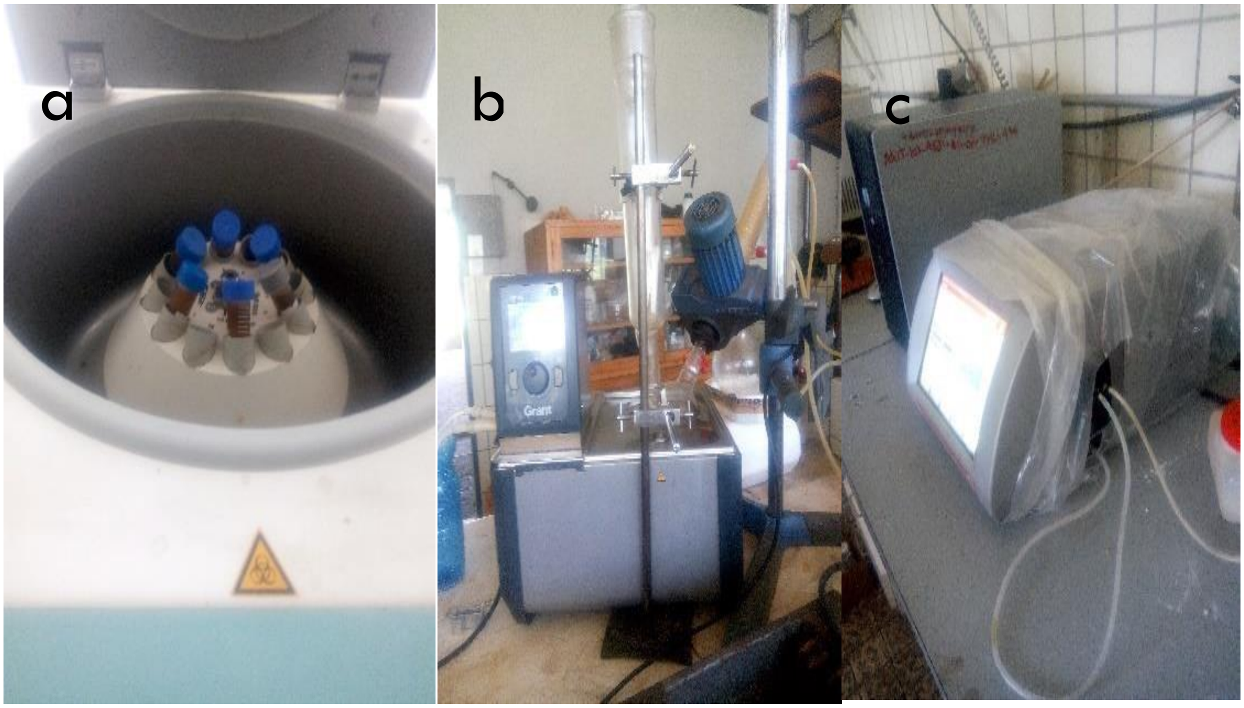

Figure 3. (a) Centrifugation (b) Rotary evaporator (c) DMA 4100 M (digital density meter)

Product Characterization

Determination of yield of ethanol

The volume percentage of ethanol was determined using digital density meter. A sample was injected to DMA 4100 M and the volume percentage of each sample was recorded. The yield (mL of ethanol per gram of sugar) was then determined as follows.

$$Actual\ volume\ of\ ethanol = \%\frac{v}{v} of\ ethanol * total\ volume\ of\ ethanol\ after\ evaporation \ldots\ldots\ldots.. (1)$$

$$Ethanol\ yield = \frac{actual\ volume\ of ethanol}{sugar\ level} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots. (2)$$

$$\%\ yield\ of\ ethanol = \left(\frac{actual\ volume\ of\ ethanol * density\ of\ pure\ ethanol}{sugar\ level}\right) * 100 \ldots (3)$$

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Characterization of ethanol using FTIR

The sample that contained optimal ethanol percentage during the volume percentage measurement was tasted for its functional group. The analysis was done in Addis pharmaceutical factory S.C, Adigrat, Ethiopia. A drop of the sample was put in to the sample holder, potassium bromide, and the percentage transmittance versus wave number were recorded and the spectrum was plotted.

Statistical Analysis

The analysis of variance, and the effects of the three fermentation parameters on ethanol yield was done using design expert version 11

RESULTS AND DISCUSSION

Physiochemical Composition of Sorghum Straw

The moisture and total solids of the sorghum straw were obtained to be 7.572 and 92.428 % respectively. It was obtained that 98.75 % of the solid part of the straw was organic, while the remaining 1.25 % was ash. These results are relatively similar to the finding of Mutepe (2012). A moisture content of 91.8 % and 2.9 % of ash were obtained (Haghighi Mood et al., 2013). Similarly, the extractives, lignin, Hemicellulose, and cellulose contents of the sorghum straw on dry basis were obtained to be 13.4 %, 16.41 %, 30.3 %, and 38.6 % respectively. The high extractives content is attributed to the presence of sucrose in the inner part of the straw (Phutela & Kaur, 2014). The above results are relatively different from earlier studies by sikgor & Becer (2015) who obtained the compositions of sorghum straw bagasse as 32 -35 % cellulose, 24-27 % hemicellulose, and 15-21 % lignin and (Haghighi Mood et al., 2013) who found the cellulose, hemicellulose, and lignin contents of sweet sorghum bagasse to be 28.53, 19.07, and 3.93 respectively. The small difference comes from the type of cultivars of sorghum (Pimentel et al., 2017).

Total Carbohydrates

The total carbohydrate content of the hydrolysates produced via sequential acid-alkali pretreatment and subsequent hydrolysis was determined using phenol sulfuric acid method. The standard curve prepared by fitting the concentration vs absorbance linearly is shown in Figure 4.

The concentrations of the hydrolysates were calculated from the equation of the standard curve.

$$\boldsymbol{X} = \frac{(\boldsymbol{Y} + \mathbf{0.0073})}{\mathbf{0.095}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots .. (4)$$

Where, X and Y represent concentration and absorbance respectively. The present study obtained 0.424 g/g of sugar yield, while previous researches on sweet sorghum bagasse showed a glucose concentration of 0.68 g/g (Haghighi Mood et al., 2013). The difference could be due to the difference in sorghum variety, the use of straw instead of the bagasse only, and the efficiency and sophistication of the equipment employed. This is because the sequential pretreatment provides efficient removal of lignin and efficient degradation of hemicellulose to monomeric sugars (Guo et al., 2013).

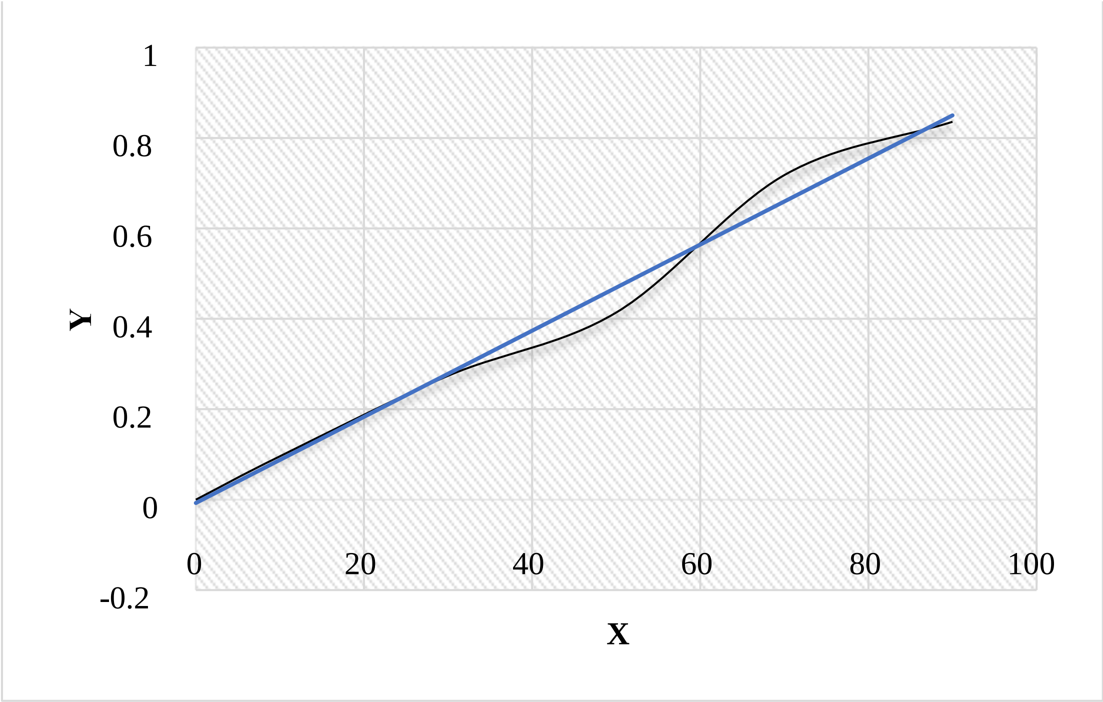

Figure 4. Standard glucose curve

Characterization of Ethanol

Ethanol yield

A maximum 0.48742 g of ethanol per g of glucose was obtained. The result is relatively higher than the yield obtained in previous researches. Mutepe (2012) obtained an ethanol yield 0.45 g per g of sugar from sweet sorghum bagasse fermentation with Saccharomyces Cerevisiae.

FTIR spectrum

Alcohols have a characteristic infrared spectrum associated with O-H, C-O and C-H stretching vibrations. When run as a liquid film, a region with a very intense and broad band at 3500 - 3200 cm^{-1} indicate the OH stretch of alcohols, while the region 1260 - 1050 cm^{-1} confirms the C-O stretch of alcohol. The bands at around 2880 and 2930 cm^{-1} were assigned as the symmetric stretching modes of the – CH_2 and – CH_3 groups, respectively (Radu et al., 2012). FTIR Spectrum analysis of ethanol produced from sorghum straw showed O-H, C-H, and C-O stretch looks like the standard ethanol.

The Results of Statistical Analysis

The regression Model

The model equations, which relates the response yield of ethanol to the independent variables is given below. The yield (ml of ethanol per g of glucose) is represented by Y_{PS} for brevity.

$$YPS = 1.144913 * sugar\ level + 0.008592 * inoculum\ size + 5.60168 * pH + 0.001055 * sugar\ level * inoculum\ size - 0.061654 * sugar\ level * pH - 0.0012 * inoculum\ size\ pH - 0.017423 * sugar\ level2 - 0.001332 \quad (5)$$

Effect of sugar concentration on ethanol yield

The effect of sugar level on ethanol yield when the inoculum size and pH are held at 10 % and 5 respectively is shown in Figure 5 and Figure 6.

The ethanol yield at low sugar level is too low. This is because of the low concentration of fermentable sugars (Haghighi Mood et sal., 2013). It is observed that the ethanol yield at sugar level of 3.2613 is 0.554696 mL/g, which is higher than the sugar yield at sugar level of 3.29875

(0.553016 mL/g). This is because of the negative effect caused by osmotic pressure at higher sugar concentration (Ratnam et al., 2005). These results agree with that of Adelabu et al. (2018). Ethanol productions at different substrate concentration of sorghum straw by two yeasts were observed. The ethanol yield increased gradually with the use of 5 % to 7.5 % and thereafter declined (Adelabu et al., 2018).

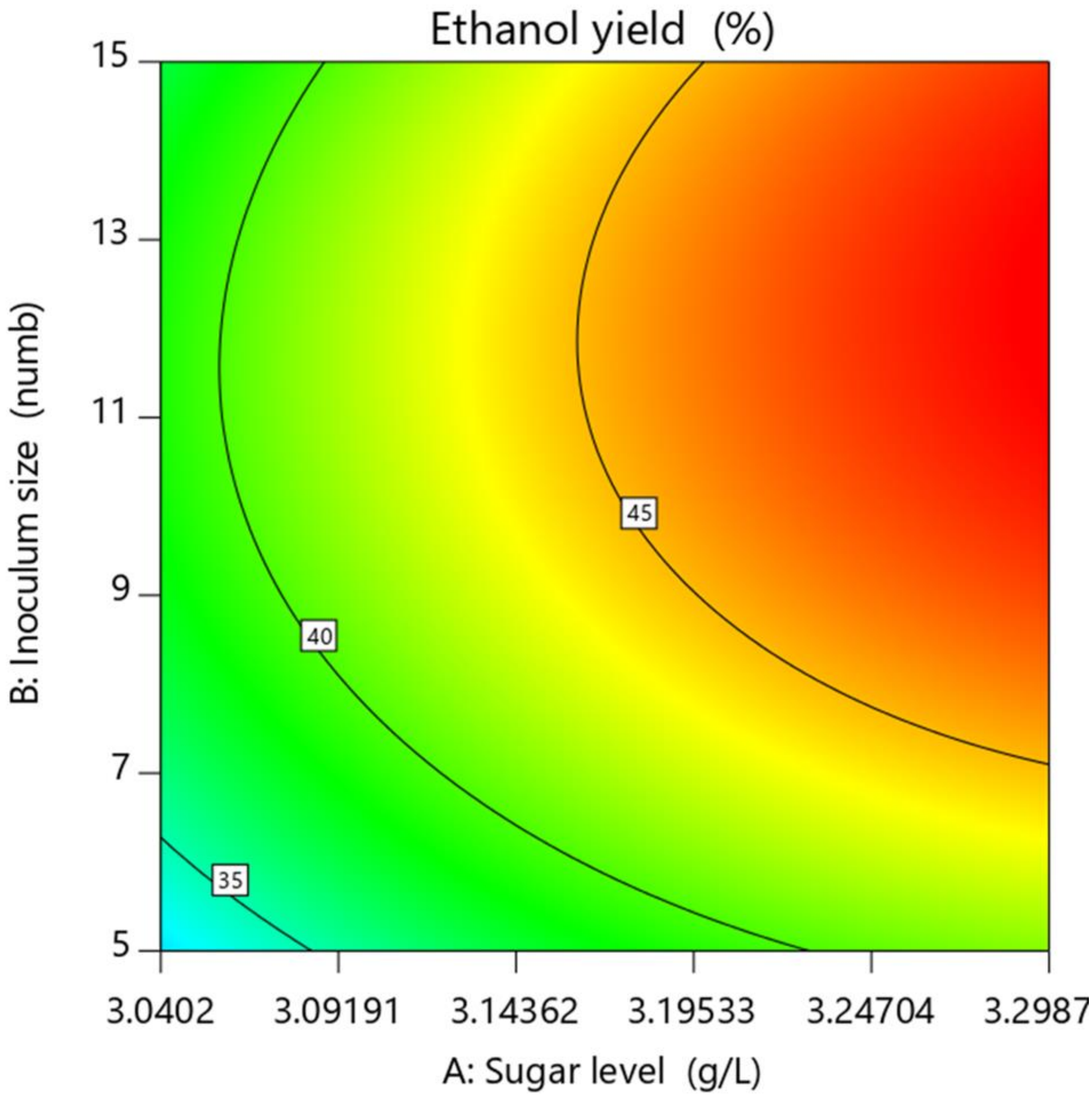

Figure 5. Effect of sugar level on ethanol yield (contour plot)

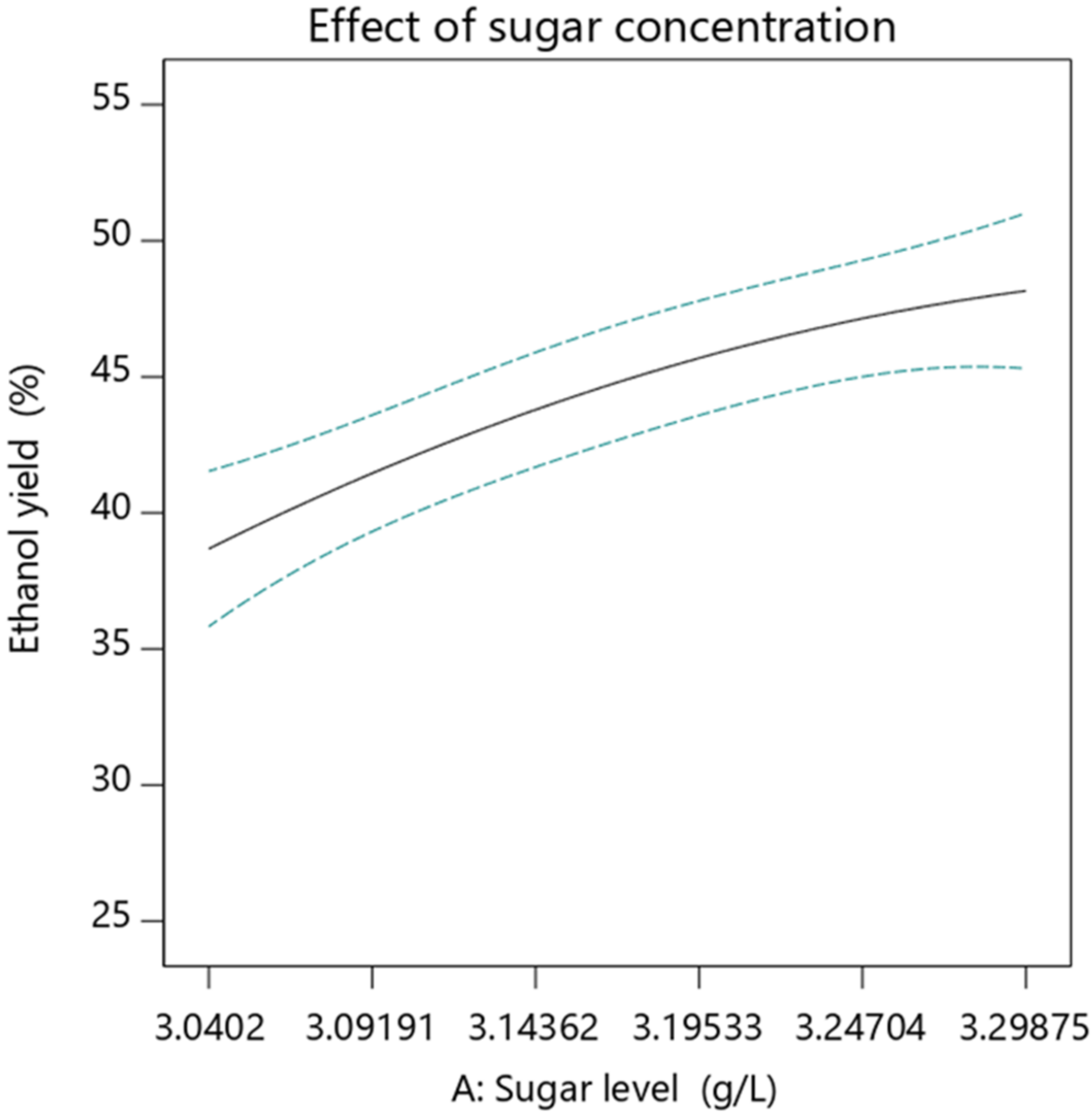

Figure 6. Effect of sugar level on ethanol yield (one factor effect)

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Effect of inoculum size on ethanol yield

Figure 7 and Figure 8 shows the effect of inoculum size on ethanol yield when sugar yield and pH are held constant at 5 and 3.17 g, respectively.

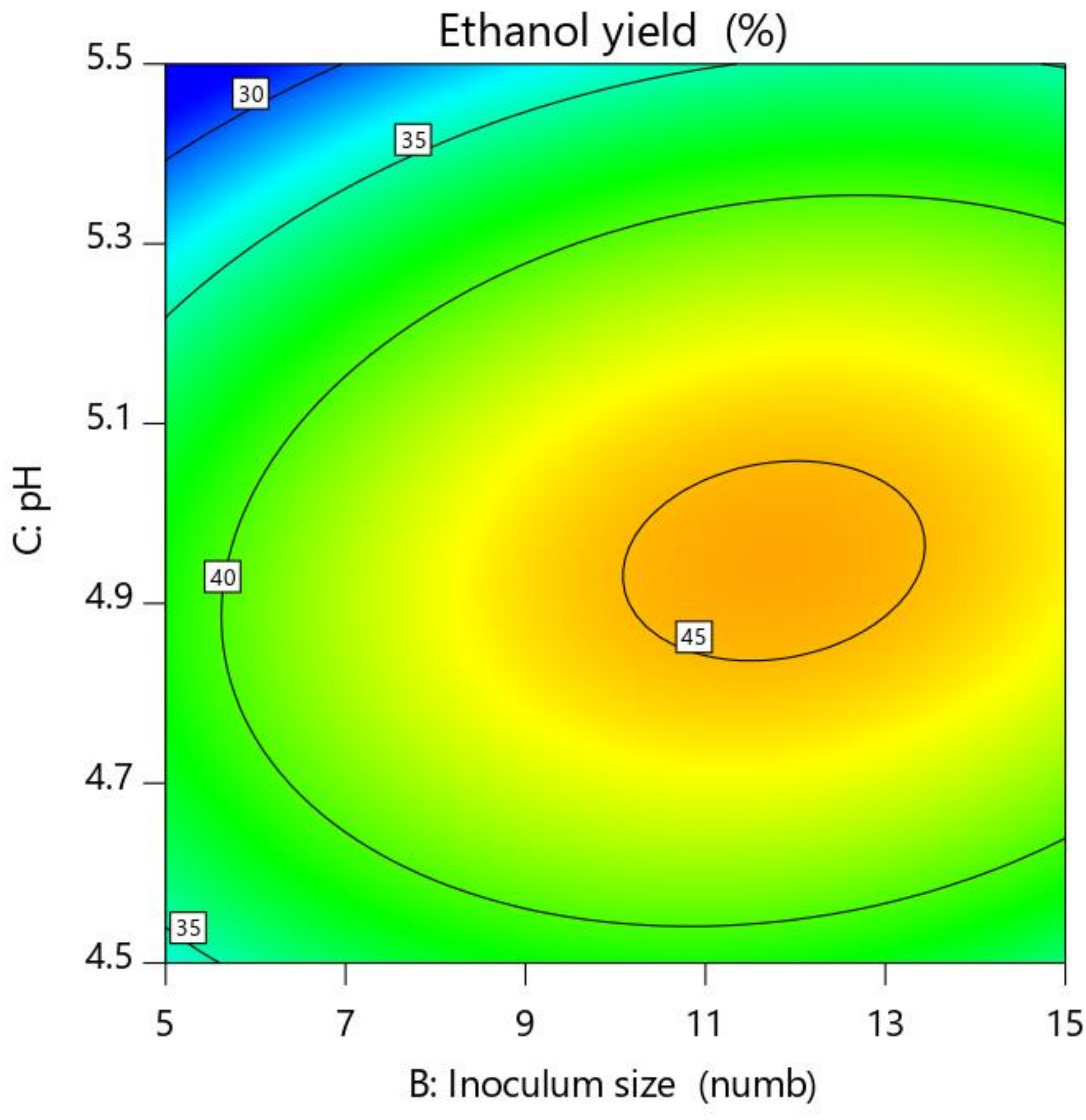

Figure 7. Effect of inoculum size on ethanol yield (contour plot)

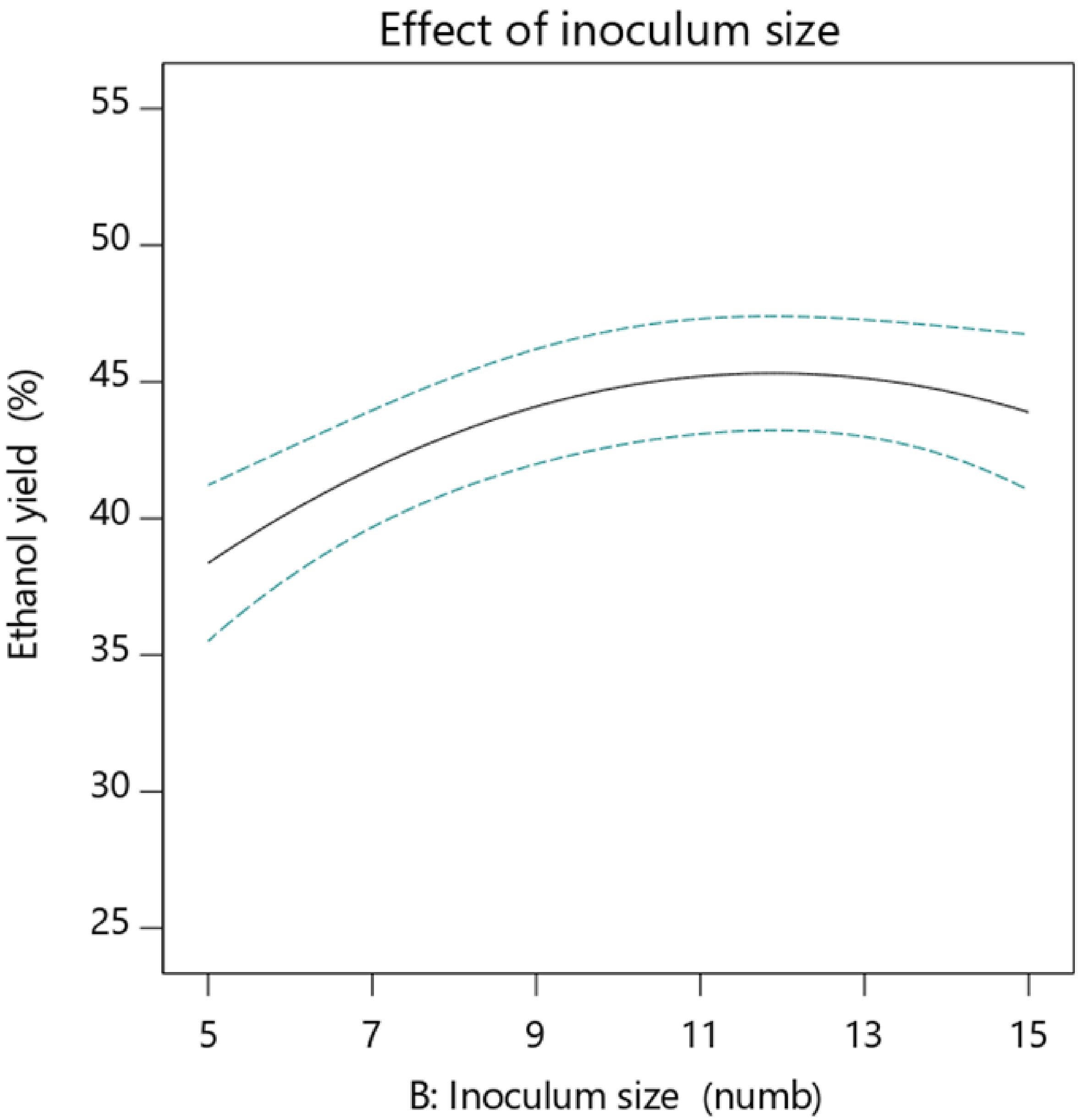

Figure 8. Effect of inoculum size on ethanol yield (one factor effect)

The results show that the ethanol yield increased with increasing inoculum size and decline at very high inoculum size. These results coincide with previous studies by giving maximum ethanol yield at some point optimum which is neither too low nor too high. Elhussieny, 2014 showed that there was observed higher ethanol yield at 2.5 mL inoculum size than 0.5 and 3 mL (Boudjema et al., 2015) while Adelabu et eal, 2018 showed that highest volume of ethanol was produced with 7.5 % inoculum concentration, followed by 5 % inoculum concentration while 15 % produced the least bioethanol (Adelabu et al., 2018). Lower ethanol production at lower inoculum size is

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probably due to the less cells which are insufficient to use the fermentation medium for yeast optimal activity, while the decreased yield at higher inoculum size might probably due to nutritional imbalance caused by tremendous growth resulting in autolysis of cells (Adelabu et al., 2018).

Effect of pH on ethanol yield

The ethanol yield was maximum at pH 5 followed by pH 4.5 and 5.5. Yield was sharply increased at the glance and reaches its maximum point at the middle. Then it goes decreasing with increasing pH. The plot where sugar level and inoculum size were held constant at 3.042 g and 15 % respectively is shown on Figure 9 and Figure 10.

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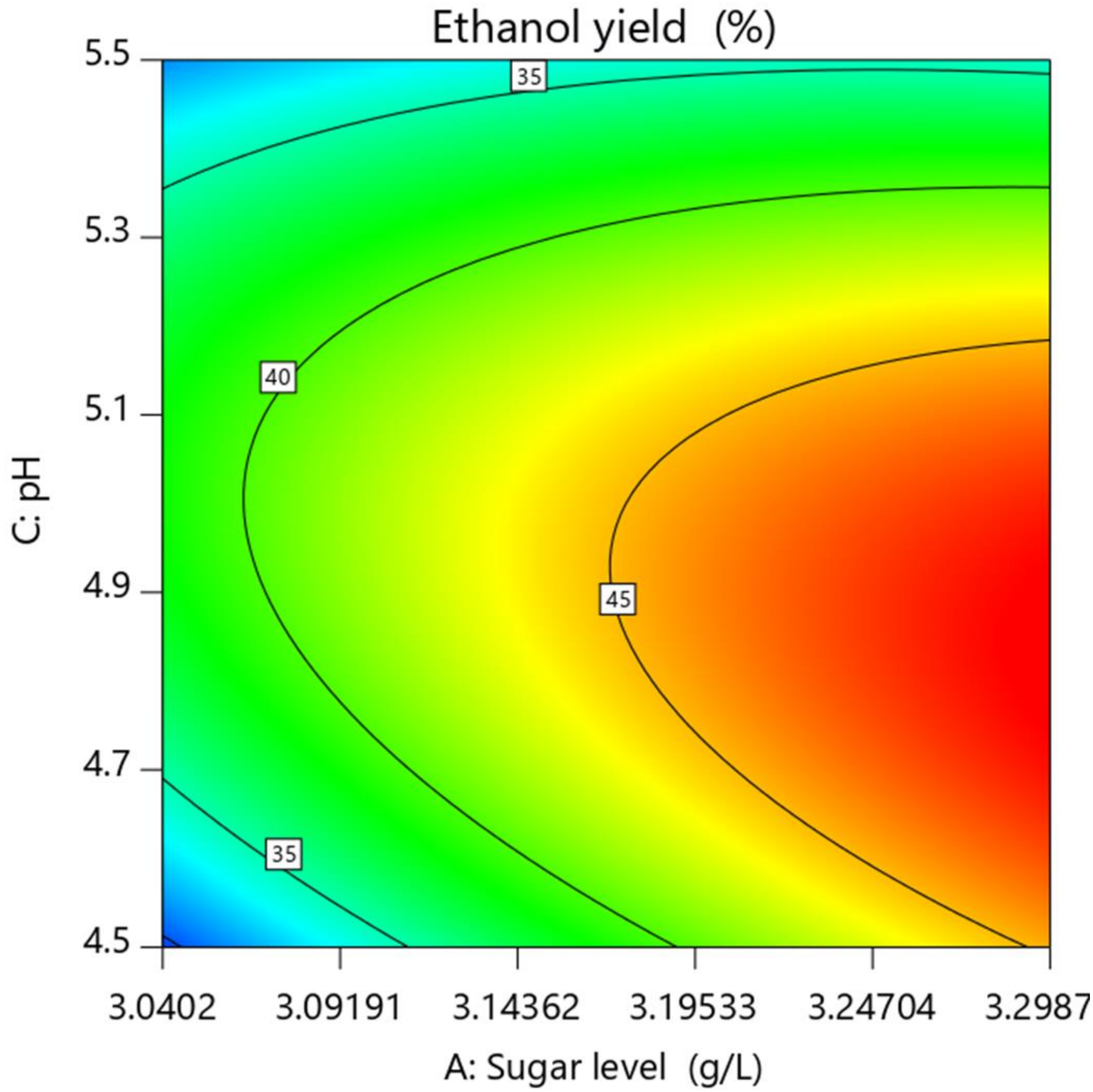

Figure 9. Effect of pH on ethanol yield (contour plot)

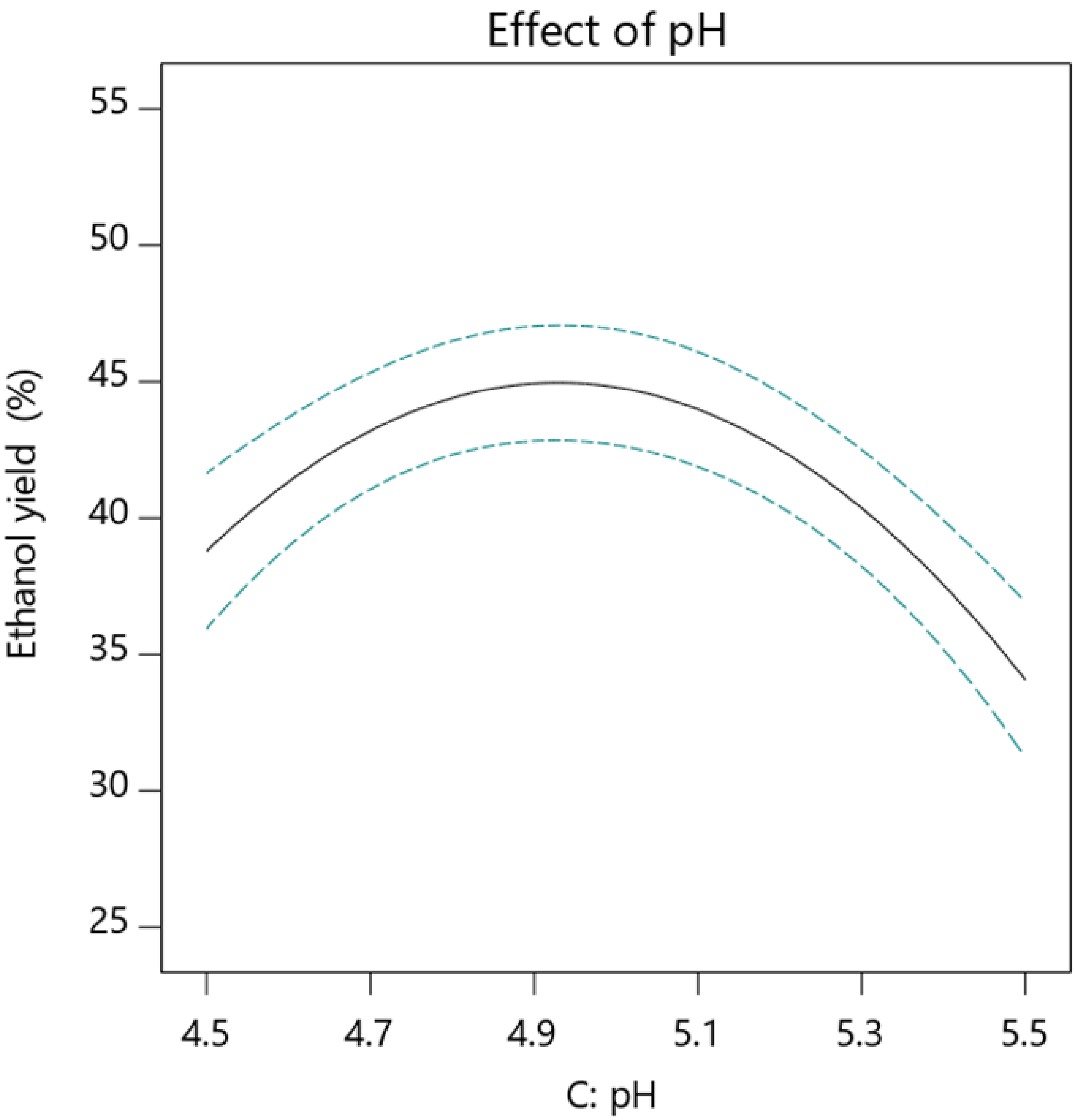

Figure 10. Effect of pH on ethanol yield (one factor effect)

As observed from the graphs, the ethanol yield is high at pH around 5 and relatively low yield was observed at pH 4.5 and 5.5.

CONCLUSIONS

In this study, sugar level, pH, and inoculum size were varied from 3.042 – 3.29875 g, 5 % - 15 %, and 4.5 - 5.5 respectively, and the results show all these three variables affect ethanol yield significantly. The maximum ethanol yield of 0.617 mL/g was obtained at pH 5, sugar level of 3.29875 g, and inoculum size of 15 %. The FTIR spectrum of the product indicated the presence of O-H, C-H, and C-O stretches similar to the standard ethanol's FTIR spectrum. This confirmed that product obtained is ethanol. On other hand, the compositional analysis of the sorghum straw gave high amount of cellulose and hemicellulose relative to lignin. This shows that sorghum straw is a good source of sugar for ethanol production. In conclusion, controlling fermentation parameters during the production of ethanol from sequential acidic-alkaline pretreated sorghum straw is a good choice in view of increasing yields of sugar and ethanol.

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Credit authorship Contribution statement

Fseha Gebremichael Gebrihet: He is the first author who conducted the experiment, collected and analyzed data, and drafted the manuscript paper. **Abnet Mengesha Dube** revised the manuscript step by step in accordance with the requirements of the journal and the reviewers' remarks.

Sincerely.

Fseha Gebremichael

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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