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Bioethanol Yield from Farm Residue of *Abelmoschus esculentus* (Okra) Using *Saccharomyces* Cerevisiae: A Preliminary Assessment

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Abstract

The use of agricultural wastes and cellulosic materials for bioethanol is increasingly gaining acceptance due to its environmental friendliness and economic advantage among other factors. The present study assesses the potentialsand usability of dry okra (*Abelmoschus esculentus* Moench) straws in ethanol production using *Saccharomyces cerevisiae*.Rate of sugar consumption and ethanol yield of the fermenting system were determined every 24 hours for 5 days. Fermentation efficiency at different pH and temperature, amount of ethanol distilled from the fermented syrup and its purity were also determined. The results showed sugar consumption was directly related to ethanol yield which increased progressively to climax at 96 hours beyond which it declined. However, pH decreases with increase in fermentation time. Optimum sugar consumption and ethanol yield was achieved at 96 hours with highest fermentation efficiencywithin pH 4.2-3.7 and at temperature 37^{o}C.Final distilled ethanol yield (collected at

80-84^{o}C)from250 mlfermented syrup of okra was 31.8 ml (12.58%). Percentage purity was found to be about 93% with specific gravity of 0.7618 KgL^{-1}. The study concluded that if fermentation of okra straw is optimized, it could be a potential raw material to supplement the use of feedstocksfor bioethanol production.

Keywords: *Abelmoschus esculentus*, biofuel, ethanol yield, fermentation, *Saccharomyces cerevisiae*

Introduction

In the recent decades, the global focus has been on safer and environmental friendly energy sources. Increase in the fossil fuel price, its implication in global warming and increase in energy demand are major impetus for alternative, cheap and clean energy. The research for new sourcesof energy remains a worthwhile activity due to debate on renewable energy, particularly, biofuel production technologies (Lin and Tanaka, 2006). The quest for global biofuel industry in the new millennium were driven by array of government policies and interventions to address domestic energy crises, ameliorate global warming, reduce pollution, and enhance economic growth. Among the biofuels of choice to replace or supplement fossil fuels is bioethanol from different biological materials.

The trend in biofuel production has been progressive and its utilization isincreasingly gaining acceptance with more countries and business interests promoting and investing in biofuel production. Currently, to save cost on petrol and reduce emission of poisonous gasesinto the atmosphere, there is advocacy for addition of percentage ethyl alcohol to petrol to ensure full combustion which consequently reduce vehicular emission (Ward and Singh, 2002). In 2009, the estimated world ethanol production was 20 billion gallons. The

United States of Americaaccounted for about 54%, Brazil 34% and the European Union (EU)approximately 5% of the global production (RFA, 2011). The global biodiesel production on the other hand was dominated by the EU with about 9 million tons representing 65% of world production in the same year (EEB, 2010).

Although cellulose rich materials could play a major role and replace agro-food sources in future utilisation for biofuel with the major source of raw materials for biofuel industries currently are agricultural products, which could also serve as food for teaming population of the rural poor or as forage to animals. It was estimated that 51% of the global ethanol produced in 2008-2010 were sourced from feedstock grains, especially from cereals while sugarcane and molasses accounted for 29% (OECD-FAO, 2011). In Europe, the use of vegetable oil for biofuel production is a common practice. For instance, rapeseed is grown extensively for biodiesel production while USA utilises soybean for the same purpose.However, recently priority is given to production of bioethanol from agricultural residues (Hahn-Hagerdal*et al*., 2006).According to OECD-FAO (2011), about 21% of the molasses produced in the years 2008-2011 was used as raw material for ethanol production. A large proportion of corn grown in America fed the biofuel companies, which produced several million gallon of ethanol and more than half (55%) of the total sugarcane production in Brazil were used to produce ethanol.

Generally, production of bio-ethanol involves bio-fermentation of agricultural feedstocks; fruits, vegetables, and cereals using microorganisms to convert sugar to alcohol and gasohol.Ethanol is a byproduct in sugar production by fermentation of molasses (Ribereau-Gayon, 1985; Periyasamy*et al.*, 2009). Currently, about 80% of total world ethanol production is

obtained from the fermentation of sugar by yeast (Lin and Tanaka, 2006). Usually, yeast cells cannot produce ethanol higher than 7% (v/v), higher volume would inhibit the cell growth by producing osmostress across the cell wall. For higher ethanol production therefore, various modifications have been carried out like mutation of yeast strain and modifications in nutrient composition of the medium. Since ethanol yield depends on the type of yeast strain, physiology, media composition, pH and temperature as pointed out by Arvindekar (1995). Addition of assimilable nitrogen source can increase the rate of fermentation by reducing the time required for completion of fermentation.In order to increase fermentation efficiency, sugar tolerant yeast strains were isolated from sugar fermented vegetable extract (Tain and Hasninaga, 1997).

Considering the amount of agriculturalfood materials that are converted into bio-diesel annually and the resultant pressure on food security, research for alternative non-food biological raw material is imperative. Cellulosic biomass, derived from non-food sources, such as trees, grasses and post-harvest farm wastecould be developed as feedstock for ethanol production. For instance, attempt was made by Krishnamurthy *et al.* (2014) to extract ethanol from pulps of *Ficuscarica* and *Phoenix dactylifera* fruits using *Saccharomyces ceresvisiaes.*

Okra (*Abelmoschus esculentus*Moench), is an economically important member of Malvaceae family. It is grown for its edible green seed pods, which are used as vegetable. The crop is cultivated in tropical, subtropical and warm temperate regions. Several million of hectares of land was estimated to be used for okra cultivation (FAOSTAT, 2008), creating enormous fibrous post-harvest waste that could be converted to bio-energy. The present study, therefore, is an attempt to assess production of bio-ethanol from post-harvest okra straws

and leaves as a potential alternative non-food material for ethanol production.

Materials and methods

Materials

Okra straw collection and preparation

The stems and leaves of okra (post-harvest biomass) were collected from abandoned okra plantation, in Bardoli, Surat, India. The biomass was sun-dried for 2 weeks and then made into powder using domestic grinding machine (Binatone). The experiment was conducted in the Biotechnology Laboratory, C. G. Bhakta Institute of Biotechnology, UkaTarsadiaUniversity, Bardoli, Gujarat, India.

Yeast, chemicals and reagents

The yeast (*Saccharomyces cerevisiae* NCIM strain No. 3228) used for the fermentation process was obtained in active culture form from Industrial Microorganisms Laboratory at National Chemical Laboratory (NCL), Pune, India. Sucrose was purchased from BDH limited, Poole, England while Sodium hydroxide (NaOH) and other reagents were obtained from SD Fine CHEM Ltd, Mumbai, India. All the chemicals, reagents and glasswaresused in the present study were of high quality of analytical grades.

Methods

Maintenance of yeast (*Saccharomyces cerevisiae* NCIM strain No. 3228)

The active yeast life culture was maintained as described by Krishnamurthy *et al,* (2014).The yeast (NCIM strain No. 3228) was maintained on yeast media by inoculating wire loop of the culture into the yeast potato dextrose agar (YPD) broth, sealed with sterile mineral oil at room temperature (25^{o}C) and kept for growth for three days when ready for use.

The determination of total carbohydrate

The total carbohydrate in the powdered okra leaves and straw was determined by Anthrone method as described by Gerhardt *et al*,(1994) with modifications. 100 mg of the powdered okra straw and leaves was added into a boiling tube for 3 hours to hydrolyze into simple sugars using 5 mL of 2.5 N-hydrochloric acid and cooled to room temperature. Solid sodium carbonate was added until the effervescence ceases and the volume made up to 100 ml. About 0.5-1 ml supernatant collected after centrifuge was used in aliquots for analysis. Standards were prepared (0.2, 0.4, 0.6, 0.8 and 1 ml) while 0 ml serves as blank, the volumes were made up to 1 ml and 4 ml of Anthronewas added in boiling state, then cooled. Carbohydrate compound forms a green coloured precipitate with anthroneat maximum absorption at 630 nm. The amount of carbohydrate present was determined by the relation

Amount of carbohydrate present in 100 mg of the sample= (mg of glucose/volume of test sample) X 100

Fermentation and ethanol estimation Process

20 g of grounded okra biomass was measuredinto 500 ml flask in three replicates. Fermentation was carried out as described by Igwe*et al.* (2012). *S. cerevisiae* was introduced nutrient agar (NA) broth and incubated at 37^{o}C for 24 hours, yeast pre-culture was conducted by method of Zhan*et al.* (2003)and **t**he pre-cultured media concentration checked by Spectrophotometer at absorbance of A_{600}. 5 ml yeast was addedto the media and the volume made up to 500 mL with distilled water. The mixture was placed on rotatory shaker to thoroughly mix the substrate with the yeast for effective fermentation.The pH of the fermenting system was adjusted to 6 and fermentation allowed for 120 hours. Sugars content was determined according to

Miller's method as modified by Abu, *et al*. (2005)andalcohol contentwas estimated every 24 hours for five days. by dichromate method (Wang *et al.*, 1997).Sugar consumption at pH 6, 5, 4 and 3 as well astemperaturesat 25, 37 and 42 ^{o}C were determined.

Ethanol distillation and determination of other parameters

After 120 hours, when fermentation process was observed to have ceased (drastic reduction of carbon (iv) oxide gas evolving form the fermenting mixture), the fermented syrupwas filtered into 500 ml distillation flasks and boiling glass chips added to reduce side swerving of the filtrates. Distillation was carried out according to Igwe*et al.* (2012) and the distilled ethanol was collected at 80 – 88 ^{o}C after second round of distillation at 80-83 ^{o}C, raw percentage ethanol yield was determined and ethanol collected. The ethanol was purified by addition of sodium hydroxide and kept overnight to increase ethanol purity (90 – 94%). Fermentation efficiencywas determined by percentage ratio of practical value to theoretical value (Practical value = O.D × dilution factor, theoretical value = 180 g glucose gives 92 g ethanol) (Fadel*et al.*, 2013) andspecific gravity of the ethanol was determined using pycnometer (SG-16A 2000, Gilson company Inc.) while the boiling point was determined by using laboratory thermometer.

Statistical Analysis

The data obtained from the experiment were analyzed usingMicrosoft Excel (2013) for Microsoft Window Operating System**.**

Results

The results of quantification of the total carbohydrate in the okra straw and leaves used for this study by Anthrone method is shown in Table 1. The amount of carbohydrate determined by the relation of carbohydrate present in 5 mg of grounded

dried okra sample was 0.023 mg.The change in pH per time interval as it affects sugar consumption and utilisation by the yeast in the fermenting system and the production is presented in Fig 1. Within the first 24 hours, ethanol content of the system was very small (0.83 w/v) and barely detectable. At each time and pH of the fermenting medium, ethanol produced was proportional to rate of sugar consumption. Reduction in pH increases the rate of sugar consumption and conversion of sugar to ethanol.Increase in fermentation time brought about progressive decrease in pH of the system and acidity of the medium (Fig 1). The pH and corresponding trend of sugar consumption every 24 hour is shown in Fig 2. The cumulative sugar consumption by *S. cerevisiae* from okra substrate at pH 6 increased with the fermentation time (0.96 – 4.10 w/v). However, a decline in rate of sugar consumption occurred beyond 96 hours of fermentation. This trend was similar for other pH (5, 4 and 3) evaluated, but optimum sugar consumption was obtained at pH 5.

At room temperature (25^{o}C) sugar consumption by *S. cerevisiae*ranged from 0.96 -3.91w/v while it ranged from 1.76 – 4.38 w/v and 1.67 – 4.08w/v at 37 and 42^{o}C respectively (Fig. 3). The optimum temperature for sugar consumption by the yeast was 37^{o}C. The rate of sugar consumption in the fermenting medium by the yeast for temperatures of 25 ^{o}C, 37 ^{o}C and 42^{o}C progressively increased from 0 hour to 96 hours and then declined after 96 hours (Fig.3). The theoretical and practical values as well as fermentation efficiency of the fermenting medium under different pH and temperatures condition is shown in Table 3. Optimum efficiency (61.27%) was obtained when the fermenting medium was at pH which corresponds to 96 hours of fermentation (Table 3). Similarly, at37^{o}C, highest fermentation efficiency (45.04%)

was obtained while temperatures of 25 ^{o}C and 42 ^{o}C decreased fermentation efficiency.

From the distillation process starting with 250 mL of the fermented syrup,ethanol collected at 80-88 ^{o}C (1st distillation) was 46.20 ml. On further distillation of the distilled (2nd distillation) at 81 -84 ^{o}C, 31.45 mlof ethanol was collected (Fig.4). After the two temperatures regimes of distillation, the percentage ethanol yield determined was 12.58%. The percentage purity of ethanolobtained after reflux distillation of the fermented syrup of okra was found to be 93.4% and the specific gravity was 0.7618 while the boiling temperature was between 80^{o}C and 91^{o}C.

Table 1: Estimation of carbohydrate by Anthrone method using Standard glucose concentration of 0.1 mg/ml.

Std. glucose(ml)	Distilled Water (ml)	Anthrone (ml)	Concentration (mg/ml)	O.D620 nm
Blank	1.0 ml	4 ml	0.0	0.00
0.2 ml	0.8 ml	4 ml	0.02	1.35
0.4 ml	0.6 ml	4 ml	0.04	1.43
0.6 ml	0.4 ml	4 ml	0.06	1.51
0.8 ml	0.2 ml	4 ml	0.08	1.73
1.0 ml	0.0 ml	4 ml	0.1	1.90
Sample 0.1 ml	0.9 ml	4 ml	0.023	1.36

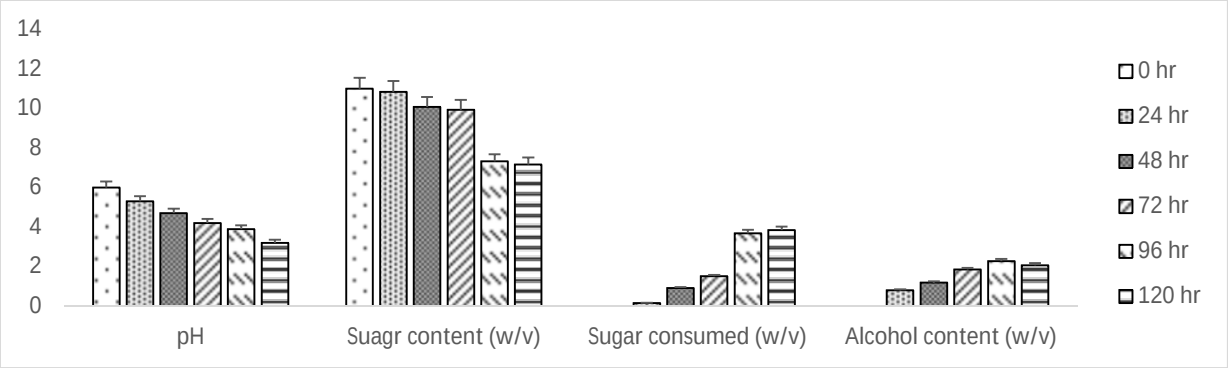

Fig. 1: Measurement of pH, sugar content, amount of sugar consumed and total alcohol content in Okra substrates at 24 hours of fermentation.

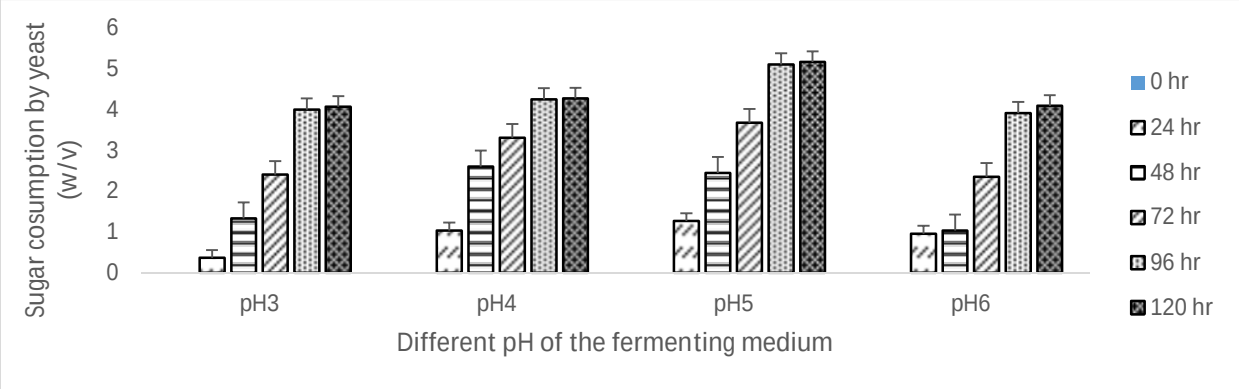

Fig. 2: Sugar consumption in okra wastes by *S. cerevisiae* at different pH of the fermenting medium

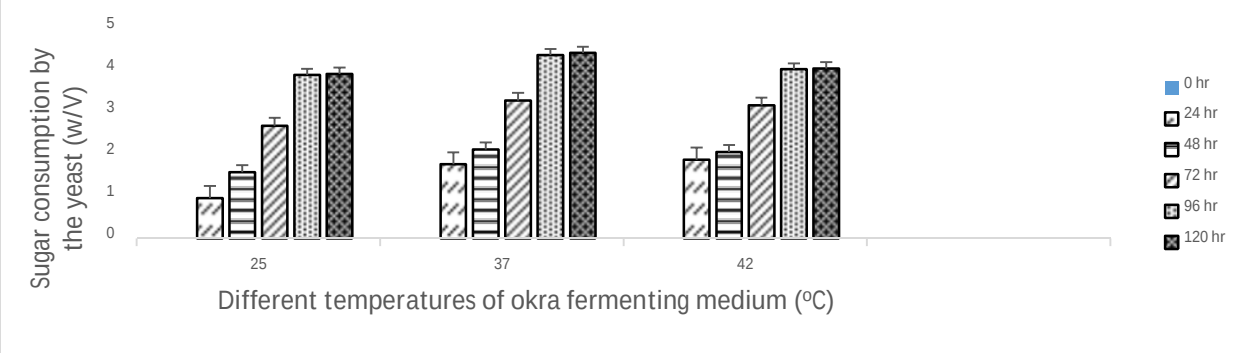

Fig. 3: Comparison of sugar consumed in okra by *S. cerevisiae* at different temperatures

Table 3: Comparison of Theoretical value, Practical value and Fermentation efficiency of okra substrate by *S. cerevisiae* at different pH and temperatures

Parameter	pH				Temperature (^{o}C)		
	3	4	5	6	25	37	42
Theoretical value (g)	2.04	2.17	2.61	2.01	1.96	2.22	2.07
Practical value (g)	1.25	0.50	0.95	0.70	0.75	1.0	0.45
Fermentation efficiency (%)	61.27	23.04	36.39	43.83	38.26	45.04	21.73

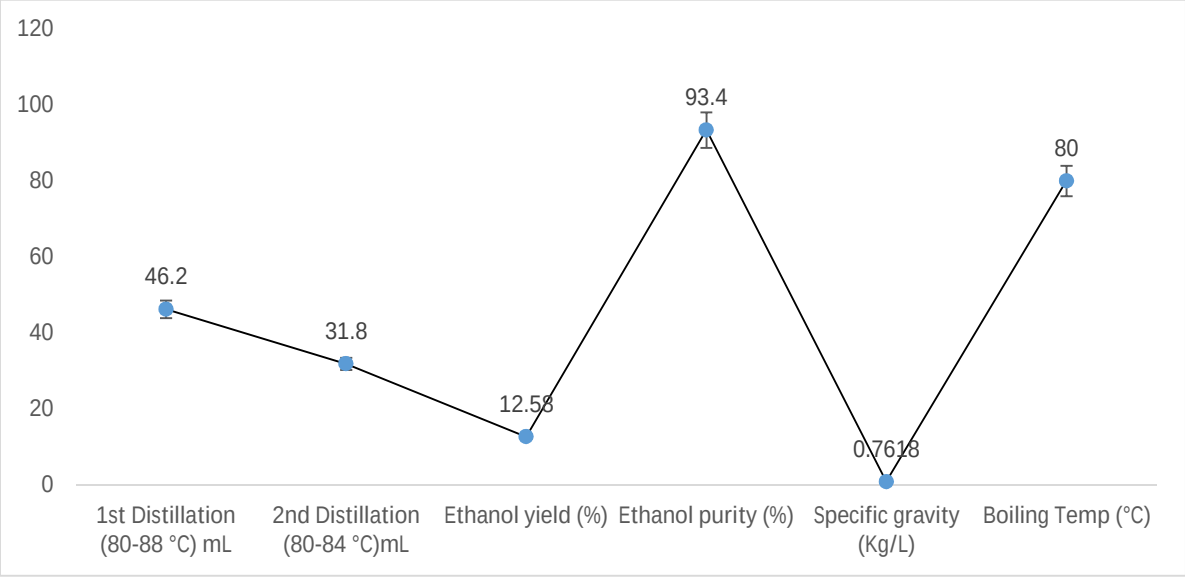

Fig. 4: Amount of distilled, percentage yield and purity of ethanol from 250 ml of okra fermented syrup using yeast (*S. cerevisiae*)

Discussion

The present study assessed potential of bioethanol production from okra straw using yeast. The amount of carbohydrate in the raw material of the okra substrate was less (about 0.23 g/ml) compared with oats (Thomas and Ingledew, 1995) and other cereal such as barley (Thomas*et al.*, 1995) due to low sugar but high fibre content of the okra straw. Sugar consumption by the

yeast led to decreasedpH of the reacting medium as a result of increase in CO_2 within the system. Within the first 24 hours, sugar consumption was low, because the inoculated yeast cell needed to adjust to the medium, thus resulting in little sugar consumption and consequently, ethanol yield within the first 24 hours of the fermentation was low. The low quantity of ethanol was as a result of low sugar consumption, since amount of sugar consumed by yeast in a fermenting system is directly proportional to ethanol produced. A similar observation was reported by Wu *et al.* (2006) while utilising*S. cerevisiae* in the fermentation of pearl millet for ethanol production. By 96 hours, sugar consumption and ethanol yield had declined, because the fermenter (yeast) had grown exponentially to attain equilibrium, in the absence of additional sugar for the fermenting medium, nutrient depletion and accumulation of CO_2 led to reduced microbial activities in the medium. This consequently reduced ethanol production from the system beyond 96 hours.

As the sugar content of the fermenting medium reduces, other fermentable sugars such as maltose and dextrins could be hydrolysed into glucose to sustain ethanol production after the original glucose in the substrate was utilized. Therefore, at 96 hours and beyond, glucoamylase conversion of dextrin and maltose could not sustain sugar requirement for the fermentation process, hence the process became slow and come to a stop.Joekes*et al.* (1998) reported that weight of fermenting mixture did not decrease further after 30 hours of fermentation. The time for optimal fermentation, however would depend on the amount of the substrate, sugar content of the medium, nature of the organism, pH and temperature. Weight loss from escaped CO_2 has been used to monitor fermentation and ethanol

yield using microoganisms (Fujita *et al*., 2001).

With respect to thermochemical factors such as pH and temperature, optimum fermentation was achieved at pH of 4.2- 3.9, sugar consumption was highest, which corroborates the earlier finding of Wu *et al*. (2006). They reportedpH range of 4 - 3.9 enhanced sugar consumption an ethanol yield in fermentation of pearl millet using *S. cerevisiae*. They further opined that lower pH values could indicate contamination of lactic acid bacteria in the fermenting system. Similarly, Nigam, (1999) noted that the maximum ethanol productivity was achieved at pH of 4.2 to 4.5.Highest sugar consumption at 37 ^{o}C revealed that the temperature was most suitable for microbial activities. This implies that temperature below or higher than 37 ^{o}C could adversely affect yeast sugar consumption and conversion to ethanol. It was argued that lower or higher temperature reduces percentage ethanol production due to inactivation or denature of the yeast cells (Bai *et al*., 2004).

The fermentation efficiencies obtained for the okra straw and leaves in this study (23.04 – 61.27% under various pH and 21.73 – 45.04% for different temp.) are far less compared with fermentation efficiencies of higher carbohydrates substrates from barley: 94.6% (Thomas *et al*., 1995) and oats; 82-87% (Thomas and Ingledew, 1995). Although, the theoretical optimal efficiency of fermentation using *S. cerevisiea* is 95% as postulated by O'Connor-Cox *et al.* (1991), the obtained values in practical term are usually lower (Thomas *et al*., 1996). The difference in theoretical and practical values in determining fermentation efficiency could greatly be affected by pH, temperature, nature of the fermenter, sugar type and content of the substrate among others (Ozmihci and Kargi, 2007; Fadel*et al.*, 2013). The results of the fermentation

efficiency of the okra substrate revealed that the pH is an important factor in determination of ethanol yield from a fermenting medium using yeast. Fermentation efficiency above 60% as obtained in this study at pH 3 implies that optimal ethanol yield was achieved. However, other physical conditions were also important. If the fermentation of the okra materials is carried out under optimal temperature, pH and other factors, higher efficiency is achievable.

Distillatecollected at 80-88 ^{o}C contained high percentage of water and other impurities due to wider range of collection temperature.Addition of sodium hydroxide (NaOH) and second reflux distillation where ethanol was collected at 80 ^{o}C eliminated most of the impurities.Igwe*et al*. (2012)reported the presence of water and impurities in alcohol distilled and collected above 80 ^{o}C using *Saccharumofficinarum, Costusafer*and *Pennisetum purpureum* as substrates. Ethanol yield from the present study was about 12.5% of the 250 mlfermented syrup. The specific gravity of 0.7618of ethanol and boiling temperatures between 80-91^{o}C was similar to the values reported by Igwe*et al,* (2012). The yield could be improved by fermenting at optimal conditions of pH 3 at 37 ^{o}C while utilising yeast strain with high physiological properties. Also, sugar or glucose could be intermittently added to the fermenting system to ensure adequate sugar in the medium.

Conclusions

The okra straw and leaves evaluated in the present study for bioethanol production using *S. cerevisiae* showed that sugar consumption by the yeast is directly related to ethanol yield. However, factors such as temperature, pH, sugar content and microbial physiologyof the fermenter are important for yield and fermentation efficiency of the process. In this study,

optimal pH 4 -3.9 and temperature (37 ^{o}C), enhances fermentation efficiency. Reflux distillation and addition of NaOH increasedpurity of the ethanol. The study concluded that okra straw and leaves could be utilised in bioethanol production, however, further studyto maximise yield and fermentation efficiency would help to unlock the great potential of okra waste as alternative source for biofuel production.

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