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Production and Spectrophotometric Quantification of Bioethanol from Pineapple Fruit Skin

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Abstract

This study investigated the potentials of pineapple waste (fruit skin) as an alternative and cost-effective lignocellulose for bioethanol production by *Zymomonas mobilis* and *Saccharomyces cerevisiae*. The substrate was pretreated using dilute hydrochloric acid (HCL) to alter the complex structure of the carbohydrate polymers to removing lignin and hemicelluloses, reduce cellulose crystallinity and increase the porosity of the materials. Enzymatic hydrolysis was carried out to further depolymerize the cellulose component to simple sugars. Hydrolysis and fermentation lasted for five days. Fermentation parameters such as pH, temperature, reducing sugar (brix level) and specific gravity were monitored for five days. The concentrations of reducing sugar (brix level) were calculated based on the relationship: Brix = 261.3×(1–1/S.G).The specific gravity of the wort was determined before and during fermentation using the specific gravity bottle of known weight. The pH and temperature of the wort was determined using calibrated HANNA multi parameter probe (HI9811-5) while ethanol content was determined spectrophotometrically using acid dichromate solution. The specific gravity, pH, temperature and reducing sugar of each of the substrates decreased as the fermentation time increases. The substrate recorded a total reducing sugar content of 17.5mg/ml. The pH of the broth for the substrate decreased during the five days fermentation period with optimum pH for ethanol production ranging from 4.9 to 5.2 for the yeast and 5.0 to 5.8 for the bacterium at 72hrs incubation. Fermentation using *S. cerevisiae* was slow and required three days to complete with maximum ethanol yield of 51%. The fermentation with *Z. mobilis* proceeded very rapidly and was completed in three days with maximum ethanol yield of 78%. Sugar utilization was faster in *Z. mobilis* than in *S. cerevisiae* with a corresponding increase in ethanol yield. Conclusively, *Z. mobilis* could be considered a better microorganism for bioethanol production.
Keywords: Bioethanol; Fermentation; Pineapple fruit skin.

1. Introduction

Ethanol is a colourless volatile flammable liquid used as a solvent and fuel. Ethanol from biomass can provide a sustainable alternative to oil to mitigate the global energy problem associated with fossil fuels exhaustion and greenhouse gas emissions [1]. Using ethanol as a gasoline fuel additive as well as transportation fuel helps to alleviate global warming and environmental pollution.

Bioethanol or ethyl alcohol is produced either through fermentation of sugar or starch based crops through hydrolysis and subsequent fermentation of ligno-cellulose material forming the basic structural components of plant dry matter, e.g. agricultural residues [2-5] or from algal biomass which is at an early stage of investigation [6], but has a very distinctive growth yield as compared with classical lignocellulosic biomass [7]. Ethanol can also be manufactured by the chemical process of reacting ethylene with steam [8].

Considerable attention has been focused on alternative energy resources due to the continuous rising in cost of petroleum, fast depletion of the world's energy source and dependence upon fossil fuel resources.

Fermentable carbohydrates such as pineapple fruit skin is low valued products that are currently disposed off as wastes [9]. However, they are a rich source of lignocelluloses, which may be converted to fermentable sugars for the production of bioethanol and can be blended with petrol or used as a pure fuel in certain engines. The advantages pineapple wastes has as a raw materials for ethanol production is that it is abundant and can be gotten at a little or no cost, which makes it relatively cheaper than those gotten from sugar or starch based food/feed stocks. This production of bioethanol from lignocellulose involves pretreatment of the materials through acid and enzyme hydrolysis to alter the complex structure of the carbohydrate polymers, thereby removing lignin and hemicellulose, reducing cellulose crystallinity, increasing the porosity of the materials [10] and exposing the simple sugars which the yeast and the bacterium can utilize during fermentation [11]. Several microorganisms, such as *Clostridium* species, have been considered as ethanologenic microbes, the yeast *S. cerevisiae* and facultative bacterium *Z. mobilis* are better microbes for industrial alcohol production [12]. Traditionally, *S. cerevisiae* has been used for the

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production of ethanol; however, it has been associated with low alcohol tolerance and low productivity which for efficient ethanol production requires improvement. *Z. mobilis*, a gram negative bacterium possesses advantages over *S. cerevisiae* with respect to ethanol productivity and tolerance. *Z. mobilis* strain grown under anaerobic conditions can produce about 1.5 to 1.9 mol of ethanol from each mol of glucose, which is much better than ethanol produced by *S. cerevisiae [13]*. *Z. mobilis* grows and ferments glucose very fast; its preference for low pH prevents contamination and grows in high glucose and ethanol concentration [14].

This study reported on the potentials of pineapple fruit skin as a substrate for both *Z. mobilis* and *S. cerevisiae* for bioethanol production. Parameters such as pH, temperature, reducing sugar (brix level) and specific gravity were also determined.

2. Materials and Methods

2.1. Sample Collection

Pineapple wastes (fruit skin) were obtained from processed pineapple from pineapple sellers at fruit garden located in Ihiagwa, Imo State, Nigeria.

2.2. Microbial Sources

Spent yeast *S. cerevisiae* was obtained from 33 Consolidated Brewery Awommama, Imo State, Nigeria. The yeast was characterized in order to ascertain the viability, purity and fermentative capability. Cultural and microscopic characterization as well as few biochemical tests was done to confirm the identity of the yeast [15, 16].

Z. mobilis was isolated from freshly tapped palm wine from *Raphia hookeri* using the method described by Cheesbrough [16]. The bacterium was isolated using formulated RM media (glucose 20.0 g, yeast extract 10.0 g, K_2HPO_4 2.0 g, agar 15.0 g) in 1000 ml of distilled water and was characterized with reference to Buchannan and Gibbon [17].

2.3. Pretreatment of the Samples

2.3.1. Physical Pretreatment

The first step in bioethanol production using pineapple wastes was size reduction. The sample (pineapple wastes) was oven dried at 50°C for 96 hrs and was ground into semi powdered form using a stainless-steel grinder and then stored in well labeled transparent polyethylene bags at room temperature.

2.4. Acid Hydrolysis

Acid pretreatment was done by dissolving 10 g, 15 g and 20 g of each of the samples in duplicates into 250 ml of 2% H_2SO_4 in a 500 ml conical flask. All this samples were soaked in dilute sulfuric acid for 24 hrs. The bottles were then capped with cotton plugs and aluminum foil. The mixture was hydrolyzed by autoclaving at 121°C for 15 mins. The pretreated samples were filtered using a 24 cm pleated filter paper in a 500 ml conical flask [18]. Acid hydrolysis was done to achieve delignification. The removal of lignin was necessary for cellulose to become readily available for the bacteria and yeast to convert the sugars e.g, glucose to ethanol [18].

2.5. Adjustment of pH

The pH of the samples was adjusted using 0.1M of NaOH or HCL prior to the addition of enzymes and inoculation of the test organisms. Otherwise the enzyme or microorganism will be deactivated in hyper acidic or basic state. A pH of around 3.5-4.0 was maintained during enzyme hydrolysis and 5.0-6.5 during fermentation. This NaOH solution was added dropwise to the sample flasks with constant stirring until the desired pH was obtained. If the pH becomes basic, concentrated HCl was added drop wise to maintain the pH in the range.

2.6. Enzymatic Hydrolysis and Saccharification Process

Aliquot (0.2 ml) of amylase and neutrase was added to each of the 500 ml conical flask containing the samples. These enzymes break down the cellulose into simple sugar (glucose) which the yeast and bacteria can utilize [19]. The enzymatic hydrolysis was carried out for 24 hrs and autoclaved to halt enzyme activity, then filtered before fermentation.

2.7. Fermentation Process

The fermentation procedures were adopted from Dowe and McMillan [20].One milliliter (1 ml) of the inocula were transferred into each of the wort contained in a well labeled 500 ml conical flask and aerated by placing on a digital shaker. Fermentation lasted for 5 days at 25-30°C. At 24 hours interval 4ml of samples were aseptically taken from the fermentation flask using a micro pipette to determine the reducing sugar content (brix level), pH, specific gravity and percentage alcohol by volume.

2.8. Determination and Quantification of Ethanol Produced

Ethanol production was determined by colorimetric method using spectrophotometer as reported by Sumbhate, *et al.* [21].

2.9. Spectrophotometric Method Using Acid Dichromate Solution

Modified method of Sumbhate*, et al.* [21] was adopted which involves the followings:

2.10. Preparation of Standard Solution

A graded concentration (0.5-4.5 mg/ml) of known ethanol concentration was prepared. The ethanol was poured in volumetric flask containing water to prevent loss due to volatility. The ethanol stock solution was freshly prepared prior to use during the fermentation period.

Table-3.1. Protocol Table for Ethanol Standard Solution

Ethanol (%)	**0**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**
Stock (Absolute Ethanol)	0	0.020	0.040	0.060	0.080	0.100	0.120	0.140	0.160	0.181	0.201
Water	4	3.980	3.960	3.940	3.920	3.900	3.880	3.860	3.840	3.819	3.799
Final Volume	4	4	4	4	4	4	4	4	4	4	4

2.11. Preparation of Sodium Dichromate Reagent

Four grams (4 g) of Sodium dichromate was used to prepare 100 mg/ml stock solution in distilled water.

2.12. Preparation of Acetate Buffer (Ph 4.3)

The acetate buffer pH 4.3 was prepared using US pharmacopoeia specification [22].

2.13. Preparation of 1N Sulfuric Acid

One thousand milliliters (1000 ml) of 1N H_2SO_4 was prepared by accurately measuring 49 ml of sulfuric acid into 951 ml of water.

2.14. Colour Reaction and Development of Colorimetric Method

An aliquot of standard stock solution containing different known concentrations of ethanol (ranging from 0.5 - 4.5 mg/ml), 5 ml of sodium dichromate solution, 5 ml of acetate buffer pH 4.3 and 25 ml of 1N sulfuric acid were added into a 50 ml of volumetric flask. The mixture was shaken gently for 1 min and allowed to stand for 120 mins as incubation period at room temperature. This reaction resulted in the formation of green coloured solution. After the incubation period, the absorbance of the green coloured solution was read on UNISPEC 23D spectrophotometer at 578 nm. This procedure was repeated in triplicates for each of the two samples [21]. A linear graph of optical density against concentration was plotted as standard ethanol plot and ethanol concentration of sample was calculated using equation obtained from the plot [21].

2.15. Determination of Bioethanol Production

The fermentation process was monitored by measuring the following parameters:

2.16. Determination of Specific Gravity

The specific gravity of the wort was determined before and during fermentation using the specific gravity bottle of known weight. Equal volume of the wort and water was weighed and the ratio between the two weights was calculated [23].

The results were calculated using the formula:

$$S = \frac{\text{Density of solution (wort)}}{\text{Density of water}}$$

2.17. Total Reducing Sugar (Brix level)

The concentration of reducing sugar (brix level) was calculated based on the relationship:

Brix = 261.3× (1 – 1/S.G) [24].

The result obtained was multiplied by the constant 50 and expressed in EBU (European brewery unit) (European brewery convention manual).

2.18. Determination of Ph and Temperature

The pH and temperature of the sample was determined using calibrated HANNA multi parameter probe (HI9811-5). Ten milliliters of each of the solution was pipetted into a 50 ml flask and electrodes were dipped into it. The function selector was then turned from standby to pH and then to temperature (^{O}C). This reading was done in triplicates and the mean recorded.

3. Results

Tables 1 and 2 shows the results of parameters (pH, temperature, specific gravity and brix level) determined for different mass (10 g, 15 g and 20g) of the substrate (pineapple fruit skin) fermented by *S. cerevisiae* and *Z. mobilis*

for five (5) days. There was a decrease in pH and temperature during the five days of fermentation. The sugar (brix) level decreased appreciably as the specific gravity decreases throughout the fermentation.Fig.1 shows the Standard Ethanol plot used in calculating the concentration of ethanol produced after fermentation. Optical density was plotted against concentration and ethanol concentration of sample was calculated using equation obtained from the plot. Figs. 2 and 3 shows the ethanol yield in percentage (%) produced by the two microorganisms. The ethanol yield reached its maximum at 72 hrs and then declined. The ethanol yield by *S. cerevisiae* was significantly lower (Fig.2) compared to *Z. mobilis* (Fig. 3). Fig. 4 shows the amount of sugar utilized by the microorganisms during the fermentation period *Z. mobilis* utilized more sugar and also faster than *S. cerevisiae*. Fig.5 shows the effect of time duration on ethanol production by the microorganisms. There was a sharp increase in ethanol production and climaxed in 72 hrs and dropped significantly thereafter. Fig.6 compares the percentage yield of ethanol by the microorganisms during the fermentation period. *Z. mobilis* produced higher percent of ethanol than *S. cerevisiae*.

Table-1. Physical parameters obtained after fermentation of pineapple fruit skin by *S. cerevisiae*

Mass of Substrate (g)	**10**				**15**				**20**			
Fermentation time(hours)	pH	Temp (^oC)	Specific Gravity	Brix Level	pH	Temp	Specific Gravity	Brix Level	pH	Temp	Specific Gravity	Brix Level
0	6.0	29.4	1.067	16.4	6.0	29.3	1.070	17.1	5.9	28.0	1.072	17.5
24	5.9	27.8	1.056	13.8	5.7	28.1	1.057	14.1	5.6	27.4	1.056	13.8
48	5.7	26.1	1.044	11.0	5.2	26.0	1.048	12.0	5.4	26.1	1.040	10.0
72	5.2	25.7	1.032	8.1	5.0	25.8	1.033	8.3	5.1	26.0	1.032	8.1
96	4.6	25.4	1.027	6.9	4.7	25.6	1.028	7.1	4.4	25.8	1.027	6.9
120	4.2	25.1	1.023	5.9	4.4	25.2	1.024	6.1	4.1	25.6	1.02	5.1

Table-2. Physical parameters obtained after fermentation of pineapple fruit skin by *Z. mobilis*

Mass of Substrate (g)	**10**				**15**				**20**			
Fermentation time(hours)	pH	Temp	Specific Gravity	Brix Level	Ph	Temp	Specific Gravity	Brix Level	pH	Temp	Specific Gravity	Brix Level
0	6.2	29.2	1.066	16.2	6.5	29.2	1.067	16.4	6.4	29.2	1.070	17.1
24	5.7	27.8	1.042	10.5	6.2	28.1	1.044	11.0	5.8	27.9	1.042	10.5
48	5.2	26.2	1.028	7.1	5.5	26.3	1.026	6.6	5.3	26.2	1.022	5.6
72	5.0	25.7	1.022	5.6	5.1	25.9	1.015	3.9	5.0	25.9	1.009	2.3
96	4.7	25.4	1.015	3.9	4.6	25.5	1.012	3.1	4.7	25.5	1.007	1.8
120	4.4	25.0	1.01	2.6	4.6	25.1	1.008	2.1	4.5	25.3	1.005	1.3

Fig-1. Ethanol Standard Plot

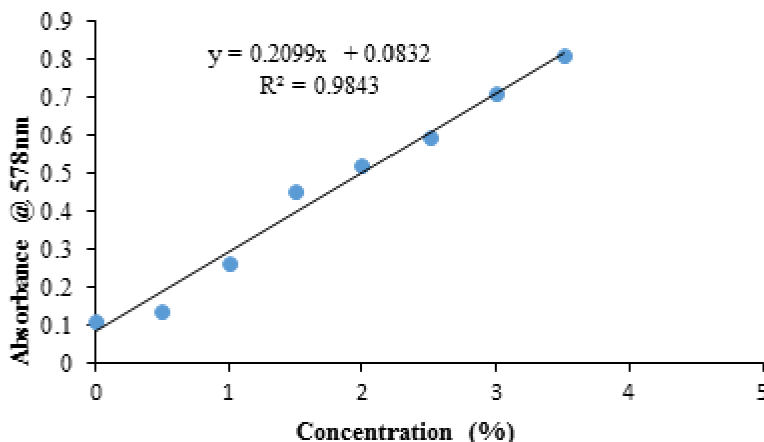


Fig-2. Ethanol produced by *Saccharomyces cerevisiae* using pineapple fruit skin as substrate

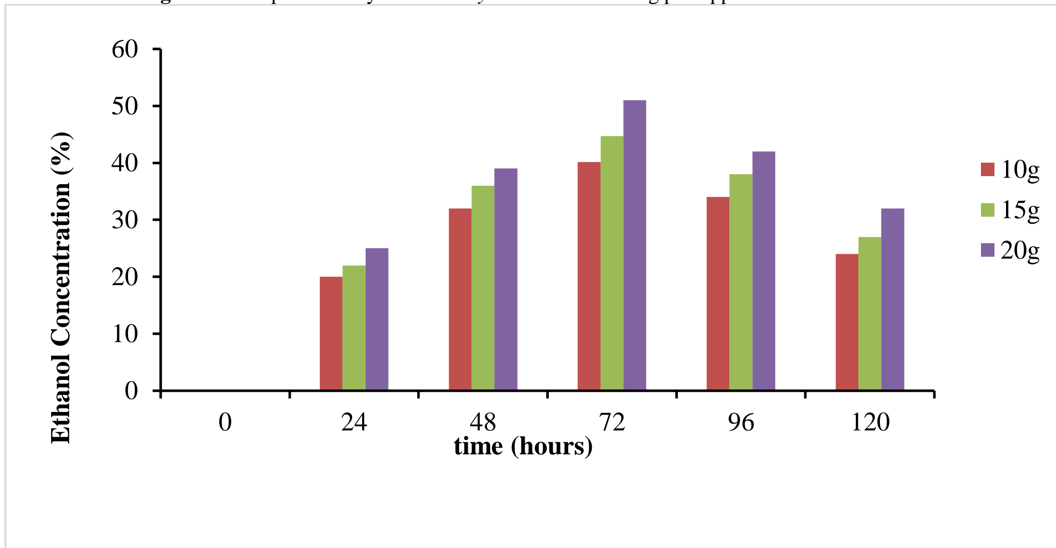


Fig-3. Ethanol produced by Zymomonas mobilis using pineaple fruit Skin as substrate

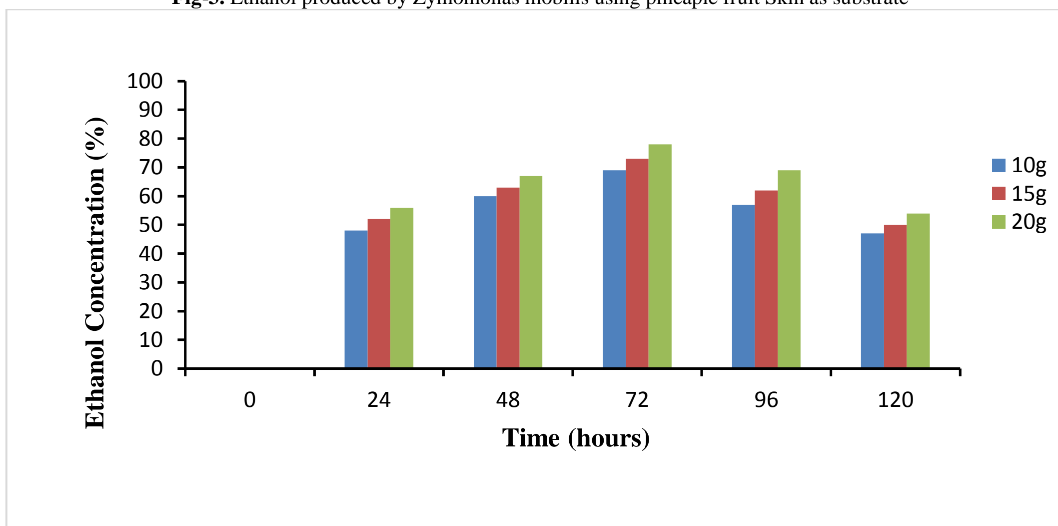


Fig-4. Sugar utilization by S. cerevisiae and Z. mobilis using pineaple fruit skin as substrate

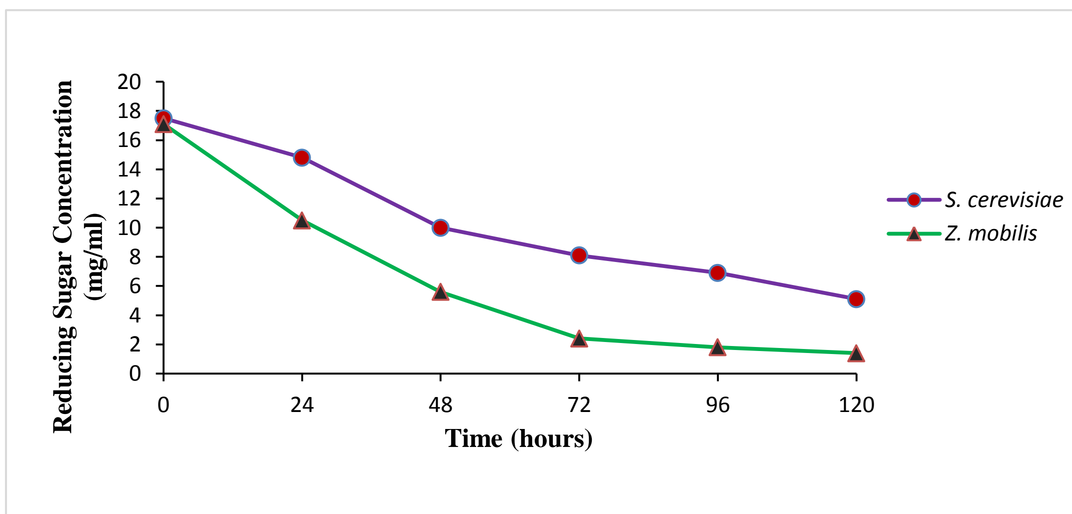


Fig-5. Effect of time duration on ethanol production by Z. mobilis and S. cerevisiae using pineaple fruit skin as substrate

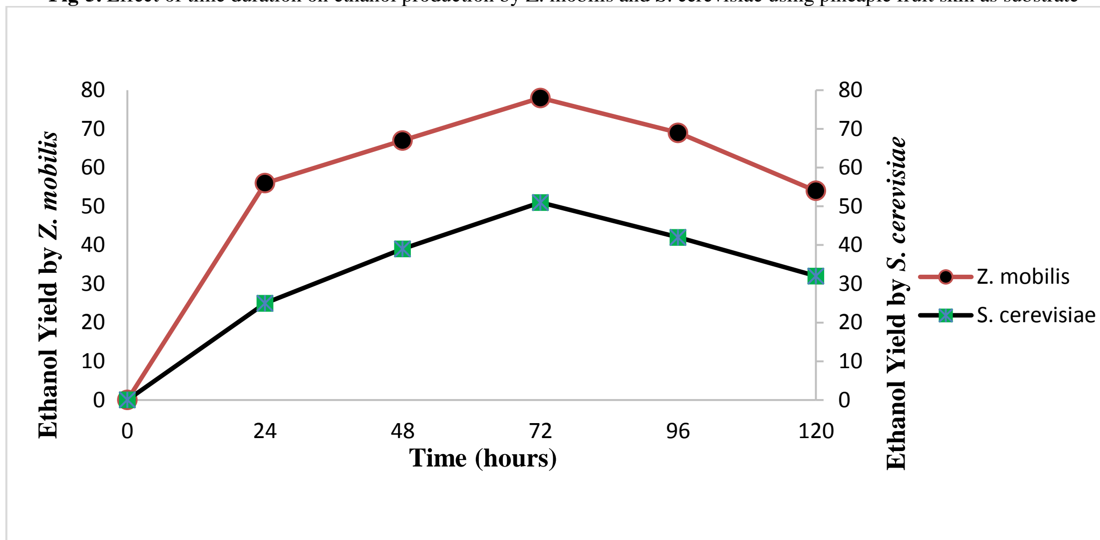
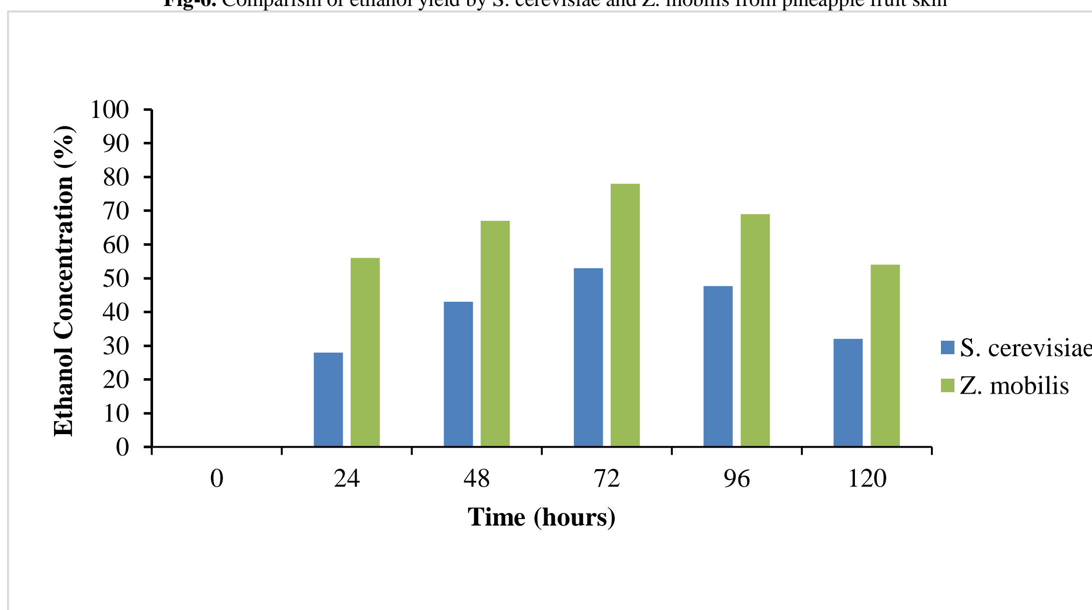


Fig-6. Comparism of ethanol yield by S. cerevisiae and Z. mobilis from pineapple fruit skin



4. Discussion

The production of bioethanol from pineapple fruit skin as an alternative and cost-effective lignocellulose, involved pretreatment of the materials via acid and enzyme hydrolysis to alter the complex structure of the carbohydrate polymers, thereby removing lignin and hemicellulose, reducing cellulose crystallinity, increasing the porosity of the materials [10] and exposing the simple sugars which the yeast and the bacterium utilizes [11]. This pretreatment method was followed by five days fermentation by the yeast, *S. cerevisiae* and a bacterium *Z. mobilis* which utilizes the sugar content of the lignocelluloses as nutrients and ends up converting the sugar to ethanol under anaerobic condition.

Separate Hydrolysis and Fermentation (SHF) approach was adopted. The pretreated biomass first undergoes enzymatic hydrolysis (saccharification) followed by ethanolic fermentation [25]. Lignocellulosic biomass cannot be saccharified by enzymes to higher yields without a pretreatment, mainly because the lignin in plant cell walls forms a barrier against enzymatic attack [26].

This study reports that the pH of the broth generally decreased during the five days fermentation period with optimum pH for maximum ethanol production ranging from 4.9 to 5.2 for the yeast and 5.0 to 5.8 for the bacterium at 72 hrs incubation. This co-relates with the work of Braide*, et al.* [4] and Braide*, et al.* [5]. As the pH decreases, the fermenting broth became more acidic, thus changing the metabolic activities of the microorganisms for increased ethanol production. The low ethanol concentration at low pH can be attributed to the fact that enzyme activity was inactivated as enzymes are pH specific. pH is regarded as one of the most important fermentation parameters due to its effect on growth of microorganism, fermentation rate and by-product formation [27]. The sugar (brix) level decreased appreciably as the specific gravity decreases throughout the fermentation. The decrease in specific gravity could be attributed to the decrease in the total soluble solids as the sugar present in the broth was fermented to alcohol and other by-products such as glycerol and CO_2 [28].

In both organisms (*S. cerevisiae* and *Z. mobilis*), a continuous increase in ethanol yield was accompanied with decreased reducing sugar concentration during and throughout the fermentation (Fig. 5). Fermentation with *S.*

cerevisiae was slow and required three days to complete with an ethanol yield of 51% (Fig. 2). The fermentation with *Z. mobilis* proceeded very rapidly and was essentially completed in three days with maximum ethanol yield 78% (Fig. 3). In all cases, the sugar utilization was faster with *Z. mobilis* than in *S. cerevisiae* with a significant difference (Fig. 4).

Bacteria are known to multiply faster than yeast thus; *Z. mobilis* might reach the lag phase earlier than *S. cerevisiae* and therefore utilized its substrate faster. The ethanol yield for *Z. mobilis* was higher than that of *S. cerevisiae* at all fermentation periods. *S. cerevisiae* is known to employ the Embden Meyerhof Parnas (EMP) pathway to metabolize glucose producing 2 moles of ATP from 1 mole of glucose whereas *Z. mobilis* employing the Entner-Doudoroff (E-D) pathway produces 1 mole of ATP from 1 mole of glucose Wang, *et al.* [29]. Ming, *et al.* [30] reported that a significant amount of the carbon source is converted into biomass as a result of the E-D pathway used by *Z. mobilis*. All the enzymes involved in fermentation are expressed constitutively, and fermentation enzymes comprise as much as 50% of the cells' total protein [30]. Clark, *et al.* [31] reported that there is linearity between maximum cell growth and ethanol production with *S. cerevisiae* strains. However, *Z. mobilis* perform less biomass formation and efficient production of ethanol compared to *S. cerevisiae* [32]. The low ethanol conversion efficiency by *S. cerevisiae* might therefore be due to the fact that a portion of the substrate was converted to cell mass and other products. Generally, there was a decline of ethanol production after three days (72 hrs) for both organisms which could be attributed to the build-up of inhibitory toxins produced in the fermentation medium as reported previously by Zakpaa, *et al.* [33]. Although liquefaction and saccharification might probably kill some microorganism that might cause contamination, both organisms were able to metabolize their substrate thus competitively inhibiting the growth of other microorganisms. They can therefore be used to produce ethanol using non-sterile substrate. This could reduce energy cost involved in sterilizing the substrate. According to Tao, *et al.* [34] and Aggarwal, *et al.* [35], cheap raw material, low processing cost and high productivity are the main considerations for most ethanol production. This work therefore shows that under favourable conditions pineapple fruit skin which is usually disposed off as waste can be used as alternative and cost-effective lignocelluloses for ethanol production.

5. Conclusion

Pineapple fruit skin was a suitable substrate for bioethanol production by *S. cerevisiae* and *Z. mobilis* as high yield of ethanol was produced from its high sugar content. However, *Z. mobilis* demonstrated higher biomass conversion efficiency, hence higher ethanol concentration compared to *S. cerevisiae*. Engineering the ethanologenic species *Z. mobilis* and *S. cerevisiae* with pentose-metabolizing pathways can be developed for developing recombinants to ferment both pentose and hexose sugars in the hydrolysate into ethanol [36]. This substrate could therefore be used for large scale bioethanol production, hence reducing its threat to the environment.

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