

Production of Bioethanol from Pineapple Rinds by Saccharification and Fermentation Using *Saccharomyces cerevisiae*

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ABSTRACT

Ethanol produced by fermentation of plant biomass is considered to be an environmentally friendly alternative to fossil fuels. This research aimed to study the effect of pivotal operating variables (incubation temperature, microbial inoculum amount and the agitation rate) on the production of bioethanol from pineapple rinds as a renewable biomass by acid saccharification and fermentation using *Saccharomyces cerevisiae*. A considerable amount of glucose, the main fermentable sugar, was released from acid (10.31 mg/mL) which was obtained through saccharification of Josapine pineapple peel powder with 72% sulfuric acid. The prominent conditions for maximum ethanol production using 1.0 g of glucose, temperature at 30°C, inoculum size of 6% (v/v) *S. cerevisiae*, incubation period of 48 hours with agitation rate 150 rpm for the first 24 hours, resulting in ethanol yield and fermentation efficiency of 0.44 g/g and 86.27% respectively. The interaction effect between incubation temperature and inoculum amount was highly significant ($P < 0.01$).

Key words: Bioethanol, pineapple rinds, saccharification, fermentation,
Saccharomyces cerevisiae

Introduction

Depletion of world's non-renewable energy sources has given rise to investigations and studies on alternative sources of energy worldwide. In contrast to fossil fuels, bioethanol is renewable energy source produced through fermentation of sugars obtained from lignocellulosics such as rice straw, corn stalk, fruit rinds and other

agricultural biomass (Naresh et al., 2007). Lignocellulosic biomass is the most economical and highly renewable natural resource in the world, the word lignocellulose refers to plant dry matter that is mainly composed of cellulose, hemicellulose and lignin. Organic matter subsuming fruit rinds are part of wastes largely generated by human activities in households, food industries, agriculture and factories, which accumulates and result in pollution of the environment. Pineapple is an important and among the major tropical fruits of the planet. According to Food and Agricultural Organization (FAO) online database, Malaysia is the third most pineapple consuming country all over the world with the rate of 255,000 tonnes annually, thereby generating high amount of residues after consumption in the form of peels and crowns yet not being fully utilised to a maximum production (Zawawi et al., 2014). A research study conducted on pineapple peels reported that each 100 g of dried pineapple peels powder (PPP) contains approximately 55 g of carbohydrates (Feumba et al., 2016). Pineapple peel residue being rich in carbohydrates can serve as a potential for inexorable production of bioethanol as well as minimizing the accumulation of pineapple peel waste in a sustainable way.

Most of the available literature on production of bioethanol from fruit peels substrate uses either chemical (acid) or enzymatic saccharification (hydrolysis) to breakdown cellulose in substrates to reducing sugars which are subsequently fermented to bioethanol. In case of chemical saccharification, bioethanol is produced through Separate Hydrolysis and Fermentation (SHF) and on the other hand using enzymatic saccharification, it is produced through Simultaneous Saccharification and Fermentation (SSF) and sometimes SHF (Gary et al., 2011). A good ethanol yield is influenced by fermentation parameters such as temperature, incubation period, agitation, inoculum size and substrate concentration. Thus, this research is aimed at economic and sustainable bioethanol production with the objective of optimizing fermentation parameters like temperature and incubation period using PPP through acid saccharification for higher ethanol yield by *Saccharomyces cerevisiae*. The objectives of this research are to determine the amount of glucose produced by Josapine pineapple peel through acid saccharification using dinitrosalicylic acid (DNS) assay and to detect and quantify bioethanol produced through optimization of fermentation parameters resembling temperature and incubation period via potassium dichromate method.

Bioethanol

Bioethanol is an alcoholic compound produced technologically through fermentation of plant sugars obtained from agricultural biomass such as fruit and vegetable waste, forestry waste and agro-residues. Bioethanol is a renewable bio-energy source; it is biodegradable and eco-friendly potential fuel with great calorific value. Bioethanol can be produced in both liquid and gaseous states (Mirza, 2015).

Production of pure ethanol apparently begins in the 12 to 14th century, during that time alcohol was mainly used for production of medicinal drugs and manufacture of painting pigments (Goyal, 2015). Ethanol was used to power an engine in 1826, and

in 1876, Nicolaus Otto used ethanol to power an early engine (Abdel-salam, 2018). In 1850's, ethanol was used as lighting fuel (Cole, 2010). Bioethanol become known to substitute fossil fuels at the beginning of 20th century, and in 1970's bioethanol fuel production in industry began using corn as the predominant feedstock due to its high availability and ease of transformation to alcohol in 2006 its market expands to about 13.5 billion gallons (Shinnosuke et al., 2008; Goyal, 2015). Bioethanol fuel is expected to increase to 36 billion gallons per year by 2022 (Cole, 2010).

The United States is the world's largest producer of bioethanol followed by Brazil; the two countries produce a total sum of 85% of the world's ethanol (U.S department of energy, 2016). Ethanol production outreached about 14.3 billion gallons in 2014 annually in the United States through saccharification and fermentation of corn as the primary feedstock (Shinnosuke et al., 2016). Ethanol used as fuel in Brazil is derived from sugarcane and used either pure ethanol or gasohol (a mixture of 24% ethanol and 76% gasoline) (Marcelo et al., 2005). Bioethanol is produced from different substrates, corn, wheat and sugarcane in United States, Canada and Brazil respectively. In Europe and many Asian countries like China, India, Japan and Indonesia, bioconversion processes (saccharification and fermentation) are used to produce bioethanol from other lignocellulosic biomass such as barley, sugar beet, rice and fruit peels.

Bioethanol or ethyl alcohol is made through bioconversion processes by microorganisms has a high calorific value Bioethanol has been used as replacement for petroleum fuel in cars since early 1900's when Henry Ford designed a T-Ford model car that operates with ethanol (Graeme, 2011)..

Fruit wastes as a source of bioethanol

Fruits are one of the most utilized commodities among all horticultural crops therefore generating large amount of lignocellulosic waste in form of rinds, seeds, pomace and pulps all over the world rich in carbohydrates. Production of ethanol from fruit rinds is a technological process of obtaining bioethanol from lignocellulosic biomass through bioconversion processes which includes pretreatment of the biomass, hydrolysis of cellulose to obtain fermentable glucose sugar (saccharification), fermentation of glucose to ethanol by microorganisms (yeast or bacteria) and ethanol recovery (Wenjuan, 2010).

Many researches have been conducted on bioethanol and stated that significant amount of reducing sugars have been produced after saccharification which are subsequently or simultaneously fermented to bioethanol. Suhas et al., (2013) utilized rinds of four fruits; pineapple, jackfruit, watermelon and muskmelon for production of bioethanol by *Trichoderma viride* and fermentation by *S. cerevisiae*. Harinder et al., (2010) evaluated orange peels as fermentation feedstock for ethanol production through two-stage acid hydrolysis (primary and secondary hydrolysis) and separate fermentation employing parameters optimized through Response Surface Methodology and a two-level central composite design. A promising ethanol productivity of 3.37 g/L/h was obtained. Reddy et al., (2011) investigated the ability of dried mango peel for ethanol production, they reported mango peel contains significant amount of reducing sugars up to 40% (w/v) and produced 5.13% (w/v) of

ethanol through direct fermentation with *S. cerevisiae*. Gaddafi et al., (2016) studied bioethanol production using banana peels by three-days fermentation with *S.cerevisiae*. Bioethanol concentrations were detected by Gas Chromatography with Flame-Ionization Detection (GC-FID) with ethanol from alkaline pretreatment as the highest, 80 ppm.

Pineapple and pineapple rinds

Pineapple (*Ananas comosus*) is a terrestrial tropical plant consisting coalesced berries, it is a leading edible member from the family of *bromeliaceae* and belongs to the genus *Ananas*. It is worldwide known due to its important bioactive component such as bromelain and its industrial use for bioethanol production. (Amar et al., 2015).

Pineapple wastes such as rinds and core are generated in a vast amount which is equal to approximately 59.36% based on raw material (Vibhavee & Suchada, 2018). As of a research conducted in 2016, it stated that in each 100g of dried pineapple peels there is approximately 55g of carbohydrates (Feumba et al., 2016) it can be utilized as a good substrate for sustainable ethanol production in a cheap and simple process.

Josapine pineapple

Josapine pineapple is a locally developed cultivar of Malaysia, a hybrid of the Johor (Spanish) and Sarawak (Cayenne) pineapple which was introduced a decade ago by Malaysian Agriculture Research and Development Institute (MARDI) researchers (Mohd & Abu-Kasim, 2005). Shamsudin et al. (2007) reported that Josapine pineapple has an average weight between 1.1 - 1.3 kg with an orange-red upon ripening. Josapine pineapple is potentially produce significant amount of reducing sugars for bioethanol production. For this reason, Josapine pineapple was selected as the substrate for bioethanol production in this study.

Lignocellulose composition and structure

Pineapple peels are made up of lignocellulose carbohydrates and other carbohydrates (Saravanan, 2015; Bronwen & Philip, 1995). Adulsman et al., (2017) was conducted a research work on pineapple peels and they found out that it has cellulose $23.03 \pm 2.14\%$, hemicellulose $59.26 \pm 0.63\%$ and lignin $4.19 \pm 0.56\%$.

Cellulose is an organic compound with the chemical formula of $(C_6H_{10}O_5)_n$, it is a polysaccharide (complex carbohydrate molecule) composed of linear chain of β D-glucose molecules (β -D-glucopyranose) linked by β -1,4-glycosidic bond with cellobiose residues as the repeating unit (Mirza, 2015). Cellulose is found in plant cell walls into microfibrils (Sridhar et al., 2017; Mirza, 2015). The hydrogen bonds and crystalline structure of cellulose make it insoluble in water and most organic solvents (Suhas et al., 2016). Despite cellulose has tensile strength due to its crystalline and insolubility, it can be hydrolysed to produce fermentable sugar (D-glucose) through acid action or cellulase enzyme (Yao-Bing & Yao, 2013; Bin et al., 2011).

Hemicelluloses are group of plant cell wall polysaccharides with β -(1-4) linked backbones structure of pentose (C5) sugars such as ribose, deoxyribose, arabinose and hexose (C6) sugars such as glucose and mannose linked in equatorial configuration. Hemicelluloses are chemically heterogeneous consisting of more than one type of sugar unit and referred accordingly such as Galactoglucomannan, Arabinoglucuronoxylan and Glucocomannan (Goyal, 2015). Xyloglucans, xylans, mannans and glucomannans, and β -(1 $\rightarrow$ 3,1 $\rightarrow$ 4)-glucans are the types of hemicelluloses found in the cell walls of all terrestrial plants including pineapple plants (Scheller & Ulvskov, 2010).

Lignin is one of the compositions of lignocellulose of plants cell wall and is found in lignocellulosic biomass such as agro-industrial residues, hardwoods and paper. It is a natural polymer highly branched with phenolic acid, the polymerisation of lignin is performed mainly with three types of monolignols which are sinapyl alcohol, S unit; coniferyl alcohol, G unit and *p*-coumaryl alcohol, H unit which gives rise to syringyl

(S) unit; Guaiacyl (G) unit and *p*-hydroxyphenyl (H) unit respectively (Qingquan et al., 2018; Goyal, 2015 and Ana et al., 2016). Lignin content in pineapple peel cell walls is $4.19 \pm 0.56\%$ (Adulsman et al., 2017).

Acid saccharification

Acid saccharification is the hydrolysis or cleavage of a chemical bond of polysaccharides, complex carbohydrate molecules to produce monosaccharides, simple carbohydrate molecules with the use of protic acids. Concentrated acid hydrolysis is a process where the crystalline cellulose is completely dissolved in 72% sulfuric acid or 42% hydrochloric acid or 77-83% phosphoric acid at a lower temperature, resulting in the homogeneous hydrolysis of cellulose (Hongzhang, 2015). The hydrolysis of crystalline cellulose in dilute acid hydrolysis takes place in temperature of about 240 °C (Keith, 2010). Dilute sulfuric acid is the most commonly used acid for high efficiency cellulose hydrolysis. Diogo et al.,(2011) estimated maximum ethanol production from brazilian woods pretreated (hydrolysed) with sulfuric acid amongst other biomass hydrolysed with other mineral acids. The advantage of dilute acid hydrolysis is the usage of low concentration of acid, low impact on the environment and low raw material cost (Hongzhang, 2015).

Fermentation

Fermentation is the anaerobic decomposition of glucose molecules to produce ethanol and carbon dioxide as by products. Bioethanol production through fermentation of reducing sugars (glucose) obtained from lignocellulosic biomass is one of the most efficient technologies. Cellulose is the most abundant organic polymer on earth. It is composed in the cell wall of plant and also produced by some bacteria such as *Acetobacter xylinum* (Bela & Timothy, 2017). Fruit peels are lignocellulosic biomass constituting cellulose. Bioethanol production from fruit peels is an alternative to fossil fuels and has been successfully conducted using different fruit peels substrates such as orange peels, mango peels, watermelon peels and so on.

Ethanol fermentation occurs subsequently after hydrolysis (saccharification) of cellulose in lignocellulosic biomass to glucose in either of the two ways; SHF, where cellulose the leading polymer of lignocellulosic biomass is hydrolyzed into its monomers (glucose) and then subjected to fermentation or alternatively SSF, where both hydrolysis of cellulose in lignocellulosic biomass and fermentation of glucose to ethanol is performed at the same time (Alfani et al., 2000). Fermentation is enhanced by the metabolic activities of microorganisms such as yeast and bacteria, one of the most commonly used organisms for fermentation is *S. cerevisiae* (baker's yeast) (Simon et al., 2000). Bioethanol produced after fermentation can be recovered through so many processes such as gas chromatography, distillation and use of chemicals such as potassium dichromate method.

Separate Hydrolysis and Fermentation (SHF)

Separate Hydrolysis and Fermentation (SHF) is a process whereby saccharification is performed separately from fermentation. This process involves the enzymatic or acidic hydrolysis of cellulose in fruit peels to produce glucose molecules in a particular step, glucose produced is then subjected to fermentation at another step. Two-stage SHF was performed by Harinder et al., (2010) to produce bioethanol from orange peels. The main advantage for SHF is that both hydrolysis and fermentation processes can be performed at their optimum operating conditions hence; SHF was used for bioethanol production from PPP in this research work.

Simultaneous Saccharification and Fermentation (SSF)

Simultaneous Saccharification and Fermentation (SSF) is another process for bioethanol production from fruit rinds, in this type of process the enzymatic hydrolysis of cellulose to glucose and fermentation of the produced glucose takes place simultaneously in a single process. Reducing sugars (glucose) produced are immediately converted to ethanol by the fermenting microorganisms, in this case the effect of end-products inhibition of cellulase enzyme is overcome (Kim et al., 2008). SSF has been used to produce bioethanol from citrus and banana peels (Mark et al., 2007; Naresh et al., 2007). The major advantages of SSF are reduced process time for bioethanol production because both hydrolysis and fermentation are performed simultaneously and reduced contamination rate.

Saccharomyces cerevisiae

S. cerevisiae is a microorganism widely used as a model for biotechnology studies. It is also called by several authors as a microbial cell factory (Si et al. 2014). This cellular species has been the most used cellular organism in the last decades for the industrial production of several bioproducts, as it is considered a robust organism and well adapted to industrial conditions. In addition, several specific platforms for the *S. cerevisiae* species have been developed to allow the production of new chemicals and fuels (Hong and Nielsen 2012). *S. cerevisiae* is a eukaryotic model organism widely used in the production of bioethanol and biotechnological processes (Simon et

al., 2000). *S. cerevisiae* is a very effective in production of bioethanol through fermentation of glucose obtained from different fruit peel substrates such as citrus and banana (Mark et al., 2007; Naresh et al., 2007). Fermentation of glucose to ethanol by *S. cerevisiae* is necessarily performed in an anaerobic condition where there is absence of oxygen molecules (Ishtar & Yde, 2006).

The detection methods of fermentation are by Dinitrosalicylic acid (DNS) assay is a colorimetric method to quantify the amount of reducing sugars produced after enzymatic saccharification of pineapple, watermelon, jackfruit and muskmelon rinds (Suhas et al., 2013).

Potassium dichromate method which has been used to detect the presence of ethanol produced through saccharification and fermentation of banana, orange and pineapple peels using *Aspergillus niger* and *S. cerevisiae* respectively (Shilpa et al., 2013).

METHODOLOGY

Microorganism and culture media

Saccharomyces cerevisiae was cultured on YPD agar to revive the organisms from glycerol stock preserved at -08°C. The cultured YPD plates were incubated at 37°C for 30 hours. The cultures were stored at 4°C and subcultured every 30 days. To grow *S. cerevisiae* in broth, single colonies are picked from YPD plates using sterile loop and aseptically transferred into 50 mL YPD broth in 250 mL conical flasks. The conical flasks were incubated at 37°C with 150 rpm until organisms grow to optical density O.D₆₀₀ nm equal to 1.0 after about 11-12 hours.

Preparation of substrate

The fruit rinds of Josapine pineapple (*Ananas comosus*) were washed three times with distilled water to remove visible impurities. After washed clean, rinds were pat dried using paper towel to remove excess water and were dried in hot air oven at 65°C for 48 hours. After drying, the rinds were powdered using a grinder. The powdered rinds are the substrates used for acid saccharification.

Acid saccharification

Acid saccharification was performed in triplicate. One gram (1 g) of the processed pineapple rinds in 500 mL beakers were added with 10 mL of 72% sulfuric acid and incubated at 30°C for 1 hour. After incubation, 280 mL of distilled water was added to each beaker and autoclave at 120°C for 1 hour. The beakers were taken out, let to cool down to room temperature and samples were filtered to separate the residue (lignin). The filtrate was neutralized with calcium carbonate until the pH was adjusted to between 5 - 6. The samples were let to sit for 1 hour to allow calcium sulphate to sediment (calcium sulphate is produced as a result of chemical reaction between sulfuric acid and calcium carbonate as shown in Equation 3.1 below). Glucose produced was obtained in a second layer and the amount was determined using DNS assay.



Calcium carbonate + Sulfuric acid → Calcium sulfate + Water + Carbondioxide

(Equation 3.1)

Reducing sugar assay

Reducing sugar assay to determine the amount of glucose produced after acid saccharification was done using DNS method as previously used by Marisa et al., in 2017 with slight modification. Distilled water was used as the control for the assay. From the triplicate beakers after acid saccharification, 200 µl of glucose produced was aliquoted from each beaker and placed in microcentrifuge tubes, 200µl of DNS reagent and 20µl of 1 N sodium hydroxide were added to the tubes. The tubes were vortex to mix well and heated together with the control in boiling water for 5 minutes. Eight hundred (800) µl of distilled water was added to all the tubes. The optical densities of the samples were measured against the blank at 540 nm. The glucose concentration was then calculated using the formula (Equation 3.2) from glucose standard curve. Amount of glucose produced is reported in the results section.

$$y = 0.1309x + 0.1241$$

(Equation 3.2)

Batch Fermentation of Josapine pineapple syrup

After reducing sugar assay, the amount of glucose in volume required to obtain 1.0 g of glucose was calculated for each replicate, amount vary due to different glucose concentration per replicate. Fermentation was carried out in triplicate using 3 pairs of autoclaved fermentation set-up. Each fermentation set-up consists of 500 mL air exclusion flask added with 200 mL of distilled water covered with a visible layer of vegetable oil, and a 500 mL fermentation flask. Fermentation was executed with 200 mL working volume; each fermentation flask was added with its respective amount of glucose and adjusted to 188 mL with yeast extract and peptone solution. The fermentation flasks were then inoculated with 12 mL overnight grown *S. cerevisiae* at O.D₆₀₀ nm equal to 1.0. All the conical flasks were sealed completely with aluminium foil to induce anaerobic condition for efficient fermentation and incubated at 30°C with 150 rpm for the first 24 hours and sampling after every 12 hours until 72 hours (3 days). Sampling obtained after every 12 hours were used for glucose residual determination and ethanol assay. Same fermentation procedure was repeated using 37°C and 40°C, all in triplicate.

Glucose residual determination

Glucose residual determination wNJNas performed through series of sampling after every 12 hours during fermentation until after 72 hours (3 days). For each of the replicate 300 µl of the fermentation mixture is aliquoted into microcentrifuge tubes and centrifuged at 10,000 ×g for 30 minutes. Two hundred (200) µl of the supernatant were transferred to new microcentrifuge tubes and glucose residues were evaluated by DNS assay.

Dinitrosalicylic acid (DNS) assay is a colorimetric method for estimation of reducing sugars containing free carbonyl group such as glucose. The principle of DNS assay is oxidation reduction (redox) reaction, when 3,5-dinitrosalicylic acid reacts with glucose molecules, the free carbonyl group of glucose becomes oxidized and gluconic acid is formed while 3,5-dinitrosalicylic acid is simultaneously reduced to 3-amino-5-nitrosalicylic acid with red-brown colour. The intensity of the colour is an index of reducing sugar, the more amounts of glucose molecules in sample the more intense the colour becomes. Suhas et al., used DNS method to quantify the amount of reducing sugars produced after enzymatic saccharification of pineapple, watermelon, jackfruit and muskmelon rinds (Suhas et al., 2013).

Ethanol assay

Ethanol produced during fermentation was estimated using potassium dichromate method as used by Nitesh et al., (2013) with slight modification. Sampling was performed every 12 hours until the end of the fermentation process (after 72 hours). During each sampling, 1100 µl of the fermentation mixture from each replicate were aliquoted to microcentrifuge tubes and centrifuged at 10,000×g for 30 minutes. One thousand (1000) µl of the supernatant were transferred to fresh microcentrifuge tubes and added with 300 µl of chromic acid. The mixtures were then heated for 10 minutes in a water bath set at 90°C. 100 µl of 40% potassium sodium tartrate (Rochelle salt) were added. The absorbance of the solutions was measured at 600 nm. Amount of ethanol produced was calculated using the formula (Equation 3.3) from ethanol standard curve (Figure 2).

$$y = 0.3138x + 0.0173$$

(Equation 3.3)

3.2.8 Analytical methods

The results in this experiment obtained from triplicate were expressed as mean ± standard deviation. Glucose and ethanol amounts were determined using glucose standard curve and ethanol standard curve respectively. The data has been analysed using Microsoft Excel Software. Statistical analysis was carried out using Two-way ANOVA of Analysis ToolPak program in Microsoft Excel Software to examine the effect of temperature and incubation period on bioethanol production. Fermentation efficiency was calculated as:

Ethanol produced / Theoretical maximum ethanol yield from sugar × 100% Theoretical maximum ethanol yield = 0.51 g ethanol per gram glucose

4. Results & Discussion

Macroscopic and microscopic observation of *S. cerevisiae*

S. cerevisiae showed flat, moist and cream colonies on YPD agar plates after 2 days of incubation at 25°C. The morphology of cells viewed under light microscope showed most of the cells shaped in pear-like schmoo morphology, a small ovoid

shaped bud (daughter cell) projected from the surface of another cell (parent cell), while some of the cells are ovoid shaped without buds. They are flat, smooth, moist, glistening or dull, and cream in color. Microscopic observation revealed *S. cerevisiae* cells are elliptical to ovoid in shape, with some spherical and elongated cells as well.

4.2 Acid saccharification of biomass and reducing sugar assay

This research study was aimed at producing bioethanol from Josapine PPP through acid saccharification and batch fermentation with *S. cerevisiae*. Josapine pineapple peel is a lignocellulosic biomass naturally composed of carbohydrates such as cellulose. Cellulose, the main structural component of plants cell wall is a polymer of glucose molecules which can be broken down enzymatically or chemically to obtain glucose monomers. Acid saccharification of Josapine PPP was conducted to produce glucose which was later fermented to bioethanol. The saccharification process was carried out in a highly acidic condition at low temperature, 72% w/w sulfuric acid at 30°C for 1 hour.

In this condition, cellulose is broken down to cellotetraose (four-glucose polymers), changing the solution to very intense brown colour as shown in Figure D.1. The solution was added with water and autoclaved at 120°C for 1 hour. This is a pretreatment step which is basically to enhance rapid and efficient hydrolysis and digestibility of cellotetraose to produce glucose (fermentable sugar) (Hendricks & Zeeman, 2009; Joseph & Ronald, 2010). Immediately after acid saccharification, reducing sugar assay using DNS method (Section 3.2.4) was performed to determine the amount of glucose produced from Josapine PPP. Glucose is a reducing sugar; it is capable of acting as a reducing agent because it has a free carbonyl group in its structure. DNS assay is a colorimetric method which tests the presence of reducing sugar such as glucose in oxidation reduction reaction where glucose serves as a reducing agent while 3,5-dinitrosalicylic acid serves as an oxidizing agent. This involves oxidation of the free carbonyl group of glucose to produce gluconic acid while simultaneously reducing 3,5-dinitrosalicylic acid to produce 3-amino-5-nitrosalicylic acid. During reducing sugar assay in this experiment, before DNS reagent was added, the colour of glucose was yellow to orange. After DNS reagent was added and heated for 5 minutes in boiling water the colour changed to red-brown. This indicates the presence of glucose which was oxidized to gluconic acid by 3,5-dinitrosalicylic acid.

The absorbance of the glucose samples was measured at 540 nm and recorded (Table 4.1). The amount of glucose produced was calculated using (Equation 3.2) obtained from glucose standard curve (Figure 4.6). Glucose amount was found to be 10.31 mg/mL, which was obtained from the average of six replicates as illustrated in Table 4.2.

Table 4.1 Absorbance readings of glucose samples at 540 nm.

Samples	Reading 1	Reading 2	Reading 3	Mean
Replicate 1	1.4098	1.4212	1.4184	1.4164
Replicate 2	1.3781	1.3884	1.3845	1.3836
Replicate 3	1.3928	1.3943	1.3930	1.3933
Replicate 4	1.6038	1.6076	1.6063	1.6059

Replicate 5	1.5604	1.5666	1.5614	1.5628
Replicate 6	1.4831	1.4212	1.4184	1.4840

$$\text{Equation 3.2: } y = 0.1309x + 0.1241$$

Where y refers to mean of absorbance at 540 nm and x refers to glucose amount.

$$\text{Replicate 1: } x = \frac{1.4164 - 0.1241}{0.1309} = 9.87 \text{ mg/mL}$$

$$0.1309$$

$$\text{Replicate 2: } x = \frac{1.3836 - 0.1241}{0.1309} = 9.62 \text{ mg/mL}$$

$$0.1309$$

$$\text{Replicate 3: } x = \frac{1.3933 - 0.1241}{0.1309} = 9.70 \text{ mg/mL}$$

$$0.1309$$

$$\text{Replicate 4: } x = \frac{1.6059 - 0.1241}{0.1309} = 11.32 \text{ mg/mL}$$

$$0.1309$$

$$\text{Replicate 5: } x = \frac{1.5628 - 0.1241}{0.1309} = 10.99 \text{ mg/mL}$$

$$0.1309$$

$$\text{Replicate 6: } x = \frac{1.4840 - 0.1241}{0.1309} = 10.39 \text{ mg/mL}$$

$$0.1309$$

Table 4.2 Amount of glucose produced from Josapine PPP

Samples	Absorbance at 540 nm	Glucose amount	Average glucose amount
Replicate 1	1.4164	9.87 mg/mL	8.846/6 = 10.31 mg/mL
Replicate 2	1.3836	9.62 mg/mL	
Replicate 3	1.3933	9.70 mg/mL	
Replicate 4	1.6059	11.32 mg/mL	
Replicate 5	1.5628	10.99 mg/mL	
Replicate 6	1.4840	10.39 mg/mL	

Amount of glucose produced from Josapine PPP (10.31 mg/mL) is similar to that obtained by Suhas et al., (2013). Who reported significant amount of reducing sugar, 10.18 mg/mL was obtained after 6 days enzymatic saccharification of pineapple peels with *Trichoderma viride*. However, the amount of glucose produced from pineapple peel as reported by Omojasola et al., is significantly lower than that produced in this experiment. Based on their results, the highest amount of glucose produced among cultures was 0.92 mg/0.5mL, obtained from *Trichoderma*

longibranchiatum (Omojasola et al., 2008). Differences in glucose production can be ascribed to the different species of fruit rinds, organisms and methods used.

4.3 Effect of temperature on bioethanol production

Temperature is one of the most important factors affecting bioethanol fermentation; it has profound effects upon microorganisms. Bioethanol fermentation is a bioconversion process accomplished using organisms such as *S. cerevisiae* in an enzyme-catalyzed reaction. Enzyme-catalyzed reactions are sensitive to small changes in temperature and because of this, the metabolism of *S. cerevisiae*, a *poikilothermic* organism whose internal body temperature is determined by its environment, is often determined by the surrounding temperature. In this research work, the effect of incubation time and three different temperatures, 30, 37 and 40°C were tested on bioethanol production using *S. cerevisiae*. Potassium dichromate method was used as the detection method for ethanol assay as mentioned in section 3.2.7. Ethanol yield at different incubation time using all three temperatures was calculated using (Equation 3.3) obtained from ethanol standard curve (Appendix E.4). Ethanol yield and fermentation efficiency for all temperatures are reported in Table 4.3.

$$\text{Equation 3.3: } y = 0.3138x + 0.0173$$

Where y refers to absorbance at 600 nm and x refers to ethanol yield.

Fermentation efficiency:

Ethanol produced / Theoretical maximum ethanol yield from sugar $\times 100\%$

Theoretical maximum ethanol yield = 0.51 g ethanol per gram glucose

Table 4.3: Effect of temperature and incubation period on ethanol yield and fermentation efficiency

Temperature	Incubation period	Ethanol yield	Fermentation efficiency
30°C	12 hours	0.284 g/g	55.68%
	24 hours	0.363 g/g	71.17%
	36 hours	0.394 g/g	77.25%
	48 hours	0.44 g/g	86.27%
	60 hours	0.384 g/g	75.29%
	72 hours	0.369 g/g	72.35%
37°C	12 hours	0.335 g/g	65.68%

	24 hours	0.362 g/g	70.98%
	36 hours	0.396 g/g	77.64%
	48 hours	0.338 g/g	66.27%
	60 hours	0.324 g/g	63.52%
	72 hours	0.263 g/g	51.56%
40°C	12 hours	0.343 g/g	67.25%
	24 hours	0.356 g/g	69.80%
	36 hours	0.421 g/g	82.54%
	48 hours	0.395 g/g	77.45%
	60 hours	0.363 g/g	71.17%
	72 hours	0.307 g/g	60.19%

The effect of temperature with three levels of sample groups, 30°C, 37°C and 40°C and incubation period with six levels of sample group, 12, 24, 36, 48, 60 and 72 hours were statistically tested using Two-way ANOVA in Excel 2013 (Appendix F). The main effect of temperature on bioethanol production was measured separately by the analysis of any significant difference among the means of its three levels of sample group. From the output results of Two-way ANOVA using Excel 2013 (Table 4.4), the main effect of temperature is referred to as “sample”. The F-value (F) (a measure of significant difference between the means of different samples) of temperature variable, 6.58997 is more than its respective F-critical value (F_{crit}), 3.259446 for a significance level of 0.05. This indicates at least one temperature among the three different temperatures tested for bioethanol production in this experiment is significantly different from the two others, it belongs to a totally different population. Therefore, the null hypothesis which states temperature has no profound effect on bioethanol production is rejected. Temperature has a statistical significant effect on bioethanol production.

Table 4.4: Results of Two-way ANOVA in Excel 2013

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0.0129362		0.006468	6.58997	0.003641	3.259446

Columns	0.059438	5	0.011888	12.11176	6.4E-07	2.477169
Interaction	0.032857	10	0.003286	3.347609	0.003622	2.106054
Within	0.035334	36	0.000982			
Total	0.140565	53				

The results in Figure 4.1 indicates that maximum ethanol yield of 0.44 g/g was produced at temperature of 30°C. The ethanol yield increased with increase in incubation period up to 48 hours of incubation after which it reduced, this is in accordance with the discovery of Naresh et al., (2007). Who reported maximum ethanol production from kinnow waste and banana peels using co-cultures of *S.cerevisiae* and *Pachysolen tannophilus* at 30°C after 48 hours of incubation. Verma et al., (2000) also reported incubation of 48 hours at 30°C as the condition for maximum ethanol production during his research study on fermentation of raw un-hydrolysed starch by a co-culture of *Saccharomyces diastaticus* and *S. cerevisiae*. Maximum ethanol production from Josapine PPP was obtained at 30°C after 48 hours of incubation. At higher temperatures, 37 and 40°C ethanol production increased with increase in incubation time up to 36 hours before it begin to reduce. The reduction of ethanol production is probable to be due to toxicity of accumulated ethanol on yeast cells. Elsayed et al., Reported reduction of ethanol production after 3 days of solid state fermentation using potato waste substrate which he related the condition to toxic effect on growth of yeast cells (Elsayed et al., 2015).

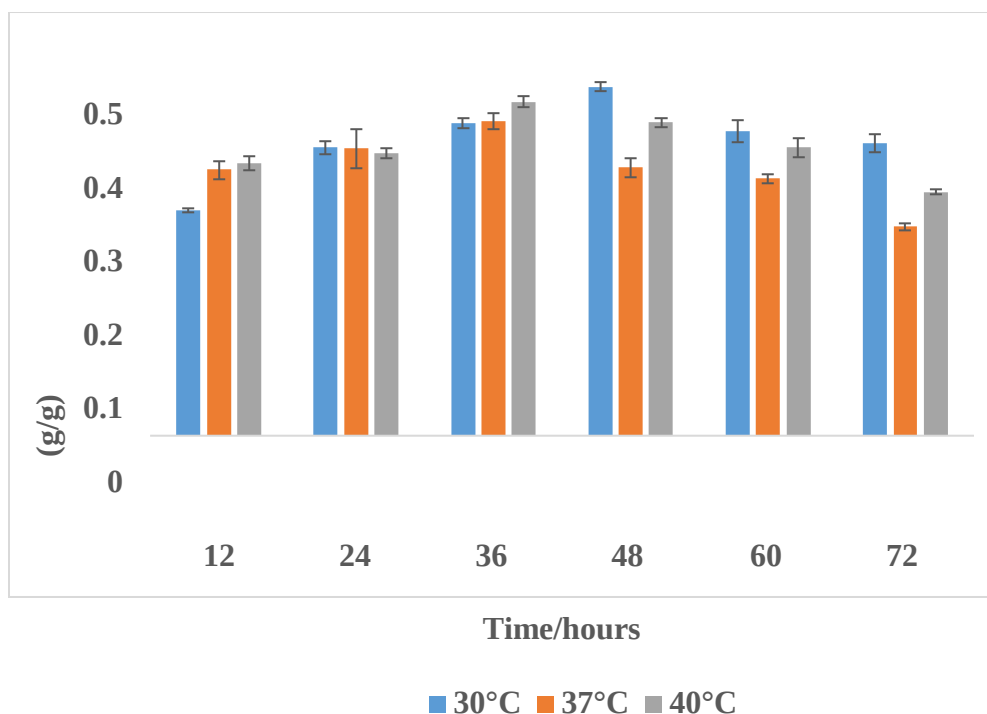


Figure 4.1: Effect of different temperature and incubation period on ethanol yield. Ethanol yield oreach temperature at each incubation period was calculated based on ethanol standard curve. The amount shown for each ethanol yield is mean of triplicate expressed as mean $\pm$ standard deviation

4.4 Effect of incubation period on bioethanol production

The effect of incubation period on bioethanol production from Josapine PPP was tested in this research work, where incubation period is the independent variable against bioethanol production, the dependent variable. Bioethanol fermentation was conducted for a total duration of 3 days (72 hours) with sampling after each 12 hours to check the amount of ethanol produced and how incubation period affected the yield. Incubation period is statistically proven to have effect on bioethanol production from Josapine PPP using two-way ANOVA. The main effect of incubation period was measured by analysis of any significant difference among the means of its six levels of sample groups. It is referred to as “columns” in the output result (Appendix F). The F-value of incubation period, 12.11176 is more than the F-critical value, 2.477169 for a significance level of 0.05. This signifies that ethanol production differs with incubation period. In other words, different amount of ethanol was obtained at different incubation period. Thus, the null hypothesis is rejected. Incubation period has significant effect on ethanol production.

In accord with data in Figure 4.1, the most effective incubation period with maximum ethanol production is 48 hours at temperature 30°C ethanol yield of 0.44 g/g and fermentation efficiency of 86.27% (Table 4.3). El-Refai et al., (1992) studied maximum ethanol productivity attained after 48 hours incubation at 30°C using mud-free, H₂SO₄-treated beet molasses for fermentation by *S. cerevisiae* Y-7.

4.5 Interaction effect of temperature and incubation period on bioethanol production

Temperature and incubation period are the two independent variables tested on bioethanol production, the dependent variable. Temperature has three levels of sample group, 30, 37 and 40°C while incubation period has six levels of sample group, 12, 24, 36, 48, 60 and 72 hours. Statistical analysis was carried out using Two-way ANOVA which measured the main effect of temperature and incubation time on bioethanol production separately as well as the interaction effect of both factors on bioethanol production. Measuring the main effects of temperature and incubation period on bioethanol production separately involves calculating any significant difference among the means of the three levels of sample group for temperature and six levels of sample group for incubation period. The main effect of temperature and incubation period demonstrated both factors have statistical significant effect on ethanol production as mentioned earlier.

The interaction effect of the Two-way ANOVA analysis is where both temperature and incubation time were considered at the same time. This was accomplished using not three or six levels sample group of temperature and incubation time respectively but, using eighteen levels of sample group based on different permutations of temperature and incubation period and each of the eighteen different groups has a sample size of three as shown in contingency table (Appendix F.1). According to the output result of Two-way ANOVA, the F-value of interaction effect between

temperature and incubation period, 3.347609 is more than its respective F-critical

value, 2.106054 for a significance level of 0.05. This means that temperature and incubation period have combined effect on bioethanol production, the two factors have effect on each other and interaction effect between the two factors on ethanol production exists. Thus, the null hypothesis is rejected.

4.6: Glucose content at different temperature and incubation time

Glucose has direct effect on fermentation and microbial cells. The initial glucose concentration used throughout fermentation at different temperature in this experiment was standardized to 1 g. During fermentation, sampling was obtained after every 12 hours until 72 hours, glucose content during each sampling was estimated using DNS method.

At 30°C, massive fall-off of glucose was observed after 48 hours of incubation which corresponds with the incubation time at which maximum ethanol production was recorded for that particular temperature as shown in Figure 4.2. Glucose content continues to reduce with increase in incubation time before the fermentation process was terminated at 72 hours. At 37°C, 36 hours of incubation was prominent for glucose reduction, simultaneously highest amount of ethanol was yielded at the same incubation time as reported in Figure 4.3. Glucose content completely declined to 0.1 mg/mL at 72 hours of incubation. Glucose reduction at 40°C was consistent before it begins to diminish to a concentration below 1.0 mg/mL after 36 hours of incubation as illustrated in Figure 4.4. The maximum ethanol yield at 40°C is at 36 hours of incubation.

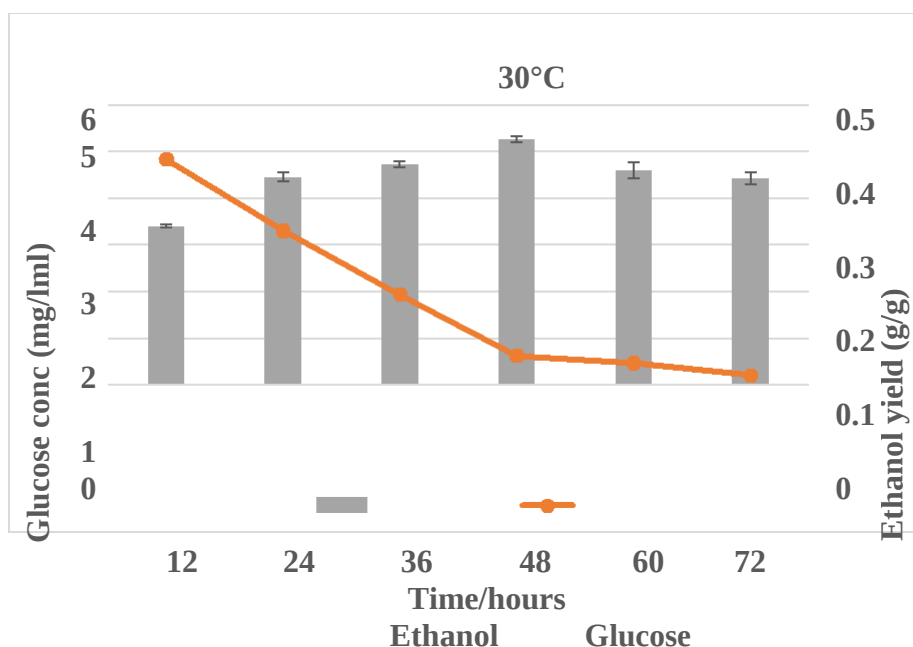


Figure 4.2: Glucose content during fermentation at 30°C

37°C

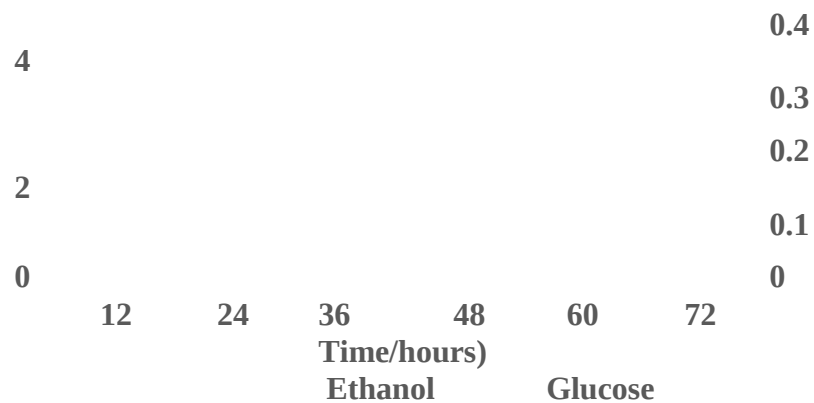


Figure 4.3: Glucose content during fermentation at 37°C

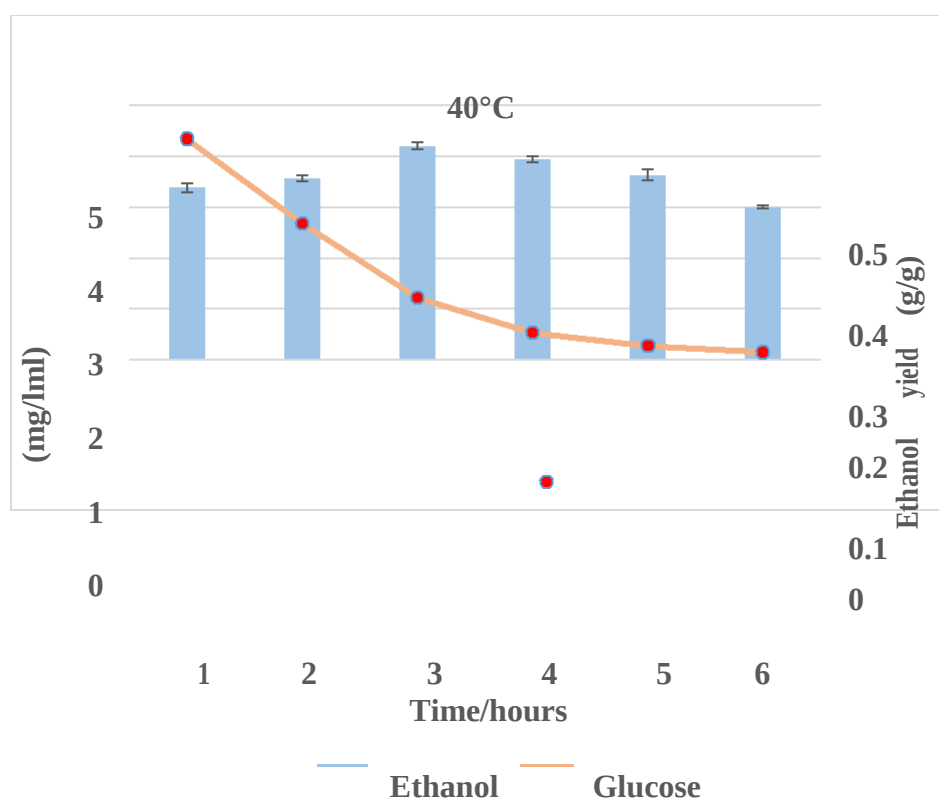


Figure 4.4: Glucose content during fermentation at 40° C

CONCLUSION AND RECOMMENDATION

This research study aimed at production of bioethanol from Josapine pineapple waste could establish that the substrate could be used effectively for bioethanol production through concentrated sulfuric acid saccharification and fermentation with *S.cerevisiae*. It could as well show a great way of minimizing and managing pineapplefruit waste in a sustainable way.

The objectives of this research work were achieved. Amount of glucose produced by Josapine PPP was determined; 10.31 mg/mL of glucose was obtained per gram of

PPP. Statistical analysis on the effect of temperature and incubation period on bioethanol production revealed the two factors have main effect as well as interaction effect on bioethanol production. Optimum temperature for bioethanol production from Josapine PPP by acid saccharification and fermentation with *S. cerevisiae* was found to be 30°C with maximum ethanol yield of 0.44 g/g and fermentation efficiency of 86.27% at 48 hours of incubation.
QQQ.

It would be of great advantage if further research work on bioethanol production using Josapine pineapple waste is carried out after determining the chemical composition of the substrate, to know the amount of carbohydrates it contains especially cellulose which is the main carbohydrate involved in glucose production.

Other fermentation parameters such as agitation, pH, different substrate and inoculum concentration could be tested to discover their effect and to conclude optimum condition for bioethanol production using Josapine pineapple substrate. Other procedures could also be established such as SSF, or the use of different strains of microorganisms for enzymatic saccharification instead of acidic, to discover which method produces higher glucose yield resulting to higher bioethanol production.

Glucose standard curve

Glucose standard curve was prepared using DNS method as described previously. Glucose solutions of different concentrations obtained by dilutions from a stock solution of 15 mg/mL were prepared as shown in (Table 1). The absorbance readings of the solutions at 540 nm were measured. Glucose standard curve was prepared in triplicate, the average readings were used to plot graph as well as calculating the standard deviation.

Table 4.5: Dilutions for glucose standard curve

Solution samples	Volumes of glucose stock (15 mg/mL)	Volumes of distilled water	Total volume

Blank	0 μl	200 μl	200 μl
1	20 μl	180 μl	200 μl
2	40 μl	160 μl	200 μl
3	60 μl	140 μl	200 μl
4	80 μl	120 μl	200 μl
5	100 μl	100 μl	200 μl
6	120 μl	80 μl	200 μl
7	140 μl	60 μl	200 μl
8	160 μl	40 μl	200 μl
9	180 μl	20 μl	200 μl

10	200 μ l	0 μ l	200 μ l
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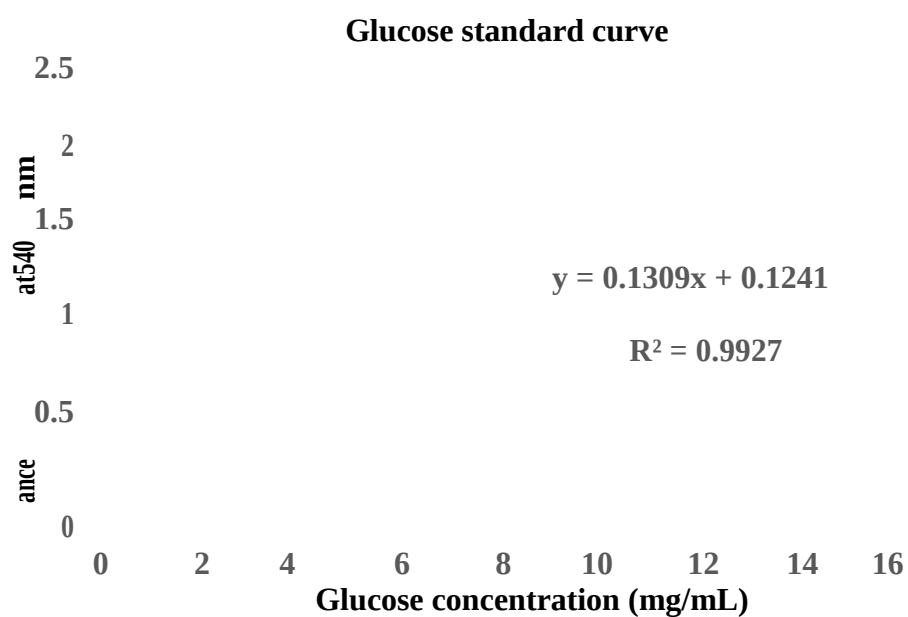
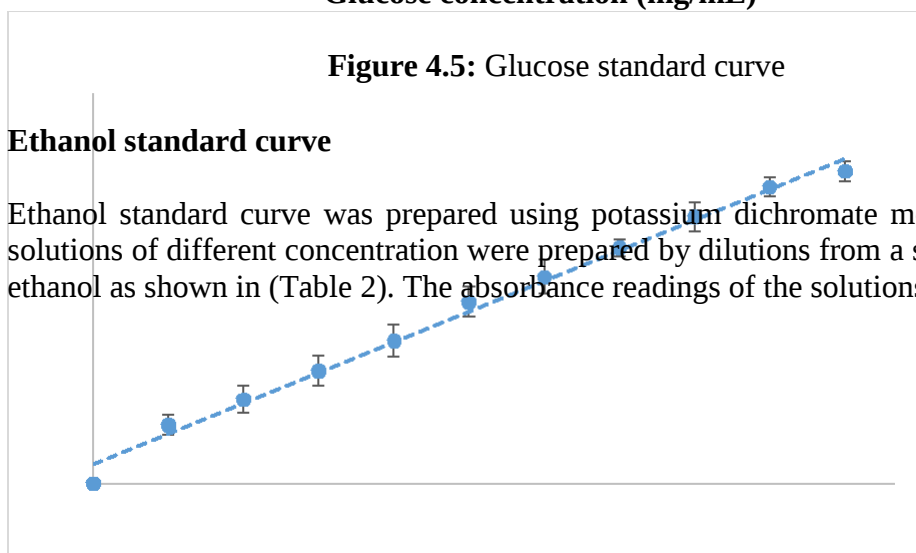


Figure 4.5: Glucose standard curve

Ethanol standard curve

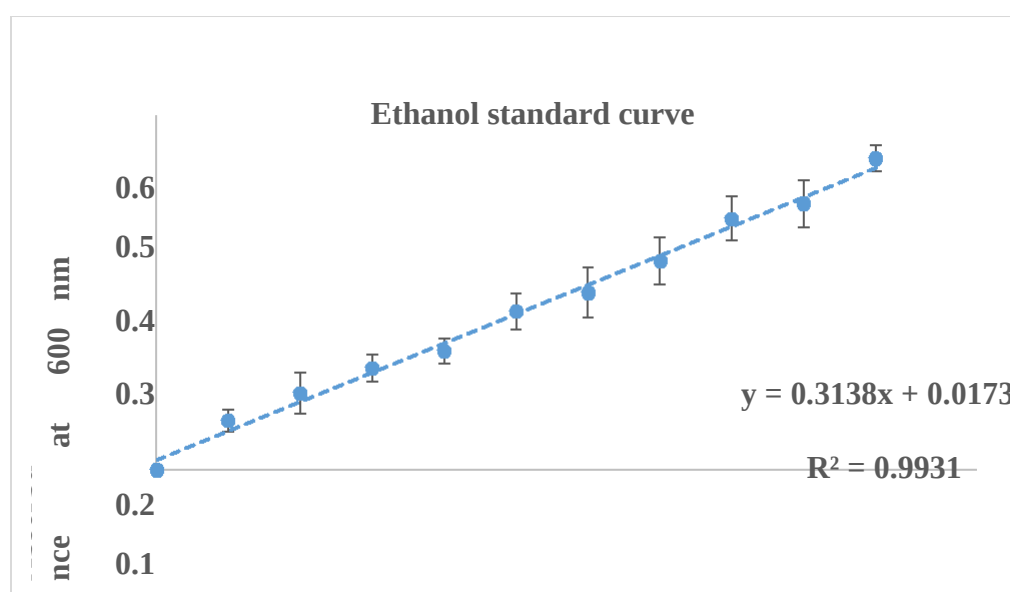
Ethanol standard curve was prepared using potassium dichromate method. Ethanol solutions of different concentration were prepared by dilutions from a stock of 99.8% ethanol as shown in (Table 2). The absorbance readings of the solutions at 600 nm



were measured. Ethanol standard curve was prepared in triplicate, the average readings were used to plot graph as well as calculating the standard deviation (Figure 2).

Table 4.6: Dilutions for ethanol standard curve

Solution samples	Volumes of ethanol (99.8%)	Volumes of distilled water	Total volume
Blank	0 mL	10 mL	10 mL
1	0.2 mL	9.8 mL	10 mL
2	0.4 mL	9.6 mL	10 mL
3	0.6 mL	9.4 mL	10 mL
4	0.8 mL	9.2 mL	10 mL
5	1.0 mL	9.0 mL	10 mL
6	1.2 mL	8.8 mL	10 mL
7	1.4 mL	8.6 mL	10 mL
8	1.6 mL	8.4 mL	10 mL
9	1.8 mL	8.2 mL	10 mL
10	2.0 mL	8.0 mL	10 mL



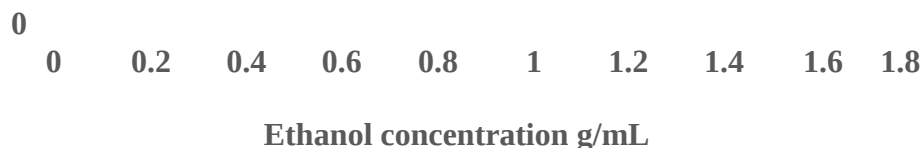


Figure 4.6: Ethanol standard curve

Table 4.7: Output of Two-way ANOVA using Excel 2013

Anova: Two-Factor With Replication

SUMMARY	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr	Total
<i>30° C</i>							
Count	3	3	3	3	3	3	18
Sum	0.852	1.089	1.184	1.32	1.154	1.108	6.707
Average	0.284	0.363	0.394667	0.44	0.384667	0.369333	0.372611
Variance	6.7E-05	0.000652	0.000386	0.000343	0.002025	0.001233	0.00287
<i>37° C</i>							
Count	3	3	3	3	3	3	18
Sum	1.005	1.086	1.188	1.015	0.972	0.791	6.057
Average	0.335	0.362	0.396	0.338333	0.324	0.263667	0.3365
Variance	0.001381	0.005979	0.000967	0.000734	0.000304	0.000184	0.002828
<i>40° C</i>							
Count	3	3	3	3	3	3	18
Sum	1.03	1.07	1.263	1.187	1.091	0.921	6.562
Average	0.343333	0.356667	0.421	0.395667	0.363667	0.307	0.364556

Variance	0.000646	0.00039	0.000523	0.000325	0.001408	0.000117	0.001809
<i>Total</i>							
Count	9	9	9	9	9	9	
Sum	2.887	3.245	3.635	3.522	3.217	2.82	
Average	0.320778	0.360556	0.403889	0.391333	0.357444	0.313333	
Variance	0.001297	0.001764	0.000634	0.002299	0.001646	0.0025	
ANOVA							
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>	
Sample	0.012936	2	0.006468	6.58997	0.003641	3.259446	
Columns	0.059438	5	0.011888	12.11176	6.4E-07	2.477169	
Interaction	0.032857	10	0.003286	3.347609	0.003622	2.106054	
Within	0.035334	36	0.000982				
Total	0.140565	53					

Table 4.8: Contingency table for Two-way ANOVA in Excel 2013

Factor 1	Factor 2					
Temperature	Incubation period (hours)					
	12	24	36	48	60	72
30°C	0.291	0.341	0.407	0.426	0.366	0.336
	0.286	0.357	0.405	0.461	0.352	0.336
	0.275	0.391	0.372	0.433	0.436	0.406
37°C	0.3	0.375	0.43	0.31	0.336	0.251
	0.331	0.279	0.369	0.364	0.304	0.278
	0.374	0.432	0.389	0.341	0.332	0.262
40°C	0.371	0.378	0.412	0.413	0.362	0.31
	0.321	0.339	0.404	0.377	0.402	0.316
	0.338	0.353	0.447	0.397	0.327	0.295

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