Microbial Journal

Bio-Ethanol Production from Wheat Straw Using Yeast Isolates

Mesfin Wondaferew[1], Asmamaw Tesfaw[2]

[1]Department of Biology, Debre Berhan University, Email meswon27@gmail.com
[2]Department of Biology, Debre Berhan University, email asmamawtesfawbelay2004@gmail.com

DOI: https://doi.org/10.59411/jhp3pb09

How to Cite:
Wondaferew, M., & Tesfaw, A. (2023). Bio-Ethanol Production from Wheat Straw Using Yeast Isolates. *Microbial Journal*, *3*(1). https://doi.org/10.59411/jhp3pb09

Abstract

In the first hand, the cost of fossil fuel is increasing alarmingly. On the second hand, combustion of fossil fuels contributes for global warming. Therefore, it need to strength the production of renewable energies. The aim of this study was to produce bioethanol from wheat straw using yeast isolates. The isolates were isolated from decomposed soil, termite soil and rotten wood samples using yeast extract peptone dextrose media (YPD) and characterized chemically and morphologically. The wheat straws were powdered and hydrolyzed with dilute sulfuric acids. After neutralization, it was used to produce ethanol. Response surface methodology was employed to optimize the ethanol production process from wheat straws. The isolates were grown optimally at a temperature of 30^{o}C, pH nearly 5, and sugar concentration 70 to 120 g/L. Among hydrolysis conditions, lower acid concentration (less than 1.5%) and temperature of 60^{o}C resulted higher reducing sugars. The optimization study showed that the highest bio-ethanol concentration of 6.8g/l was observed by SWX under the optimum conditions of with 1% H2S04, 60oC temperature and 52.5-minute time hydrolysis at 30°C for 48 hour incubation time. Wheat straws could be good candidate for ethanol production.

Key words: Ethanol, Fossil fuel, Global warming

Microbial Journal

1. Introduction

Nowadays world primary energy source is dominated by fossil fuels (coal, crude oil, oil and natural gas), mainly biofuels, hydropower, geothermal, wind and solar energy are the renewable energy sources; currently represent less percentage of the primary energy use. In contract the energies play a great comprising role as an energy source and lower supplies of the world energy consumption. However, ethanol can be produced from several biomasses of plant derived materials, agricultural crops and trees, wood and wood residues, municipal residues and other waste materials (Adeeyo *et al*., 2015).

Unlike bioethanol, fossil fuels (such as coal, natural gas, and oil) are not only finite resources, their consumption having environmental impacts. Variety of chemicals such as, carbon dioxide, nitrogen oxides, heavy metals, sulfur dioxide, and volatile organic compounds release into the air. However most of the renewable energies have little environmental impact and in most cases their social impacts is very low (sun and change, 2002). In other hands, reducing the use of fossil fuels also is reducing the amount of carbon dioxide produced, as well as reduces the levels of pollutants (Demirbas, 2005).

Production of bioethanol involves the step of pretreatment, hydrolysis, fermentation, and distillation. Additionally, pretreatments can be physical (downsizing), physic-chemical (liquid hot water, steam explosion, ammonia fiber explosion), chemical (acids, alkaline, oxidative alkaline, wet oxidation, ozonolysis) and biological pretreatments (Silvaa *et al*., 2016).

Bacterial, yeast and filamentous fungi able to ferment pentose; however it is conducted by many microorganisms. The most promising yeast species identified so far, are *Candida shehatae*, *Pichia stipites* and *Pachysolen tannophilus (*Kuhad *et al.*, 2011). *P. stipites* yeast mainly used to produce ethanol from many of the sugars found in lignocellulosic material. However, almost one-third of the reducing sugars obtained from hydrolyzed lignocellulosic materials are pentose, it composed primarily of xylose (Ali *et al.*, 2012). It is a native xylose-fermenting (the most abundant sugar in hemicelluloses) yeast that can yield up to 0.42-0.47 gram of ethanol per gram of substrate used (Ali *et al*., 2012).

Microbial Journal

The increasing industrialization and motorization of the world has led to a steep rise for the demand of petroleum-based fuels. Today fossil fuels take up 80% of the primary energy consumed in the world, of which 58% is consumed by the transport sector. The sources of these fossil fuels are becoming exhausted and found major contribution in greenhouse gas (GHG) emissions by consumption of fossil fuels to fulfill the energy demand, which leads to many negative effects including climate change, retreating of glaciers, rise in sea level, and loss of biodiversity(Chan *et al*., 2007).

Ethiopia imports ethanol products for its fuel requirements, and the demand for fuel is rapidly increasing, which is associated with its growing economy and expanding infrastructure. Due to be paid to such phenomenon, and indeed in view of the recent trends in the increase price of the traditional petro-fuel, biofuel has been gaining greater attention by the Ethiopian government. Most countries depend mainly, in some cases almost completely, on fossil fuels (Nikolić *et al*., 2016). Thus, security of petroleum supply or other sources of energy which can replace petroleum is critical for Ethiopia to diversify the energy. However, the future of petroleum products reserve is uncertain with increase in price that makes the foreign currency expenditure intolerably high and affect transport tariff and price of other commodities negatively. Moreover, due to environmental concerns about air pollution caused by the combustion of fossil fuels, the search for alternative fuels will gain importance.

2. Materials and Methods

2.1. Sample Collection and Preparation

In this study wheat straw used as a substrate to produce ethanol. It was collected from local farmers of Moretina Jiru. The Samples used for this study was prepared in Debre Berhan university microbiology laboratory. 3kg of wheat straw was washed in order to remove unwanted matter and dried at 60^{o}C for 24h until the weight remain constant . Then the dried sample was sieved and milled the over size in to appropriate particle size which is less than 1mm.

2.2. Moisture Content Determination

Microbial Journal

The moisture content of the samples was determined by oven drying method. The samples were weighed with glass crucible and placed in the air drying oven for 24 hr. at 60°C and cooled to room temperature and weighed. The process was repeated until constant weights was achieved and made it free of moisture content. The moisture content was calculated as follows:

$$\text{Moisture Content (\%)} = \frac{W2-W3}{W2-w1} \times 100$$

Where: W_1 = mass of the sample container in gram

W_2 = mass in gram of sample + sample container before drying

W_3 = mass in gram of sample + sample container after drying

2.3. Isolation of yeasts

Plant decomposed soil, termite soil and rotten wood samples were collected from Ankober, sheep dung compost in Debre Berhan sheep breeding from three sites. Six fruit samples (mango, avocado, banana, orange, papaya and pine apple) were collected from local market; six soil samples were from Debre Sina; three samples of animal dung from Debre Berhan University. The samples were stored in sterile plastic bags and transported under aseptic to the laboratory within 24 h. The samples were serially diluted and mixed using vortex. The YPXA and YPAA media used for isolation of yeast in 300 mL of flask by compose of yeast extract (1%), peptone (2%), xylose/arabinose (1%) and agar (2%). The media was sterilized at 121^{o}c for 15 min. From serially diluted samples, 0.1 ml was inoculated to the media and incubated at 30^{o}C for 3 days.

2.4. Yeast purification

To obtain pure yeast, each of emerging colonies was streaked aseptically to fresh yeast extract, peptone, xylose and agar (YPXA). Then, it was kept in the refrigerator at 4^{0}C for further study.

2.5. Yeast Isolates Screening (test of CO_2 production)

Screening was done by using xylose and arabinose containing fermentation. Fermentation test was made using Durham tube and the effective four isolates were selected. The yeast isolates were identified for the production of CO_2 within 24 hours (El-Banna *et al.*, 2012).

2.6. Morphological identification

The morphologic characteristics of the isolated yeasts were examined after growth on yeast peptone xylose agar (YPX) media at 30°C for 48hr, its colony morphology, form, size, elevation, margin/edge; colony color was observed using hand lens and microscope. A sample of yeast was mixed in a droplet of sterile distilled water on glass slide and smeared until the smear dried off. The smear was then stained using diluted methylene blue dye, air dried and observed under light microscope.

2.7. Ethanol tolerances

The ability of isolates to tolerate various concentrations of alcohol was tested. To determine ethanol tolerance capacity of isolate, yeast isolates were inoculated into 100 ml YPX broth with 5, 10, 15% and 20% (v/v) ethanol and incubated at 30°C for 72 h (Osho,2005).

2.8. Effect of Temperature on Yeast Growth

The effect of temperature on yeast biomass yield of the four isolates was tested. The process of propagation was undertaken at temperatures of 20^0C, 30^0C, 40^0C and 50^0C using 20% w/v media. The pH of the medium was maintained at 5. Yeast biomass yield was recorded after 24hrs growth.

2.9. Effect of sugar concentration

The effect of sugar concentration on growth and ethanol production was tested by incorporating 10, 20, 30, 40 and 50 percent in xylose and arabinose of ethanol production media. Fermentation was carried out in 500 ml conical flasks. Samples were distilled after every 24-hour interval for determination of ethanol content in the media (Caputi *et al.*, 1968). The initial sugar

concentration that was efficiently utilized by the isolates was selected and maintained in fermentation media analyzing the other parameters.

2.10. The effect of pH

The of pH on ethanol production was tested on the same media by adjusting the pH 3 to 4, 5 and pH 6 and incubated at the temperature of 30^0C for 72 hours.

2.11. Determination of sedimentation/flocculation

Following the methods used by Campelo and Belo (2004) after completion of fermentation, the fermentation broth in each was filtered and the suspension in each flask were centrifuged at 5000X for 10 minutes several times with intermittent washing with cold distilled water. Each yeast biomass was buffered at pH 5 and measured using spectrophotometer.

2.12. Dilute acid hydrolysis

Samples based on the wheat straw were put in flask. The ratio between wheat straw and acid solution was 1:10 W/V and the mixture of wheat straw and acid solution made 500 ml in flask. The acid hydrolysis procedure of the experiment started with adding of (1-3% v/v) diluted sulfuric acid to the non-soluble component from pretreatment steps and the wheat straws were hydrolyzing in the autoclave at a time of (15-90min). The mixtures were allowing standing for 10 min at room temperature in order to equilibrate the acid concentrations between the bulk phase and the biomass. The ratio between wheat straw and acid solution was 1:10 W/V (solid to liquid ratio). Hydrolysis was performed in an autoclave at (30-90°C) for several minute (Mussatto and Roberto, 2005). The filtered hydrolyzate was neutralized with NaOH until the pH became in a range of 5. The response variable was sugar content after hydrolysis and ethanol yield after fermentation.

Table 1 Experimental design formulated for acid hydrolysis stage

Std.	Run number	Factor 1 Acid Conc. (%)	Factor 2 temperature (^{0}C)	Factor 3 Time (min)
17	1	2.00	60.00	52.50

20	2	2.00	60.00	52.50
11	3	2.00	30.00	52.50
1	4	1.00	30.00	15.00
8	5	3.00	90.00	90.00
14	6	2.00	60.00	90.00
18	7	2.00	60.00	52.50
16	8	2.00	60.00	52.50
13	9	2.00	60.00	15.00
7	10	1.00	90.00	90.00
2	11	3.00	30.00	15.00
10	12	3.00	60.00	52.50
5	13	1.00	30.00	90.00
4	14	3.00	90.00	15.00
19	15	2.00	60.00	52.50
15	16	2.00	60.00	52.50
3	17	1.00	90.00	15.00
6	18	3.00	30.00	90.00
12	19	2.00	90.00	52.50
9	20	1.00	60.00	52.50

2.13. Filtration

The lignin and degraded cellulose which is called monomeric sugar was separated by using filter funnel. Then the sugar solution or filtrate was neutralized and introduced into fermentation. The lignin which obtains from this filtration process was measured before use for another purpose.

2.14. pH adjustment

Before addition of any micro-organism to the prepared samples, pH of these samples was adjusted TO WHAT?. A pH 5 was employed for selection of optimum pH condition to ethanol production (Periyasamy *et al*., 2009).

2.15. Determination of Sugar content

DNS solution was prepared by using DNS (5g), Phenol (1g), Sodium sulfite (0.25g), NaOH (500ml) and Na^+ tartarate (40g).

DNS solution was designed to detect the presence of reducing sugars. As the reaction proceeds, the color of the reaction mixture changes progressively from yellow to red color. When the conditions are carefully controlled, the coloration developed and the amount of precipitate formed depends upon the amount of reducing sugars present. Hence, in most conditions, a sufficiently good estimation of the concentration of xylose-equivalent reducing sugars present in a sample can be obtained. Standard curve was plotted from known concentration of standard glucose and DNS solution; so that concentration of sugar yields in hydrolysate which obtained from hydrolysis was determined using digital spectrophotometer by measuring absorbance versus sugar concentration at 540nm wave length.

Plotting calibration curve Standard glucose dilution series solution was prepared at different concentration of 0, 2%, 4%, 6%, 8% and 10%. One ml of each of the standard glucose solution is added into labeled test tubes, each containing 1.5 ml of DNS solution and mixed by shaking. The labeled test tubes were heated in water bath for 15 minutes 90^oc. 0.5 Na^+-tartrate was added after boiling the mixture to stop the reaction. Then the sugar content of hydrolysate was determined using spectrophotometer by measuring absorbance versus sugar concentration at 540nm wave length. Finally, plot a calibration curve to show the % of absorbance of red light by the standard glucose solution (Miller, 1959).

2.16. Sterilization

The reactor and all the equipment that were used for fermentation purposes were sterilized (autoclaved). The sterilization was carried out at a temperature of 121℃, for 15 minutes.

2.17. Fermentation process

All fermentations were carried out in 250 ml Erlenmeyer flasks with a 100 ml working volume. The substrates were supplemented with YP. An inoculum was growing for 24 hours in the same media as the fermentation media. The flasks were covered with aluminum foil and seal with Para

film incubated at 30^{o}C. 1ml of yeast isolate culture was added to all flasks for fermentative production of bioethanol. The adapted media with the proportion of 1:10 to the soluble sample was added. The incubator was set at parameters of fermentation timeand fermentation temperature of 72 hour, 10% (with the proportion of 1:10 that is the prepared media and sample) and 30^{o}C respectively. After 72 hours of fermentation; the samples were taken out and distilled (Sumphanwanich *et al*., 2008). The volume of the sample was 200ml and 20ml of media was added based on the proportion above.

2.18. Distillation

The distillate (ethanol+water) was analyzed by density (Ademiluyi and Mepba, 2013). Distillation is required to generate concentrated and purified ethanol from fermentation products. The principle of distillation depends upon the boiling points of components in the mixture; lower boiling components preferentially vaporize at lower temperatures. In bioethanol distillation, ethanol vaporizes before water as it has a lower boiling point (78.5°C) compared to that of water (100^{0}C) , the ethanol turns into the vapor state before the water and it was condensed and separated. Fermented mixtures were heated using a simple distillation to obtain pure ethanol from the sugars.

2.19. Density of bioethanol

Density of the liquid or solution is defined as the mass of unit volume; density is also termed specific gravity. Density was determination by pycnometer cylinder is a very precise method (Ademiluyi and Mepba, 2013). The expression for specific gravity is:

$$\text{Specific gravity} = \frac{w1-wo}{w2-wo}$$

Where: W_0- weight (g) of empty bottle

W_1- weight (g) of bottle + sample

W_2 - weight (g) of bottle + water

Microbial Journal

2.20. Data Analysis

The effects of the process variables; temperature (30-90^{o}C), reaction time (15-90 min) and acid concentration (1-3%) were analyzed by Design Expert version 7.0 software CCD (central composite design). The response variable were sugar content after hydrolysis and ethanol yield after fermentation.

3. RESULTS AND DISCUSSION

3.1. Yeast isolation

A total of thirty (30) yeast isolates were retrieved from ten (10) plant decomposed soil found in Ankober and Debresina (Wofwasha), termite soil and three (3) rotten wood samples were collected from Ankober, four (6) sheep dung compost in Debre Berhan sheep breeding from three sites. Six (6) fruit samples (mango, avocado, banana, orange, papaya and pine apple) were collected from local market. Three (3) samples of animal dung from Debre Berhan University. Two (2) samples of fermented dough (Teff and Wheat) were conducted to obtain the microorganisms. Most of them showed smooth surfaces with circular margins, and creamy white texture. However, a few isolates showed slightly red and pinkish colonies.

3.2. Yeast Purification

A total of fifteen (15) yeast isolates were recovered from a total isolate samples (30) of three from avocado, three from rotten woods, five soil, two from termite soil, one from papaya and one from animal dung. The selection was done based on color shape and size. Most of the yeast colonies exhibited smooth surfaces with circular margins. The color of the pure colonies was showed creamy and white. The cells were found to be of various shapes such as round, oval and spherical.

Microbial Journal

3.3. Morphological and physiological characteristics

After 3 days of incubation at 30^0C, the isolate show that white and cream color on both agars. The cell morphology of the isolates LCX, SWX, LCA and LWA under compound microscope was elongated and budding cells were present and pseudo mycelia were also developed

Table 2 Morphological and physiological characteristics

Characteristics	Isolates			
	LCX	SWX	LCA	LWA
Color	Creamy	White	Creamy	White
Surface	Smooth	Smooth	Smooth	Smooth
Elevation	Large	Small	Large	Large
Ascospores	+	+	+	+

3.4. Yeast Isolates Screening

In this study isolates showed variation of utilization of two different sugars (Table .3). Ten of the isolates utilized xylose and arabinose (flout and full CO_2 in Durham tube) and the others five isolates not capable to complete metabolizing these sugars (not flout and full CO_2 in Durham tube). LCX, SWX LCA and LWA were able to show rapid fermentative rate on sugars, but some of fermentation show on isolates of CWA, MWA, TMA, LYX, SYX and LWX within long time interval. The other isolates RDA, SWA, LBA, MWX and SCX were started to fermentation but they were not completed.

Microbial Journal

Table 3 Yeast Isolates Screening

Isolates	Yeast Isolates														
	CWA	LWA	RDA	MWA	TMA	LCA	SWA	LBA	LYX	LCX	MWX	SWX	SCX	SYX	LWX
Time	+	++	-	+	+	++	-	-	+	++	-	++	-	+	+

_ No fermentation

\+ Good fermentation

\+ + Very good fermentation

3.5. Ethanol tolerance of yeast isolates

The isolates which have rapid fermentative were selected for yeast ethanol tolerance (figure 1). Ethanol inhibits alcoholic fermentation, which decrease the concentration of ethanol but, which can be produced up to 12% ethanol concentration (Wayman and Rarekh, 1990). The highest ethanol tolerant was occurring by LWA and LCX isolate was 2.27 % and 2.9 % (v/v) resistant to ethanol in the media of 20%. *Saccharomyces cerevisiae* tolerate up to 15% of ethanol in the medium (Sathees Kumar *et al.*, 2011). The stage of inhibition is also related to other environmental factors, like high sugar concentration and high temperature which reasons to the limitation of ethanol fermentation. High ethanol concentrations reduce cell vitality and increase cell death (Stanley *et al*., 2010). In this study the ethanol tolerance of LCA and SWX were 1.09% and 0.83% at 20% ethanol concentration of media respectively. This factor may be due to toxic effect of ethanol has also been attributed to damaging the cell membrane or changing its properties. Khaing *et al.* (2008) have also reported that some yeast isolates have tolerated up to 15% of ethanol in the medium and other yeast isolate were tolerated up to 20% of ethanol and maintained maximum ethanol production over a long incubation period.

Microbial Journal

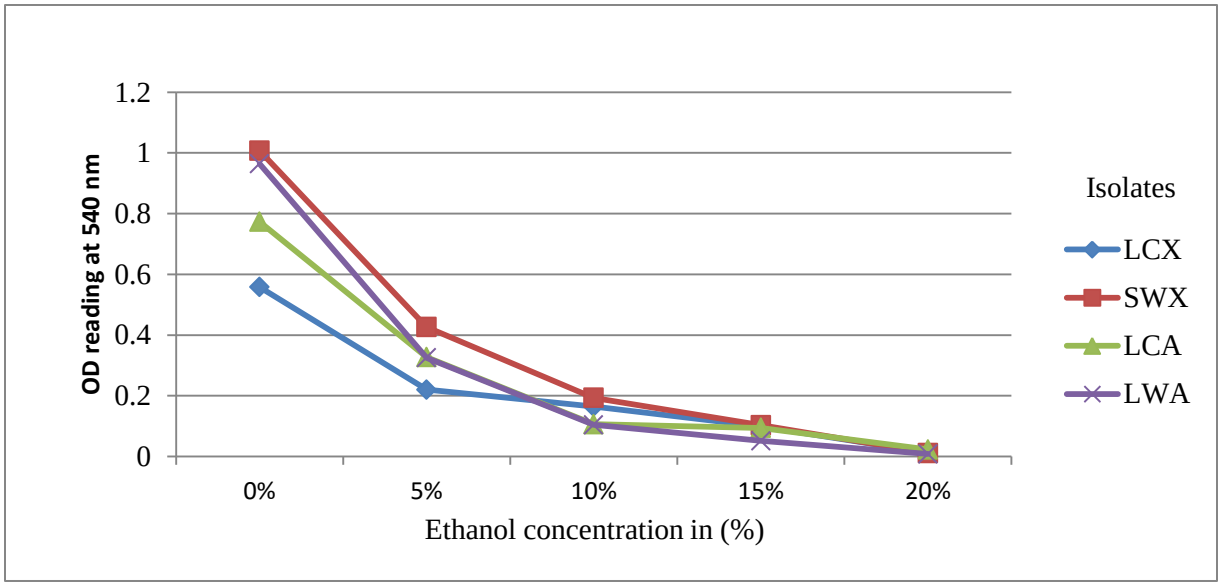

Figure 1 Ethanol tolerance of yeast isolates

3.6. Effect of pH on ethanol production

Initial sugar concentration of 20% and temperature of 30°C was selected for further studies and subjected to pH treatments 3, 4, 5 and 6 .The results were shown in figure 2. At pH 3 fermentation took place but it gave low ethanol content. Best results were obtained at pH 5 where maximum ethanol production was noticed for the isolates of SWX and LWA yeast which 8.44g/l and 8.21g/l of ethanol respectively. However, yeast isolates was not much affected by pH in range 4 and 6 as indicated in figure 2. As literature Lu *et al*., (2017) the growth of yeast cultures has wide range but, optimum pH was in the range of 4.0–5.0 and it affects the final ratio of organic waste products produced by yeast cultures. Isolate LCA has high production at P^H4 was 6.36g/l ethanol but it was decrease when it run to pH 5 was 5.76g/l ethanol and so on. Decrease in pH from 5 to 3 was found to the minimum fermentation of sugars.

Microbial Journal

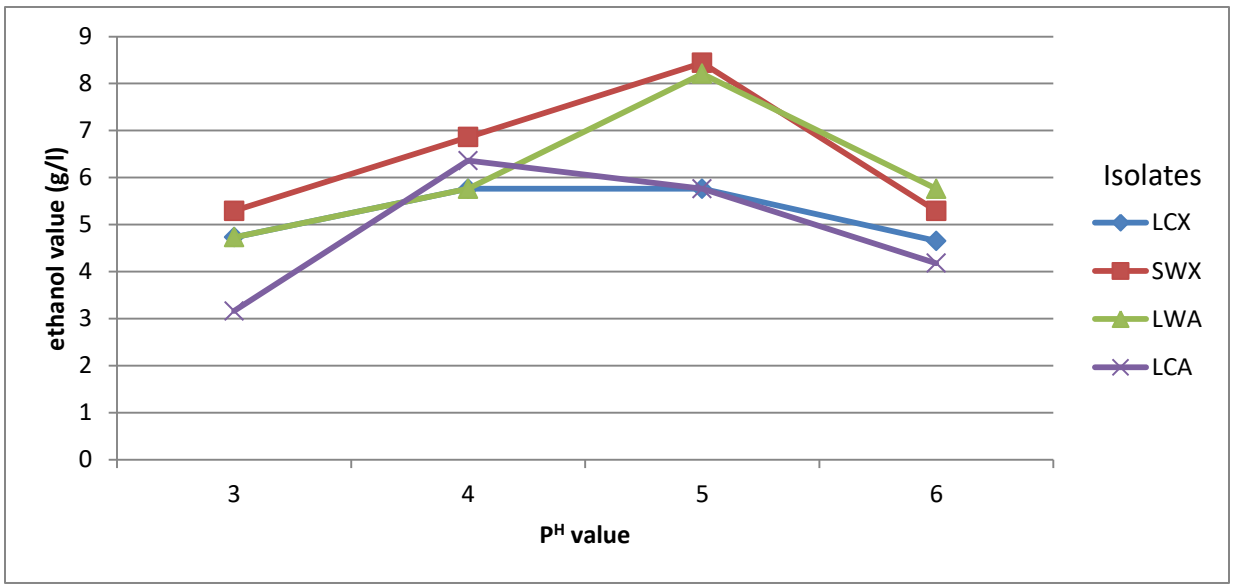

Figure 2 Effect of pH on ethanol production

3.7. Effect of sugar concentration

The growth of isolate gradually increasing concentrations of sugar showed an increase in ethanol production as increase sugar concentration in YPX broth medium as shown in figure 3. Samples were taken within 24 hours as increasing time with increase concentration for the study and also increase the time of incubation to obtained higher ethanol yield, because high initial sugar concentration takes longer fermentation time (Laopaiboon *et al.*, 2007).. However, at the sugar concentration of 1% and 2% the ethanol was less in the first 24hrs. As the concentration sugars increase from 2% to 3% and 10% the ethanol yield of all the isolates were increase until certain value of sugar concentration and decrease gradually. According to Chang *et al.*, (2018) too high sugar concentrations can inhibit metabolism due to increased osmotic stress and too low sugars may limit the rate of ethanol production. In this study when initial sugar concentration goes to 10% SWX isolate produce high ethanol 16.5g/l but after this the production was decreased 11.68g/l at 15% of sugar concentration. It was occurs due to the reasons that discussed to the above that is osmotic pressure or stress.

Microbial Journal

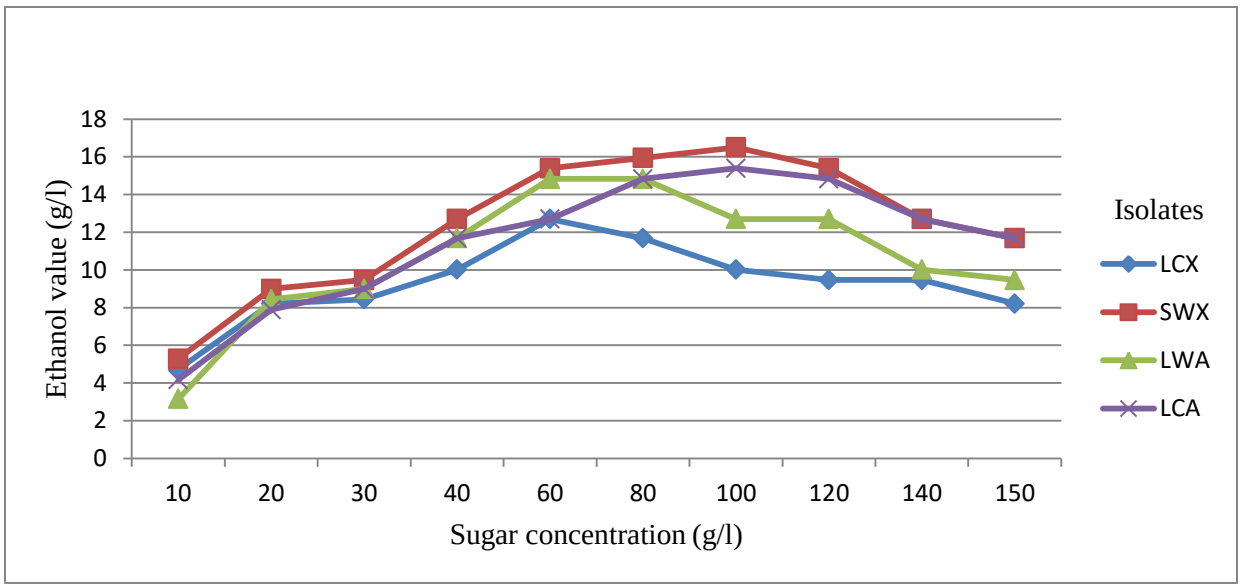

Figure 3 Effect of sugar concentration on ethanol production

3.8. Effect of temperature on ethanol production

Temperature is one of the major factors that determine the ethanol production. To know the optimum temperature for ethanol fermentation, the solutions were incubated at 20, 30, 40 and 50^{o}C with 20% initial sugar concentration. Samples were taken every 24 hours of incubation time to fermentation. A low ethanol yield, 2.90 % (v/v) was observed at 50°C in 24hr by the LCA isolates. High temperature is considered as a stress factor for microorganisms, which is unfavorable for their growth due to, denatures their structure and enzymatic activity (Lin *et al.*, 2012). As shown in figure 4 at 30°C ethanol yield was high and produced 8.44 % (v/v) by the isolate SWX compared to other temperature ranges. Too high temperature kills yeast, and low temperature slows down yeast activity. Therefore, specific range of temperature which is 30°C is required (Demirbas, 2005). However increasing the temperature beyond 30°C the isolate SWX growth as well as production of alcohol decreased. It occurs because of various types of yeast play for ethanol production is mesophilic organisms to growth (Ho and Powell, 2014).

Microbial Journal

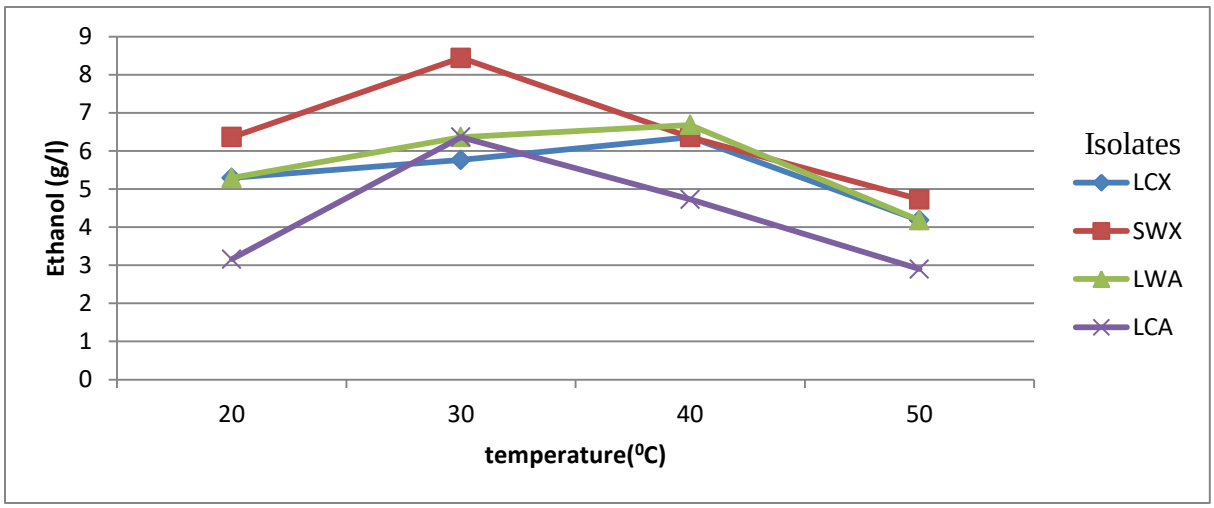

Figure 4 Effect of temperature on ethanol production.

3.9. Determination of sedimentation / flocculation

The yeast flocculation was determined after the end of fermentation at 72 hours. The maximum cell density was recorded for SWX in the fermentation was determined with initial sugar concentration of 20% g/l as it was indicated in figure 5. The incubated isolates was centrifuged and buffered with pH 5 to measure by spectrophotometer. As reported by Stratford, (1996) flocculation of yeast has wide range (2.5-9.0) but, the optimum range was taken place between 4.0 and 5 because it affects ionization of lectin amino acid (only present in flocculent cells) with the consequence of change in its conformation. From this study, the rate was rapid to isolates SWX and LWA within two hour reading interval for four hours 72.1% and 71.22% sedimentation rate respectively. Flocculation of yeast phenomenon was affected by physiological; environmental the nature of structure effects (Stewart, 2015). Flocculation may enhance the survival of yeast cell during adverse (e.g. Starvation) condition due to this sedimentation is important in an environment with limited nutrient because the death and autolysis of the cell inside the flocs can provide further nutrient to cell in the surrounding environment (Jin and Speers, 2000). Isolate LCA was able to show slow rate within the same time of other isolates which 62.1% and it may be due to the above discussed reasons. The rate of sedimentation depends on particle size and generation, which is smaller and younger yeast cell was settle slowly than larger and older cell (Stratford, 1996).

Microbial Journal

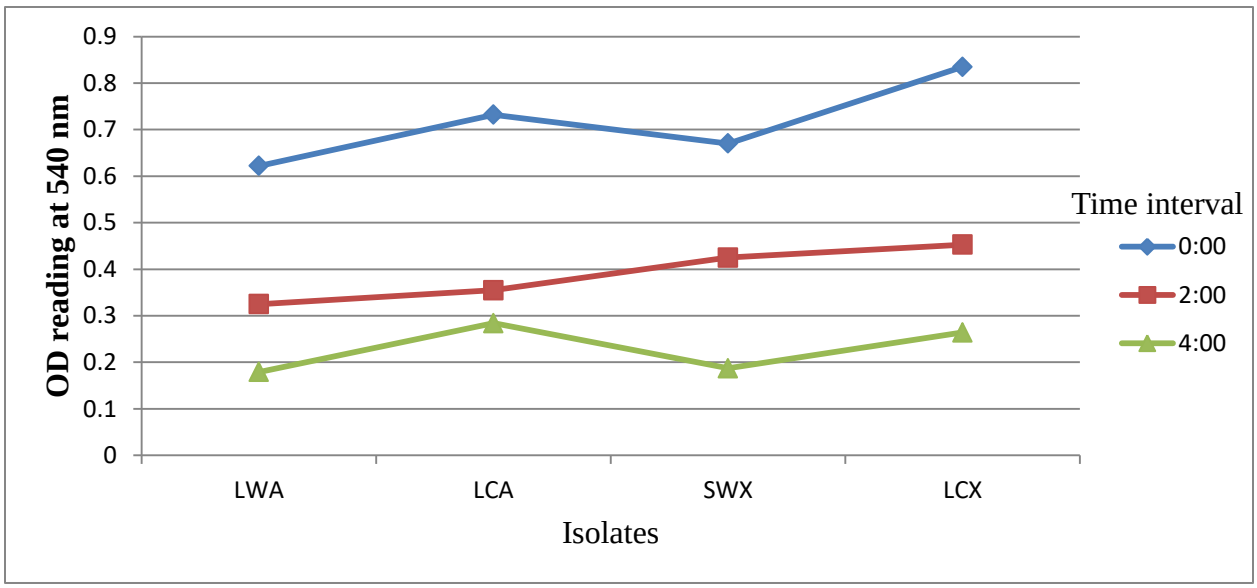

Figure 5 Sedimentation / flocculation

3.10. Moisture content of wheat straw

The fresh wheat straw was collected from farmers and THEY were milled in Debre Berhan Agricultural Research Center. Two samples (523g and 486g) were prepared to determine the moisture content 60^{o}C and the moisture content of the sample was presented in Table 5. Drying process was terminated at 72hr as the weight of the sample was approximately equal with the weight at 24hr.

Table 5 Determination of moisture contents

Sample weight	Drying time (hour)						Percentage (%)
	0	12	24	36	48	72	
	523	512.1	507.98	503.50	503.46	503.45	6.52%
	486	476.71	474.58	470.26	470.17	470.16	6.21%

3.11. Total reducing Sugar determination

Total reducing sugar (TRS) of the hydrolysate sample was determined using Spectrophometer, which measures the intensity of light. Standard curve was plotted from known concentration of

standard glucose and DNS solution reagent in digital spectrophotometer at 540nm wavelength and the corresponding concentration of sample was determined.

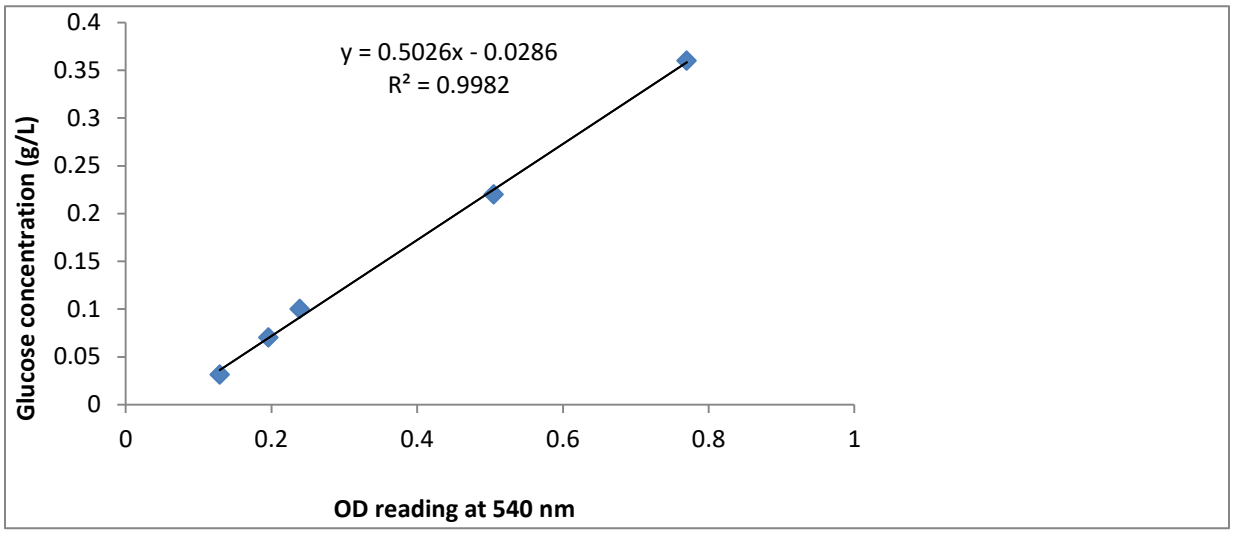

Figure 6 Calibration curve of glucose standard for determination of total sugar content

3.12. Dilute acid hydrolysis

Table 6 Yield of reducing sugar and bioethanol hydolysate of wheat straw

Run	Acid (%)	Temp. (^{0}c)	Time (min)	Absorbance (450nm)	Reducing sugar (g/l)	Ethanol (g/l)
1	2.00	60.00	52.50	0.64	12.84	5.3
2	2.00	60.00	52.50	0.62	12.44	5.1
3	2.00	30.00	52.50	0.42	8.41	2.8
4	1.00	30.00	15.00	0.41	8.21	3.4
5	3.00	90.00	90.00	0.28	5.61	1.6
6	2.00	60.00	90.00	0.52	10.43	4.2
7	2.00	60.00	52.50	0.65	13.04	5.3

8	2.00	60.00	52.50	0.72	14.44	5.8
9	2.00	60.00	15.00	0.54	10.42	4.3
10	1.00	90.00	90.00	0.50	10.02	3.7
11	3.00	30.00	15.00	0.26	5.19	1.1
12	3.00	60.00	52.50	0.37	7.41	2.6
13	1.00	30.00	90.00	0.76	15.25	6.4
14	3.00	90.00	15.00	0.21	4.19	1.03
15	2.00	60.00	52.50	0.59	13.04	5.3
16	2.00	60.00	52.50	0.58	11.63	4.2
17	1.00	90.00	15.00	0.52	10.42	3.7
18	3.00	30.00	90.00	0.23	4.59	1.3
19	2.00	90.00	52.50	0.31	6.21	2.6
20	1.00	60.00	52.50	0.78	15.65	6.8

Hydrolysis of wheat straw using dilute sulfuric acid the produced glucose concentration increases with increasing time and temperature as shown in the table 6. Based on this, the maximum yield of glucose and ethanol were noted for 1% of dilute acid concentration, at a temperature of 60°C and hydrolysis time of 52.5 min. For this condition the obtained glucose and ethanol yield were 15.65g/l and 6.8g/l respectively. But the glucose concentration and ethanol yield were observed to decrease at high acid concentration, and high temperature and low time (Balat, 2011). This may be due to formation of other intermediates products (Liu *et al*., 2009). To analyze the experimental results, Design expert® 7.0.0 software was used. The minimum yield glucose 4.19g/l and minimum yield of ethanol 1.03g/l were obtained experiment number 14 at a temperature of 90℃, 3% acid concentration and 15 minutes of hydrolysis time. The decrease and increase of the yield was depending on the level of factors. The dependent variable used as a response parameter was the glucose yield and ethanol. Overall, these results indicate that over acid concentration had an unfavorable effect on sugar conversion of wheat straw (Nutawan *et al*., 2010). Pretreatment typically breaks down the macroscopic rigidity of biomass and reduces the physical barriers of mass transport (Liu *et al*., 2009). Among the different hydrolysis method, dilute acid hydrolysis at high temperature is effective. The experiment showed that hydrolysis of

wheat straw by dilute sulfuric acid solution caused considerable rise of utilization rate and higher yield of reducing sugar.

3.13. Yield of reducing sugar from dilute acid hydrolysate

3.13.1. ANOVA for Response Surface Quadratic Model

To determine whether or not the quadratic model is significant, it was important to perform analysis of variance (ANOVA), table 7below. The probability values (P-values) were used to perform as a device to check the significance of each coefficient, which also showed the interaction strength of each parameter. The smaller the p- values are, the bigger the significance of the corresponding coefficient.

Table 7 Analysis of variance (ANOVA) for Response surface reducing sugar

Source	Sum of square	Df	Mean square	F-value	P-value prop>F	
Model	218.85	9	24.32	8.60	0.0012	Significant
A-Acid	106.02	1	106.02	37.51	0.0001	
B-Temp.	2.70	1	2.70	0.96	0.3511	
C-Time	5.58	1	5.58	1.97	0.1903	
AB	1.16	1	1.16	0.41	0.5370	
AC	4.23	1	4.23	1.50	0.2491	
BC	3.67	1	3.67	1.30	0.2809	
A^2	0.18	1	0.18	0.063	0.8072	
B^2	43.25	1	43.25	15.30	0.0029	
C^2	1.99	1	1.99	0.70	0.4209	
Residual	28.27	10	2.83			
Lack of Fit	24.03	5	4.81	5.67	0.0399	Significant
Pure Error	4.24	5	0.85			
Cor Total	247.12	19				

Microbial Journal

The Model F-value of 8.60 implies the model is significant. There is only a 0.12% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 5.67 implies the Lack of Fit is significant. There is only a 3.99% chance that a "Lack of Fit F-value" this large could occur due to noise.

Table 8 Model adequacy measures of yield of reducing sugar

Std. Dev.	1.68	R-Squared	0.8856
Mean	9.97	Adj R-Squared	0.7827
C.V. %	16.86	Pred R-Squared	-0.3566
PRESS	335.24	Adeq Precision	10.201

The regression coefficient (R^2) evaluates the correlation between the experimental data and the predicted responses. Results of R^2 = 0.8856 and Adj-R^2 = 0.7827 obtained explicates that the predicted values were found to be in good agreement with experimental values. Since the R^2 value is closer to 1.0 it indicates that the regression line perfectly fits the data. Results imply that the predicted values were found to be in good agreement with experimental values (R^2 = 0.8856 and Adj-R^2 = 0.7827), indicating the achievement of the RSM. "Adeq Precision" measures the signal to noise ratio. In general, a high value of R^2 indicates that there is good fit between the predicted data and experimental data. A ratio greater than 4 is desirable. Your ratio of 10.201 indicates an adequate signal. The coefficient of variation (C.V.) was found to be 16.86%, this value of CV indicating that the deviations between the predicted data and experimental data were small, that means the experiments were precise and reliable. This model can be used to navigate the design space

Final Equation in Terms of Actual Factors:

Reducing sugar = -0.16949 -4.01386 * Acid+0.51774 * Temp.+0.15839 * Time +0.012667 * Acid* Temp.-0.019400 * Acid * Time -6.02222E-004 * Temp. * Time+0.25409 * $Acid^2$ – 4.40657E-003 * $Temp.^2$ -6.05091E-004 * $Time^2$

Microbial Journal

Final Equation in Terms of Coded Factors:

Reducing sugar = +12.25 -3.26 * A-0.52 * B+0.75 * C +0.38 * A * B -0.73 * A * C-0.68 * B *C +0.25 * A^2-3.97 * B^2-0.85 * C^2

3.13.2. Diagnostics Case Statistics

Table 9 Actual versus model Predicted values ofreducing sugar yield

Actual Value	Predicted Value	Residual
8.21	9.69	-1.48
5.19	3.88	1.31
10.42	9.25	1.17
4.19	4.95	-0.76
15.25	14.00	1.25
4.59	5.27	-0.68
10.02	10.84	-0.82
5.61	3.64	1.97
15.65	15.76	-0.11
7.41	9.25	-1.84
8.41	8.81	-0.40
6.21	7.77	-1.56
10.42	10.66	-0.24
10.43	12.15	-1.72
11.63	12.25	-0.62
14.44	12.25	2.19
12.84	12.25	0.59
13.04	12.25	0.79
13.04	12.25	0.79
12.44	12.25	0.19

Microbial Journal

3.13.3. Normal probability plot

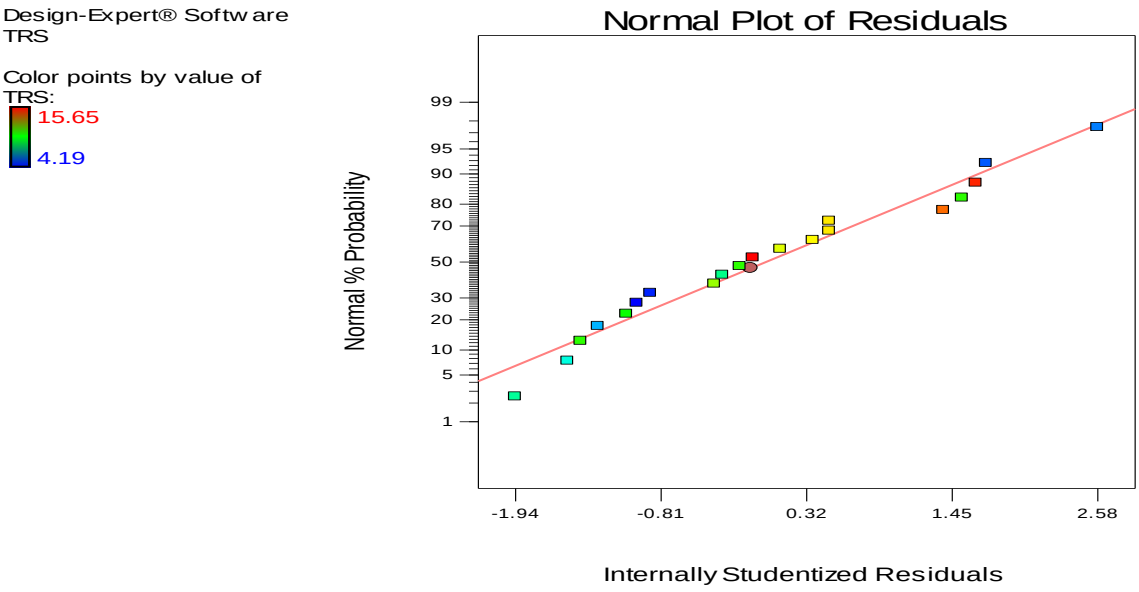

Figure 7 Normal plots of residuals

From the plot as shown above, the normal probability plot indicates the residuals following a normal distribution, in the case of this experiment the points in the plots shows fit to a straight line in the figure 7, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal. This could be useful to know how much the model is acceptable.

3.13.4. Response Surface and Contour plot on the experimental variables for reducing sugar

In order to analyze the regression equation of the model, three-dimensional surface and contour plots were obtained by plotting the response (yield of reducing sugar) on the Z-axis against any two variables while keeping the other variable at zero level. These plots are created to analyze the change in the response surface. Conical shape response surface plot indicates optimum operating conditions. The response optimized value for the production of bioethanol was based on the two process variables described on the response surface plot. The effect of the independent variables and their mutual interaction on the yield of ethanol can be seen in Figures 7, 8, and 9.

Microbial Journal

3.13.4.1. The effects of hydrolysis temperature and acid concentration on reducing sugar yield

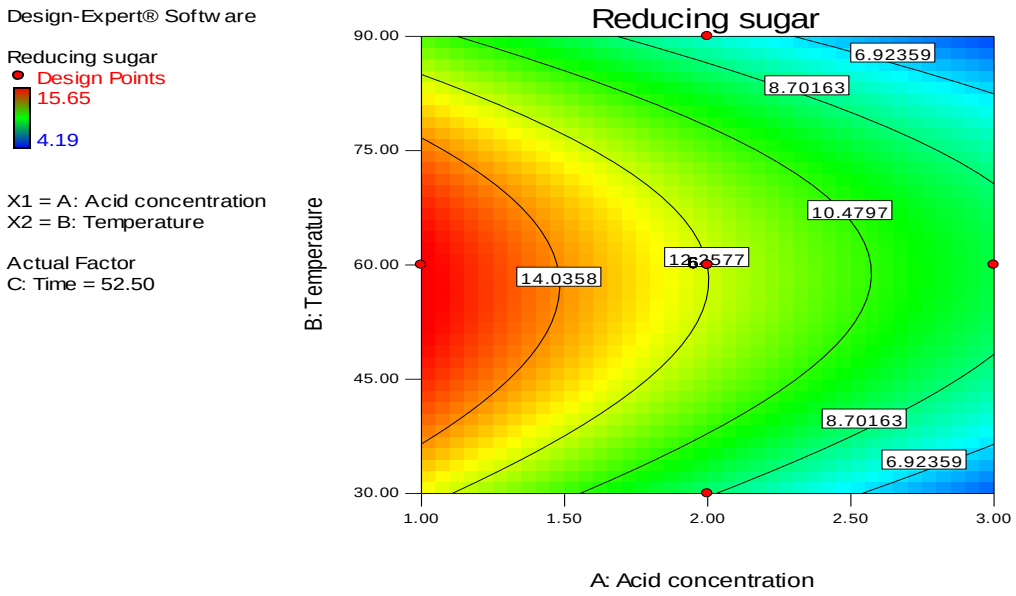

Figure 8 Contour plots of the effects of acid concentration and temperature on the yield of reducing sugar.

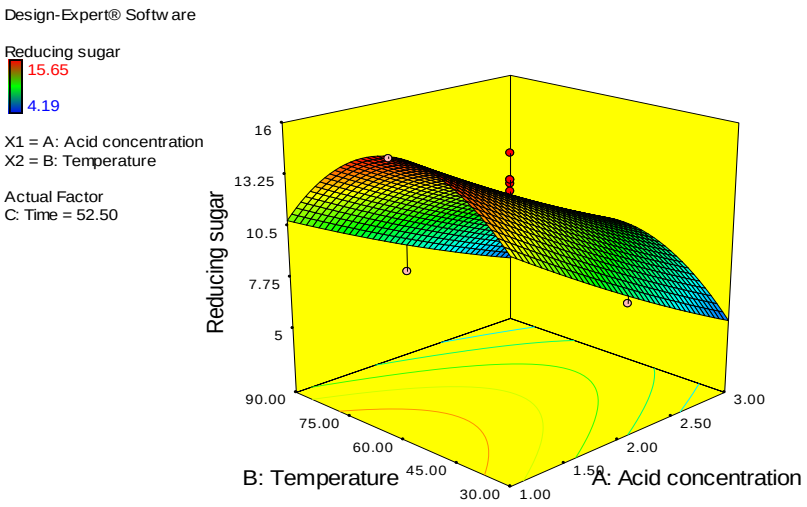

Figure 9 Surface plots of effects of temperature and acid concentration on the yield of reducing sugar.

Microbial Journal

In contour and 3D surfaces graph figures 8 and 9 also shown that the effect of acid concentration and temperature on the glucose yield. At the lower and higher levels of temperature, the production level of glucose yield decrease. However, at lower acid concentration increases the yield. This is because it has effect of the hydrolysis treatment. At lower temperature the cellulose might not hydrolysis to simple glucose and at higher acid concentration and temperature cellulose forms other degradation products. Hence both acid concentration and temperature have strong relationship for the yield of glucose production. When the levels of temperature increase hydrolysis resulted in higher yield of ethanol. However, as it was seen from the graph after some increments of temperature, the yield of ethanol became decreases since the possible formation of other molecules instead of glucose formation due to high temperature. However, at the higher levels of both temperature and acid concentration, the yield of glucose declined as a result ethanol yield also decreases. The physical barrier of lignin remains bound to cellulose or hemicellulose after pretreatment reduction the in surface area of cellulose site available for hydrolysis may cause for lower yield of reducing sugar (Moilanen *et al*,. 2011). Certainly this is due to presence of the strong interaction between these two variables. The highest yield of reducing sugar was obtained around 52-minute hydrolysis time, $60^{o}C$ temperature and 1% H_2SO_4 concentration was 15.65g/l. In this case all time, temperature and concentration are factors which affect the yield of reducing sugar. When temperature and concentration increased the amount of reducing sugar was decreased.

Microbial Journal

3.13.4.2. The effects of hydrolysis time and acid concentration on reducing sugar yields

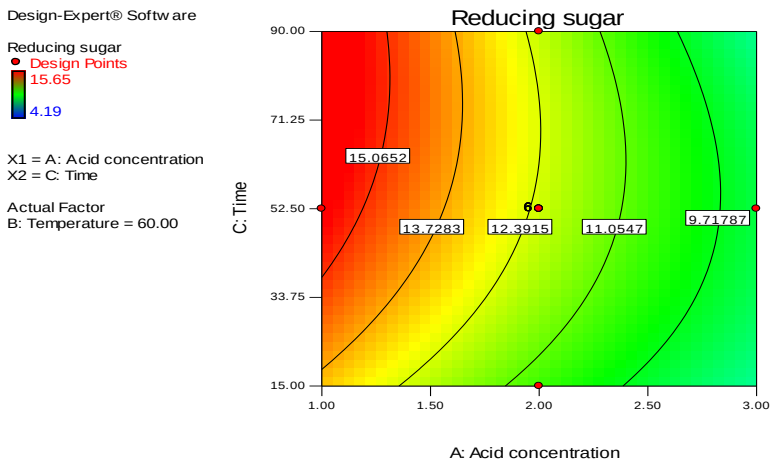

Figure 10 Contour plot of the effects of acid concentration and time on the yield of sugar

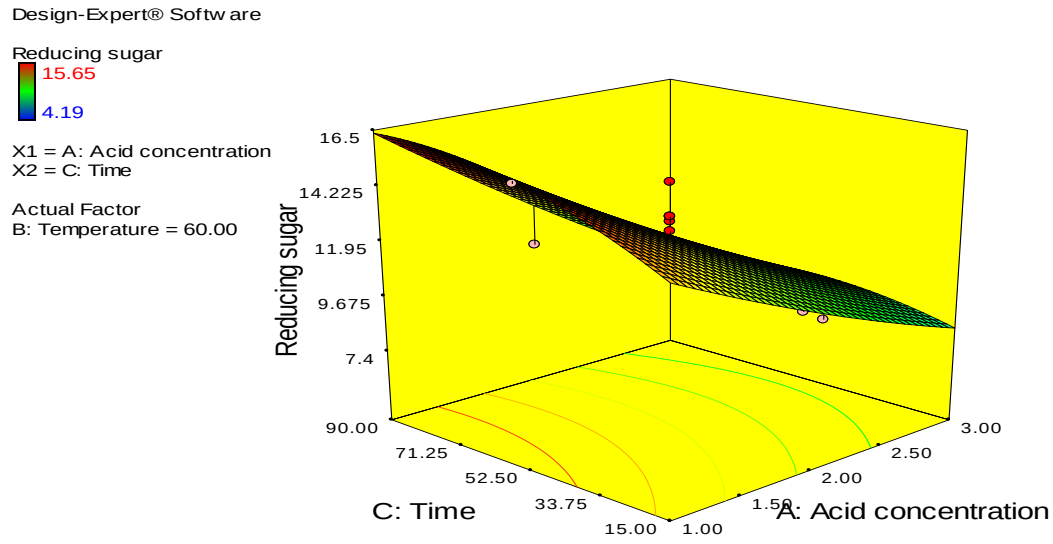

Figure 11 Surface plot of the effects of time and acid concentration on the yield of sugar

Microbial Journal

The effects of acid concentration and time on the yield of glucose, temperature was selected at the center point, are shown in figure 11. The maximum yield of glucose was observed at lower acid concentration and medium hydrolysis time. At increasing acid concentration, and time decreasing the yield of glucose became decreases since the possible formation of other molecules instead of glucose formation or the conversion glucose in to other fermentation inhibitors such as furfural. At the lower and higher levels of acid concentration and time, the yield of sugar level decrease since it has effect of the hydrolysis treatment. This decrease in sugar concentration may account for the further sugar degradation that occurred under the severe acidity. Overall, these results indicate that extreme acidity had an unfavorable effect on sugar conversion of wheat straw (Nutawan *et al.*, 2010). At lower acid concentration and time the cellulose might not hydrolysis to simple glucose and at higher acid concentration and time the cellulose might convert to other molecules which might not be fermentable.

3.13.4.3. The effects of hydrolysis temperature and time on reducing sugar yields

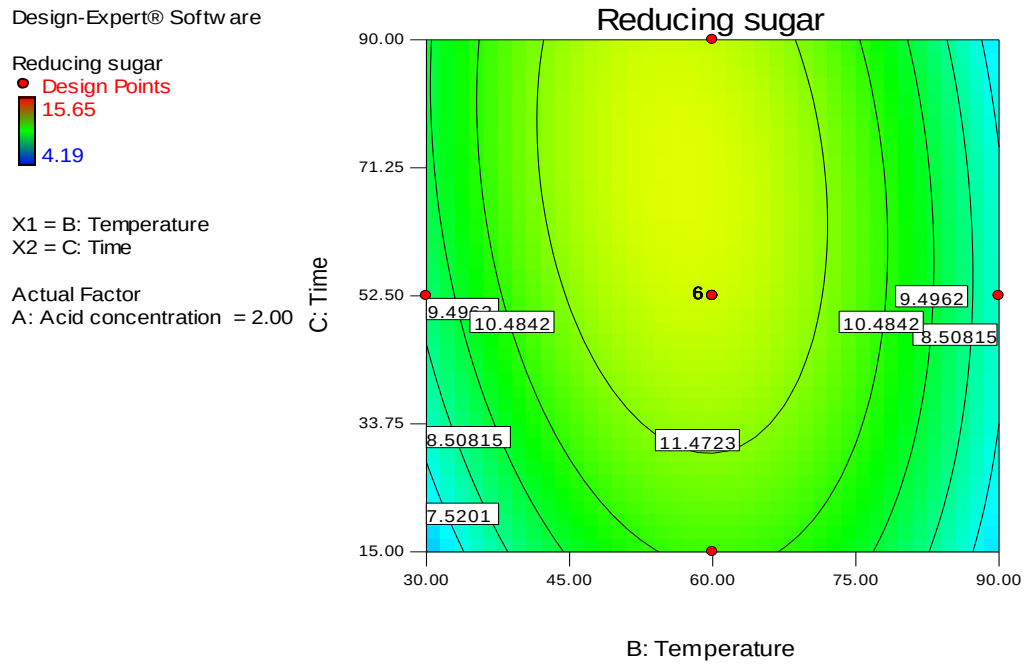

Figure 12 Contour plot of effects of temperature and time on the yield of reducing sugar

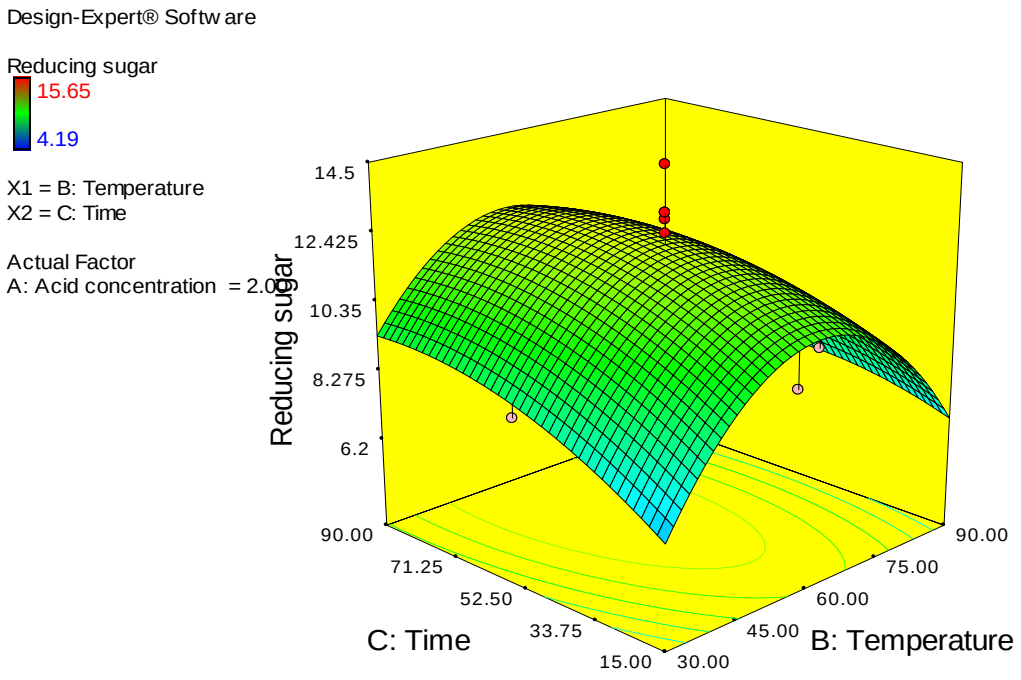

Figure 13 Surface plots of the effects of temperature and time on the yield of reducing sugar

The effects of acid concentration and time on the yield of glucose, acid concentration was selected at the center point, are shown in figure 13 and 12. The maximum yield of glucose was observed at medium temperature and medium hydrolysis time. At increasing/decreasing temperature, and time from the medium the yield of glucose became decreases since the possible formation of other molecules instead of glucose formation or the conversion sugars such as glucose, arabinos, galactose, frurural and xylose in to other fermentation inhibitors.

3.14. Yield of bioethanol from acid hydrolysate of wheat straw

The yield of bioethanol was determined using pycnometer cylinder and the density was related with concentration of bioethanol using standard reference table. For this hydrolysate fermentation use the isolate SWX due to its good performance of in most aspects of optimization from the other isolates.

Microbial Journal

3.14.1. ANOVA for bio-ethanol yield from dilute acid hydrolysate of wheat straw

Table 10 Analysis of variance (ANOVA) for the quadratic model

Source	Sum of square	Df	Mean square	F-value	P-value prop>F
Model	54.11	9	6.01	12.62	0.0002 significant
A-Acid	26.80	1	26.80	56.26	< 0.0001
B-Temp.	0.56	1	0.56	1.18	0.3030
C-Time	1.35	1	1.35	2.83	0.1236
AB	0.86	1	0.86	1.82	0.2076
AC	0.62	1	0.62	1.30	0.2799
BC	0.86	1	0.86	1.82	0.2076
A^2	0.012	1	0.012	0.024	0.8790
B^2	10.30	1	10.30	21.62	0.0009
C^2	0.41	1	0.41	0.86	0.3767
Residual	4.76	10	0.48		
Lack of Fit	3.37	5	0.67	2.42	0.1773 not significant
Pure Error	1.39	5	0.28		
Cor Total	58.87	19			

In Table 10, the Model F-value of 12.62 implies the model is significant. There is only a 0.02% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 2.42 implies the Lack of Fit is not significant relative to the pure error. There is a 17.73% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Microbial Journal

Table 11 Model adequacy measures for yield of bio-ethanol from wheat straw

Std. Dev.	0.69	R-Squared	0.9191
Mean	3.83	Adj R-Squared	0.8463
C.V. %	18.04	Pred R-Squared	0.2055
PRESS	46.77	Adeq Precision	12.505

The model was tested for adequacy by analysis of variance. The regression model was found to be highly significant with the correlation coefficients of determination of R^2, adjusted R^2 and predicted R^2 having a value of 0.9191, 0.8463 and 0.2055 respectively (table 11). The quality of the model developed could be evaluated from their coefficients of correlation. The value of R^2 for the developed correlation is 0.9191. It implies that 91.91% of the total variation in the percentage of yield is attributed to the experimental variables studied. The results demonstrated that the regression model equation provided a very accurate description of the experimental data, in which all the points are very close to the line of perfect fit. This result indicates that it was successful in capturing the correlation between the three hydrolysis reaction process variables to the percentage of bioethanol yield. The adequacy of the model was further checked with analysis of variance (ANOVA) based on a 95% confidence level, F – value is a test for comparing model variance with residual (error) variance.

Final Equation in Terms of Actual Factors:

Ethanol =-0.43240 -2.16425 * Acid +0.24352* Temp. +0.070933 * Time +0.010958 * Acid * Temp.-7.43333E-003* Acid * Time -2.92222E-004*Temp. * Time +0.065000 * $Acid^2$-2.15000E-003 * $Temp^2$ -2.73778E-004*$Time^2$

Microbial Journal

3.14.2. Diagnostics Case Statistics

Table 12 Actual versus model Predicted values of bio-ethanol yield

Actual Value	Predicted Value	Residual
3.40	3.93	-0.53
1.10	0.55	0.55
3.70	3.45	0.25
1.03	1.39	-0.36
6.40	5.88	0.52
1.30	1.39	-0.087
3.70	4.09	-0.39
1.60	0.91	0.69
6.80	6.66	0.14
2.60	3.38	-0.78
2.80	3.26	-0.46
2.60	2.78	-0.18
4.30	4.20	0.098
4.20	4.94	-0.74
4.20	4.95	-0.75
5.80	4.95	0.85
5.30	4.95	0.35
5.30	4.95	0.35
5.30	4.95	0.35
5.10	4.95	0.15

Microbial Journal

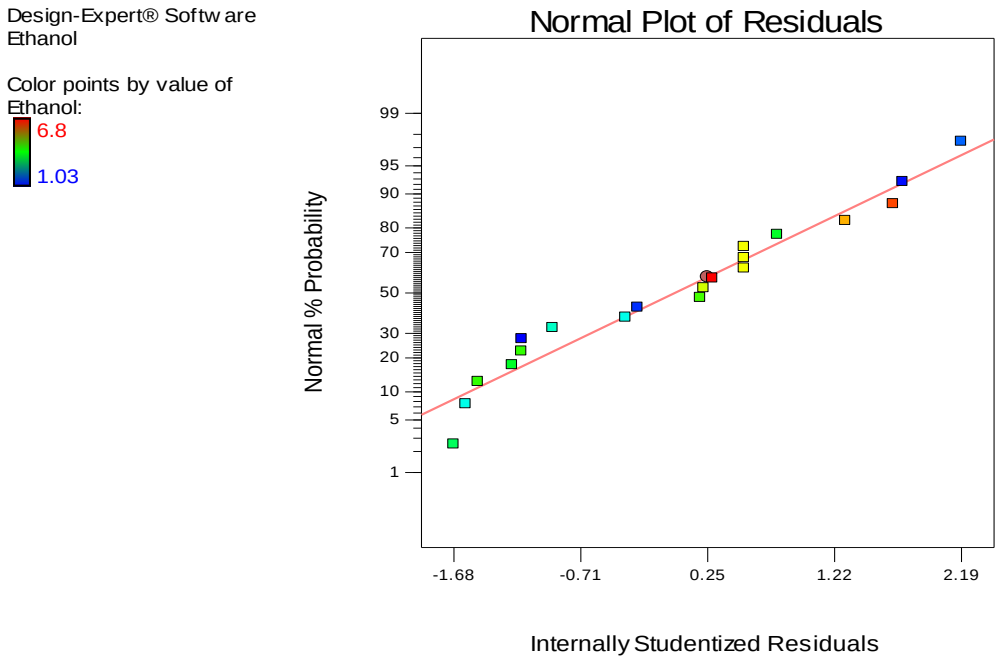

Figure 14 Predicted versus actual percentage yield of bio-ethanol in hydrolysis of wheat straw.

From the plot as shown above, the normal probability plot indicates the residuals following by the normal % probability distribution, in the case of this experimental data the points in the plots shows fitted to the straight line in the figure, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal (figure 14).

3.14.3. Response Surface and Contour plot on the Experimental Variables

3.14.3.1. The effects of hydrolysis temperature and acid concentration on bio-ethanol yield

The effects of hydrolysis temperature and acid concentration on bioethanol yield were shown in figures 15 and 16 by holding the hydrolysis time at middle. For the interaction, black and red line indicates low and high level of parameters respectively.

Microbial Journal

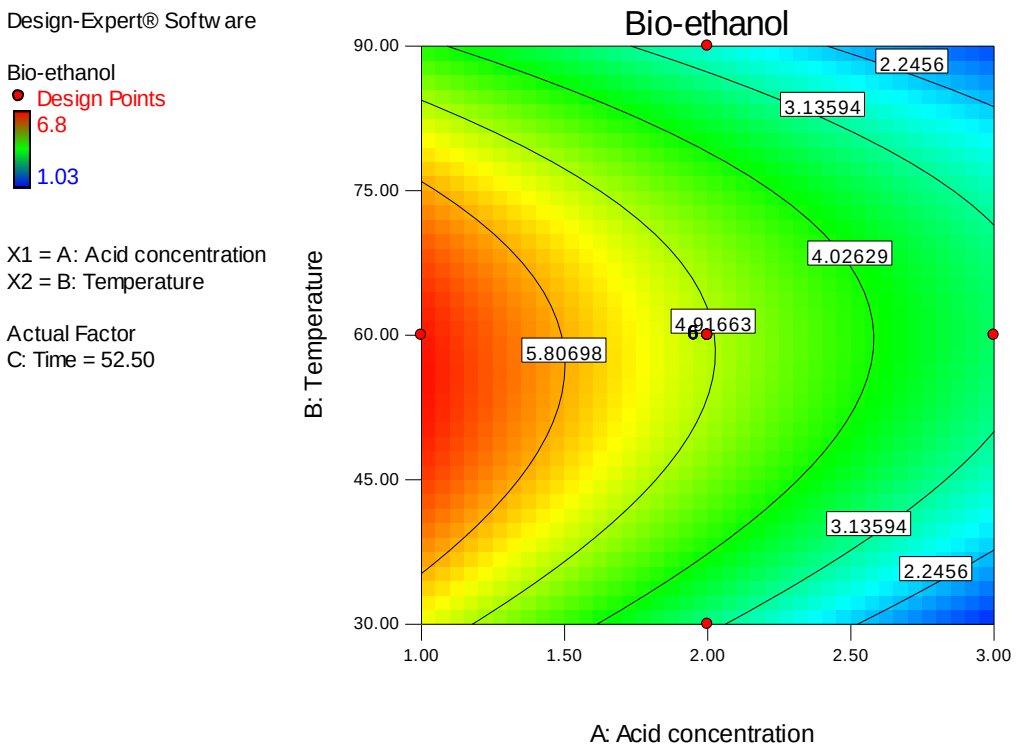

Figure 15 Contour plot of the effects of acid concentration and temperature on the yield of ethanol.

The effects of processing variables on ethanol yield were analyzed using contour plots as well. Figure 15 shows the effects of two independent variables on the response while the other one variable was held constant at the middle range. So, the contour plot graph in the above shows predicted response of ethanol yield as a function of hydrolysis temperature and acid concentration. As hydrolysis temperature and acid concentration increases towards the center, the yield was registered higher value as shown from the graph above (yellow red color). Further increasing the value of the parameters (hydrolysis temperature and acid concentration) the yield starts to decrease as shown from the figure 15 and 16 (blue green color).

Microbial Journal

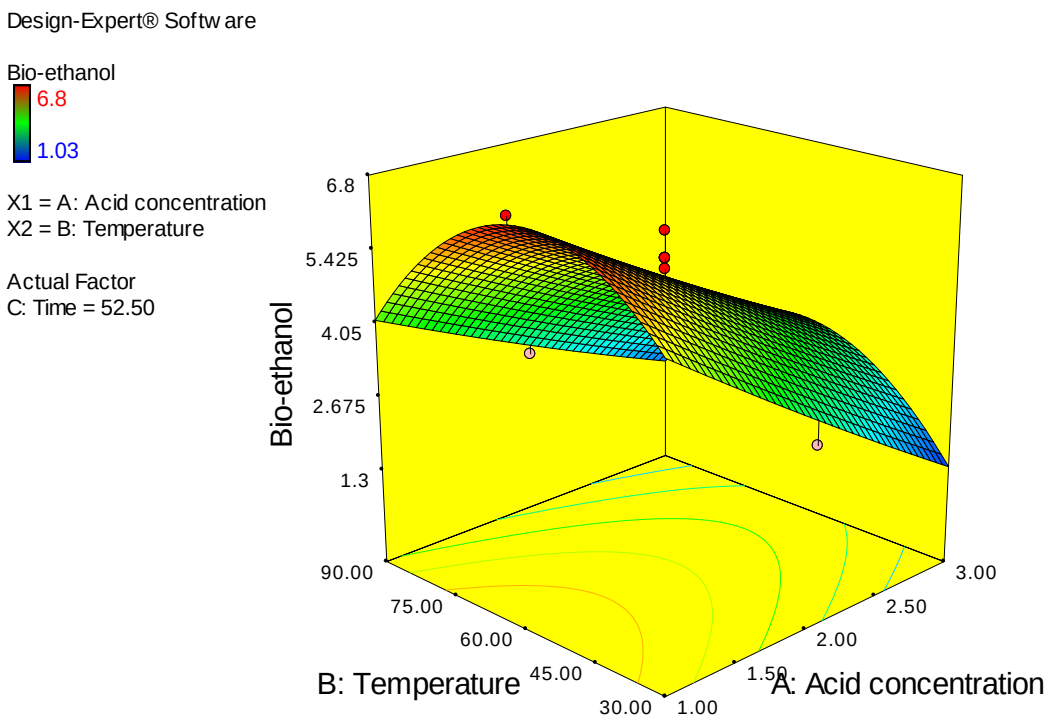

Figure 16 Surface plots of the effects of temperature and acid concentration on the yield of ethanol.

The best way to show the effects of parameters for ethanol yield is to generate response surface plots. The response surface plot figure 16 obtained from hydrolysis temperature and acid concentration was conical shape. This response surface shows that, at the minimum value of temperature and acid concentration the yield was minimum around 6.8g/l (blue green color) at the corners (blue color) and at the center the yield becomes maximum 1.3g/l (red color).n However, at the higher levels of both temperature and acid concentration, the yield of ethanol decreases. Certainly this is due to presence of the strong interaction between these two variables. As temperature increased from 30℃ to 60℃ and 1% acid concentration, the yield of ethanol also increases to its optimal 6.8g/l. But when the temperature increased beyond 60℃ there was decrease in the yield of ethanol. This is because, sugar degradation products such as pentose sugar monomers may dehydrate to the inhibitor furfural, hexose sugars (e.g. glucose) may degrade to the toxic hydroxymethyl-furfural (HMF) which leads to decreased glucose yield.

Microbial Journal

These inhibitors have toxic effects on the fermenting organisms, thus reducing the ethanol yield and productivity (Harmsen *et al.*, 2010).

3.14.3.2. The effects of hydrolysis time and acid concentration on bio-ethanol yield

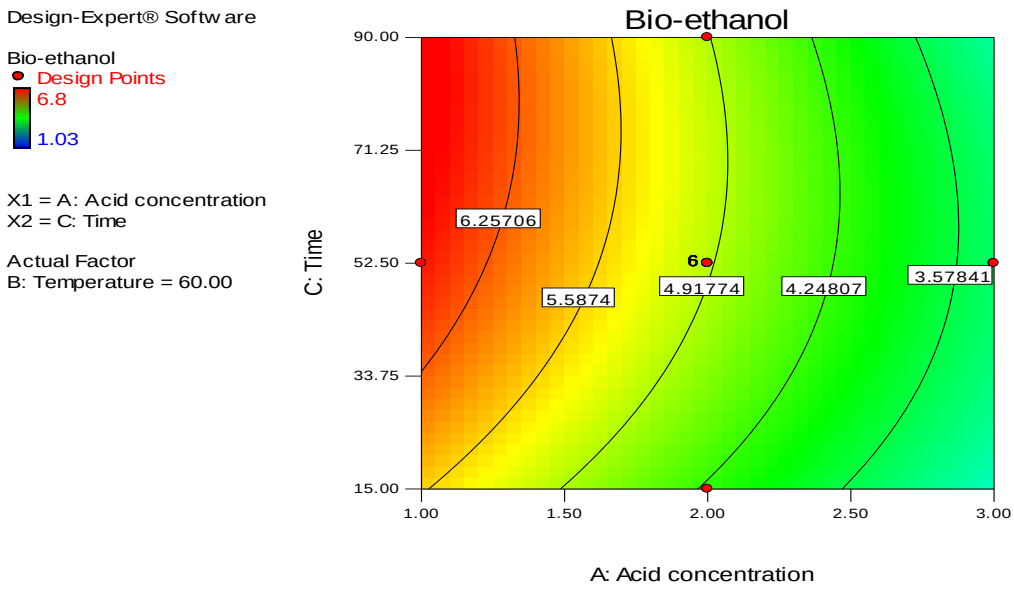

Figure 17 contour plot of the effects of time and acid concentration on the yield of ethanol.

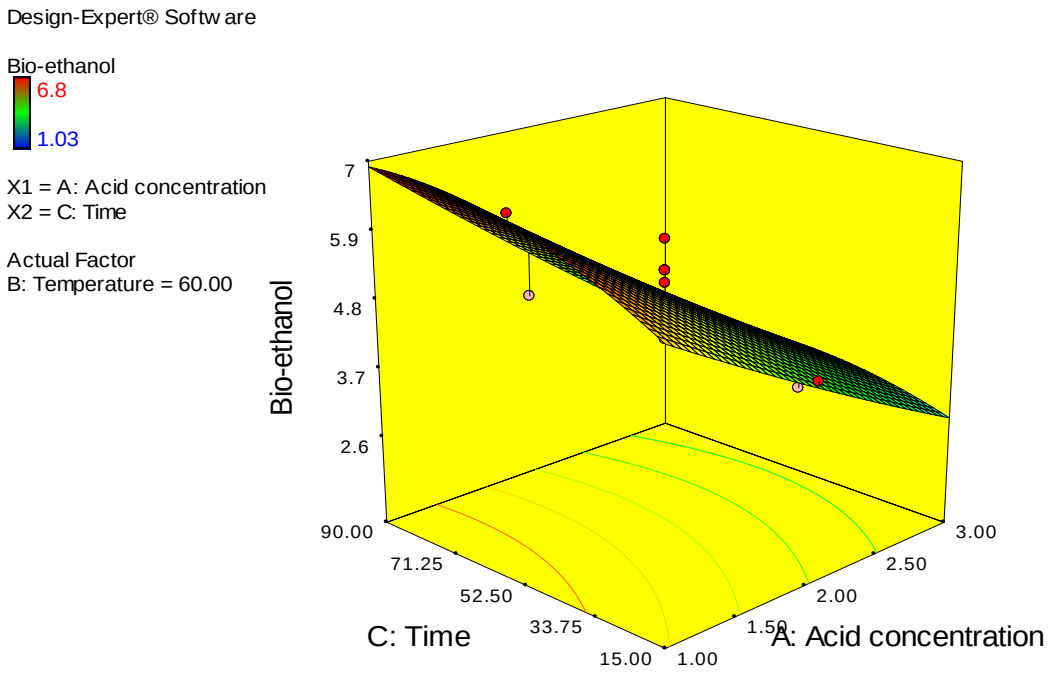

Figure 18 Surface plots of the effects of time and acid concentration on the yield of ethanol.

It can also possible to analyze the effects of processing variables on ethanol yield using contour plots and 3D plots which show predicted response of ethanol yield as a function of hydrolysis time and acid concentration as shown in figure 17 and 18. To contour plot shows higher ethanol yield at the center (yellow red color) when both hydrolysis time and acid concentration increase towards the center. But at lower and higher-level acid concentration and hydrolysis time, the value of ethanol yield becomes lower as shown from the graph (blue green color). In Surface response plot from above graph at the corners that means at minimum and maximum values of hydrolysis time and acid concentration shows minimum value of ethanol yield (blue green color) and at the center point (middle point of parameters) the graph shows maximum yield of ethanol. In general, it is simple to understand the effect of process parameters of acid concentration and time on the yield of ethanol, temperature was selected at the center point, are shown in figure 17 and 18. The maximum yield of ethanol was observed at low acid concentration and middle hydrolysis time. At increasing acid concentration, and time the yield of ethanol became decreases since the possible formation of other molecules instead of glucose formation or the

conversion sugars such as glucose and xylose in to other fermentation inhibiters. Soluble aromatic phenols produced by the depolymerisation of lignin during pretreatment inhibit fermentation of sugars in the production of bioethanol. Small concentrations of these inhibitors have been found to destroy the integrity of the yeast membrane systems preventing growth and sugar assimilation (Palmqvist *et al.*, 2000).

3.14.3.3. The effects of hydrolysis time and temperature on bio-ethanol yield

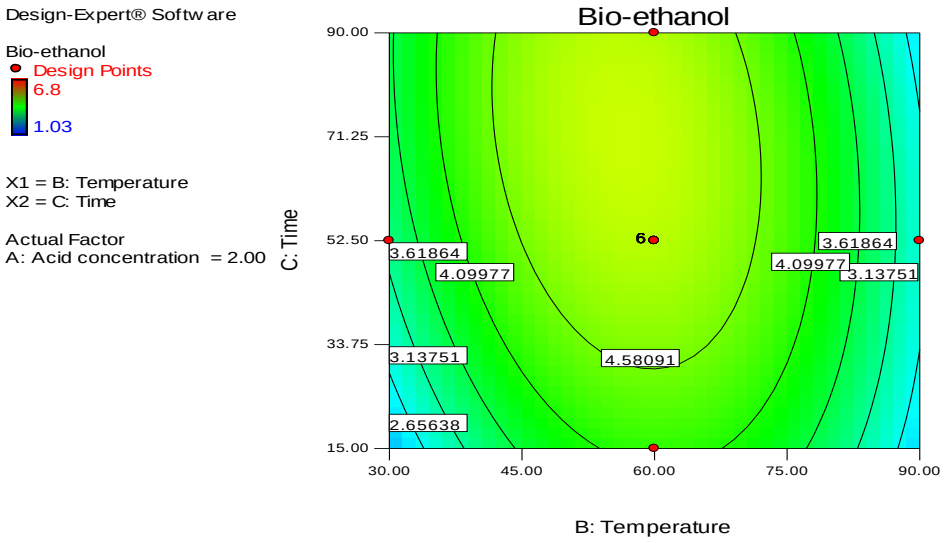

Figure 19 Contour plots of the effects of time and temperature on the yield of ethanol.

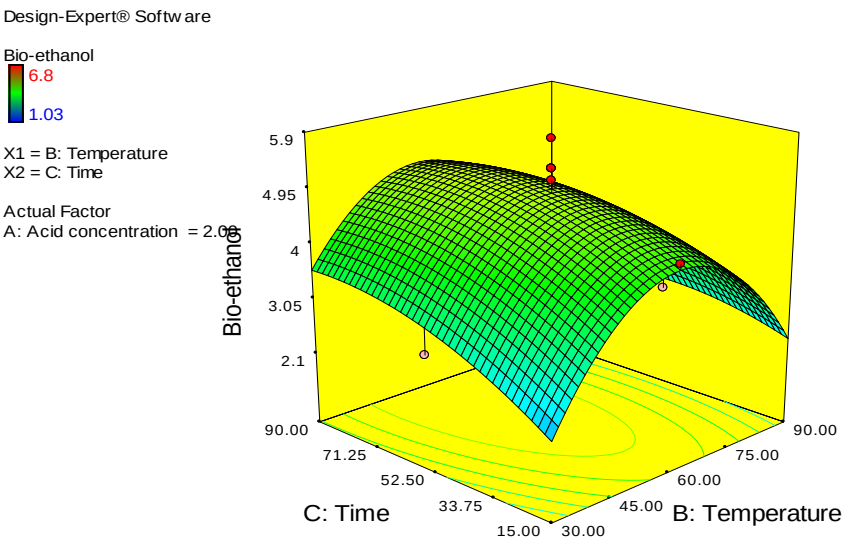

Figure 20 Surface plots of the effects of time and temperature on the yield of ethanol.

From the contour plot graph showing predicted response of ethanol yield as a function of hydrolysis time and hydrolysis temperature was shown in figure 19 and 20. As hydrolysis time increases at lower level temperature gives positive effect on the yield of ethanol and it decrease when the hydrolysis time and temperature became higher and higher. The response surface Figure 20, obtained from hydrolysis time and hydrolysis temperature was conical shape. It suggests that there were well-defined optimum operating conditions. The response optimized value for the production of ethanol from wheat straw was based on both in hydrolysis time and temperature. The effects of acid concentration and time on the yield of glucose, acid concentration was selected at the center point (figure 19 and 20. The maximum yield of glucose was observed at medium temperature and medium hydrolysis time. At increasing/decreasing temperature, and time from the medium the yield of glucose became decreases since the possible formation of other molecules instead of glucose formation. The decrease of sugar content in acid treated samples with increasing of acid concentration is may be because of degradation of monomeric sugars (xylose, glucose) in furfural and hydroxymethyl furfural. These substances are toxic substances for yeast and can inhibit the yeast growth (Nutawan *et al.*, 2010).

4. Conclusion

Considering on morphological and physiological characteristics, the four yeast isolates were selected to further optimization of the results. Four yeast isolates showed best growth, at 30℃ at pH 5 and 20% glucose concentration. In this study diluted acid hydrolysis were used and the effect of the hydrolysis process variable (time, temperature and dilute acid concentration) in the yield of reducing sugar/ethanol was investigated and optimized using response surface methodology (RSM) based on central composite design (CCD). The bio-ethanol production from wheat straw and optimization test showed that 1% H_2S0_4 was preferable than other dilute acid hydrolysis values. The optimization study showed that the highest bio-ethanol concentration of 6.8g/l was observed by SWX under the optimum conditions of with 1% $H_2S0_{4,}$ 60^{o}C temperature and 52.5 minute time hydrolysis at 30°C for 48 hour incubation time. The minimum yield was obtained at 3% acid concentration, 90^{o}C temperature and 15 minute time which were 4.19 g/l of

reducing sugar and 1.03 g/l of ethanol yield. Samples containing high amount of reducing sugar concentration produced high amount of ethanol. Based on analysis of variance (ANOVA) hydrolysis temperature and acid concentration interaction have significant effect on the yield of ethanol. Generally, the ethanol production from wheat straw may gain the fuel availability and it may lead to the sustained development.

5. References

Adeeyo, O., Oresegun, O. M., Oladimeji, T. E. (2015). Compositional analysis of lignocellulosic materials: Evaluation of an economically viable method suitable for woody and non-woody biomass. *American Journal of Engineering Research* (AJER). 4: 14-19.

Ali, W.R., Rasul, Aziz, K., Bujag, A. and Shamsiah, D. S. (2012). Production of bio cellulosic ethanol from wheat straw. *Acta Polytechnica*. 52: 143-151

Ali Z., Hussain, M., Arshad, M. (2014). Saccharification of corn cobs an agro-industrial waste by sulphuric acid for the production of monomeric sugars: International Journal of Biosciences. 5 (3): 204-213.

Alicia (2013). Sodium hydroxide pretreatment of corn Stover and subsequent enzymatic hydrolysis: An investigation of yields, kinetic modeling and glucose recovery. *International Journal of Biosciences*. 12: 52-61

Arenas-Cárdenas, P., López-López, A., Moeller-Chávez, G. E. and León-Becerril, E. (2017). Current pretreatments of lignocellulosic residues in the production of bioethanol. *Waste and Biomass Valorization.* 8: 161-181.

Azhar, S. H. M., Abdulla, R., Jumbo, S. A., Marwari, H., Gansu, J. A., Fail, A. A. M. and Rodrigues , K. F. (2017). Yeasts in sustainable bioethanol production. *Biochemistry and biophysics reports.* 10: 52-61.

Balat, M. (2011). Production of bioethanol from lignocellulosic materials via the biochemical pathway. *Energy conversion and management*. 52: 858-875.

Belisa, B., Shied B., Conclaves, D. L., Knychala, M. M., Matsushika, A., Bon E. P. and Stambuk, B. U. (2015). Cloning novel sugar transporters from *Scheffersomyces (Pichia) stipitis* allowing d-xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Biotechnology letters*. 37: 1973-1982.

Breisha, G. Z. (2010). Production of 16% ethanol from 35% sucrose. *Biomass and bioenergy*. 34: 1243-1249.

Campelo, A.F. and Belo, I. (2004). Fermentative capacity of baker's yeast exposed to hyperbaric stress. *Biotechnol Lett*. 26(15): 1237-1240.

Chan, ES. Chandel, A. K., Rudravaram, R., Narasu, M. L, Rao, L. V. and Ravindra Pogaku (2007). Economics and environmental impact of bioethanol production technologies: an appraisal. *Biotechnology and Molecular Biology Reviews*. 2(1):14-32.

Chang, Y.H., Chang, K.S., Chen, C.Y., Hsu, C.L., Chang T.C. and Jang, H.D. (2018). Enhancement of the Efficiency of Bioethanol Production by *Saccharomyces cerevisiae* via Gradually Batch-Wise and Fed-Batch Increasing the Glucose Concentration. *Fermentation*. 4: 45-56.

Davidi, L., Morais, S., Artzi, L., Knop, D., Hadar, Y., Arfi, Y., Bayer, E.-A. (2016). Toward Combined Delignification and Saccharification of Wheat Straw by a Lactase-Containing Designer Cellulose. *Proceedings of the National Academy of Sciences of the United States of America*. 113(39), 10854–10859.

De Souza Abdu, A. K., de Souza da Silva, A. and Martins de Oliveira Junior, A. (2017). Effect of inhibitors on ethanol production by *Pichia stipitis* in a complex culture media. *Acta Scientiarum. Technology*: 39 (2):1806-2563.

Demirbaş, A. (2005). Bioethanol from cellulosic materials: a renewable motor fuel from biomass. *Energy sources*. 27: 327-337.

Donghai, S., Junshe, S., Ping, L. and Yanping, L. (2006). Effects of Different Pretreatment Modes on the Enzymatic Digestibility of Corn Leaf and Corn Stalk1. *Chinese Journal of Chemical Engineering.* 14:796-801.

ESC (Ethiopian Sugar Corporation, (2011). The five years strategic plan of the Ethiopian sugar corporation. Publish Ethiopia.

El-Sheikh, H. H. (2016). Factors Affecting Bioethanol Production from Hydrolyzed Bagasse. *Int. J. Adv. Res. Biol. Sci.* 3: 130-138.

Flores-Cosío, G., Arellano-Plaza, M., Gschaedler-Mathis, A., Amaya-Delgado, L.(2018). Physiological responses to furan derivatives stress by *Kluyveromyces marxianus* SLP1 in ethanol production. *Revista mexicana de ingeniería química*. 17:189-202

Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, MF. (2007).Towards industrial pentose-fermenting yeast strains. *Appl Microbiol Biotechnol*. 74:937–953.

Harmsen P., Huijgen W., Bermudez L., Bakker R. (2010).Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. In *Biosynergy*. Edited by. Wageningen UR Food and Biobased Research;

Harris, J., Baker, A. and Zerbe, J. (1984). Two-stage, dilute sulfuric acid hydrolysis of hardwood for ethanol production. *Energy biomass wastes*. 8: 1151-1170.

Ho, D. and Powell, C. (2014). The Effect Temperature on the Growth Characteristic of Ethanol Producing Yeast Strain. *International Journal of Renewable Energy and Environmenta Engineering*. 2: 1-6.

Hossain, Z., Golam, F., Jaya, N. S., Mohd S. A., Rosli, H. and Amru, N. B. (2014). Bioethanol Production from Fermentable Sugar Juice. *The Scientific World Journal*. 11:7

Jemal Fito, Nurelegne Tefera, Sisay Demeku, Helmut, K. (2017). Water Footprint as an Emerging Environmental Tool for Assessing Sustainable Water Use of the Bioethanol

Distillery at Metahara Sugarcane Farm, Oromiya Region, Ethiopia. *Water Conservation Science and Engineering.* 2: 165-176.

Jin, M., Lau, M. W., Balan, V. and Dale, B. E. (2010). Two-step SSCF to convert AFEX-treated switchgrass to ethanol using commercial enzymes and *Saccharomyces cerevisiae* 424A (LNH-ST). *Bioresource technology*. 10: 8171-8178.

Jin, Y., Speers, R.A. (2000). Effect of environmental condition on flocculation of *saccharomyces cervices*. *J. Am. Soc. Brew. Chem*. 58: 108-116.

Kalhorinia, S., Goli, J. K. and Rao, L. V. (2014). Screening and parameters optimization of pentose fermenting yeasts for ethanol production using simulated media. *Bioscien Biotechnol Resear Asia.* 11: 641-648.

Khaing ,T. W., Weine, N. and Mya, M.O. (2008). Isolation, characterization and screening of thermo tolerant, ethanol tolerant indigenous yeasts and study on the effectiveness of immobilized cell for ethanol production. *Asian J. Sci. Technol*. 1: 12-14.

Kim, S., Dale, B.E. (2004). Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy*. 26: 361–375.

Kordowska-Wiater, M. (2015). Production of arabitol by yeasts: current status and future prospects. *Applied Microbiology*. 119(2):303–314.

Koti, S., Govumoni, S. P., Gentela, J. and Rao L. V. (2016). Enhanced bioethanol production from wheat straw hemicellulose by mutant strains of pentose fermenting organisms *Pichia stipitis* and *Candida shehatae*, 5: 15-45.

Kuhad, R. C., Gupta, R., Khasa, Y.P., Singh, A. and Zhang, Y.H. P. (2011). Bioethanol production from pentose sugars: Current status and future prospects. *Renewable and Sustainable Energy Reviews*. 15: 4950-4962.

Kumar, A.K., Sharma, S. (2017) Recent Updates on Different Methods of Pretreatment of Lignocellulosic Feedstocks: A Review. *Bioresources and Bioprocessings*. 4(1), 7-9.

Kumar, M. N., Ravikumar, R., Thenmozhi, S., Kumar, M. R. and Shankar, M. K. (2018). Choice of Pretreatment Technology for Sustainable Production of Bioethanol from Lignocellulosic Biomass: Bottle Necks and Recommendations. *Waste and Biomass Valorization*. 8 (1):1-17.

Kumar, P., Barrett, D.M., Delwiche, M.J. and Stroeve, P. (2009). Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. *Industrial and engineering chemistry research.* 48(8): 3713-3729.

Laopaiboon, L., Thanonkeo, P., Jaisil, P. and Laopaiboon, P. (2007). Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by *Saccharomyces cerevisiae. World Journal of Microbiology and Biotechnology*. 23: 1497-1501.

Lara-Hidalgo, C., Grajales-Lagunes, A., Ruiz-Cabrera, M.A., Ventura-Canseco, C., GutiérrezMiceli, F.A., Ruiz-Valdiviezo, V.M., Archila, M.A., Guillén-Navarro, K., Sánchez, J.E. (2017). Agave Americana honey fermentation by *Kluyveromyces marxianus* "comiteco" production, aspirit from Mexican southeast. *Revista Mexicana de Ingeniería Química.* 16: 771-779

Lenihan, P., Orozco, A., O'Neil, E., Ahmad, M.N.M., Rooney, D.W. and Walker, G.M. (2010). Dilute acid hydrolysis of lignocellulosic biomass. *Chemical Engineering Journal*. 156 (2): 395-403.

Liu, L., Ye, X.P., Womac, AR, and Sokhansanj, S. (2010).Variability of biomass chemical composition and rapid analysis using FT-NIR techniques. *Carbohydr Polym*. 81:820–829.

Lin, Y., Zhang, W., Li, C., Sakakibara, K., Tanaka, S. and Kong, H. (2012). Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. *Biomass and bioenergy*. 47: 395-401.

Liu, R. and Shen, F. (2008). Impacts of main factors on bioethanol fermentation from stalk juice of sweet sorghum by immobilized *Saccharomyces cerevisiae* (CICC 1308). *Bioresource technology*. 99: 847-854.

Lopes, D. D., Rosa, C. A., Hector, R. E., Dien, B. S., Mertens J. A. and Ayub, M. A. Z. (2017). Influence of genetic background of engineered xylose-fermenting industrial *Saccharomyces cerevisiae* strains for ethanol production from lignocellulosic hydrolysates. *Journal of industrial microbiology & biotechnology*. 44: 1575-1588.

Louhichi, B., Belgaib, J. Benamor, H. and Hajji, N. (2013). Production of bio-ethanol from three varieties of dates. *Renewable Energy*. 51: 170–174.

Lu, Y., Voon, M. K. W., Huang, D., Lee, P.R. and Liu, S.Q. (2017). Combined effects of fermentation temperature and pH on kinetic changes of chemical constituents of durian wine fermented with *Saccharomyces cerevisiae*. *Applied microbiology and biotechnology*. 101: 3005-3014.

Maitan-Alfenas, G. P., Visser, E. M. and Guimarães, V. M. (2015). enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products. *Current Opinion in Food Science*. 1: 44-49.

Miller, G. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 31:426-428.

Moilanen U, Kellock M, Galkin S, Viikari L. (2011). The laccase-catalyzed modification of lignin for enzymatic hydrolysis. *Enzyme and Microbial Technology* 49:492- 498.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M. and Ladisch, M. (2005) Features of promising technologies for pretreatment of lignocellulose biomass. *Bioresource Technology* 96: 673–686.

Mussatto, S. I. and Roberto, I. C. (2005). Acid hydrolysis and fermentation of brewer's spent grain to produce xylitol. *Journal of the Science of Food and Agriculture*. 85: 2453-2460.

Nadir, N., Mel, M., Karim, A., Ismail, M. and Mohd Yunus, R. (2009). Comparison of sweet sorghum and cassava for ethanol production by using *Saccharomyces cerevisiae*. *Journal of Applied Sciences.* 9: 3068-3073.

Nambu-Nishida, Y., Nishida, K., Hasunuma, T., Kondo, A. (2017). Development of a comprehensive set of tools for genome engineering in a cold- and thermo-tolerant *Kluyveromyces marxianus* yeast strain. *Scientific Reports*. 7:1-7.

Nanda, S., Mohammad, J., Reddy, S. N., Kozinski J. A. and Dalai, A. K. (2014). Pathways of lignocellulosic biomass conversion to renewable fuels. *Biomass Conversion Biorefinery*. 4: 157-191.

Nigam, P. S. and Singh A. (2011). Production of liquid biofuels from renewable resources. *Progress in energy and combustion science*. 37: 52-68.

Nikolić, S., Pejin, J. and Mojović, L. (2016). Challenges in bioethanol production: utilization of cotton fabrics feedstock. *Chemical Industry and Chemical Engineering Quarterly*. 22(00):1-1

Niven, A. and Robert, K. (2005). Ethanol in gasoline: environmental impacts and sustainability review. *Renewable and sustainable energy reviews*. 9(6): 535-555.

Nutawan Y., Phattayawadee P., Pattranit T. and Mohammad N. E. (2010). Bioethanol Production from Rice Straw. *Energy Research Journal* 1 (1): 26-31.

Ortiz, R., Sayre, K.D., Govaerts, B., Gupta, R., Subbarao, G.V., Ban, T., Hodson, D., Dixon, J.M., Ivan Ortiz-Monasterio, J., Reynolds, M. (2008). Climate change: can wheat beat the heat. *Agriculture, Ecosystems and Environment* 126: 46–58.

Osho, A. (2005). Isolation, identification and screening of the yeast flora from indian cashew apple for sugar and ethanol tolerance. *Int. J. Biotechnol. Wel. Indust.* 6*:* 259-265.

Palmqvist E., Hahn-Hagerdal B. (2000). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technology*. 74:25-33.

Pavlečić, M., Rezić, T., Šantek, M. I., Horvat, P. and Šantek, B. (2017). Bioethanol production from raw sugar beet cossettes in horizontal rotating tubular bioreactor. *Bioprocess and biosystems engineering.* 40: 1679-1688.

Perez, J., Munoz-Dorado, J., Rubia, T.-D.-L., Martinez, J. (2012). Biodegradation and Biological Treatments of Cellulose, Hemicellulose, and Lignin: An Overview. *International Microbiology*. 5: 53–63.

Radhika, K., Ravinder, R. and Ravindra, P. (2011). Bioconversion of pentose sugars into ethanol: a review and future directions. *Biotechnology and Molecular Biology Reviews*. 6: 8-20.

Rao, R.S., Bhadra, B. and Shivaji, S. (2008). Isolation and characterization of ethanol-producing yeastsfrom fruits and tree barks. *Letters in Applied Microbiology*. 47:19–24

Ratnavathi, C. V., Chakravarthy, S. K., Komala, V. V., Chavan, U. D. And Patil, J. V. (2011) "Sweet sorghum as feedstock for biofuel production: a review," *Sugar Technology*.13 (4): 399–407.

Rodrussamee, N., Sattayawat, P. and Yamada, M. (2018). Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* Cmuwf1–2. *BMC microbiology*. 18: 73-82.

Saini, J. K., Saini, R. and Tewari, L. (2015). Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *Biotech*. 5: 337-353.

Sathees Kumar, R., Shankar, T. and Anandapandian K. T. K. (2011). Characterization of alcohol resistant yeast *Saccharomyces cerevisiae* isolated from Toddy. *Int.l Res.J. Microbiolo.* 26: 399-405.

Senthilkumar, V. and Gunasekaran, P. (2005). Bioethanol production from cellulosic substrates: Engineered bacteria and process integration challenges. *Journal of Scientific and Industrial Research.* 64: 845-853.

Silvaa, A. S., Júniora, A. M. O., de Farias Silvab, C. E. and Baud, A. K. S. (2016). Inhibitors Influence on Ethanol Fermentation by *Pichia Stipitis*. *Chemical Engineering*, 49.

Sime, W., Kasirajan, R., Latebo, S., Mohammed, A., Seraw, E., and Awoke, W. (2017). Coffee Husk Highly Available in Ethiopia as an Alternative Waste Source for Biofuel Production, 8(7), 1874–1880.

Slininger, P. J., Shea-Andersh, M. A., Thompson, S. R., Dien, B. S., Kurtzman, C. P., Balan,V., da Costa Sousa, L., Uppugundla, N., Dale, B. E. and Cotta, M. A. (2015). Evolved strains of *Scheffersomyces stipitis* achieving high ethanol productivity on acid-and base-pretreated biomass hydrolyzate high solids loading. *Biotechnology for biofuels*. 8: 60-69.

Stanley, D., Bandara, A., Fraser, S., Chambers P. and Stanley G. A. (2010) .The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. *Journal of applied microbiology*. 109: 13-24.

Stewart, G.G. (2015). The concept of nature-nurture applied to brewer's yeast and wort fermentation. *Tech. Quart. Master Brew. Assoc. Am*, 52: 146-151.

Stratford, M. (1992). Yeast flocculation: new perspectives. *Adv. Microb. Physiol*. 33:1-71.

Sumphanwanich J., Leepipatpiboon N., Srinorakutara T., Akaracharanya A. (2008). Evaluation of dilute-acid pretreated bagasse, corn cob and rice straw for ethanol fermentation by *Saccharomyces cerevisiae: Annals of Microbiology*, 58 (2) 219-225.

Sun, Y., Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review, *Bioresource technology*. 83(1): 1-11.

Taherzadeh, M. and Karimi, K. (2007). Acid- based hydrolysis processes for ethanol from lignocellulosic materials: *A Review. BioRes*. 2: 472-499.

Ting, C. L., Makarov, D.E. and Wang, Z.G. (2009). A Kinetic Model for the Enzymatic Action of Cellulase. *The Journal of Physical Chemistry*. 113(14): 4970–4977.

Tutt, M. (2015). Factors affecting biochemical composition of lignocellulosic biomass and its effect on selection of pretreatment method and on bioethanol production potential. *Eesti Maaülikool*. 10(1): 269 - 276.

Vohra, M. Manwar, J., Manmode, R., Padgilwar, S.and Patil S. (2014). Bioethanol production: feedstock and current technologies. *Journal of Environmental Chemical Engineering*. 2: 573-584.

Watanabe, S., Shimada, N., Tajima, K., Kodaki, T. and Makino, K. (2006). Identification and characterization of L-arabonate dehydratase, L-2-keto-3-deoxyarabonate dehydratase and L-arabinolactonase involved in an alternative pathway of L-arabinose metabolism: novel evolutionary insight into sugar metabolism. *Journal of Biological Chemistry.* 281(44):33521-36.

Zhu, J.Y., Pan, X. J. (2010). Woody biomass pretreatment for cellulosic ethanol production: Technology and energy consumption evaluation. *Bioresource Technology.* 101(13): 4992-5002.