
Acid Hydrolysis Optimization of *Prosopis Juliflora* Stem for Bioethanol Production

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Abstract: Ethanol producing from biomass has become an interesting substitute to petroleum in the struggle against raised levels of fossil carbon dioxide emission to the atmosphere. The biotechnological conversion of biomass into fuels requires hydrolysis of the polysaccharide fraction into monomeric sugars. This study was aimed at the optimization of the processing conditions of the hydrolysis step of bio-ethanol production to produce high fermentable sugar. In this study dilute acid hydrolysis, temperature and residence time were used as process parameters. Response Surface Methodology (RSM) was employed for the optimization of hydrolysis conditions. A 3 (three) level design was used to develop a statistical model for the optimization of process variables. The raw material, *P. juliflora* stem, was collected from Afar region eastern part of Ethiopia specifically Wrer Agricultural Research Center. The optimal hydrolysis conditions that resulting for the maximum total reducing sugar concentration were at acid concentration; 2% (v/v), temperature; 128.01°C and hydrolysis time; 55 minutes. Under these conditions, the total reducing sugar concentration was obtained to be 184.716mg/g.

Keywords: Ethanol, *Prosopis Juliflora*, Reducing Sugar, Hydrolysis

1. Introduction

1.1. Back Ground

Fuels obtained from renewable resources have deserved a great deal of interest during the past decades mainly due to concerns about fossil fuels depletion and environment pollution. Research efforts have been multiplied in the last years as a consequence of constant increasing costs and environmental impact derived from the use of crude-based fuels (Gray & Emptage, 2006). Production of fuel from renewable resources has advantages over fossil fuel in that less environmental impact and sustainability of resource.

Shortage of oil supplies, the growing pressure on reduction in the net emission of CO₂, global warming, and the use of food crops for ethanol production resulted in global food crises have maintained research and development on the ethanol production from non edible renewable raw materials (Himmel, 2007). Although much progress has been made in many areas of biomass biorefinery, efficient production of cellulosic ethanol from plant biomass remains a challenge.

Prosopis juliflora (SW) D. C., Leguminosae, a popular plant known as mesquite, is native to Central and South America and has spread to North America.

In East Africa, *P. Juliflora* was introduced in the 1970s' through collaborative projects involving local governments and outside agencies (Coppock, 2005). In the Afar region of Ethiopia, where *P. juliflora* is having dramatic impacts across the landscape, its spread and impacts on resources has been ranked as one of the leading threats to the range lands and biodiversity, ranked as the most problematic plant invader in Ethiopia (Tessema, 2007; EEP, 2006). The potential use of *P. juliflora* is that it is a fast growing plant, which can grow on any marginalized areas; this may be taken as advantageous to use as a sustainable feedstock for bioethanol production. Hydrolysis of the pod of *P. juliflora*, into simple sugar with water in the absence of acid shows that the potential as feed stock for ethanol production (Negusu, 2009).

The *P. juliflora* (Mesquite) has recently been suggested to be used as raw material for long-term sustainable production of cellulosic ethanol (Hopkins, 2007). Its nature to tolerate drought, grazing, heavy soil, sand as well as saline dry flats

and indigestibility by animals made it a potential feedstock for ethanol production.

1.2. Objective of the Study

The aim of this work was to determine optimum condition for the hydrolysis of *P. juliflora* stem to release fermentable sugar for ethanol production.

2. Method

2.1. Sample Collection and Preparation

Samples of *P. juliflora* stem were collected from Afar region (Werer) in February, 2013. They were collected by cutting with large knife from the upright part of the plant having approximately 3-4 mm in diameter.

The stems of *P. juliflora* were chopped to suitable size for drying and for further milling. The chopped stems were washed in tap water to remove impurity from outer part of the stem. Sample drying was carried out in oven at a temperature of 65°C for 48h.

Oven dried samples were milled in hammer mill (Brazile, model TRAPP, TRF300G) to 2-3mm size then in a disk mill (German, RETSCH, SK1) to more smaller sizes where sieved to a particle size of 1.4 mm and 0.5 mm. Milled material was stored at room temperature in air tight containers and dried in a laboratory oven at 50°C for 24h prior to use.

2.2. Sample Characterization

2.2.1. Moisture Analysis

Three grams of milled *P. juliflora* were taken for moisture determination and put in an oven working in forced hot air purging system at 105°C in a convection oven and the result of moisture content is expressed in percentage weight form.

2.2.2. Ash Analysis

The crucible was marked with pencil and it was placed in the muffle furnace at 550°C until a constant weight gained. Three samples having 7.4, 8.03 and 9.17 g were taken for ash determination. The samples were inserted in the muffle furnace and ignited at 550°C for three hours.

2.2.3. Extractives Analysis

Ten gram of milled sample was taken for the analysis of extractive contents and extracted with 98% ethanol refluxing in a soxhlet apparatus for 24 hr. The extract was concentrated in a rotary evaporator and dried in a vacuum oven at 40°C for 24 hr. The amount of extractive substances expressed in percent on dry weight bases (ASTM D1105-84, 1993).

2.2.4. Acid Insoluble Lignin

The acid insoluble lignin amount was determined following the ASTM E 1721-95 (ASTM, 1995). Ten ml of Sulfuric acid (H₂SO₄) at a concentration of 72% was used to hydrolyze 3g of extract free sample in 100 ml beaker and the mixture was stirred and dispersed thoroughly with a glass rod and held for 2 hr at 30°C in a water bath. Then the solution was transferred to 250 ml Erlenmeyer flask and diluted to 3% and refluxed for

2 hr. The hydrolyzed substrate was filtered through fritted glass filter. The remaining residue was dried to constant weight and the dried residue was then placed in a furnace at 575°C for 3 hr and the residue ash was weighed.

2.2.5. Determination of Neutral Detergent Fiber (NDF) Procedure

Two gram sample of the *P. juliflora* stem was taken in a conical flask of 500 ml capacity and 100 ml of neutral detergent solution was added to it. Then 0.5 g sodium sulphate was added and refluxed for 1hr. The contents were filtered comprehensively, by washing its residues two times with distilled water. The residues were shifted to tared crucibles and kept in an oven at 105°C for 24 hr until constant weight achieved. Then, the Neutral Detergent Fiber was determined and expressed in percentage weight bases AOAC (Association of Official Analytical Chemists, 1972).

2.2.6. Acid-Detergent Fiber Procedure

Acid detergent solution:

Acid detergent solution was prepared by dissolving 20 g Cetyltrimethylammonium bromide (CTAB) in 1.0 N sulfuric acid. The residues of NDF were transferred to a 500ml conical flask, fitted over with an air condenser, and added 100 ml of the acid detergent solution. The flask was heated to boiling for 6 minutes, and allowed to reflux fiber for 60 minutes. The flask-contents were filtered, thoroughly, using a suction pump by washing twice with the acetone. The residues were transferred to a dried crucible and kept in the oven, at 105°C for 24 hr.

The oven-dried crucibles were taken out of the oven, desiccated and allowed to cool. The weight of the crucible along with dried residues was recorded.

2.2.7. Determination of Cellulose Contents (%)

Acid detergent fiber residues, in dried form, were taken in a crucible, weighed and placed in a beaker, to which 10ml of 24 N H₂SO₄ was added and allowed to stand for one hour. After one hour, further 10 ml of 24 N H₂SO₄ was added and then again allowed to stand for one hour. This process was repeated for the third time. After that distilled water was added to get 100 ml of the volume. The solution was, then, filtered and washed with distilled water. The residues left were transferred to tared crucibles and were kept in the oven at 105°C for 8 hours until constant weight of the dried residues reached.

2.2.8. Determination of Hemicellulose (%)

The weights of the dried residues of ADF and NDF, discussed previously, were taken.

The difference among them was determined and resulted Hemicellulose value.

2.3. Acid Hydrolysis

10% (w/v) of Milled wood samples at a solid loading was soaked in sulphuric acid (H₂SO₄) of 2, 4 and 6% (v/v). Acid hydrolysis was performed in triplicate at 110, 125 and 140°C in autoclave with residence times of 15, 35 and 55 min. After hydrolysis, the reaction vessels were rapidly cooled in a cold

water bath. The pH of the hydrolyze material was adjusted to pH 5.2 with 2M NaOH solution. The hydrolysate was filtrated to separate liquid and solid phase by suction with vacuum. Then, the filtrate fraction was collected for sugar analysis by using Titration (Canettieri, 2007; Melsert, 2007).

2.3.1. Determination of Sugar

The total reducing sugar from hydrolysis of *P. juliflora* were determined using standardisation of Fehling's Solution

2.3.2. Experimental Design and Data Analysis

The RSM with three level factorial was chosen to determine the effect of three operating factors of the hydrolysis temperature, time, and acid concentration. One response variables which were yield of reducing sugars produced per total holocellulose dry mass, on the operating condition, which has a significant influence on the acid hydrolysis process. Mean values of the treatments were compared at $p=0.05\%$ confidence level with experimental designe 10.0 program.

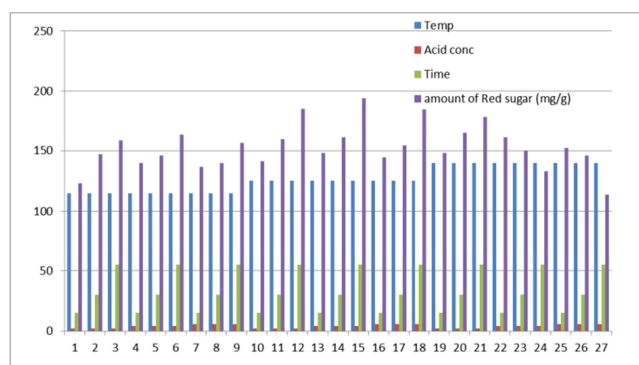
3. Result and Discussion

3.1. Proximate Chemical Composition of *P. Juliflora*

The chemical components for *P. juliflora* are comparable to other biomass source used for the same objective, for instance, for *Eucalyptus grandis* residue 40%, 16% and 23% for cellulose, hemicelluloses and insoluble lignin, respectively Canettieri *et al.*, (2007); loblolly pine also showed that 43.6%, 21.2% and 26.8% cellulose, hemicelluloses and insoluble lignin, respectively (Puneet *et al.*, 2009). Whereas for Cotton Stems, 37.9%, 20.4% and 24.0% for cellulose, hemicelluloses and insoluble lignin, respectively which is lower in cellulose content (Beck & Clements., 1982).

The considerably high cellulose and hemicelluloses content in *P. juliflora* indicates the potential for production of a great variety of products, as ethanol, which may be used as a fuel or raw material for synthesis of different chemicals, (Werpy & Petersen, 2004).

3.2. Total Reucing Sugar Yield



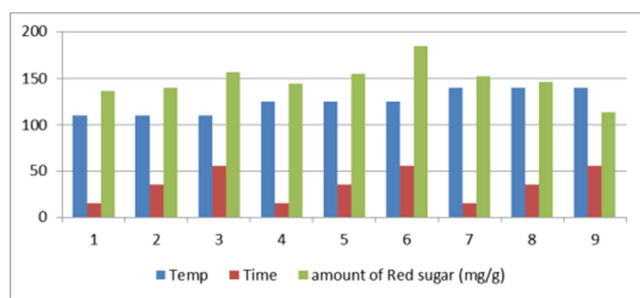
c) Six percent H₂SO₄**Figure 2.** TRS yield with respect at different acid concentration as a function of temperature and resident time.

Figure 2a shows the experiment carried out at 2% H₂SO₄ as a function of resident time and temperature. As it is shown in the figure, at 115°C, 125°C and 140°C, the amount of free sugar released were constantly increased as the resident time increases as shown in figure 2a. In addition, in all the three cases of the same residential time, the amounts of free sugar produced were increased as the temperature increases except in one case where the highest amount of free sugar production is attained figure 2a. The highest amount of free sugar release was found at a temperature of 125°C and

resident time of 55 min, indicating at 2% of H₂SO₄ concentration figure 2b. An increase in temperature around 140°C, increase the formation of toxic compounds such as furfural and 5-hydroxymethyl-furfural (HMF). In addition, these compounds lead to reduction of fermentable sugars (Kootstra *et al.*, 2009).

As depicted in Figure 2b, the yield of hydrolysate at 110°C and 125°C with increasing resident time had a positive effect on TRS yield. But further increase in temperature leads to decline the TRS yield even for a short resident time. This is an indication that at high temperature, in this case 140°C, leading to degradation of sugar. Thus high temperature, with increasing the reaction time reduced the yield of TRS which means that the reaction require resident time of less than 15 min. The studies of Ojumu & Ogunkunle (2005) also found that, the glucose yield gradually decreases after the 20 min of reaction due to its decomposition.

In the case of 6% H₂SO₄ figure 2c, the release of hydrolyzed sugar showed similar trend as 4% acid concentration. For a minimum temperature 110°C the influence of residence time is insignificant when compared to 2% and 4% H₂SO₄. Hydrolysis the prosopis stem with 2% and 4% of H₂SO₄ at 125°C the maximum reducing sugars were released at hydrolysis time of 55 min.

3.3. Statistical Analysis of the Experimental Result

Table 1. Coded (A, B and C), Respective Actual Levels (Temp, Acide and Time) and the response value.

Test no.	Coded value			Actual value			Yield of total reducing sugars (mg/g)
	A	B	C	Time (min)	Acid conc (%v/v).	Temp (°C)	
13	-1.00	-1.00	-1.00	15	2	110	123.29
27	0.00	-1.00	-1.00	35	2	110	147.06
26	1.00	-1.00	-1.00	55	2	110	158.66
15	-1.00	0.00	-1.00	15	4	110	139.63
7	0.00	0.00	-1.00	35	4	110	146.21
4	1.00	0.00	-1.00	55	4	110	163.04
1	-1.00	1.00	-1.00	15	6	110	136.44
31	0.00	1.00	-1.00	35	6	110	139.93
9	1.00	1.00	-1.00	55	6	110	156.25
8	-1.00	-1.00	0.00	15	2	125	141.51
2	0.00	-1.00	0.00	35	2	125	159.57
22	1.00	-1.00	0.00	55	2	125	185.64
20	-1.00	0.00	0.00	15	4	125	148.22
25	0.00	0.00	0.00	35	4	125	160.94
23	1.00	0.00	0.00	55	4	125	194.30
29	-1.00	1.00	0.00	15	6	125	144.23
18	0.00	1.00	0.00	35	6	125	154.32
32	1.00	1.00	0.00	55	6	125	184.73
10	-1.00	-1.00	1.00	15	2	140	148.22
5	0.00	-1.00	1.00	35	2	140	164.47
16	1.00	-1.00	1.00	55	2	140	178.50
17	-1.00	0.00	1.00	15	4	140	160.94
12	0.00	0.00	1.00	35	4	140	150.00
21	1.00	0.00	1.00	55	4	140	132.98
11	-1.00	1.00	1.00	15	6	140	152.44
28	0.00	1.00	1.00	35	6	140	145.91
14	1.00	1.00	1.00	55	6	140	113.64
24	0.00	0.00	0.00	35	4	125	159.23
6	0.00	0.00	0.00	35	4	125	158.89
30	0.00	0.00	0.00	35	4	125	161.23
19	0.00	0.00	0.00	35	4	125	161.23
3	0.00	0.00	0.00	35	4	125	161.12

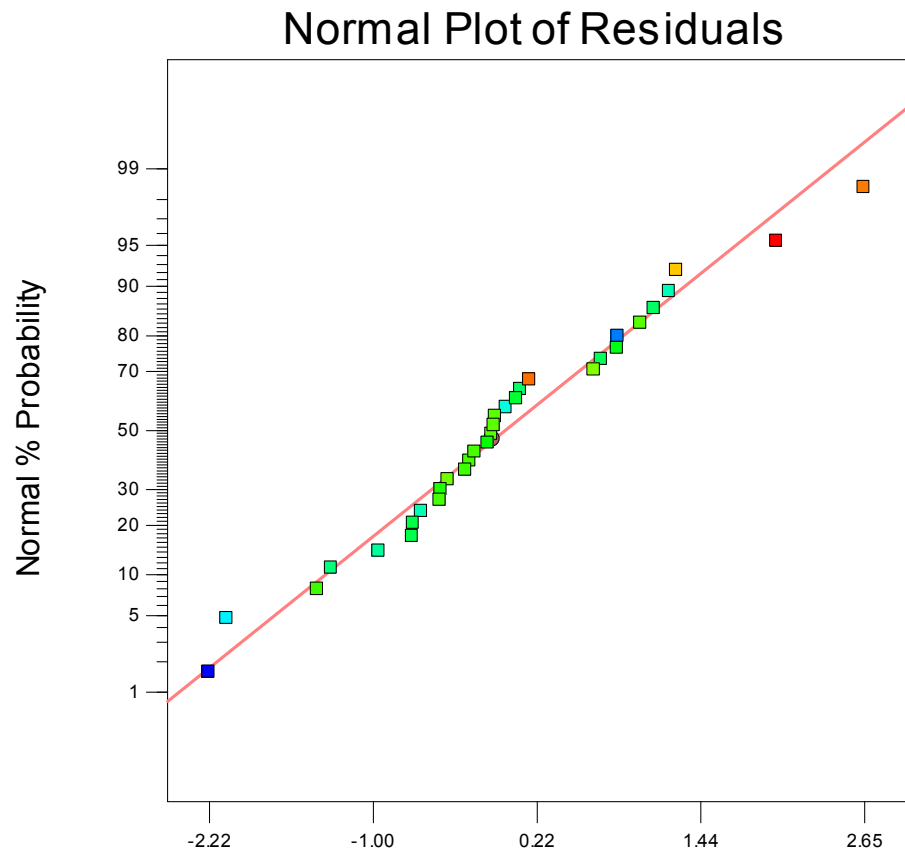
The ANOVA results from quadratic equation described in table 2 below indicate the model was significant due to high F-Value of 5.77 and have a low Probability value of 0.0004.

Table 2. Analysis of Variance (ANOVA) for Quadratic Models of TRS.

Source	Sum of square	Degree of freedom	Mean square	F-value	P-value, Prob>F
Model	6545.80	10	654.58	5.77	0.0004 significant
A-Time	1660.61	1	14.55	14.55	0.0010
B-Acid conc.	347.60	1	3.05	3.05	0.0095
C-Temp.	74	1	0.65	0.65	0.4276
AB	650.33	1	5.70	5.70	0.0264
AC	1102.08	1	9.66	9.70	0.0053
BC	572.42	1	5.02	5.02	0.0361
A ²	49.05	1	0.43	0.40	0.5192
B ²	29.29	1	0.26	0.28	0.6177
C ²	1586.08	1	13.90	14.09	0.0012
ABC	358.99	1	3.15	3.15	0.0906
Residual	2390.83	21	114.11		
Pure Error	5.42	5	1.08		
Cor Total	8942.5	31			

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Figure 3. Normal plots of residuals.

By applying multiple regression the three variable and total reducing sugar produced relationship was found by second degree polynomial.

Red. sugar (mg/g) = 161.87 + 9.60 A - 4.39 B + 2.04 C - 7.36 AB - 9.58 AC - 6.96 BC + 2.74 A² - 1.90 B² - 14.77 C² - 6.70 ABC 3.1.

Red. Sugar (mg/g) = -973.40886 - 0.85175 * Time - 12.01358 * Acidconc + 17.0243 * Temp + 1.21153 * Time * Acidconc + 0.012714 Time * Temp + 0.16056 * Acidconc * Temp + 6.84234E - 003 * Time² - 0.47618 * Acidconc² - 0.065651 * Temp² - 0.011165 * Time * Acid conc * Temp 3.2

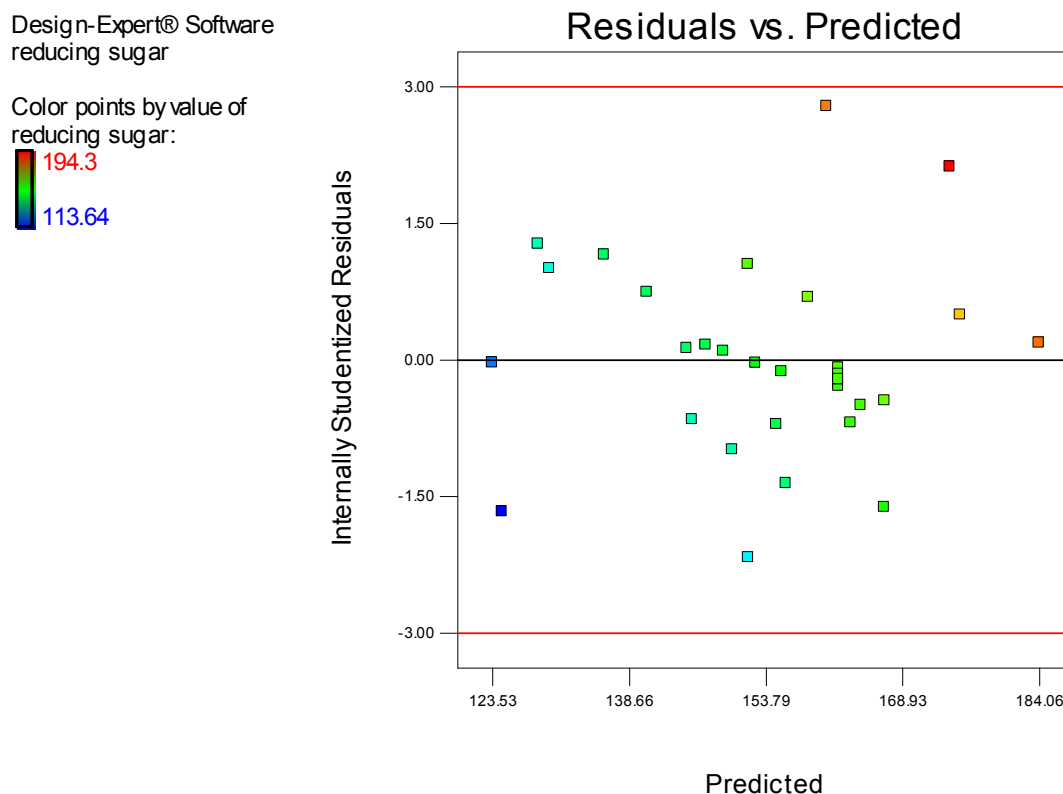


Figure 4. Residual versus predicted values.

The plot as shown in figure 3, the residuals follow a normal distribution, indicating that quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA).

The plot in figure 4 shows constant range of residuals across the graph which is justifiable no need for a transformation to minimize personal error.

3.4. Effects of Experimental Variables on Hydrolysis Results

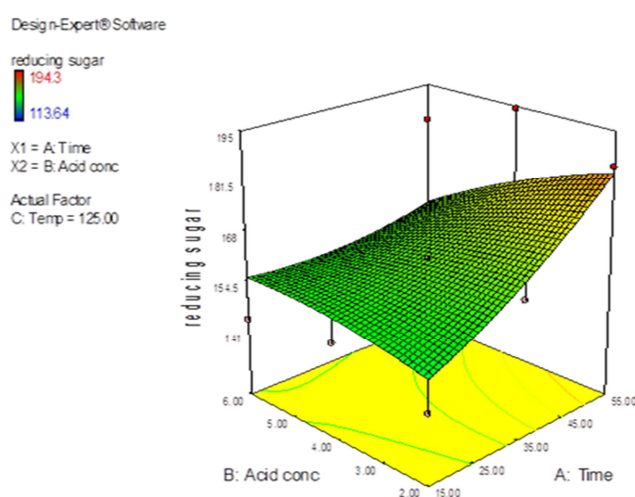


Figure 5. Effect of acid concentration and Time on reducing sugar yield when temperature was at the center point.

For lower value of acid concentration and reaction time had resulted in smaller conversion of *Prosopis juliflora* stem

in to fermentable reducing sugar. For lower residence time increasing of the acid concentration had small effect on TRS yield and the plot indicates that for higher value of acid concentration TRS yield decreased from its value across the resident time. Due to liability of sugar components in higher concentration of acid better amount of TRS yield was obtained at lower acid concentration (2%) and highest residence time (55min) At higher concentration the formation of HMF and its yield increases (Talebnia, 2008). The formation of inhibitors such as furfural and HMF from the decomposition of liberated sugars through the secondary reactions (Bienkowski et al. 1987; Taherzadeh et al. 2000; Azhar et al. 1981). Formation of HMF during dilute-acid hydrolysis is a sequential reaction where cellulose and hemicellulose are first hydrolyzed to their hexoses and pentoses monomers with subsequent decomposition to furfural and HMF. These two reactions are influenced by temperature and acid concentration. The higher ratio of the first reaction rate constant compared to the second one increasing the yield of total liberating sugars.

These facts suggest that the conditions for dilute-acid hydrolysis and the variables affecting this process should be carefully selected and optimized to yield the highest hydrolyzate and extent of depolymerization of the carbohydrate polymers and maximum release of sugars, while the formation of inhibitory compounds is minimized. The hydrolysis is shown higher value at the corner of low value acid concentration and higher value of reaction time in which the temperature keeps at the centre point.

At lower temperature increasing of time had resulted in a

considerable change with linear increment of TRS yield as a result of slow hydrolysis of the polysaccharides; whereas, at higher temperature the influence of increasing hydrolysis time had negative effect for TRS yield with inter molecular condensation of the released fermentable sugars to their corresponding furfural and HMF figure 6. As the temperature increased for each level of time the TRS yield had increased; however, further increasing of the reaction temperature resulted in a lower TRS yield, which might be due to the decomposition of TRS to form HMF, furfural, levulinic acid, and acetic acid (Change *et al.*, 2007, Talebnia, 2008). In the other case, at lower residence time increasing of temperature to optimum level has a strong positive effect on the formation of TRS which followed by a decline of released sugar as the temperature increased beyond the suitable temperature value. Similarly, for higher residence time increasing temperature showed a moderate increment in the formation of TRS to its maximum yield for initial increasing temperature and reached maximum not far away from 120°C, then further increasing temperature decline the yield of TRS. Thus, more resident time is required if the hydrolyzate reaction takes place at minimum temperature. This graph also indicates that the maximum temperature at which higher TRS yield was obtained to be around 130°C. In this work, the maximum TRS yield was achieved with a reaction time of 55 min.

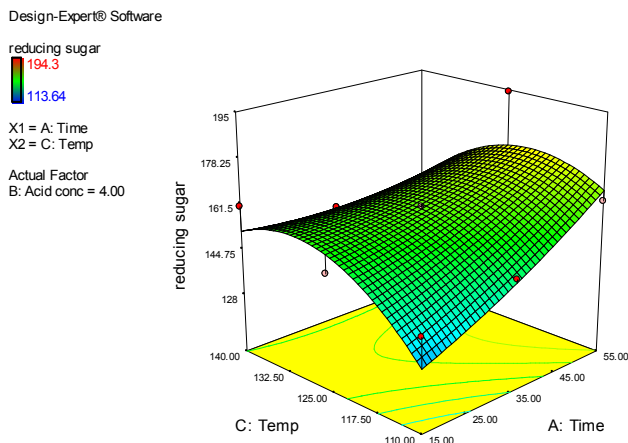


Figure 6. Effect of time and temperature on sugar yield when acid concentration was at the center point.

When time was kept at the center point 35 min the interaction of temperature and acid concentration, at the lower levels of temperature in figure 7 an increase in acid concentration resulted in slightly more yield as stated earlier in figure 5 were an increase of acid concentration resulted in negative effect on TRS even for lower temperature the yield was not satisfactory. In other way, at higher levels of temperature, increase in acid concentration results continuous declination of sugar yield. Furthermore, the declination of TRS started as the temperature goes beyond 130°C at 2% acid concentration. This situation similarly observed in the previous studies of Talebnia, (2008), at the higher levels of both temperature and acid concentration, the yield of sugars declined, certainly due to the decomposition of sugar

components in furfural and HMF (Amarasekara, 2011).

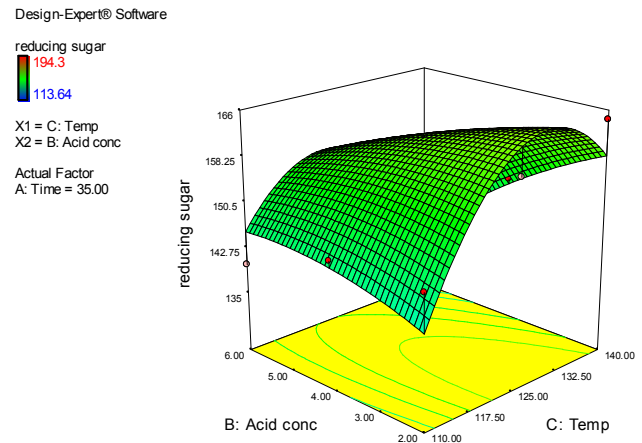


Figure 7. Effect of temperature & acid concentration on TRS yield when time was at the center point.

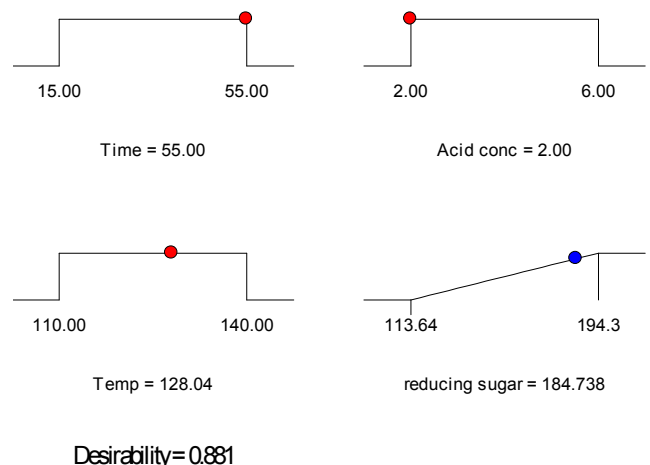
However, increasing of temperature beyond 125°C the sugar yield generation by all the acid concentration was start to decline sharply. It can conclude from figure 4 at the higher levels of both temperature and acid concentration, the yield of sugars declined, certainly due to formation of HMF and furfural (Talebnia, 2008). Theoretically, higher temperature could accelerate the rate of cellulose hydrolysis to TRS; however, unwanted side reaction could also be produced at elevated temperature (Amarasekara & Wiredu, 2012; Chang *et al.* 2007; Li *et al.* 2008).

3.5. Optimization

Table 3. Optimization criteria for optimum sugar yield.

Name	Goal	Lower Limit	Upper Limit
Time (min)	In range	15	55
Acid concentration (%v/v)	In range	2	6
Temperature (°C)	In range	110	140
Reducing sugar (mg/g)	Maximize	113.64	194.3

The optimum possible solutions in hydrolysis for different independent variables for reducing sugar yield and the corresponding surface plot are shown in table 4 and figure 8 below.



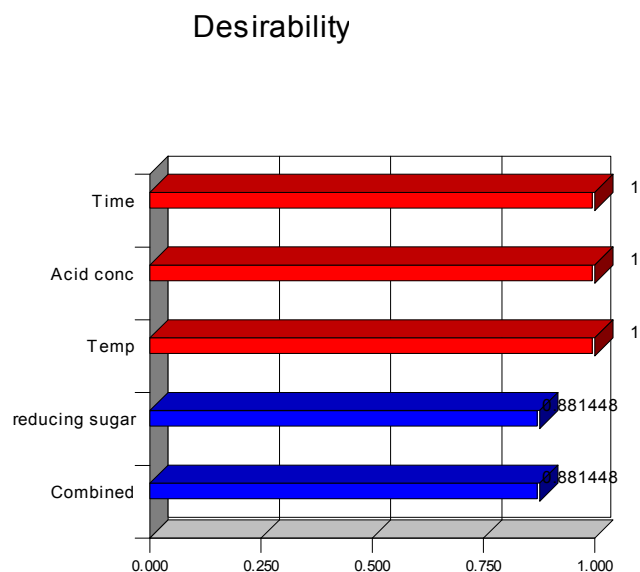


Figure 8. Ramp and histogram display of desirability.

Table 4. Solution for optimization.

Number	Time	Acid conc	Temp	reducing sugar	Desirability
1	55.00	2.00	128.01	184.716	0.881
2	55.00	2.00	128.18	184.714	0.881
3	55.00	2.00	127.91	184.712	0.881
4	55.00	2.00	128.43	184.704	0.881
5	55.00	2.00	127.61	184.704	0.881
6	55.00	2.00	128.55	184.696	0.881
7	55.00	2.01	128.53	184.622	0.880

The ramp display combines the individual graphs for interpretation. The dot on each ramp reflects the factor setting or response prediction for optimum solution. The height of the dot shows how desirable it is. The histogram shows how well each variable satisfied the criteria: values near one are good.

In the present work, dilute sulphuric acid hydrolysis of milled wood of *P. juliflora* was optimized to achieve maximum sugar yield at minimum severity conditions. The optimum hydrolysis conditions i.e. 2.0% (v/v) acid at 128.01°C for 55 min, gave saccharification yield of 184.716 mg/g. This value is comparable with other studies, for example acid hydrolysis of bagasse from *Agave tequilana* weber (Saucedo et al. 2010) fermentable sugar yield was 200mg/g of agave bagasse treated when the reaction is carried out at 150°C, reaction time 10 min and 2% of catalyst. While any further increase in hydrolysis stringency caused the increase in release of toxic compounds without much effect on sugar yield.

3.6. Validation of the Model

The suitability of the model equation for predicting the optimum response values was tested using the optimum conditions mentioned above. The results obtained from three replications demonstrated that the maximum total sugar concentration 194mg/g obtained was close to the predicted value 191mg/g. This result indicates that there is excellent

correlation between experimental and predicted values and in turn proves the validity of the model.

4. Conclusion

This research was designed to utilize *P. juliflora* stem for ethanol production. The optimization results of *P. juliflora* stem clearly showed that the plant's stem is a promising raw material for production ethanol. The following conclusions are drawn from the study:

- *P. juliflora* containing 59% (w/w) holocellulose is used as a low-cost feedstock for bioethanol production
- Hydrolysis of *P. juliflora* stem is influenced by the concentration of acid, time and temperature.
- The concentration of total reducing sugars produced during hydrolysis is related to acid concentration, time and temperature by a validated quadratic polynomial regression model.
- The optimum processing conditions drawn from RSM were an acid concentration of 2% (v/v), temperature of 128.01°C, and time of 55 minutes. Under these conditions, the maximum concentration of total reducing sugar obtained was 184.72mg/g.

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