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Statistical BBD (Box-Behnken Design) for sugarcane bagasse pretreatment condition optimization using liquid ammonia and saccharification of bagasse

Rama Mohan P and Boonsawang P

Abstract

An ongoing study was directed to detect the best possible pretreatment process to improve the cellulose and reduce the total lignin yield in liquid ammonia (NH_3)-pretreated bagasse. To examine the individual and cooperative effects of process variables: substrate concentration, NH_3 loading, pretreatment temperature and time on the overall yield of cellulose, hemicellulose and lignin in resultant bagasse, statistical response surface methodology (RSM) design was applied. Tests were done by Box-Behnken Design (BBD) to set up the ideal pretreatment process conditions for bagasse. Results of the response surface design analysis indicate that ideal conditions to achieve the highest cellulose yield (44.6%) with decreased lignin yield (8.7%) at a substrate bagasse concentration is around 2%, NH_3 loading is around 20%, treatment temperature is 120 ºC and retention time for 75 min. The good correlation noticed among the observed and predicted experimental results indicate the present model was useful to pretreat the lignocellulosic substrates. Model verification is specified, there is no disagreement among predicted and observed values. Higher reducing sugar (39.6 mg/g) yields were observed in high NH_3 loaded treated bagasse than the low NH_3 loaded pretreated bagasse (26.8 mg/g).

Keywords: Liquid ammonia (NH_3), sugarcane bagasse, BBD, pretreatment, optimization, cellulose yield

Introduction

Lignocellulosic biomass, for example, agricultural deposits (corn stover, wheat straw and sugarcane bagasse), energy crops and wood, is an attractive material for bioethanol making since it is an immense renewable resource on the earth (Wyman, 1994) [1], and usually contain higher levels of cellulose and hemicellulose (75–80%) along with lignin, which can't be easily changed to simple monomeric sugars due to their recalcitrant nature (Pala *et al*., 2007) [2]. The natural procedure for changing the lignocellulosic substrates into bioethanol requires: delignification to free hemicelluloses and cellulose from their complex association with lignin polymer, depolymerization of the polysaccharides to provide free sugars, and subsequent fermentation of combined pentose and hexose sugars to generate ethanol (Saha and Bothast, 1997) [3].

On account of the complex structure of biomass and its recalcitrant nature, lignocellulosic material needs destructive pretreatment to yield a substrate that can be effectively hydrolyzed via industrial cellulolytic enzymes or by enzyme-producing microbes to unleash sugars for fermentation (Agbor *et al*., 2011) [4]. The reason for pretreatment is to open up the biomass structure, to develop the available surface area, to lessen the cellulose crystallinity and to develop the porosity, pore size and pore volume. There are a number of pretreatment procedures accessible for different biomasses with varying degrees of achievement: acid pretreatment, alkaline pretreatment, wet oxidation, steam explosion, hot water and organic solvent pretreatment (Morjanoff and Gray, 1987) [5].

Among various chemical pretreatments, anhydrous liquid ammonia is widely utilised chemical and the price is about one-fourth the price of sulphuric acid on a molar basis suggesting the AFEX/ARP pretreatment is less expensive than DAP (Kim *et al*., 2003) [6]. Considering pretreatment chemicals, ammonia has several useful characteristics: greatly selective delignification reaction, swelling of cellulosic materials, low interface with carbohydrates and elevated volatility (Kim *et al*., 2003) [7]. The ammonia fibre explosion (AFEX) method can also alter or efficiently diminish the lignin portion of the lignocellulosic materials, whereas the hemicelluloses and cellulose portions may just remain together. At ideal conditions, AFEX can considerably promote the enzymatic hydrolysis.

The main benefit of the AFEX process that it does not generate some forms of inhibitory pretreatment by-products, which were generated during the other pretreatment procedures, for example furans in steam explosion and dilute-acid pretreatment. Although a fraction of the phenolic parts of the lignin complex and additional cell wall extractives may stay on the cellulosic surface (Taherzadeh and Karimi, 2008) [8]. AFEX pretreatment can considerably enhance the saccharification rates of different grasses and herbaceous crops and be utilised for the treatment of numerous lignocellulosic substrates including corn stover (Kim *et al*., 2003) [6], rice straw (Vlasenko *et al*., 1997) [9], wastepaper (Kim *et al*., 2000) [10] and herbaceous biomass (Iyer *et al*., 1996) [11]. AFEX process pretreatment does not require little molecule size for efficacy (Vlasenko *et al*., 1997) [9].

Some of the principal cellulosic agro-industrial by products are sugarcane bagasse, a dry pulpy-fibrous residue of cane stalks available after the distruction and extraction of the rum from the sugarcane. Bagasse is a proper cellulosic substrate for a bioconversion procedure because of its moderately low lignin portion and its availability at central collection sites (Dawson and Boopathy, 2007) [12]. Fractionation of bagasse constituents and their conversion into useful fermentable sugars are important in allowing this renewable feedstock to be used for bioethanol generation (Rezende *et al*., 201) [13]. For the essential utilisation of lignocellulosic feedstock, it is essential to establish a pretreatment procedure for the specific and effective fractionation of its principle polysaccharide components, which must undergo hydrolysis to generate a higher concentration of sugar monomers, which can then be utilised as substrates for biotechnological and chemical processes (Mussato and Roberto, 2002) [14].

Another important level in the progress of an efficient and financial pretreatment system is optimizing the pretreatment conditions. The conventional "one variable at a time" (OVAT) procedure is time-consuming; also, the interactions between independent variables are usually not considered (Karunanithy and Muthukumarappan, 2011) [15]. RSM is a helpful strategy for testing multiple process variables, since fewer experimental trials are required in comparison with the study of one variable at a time. Besides, interactions between factors can be recognised and measured by such a method. Currently, RSM has been well applied to biomass pretreatment by many researchers (Rodrigues *et al*., 2003; Karunanithy and Muthukumarappan, 2011) [16; 15]. Even though biomass pretreatment research has been conducted for over three decades, no impeccable conversion process has been described for biofuels generation from biomass on a profitable scale (De Leon and Coors, 2008) [17].

Proximate analysis of untreated bagasse demonstrates that bagasse contains cellulose of around 42.7%, hemicellulose of 34.4% and lignin of around 18.2% (Mohan *et al*., 2013) [18]. The essential target of the current study was to utilise RSM, particularly BBD, for finding improved pretreatment process circumstances to enhance the yield of cellulose and decrease lignin yield in liquid NH_3-pretreated bagasse.

Material and Methods

Raw bagasse substrate

Sugarcane bagasse solid residue was kindly given by S.V. Sugar Factory, Tirupati, India. Then bagasse was kept in an oven to dry for 7-8 hours at 70-80 ºC and the oven dried material was collected, sieved, milled, and utilised in granular form.

Experimental method design

BBD with the quadratic model (Box & Wilson, 1951) [19] was implemented for bagasse pretreatment process optimization. Four variables, such as substrate concentration (X_1% w/v), NH_3 loading (X_2% v/v), pretreatment process temperature (X_3 ºC) and pretreatment time (X_4 min), were chosen as the free independent variables represented in Table 1. Cellulose (Y_1%), hemicellulose (Y_2%) and lignin (Y_3%) yields were used as response-dependent outcome variables (Y). In BBD, the levels and range of the variables tested in present study shown in Table 1. Total 29 experiment trails were tested according to Table 2. The statistical analysis of design was completed by Design-Expert 8.0.6.1 (Stat-Ease Inc., Minneapolis, USA) version software. Three-dimensional (3D) surface plots and their connected contour plots were achieved to observe the interaction of one variable with another.

Bagasse saccharification

Saccharification tests were conducted as duplicate runs in 50 mL Erlenmeyer flasks comprising high (20%) and low (2%) NH_3 pretreated 1g bagasse, crude cellulose mixture (Mohan *et al*., 2013) [18] and Tween-80 (polysorbate 80) in 0.5 M Citric acid – Sodium citrate buffer (pH 4.8-5.0) were added to 1% (v/v) of penicillin-streptomycin antibiotic solution. Then flasks were aseptically incubated in a rectangular incubator shaker at 50 °C with 200 rpm for 60-90 h. Samples were taken at regular intervals, then centrifuged (12000 rpm) for 12-15 minutes and the obtained supernatant liquid was used for sugar estimation.

Experimental analysis

For the individual separation of cellulose, hemicellulose and additionally lignin from the different components of the lignocellulosic biomass, the procedure (AOAC, 2005) [20] involving multifunction procedures was used. Procedure of measurement of neutral detergent fiber (NDF) report for the hemicellulose, lignin and cellulose content and express mainly the primary cell wall fibers in plant biomass. After NDF estimation, the obtained residue was further utilized for acid detergent fiber (ADF) estimation. The content of hemicellulose was calculated through subtracting the ADF value from the NDF value (Jung and Vogel, 1992) [21]. The material treated by ADF and NDF was additionally hydrolyzed by using 72% concentrated H_2SO_4 to estimate the cellulose percentage. Lignin content was attained through the ashing of hydrolyzed solid residue. Reducing sugar and glucose were assessed using the 3,5-dinitrosalicylic acid (DNSA) procedure (Miller, 1959) [22]. The content of arabinose and xylose was valued by the procedure previously described (Khabarov *et al*., 2006) [23].

Results

Process optimization trials are planned to provide intensive knowledge about just some factors recognised through exploration as having the highest effect on efficacy. Finally, under particular experimental conditions, the conformation of experiments is utilised to confirm the results (Chen *et al*., 2002) [24]. The impact of four process variables on the yield of cellulose, hemicellulose and lignin was examined employing BBD. Table 1 and Table 2, display the experimental results. Influence of every variable and their associations were examined by applying the statistical ANOVA (analysis of variance) and chi-squared test (X_2) as suitable to the current experimental model being utilized.

Table 1: Coded and actual values of the factors in Box-Behnken Design (BBD).

Factor	Name	Low actual	Middle actual	High actual	Low coded	Middle coded	High coded
X_1	Substrate (%, w/v)	5		20	-1	0	1
X_2	Liquid NH_3 (%,v/v)	2		20	-1	0	1
X_3	Temperature (°C)	60	90	120	-1	0	1
X_4	Time (min)	30	75	120	-1	0	1
Response	Name	Units	obs[a]	Min.	Max.	Mean	Std. Dev.
Y_1	Cellulose	%	29	30.2	44.6	37.8276	10.2
Y_2	Hemicellulose	%	29	14.8	23.6	17.1931	12.4
Y_3	Lignin	%	29	8.7	19.8	13.9448	3.78

[a]Observed run values

Considering optimised treatment conditions, an estimated regression equation supported the conclusion that the yield of cellulose (Y_1%), hemicellulose (Y_2%) and lignin (Y_3%) is a characteristic of the experimental substrate concentration (X_1%), NH_3 loading (X_2%), pretreatment process temperature (X_3 °C) and pretreatment time (X_4 min). Using multiple regression on the present trial data result, the next second-degree polynomial equation used to be considered to signify the yield of cellulose, hemicellulose and lignin appropriately.

Cellulose (%) $$Y_1=36.8–0.73X_1+2.09X_2+2.69X_3+3.58X_4–1.03X_1^2+1.0X_2^2+1.4X_3^2+1.12X_4^2+0.1X_1X_2+0.2X_1X_3+0.58X_1X_4–1.92X_2X_3+0.35X_2X_4–1.05X_3X_4 \quad (1)$$

Hemicellulose (%) $$Y_2=15.2+0.13X_1+0.40X_2+0.92X_3+2.05X_4+0.28X_1^2+2.28X_2^2+2.48X_3^2–0.067X_4^2–0.87X_1X_2+0.22X_1X_3–0.6X_1X_4+0.1X_2X_3–0.025X_2X_4–0.075X_3X_4 \quad (2)$$

Lignin (%) $$Y_3=13.4+0.91X_1–0.99X_2–0.70X_3–2.92X_4+1.90X_1^2–0.82X_2^2–1.46X_3^2+1.69X_4^2+0.82X_1X_2+0.30X_1X_3+0.35X_1X_4+0.075X_2X_3+0.22X_2X_4+0.77X_3X_4 \quad (3)$$

By utilising the mentioned equations, the anticipated levels for yield of cellulose, hemicellulose and lignin in bagasse are presented in Table 3. The integrity of the present design can be tested using various criteria. The determination coefficient (R^2) values for three process- dependent (response) variables were greater than 0.90, representing that the regression analysis model clarified the process properly and confirmed that the models for every dependent variable were very well fitted to clarify the relationships between the variables.

ANOVA for the present quadratic regression model proved that Eqs. 1–3 are greatly statistically important models for yield of cellulose, hemicellulose and lignin responses in resultant bagasse, as it was noticeable from the F-test with a low probability (p) value. By using R^2, the present model's goodness-of-fit was examined.

Cellulose yield

Linear polysaccharide cellulose may be divided into glucose monomers by both enzymatically through the action of cellulase enzymes or chemically with sulfuric or different concentrated acids. Acids or microbial hemicellulases are involved in the hydrolysis of heterogeneous hemicellulose (polyose) polymer to liberate its constituent sugar residues (Mosier *et al.*, 2005) [25]. For cellulose yield based on the regression analysis (Y_1), the R^2 value is 0.9239, specifies that simply 7.61% of the aggregate changes were not clarified by the current model. Also, the value of the present model was supported by the high adjusted R^2 value of around 0.8478. Table 3 shows that X_3 and X_4 are considerable model terms with 99% probability. Because of the significant interaction among X_2 and X_3, this will improve the cellulose percentage in bagasse.

Table 2: Box-Behnken design matrix

Std.	Substrate (%, w/v)	NH_3 (%, v/v)	Temp. (°C)	Time (min)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
	X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3
1	-1	-1	0	0	35.1 (35.4)	16.8 (16.3)	15.2 (15.3)
2	1	-1	0	0	32.8 (33.9)	17.6 (18.3)	17.1 (15.5)
3	-1	1	0	0	39.2 (39.5)	18.9 (18.9)	11.6 (11.7)
4	1	1	0	0	36.9 (38.1)	16.2 (17.4)	16.8 (15.2)
5	0	0	-1	-1	30.2 (31.0)	18.6 (17.5)	16.4 (17.0)
6	0	0	1	-1	38.6 (39.4)	16.4 (16.5)	15.9 (15.0)
7	0	0	-1	1	40.6 (41.2)	18.2 (18.8)	11.2 (10.6)
8	0	0	1	1	43.8 (44.5)	19.7 (20.5)	11.4 (10.8)
9	-1	0	0	-1	34.6 (34.6)	12.2(12.6)	14.2 (13.7)
10	1	0	0	-1	33.4 (32.0)	14.8 (14.1)	16.2 (15.4)
11	-1	0	0	1	41.5 (40.5)	18.2 (17.9)	11.8 (11.4)
12	1	0	0	1	42.6 (40.3)	17.9 (17.0)	10.4 (11.3)
13	0	-1	-1	0	32.2 (32.4)	20.2 (20.1)	12.6 (12.8)
14	0	1	-1	0	41.2 (40.5)	24.6 (23.9)	10.8 (10.7)
15	0	-1	1	0	43.3 (41.2)	22.8 (21.5)	9.2 (9.6)
16	0	1	1	0	44.6 (42.0)	23.6 (23.0)	8.7 (9.5)
17	-1	0	-1	0	36.2 (35.4)	17.0 (16.8)	13.9 (14.2)
18	1	0	-1	0	33.8 (33.5)	17.2 (16.9)	14.2 (13.9)
19	-1	0	1	0	39.4 (40.3)	17.6 (18.5)	13.9 (14.5)
20	1	0	1	0	37.8 (39.3)	18.9 (19.2)	14.2 (13.9)
21	0	-1	0	-1	34.2 (33.6)	14.7 (14.9)	17.4 (18.3)

22	0	1	0	-1	36.8 (37.0)	16.4 (15.7)	15.6 (15.9)
23	0	-1	0	1	39.6 (40.0)	19.8 (18.6)	11.6 (11.6)
24	0	1	0	1	43.6 (44.9)	16.4 (15.7)	14.3 (13.8)
25^{a}	0	0	0	0	36.8 (36.8)	15.2 (15.2)	13.4 (13.4)
26^{a}	0	0	0	0	36.8 (36.8)	15.2 (15.2)	13.4 (13.4)
27^{a}	0	0	0	0	36.8 (36.8)	15.2 (15.2)	13.4 (13.4)
28^{a}	0	0	0	0	36.8 (36.8)	15.2 (15.2)	13.4 (13.4)
29^{a}	0	0	0	0	36.8 (36.8)	15.2 (15.2)	13.4 (13.4)

Std: Standerd run order; ^{a}= Central value.

Hemicellulose yield

Pretreatment severity conditions are typically a compromise to maximise sugar recovery, depending upon what kind of pretreatment method is utilized. Hemicellulose could be obtained either as a solid fraction or as a blend of both liquid and solid fractions (Chandra *et al*., 2007) [26]. From the experiments, the model R^2 value for hemicellulose is around 0.9210, which specifies only 7.9% of total variations were not clarified, and the adjusted R^2 is 0.8401 in the present design, which has greater significance. Table 3 shows that X_4, X_2^2 and X_3^2, are considerable model terms with 99% probability and X_3 is an important model term with a percent probability of around 95%. However, no important interaction was observed between the method variables on bagasse hemicelluloses yield.

Table 3: Analysis of variance for the experimental results of the Box-Behnken design

		F-Value			P-value		
Source	**df**	**Y_1**	**Y_2**	**Y_3**	**Y_1**	**Y_2**	**Y_3**
Model	14	12.14	11.5	10.99	< 0.0001	< 0.0001	< 0.0001
X_1	1	3.01	0.25	7.3	0.1049$^{\#}$	0.6219$^{\#}$	0.0172*
X_2	1	25.03	2.29	8.71	0.0002$^{\alpha}$	0.1526$^{\#}$	0.0105*
X_3	1	41.45	12.02	4.34	< 0.0001$^{\beta}$	0.0038	0.0561$^{\#}$
X_4	1	73.12	60.1	75.32	< 0.0001$^{\beta}$	< 0.0001$^{\beta}$	< 0.0001$^{\beta}$
X_1X_2	1	2.71	3.65	2.01	1$^{\#}$	0.0768	0.1783$^{\#}$
X_1X_3	1	0.076	0.24	0.27	0.7864$^{\#}$	0.6308$^{\#}$	0.6143$^{\#}$
X_1X_4	1	0.63	1.72	0.36	0.4404$^{\#}$	0.2113$^{\#}$	0.5573$^{\#}$
X_2X_3	1	7.07	-6.78	0.017	0.0187*	1$^{\#}$	0.8993$^{\#}$
X_2X_4	1	0.23	2.98	0.15	0.6363$^{\#}$	9.57$^{\#}$	0.7049$^{\#}$
X_3X_4	1	2.1	2.70	1.77	0.1691$^{\#}$	8.72$^{\#}$	0.2043$^{\#}$
X_1^2	1	3.28	0.62	17.35	0.0918$^{\#}$	0.444$^{\#}$	0.001*
X_2^2	1	3.07	40.31	3.22	0.1018$^{\#}$	< 0.0001$^{\beta}$	0.0942$^{\#}$
X_3^2	1	6.02	47.68	10.18	0.0278*	< 0.0001$^{\beta}$	0.0065*
X_4^2	1	3.88	0.034	13.7	0.0688$^{\#}$	0.8556$^{\#}$	0.0024*
Residual	14						
Lack of fit	10						
Pure error	4						
Cor total	28						

Y_1=Cellulose, Y_2=Hemicellulose, Y_3=Lignin; *p<0.05–significant at 5% level, $^{\alpha}p$<0.001–significant at 1% level, $^{\beta}p$<0.0001 significant at 0.1% level, $^{\#}$not significant

Lignin yield

Delignification (removal of lignin using chemicals) leads to biomass swelling, a disorder of lignin structure, increases in the internal surface region and improved availability of cellulolytic enzymes to cellulose fibres (Agbor *et al*., 2011) [4]. Though not all pretreatment procedures result in considerable delignification, the lignin polymer structure might be altered without extraction because of changes in the chemical properties of the lignin. In this research, considering the R^2 for lignin yield is 0.9166 and only 8.34% of the entire changes were not clarified with this model. The importance of the present model was supported on by getting the high adjusted R^2 value of around 0.8332. X_4 is a considerable model term with a percent probability of 99%. Because of the significant interaction among X_2 and X_3 (Table 3) the cellulose yield in bagasse increases. There is no considerable interaction observed among independent variables to reduce the lignin percentage in the final bagasse obtained.

From ANOVA Table 3, it can be understood that both pretreatment process temperature and retention time had the strongest effect on the yield of cellulose and hemicellulose and retention time showed a considerable effect on lignin yield. Temperature increases induce the cleavage of the lignin-carbohydrate bonds. A lower yield of lignin was accomplished at the temperature at its middle level (75 min).

Optimization

The experimental response surface designs displayed the effects of four individual method variables on the total yield of cellulose, hemicellulose and lignin in the obtained bagasse. Result denotes that the response surface designs of both cellulose and hemicellulose had the highest position along with lignin at the restricting point. Box-Behnken designs were helpful in showing the way with which to modify the factors to increase the cellulose and reduce the hemicellulose and lignin yields. To cellulose, the evaluation of the predicted values with the observed actual values are in acceptable agreement. The improved level of cellulose content was accomplished at a substrate concentration of 12.5% with a pretreatment retention time of 75 min. There is a substantial and significant interaction observed between NH_3 loading and pretreatment temperature (Figure 1), which shows that the increased temperature alongside increased NH_3 loading results in better cellulose yield in the resultant bagasse.

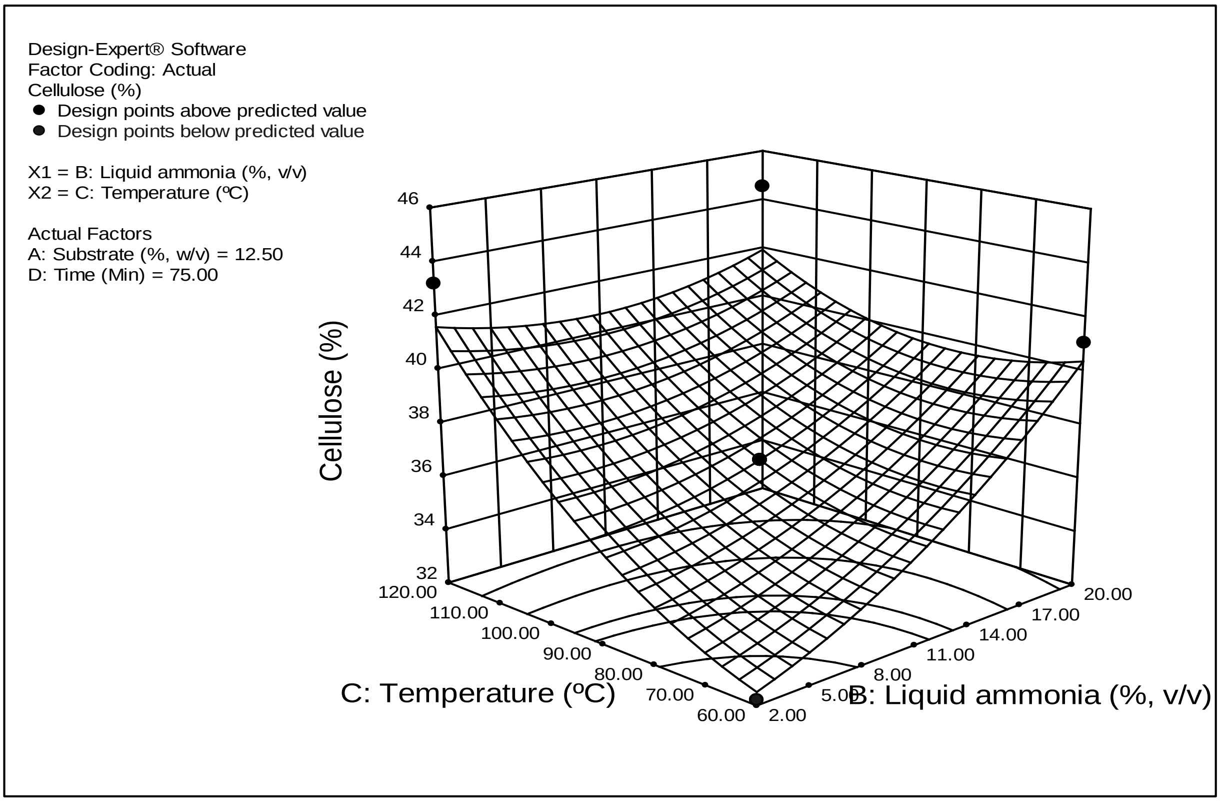

Fig 1: Response surface plot show the interactive effect of liquid ammonia (%) and pretreatment temperature (ºC) on cellulose yield.

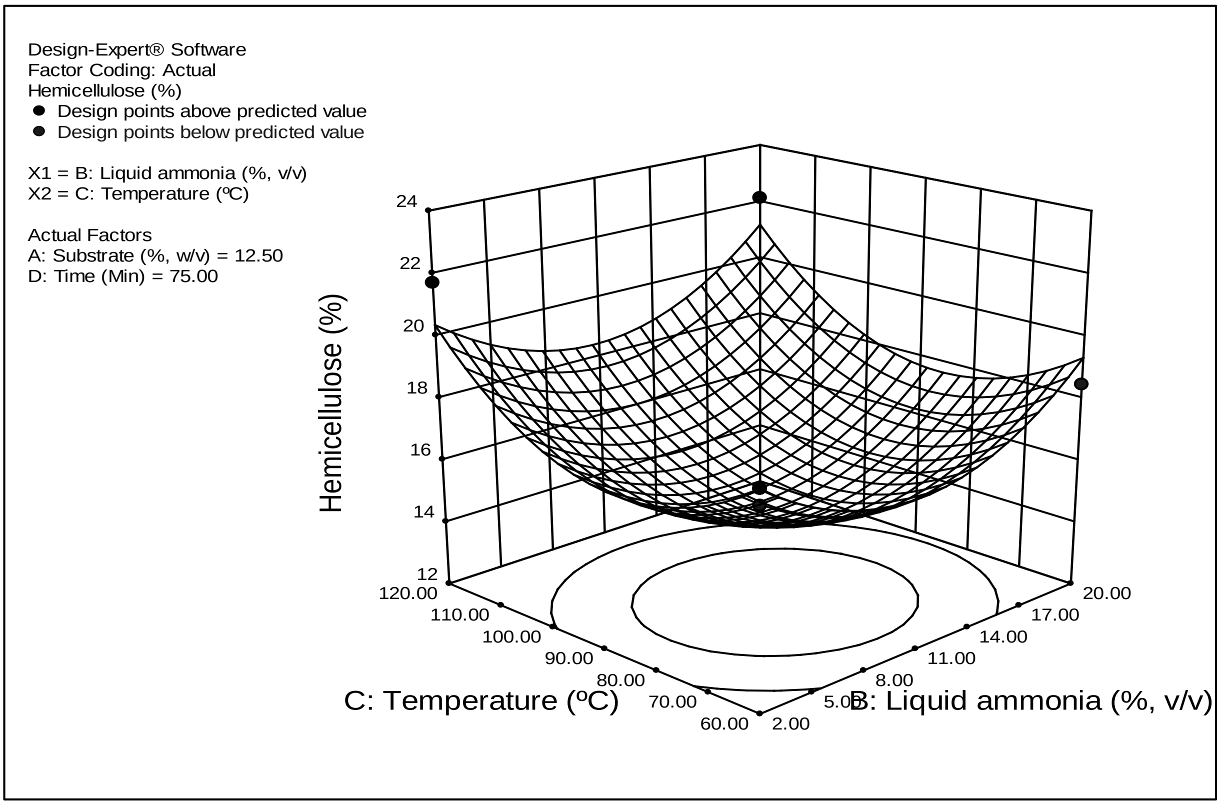

Fig 2: Response surface plot show the interactive effect of liquid ammonia (%) and pretreatment temperature (ºC) on hemicellulose yield.

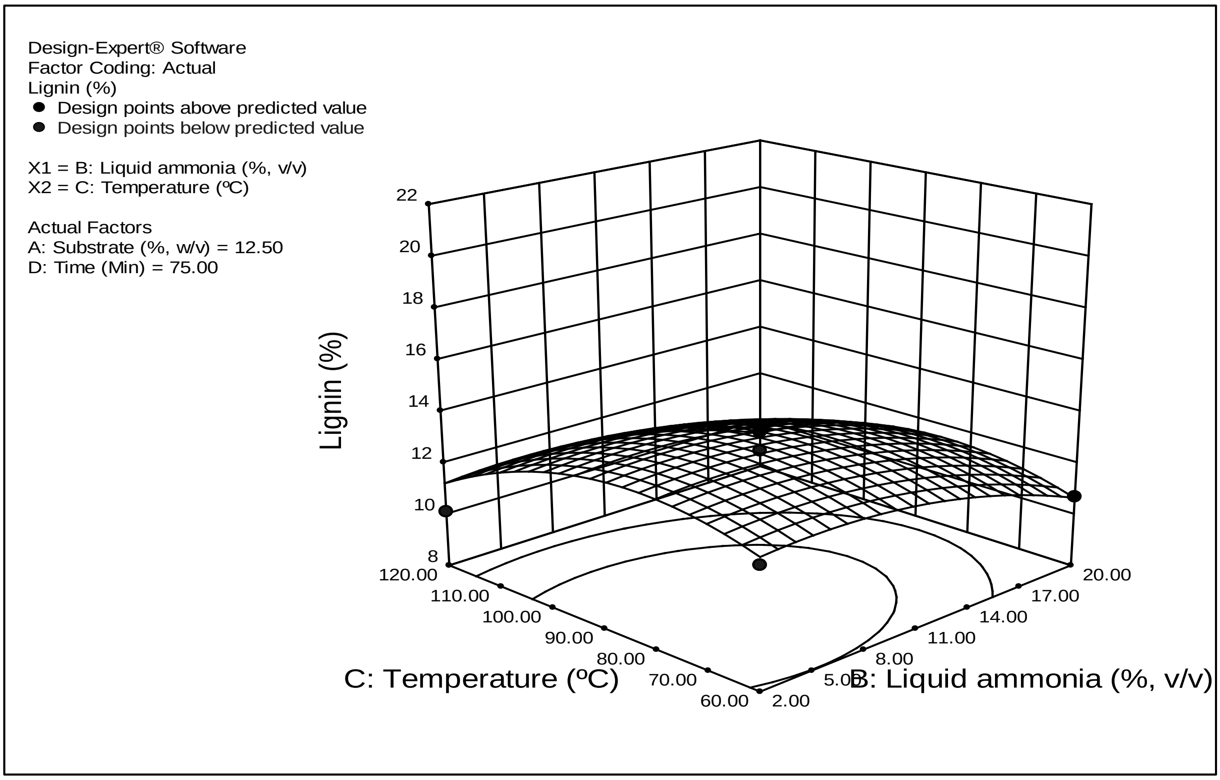

Fig 3: Response surface plot show the interactive effect of liquid ammonia (%) and pretreatment temperature (ºC) lignin yield.

For hemicellulose, yield was obtained, and the research result shows that there is a fine association among that observed and predicted values. The response surface design of hemicelluloses shows the higher NH_3 loading at higher temperatures increases the hemicellulose yield (Figure 2). For lignin yield, the results of predicted and actual values show a good correlation regarding experimental results. The response surface graph (Figure 3) demonstrated that increased concentration of NH_3 with increased temperature results in reduced bagasse lignin yield. The lowest yield of both cellulose and hemicellulose with low lignin was attained under the pretreatment optimised conditions: substrate concentration of 12.5%, NH_3 loading at 11%, pretreatment temperature of 60 ºC and pretreatment time of 30 min was 30.2, 18.6 and 16.4%, respectively. For choosing the optimal conditions and range, the models were analyzed individually. To confirm the predicted optimum continuous trails, they were performed. The outcome from three verification experiments was consistent with the fitted value and the model used was demonstrated to be sufficient. The highest yield of both cellulose and hemicellulose, in addition to low lignin, was achieved with the ideal conditions: substrate concentration of 12.5%, NH_3 loading at 20%, pretreatment temperature of 120 ºC and pretreatment time of 75 min was 44.6, 23.6 and 8.7%, respectively. Highest predicted response value from the present model was 42.0, 22.2 and 9.5%. By comparison, the experimental result showed that the yield of cellulose and hemicellulose increased from 30.2% to 44.6%, 18.6% to 23.6%, respectively, and the yield of lignin was reduced from 16.4% to 8.7%.

At the temperature of 120 ºC with pretreatment time for75 min highest cellulose content was obtained. The higher cellulose content (44.6%) shows that, the pretreatment process at 120 ºC or higher effectually reduce the overall lignin content in final obtained pretreated bagasse. The efficiency of the present model was approved via conducting confirmation trails within the experimental values. The results of the confirmation/validation runs have been additionally statistically examined to identify the correlation among predicted and observed values.

Bagasse saccharification

A pretreatment process is essential to facilitate the release of sugars from lignocellulosic biomass prior to fermentation. A comparison for relating sugar yields between the low and higher NH_3- treated bagasse is presented (Table 4) and revealed that sugar yields are higher in the pretreated bagasse at 120 ºC for 75 min in comparison with the bagasse treated at 60 °C for 30 min. Pretreatment with higher NH_3 loading enhances the cellulose and reduces the lignin yield as compared with the other experimental runs. Diminishing the lignin portion in pretreated substrates permits almost entire saccharification of the polycarbohydrates. Hence, conversion of cellulose results in an increase in the accessible sugar (39.6 mg/g) content in the hydrolysate. Compared with other samples, reducing sugar (34.2 mg/g) levels in pretreated bagasse were higher because of the higher cellulose portion of bagasse before pretreatment. Results clearly explain that following pretreatment, the easy availability of reducing sugars in pretreated substrate was higher from their specific carbohydrate portion. The greater cellulose and hemicellulose contents were mainly attached to the less available lignin. Present results demonstrate that NH_3 pretreatment could effectively disorganize the lignin polymer structure and increase the available cellulose surface area. Corresponding yields of arabinose, xylose and glucose 34.2, 18.4 and 5.8, 18.4 and 34.2 mg/g, respectively, were improved in higher NH_3 loading treated substrate compared to lower NH_3 treated bagasse with the low corresponding yields of arabinose, xylose and glucose of 4.6, 12.8 and 22.3 mg/g, respectively.

Table 4: Yield of sugars (mg/g) in pretreated bagasse after pretreatment with liquid Ammonia (NH_3)

Substrate	Cellulose (%)	Hemicellulose (%)	Reducing sugars	Glucose	Xylose	Arabinose
Low pretreated	37.4±0.8	31.9±0.6	38.5±0.8	34.8±0.2	29.5±0.1	6.8±0.3
High pretreated	72.6±0.4	56.8±0.2	78.6±0.4	70.1±0.1	52.2±0.2	8.9±0.1

Composition of percentages calculated from values on a dry-weight basis; Data represents the mean ± SEM, n=3.

Discussion

In this research study, a statistical RSM design was applied to examine the impact of various process variables, including bagasse substrate concentration, NH_3 loading, pretreatment process temperature and retention time, on total cellulose, hemicellulose and lignin yield in the resultant pretreated bagasse. Ammonia based solvents (NH_3, hydrazine), polar aprotic solvents (DMSO), metal complexes (cuoxan, cadoxen and ferric sodium tartrate) and wet oxidation additionally reduce the cellulose crystallinity, disorder the association of the lignin polymer with cellulose, and, in addition, dissolve hemicellulose (Mosier *et al*., 2005) [25]. Pretreatment with ammonia is powerful in enhancing cellulose digestion, with the advantage of ammonia being recyclable because of its high volatility (Dale and Moreira, 1982) [27]. RSM technique was efficiently implemented for xylitol generation from bagasse hemicellulosic hydrolysate through optimizing the vacuum evaporation method variables, for example temperature, pH, xylose concentration and activated charcoal treatment (Rodrigues *et al*., 2003) [16]. Ammonia pretreatment has an excessive selectivity for reaction with lignin. Ammonia recycles percolation (ARP) pretreatment is an efficient and selective delignification method that decreases 70–85% of the lignin in corn stover within 20 minutes of pretreatment and diminishes 40–60% of the hemicellulose, leaving the cellulose intact (Kim *et al*. 2003) [6].

Catalyst concentration is yet another significant parameter confirming the efficacy of pretreatment. Results from an existing study stated that the liquid NH_3 loading/concentration (20%) were higher than the substrate concentration (2%) to achieve the extreme delignification (8.7%) and the enhanced total cellulose yield in pretreated bagasse at an elevated temperature (120 ºC) with a pretreatment time of 75 min. Because of the fact that carbohydrates and more lignin were soluble at increased temperature. Moderately more lignin portion was observed at a lower temperature and with a shorter pretreatment time. This result shows that a high NH_3 concentration (20%) more effectively breaks down the lignocellulosic matrix and changes its chemical components.

In this experimental study, the most major compositional change is in lignin yield. The pretreatment diminished the yield of lignin (8.7%) in the resulting bagasse. The delignification is rapid with the pretreatment time (75 min). However, the duration of the pretreatment did not have a considerable influence on the recovery yields of the insoluble solids or the portion of lignin removal. Results also demonstrate that temperature has a profound effect on the delignification reaction, and moderately high lignin content can be achieved at lower temperatures and with shorter pretreatment times. The degree of degradation to the biomass structure depends on the temperature, and the temperature influences the delignification kinetics and also influences the substrate saccharification. The temperature of 120 ºC yielded more cellulose compared to hemicelluloses, as was noticed in this study.

The action of aqueous ammonia is reacted mainly with lignin (but not cellulose) and causes lignin depolymerization and separation of lignin-carbohydrate linkages. An extensive and modifiable degree of delignification has been reported in experiments with hardwood (Yoon *et al*., 1995) [28] and with agricultural deposits (Iyer *et al*., 1996) [11] at 160-180 °C with residence times of 14 min. It used to be rather less efficient in the pretreatment of softwood-based pulp mill sludge (Kim *et al*., 2000) [10]. Salvi *et al*. (2010) [29] reported that dilute NH_3 treatment removed 44% of the earlier lignin and 35% of the former xylan and reserved 90% of the glucan in the treated material.

Aqueous ammonia causes swelling of cellulose polysaccharide and a phase change in the crystal structure from cellulose I to cellulose III. It is assumed that ammonolysis of glucuronic cross-links makes the carbohydrate more available (Lin *et al*., 1981) [30]. The ratio between the NH_3 loading and biomass concentration influences the overall sugar yield in the resultant substrate. Increased NH_3 concentration results in enhanced sugar yields due to the fact that lignin is more solubilized in aqueous ammonia. For an ammonia to biomass ratio of 1:1, the fermentable total sugar yield was around 87% at 90 ºC for 30 min of treatment. Under conditions of an ammonia to biomass ratio of 2:1, fermentable total sugar yield improved fourfold compared to the control. The maximum yield of fermentable sugar was about 99% for a 2:1 ammonia-to-biomass ratio at 90 °C and 30 min of treatment time. Although the delignification by strong basic catalysts like ammonia causes improved availability of cellulose to cellulase for enhanced enzymatic digestibility (Ko *et al*., 2009) [31]. The corresponding yield of reducing sugars and sugars (glucose, xylose and arabinose) is higher with high-NH_3-loaded pretreated bagasse than with low-NH_3-treated bagasse. The results show that the pretreatment will effectively decrease the lignin content while increasing the available carbohydrate portion.

Conclusion

The results of the current study have evidently shown that RSM is an effective process for optimization of pretreatment conditions for to achieve the highest levels of cellulose and delignification in sugarcane bagasse. Through response surface and contour diagrams, the optimal set of method variables can be acquired graphically in order to achieve the desired levels of cellulose, hemicellulose and lignin yield during pretreatment. Pretreatment of bagasse with liquid ammonia was effective in improving the cellulose yield (44.6%) and reducing the lignin content (8.7%). Increased temperature (120 °C) and increased liquid ammonia loading result in more delignification. The experimental value (44.6%) is well in line with the predicted value (42.0%). The fermentable sugar glucose yield is also increased in bagasse treated with a higher ammonia (20%) loading.

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