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Production of Xylitol from Alkali Pre-Treated Corn Cobs Hydrolysate

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ABSTRACT

Corn cobs consist of cellulose, hemicellulose, lignin and pectin, with about 80% of fermentable sugars of which approximately 48% of sugars are derived from cellulose and 32% sugars derived from hemi cellulose. Both the compounds cellulose and hemicellulose are very important as they have wide range of applications in production of various value added products like ethanol and xylitol. When corn cobs (100 g dry weight) were treated with sodium hydroxide in alkali pretreatment method, 43.83 ± 0.13 g (95%) of hemicellulose (contains 70-90% of xylose) and 0.022 ± 0.011 mg/ml of lignin was obtained. During this process, inhibitor compounds like phenolics and furfurals were found to be 0.27 ± 0.01 mg/ml and 0.0031 ± 0.00040 mg/ml, respectively. Hydrolysate obtained by this alkali pretreatment method gave high yield of hemicellulose and less quantity of inhibitors when compared with normal acid pretreatment method. Hemicellulose pellet obtained by alkali pretreatment method yielded 32.83 ± 0.10 g of xylose (90%), which when further, subjected to fermentation with wild and adapted (20 cycles) *Candida tropicalis* WP, produced 0.63 and 0.69 g xylitol/g xylose, respectively.

Keywords: alkali pretreatment, *Candida tropicalis* WP, corn cobs, hemicellulose, lignocellulose, xylitol
Abbreviations: **$(NH_4)_2SO_4$**, di-ammonium sulphate; **K_2HPO_4**, di-potassium hydrogen phosphate; **HPLC**, high pressure liquid chromatography; **$MgSO_4$**, magnesium sulphate; **KH_2PO_4**, potassium di-hydrogen phosphate; **NaOH**, sodium hydroxide; **UV-VIS**, ultra violet and visible

INTRODUCTION

Xylitol, a five-carbon sugar alcohol is used as a natural sweetener in the food and confectionary industries. It has an anti-cariogenic effect that inhibits the growth of the tooth-decaying bacterium *Streptococcus mutans* (Makinen *et al.* 1992). Its sweetness level is equal to that of sucrose, but with 1/3 less calorific value and it can replace sucrose on a weight-to-weight basis. When dissolved in water, xylitol has low viscosity and negative heat effect, and it does not require insulin for metabolic regulation. Owing to these benefits, the use of xylitol in the food industry is growing rapidly. However, though there was lot of benefit, the cost of production by chemical synthesis has become limiting factor for xylitol usage. Efforts to develop more cost-effective methods of production include, using a cheaper and renewable agro based materials (hemicellulose), high yielding microorganisms which can efficiently convert xylose into xylitol, optimization of various conditions for high xylose yield from selected hemicellulosic substrates, removal of inhibitors, and adaptation of the microorganisms to hemicellulosic hydrolysate. *Candida tropicalis* has recently attracted attention because it accumulates xylitol, as a result of high xylose-assimilating activity (Ko *et al.* 2006). In the present study, an attempt was made for cost effective production of xylitol by using corn cobs as suitable substrate, as it has high percentage of hemicellulose (33-35%) and readily available in local area. The composition of corn cobs was found to contain cellulose-45 to 50%, hemi cellulose-33 to 35%, uronic acids-3 to 4% and ash-2%. In the present study, to obtain more xylose from corn cobs both acid pre treatment and alkali pretreatment was done. Further, we tried to produce xylitol from xylose obtained by alkali pretreatment methods using one of our labs isolate i.e. *Candida tropicalis* WP.

MATERIALS AND METHODS

Microorganisms and culture conditions

Strains of *Candida tropicalis* were isolated (R. Sreenivas Rao *et al.* 2010) from fruit pulp of orange and these strains have been screened for their efficiency to convert xylose to xylitol. Out of various isolates screened, *C. tropicalis* WP was selected for further experiments. The strain was identified using CANDIFAST KIT (International Microbio, Stago Group, France). *C. tropicalis* strains were maintained on YEPX (yeast extract-peptone-xylose, Hi-Media, Mumbai, India) agar and sub-cultured every 4 weeks.

Inoculum preparation

Inoculum (100 ml) media was prepared in 250 ml capacity flask using media consisting of yeast extract (10 g/l), peptone (20 g/l), xylose (30 g/l) (YEPX), KH_2PO_4 (0.5 g/l), K_2HPO_4 (0.5 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), $(NH_4)_2SO_4$ (2 g/l). The media was adjusted to pH = 5, sterilized by autoclaving at 11 psi for 15 min and further inoculated with *C. tropicalis* and then the flasks were incubated at 30°C for 24 h on a rotary shaker maintained at 250 rpm.

Fermentation medium

Fermentative medium (100 ml; Sigma-Aldrich, Mumbai, India) was prepared in 250 ml capacity flask by supplementing the detoxified hydrolysate with the following media components like yeast extract (10 g/l), glycerol (15 g/l), glucose (10 g/l), KH_2PO_4 (0.5 g/l), K_2HPO_4 (0.5 g/l), $MgSO_4$ (0.2 g/l), $(NH_4)_2SO_4$ (2 g/l). Adapted culture cells, which were grown in the above fermentation medium for 10 to 15 cycles (5%, 1.0 OD at 600 nm) were used to inoculate the fermentative media and further incubated at 30°C for 72 h on a rotary shaker at 250 rpm to carry out fermentation process.

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Table 1 Yield of hemicelluloses by alkali pretreatment at different temperatures.

Temp (^{0}C)	Hemicellulose (g/l)	Lignin (g/l)	Phenolics (g/l)	Furfural (g/l)
Control	19.50 ± 0.30	0.056 ± 0.017	0.14 ± 0.015	0.0016 ± 0.00015
30^{0}C	19.83 ± 0.45	0.063 ± 0.016	0.23 ± 0.02	0.0022 ± 0.00025
40^{0}C	20.83 ± 0.44	0.071 ± 0.013	0.25 ± 0.02	0.0025 ± 0.00025
50^{0}C	28 ± 0.26	0.074 ± 0.008	0.27 ± 0.02	0.0027 ± 0.00030
60^{0}C	29.16 ± 0.35	0.081 ± 0.011	0.31 ± 0.03	0.0030 ± 0.00030
70^{0}C	43.83 ± 0.13 (95%)	0.022 ± 0.011	0.27 ± 0.01	0.0031 ± 0.00040
80^{0}C	31 ± 0.39	0.117 ± 0.015	0.41 ± 0.01	0.0046 ± 0.00020

Hemicellulose extraction by alkali pretreatment method

Hemicellulose extraction by alkali pretreatment method was carried out according to Rutenberg and Herbst (1957) method. Six grams (dry weight) of corn cobs were mixed with 60 ml of extracting solution (1: 10, w/v) containing 2% of 1M NaOH and incubated for 5 h at 70°C in water bath with occasional stirring. After cooling to 30-35°C, the mixture was centrifuged at 20°C for 20 min at 18,000 × *g* and further, the supernatant was adjusted to pH 4.5-5.0 with 6 N HCl with rapid stirring. Three volumes of cold ethanol (95%, 4°C) was slowly added to the supernatant with continuous stirring for 5 min and the precipitate (crude, rubber-like xylan pellet) was collected by low speed centrifugation at 20°C for 10 min at 5000 × *g*. The bulk supernatant separated contain lignin fraction which can be further used for other applications.

Extraction of xylose from hemicellulose

Precipitate obtained by alkali pretreatment method was treated with 1.5% dilute 1M sulfuric acid and kept in water bath at 75°C for 30 min. After cooling to 30-35°C the solution was neutralized with 1M NaOH and further subjected to detoxification with 2% activated charcoal at 75°C for 30 min. Detoxified hydrolysate was filtered and analyzed by HPLC.

Analytical methods

The quantification of hemicellulose was carried out by Rutenberg and Herbst method (1957). In this method the hemi cellulose pellet obtained during alkali extraction process was quantified by taking the dry weight of pellet.

Lignin was quantified by the Iiyama and Wallis (1990) method and absorbance was measured at 280 nm using a UV-VIS spectrophotometer (Systronics 117, Naroda, Ahmedabad, Gujrat, India). The quantification of phenolics was done according to **the** Tanner and Brunner method (1987) and absorbance was measured at 750 nm using UV-VIS spectrophotometer (Systronics 117). The quantification of furfurals was done according to the Martínez *et al*. (2000) method. The test sample was suitably diluted and absorbance was measured spectrophotometrically at 280 nm using a UV-VIS spectrophotometer (Systronics 117).

Sugars in the hydrolyzate were estimated by high performance liquid chromatography (HPLC) fitted with a Rezex RPM column (8 mm × 300 mm) (Phenomenex, USA). The samples were eluted with HPLC grade water at a flow rate of 0.6 ml/min at 75°C and detected with a differential refractometer (RID).

Statistical analyses

Optimization was carried out for the extraction of hemicellulose by treating 6 g (dry weight) of corn cobs mixed with 60 ml of extraction solution (1:10 w/v) containing 2% of 1M NaOH and incubated at various temperature and time periods. The hemicellulose and the supernatant obtained after the extraction of hemicellulose for every temperature and time period were quantified for lignins, phenolics and furfurals.

To obtain maximum amount of xylose, the hemicellulose precipitated by alkali pretreatment method was further acid hydrolysed with 1M 1.5% H_2SO_4 and optimized by incubating in water bath at various temperatures for 30 min. At every temperature the acid hydrolysate was further neutralized and quantified for xylose, lignin, phenolics and furfurals.

All the above experiments were carried out three times to assess whether there was any significant difference among the mean values at various temperatures and time periods. Correlation and one-way ANOVA (*post hoc*) analysis was performed.

RESULTS

Corn cobs composition

The composition of corn cobs was found to contain 42.0% of cellulose, 38.5% of hemi-cellulose, 12.5% of lignin, 3.5% of pectin, 2.0% of uronic acids and 1.5% of ash.

Yield of hemicellulose by alkali pretreatment method

When optimization of alkali pretreatment of corn cobs was carried out at various temperatures ranging from 30-80°C and time ranging from 1-6 h respectively, maximum yield of hemicellulose was obtained at 70°C (43.83 ± 0.13 g/100 g of dry weight of corn cobs) as shown in **Table 1** when treated for 5 h (effect of time on hemicelluloses yield), shown in **Table 2**.

Yield of xylose from hemicellulose

Hemicellulose precipitate obtained after alkali pretreatment was acid hydrolysed with 1.5% sulphuric acid at different temperatures (70, 90, 100, 121°C), yield of xylose was maximum when treated at 100°C i.e. 32.83 ± 0.10 g (which is equal to 90% total xylose present in the hemicellulose pellet) as shown in **Table 3**.

Yield of xylitol with wild and adapted *Candida tropicalis* WP

When *C. tropicalis* (5%, 1.0 OD at 600 nm) was inoculated into the fermentative media (described in materials and methods) and incubated at 30°C for 72 h at 250 rpm, and

Table 2 Effect of time on yield of hemicelluloses at 70°C.

Time in h (at 70°C)	Hemicellulose (mg/ml)
Control	19.50 ± 0.15
1	21.50 ± 0.08
2	22.50 ± 0.11
3	26.16 ± 0.13
4	28.66 ± 0.08
5	43.83 ± 0.13 (95%)
6	30.50 ± 0.15

Table 3 Yield of xylose by acid pretreatment at different temperatures.

Temp (°C)	g/100 g of dry weight of corn cobs			
	Xylose	Lignin	Phenolics	Furfural
Control	18.33 ± 0.34	2.17 ± 0.12	1.4 ± 0.2	0.020 ± 0.003
70	20.33 ± 0.23	4.9 ± 0.40	2.8 ± 0.3	0.027 ± 0.003
90	22.18 ± 0.21	5.7 ± 0.40	3.6 ± 0.2	0.036 ± 0.002
100	32.83 ± 0.10	6.0 ± 0.25	3.8 ± 0.2	0.043 ± 0.003
121	20.82 ± 0.12	7.4 ± 0.20	4.3 ± 0.2	0.052 ± 0.007

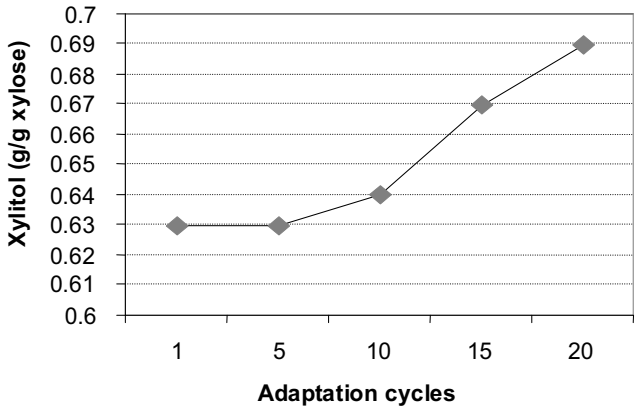

Fig. 1 Yield of xylitol with adapted *Candida tropicalis* WP.

analyzed by HPLC for xylitol, the yield was found to be 0.63 ± 0.12 g xylitol/g xylose. Whereas fermentation with adapted *C. tropicalis* (wild type *C. tropicalis* after 20 cycles of adaptation) after 72 h of incubation yield, 0.69 ± 0.08 g xylitol/g xylose, as shown in **Fig. 1**.

DISCUSSION

Iranmahboob *et al*. (2002) and Bobleter (1994) reported that biomass mainly composed of cellulose (34-50%), hemi cellulose (19-34%), lignin (11-30%) and smaller amounts of pectin, protein, extractives and ash. Composition of these components differs with the source of plant species, age and growth conditions. Pretreatment process is necessary in order to alter the structural integrity, to remove the lignin and increase the surface area to make this material available as fermentable sugars. Pretreatment can be done by either acidic method or alkali method. Conner *et al*. (1984) reported pretreatment by acid hydrolysis using different mineral acids such as sulfuric, hydrochloric acid, nitric acid, hydrofluoric acid, acetic acid and phosphoric acids at high temperature and pressure (commonly 160°C and 10 atm). Sun *et al*. (2002) and Cara *et al*. (2007) concluded that acid hydrolysis by concentrated acids is toxic, corrosive, hazardous and without any exception, all sugar liquors obtained by acid hydrolysis release more inhibitor compounds like furan derivatives, aliphatic acids and phenolic compounds. Furan derivatives, commonly known as furfurals and hydroxymethylfurfural (HMF), are produced from the degradation of pentoses and hexoses, respectively. It was also observed that, acid hydrolysis with the use of concentrated acids require high temperature, pressure and is also difficult to remove excess acid after treatment. Mussatto *et al*. (2004) demonstrated that the use of strong alkali solutions, depolymerized xylan may be extracted from lignocellulosics, but the product obtained is completely deacetylated and has very limited solubility in water hence was not the preferred hydrolyzing reagent. Garrote *et al*. (1999) proposed mild alkali treatment as an alternative method for the separation of hemicelluloses with limited solubilization of lignin. Mild alkali pretreatment produces reduced quantities of sugar derivatives like furfurals and hydroxyl-methyl furfurals (Mussatto and Roberto 2004). Alkali pretreatment performed at mild temperatures yield high mass of hemi cellulose without modifying the cellulose structure substantially, and also allow improved recovery of glucose during further processing (Montane *et al*. 1998; Shimizu *et al*. 1998). Aachary *et al*. (2009) reported 81 ± 1.5% of xylooligosaccharides from alkali pretreated corncob powder using a commercial endoxylanase, but in the present investigation we could able to obtain 90% of xylose from alkali pretreated corn fiber. In the literature, the xylitol yield from different hemicellulosic hydrolyzates by acid pretreatment require extensive detoxification process as it produce high amounts of inhibitors, so to improve xylitol production ability of fermentative yeast, the organisms must be grown

Table 4 Correlation analysis for effect of temperature on extraction of hemicellulose.

Temperature (°C)	Hemicellulose	Lignin	Phenol	Furfural
1.000	0.815**	0.295	0.832**	0.855**
	1.000	-0.295	0.456*	0.513*
		1.000	0.634**	0.568**
			1.000	0.949**
				1.000

* Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)

for number of cycles in the hydrolysate for adaptation. To improve xylitol production ability of fermentative yeast, in acid pretreated sugarcane bagasse hydrolysate containing high inhibitors, extensive detoxification was performed and adaptation of yeast cells by sub culturing (for 25 cycles) in the hydrolysate, xylitol production was increased from 0.45 g xylitol/g xylose to 0.65 g xylitol/g of xylose (Sreenivas *et al*. 2006). In another investigation Ling *et al*. (2011) reported a yield of 0.73 g xylitol/g xylose with corn cob hydrolysate after optimization. In the present study xylitol production was increased from 0.63 ± 0.12 g xylitol/g xylose to 0.69 ± 0.08 g xylitol/g xylose, with alkali pre treated hydrolysate without extensive detoxification process, with only 20 adaptation cycles and without further optimization.

Statistical evaluation of hemicellulose extracted at various temperatures and time periods from alkali treated corn cob

To test whether the hemicelluloses, lignin, phenolics and furfurals were affected by treating at different temperatures significantly from each other, one-way ANOVA was performed. The results in **Table 4** indicate that temperature has significant effect on quantity of hemicelluloses [F (6, 14) = 415.457; $P < 0.001$], lignin [F (6, 14) =189.983; $P < 0.001$], phenolics [F (6, 14) =22.453; $P < 0.001$] and furfurals [F (6, 14) = 15.157; $P < 0.001$]. Further *post-hoc* analysis revealed that quantity of hemicellulose extracted was highly significant at 70°C ($P < 0.05$; LSD) whereas quantity of lignins, phenolics and furfurals were highly significant at 80°C ($P < 0.05$; LSD).

The analytical variables i.e., hemicelluloses, lignins, phenolics and furfurals were subjected to Pearson's correlation for the measurement of linear association between the above said variables. The analysis revealed that there was a significant positive correlation between temperature and the variables. For example, temp-hemicellulose (0.815) temp-phenolics (0.634) and temp-furfurals (0.855) (**Table 5**).

Pearson's correlation and *post-hoc* multiple comparison analysis was carried out with the mean scores of hemicelluloses treated for different time periods. The analysis was done to find the effect of time period on the extraction of hemicelluloses and how they differ significantly from each other. *Post-hoc* analysis (**Table 6**) revealed that the time period of 4 h was highly significant for the extraction of maximum amount of hemicelluloses ($P < 0.05$; LSD). Correlation analysis (**Table 7**) between time period and hemicelluloses production showed that there was a significant positive correlation between these two variables (i.e., 0.881, sig-0.000; $P < 0.05$).

Statistical evaluation of xylose extracted at various temperatures from acid hydrolysis of hemicellulose

To test whether the xylose extraction, lignin, phenolics and furfurals were affected by treating at different temperatures significantly from each other, one-way ANOVA was performed. The results in **Table 8** indicate that temperature has significant effect on quantity of xylose [F (4, 10) = 57.173; $P < 0.001$], lignin [F (4, 10) = 82.479; $P < 0.001$], phenolics

Table 5 One-way ANOVA (*post-hoc*) analysis for effect of temperature on extraction of hemicellulose.

Dependent variable	(I) Temperature (°C)	(J) Temperature (°C)	Mean difference (I-J)	Std. error	Sig.	95% Confidence interval	
						Lower bound	Upper bound
Hemicellulose	27	30	-0.336	0.60029	.584	-1.624	.950
		40	-1.3300*	0.60029	.044	-2.617	-0.042
		50	-0.8500*	0.60029	.000	-9.787	-7.212
		60	-9.661*	0.60029	.000	-10.94	-8.373
		70	-24.33*	0.60029	.000	-25.61	-23.04
		80	-11.500*	0.60029	.000	-12.78	-10.21
Lignin	27	30	-0.0063*	0.00292	0.048	-0.012	-0.000
		40	-0.0143*	0.00292	0.00	-0.0206	-0.008
		50	-0.0176*	0.00292	0.00	-0.0239	-0.011
		60	-0.0246*	0.00292	0.00	-0.030	-0.018
		70	0.3433*	0.00292	0.00	0.0280	0.040
		80	-0.0606*	0.00292	0.00	-0.066	-0.054
Phenol	27	30	-0.086*	0.02377	0.003	-0.137	-0.035
		40	-0.106*	0.02377	0.001	-0.157	-0.055
		50	-0.130*	0.02377	0.00	-0.180	-0.790
		60	-0.163*	0.02377	0.00	-0.214	-0.112
		70	-0.126*	0.02377	0.00	-0.177	-0.075
		80	-0.263*	0.02377	0.00	-0.314	-0.212
Furfural	27	30	-0.0007	0.0003	0.053	-0.0014	0.000
		40	-0.001*	0.0003	0.012	-0.0017	-0.000
		50	-0.0012*	0.0003	0.003	-0.0019	-0.000
		60	-0.0015*	0.0003	0.001	-0.0022	-0.000
		70	-0.0015*	0.0003	0.000	-0.0023	-0.000
		80	-0.003*	0.0003	0.00	-0.0038	-0.002

*The mean difference is significant at the 0.05 level

Table 6 Correlation analysis for effect of time period on extraction of maximum hemicellulose.

Time period (h)	Hemicellulose
1.000	0.881**
	1.000

** Correlation is significant at the 0.01 level (2-tailed)

[F (4, 10) = 9.028; $P < 0.001$] and furfurals [F (4, 10) = 13.971; $P < 0.001$]. Further *post-hoc* analysis revealed that quantity of xylose extracted was highly significant at 100°C ($P < 0.05$; LSD) whereas quantity of lignin, phenolics and furfurals were highly significant at 121°C ($P < 0.05$; LSD).

The analytical variables i.e., xylose, lignins, phenolics and furfurals, were subjected to Pearson's correlation for the measurement of linear association between the above said variables. The analysis revealed that there was a significant positive correlation between temperature and all the variables. For example, temp-lignin (0.977) temp-phenolics (0.881) and temp-furfurals (0.885) (**Table 9**).

CONCLUSION

Generally most of the researchers prefer alkali pretreatment method only, to obtain de-lignified cellulosic hydrolyzate, but novelty in the present investigation is, alkali pre treatment was found to be the best method for separation of high amount of hemi cellulose (95%) from the lignocellulosic substrate used. When this hemi cellulose was further treated with 1.5% of sulphuric acid for its conversion into xylose, we could obtain 90% yield with less inhibitors and overcome extensive detoxification step and thereby found to be more economical when compared to acid pretreatment method. Sreenivas *et al*. (2006) reported 0.65 g xylitol/g of xylose by *C. tropicalis* after 25 cycles of adaptation from acid pretreated hydrolysate of sugarcane bagasse, but in the present investigation we could able to obtain 0.63 ± 0.12 g xylitol/g xylose by *C. tropicalis* with out any adaptation cycles, which shows suitability of alkali pretreated hydrolysate for the growth of microorganisms. Alkali pretreatment method has good scope to achieve more conversion of xylose into xylitol with improved strains and optimization.

Table 8 Correlation analysis for effect of temperature on extraction of maximum xylose from hemicellulose.

Temperature (°C)	Xylose	Lignin	Phenol	Furfural
1.000	0.464	0.977**	0.881**	0.885
	1.000	0.374	0.427	0.441
		1.000	0.868	0.856
			1.000	0.705
				1.000

** Correlation is significant at the 0.01 level (2-tailed)

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Table 7 One-way ANOVA (*post-hoc*) analysis for effect of time period on extraction of maximum hemicellulose.

Dependent variable	(I) Time period (h)	(J) Time period (h)	Mean difference (I-J)	Std. error	Sig.	95% Confidence interval	
						Lower bound	Upper bound
Hemicellulose	0	Control	-2.000	0.576	0.005	-3.255	-0.744
		1 h	-2.000	0.576	0.005	-3.255	-0.744
		2 h	-6.660	0.576	0.000	-7.915	-5.404
		3 h	-9.160	0.576	0.000	-10.41	-7.904
		4 h	-23.87	0.576	0.000	-25.12	-22.61

*The mean difference is significant at the 0.05 level

Table 9 One-way ANOVA (*post-hoc*) analysis for effect of temperature on extraction of maximum xylose from hemicellulose.

Dependent variable	Temperature (°C)	(J) Temperature (°C)	Mean difference (I-J)	Std. error	Sig.	95% Confidence interval	
						Lower bound	Upper bound
Xylose	27	70	-1.990	0.8874	0.049	-3.967	-.0125
		90	-3.840	0.8874	.001	-5.817	-1.862
		100	-14.490	0.8874	.000	-16.467	-12.512
		121	-2.713	0.8874	.012	-4.690	-.735
Lignin	27	70	-2.366	0.3656	.000	-3.181	-1.551
		90	-3.530	0.3656	.000	-4.344	-2.715
		100	-3.796	0.3656	.000	-4.611	-2.981
		121	-5.230	0.3656	.000	-6.044	-4.415
Phenol	27	70	-1.400	0.53291	.025	-2.587	-.212
		90	-2.200	0.53291	.002	-3.387	-1.012
		100	-2.400	0.53291	.001	-3.587	-1.212
		121	-2.900	0.53291	.000	-4.087	-1.712
Furfural	27	70	-0.0066	0.00475	.191	-.017	.0039
		90	-0.156	0.00475	.008	-.0262	-.0050
		100	-0.0226	0.00475	.001	-.033	-.0120
		121	-.0316	0.00475	.000	-.0422	-.0210

*The mean difference is significant at the 0.05 level

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