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RESEARCH ARTICLE

EFFECT OF PRETREATMENT FOR DELIGNIFICATION AND SUGAR RECOVERY FOR BIOETHANOL PRODUCTION FROM ARECANUT HUSK WASTE

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ABSTRACT

To meet the challenges of generating sufficient and sustainable energy, a potentially viable alternative is to use cellulosic biomass for second-generation biofuel and bioenergy production. In the present study, Arecanut husk is cheap abundant lignocellulosic raw material in the agricultural waste; it is available as alternative feedstock for bioethanol production. The moisture content of the fresh raw material (68.39 ± 0.20 %). Arecanut husk raw material were collected and dried in the hot air oven at 80^0 C for 48hours. The dried arecanut husk raw materials were subjected by powdered sequentially using hammer mill, ball mill and flour mill to obtain fine sized particles. The particles were separated by using a range of graded sews. Then sieved the different particle size such as sample-A (0.28 ± 0.01 mm), sample-B (0.43 ± 0.02 mm), and sample-C (0.64 ± 0.01 mm). The chemical analysis of arecanut husk raw material was done by using pretreatment methods. In acid hydrolysis the more amount of reducing sugar was found in the sample-A (4.12 ± 0.02 mg/g), non-reducing sugar in the sample-B (0.37 ± 0.01 mg/g), Protein in the sample-C (5.95 ± 0.06 mg/g) and Carbohydrate in the sample-B (2.82 ± 0.01 mg/g) of Arecanut fiber. In alkaline hydrolysis the more amount of reducing sugar was found in the sample-A (1.6 ± 0.10 mg/g), non-reducing sugar in the sample-A (1.02 ± 0.03 mg/g), Protein in the sample-A (7.40 ± 0.15 mg/g) and Carbohydrate in the sample-B (2.43 ± 0.02 mg/g) of Arecanut fiber.

INTRODUCTION

Cellulosic ethanol produced from various lignocellulosic materials has the potential to be a valuable substitute for present day's fuel crisis. Lignocellulosic biomass, which is most abundant and low-cost biomass world over, can be used as raw materials for production of fuel ethanol (Chen *et al.,* 2007). Production of ethanol from abundantly available diverse biomass such as Arecanut husk requires a greater amount of processing and pretreatment to make the sugar monomers available to the microorganisms that are typically used to produce ethanol by fermentation (Prasad *et al.,* 2007 and Zheng *et al.,* 2008). Arecanut husk is cheap abundant lignocellulosic materials in the agricultural waste; it is available as alternative feedstock for ethanol production (Prasad *et al.,* 2007). Many physicochemical structural and compositional factors hinder the hydrolysis of cellulose present in biomass to sugars and other organic compounds that can later be converted to fuels (Mathew and Govindarajan, 1964). The goal of pretreatment is to make the Arecanut husk to finite particles cellulose accessible to hydrolysis for conversion to ethanol (Taherzadeh and Karimi, 2008; Mosier *et al.,* 2005). Pretreatment is one of the most important and expensive operation steps in releasing sugars from hemicellulose and cellulosic ethanol production.

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The lignocellulosic biomass must be first pretreated to increase surface area, bulk density and reduce crystallinity of cellulose so as to make it accessible for hydrolysis (Dhanya *et al.,* 2010). The pretreatment of naturally resistant cellulosic materials is essential but representing about 20% of the total cost. In addition, pretreatment has pervasive impacts on all other major operations in the overall conversion scheme from choice of feedstock through to size reduction, hydrolysis, fermentation and on to product recovery, residue processing and co-product potential. Pretreatment must meet the following requirements such as improving the formation of sugars, avoid degradation or less of carbohydrates, avoid formation of by products inhibitory to subsequent hydrolysis and fermentation and should be cost effective (Dhanya *et al.,* 2010; Kumar *et al.,* 2009). There is key challenge to commercializing production of ethanol from cellulosic biomass which holds great potential due to the widespread availability, abundance and relatively low cost of cellulosic materials (Dhanya *et al.,* 2010). It is evident from preliminary studies that relatively larger particles obtained from a number of pretreatment and processing methods yielded reduced sugar on hydrolysis. In the present study, we have optimized processing techniques to obtain finite particles to change the physical and chemical structure of the lignocellulosic biomass (Lee *et al.,* 2008). In turn, improved hydrolysis rates. Lignin is the main constituent of arecanut fiber, responsible for its

stiffness. It is also partly responsible for the natural colour of the fiber (Mohan Kumar, 2008). Chemical constitution is mainly of cellulose, hemicellulose, lignin, pectin's, waxes, water soluble substances and residual ash along with the organic materials (Rajan *et al.,* 2005). The total hemicellulose content varies with the development and maturity, the mature husk containing less hemicellulose than the immature ones. The lignin content proportionately increases with the development until maturity (Raghupathy *et al.,* 2002). After chemical analysis submerged fermentation will be done for bioethanol production. This paper presents a brief overview of the effectiveness of two different acid and alkali pretreatments for maximum recovery of sugar for ethanol production from Arecanut husk waste.

MATERIALS AND METHODS

Collection and milling of Arecanut husk

Arecanut husk samples were collected from the local farmer in Shivamogga region, Karnataka, India and cleaned. Moisture content was recorded and stored in an appropriate condition. The Arecanut husk samples were air dried using cabinet tray dryer at 80^0c for 24-48h and powdered sequentially using hammer mill, ball mill and flour mill to obtain fine sized particles. The particles were separated by using a range of graded sews. Such as fine size powder (Sample-A), medium size powder (Sample-B) and coarse size powder (Sample-C) (Narayanamurthy *et al.,* 2008; Sweetha, 2010; Seema, 2008).

Pretreatment on the Arecanut husk raw material
Acid hydrolysis

100gram of the raw material was weighed into 2 liter conical flasks and 1000ml of 1N sulphuric acid was added to the conical flask. The flasks were covered with aluminum foil and heated for six hours on boiling water bath. The flask was allowed to cool and filtered. The pH was adjusted to 4.5 with 0.4M Sodium hydroxide. The total, reducing and non reducing sugar content of pretreated raw materials was estimated (Xu *et al.,* 2010; Seema, 2008).

Alkaline hydrolysis

100gram of dried sample was weighed into 2 liter conical flask and 1000ml of 0.25 M Sodium hydroxide solution was added to the conical flask. The flask was left for one hour, after which the mixture was neutralized with 0.1 M Hydrochloric acid to a pH of 4.5. The flask was allowed to cool at room temperature and filtered. The total reducing and non- reducing sugar content of pretreated raw materials was estimated (Xu *et al.,* 2010; Seema, 2008).

Analytical Methods

Determination of reducing sugar

The reducing sugars were estimated by Dinitrosalicylic acid method. The aliquots of extract were pipette out from 0.5 to 3ml in test tubes the volume was equalized to 3ml with water in all the tubes. Then 3ml of DNS reagent was added, mixed and heated for 5 min. on a boiling water bath. After the colour has developed, 1 ml of 40% Rochelle salt solution was added and mixed. The tubes were cooled under running tap water and the absorption was read at 510nm. The amount of reducing sugar in the sample was calculated using standard graph prepared from working standard Glucose (Agblevor *et al.,* 2006; Sadasivam and Manickam, 1996).

Determinations of Non-reducing Sugar

Non-reducing sugars present in the extracts were hydrolyzed with sulphuric acid to reducing sugars. Then the total reducing sugars were estimated by DNS method. About 100mg of the sample was taken and the sugars were extracted with 80% alcohol (hot) twice (5ml each time). The supernatant was collected and evaporated on water bath. Ten ml of distilled water was added to dissolve the sugars. 1ml of extract was pipette in to a test tube and 1ml of 1N H_2SO_4 was added. The mixture was hydrolyzed by heating at 49^0c for 30min and then 1 or 2 drops of methyl red indicator was added. The contents were neutralized by adding 1N NaoH drop wise from a pipette. Appropriate reagent blanks were maintained. Then total non-reducing sugar was estimated by DNS method (Agblevor *et al.,* 2006; Sadasivam and Manickam, 1996).

Determination of protein

Protein reacts with Folin Ciocalteau (FC) reagent to give a blue coloured complex. The colour so formed will be the reaction of the alkaline copper with protein and reduction of phosphomolybdic–phosphotugstic components in the FC reagent by amino acids tyrosine and tryptophan present in the protein. About 0.5g of the sample was collected. Aliquots of 0.2, 0.4, 0.6, 0.8 and 1ml of working standard solution were pipette into a series of test tubes. At the same time 0.1ml and 0.2ml of the sample extract was taken into two other test tubes. The volume was made up to 1ml using distilled water. Then 5ml of alkaline copper was added, mixed well and incubated at room temperature for 10 minutes. About 0.5ml of FC reagent was added, mixed well and incubated at room temperature in dark for 30 minutes. The absorbance was read at 660nm. Concentration of protein was calculated from standard graph (Agblevor *et al.,* 2006; Dam *et al.,* 2004; Sadasivam and Manickam, 1996).

Determination of total carbohydrate by phenol-sulphuric acid method

In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms an orange – yellow coloured product with phenol and will be had absorption maximum at 490nm. 100 milligrams of each of the sample was weighed into boiling tube and hydrolyzed by keeping it in a boiling water bath for 3hr with 5ml of 2.5N HCl and cooled to room temperature. This was neutralized with solid Sodium Carbonate until the effervescence ceased. Volume was made up to 100ml, centrifuged and supernatant was collected. About 0.1 and 0.2 ml of this supernatant was taken into two separate test tubes for analysis. A volume of 0.2, 0.4, 0.6, 0.8 and 1ml working standard were taken into series of test tubes. Volume was made up to 1ml in each tube with distilled water and a blank was set with 1ml distilled water. Then 1ml of phenol solution and 5ml of 96% sulphuric acid were added to each tube and shaken well. After 10min the contents in the tubes were shaken and placed in a water bath at $25\text{-}30^0$c for 20 min. Then the absorbance was read at 490nm. Standard graph was plotted

and the concentration of the total sugars was calculated (Sadasivam and Manickam, 1996).

Physicochemical Parameters of Arecanut husk

Proximate composition analysis was carried out for Particle Size (mm), Surface area of the Sample (m2/Kg), Density of the bed, Moisture content (%), Total solids (%), Organic carbon (%), Nitrogen (%), Cellulose (%), Hemicellulose (%) and Lignin (%) (Agu *et al.,* 1997; Seema, 2008; Sweetha, 2010).

Scanning Electron Microscopy Analysis

Scaning electron microscopy (SEM) analysis was performed with a FEI Quanta 200 operated at 20 Kv. The samples were coated (gold/palladium) with a SC7640 Suto/Manual High Resolution Sputter Coater (Indian institute of science, Bangalore).

Table: 1. Chemical composition of Arecanut husk by Acid hydrolysis

Sl. No.	Chemical Constituents	Samples (mg/gm)		
		A	B	C
1	Reducing Sugars	4.12 ± 0.02	3.18 ± 0.02	3.08 ± 0.01
2	Non-reducing Sugars	0.35 ± 0.03	0.37 ± 0.01	0.24 ± 0.01
3	Protein Content	3.35 ± 0.02	5.80 ± 0.10	5.95 ± 0.06
4	Total Carbohydrate Content	2.61 ± 0.03	2.82 ± 0.01	2.79 ± 0.01

Table: 2. Chemical composition of Arecanut husk by Alkaline Hydrolysis

Sl. No.	Chemical Constituents	Samples (mg/gm)		
		A	B	C
1	Reducing Sugars	1.6 ± 0.10	1.18 ± 0.02	0.86 ± 0.02
2	Non-reducing Sugars	1.02 ± 0.03	0.74 ± 0.03	0.72 ± 0.02
3	Protein Content	7.40 ± 0.15	6.95 ± 0.02	7.0 ± 0.12
4	Total Carbohydrate Content	1.83 ± 0.15	2.43 ± 0.02	1.86 ± 0.12

Note: A-Fine sized Powder, B-Medium sized powder & C- Coarse sized Powder

Table: 3 Physicochemical Parameters of Arecanut husk

Sl. No.		A	B	
1	Particle Size(mm)	0.28 ± 0.01	0.43 ± 0.02	0.64 ± 0.01
2	Surface area of the Sample(m2/Kg)	1.93 ± 0.02	6.77 ± 0.08	12.60 ± 0.25
3	Density of the bed	610.4 ± 4.99	493.60 ± 2.62	472.47 ± 0.98
4	Moisture content (%)	3.30 ± 0.20	2.90 ± 0.10	2.60 ± 0.15
5	Total solids (%)	83.26 ± 0.92	75.29 ± 0.61	70.86 ± 0.81
6	Organic carbon (%)	31.49 ± 0.59	29.38 ± 0.12	23.64 ± 0.22
7	Nitrogen (%)	0.52 ± 0.02	0.05 ± 0.01	0.59 ± 0.04
8	Cellulose (%)	39.0 ± 1.53	33.5 ± 0.74	28.0 ± 0.85
9	Hemicellulose (%)	8.30 ± 0.15	32.4 ± 0.19	41.6 ± 0.23
10	Lignin (%)	8.0 ± 0.45	11.5 ± 0.35	21.0 ± 0.19

Note: A-Fine sized Powder, B-Medium sized powder & C- Coarse sized Powder

RESULTS

Effects of Acid and Alkaline hydrolysis Pretreatments on different sized Arecanut husk samples

In the present study, the reducing sugar concentration was more in the sample- A (4.12mg/g) than compared to samples-B (3.28mg/g) and sample-C (3.08mg/g) in the acid hydrolysis. In alkaline hydrolysis, the reducing sugar concentration was more in the sample-A (1.6mg/g) than compared to sample-B (1.18mg/g) and sample-C (0.86mg/g) (Table 1 & 2 and Fig. 1 & 2). The non reducing sugar concentration was more in the sample-B (0.37mg/g) than compared to sample-A (0.35mg/g) and sample-C (0.24mg/g) in acid hydrolysis. In alkaline hydrolysis, the non reducing sugar was more in the sample-A (1.02mg/g) than compared to sample-B (0.74mg/g) and sample-C (0.72mg/g) (Table 1 & 2) (Fig. 3 & 4). In acid hydrolysis protein content was more in the sample- C (5.95mg/g) than compared to sample-B (5.80mg/g) and sample-A (3.35mg/g). In alkaline hydrolysis, the protein content was more in the sample- A (7.40mg/g) than the other sample-C (7.0mg/g) and sample-B (6.95mg/g) (Table 1 & 2) (Fig. 5 & 6). In acid hydrolysis, the carbohydrate content was more in the sample- B (2.82mg/g) than compared to sample-C (2.79mg/g) and sample-A (2.61mg/g). In alkaline hydrolysis, the carbohydrate content was more in the sample- B (2.43mg/g) than the other sample-C (1.86mg/g) and sample-A (1.83mg/g) (Table 1 & 2) (Fig. 7 & 8).

Physicochemical Parameters of Arecanut husk

Arecanut husk flour sample were fractionated in to three graded particles such as sample-A (0.28 ± 0.01 mm), sample-B (0.43 ± 0.02 mm) and sample-C (0.64 ± 0.01 mm). The Surface area of the Sample-A (1.93 ± 0.02 m^2/Kg), Sample-B (6.77 ± 0.08 m^2/Kg) and Sample-C (12.60 ± 0.25 m^2/Kg). The Density of the bed of the Sample-A (610.4 ± 4.99), sample-B (493.60 ± 2.62) and sample-C (472.47 ± 0.98). The Moisture content of the sample-A (3.30 ± 0.20 %), sample-B (2.90 ± 0.10 %) and sample-C (2.60 ± 0.15 %). The total solids of the sample-A (83.26 ± 0.92 %), sample-B (75.29 ± 0.61 %) and

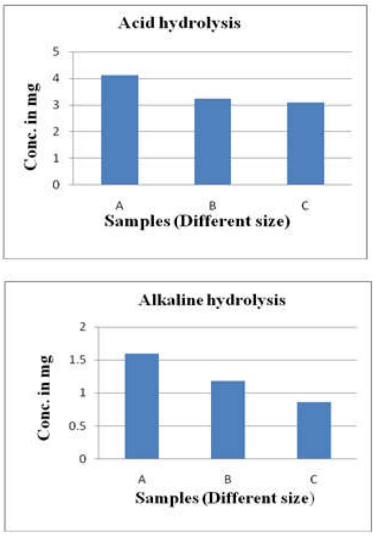
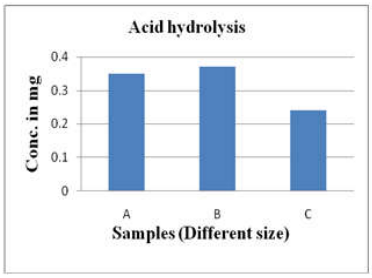


Figure 1 & 2. Reducing sugar content in different samples during acid and alkaline hydrolysis



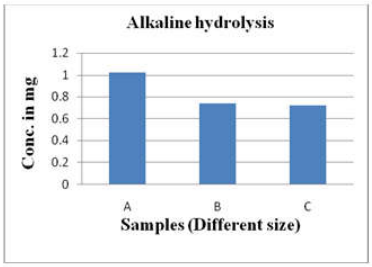
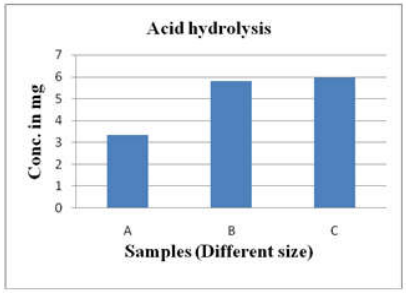


Figure 3 & 4. Non-reducing sugar content in different samples during acid and alkaline hydrolysis



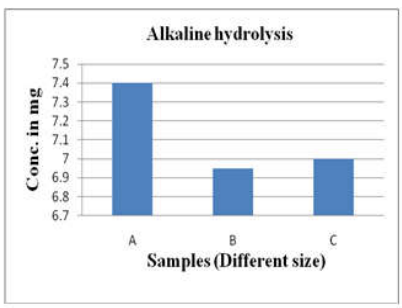
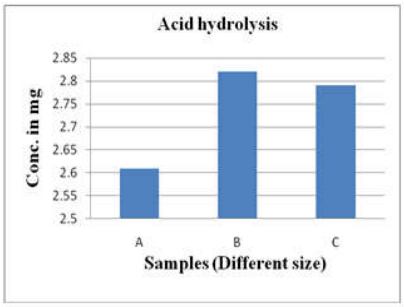


Figure 5 & 6. Protein content in different samples during acid and alkaline hydrolysis



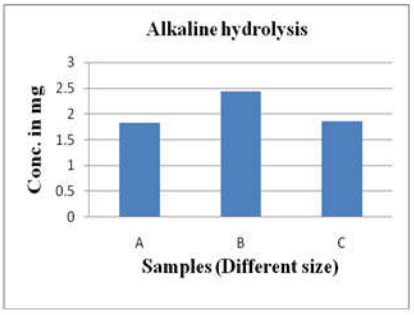
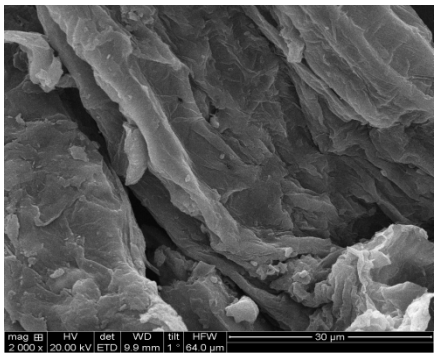
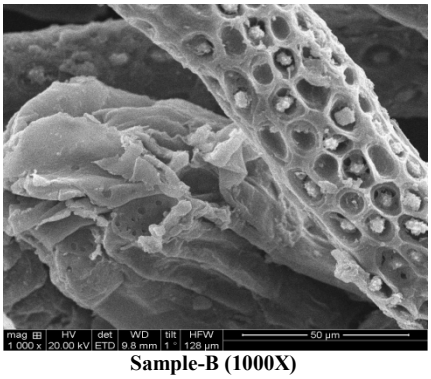


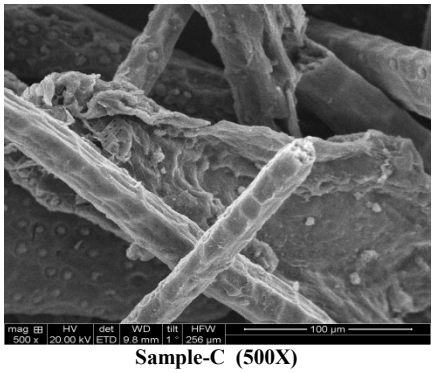
Figure 7 & 8. Total carbohydrates content in different samples during acid and alkaline hydrolysis



Sample-A (2000X)

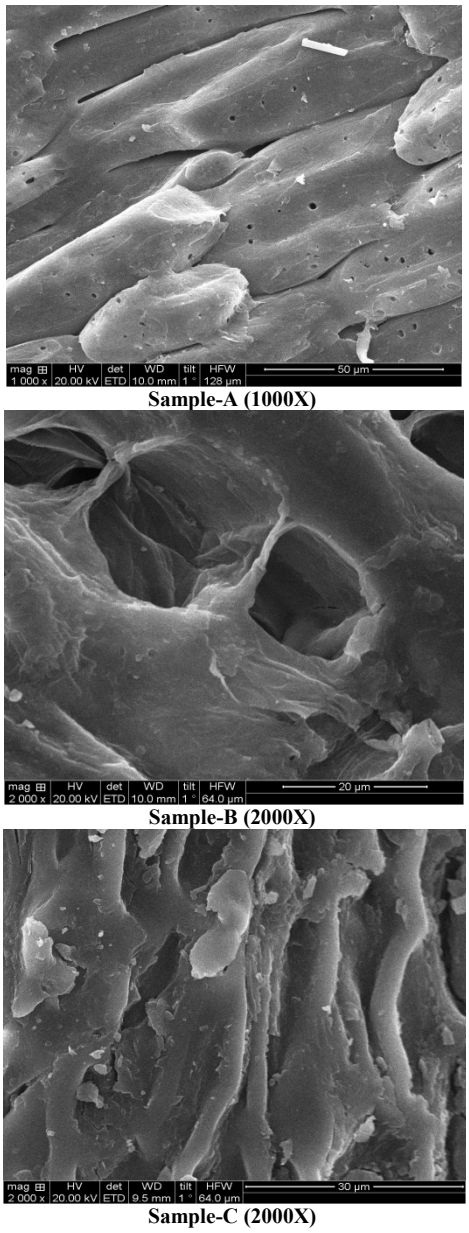


Sample-B (1000X)



Sample-C (500X)

Figure: 9. SEM image of fracture surface of different sized Arecanut husk samples in Acid hydrolysis Pretreatment



Sample-A (1000X)

Sample-B (2000X)

Sample-C (2000X)

Figure 10. SEM image of fracture surface of different sized Arecanut husk samples in Alkaline hydrolysis Pretreatment

sample-C (70.86 ± 0.81 %). The organic carbon content of the sample-A (31.49 ± 0.59), sample-B (29.38 ± 0.12) and sample-C (23.64 ± 0.22).The nitrogen content of the sample-A (0.52 ± 0.02), sample-B (0.05 ± 0.01) and sample-C (0.59 ± 0.04). The cellulose content of the sample-A (39.0 ± 1.53), sample-B (33.5 ± 0.74)and sample-C (28.0 ± 0.85).The hemicellulose content of the sample-A (8.30 ± 0.15), sample-B (32.4 ± 0.19) and sample-C (41.6 ± 0.23).The lignin content of the sample-A (8.0 ± 0.45), sample-B (11.5 ± 0.35) and sample-C (21.0 ± 0.19) (Table 3).

Scanning electron microscopy analysis

SEM images of chemical pretreated different sized Arecanut husk samples such as sample-A, sample-B and sample-C. In acid hydrolysis pretreatment shows different SEM images such as sample-A (2000X), sample-B (1000X) and sample-C (500X) (Fig. 9). In alkaline hydrolysis pretreatment shows different SEM images such as sample-A (1000X), sample-B (2000X) and sample-C (2000X) (Fig.10).

DISCUSSION

In the present study Chemical pretreatments had the primary goal of improving biodegradability of cellulose by removing lignin and or hemicelluloses and to a lesser degree decreasing the degree of polymerization and crystallinity of the cellulose component (Dhanya *et al.,* 2009). Results indicate that Arecanut husk treated for long treatment time produced more soluble sugar. Moreover, the acid hydrolysis effect on soluble sugar content of Arecanut husk is more profound than of alkali treatment (Alfani *et al.,* 2000). The total amount of reducing sugar concentration was more in the sample- A (4.12mg/gm) than compared to other samples in the acid hydrolysis. Same work has done in the relevant to estimate the reducing sugar content per gram of fiber was found to be 1.8mg/gm for dry nut fiber (Rajan *et al.,* 2005). Non reducing sugar was more in the sample-A (1.02mg/g) in alkaline hydrolysis than compared to the acid hydrolysis. Similarly, relevant to the amount of non-reducing sugar content in per gram of the fiber was found to be 0.68mg/gm for dry nut fiber (Rajan *et al.,* 2005). In this study the pretreatment methods like acid hydrolysis and alkaline hydrolysis were carried out and the breaking of sugar molecules was more in the fine powder (Mohammad *et al.,* 2008; Latif, and Rajoka, 2001). In acid hydrolysis the protein content was more in the sample-C than compared to other samples-B and C. If the more protein content present in the sample inhibits the activity of the enzyme and also decreasing the concentration of the sugar (Seema, 2008; Swetha, 2010). The total amount of carbohydrate content was more in the sample-B (2.82 ± 0.01mg/g and 2.43 ± 0.02 mg/g) than compared to other samples-A and C, both acid and alkaline hydrolysis (Seema, 2008; Sweetha, 2010).

Physicochemical Parameters of Arecanut husk, the Arecanut husk flour sample were fractionated in to three graded particles such as sample-A, sample-B and sample-C. The Surface area of the Sample, Density of the bed, Moisture content of the sample, total solids of the sample, organic carbon content of the sample, nitrogen content of the sample, cellulose content of the sample, hemicellulose content of the sample and lignin content of the sample (Seema, 2008; Sweetha, 2010). Scaning electron microscopy analysis was performed with a FEI Quanta 200 operated at 20 kV. The samples were coated (gold/palladium) with a SC7640 Suto/Manual High Resolution Sputter Coater (Indian institute of science, Bangalore). Based on the results from Scaning electron microscopies (SEM) were used to gather information on the effect of the pretreatment on the ultra structure and possible disruption of the Arecanut husk cell wall structures. SEM images of sample-A, sample-B and sample-C in acid and alkaline hydrolysis pretreatments. In coarse particles, the cell wall itself is surround by a sheath palisade (sample-A, SEM image (2000X) of acid hydrolysis) and slightly higher magnification the individual cells of the cell wall can be identified (sample-B, SEM images, (1000X) of acid hydrolysis). A high resolution of the SEM scan (amplitude image) of primary cell wall lining the cell cavity shows interwoven celluose microfibrils, partiaaly imbedded in non-cellulosic polymers. In medium size particles, the defibrating effect of the pretreatment causes the individual fibres to partially separate, as can be seen in sample-B (SEM image). Fine particles causes no further separation of fibres (1000X and 2000X of sample-A in acid hdrolysis, SEM images) but

removes most of the surface layer seen in sample-A (SEM image (2000X) in acid hydrolysis). Milling causes partially separated fibres with 90^0C compression bends (sample-B, SEM image (1000X) of acid hdrolysis) (Sweetha, 2010).

Initially, the most apparent effect of the milling pretreatment apart from a size change from coarse in to fine particles is the partial defibration, or separation of individual fibres and cell types of the Arecanut husk. Although the milled material is quite heterogeneous and contains larger pieces (up to about 1 cm) that are easily recognised as Arecanut husk particles, a significant fraction consists of cells that are either completely or partially separated from each other. Initially, milling in sample-A did not have a great effect on the overal structure of the pretreated material apart from a change in size of the particle, presumably due to the hemicellulose content of the middle lamella (Mohan Kumar, 2008; Sweetha, 2010). Surprisingly, neither the overal or fibrillar structure of the Sample-A ,sample-B and sample-C fibres seems to show large structure change such as the rupture of fibres or a visible increase of porosity, which are believed to be associated with hammer milling pretreatments (Mohan Kumar, 2008; Seema, 2008). Based on these observations, we therefore propose that the re-localization of lignin as well as partial hemicellulose removal are likely to be important factor in increasing the enzymatic digestibility of Arecanut husk particles through hammer milling pretreatment. It seems that exposing cellulose through manipulation of hemicelluloses and lignin are equally as important as altering the crystallinity and rupture of the skeletal structure of the cell wall. The cost effectiveness of the chemicals is also an important characteristic in deciding the chemicals for pretreatment. In that case Sulfuric acid and Sodium hydroxide can be used for pretreatment (Dhanya *et al.,* 2009).

Conclusion

The processing of cellulosic biomass to fuels would open up major new agricultural markets and provide societal benefits, but pretreatment operations essential to economically viable yields have a major impact on costs and performance of the entire system. Tools demonstrated here namely mass balance analysis, milling process and pretreatment methods can be used to screen process for future and alternative process for improved ethanol production. Thus, extensive work on improving the hydrolysis rate of cellulose treated with acid and alkali has to be done to promote commercialization of cellulosic ethanol.

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