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COMPARISON OF VARIOUS PRETREATMENTS ON BIOMASS FOR INCREASED ENZYMATIC SACCHARIFICATION FOR THE PRODUCTION OF BIOFUEL

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Abstract: Lignocellulosic biomass pretreatment is prerequisite to surpass recalcitrance and increase enzymatic accessibility to cellulose for maximum sugar yields. The presence of limited feedstock alternatives, nowadays research has been moved towards exploring various forest and waste feedstock. In the present study *Acacia nilotica* seeds, *Anthocephalus cadamba* leaves,whole plant of *Taraxacum officinale, Lantana indica and Parthenium hysterophorus* was used to check for the maximum monomer sugar recovery. Hot water, autoclave, acid treatment, alkaline and biological pretreatment was employed and checked for the yields. Carbohydrate compositional analysis revealed that acacia seeds were having 52% cellulose content and thereby 503 mg/ml reducing sugar content. Scanning electron microscopic analysis showed the broken linkages between carbohydrate polymers which is necessary for enzymatic saccharification.
Keywords: Fermentation, Pretreatment, Scanning electron microscopy, Saccharification.

Introduction

Increased concerns on the environmental impact of conventional fuels and their acute depletion have diverted the classical research towards bioenergy production from biomass. Biomass includes forest residues, wood residues, agricultural residues, animal and human wastes. They can be processed biologically or chemically by breaking lignocellulosic material into simple sugars which can be used in fermentation to produce bioethanol, biodiesel or methane. There are innumerable factors which results in recalcitrance of lignocellulosic material. Work is under process to obtain commercially sustainable and economically viable method of pretreatment which can contribute to enhance release of monomer sugars for downstream processing [1]. The interaction of cell wall and pretreatment method can leads to compositional and structural alterations which directly affects the enzymatic saccharification [2]. Cellulose crystallinity, its accessibility and extent of lignifications can be correlated to sugar recovery [3,4,5]. These factors in association contribute to biomass recalcitrance which can be overcome by choice of pretreatment method

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[6,7]. Because of complex interactions between pretreatment and type of biomass, a comparative evaluation of various biomass and pretreament would provide important insights into the mechanism of lowering recalcitrance and delignification. Literature reports are scant for comparative study evaluations. Pretreatment methods mainly comprise of mechanical, chemical, biological, physio-chemical, organosolv, ozonolysis, ammonia fibre explosion (AFEX) and green liquids [8] which can be applied to break recalcitrance of biomass.

Five plant biomass were taken for the study viz; *Acacia nilotica* seeds, *Taraxacum officinale ,Anthocephalus cadamba, Lantana indica and Parthenium hysterophorus. Acacia nilotica* is flowering plant which comes under the legume family, Fabaceae ; and it is commonly known as shittah or thorn trees. *Acacia* is the fastest growing trees in the Indian environment, out of 163 species 32 species found in Asian countries [9,10]. It does not require in specialized condition for their growth and development. *Acacia* plant can produce variety of products like seeds, wood, medicines, paints, perfumes and different type of alkaloids. It also helps in protection of environment through soil enrichment, soil reclamation, protection against fire and wind, and as a haven for biodiversity and ornament [11]. The foliage and the pods dropped during the dry or summer season that can be a fundamental source of nutrients in periods of feed scarcity. *T. officinale* is found growing in temperate regions of the world, in lawns, garden, roadsides and other areas with moist soils. *T. officinale* is mainly considered but it has medicinal properties. *Anthocephalus cadamba* commonly known kadam locally is a tropical tree. The growth rate of the tress is very fast and it requires 32℃ to 42°C temperature. The tree is supportive for aforestation and the absence of serious pests and diseases [12,13,14]. While, *Lantana indica* is a toxic weed which has executed a great threat to land yield, grazing livestock, biodiversity and subsequently to the overall ecology. It is a rugged evergreen shrub from the tropical area grows around 6 feet tall and spread rapidly in fields [15,16]. *Parthenium* sp. is asteraceae family plant and is native to American tropics. The common names are congress grass, gajar ghas, white top, and feverfew. This weed is found in summer rainfall areas and is capable of growth in highly resistant environment.

The present study is mainly focused on the optimization of pretreatment methods on various biomass types, which would result in monomer sugars for downstream processing. The impact of pretreatment methods was analyzed by scanning electron microscopy technique. Enzymatic saccharification was performed followed by fermentation for ethanol production.

Materials required

The biomass of *Acacia nilotica* seeds, *whole plants of Taraxacum officinale ,Anthocephalus cadamba, Lantana indica and Parthenium hysterophorus* was collected from the vicinity of School of Biotechnology, Devi Ahilya University, Indore. They were washed thoroughly with tap water to remove any impurity. Thereafter, dried at 60^{o}C in an oven for 12 h, ground to 10 mesh size and stored for further experiments. The cellulase (6.7units/mg solid) and β-glucosidase (3.40units/mg solid) enzymes were purchased from Sigma Aldrich, USA. All the other chemicals were purchased locally and were analytical grade.

Micro-organism and culture conditions

The Forise yeast culture was maintained aseptically on MGYP agar slant containing 3 g malt extract, 10 g glucose, 3 g yeast extract, 5 g peptone and 20 g agar per litre.

For experimental use, ethanol production media for yeast culture was optimized containing yeast extract 1g, KH2PO4 0.1g, (NH4)2SO4 0.1g, MgCl2 0.1g and pH was adjusted to 5.0

Random soil screening was performed for isolation of potent fungal strains which has an ability to hydrolyze polysaccharides. The cultures were grown on Czapeck-Dox medium supplemented with 2% Carboxy methyl cellulose (CMC) to isolate pure fungal cultures for the purpose. A clear zone around the fungal colony represented the production of cellulase enzyme. The pure colonies were isolated for biological treatment and 10 % glycerol stocks were also maintained for further use and identification.

Composition analysis of various biomasses

For the cellulose and hemicelluloses contents of plant residues, 0.7 gm of dried ground plant sample was taken in the clean test tube and mixed with 5 ml 72 % H_2SO_4 (v/v). The tubes were boiled for 4.5 hr to hydrolyze the cellulose and hemicelluloses contents and the lignin content remains present in the residue [17]. The content was centrifuged at 10,000 x g for 10 min and supernatant was collected for the estimation of amount of glucose (C1) using glucose oxidase-peroxidase and o-dianisidine method as described by Trinder [18] and total reducing sugar contents (C2) was estimated using DNSA method [19]. The cellulosic and hemicellulosic contents present in the plant were determined using formula:

$$\%\frac{w}{w}\,Cellulose\ content = \left(\frac{0.9}{0.96}\right) X\ C1\ X\ \left(\frac{V}{M}\right) X\ \alpha\ X10$$

Where, 0.9 - molecular weight ratio of the polymer and the monomer pentose, 0.96 is saccharification yield of xylan to xylose, C1 is glucose concentration (g/L), V is total volume

of sugar solution (L), M is dry weight of the algal biomass sample (g) and A is dilution of the sample (if any).

The hemicelluloses content was calculated using equation:

$$\%\frac{w}{w}\ Hemicellulose\ content = \left(\frac{0.88}{0.93}\right) X\ (\ C2 - C1)\ X\ \left(\frac{V}{M}\right) X\ \alpha\ X\ 10$$

Where, 0.88 is molecular weight ratio of the polymer and the monomer pentose, 0.93 is saccharification yield of xylan to xylose, C2 is reducing sugars concentration (g/L) from the DNS method, C1 is glucose concentration (g/L), V is total volume of sugar solution (L), M is dry weight of the algal biomass sample (g) and α is dilution of the sample (if any).

Pretreatment methods and Sugar estimation

All five biomass were taken and subjected to various pretreatment as mentioned below:

Hot water treatment: The plant biomasses were mixed with distilled water in 1:10 solid to liquid ratio in a flask and incubated at 90^{o}C for 1 hr.

Autoclave treatment: The plant biomasses were mixed with distilled water in 1:10 solid to liquid ratio in a flask and autoclaved for 30 min followed by centrifugation.

Acid treatment: The plant biomasses were mixed with 0.4% sulphuric acid (v/v) in 1:10 solid to liquid ratio in a flask and incubated at 50^{o}C over night.

Ammonium chloride pretreatment: The plant biomasses were mixed in 1:10 solid/liquid ratio with ammonium chloride (90g/L) and incubate at room temperature for 24 hours.

Biological treatment: The plant biomasses were mixed in 1:10 solid/liquid ratio with inoculum containing Czapeck-Dox medium and incubated at 37^{o}C for 48 hr.

After incubation, samples were centrifuged at 10,000 x g for 10 min. Supernatant were used to measure reducing sugar concentration using dinitrosalicylic acid (DNSA) method.

Scanning electron microscopy (SEM) imaging

Acacia seeds were selected for SEM analysis after reducing sugar and glucose estimation. The biomass was subjected to SEM (model JEOL JSM-5600) at a resolution of 5.9KcV-138Kcv and BIAS was at -500V. Untreated biomass was taken as control to compare extent of delignification. Before analysis, samples were dried at 60^{o}C for 24 hr.

Saccharification of pretreated biomass

Acacia seeds were having maximum reducing sugar content after acidic pretreatment, which was taken further for saccharification and fermentation process. Pretreated acacia seeds were washed three times with distilled water and residue was dried in hot air oven at 50oC over night. 2g dried biomass was mixed with 25 ml of 50mM sodium citrate buffer (pH 4.8) and

commercial cellulase enzyme at 37°C for 72 hr in an incubator. The cellulase loading was optimized to 1.25%. The content was supplemented with 2% (w/v) sodium azide to avoid microbial growth. After 72 hr, the sample was centrifuged at 10,000 x g for 10 min and supernatant was taken for reducing sugar and glucose estimations.

Fermentation and ethanol estimation

The *acacia* seed biomass after saccharification (98.75 mg/dl glucose) was taken in an Erlenmeyer flask, and mixed with 5% yeast inoculum in production media. Fermentation was performed at 35°C for 72 h and pH was kept slightly acidic. Qualitative and quantitative estimation of ethanol production was done using gas chromatography (Shimadzu, GC-14B) and sodium acetate and dichromate method at absorbance of 578 nm [20].

Theoretical ethanol yield was calculated by equation:

$$Theoretical\ maximum\ ethanol\ yield\ (\%) = \frac{\text{Ethanol produced (g)}}{\text{Initial sugar (g)} \times 0.511} \times 100$$

Results and discussion

Biomass constituent analysis

Total carbohydrate content of various biomasses viz; *Acacia nilotica* seeds, *whole plants of Taraxacum officinale, Anthocephalus cadamba, Lantana indica and Parthenium hysterophorus* was estimated as mentioned in table 1. It was found out that acacia seeds have highest cellulose content of 52.79% and hemicelluloses of 14.72 %. Pretreatment of various biomasses was performed and reducing sugar content was estimated (Table 2). Pretreatment on all the plant biomasses revealed that acacia seeds have maximum reducing sugar content of 503.3 mg/ml and thereby have maximum chances of increased glucose yields after saccharification.

Table 1: Constituent analysis of various biomasses

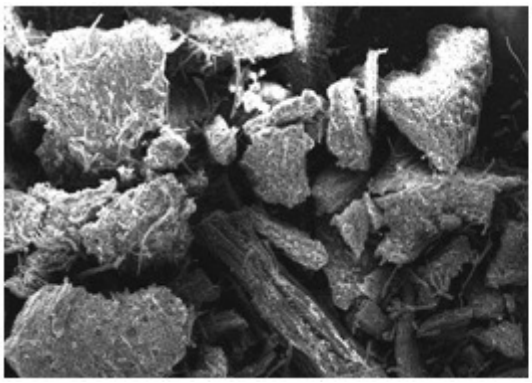
S.No.	Plant biomass	Cellulose content (%)	Hemicelluloses content (%)
1	*Parthenium hysterophorus*	29.70	21.30
2	*Acacia nilotica*	52.79	14.72
3	*Anthocephalus cadamba*	28.65	23.08
4	*Lantana indica*	29.15	27.82
5	*Taraxacum officinale*	30.08	28.84

Table 2: Reducing sugar yield after pretreatment of biomass

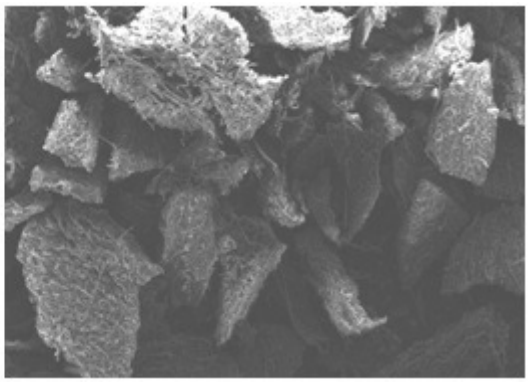
Plant biomass ↓	**Reducing sugar estimation (mg/ml)**				
Pretreatment modes →	Hot water treatment (1: 10, 100°C, 1h)	Autoclave treatment (1:10, 15 psi, 121°C, 30 min)	Acid treatment (1:10, 0.4 % H_2SO_4, 50 °C)	NH_3Cl treatment (1:10, room temp, 24 h)	Biological treatment (1:10, 37°C, 24h)
Parthenium hysterophorus	285.0	388.0	501.0	102.32	38.60
Acacia nilotica	323.0	322.0	503.3	61.40	12.10
Anthocephalus cadamba	103.14	104.5	187.9	102.9	12.10
Lantana indica	575.0	479.0	122.3	93.38	20.30
Taraxacum officinale	208.0	277.0	455.0	51.49	37.01

Scanning electron microscopic analysis of acacia seeds

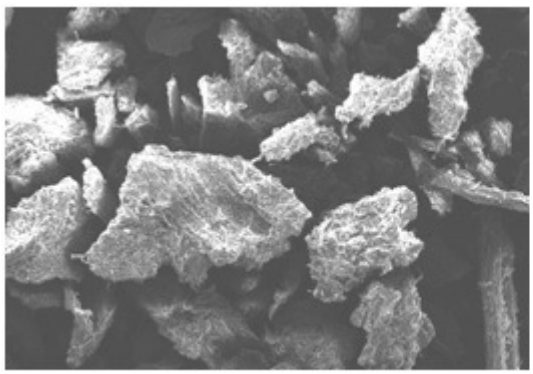
After pretreatment SEM was performed on acacia seeds and was compared with the untreated acacia seeds to check for extent of delignification. It is evident from the figures that acid pretreatment worked best on acacia seeds. There is a formation of holes and separation of polymers in the biomass structure showing release of cellulose and hemicelluloses out of concrete lignin covering (Figure 1).



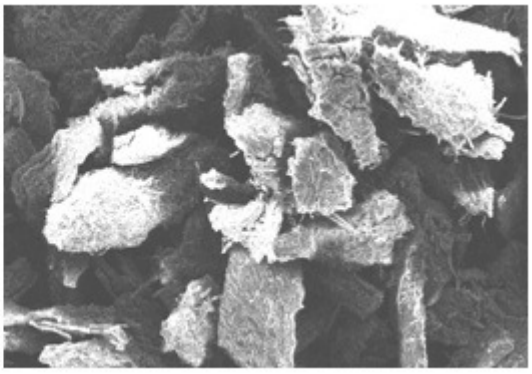
Hot water treated seeds



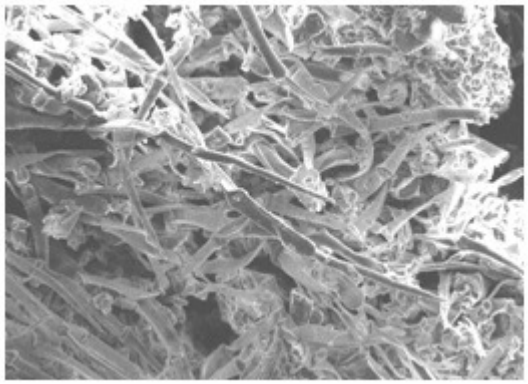
Autoclave treated seeds



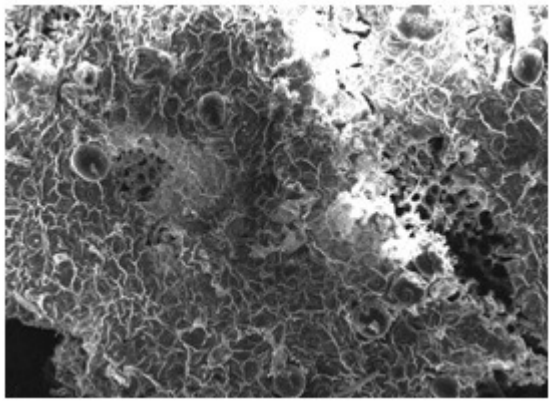
Acid treated seeds



Ammonium chloride treated seeds



Biological treated seeds



Untreated seeds

Figure 1: Scanning electron microscopic analysis of acacia seeds

Saccharification and fermentation

Saccharification on acacia seeds was performed using 1.25% cellulase in sodium citrate buffer. Hydrolysis using cellulase leads to breakdown of 1,4-β-D-glycosidic linkage in cellulose. After hydrolysis, glucose content was estimated using oxidase- peroxidase method which was 98.75 mg/dl. Hydrolysis was followed by fermentation using 15% Forise yeast inoculum in ethanol production media at 30^{o}C for 72 hr. Ethanol content was estimated to be 2.3 mg/ml which means approximately 4.89% ethanol was obtained from acacia seeds.

Conclusion

Acacia seeds are one of the good alternatives of feedstock for biofuel production, as it has high cellulose content. Mild sulphuric acid treatment provides an opportunity to obtain maximum reducing sugars and thereby ethanol after fermentation. Hot water treatment and autoclave treatments provides similar outcomes which can also be refined with combinations with other treatment methods. Biological treatment and alkaline treatment with ammonium chloride gave slight enhancement of enzymatic digestibility and delignification. Present study can led to a step forward towards exploring other advanced pretreatment methods to

investigate other aspects of acacia seeds and its utility in biofuel production.

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