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Lime Pretreatment Associated Compositional and Ultrastructural Changes in Selected Root and Vegetable Processing Residues

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Abstract— *The study aimed at exploring the suitability of processing residues from selected root and vegetables for bioethanol production, which are otherwise environmental pollutants. The effect of lime pretreatment at high (HT), low (LT) or room (RT) temperatures on compositional and ultrastructural changes in peels of root crops (sweet potato, elephant foot yam and tannia) and vegetable processing residues (peels from ash gourd and mixed vegetable waste) was studied. Pretreatment resulted in the removal of very little polysaccharides, including starch from these biomasses. Hemicellulose was removed to a higher extent in 24 h RT pretreatment (11.6-12.3%) compared to 7.3-8.5% removal in HT pretreatment. Maximum lignin removal (ca. 33-38%) occurred in RT pretreated (24 h) samples. Approximately 22-25.7% lignin was removed during HT pretreatment (121 °C) for 30 min. which increased to 28-31% when prolonged to 60 min. Pretreatment Efficiency (PE) was low (4.2-14.7%) in HT pretreatment, while 5.7-13.5% and 5.2-14.2% PE was observed in LT and RT pretreatments respectively. Scanning electron micrographs of lime pretreated biomass indicated that starch being a major ingredient of the biomass under study, preferential saccharification of starch by amylases might be necessary to expose the cellulose and hemicellulose for their subsequent saccharification to release fermentable sugars.*

Keywords—Composition, Lime pretreatment, Processing residues, Root crops, Ultrasructure, Vegetable crops.

I. INTRODUCTION

There is an ever-increasing global concern over the rapid depletion of fossil fuel resources, enhanced demand for transportation fuel in developed and developing countries and the environmental challenges caused by the emission of greenhouse gases (GHGs) resulting from the burning of coal and fuel, which is implicated as the main factor for global warming [1, 2]. Bioethanol from renewable resources is recognized as the best transportation fuel which could help reduce dependency on fossil fuels [3]. Despite the cost-effectiveness of corn and sugar based ethanol, the ethical conflicts on the diversion of food to fuel have necessitated the search for potentially cheap and inedible feedstock for bioethanol production [4-6]. Owing to the low cost and abundant availability, lignocellulosic biomass (LCB) has been widely recognized as the most viable and sustainable feedstock for biofuel production. It is reported that bioethanol from cellulosic and other biomass resources has the potential to reduce GHG emission by 86% [7]. Nevertheless, the sustainability of second generation (2G) ethanol produced from LCBs, despite its potential to replace oil-based fuels depends on the economically feasible production, by overcoming the technological barriers such as recalcitrance to degradation, enzyme costs for effective conversion to sugars, high pretreatment costs and its associated problems *viz.,* formation of inhibitors, cost of chemicals for neutralization etc. [8-10].

Although lignocellulosic materials generally comprise agricultural residues, woody biomasses and dedicated crops such as switchgrass, Bermuda grass etc., there is also a major global contribution from the processing residues due to the increased industrial activities. While as high as 90% of the LCBs are constituted by cellulose, hemicellulose and lignin, processing residues contain starch also as a main component [2, 11, 12], indicating the need for different approaches in their handling for ethanol production. The three main steps in the conversion of LCBs to ethanol are pretreatment, saccharification to monomeric sugars and fermentation. The aim of pretreatment is to detach lignin and hemicellulose from the cellulose, reduce the crystallinity and increase the porosity of cellulose, thereby enhancing its accessibility to cellulases [13, 14]. An efficient pretreatment method should reduce the formation of fermentation inhibitors, preserve the potential sugar yielding carbohydrates in the residue, improve the release of sugars prior to and during enzymatic saccharification and minimize energy requirement [14, 15-18]. Dilute acids and alkali have been used for pretreatment by several researchers on a wide variety of LCBs and extensive reviews have appeared on such techniques and their comparative advantages/disadvantages [3, 6, 14, 19-21]. Major

disadvantages of acid treatment include the need for corrosion-resistant reactors, less efficiency of lignin removal and formation of inhibitors such as furfural, 5-hydroxymethyl furfural and acetic acid [4, 22, 23]. Hence, lime (calcium hydroxide) pretreatment has been attempted for several lignocellulosic feedstocks [11, 24-27]. Lime pretreatment has regained interest as a promising pretreatment technique because it is a cheap chemical that could be safely handled, needs only low temperatures and pressures and could be recovered easily. Besides, lime also facilitates the removal of lignin and acetyl groups and reduces the chances of formation of fermentation inhibitors [11, 28, 29]. The divalent calcium ions in calcium hydroxide are reported to effectively crosslink with lignin, thereby preventing its non-productive binding with cellulase [30, 31].

Sweet potato (*Ipomoea batatas* Lam) is the second most important root crop with a world production of 103.11 million tonnes [32] and China is the leading producer accounting for almost 80% of the global production. During the processing of sweet potato for starch or flour preparation, approximately 5-6% goes as waste peel and is reported to contain 79% carbohydrate [33]. Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) is a most popular root crop grown and consumed in South Asian countries such as India, Malaysia, Indonesia and the Philippines [34] and during processing, considerable loss (*ca.* 15%) of peel occurs due to the non-uniform surface morphology of the roots. Tannia (*Xanthosoma sagittifolium* (L.) is a tropical root crop grown widely in West Africa, tropical America and Asia [35]. Processing of cormels leads to the generation of peels (10-13%) as refuse and consist of the thin skin along with the outer cortex of the roots [35, 36] and except for compositional studies, its value addition has not been reported. Ash gourd (*Benincasa hispida* Cogn.) is cultivated as a vegetable in India, Japan, China and Australia [37]. Approximately 25% goes as peel waste during commercial processing for sweet manufacture in India, causing major disposal problems [38]. It is estimated that 73-96% of the typical family's waste comprises of biodegradable materials in lower income groups and 26% in the higher groups in India [39]. Out of the biodegradable wastes generated, a major part is accounted by kitchen/domestic waste, while hotels also contribute significantly to this fraction of solid waste. With a view to exploring the potential of these processing wastes (which are also rich in starch besides cellulose and hemicellulose) for bioethanol production, the effect of lime pretreatment at high, low and room temperatures on compositional and ultrastructural alterations in three root crop processing wastes (peels from sweet potato, elephant foot yam and tannia) and two vegetable wastes such as ash gourd peel and mixed vegetable wastes (comprising the inedible parts such as peels, seeds and pulp part covering them and damaged parts of common vegetables collected from the households and restaurants) was investigated. As different from the typical LCBs, these wastes also contain appreciable amounts of starch, which comes along with the peel during the peeling operation, enabling them to be categorized as lignocellulo-starch biomass (LCSB). Nevertheless, their ultrastructural and compositional differences as well as the alterations brought about by pretreatments have hitherto not been reported. Hence this study aims at a detailed understanding of the changes brought about during lime pretreatment on the polysaccharide and lignin components so that the best treatment could be identified for further saccharification studies.

II. MATERIALS AND METHODS

2.1 Samples

Peels collected from sweet potato, elephant foot yam, tannia and ash gourd by manual peeling were washed in running tap water to remove the adhering dirt and sand, immediately drained and dried in the sun for 24-36 h, followed by drying in an air oven to reduce the moisture content to <10%. It was then powdered in a hammer mill (particle size: *ca.* 2-3 mm) and packed in air tight containers till use. In order to utilize the whole waste residues for bioethanol production, the unscreened biomass was used for the various experiments. Besides, mixed vegetable wastes were collected from households and restaurants and these were also dried, powdered and stored for further studies.

2.2. Enzyme Source

Spezyme® Xtra (α-amylase) and Stargen™ 002 (Granular starch hydrolyzing enzyme) were supplied by M/s Genencor International Inc. USA (presently Danisco US Inc., USA). Spezyme contained a thermostable α-amylase (E.C. 3.2.1.1) with an activity of 14,000 α-amylase units (AAU)/g (1.0 AAU = amount of enzyme required to hydrolyze 10.0 mg starch/min under the assay conditions) [40]. Stargen™ 002 contains *Aspergillus kawachi* α-amylase (E.C. 3.2.1.1) expressed in *Trichoderma reesei* and a gluco-amylase (E.C. 3.2.1.3) from *Trichoderma reesei* that work synergistically to hydrolyze granular starch substrate to glucose. It has an activity of 570 Glucoamylase units (GAU)/g and one GAU is the amount of enzyme that will liberate one gram of glucose per hour from soluble starch substrate under the conditions of the assay [41].

2.3. Pretreatments

Three types of lime pretreatments were attempted in this study such as (i) treatment with lime (calcium hydroxide; 0.1 g/g biomass) at high temperature (121 °C) and

pressure of 0.102 MPa for 30 min. and 60 min. (HT pretreatment) (ii) treatment at low temperature (50 °C) for 6 h and 24 h (LT pretreatment) and (iii) treatment at room temperature (30 ±1 °C) for 24 h and 48 h (RT pretreatment). In the first experiment, the unscreened biomass residues (10 g) were suspended in 100 ml lime solution (10% w/v) in a 250 ml Erlenmeyer flask and exposed to heat in a Pressure Cooker (M/s TTK Prestige India Ltd.) for 30 min. and 60 min. (as separate lots and time after the pressure build up) at 121 °C and pressure 0.102 MPa. The flasks after pH adjustment to 6.0 with Conc. Hydrochloric acid (HCl), were cooled, volume made up to the nearest and filtered. Part of the residue (2.0 g each) at each time period was lyophilized (Thermo-Savant Freeze Drying Chamber FDC-206) for ultrastructural studies using the scanning electron microscope. The remaining residue was dried in an air oven at 50 °C for 20 h followed by high temperature drying at 100 °C for 1 h and stored after cooling to room temperature for further studies.

In the second experiment, one set of biomass slurry was incubated at 50 °C in a thermostatic water bath (Julabo SW22) for 6 h, while the second set was incubated for 24 h. In the third experiment, one set of biomass slurry was incubated at room temperature (30 ±1 °C) for 24 h, while the second set was incubated for 48 h. After the incubation, the pH was adjusted to 6.0 using concentrated HCl and volume raised to the nearest. The filtrates and residues were stored as in the first experiment, for further studies.

2.4. Compositional Studies

The pretreated residues were subjected to compositional analysis comprising starch, total and reducing sugars, cellulose, hemicellulose, ash and lignin by standard procedures. Detailed compositional analyses of the native biomasses selected were reported earlier [42]. In the present study, only the composition of the pretreated biomass has been undertaken as per the methods described under:

2.4.1. Starch

Starch being a major component of the biomass residues under study, the total starch content in the pretreated biomasses was determined using the hydrolytic enzymes such as Spezyme and Stargen as per the procedure standardized earlier [43]. Biomass slurry (0.5g/20 ml) was digested with Spezyme (0.5 ml equivalent to approximately 7000 α-amylase units) for 30 min. at pH 5.5 and 90 °C after which the temperature and pH were brought to 40 °C and 4.5 respectively and digested for 24 h with Stargen (0.5 ml or 285 Glucoamylase units). The reducing sugars released were assayed by the titrimetric method of Moorthy and Padmaja [44]. Enzyme and substrate blanks were also kept to nullify the reducing sugars originally present in the enzyme and biomass samples respectively. Starch content was calculated from the reducing sugar values using the Morris factor, 0.9.

2.4.2. NDF and ADF

The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed by the method of Goering and Vansoest [45] with slight modifications to take care of the interference from starch. Native/pretreated residue (0.5 g) was mixed with 0.5 g sodium sulphite and 50 ml cold neutral detergent solution and after boiling the pH was adjusted to 5.5 and Spezyme (0.5 ml) added and boiling continued for 1 h. After incubation for 1 h, the pH and temperature were brought down to 4.5 and 40 °C respectively and incubated with 0.5 ml Stargen for 24 h. The contents after filtration through Whatman no.1 filter paper (Grade 1; 11 µm pore size) and washing with acetone were dried in an air oven at 100 °C for 8 h. The dry weight of residue (W1) was used to calculate NDF as:

$$\text{NDF (\%)} = \frac{\text{W1 x 100}}{\text{Sample weight}} \quad (1)$$

ADF was determined from the NDF fraction by treating 0.5 g of it with 50 ml acid detergent solution (20 g cetyl trimethyl ammonium bromide in 1 l of 1 N sulfuric acid) and heating for 1 h after the onset of boiling. The contents after filtration were washed and dried at 100 °C overnight. ADF in the NDF fraction was calculated from the residue weight (W2) using the formula and worked back to express as percentage of the original biomass:

$$\text{ADF (\%) in NDF} = \frac{\text{W2 x 100}}{\text{Sample weight of NDF}} \quad (2)$$

2.4.3. Structural Carbohydrates and Lignin

Hemicellulose content in the pretreated residue was determined as the difference of Neutral detergent fibre (NDF) and acid detergent fibre (ADF). Cellulose content in the ADF fraction from the pretreated residue was determined using acetic-nitric reagent by the method of Updegroff [46] with slight modification to avoid interference from starch by using the ADF fraction from the pretreated residue, which was found to give highly reliable results. Ten milliliters of acetic/nitric reagent (10:1 mix of 80 % acetic acid and concentrated nitric acid) were added to 0.5 g ADF in a long test tube which was then boiled for 30 min. at 100 °C in a boiling water bath. The slurry after dilution with de-ionized water was filtered through Whatman no. 1 filter paper and the filtrate was discarded. Residue after washing with distilled water was hydrolysed with 67% sulfuric acid (10 ml) at room temperature for 1 h. The sugars released were

estimated using anthrone reagent and cellulose content in the pretreated biomass calculated using pure cellulose standard was worked back to the original biomass based on the weight of the dry solids remaining after pretreatment. The ash content was determined in the ADF fraction from treated residue by the standard procedure [47], by keeping in a muffle furnace at 550 °C for 6 h. In order to eliminate the error due to the held up proteins in the lignin fraction, the crude protein content in the ADF fraction (from each pretreated residue) was determined by the Kjeldahl method [47] and subtracted from the ADF values to get the true ADF content. The lignin content of the pretreated biomass was calculated as:

Lignin (%) = True ADF (%) - [(cellulose + ash) %] (3)

2.4.4. Characterization of Pretreated liquor

The reducing sugar content in the filtrate was quantified by the same titrimetric method, while the reducing sugars held back in the residues were computed from the substrate blank values from starch estimation.

2.5 Pretreatment Efficiency

The total reducing sugar content (pretreated liquor + residue) after nullifying the original reducing sugar (RS) content in the native biomass was used to compute the Pretreatment Efficiency on the basis of the potential sugar yielding carbohydrates (cellulose, hemicellulose, starch and total sugars) as:

$$PE\ (\%) = \frac{[(RSpt + RSr) - Rsob] \times 100}{[C+HC+S+TS \text{ in original biomass (\% dwb)}} \quad (4)$$

Where RSpt = RS released from the biomass due to pretreatment (expressed as % of the original biomass); RSr = RS held back in the residue (expressed as % of the original biomass); RSob = RS (%) originally present in the biomass; C: cellulose; HC: hemicellulose; S: starch and TS: total sugars; (C+HC+ S+TS represent the total potential sugar yielding carbohydrate fraction).

2.6 Ultrastructural Studies

The ultrastructure of native as well as pretreated biomass was studied on HITACHI Scanning Electron Microscope S-2400). Dry powder (native) and lyophilized powder (pretreated) were applied on the double side carbon pasted on an aluminium stub. A thin gold-platinum coating was applied for 3 min. using E-1010 Ion Sputter Unit under 10 Pa vacuum and discharge current of 10 mA. The SEM photographs were visualized at 500x magnification.

2.7 Statistical Analysis

The various biochemical constituents were expressed as percentage of the original biomass based on the water insoluble residue weight obtained from each pretreatment. Three replicates were kept for each experiment and duplicate analyses were performed on each replicate. Statistical analysis was performed by Analysis of Variance (ANOVA) for statistical testing of the mean values and was followed by least significant difference (LSD) for pair-wise comparison of mean values by using the statistical package, SAS 9.3 [48].

III. RESULTS AND DISCUSSION

The effect of pretreatment of unscreened powders of selected root and vegetable processing wastes as well as mixed vegetable wastes with lime at high temperature (121 °C; 0.102 MPa) for 30 min and 60 min, low temperature (50 °C; 6 h and 24 h) and room temperature (30 ± 1 °C; 24 h and 48 h) on the compositional and ultra structural changes were studied.

3.1 Compositional Changes due to Pretreatment

3.1.1. Polysaccharides and Lignin

The changes in cellulose, hemicellulose and starch during pretreatment of peels of sweet potato (SP), elephant foot yam (EFY), tannia and ash gourd as well as mixed vegetable waste (MVW) is given in **TABLE** 1. Very small quantities of polysaccharides were removed during pretreatment. Except in SP peel and MVW, there was insignificant change in cellulose from the native, in all the three types of treatments and time periods. Cellulose removal ranged from 6.28-9.09% in the biomass residues in 24 h RT pretreatment while in the HT pretreatment (60 min.), there was only negligible removal (1.0-2.45 %). Hemicellulose was also removed to a higher extent in 24 h RT pretreatment (11.6-12.3%) compared to 7.3-8.5% removal in HT pretreatment (Fig. 1a and b). Maximum lignin reduction occurred in the RT (24 h) pretreated samples, followed by LT (24 h) treatment for most biomasses, which was insignificant with the HT pretreatment at 121 °C for 60 min. (**TABLE** 1). Chang et al. [49] reported lime loading of 0.1 g Ca $(OH)_2$ /g dry biomass for bagasse and wheat straw as optimum where no glucan or xylan removal occurred. Based on enzyme digestibility of pretreated LCBs, lime pretreatment conditions were optimized by different researchers as 120 °C for 1 h for bagasse [49], 100-120 °C for 2 h for switchgrass [50] and 120 °C for 4 h for corn stover [24]. Kim and Holtzapple [51] observed that after 16 weeks of lime (0.5g/g dry biomass) pretreatment of corn stover at 55 °C, only 6.3% glucan was solubilized, while 21% xylan was solubilized.

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Table 1: Polysaccharide and lignin changes in lime pretreated root and vegetable processing residues (expressed as g/100 g original material on dry basis).

Parameters	Original Biomass [42]	Lime pretreatment					
		High temperature (121 °C)		Low temperature (50 °C)		Room temperature (30 ±1 °C)	
		30 min.	60 min.	6 h	24 h	24 h	48 h
(a) Sweet potato peel							
Cellulose (C)	13.31^{a}	12.31^{b}	13.17^{ab}	12.53^{ab}	12.87^{ab}	12.10^{b}	12.59^{ab}
Hemicellulose (HC)	13.32^{a}	11.96^{b}	12.32^{b}	12.17^{b}	12.06^{b}	11.72^{b}	12.13^{b}
Starch(S)	32.05^{a}	31.86^{a}	31.11^{b}	31.96^{b}	30.71^{c}	30.61^{c}	31.84^{a}
Lignin (L)	8.15^{a}	6.37^{b}	5.62^{c}	6.46^{c}	5.40^{c}	5.29^{c}	5.43^{c}
(b) Elephant foot yam peel							
C	15.63^{a}	14.61^{a}	15.47^{a}	14.68^{a}	15.00^{a}	14.53^{a}	14.65^{a}
HC	14.00^{a}	12.70^{bc}	12.98^{b}	12.90^{bc}	12.59^{cd}	12.34^{d}	12.81^{bc}
S	28.96^{a}	28.71^{a}	28.21^{b}	28.71^{a}	27.73^{c}	27.51^{c}	28.67^{a}
L	7.01^{a}	5.28^{bc}	4.92^{cd}	5.73^{b}	4.60^{de}	4.37^{e}	4.66^{de}
(c) Tannia peel							
C	17.32^{a}	16.19^{a}	17.11^{a}	16.21^{a}	16.69^{a}	16.12^{a}	16.25^{a}
HC	14.48^{a}	12.97^{bc}	13.25^{bc}	13.61^{b}	12.98^{bc}	12.71^{c}	13.73^{b}
S	30.46^{a}	30.12^{b}	29.48^{c}	30.22^{ab}	29.19^{d}	29.11^{d}	30.10^{b}
L	8. 26^{a}	6.25^{c}	5.71^{d}	6.72^{b}	5.53^{d}	5.46^{d}	5.69^{d}
(d) Ash gourd peel							
C	18.67^{a}	17.55^{a}	18.21^{a}	17.60^{a}	18.12^{a}	17.49^{a}	17.63^{a}
HC	18.30^{a}	16.36 ^{b}	16.87 ^{b}	16.93 ^{b}	16.67^{b}	16.17^{b}	16.78^{b}
S	19.91^{a}	19.71^{a}	19.25^{b}	19.89 ^{a}	19.30^{b}	19.20^{b}	19.77^{a}
L	10.70^{a}	7.95^{bc}	7.55^{cd}	8.46^{b}	7.09^{d}	7.06^{d}	7.11^{d}
(e) Mixed vegetable waste							
C	11.71^{a}	11.03^{c}	11.59^{ab}	11.07^{bc}	11.30^{abc}	10.91^{c}	11.66^{a}
HC	11.97^{a}	10.70^{b}	11.00^{b}	10.99^{b}	10.78^{b}	10.50^{b}	10.92^{b}
S	28.10^{a}	27.88^{a}	27.22^{b}	27.97^{a}	27.01^{b}	26.96^{b}	27.90^{a}
L	7.55^{a}	5.80^{b}	5.41^{bc}	5.85^{b}	5.01^{c}	4.99^{c}	5.22^{bc}

*Each value is mean from three replicates; statistical comparison for each parameter for each biomass was made with the respective native untreated samples; means with different alphabets in each row are significantly different at $p < 0.05$.

Saha and Cotta [12] reported that lime (0.1g/g biomass) pretreatment of rice hulls at 121 ºC for 1 h yielded more sugars during enzymatic saccharification than lower loading rate of lime and exposure periods. Most of the starch remained unhydrolyzed in the lime pretreated biomass (**TABLE** 1). The percentage hydrolysis ranged from 3.6% to 5.0% in the RT (24 h) pretreated biomasses, while it was 2.6% to 3.3% in the HT pretreated biomasses (Fig. 1 a and b). Dilute sulfuric acid (DSA) pretreatment was earlier found to hydrolyze 85-94% of starch in these biomasses exposing the cellulose fibers for saccharification [42]. Nevertheless, lime pretreatment at 121 °C retained most of the starch along with cellulose, while 11% of the hemicellulose got solubilized. Saha and Bothast [52] reported that no glucose was released from starch during hot water pretreatment of corn fiber at 121°C for 1 h. Starch changes during pretreatment of biomasses have not hitherto been reported, as most of the LCBs do not contain starch. Lime pretreatment resulted in the retention of high percentage of solid biomass (**TABLE** 2). Except in the case of sweet potato peel and MVW, there were no significant differences in solids recovery in the various treatments. There are several reports on the high biomass recovery after lime pretreatment of sugarcane bagasse [25, 49, 52]. It was found that delignification was not influenced by high temperature, as it was non-significant for RT and LT for 24 h and HT for 60 min. for most residues. Lignin removal ranged from 34-37.6% at RT (24 h) and on prolonging the time to 48 h, there were only insignificant changes in lignin. Approximately 22-25.7% lignin was removed from the various residues during HT pretreatment (121 °C) for 30 min. and 29-31% lignin

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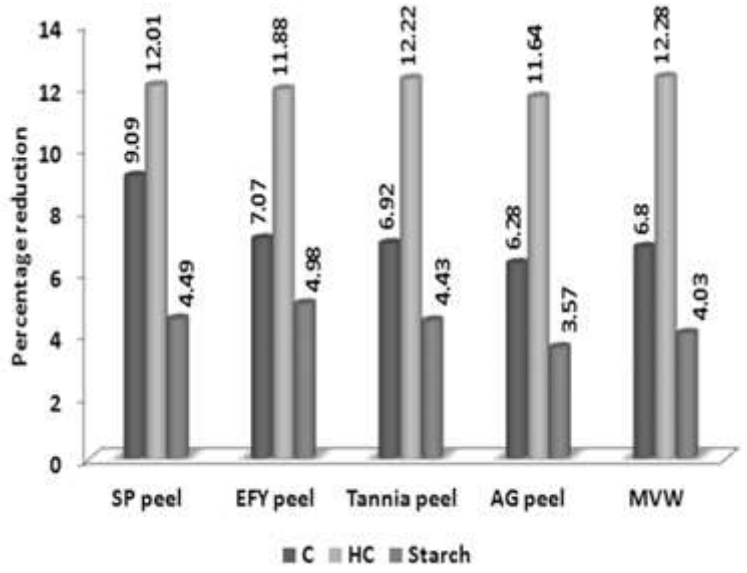

Fig. 1a: Percentage removal of C, HC and starch from biomass due to RT pretreatment with lime (24 h).

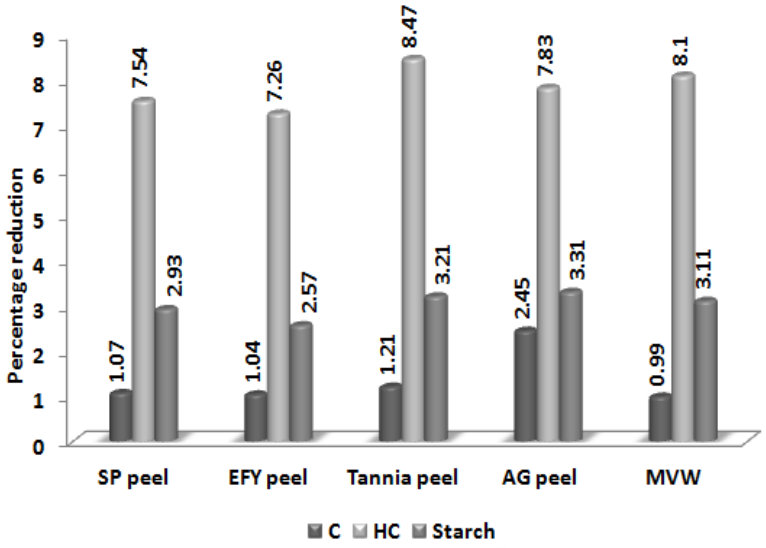

Fig.1b: Percentage removal of C, HC and starch from biomass due to HT pretreatment with lime (60 min.).

Table.2: Percentage solids remaining in lime pretreated root and vegetable processing residues.*

Biomass residue	**Percentage solids remaining after lime treatment**					
	HT (121 °C)		**LT (50 °C)**		**RT (30 ±1 °C)**	
	30 min	**60 min**	**6 h**	**24 h**	**24 h**	**48 h**
Sweet potato peel	92.50^{ab}	91.35^{ab}	94.25^{ab}	90.10^{ab}	88.13^{b}	95.79^{a}
Elephant foot yam peel	95.85^{a}	93.60^{a}	96.08^{a}	92.48^{a}	91.55^{a}	97.50^{a}
Tannia peel	90.90^{a}	90.00^{a}	92.25^{a}	89.00^{a}	87.40^{a}	93.50^{a}
Ash gourd peel	94.80^{a}	91.43^{a}	95.73^{a}	92.23^{a}	91.03^{a}	97.04^{a}
Mixed vegetable waste	95.00^{bc}	94.73^{dc}	95.75^{b}	94.01^{d}	91.00^{e}	98.64^{a}

*Each value is mean from three replicates; means with different alphabets in each row are significantly different at $p < 0.05$.

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removal occurred from different biomasses by extending the time to 60 min. (Fig. 2a-e). Lignin removal was much less (18.3-22.5%) when biomass residues were pretreated with lime at 50 °C for 6 h. Nevertheless, on prolonging the reaction time to 24 h, 33-34% removal was observed. Although high temperature is reported to remove more lignin from biomass, the lower extent of removal in the present study might have resulted from the lower exposure time at HT compared to 24 or 48 h at RT.

Kim and Holtzapple [51] found that lignin and hemicellulose were selectively removed and cellulose crystallinity increased with delignification of lime pretreated corn stover. There are several reports that the divalent calcium ions of lime have high affinity for lignin and could effectively crosslink lignin [30, 31]. Lime is also reported to remove acetyl groups and lignin-carbohydrate ester linkages, thereby enhancing cellulose digestibility [14]. Xu et al. [26] reported that although calcium ions cross linked lignin under alkaline conditions, lignin complex remained in the residue without getting solubilised and hence the lignin content in the pretreated residue was high. They had also found that only 16-35% reduction in lignin occurred in lime pretreated switchgrass which corroborated with our results. Under alkaline conditions, lignin molecules become negatively charged due to the ionization of carboxyl, methoxy and hydroxyl groups, which then have a high affinity for calcium [3].

3.2. Reducing sugars and Pretreatment Efficiency

Reducing sugars in the pretreated liquor from lime pretreated residues indicated that there was only small increase from the original value in all the three pretreatments, which resulted primarily from the hemicellulose hydrolysis, followed by the mild starch hydrolysis leading to exposure of reducing groups (**TABLES** 3 and 1). In the case of the various biomasses, maximum increase was noticed in RT (24 h) pretreatment followed by LT (24 h).

Accordingly, the Pretreatment Efficiency (PE) computed based on the potential sugar yielding carbohydrates was also low for the various treatments (**TABLE** 4). Approximately 4.2-14.7% PE was observed in the HT pretreatment, while 4.6-13.5% and 5.2-14.2% PE were observed in LT and RT pretreatments respectively. Among the biomasses, the lowest PE was observed for EFY peel, which might be due to the structural variations among the biomasses. Prolonging the reaction time for all the treatments resulted in significant decrease in PE for RT and HT pretreatments, possibly as a consequence of conformational changes in starch whereby some of the exposed reducing groups were reverted. This is also supported by the low RS values in the pretreated liquor from RT (48 h) and HT (60 min.) for most biomasses. Kim and Holtzapple [51] reported that delignification and deacetylation could remove the barriers to enzymatic hydrolysis and even though the crystallinity of biomass was increased slightly on delignification, it had less effect on the ultimate sugar yields. Wang et al. [7] reported much lower solid loss in lime pretreatment of coastal Bermuda grass than NaOH pretreatment. They also found that reducing sugar release during enzymatic saccharification of lime (0.1g/g biomass) pretreated (room temperature) Bermuda grass was less at 48 h, compared to 34 h and also lower at 24 h compared to 6 h at 50 °C. Based on the delignification, slightly higher starch hydrolysis and energy expenditure considerations, RT pretreatment with lime (24 h) and HT pretreatment (60 min.) could be considered as the best pretreatments. Even though energy expenditure is more on the HT pretreatment for 60 min., starch gelatinization occurring at 121 °C might be advantageous for effective saccharification in the next stage. Nevertheless, saccharification studies presently underway could only confirm the relative advantage of lime pretreatment techniques over others such as dilute sulfuric acid and steam pretreatment reported earlier for these residues [42].

Removal of the pretreated liquor by filtration before saccharification might be more difficult due to starch gelatinization. However, since the biomasses under study have a high percentage of starch, treatment of pretreated slurry as a whole might be advantageous compared to the treatment of water insoluble solids.

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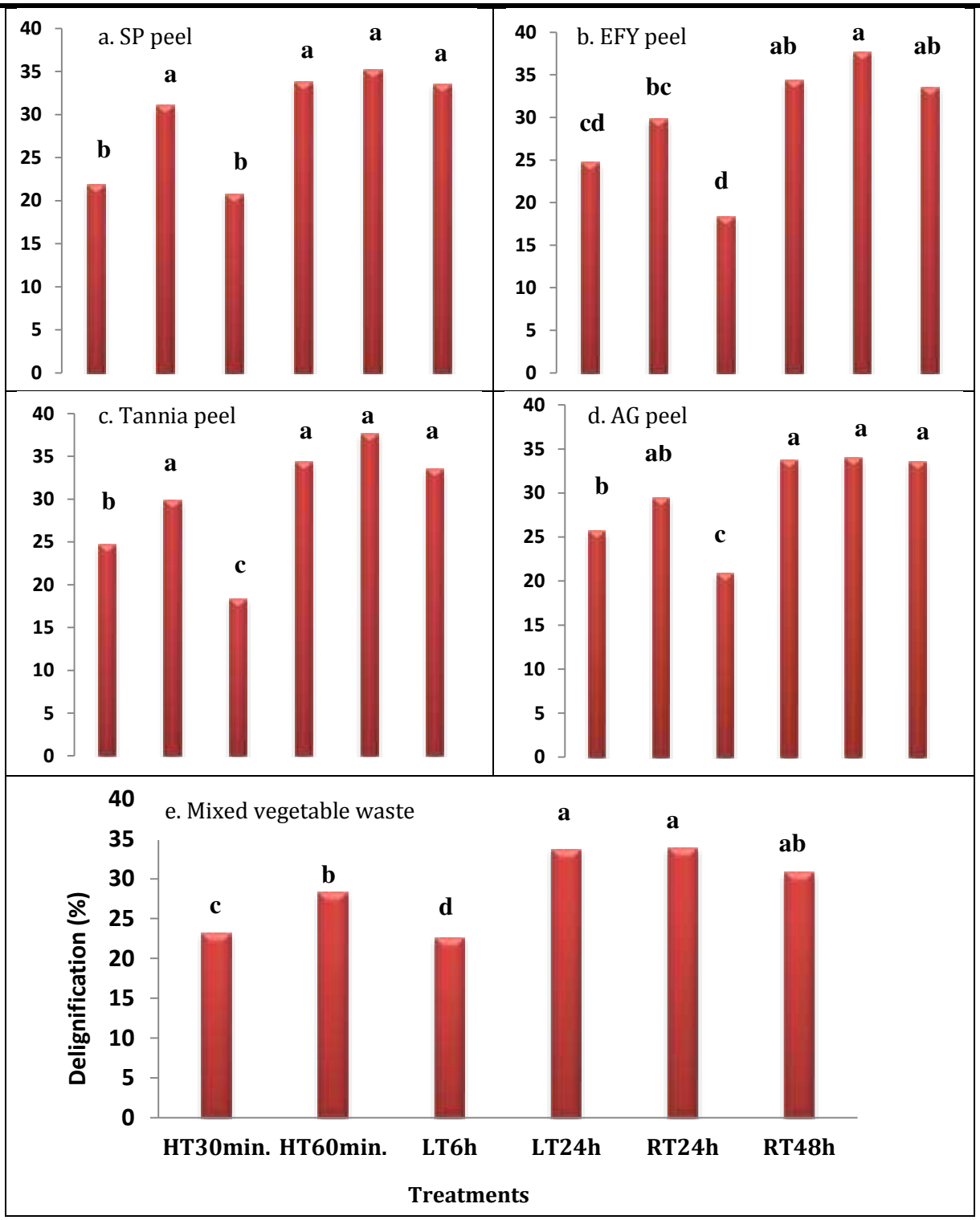

Fig. 2a-e. Delignification in processing residues subjected to lime pretreatment. Statistical comparison was between treatments and bars with different alphabets on the top are significant at $p < 0.05$.

Table 3: Reducing sugar content (g/L) in the pretreated liquor from lime pretreated residues.*

Type of lime pretreatment and time	Sweet potato peel	Elephant foot yam peel	Tannia peel	Ash gourd peel	Mixed vegetable waste
(a) Native biomass without pretreatment [42]					
Initial	6.22^{f}	2.58^{f}	1.34^{f}	5.19^{f}	7.50^{f}

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(b) HT pretreatment (121° C and 0.102 MPa)					
30 min	8.73^{c}	5.18^{c}	1.67^{e}	8.52^{b}	9.52^{c}
60 min	9.20^{b}	4.44^{f}	2.32^{c}	7.72^{e}	9.20^{e}
(c) LT pretreatment (50 ° C)					
6 h	8.19^{e}	4.78^{e}	1.56^{f}	7.65^{f}	9.27^{d}
24 h	9.20^{b}	5.74^{b}	2.60^{b}	7.97^{c}	10.05^{b}
(d) RT pretreatment (30 ±1 °C)					
24 h	9.97^{a}	6.69^{a}	2.70^{a}	9.13^{a}	10.87^{a}
48 h	8.25^{d}	4.98 ^{d}	1.70^{d}	7.82^{d}	8.93^{f}

*Statistical comparison was made for each parameter with the respective values in the original (native) biomass for each sample; means with different alphabets in each column are significant at $p < 0.05$.

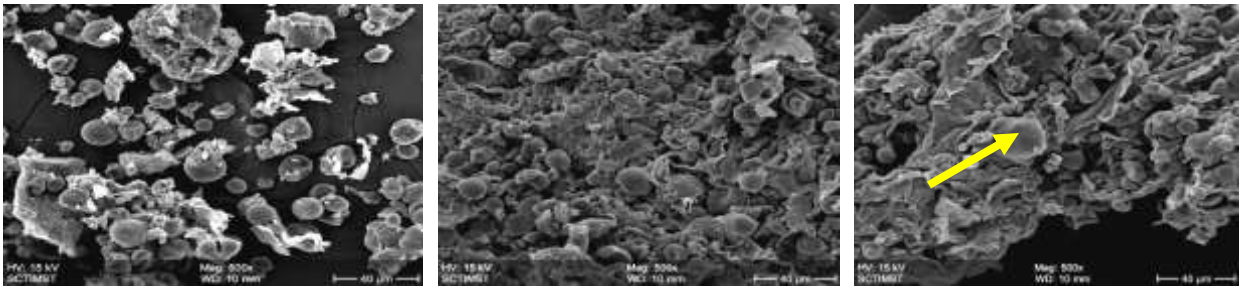
Table 4: Pretreatment Efficiency (%) in sugar release from lime pretreated biomass.*

Type of lime pretreatment and time	**Sweet potato peel**	**Elephant foot yam peel**	**Tannia peel**	**Ash gourd peel**	**Mixed vegetable waste**
(a) HT pretreatment (121 °C and 0.102 MPa)					
30 min.	12.51^{b}	5.21^{b}	6.17^{f}	14.73^{a}	13.53^{b}
60 min.	12.36^{c}	4.22^{d}	7.70^{c}	12.18^{e}	12.17^{d}
(b) LT pretreatment (50 °C)					
6 h	11.18^{e}	4.60^{c}	6.68^{e}	12.53^{d}	11.62^{e}
24 h	11.61^{d}	5.73^{b}	8.65^{a}	11.84^{f}	13.46^{c}
(c) RT pretreatment (30 ± 1 °C)					
24 h	12.67^{a}	7.94^{a}	8.55^{b}	13.72^{b}	14.19^{a}
48 h	11.12^{f}	5.17^{b}	7.22^{d}	12.59^{c}	11.49^{f}

* Computed as given in Methods (Equation 4) based on the potential sugar yielding carbohydrates; means with different alphabets in each column are significant at $p < 0.05$.

3.3 Ultrastructure of Pretreated Biomass

Scanning electron microscopy (x500) was done to understand the ultrastructural changes brought about in the biomass due to lime pretreatment. In the case of peel residues from the three root crops, large number of intact and deformed starch granules was visible (Fig. 3). Starch damage occurring during the grinding and milling operations might have led to alteration in the morphology of starch granules [42]. Broken cell structures were also evident, indicating the absence of rigid fibers in the native biomasses under study, as different from the typical LCBs. Rigid fibrous pattern was earlier reported for cassava leaf and stem powders from our laboratory, while such structures were absent in the peel samples which were dominated by starch [53]. Native ash gourd peel presented a surface morphology with open holes and broken fibers. Such holes normally found on removal of hemicellulose and lignin during pretreatment indicated the possibility of native enzymes which might be acting during the drying time (24 h). Nevertheless, the compositional profile indicated the presence of 18.3% hemicellulose and 10.7 % lignin in ash gourd peel powder with slightly lower starch content (19.9%) than the other residues (28-32%). Mixed vegetable waste also had open pores, with many pores being plugged in by starch granules (Fig. 3m).



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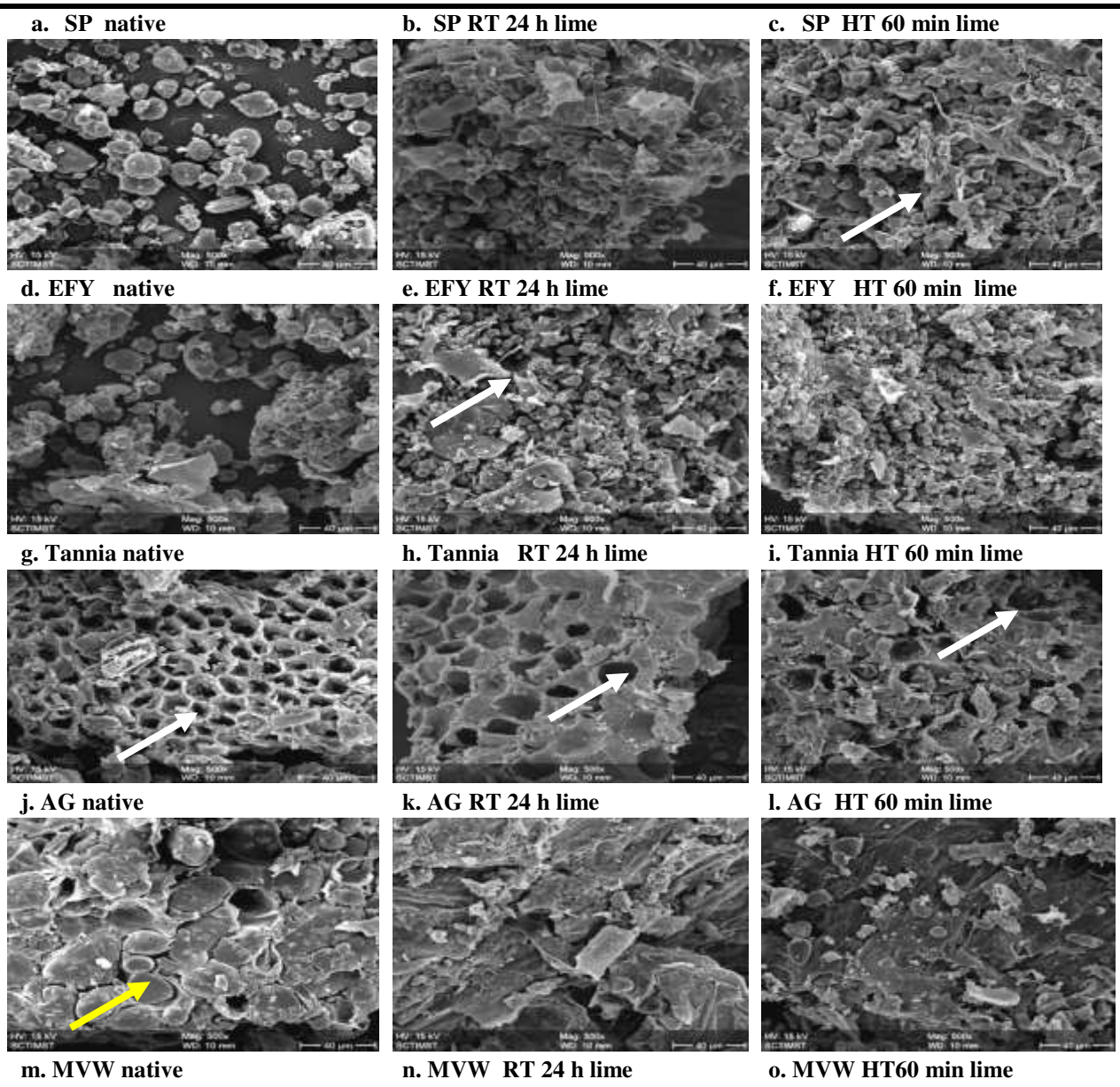

Fig. 3 (a-o): SEM photographs of lime pretreated (RT for 24 h and HT for 60 min.) biomass samples (x500); white arrows indicate the deformed cell pores; yellow arrows indicate the plugging of holes by starch

Lime pretreatment at room temperature resulted in greater distribution of intact and broken starch granules on the surface in the case of the root crop peels (Fig. 3 b, e and h). Apertures resulting from the removal of hemicellulose and lignin as reported in the case of lime (0.5g/g biomass at 55 °C) pretreatment of poplar [29] or for 2.5% potassium hydroxide (KOH) treated sugarcane bagasse [54] or 2.0% KOH pretreated corn cobs [55], were not visible in root crop residues subjected to RT lime pretreatment, probably because of the masking of the pores by the enormous starch granules. Broken fiber particles were evident especially in EFY and tannia peels (Fig. 3 e and h). Ash gourd peel which had several well defined holes in the native biomass, changed to a surface morphology having stretched holes with larger diameter. Besides, some of the pores were sealed by filmy material, which might be partially solubilized hemicellulose/starch. Ash gourd peel was reported to contain *ca.* 8.5% ash, a major part of which was contributed by the chalky wax on the peels. Ghosh and Baghel [56] reported that the wax coating contained pentacyclic triterpene, isomultiferol acetate etc. as major components. Besides a number of methyl pyrazines have been reported from the extracts of the whole fruit (with peel) [56]. The interaction of such compounds with lignin or carbohydrates during lime pretreatment is not understood. The starch plugged cavities seen in the native MVW disappeared on lime pretreatment at room temperature. Largely fragmented or broken fibers were seen with a few starch granules (Fig. 3 m and n). Deacetylation during lime treatment might have

facilitated the deconstruction of cellulose changing to amorphous form, without much change in the absolute content of cellulose. Gelatinized and swollen starch granules were seen in the HT pretreated (60 min.) biomass samples (Fig. 3 c, f, i, l and o). As the gelatinized starch was spread over the surface, broken fiber structures were not very clear, especially in the SEM of root crop peels. In the case of ash gourd peel, open pores were all deformed with coating of gelatinized starch over some of the holes (Fig. 3 l). Swollen starch along with fiber particles were seen in HT pretreated MVW (Fig. 3 o). Scanning electron micrographs of lime pretreated biomass indicated that starch being a major ingredient of the biomass under study, preferential saccharification of starch by amylases might be necessary to expose the cellulose and hemicellulose for their subsequent hydrolysis by cellulases.

IV. CONCLUSION

The present study dealing with a novel approach on the understanding of lime pretreatment effect on starch containing lignocellulosic biomass hitherto not known, showed that RT and HT pretreatments of the biomasses gave high biomass yield coupled with high delignification (34-38% and 29-31% respectively) when compared to the other treatments. Considering the low energy expenditure, slightly higher starch hydrolysis and high lignin removal, these pretreatments are considered the best for the biomasses in the present study. Two clear indications from the compositional and ultrastructural studies were (i) preferential hydrolysis of starch during enzymatic saccharification shall be advantageous, as it exposes the cellulose and hemicellulose for further enzymatic cleavage and (ii) starch swelling in RT pretreatment and gelatinization in HT pretreatment being major changes, whole slurry saccharification would be necessary to get high fermentable sugar yield.

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