

# Microwave-Assisted Alkali Delignification Coupled with Non-Ionic Surfactant Effect on the Fermentable Sugar Yield from Agricultural Residues of Cassava

Pooja N. S, Padmaja G

Division of Crop Utilization, ICAR- Central Tuber Crops Research Institute, Kerala, India

**Abstract**— *Cassava stem, leaves and peel are agricultural residues generated as waste biomass during the cultivation and processing of cassava. The potential of these biomasses as feedstock for ethanol production depends on the effective deconstruction via pretreatment and saccharification. The effect of alkaline hydrogen peroxide (AHP) treatment on microwave (MW)-irradiated or steam-exposed aqueous slurry was compared with MW-irradiation (300 W) of alkali slurry in delignifying the biomass and degrading the polysaccharides. Cellulose was degraded to a higher extent than hemicellulose in the AHP treatments. The steam-exposed and AHP pretreated residues on saccharification with Cellic (Cellulase complex) alone or Cellic along with Tween 20 resulted in high conversion of carbohydrate to reducing sugars (RS) in leaves (64-70%) and peel (74-78%), with slightly lower conversion in stem. MW-irradiation of alkali slurry (5 min.) followed by Tween 20 supplemented saccharification was a better strategy degrading cellulose and hemicellulose to very high extent. Tween 20 supplementation was beneficial in enhancing the RS release from the biomasses even when Cellic dosage was halved. Ultrastructural studies indicated the disappearance of starch granules from stem and peel samples after MW-irradiation and saccharification, while fragmented cellulose fibers were visible in leaf samples. The study showed that MW-assisted alkali pretreatment followed by saccharification with Cellic in presence of Tween 20 was very effective in releasing maximum sugars from these biomasses.*

**Keywords**—*Cassava processing residues, Composition, Microwave-alkali pretreatment, Saccharification, Tween 20, Ultrastructure.*

## I. INTRODUCTION

Lignocellulosic biomass (LCB) comprising wood, agricultural residues and dedicated grasses such as switchgrass, Bermuda grass or *Miscanthus* sp. is considered as the most advantageous feedstock for

biofuel production, owing to the cheap and abundant availability [1]. Nevertheless, the cost effective production is still a major challenge due to the highly recalcitrant nature, and other technological barriers such as high enzyme costs, low conversion rate, generation of saccharification/fermentation inhibitors etc. [2-4]. Lignin contributes to the high recalcitrance, protecting the cellulose and hemicellulose from degradation. Besides its unproductive binding to cellulase making only low quantity of enzyme for hydrolysis, lignin also creates a barrier to the free entry of enzymes for high degree of hydrolysis [3, 5]. Parameters such as cellulose crystallinity, accessible surface area, degree of cellulose polymerization and acetylation of hemicellulose etc. have been reported as major bottlenecks in the successful enzymatic cleavage of polysaccharides to fermentable sugars [1]. Pretreatment aims at breaking down the lignin-hemicellulose matrix and reducing cellulose crystallinity so that it becomes susceptible to enzymatic hydrolysis [6, 7]. It should result in high yield of fermentable sugars after saccharification, reduce operating costs and restrict the formation of fermentation/saccharification inhibitors [6]. Although several pretreatment techniques have been developed during the past three decades such as mechanical, physico-chemical, chemical, biological and organosolv treatments, most of them require severe processing conditions, costly chemicals or sophisticated set up besides low efficiency [4, 6, 7]. Further, the type of pretreatment varies with biomass depending on its composition and hence necessitates optimization for each biomass [8].

Alkaline hydrogen peroxide (AHP) is reported to delignify agricultural biomass and increase its saccharification efficiency and the main effect is solubilisation and separation of lignin from the hemicellulose matrix [9]. Very high fermentable sugar yields have been reported from AHP-pretreated wheat straw, rice hulls and barley straw [10-12]. Alkaline hydrogen peroxide pretreatment has also been suggested

as a promising approach for enhancing the saccharification of rice straw, sugarcane bagasse, corn stover etc. [13-15]. There has been a recent upsurge in interest in the use of microwave irradiation as a feasible option for the pretreatment of biomass and several reviews have appeared on this topic [16-18]. Microwaves interact with both polar molecules and ions in LCBs and produce thermal and non-thermal effects, which help breakdown the biomass in a shorter time compared to conventional heating [19, 20]. Microwave (MW)-assisted alkali pretreatment has been attempted by many researchers as an effective tool to delignify the biomass [21, 22]. Hydrogen peroxide has been reported as an activator of MW irradiation as it degrades easily to water and oxygen [23].

Cassava (*Manihot esculenta* Crantz) is a tropical root crop cultivated in approximately 102 countries of the world for its starchy tubers capable of providing energy [24]. During the harvesting of the crop for human consumption or industrial processing of the roots for starch production, three types of wastes are generated such as peels, leaves and stems (constituting approximately 44% of the total plant biomass) [25]. We had earlier reported the relative content of polysaccharides such as cellulose, hemicellulose and starch in these biomasses and found that the peel contained *ca.* 30% starch, besides 14% cellulose and 23% hemicellulose, while the stem contained 15% starch, 23% cellulose and 28% hemicellulose. Leaves had the least content of starch (2.4%) besides 17% cellulose and 27% hemicellulose [25]. As starch contributes substantially towards the fermentable sugar yield in these residues especially the peel and stem, they require alternative pretreatment and saccharification approaches. Steam pretreatment of moist samples or MW-assisted dilute sulfuric acid pretreatment were earlier reported from our laboratory as effective techniques to enhance the fermentable sugar yield from cassava peel, but not optimal for the other two residues during saccharification with Accellerase [25]. Subsequent studies using another cellulolytic complex, Cellic CTec 2 also showed that very high yield of sugars was possible from peel. Nevertheless, optimum hydrolysis of polysaccharides could not be achieved for the other two biomasses [26].

Non-ionic surfactants such as Tween 20 (polyethylene glycol sorbitan monolaurate) and Tween 80 (polyethylene glycol sorbitan monooleate) have been reported to prevent the non-productive binding of lignin to cellulases [27]. Various effects have been reported for surfactants including alteration in the structure [28, 29], stabilization of enzymes thereby preventing their denaturation [29], positive interaction between substrate and enzymes etc. [27]. Surfactants with high hydrophilic-lipophilic balance

(HLB) such as Tween 20 (HLB 16.7) have been reported to be more effective in extracting hydrophobic lignin degradation products into the soluble phase [30]. Hence, the aim of the present study was to compare the fermentable sugar yield from agricultural residues of cassava in three pretreated systems such as AHP pretreatment on steam-exposed or MW-irradiated biomass slurry as well as MW-assisted alkali pretreatment. The effect of supplementing Tween 20 at the saccharification stage in steam-exposed AHP and MW-assisted alkali pretreatments in enhancing the sugar yield was also studied. The extent of delignification in the various treatments, compositional changes in cellulose and hemicellulose and the ultrastructural alterations brought about in the effective combinations were also studied.

## II. MATERIALS AND METHODS

### 2.1 Samples

Stems and leaves were collected from fully mature and healthy cassava plants (variety: Sree Jaya) grown at the Institute farm. Leaves along with the stalk were separated from the stems and allowed to wilt in the shade for 18 h to facilitate the elimination of cyanoglucosides to the maximum. Stems were chopped to small pieces (*ca.* 5.0 cm long) and both wilted leaves and stems were dried in the sun for 36–48 h. Dry stems and leaves were powdered in a hammer mill to particle size of *ca.* 2-3 mm and the powder was used without further sieving (unscreened) for the study. Peels (skin  $\pm$  rind) were manually separated from the roots, chopped into pieces of *ca.* 2-3 cm length and dried in the sun for 36-48 h. Dry peels were powdered in a hammer mill to particles of similar size as before and stored in airtight bottles until use.

### 2.2 Enzyme Source

Cellic® CTec 2, an improved cellulase enzyme cocktail from M/s Novozymes, Bagsvaerd, Denmark, containing beta-glucosidase as well as xylanase, with reportedly high tolerance to product inhibition was used for saccharification [31]. The optimum temperature and pH of Cellic were standardized in our laboratory on these biomasses and were found to be 50 °C and 5.5 respectively [26].

### 2.3 Pretreatment

#### 2.3.1. Experiment 1: Alkaline hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) pretreatment of steam-exposed/MW-irradiated biomass *MW-irradiated slurry*

Ten percent (w/v) slurry of the dry biomass powders were prepared in distilled water and exposed to MW irradiation at 300 W for 20 min. in a general purpose Microwave Oven (M/s Samsung, Thailand). The equipment had MW radiation at power levels ranging from 100 W to 900 W. The flasks after exposure were treated with 2.5 ml and 5.0

ml 35% H<sub>2</sub>O<sub>2</sub> and pH adjusted to 11.5 with 4M NaOH and volume raised to 100 ml. The microwave power regime was chosen based on our earlier studies [25, 26]. The flasks were then incubated either for 4 h at 50 °C or 24 h at room temperature (30±1 °C) in a thermostatic water bath (M/s Julabo SW22).

#### *Steam-exposed biomass slurry*

The powdered samples (10.0 g each of dry powder of stems, leaves and peels) were moistened with distilled water to raise their moisture contents (MC) to *ca.* 40 % and exposed to steam in a Vegetable Steamer for 30 min. at 100 °C as per the techniques standardized earlier [25]. The steam treated (ST30) slurry (equivalent to 10% w/v original dry biomass) was treated with 2.5 ml 35% H<sub>2</sub>O<sub>2</sub> and the pH was raised to 11.5 using 4M sodium hydroxide (NaOH) and volume raised to 100 ml (Set 1). Another set of flasks containing the same quantity of each biomass along with 5.0 ml H<sub>2</sub>O<sub>2</sub> was pH adjusted to 11.5 and volume raised to 100 ml (Set 2). Two flasks from each set (for each biomass) were incubated for 4 h at 50 °C in a thermostatic water bath while another two sets were incubated at room temperature for 24 h in the water bath.

#### *Compositional analysis*

The samples after the respective pretreatments were first squeezed through a muslin cloth and then through Whatman No. 1 filter paper and the residues were dried in an Oven at 60 °C for 18 h. The dry weight of the pretreated residues was assessed and they were further analysed for the compositional changes such as cellulose, hemicellulose and lignin as per the methods reported earlier [25].

Cellulose content was determined in the residue using acetic-nitric reagent by the method of Updegroff [32]. Hemicellulose content was determined as the difference of Neutral detergent fibre (NDF) and acid detergent fibre (ADF). The ADF and NDF were analyzed as described by Goering and Vansoest [33]. The ash content of the biomasses was determined by the standard procedure [34] and the lignin content of pretreated biomass was calculated as the difference of the sum of cellulose and ash from the acid detergent fiber [25].

#### *Enzyme saccharification*

Based on the compositional analysis, the best pretreatment with regard to lignin reduction was found to be steam pretreatment and hence this alone was carried over to further enzymatic saccharification studies. In the case of stem and leaf samples, 5.0 ml H<sub>2</sub>O<sub>2</sub> for 24 h at RT group gave the pretreated biomass with low lignin content and hence these were selected. Nevertheless, for peel

samples, 2.5 ml H<sub>2</sub>O<sub>2</sub> for 24 h at RT group had the lowest lignin retention and hence this was used.

Steam pretreatment was done as described above and the slurry from each biomass was adjusted to pH 5.5 and equilibrated at 50 °C for 10 min. Twenty milligrams of sodium azide were added to each flask to prevent microbial contamination during incubation. Cellic equivalent to 500 mg enzyme protein was added to each sample and incubated at 50 °C for 120 h (full dose enzyme set, T1).

In another set of flasks, Cellic was added along with 250 mg Tween 20 (T2) to find out the enhancing effect on sugar yield due to the prevention of non-productive binding of lignin to cellulases by the non-ionic surfactant. In a third set of flasks, half the dose of Cellic (250 mg enzyme protein) was added along with 250 mg Tween 20 (T3) to find out whether the enzyme dosage could be reduced in the presence of Tween 20.

All the flasks were incubated for 120 h after which the reducing sugars released in the supernatant fraction was assayed by Nelson-Somogyi method [35] and the composition of the enzyme saccharified residues was further determined to evaluate the extent of retention of cellulose, hemicellulose and lignin.

#### **2.3.2. Experiment 2: MW-assisted alkali pretreatment of biomass and enzymatic saccharification**

This experiment was conducted to find out whether alkali pretreatment alone under MW irradiation was better than initial exposure of aqueous slurry to MW and further pretreatment with H<sub>2</sub>O<sub>2</sub> and alkali (Experiment 1). Preliminary trials showed that there was extensive swelling and volume reduction when alkali slurry was exposed to MW for 20 min. and as free movement of water was necessary for effective MW irradiation, the pretreatment time was reduced to 5 min. at 300 W MW power. Biomass slurry (10% w/v) in 3% NaOH was prepared for each sample and the alkaline slurry was exposed to MW irradiation at 300 W for 5 min.

The slurry was then enzymatically saccharified as described earlier using full dose of Cellic (T1), full dose with Tween 20 (T2) or half dose of Cellic with Tween 20 (T3). The composition of the saccharified residue as well as the RS content in the supernatant was studied.

#### **2.4 Ultrastructural Studies**

The changes in the reorientation/structural alterations of cellulose, hemicellulose and lignin due to MW-assisted alkali pretreatment/enzymatic saccharification (adjudged as the best from the RS values) were studied using Scanning Electron Microscopy (SEM; HITACHI Scanning Electron Microscope Model S- 2400). The dry residues after pretreatment as well as after enzymatic

saccharification were subjected to SEM. The samples were applied on the double side carbon pasted on an aluminium stub. A thin gold-platinum coating (10-15 nm thick) was applied for 3 min. using E-1010 Ion Sputter Unit under 15 kV and 10 Pa vacuum and discharge current of 10 mA and the SEM photographs were visualized at 1500x magnification.

## 2.5 Statistical Analysis

The various biochemical constituents in the pretreated/saccharified residue were expressed as percentage of the original biomass, based on the water insoluble residue weight obtained from each pretreatment. Two replicates were kept for each experiment and duplicate analyses were performed on each replicate. The data were subjected to 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 [36].

## III. RESULTS AND DISCUSSION

### 3.1 Alkaline hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) pretreatment of steam-exposed/MW-irradiated biomass

3.1.1 Structural polysaccharide changes in the AHP pretreated agricultural residues from cassava

Compositional alterations in the structural polysaccharides (cellulose and hemicellulose) consequent

to alkaline hydrogen peroxide (AHP) pretreatment of microwave (MW)-irradiated and steam-exposed biomass (stem, leaves and peels of cassava) are given in **TABLE 1**. AHP pretreatment at higher temperature (50 °C) for 4 h had a significant effect in lowering the cellulose content of MW-irradiated stem and peel at both levels (2.5 and 5.0% v/v) of H<sub>2</sub>O<sub>2</sub>, while in the case of leaves, significant effect was noticed only for 5.0 % level. Both room temperature (RT) exposure for 24 h and high temperature (HT) exposure for 4 h to AHP (5.0% v/v) were not significantly different for stem. Steam-exposed samples when exposed to AHP pretreatment brought about highly significant cellulose removal from cassava peel compared to the other two biomasses (**TABLE 1**) and maximum reduction was observed for T6 (2.5% AHP for 24 h at RT). There was no significant change in hemicellulose content in peel samples subjected to AHP pretreatment after MW-irradiation of aqueous slurry for 20 min. Nevertheless, in the case of stem and leaf samples, significant reduction in hemicellulose was observed for most MW-irradiation treatments. On the contrary, hemicellulose content was significantly reduced for peel samples only when the steam-exposed samples were subjected to AHP treatment (**TABLE 1**). In the case of stem, HT (50 °C) facilitated removal of HC from steam-exposed AHP pretreated samples.

Table.1: Structural polysaccharide changes in pretreated biomass subjected to AHP treatment\*.

Treatments	H <sub>2</sub> O <sub>2</sub> levels (% v/v)	Cellulose content (g/100 g original biomass on dry basis)			Hemicellulose content (g/100g original biomass on dry basis)		
		Stem	Leaves	Peel	Stem	Leaves	Peel
Initial (without any treatment)**		22.8 <sup>a</sup>	17.3 <sup>a</sup>	14.17 <sup>a</sup>	28.8 <sup>a</sup>	27.6 <sup>a</sup>	23.4 <sup>a</sup>
MW-irradiated samples							
T1	2.50	16.90 <sup>c</sup>	16.67 <sup>a</sup>	10.82 <sup>b</sup>	27.10 <sup>ab</sup>	25.33 <sup>b</sup>	23.30 <sup>a</sup>
T2		18.26 <sup>b</sup>	17.35 <sup>a</sup>	12.19 <sup>ab</sup>	26.00 <sup>b</sup>	25.63 <sup>b</sup>	22.63 <sup>a</sup>
T3	5.00	18.00 <sup>b</sup>	15.73 <sup>b</sup>	11.84 <sup>b</sup>	26.50 <sup>b</sup>	24.33 <sup>c</sup>	23.13 <sup>a</sup>
T4		19.08 <sup>b</sup>	16.71 <sup>a</sup>	13.13 <sup>a</sup>	25.30 <sup>c</sup>	25.53 <sup>b</sup>	22.17 <sup>a</sup>
Steam-exposed samples							
T5	2.50	19.77 <sup>b</sup>	15.35 <sup>b</sup>	10.69 <sup>c</sup>	27.00 <sup>b</sup>	27.40 <sup>a</sup>	19.20 <sup>b</sup>
T6		20.91 <sup>ab</sup>	16.85 <sup>a</sup>	9.13 <sup>d</sup>	28.50 <sup>a</sup>	25.37 <sup>b</sup>	18.50 <sup>b</sup>
T7	5.00	22.40 <sup>a</sup>	17.47 <sup>a</sup>	10.22 <sup>c</sup>	26.80 <sup>b</sup>	27.20 <sup>a</sup>	19.20 <sup>b</sup>
T8		21.98 <sup>a</sup>	16.65 <sup>a</sup>	11.66 <sup>b</sup>	28.10 <sup>a</sup>	26.83 <sup>a</sup>	17.20 <sup>c</sup>

\* Treatments T1, T3, T5 and T7 were exposed to AHP for 4 h at 50 °C; T2, T4, T6 and T8 were exposed to AHP for 24 h at room temperature (30±1 °C); \*\* Ref. [26]; statistical comparison was made within each column and values with different superscripts are significant at p < 0.05.

Alkaline peroxide pretreatment is known to decrystallise cellulose [9]. Banerjee et al. [37] reported that AHP pretreatment was beneficial in enhancing lignocelluloses deconstruction at low temperature and atmospheric pressure and had less environmental effect as well.

### 3.1.2 Lignin changes

Highest delignification occurred when steam-exposed stem samples were subjected to AHP treatment using 5.0% v/v H<sub>2</sub>O<sub>2</sub> followed by MW-irradiation and AHP (5.0% v/v H<sub>2</sub>O<sub>2</sub>) treatment (Fig. 1). Nevertheless, lignin changes in cassava leaves subjected to various treatments (T1- T7) were insignificant and significant reduction was noticed only in T8 (5.0% v/v for 24 h at RT). In the case

of peel, delignification was the highest for steam-exposed sample subjected to AHP (2.5% v/v H<sub>2</sub>O<sub>2</sub> for 24 h at RT), which was not significantly different from the MW-irradiated sample (T2). It was found in the present study that steam-exposed samples subjected to AHP treatment had better delignification than MW-irradiated samples. Singh et al. [38] reported that MW-assisted H<sub>2</sub>O<sub>2</sub> treatment of rice straw disrupted the ester linkages between carbohydrates and lignin and helped in efficient delignification. Prolonged reaction time at 30 °C for 24 h was beneficial than 50 °C in eliminating lignin from steam-exposed and AHP pretreated agricultural residues (Fig. 1).

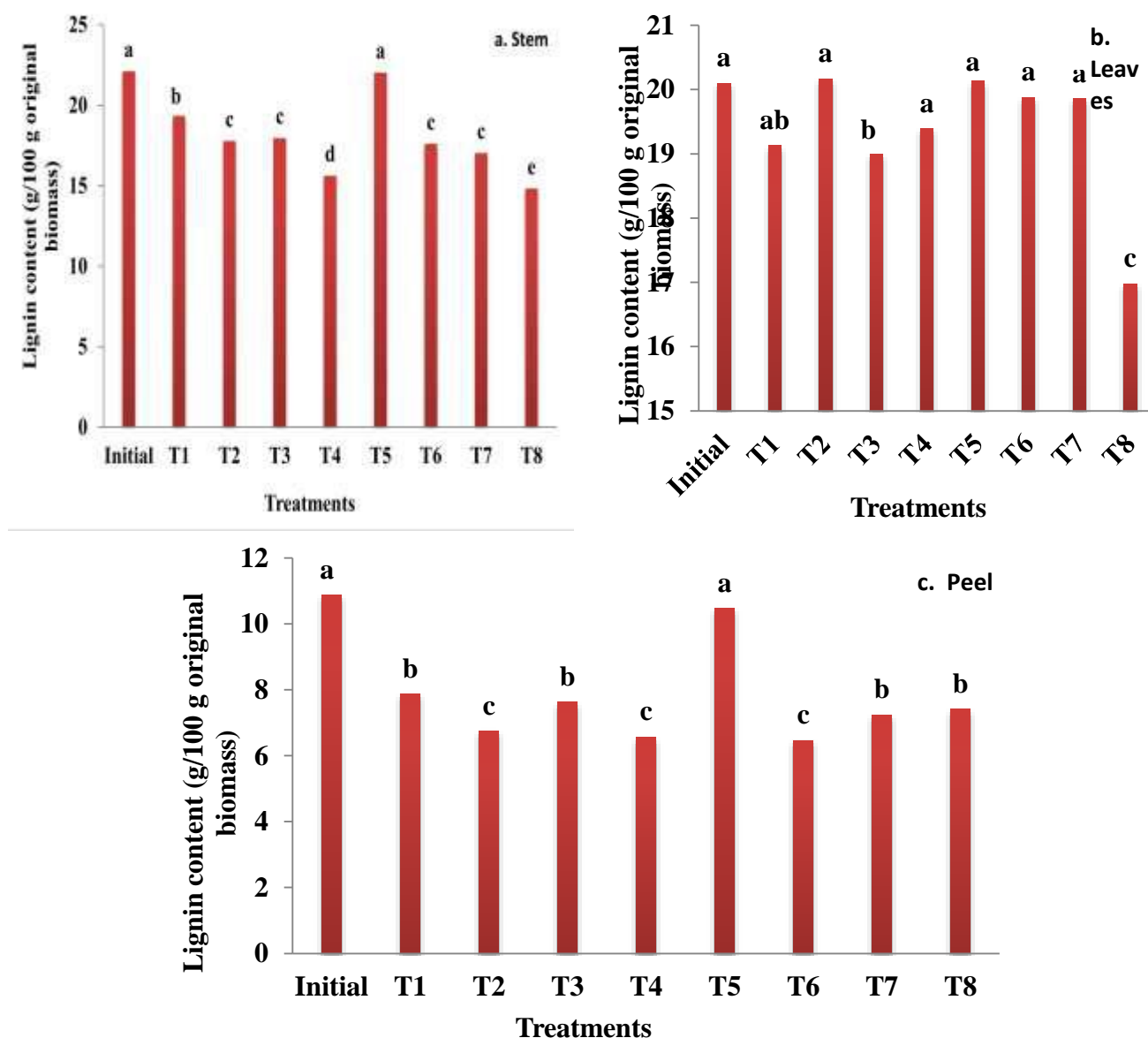


Fig.1 (a-c): Lignin changes in MW-irradiated or steam-exposed biomass subjected to AHP treatment; bars with different alphabets are significant at  $p < 0.05$ .



### 3.1.3 Enzymatic saccharification

#### *Structural polysaccharides in residue after saccharification*

Based on delignification, the steam-exposed biomasses subjected to AHP treatment alone were used further for saccharification. The AHP levels found optimum for cassava stem and leaves were 5.0% v/v and 2.5% v/v for cassava peel and the reaction time was 24 h at room temperature. The effect of Tween 20 supplementation along with full dose of Cellic CTec 2 (T2) or half dose of enzyme (T3) was studied to understand whether enzyme saving was possible in Tween 20 supplemented system. Significant hydrolysis of cellulose occurred after enzymatic saccharification in stem and leaves when Cellic-based system was supplemented with Tween 20. Nevertheless, when Cellic dosage was reduced to half (250 mg enzyme protein/10g biomass), there was significant reduction in cellulose hydrolysis in these two biomasses (TABLE 2).

However, in the case of peel, neither Tween-20 supplementation nor Cellic dosage reduction had any significant effect in cellulose hydrolysis and *ca.* 80-86% cellulose hydrolysis was observed after 120 h saccharification in T1-T3. Hemicellulose was hydrolysed to the extent of 45%, 60% and 63% respectively from stem, leaves and peel, when saccharified with Cellic (500 mg enzyme protein/10g biomass). There was no significant variation in hemicellulose hydrolysis in

cassava leaves, when Tween 20 was supplemented or Cellic dosage was halved, indicating that the effect of Tween 20 was more on enhancing cellulose hydrolysis. Tween 20 supplementation was found to negatively impact hemicellulose hydrolysis in cassava stem and only 25% hydrolysis occurred. In the case of leaves and peel, significant alterations were not noticed due to Tween supplementation. Saha and Cotta [10] also reported that alkaline peroxide pretreated wheat straw could be converted to fermentable sugars with excellent yield. The commercial enzyme preparation Cellic® CTec 2 was reported to contain very high beta-glucosidase activity (*ca.* 34,495 ± 2,935 nkat/g) [39]. The hemicellulase co-activities of cellulase enzyme complex might be remaining unaffected even after Tween 20 supplementation. Karagoz et al. [40] studied the advantage of AHP pretreatment for enhancing bioethanol production from rapeseed straw and found that 5% (v/v) H<sub>2</sub>O<sub>2</sub> at 50 °C for 1 h was the optimal pretreatment condition. They found that as high as 94% of cellulose was digested by enzymes during this pretreatment and saccharification. One of the greatest advantages of AHP pretreatment was that the pretreatment and enzymatic hydrolysis could be performed in the same reactor [41]. They used a lower concentration of H<sub>2</sub>O<sub>2</sub> (1.25%) than the present study and reported that 75% glucose and 71% xylose were released from AHP pretreated corn stover after 48 h enzymatic saccharification.

Table.2: Structural polysaccharide changes after saccharification (120 h) of steam-exposed and AHP pretreated biomass with or without surfactant supplementation\*.

Enzyme treatments	Cellulose content (g/100g original biomass)			Hemicellulose content (g/100g original biomass)		
	Stem	Leaves	Peel	Stem	Leaves	Peel
Cellic alone (T1)	7.90 <sup>b</sup> (65.35)**	5.39 <sup>a</sup> (68.84)	2.04 <sup>a</sup> (85.60)	15.78 <sup>c</sup> (45.21)	10.9 <sup>a</sup> (60.51)	8.61 <sup>b</sup> (63.21)
Cellic+ Tween 20 (T2)	7.03 <sup>b</sup> (69.17)	3.40 <sup>c</sup> (80.35)	2.00 <sup>a</sup> (85.89)	21.48 <sup>b</sup> (25.42)	10.00 <sup>a</sup> (63.77)	8.33 <sup>b</sup> (64.40)
Cellic (half dose) + Tween 20 (T3)	9.30 <sup>a</sup> (59.21)	4.39 <sup>b</sup> (74.62)	2.83 <sup>a</sup> (80.03)	23.80 <sup>a</sup> (17.36)	9.56 <sup>a</sup> (65.36)	11.40 <sup>a</sup> (51.28)

\* Statistical comparison was made within each column and values with different superscripts are significant at  $p < 0.05$ ;

\*\* indicates the percentage decrease from the original value in the respective biomasses.

#### *Residual lignin*

Residual lignin after enzymatic saccharification (expressed as % of the original biomass) indicated significant reduction in levels in the three biomasses (Fig. 2). Except in the case of leaves, saccharified with full dose of Cellic (T4), there were no significant differences in the lignin content among the various enzyme treatments with or without Tween supplementation. Deconstruction of lignocelluloses coupled with the hydrolysis of cellulose and hemicellulose along with the

starch present in stem and peel might have resulted in further release and solubilisation of lignin hydrolytic products into the supernatant. Gould [9] reported the use of alkaline H<sub>2</sub>O<sub>2</sub> (pH 11.5) in delignification of LCBs and the same pH was adopted in the present study as well. Lignin degradation releases soluble phenolics into the supernatant liquid which are reported to be inhibitory to cellulases [42, 43]. Surfactants such as Tween and polyethylene glycol have been reported to reduce the levels of lignin hydrolytic products and enhance

saccharification [27, 44]. Previous studies showed that Tween 20 was highly effective in removing up to 80% phenolics from the prehydrolysates from lignocellulose-starch biomasses, by possible complex formation with them [44].

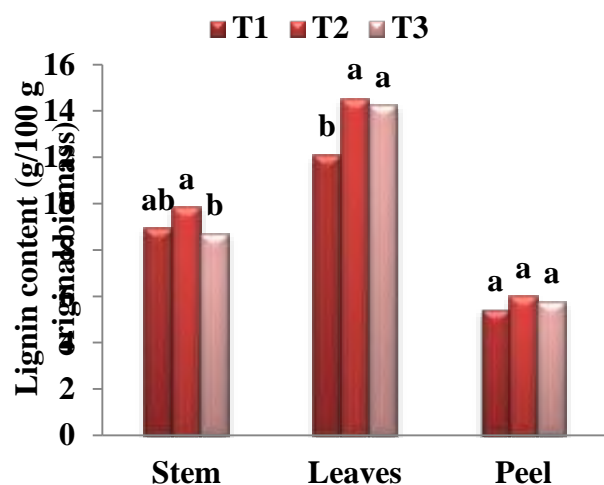


Fig.2: Lignin changes after saccharification (120 h) of steam exposed and AHP pretreated biomass with or without surfactant supplementation; bars with different alphabets are significant at  $p < 0.05$

#### Reducing sugar content and Overall Conversion Efficiency (OCE)

The reducing sugar content in the saccharified mash as given in TABLE 3 indicates that the highest RS release was obtained for cassava peels, which was evidently due to its high total carbohydrate content (71.77 % on dry basis) [25]. Highest RS release was obtained from Tween

20 supplemented system with full dose of Cellic for peel and stem, although there was no difference between full and half dose in the case of leaves. The OCE expressed as percentage conversion of total carbohydrate to RS was also the highest for cassava peel. A higher conversion of carbohydrate to RS was observed for leaves compared to stem and the effect of Tween 20 in enhancing the RS yield was evident for the three biomasses (TABLE 3). Divya Nair et al. [45] found that Tween 20 supplementation during saccharification of steam or dilute acid pretreated cassava starch factory residue significantly enhanced the RS and ethanol yield from it. The beneficial effect of surfactants such as Tween 20 in preventing the non-productive binding of lignin onto cellulase by forming complex with lignin and resulting enhanced saccharification yield has been reported by several researchers [30, 45, 46]. Previous studies indicated that the use of Cellic alone during saccharification of steam-exposed (30 min.) slurry from cassava stem or peel released ca. 38 and 73 g/L RS after 120 h saccharification, while ca. 27 g/L was only released from the leaves [26]. This showed that AHP treatment of steam-exposed slurry was superior to simple steam pretreatment in the case of leaves only. Saha and Cotta [11] reported that the addition of Tween 20 @ 4.3 g/L in AHP treated rice hull hydrolysate was not effective in enhancing the saccharification yield, while Kaar and Holtzaple [29] reported the additive effect of Tween 20. The low OCE obtained for leaves and stem indicated the need to improve the saccharification yield by modifying the pretreatment strategies. Hence the effect of microwave-assisted alkali pretreatment followed by enzymatic saccharification was attempted.

Table.3: Reducing sugar content (g/L) and Overall Conversion Efficiency (%) after saccharification of steam-exposed and AHP pretreated biomass with or without surfactant supplementation

Enzyme treatments	Reducing sugar content (g/L of saccharified mash)			Overall Conversion Efficiency (%)		
	Stem	Leaves	Peel	Stem	Leaves	Peel
Cellic alone (T1)	35.31 <sup>b</sup>	31.74 <sup>b</sup>	53.43 <sup>b</sup>	51.43 <sup>b</sup>	64.21 <sup>b</sup>	74.43 <sup>b</sup>
Cellic+ Tween 20 (T2)	37.74 <sup>a</sup>	34.96 <sup>a</sup>	56.20 <sup>a</sup>	54.96 <sup>a</sup>	70.72 <sup>a</sup>	78.29 <sup>a</sup>
Cellic (half dose) + Tween 20 (T3)	31.56 <sup>c</sup>	34.37 <sup>a</sup>	48.99 <sup>c</sup>	45.96 <sup>c</sup>	69.53 <sup>c</sup>	68.24 <sup>c</sup>

\* Statistical comparison was made within each column and values with different superscripts are significant at  $p < 0.05$ .

### 3.2. Microwave-assisted alkali pretreatment and saccharification

#### 3.2.1 Structural carbohydrates and lignin in saccharified residues

There was significantly higher degradation of cellulose, hemicellulose and lignin in MW-assisted alkali

pretreated biomass saccharified using Cellic compared to steam-exposed and AHP pretreatment followed by saccharification (TABLE 1 vs. TABLE 4). The percentage reduction in cellulose ranged from 90-93% when Cellic alone was used which increased to 91-95% when Tween 20 was also supplemented. Nevertheless,

when Cellic dosage was reduced to half, there was significantly lower hydrolysis. A similar trend was observed in the case of hemicellulose also, although the hydrolysis was less than cellulose. Cellulose and hemicellulose hydrolysis could be significantly improved from both stem and leaves when MW-assisted alkali pretreated residue was saccharified (TABLE 4). Tween 20 supplementation helped to enhance the breakdown of both cellulose and hemicellulose. Even when the Cellic dosage was halved, there was 84-88% hydrolysis of cellulose and 77-85% hydrolysis of hemicellulose from the various residues, whereas the extent of hydrolysis under similar conditions of saccharification in steam-exposed AHP pretreatment was only 59-80% for cellulose and 17-65% for hemicellulose (TABLE 4 vs. TABLE 2). This indicated that MW-assisted alkali pretreatment was superior to steam-exposure followed by AHP treatment of the selected biomasses. Singh et al. [47] observed that alkali concentration, MW-irradiation time and substrate concentration were important for the optimum pretreatment efficiency of rice straw and reported the optimum values as 2.75%, 22.5 min and 30g/L respectively. However, much lower irradiation time (5 min.) and higher substrate concentration (100 g/L) was

used in the present study. Zhu et al. [48] also reported higher fermentable sugar yield from MW (700 W)-assisted alkali (1% NaOH) pretreatment for 25 min followed by enzymatic saccharification of wheat straw, compared to conventional alkali treatment. Nevertheless, as compared to the reported results for wheat or rice straw, very high hydrolysis of both cellulose and hemicellulose was observed in the present study for the biomasses when 3% alkali slurry was subjected to MW-irradiation at 300 W for 5 min. Partial hydrolysis of hemicellulose during pretreatment is reported to result in high levels of xylooligomers in the pretreated liquor, which are strongly inhibitory to cellulase [49]. The very high hydrolysis of cellulose and hemicellulose obtained indicated that the possibility of such inhibition was negligible in the treated biomasses. Zhu et al. [50] obtained 12 times more sugar yield from *Miscanthus* sp. subjected to MW (300 W)-assisted alkali (0.2M NaOH) for approximately 10 min. compared to conventional alkali or dilute acid pretreatment. Budarin et al. [51] reported that 180 °C was the crucial turning point in the MW degradation of cellulose and this temperature is achieved in MW oven at 300 W and the same was used in the present study as well.

Table.4: Structural polysaccharide changes after saccharification (120 h) of MW- assisted alkali pretreated biomass with or without surfactant supplementation\*.

Enzyme treatments	Cellulose content (g/100g original biomass)			Hemicellulose content (g/100g original biomass)		
	Stem	Leaves	Peel	Stem	Leaves	Peel
Cellic alone (T1)	2.37 <sup>a</sup> (89.61)**	1.40 <sup>b</sup> (91.91)	1.02 <sup>a</sup> (92.80)	6.89 <sup>a</sup> (76.08)	3.92 <sup>a</sup> (85.80)	3.85 <sup>a</sup> (83.55)
Cellic+ Tween 20 (T2)	2.05 <sup>a</sup> (91.01)	1.21 <sup>b</sup> (93.01)	0.64 <sup>a</sup> (95.48)	4.80 <sup>b</sup> (83.33)	3.67 <sup>a</sup> (86.70)	3.34 <sup>a</sup> (85.73)
Cellic (half dose) + Tween 20 (T3)	2.73 <sup>a</sup> (88.03)	2.73 <sup>a</sup> (84.22)	1.69 <sup>a</sup> (88.07)	6.73 <sup>a</sup> (76.63)	4.08 <sup>a</sup> (85.22)	3.98 <sup>a</sup> (82.99)

\* statistical comparison was made within each column and values with different superscripts are significant at  $p < 0.05$ ;

\*\* indicates the percentage decrease from the original value in the respective biomasses.

Residual lignin in the three biomasses subjected to MW-assisted alkali pretreatment followed by enzymatic saccharification (120 h) showed that Tween 20 supplementation facilitated the removal of more lignin from the biomass possibly through complex formation with it (Fig. 3). While lignin was removed to a greater extent from MW-assisted alkali treated and saccharified cassava leaf and peel samples compared to AHP treatment (Fig. 2), higher retention was observed in the

respective stem samples treated with either Cellic alone or with half dose of Cellic and Tween 20. High removal of lignin from MW-assisted alkali pretreated biomass has been reported by several researchers [47, 48, 52]. The ester linkages between lignin and hemicellulose are broken down during MW-irradiation which then facilitates rapid hydrolysis of polysaccharides during saccharification.



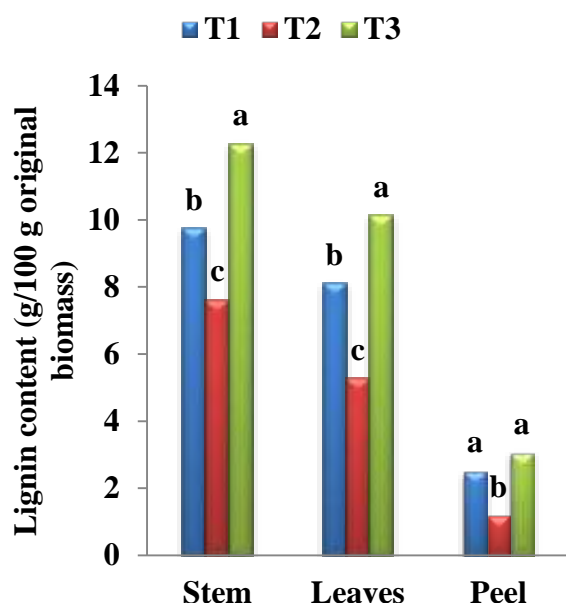


Fig.3: Lignin changes after saccharification (120 h) of MW-assisted alkali pretreated biomass with or without surfactant supplementation; bars with different alphabets are significant at  $p < 0.05$

### 3.2.2 Reducing sugars and Overall Conversion Efficiency

The saccharified mash from MW-assisted alkali pretreated biomass had significantly higher RS content

compared to steam-exposed and AHP pretreated biomass (TABLE 5 and TABLE 3). There was evidently higher release of RS from Tween 20 supplemented system with full dose of Cellic (TABLE 5). Accordingly the OCE was also high for the biomass residues with as high as 82-94% of the potential carbohydrates getting hydrolyzed after 120 h saccharification. Hu and Wen [22] also reported 90% conversion of carbohydrates to sugars when MW-assisted alkali (1% NaOH) pretreated switchgrass was saccharified. Singh et al. [47] observed that MW-assisted alkali pretreated rice hull had low cellulose crystallinity which facilitated its effective hydrolysis during the saccharification step. Lignin hydrolytic products especially low molecular weight phenols have been reported to be inhibitory to cellulases [43] and the effect of Tween 20 in preventing the non-productive binding of cellulases to lignin has been reported [27, 46]. The very high OCE obtained in the present study in Tween supplemented system is evidently due to lignin channeling effect. Haven and Jorgensen [39] found that as high as 65% the  $\beta$ -glucosidase activity in Cellic CTec 2 was adsorbed onto lignin from pretreated wheat straw and such adsorption could be reduced by supplementing the system with bovine serum albumin or polyethylene glycol.

Table.5: Reducing sugar content (g/L) and Overall Conversion Efficiency (%) after saccharification of MW-assisted alkali pretreated biomass with or without surfactant supplementation\*.

Enzyme treatment	Reducing sugar content (g/L of saccharified mash)			Overall Conversion Efficiency (%)		
	Stem	Leaves	Peel	Stem	Leaves	Peel
Cellic alone (T1)	54.12 <sup>b</sup>	41.60 <sup>c</sup>	56.25 <sup>b</sup>	78.82 <sup>b</sup>	84.16 <sup>c</sup>	78.36 <sup>b</sup>
Cellic+ Tween 20 (T2)	57.79 <sup>a</sup>	46.23 <sup>a</sup>	59.55 <sup>a</sup>	84.17 <sup>a</sup>	93.52 <sup>a</sup>	82.95 <sup>a</sup>
Cellic (half dose) + Tween 20 (T3)	53.76 <sup>b</sup>	44.12 <sup>b</sup>	54.97 <sup>b</sup>	78.30 <sup>b</sup>	89.25 <sup>b</sup>	76.57 <sup>c</sup>

\* Statistical comparison was made within each column and values with different superscripts are significant at  $p < 0.05$

### 3.3 Ultrastructural studies

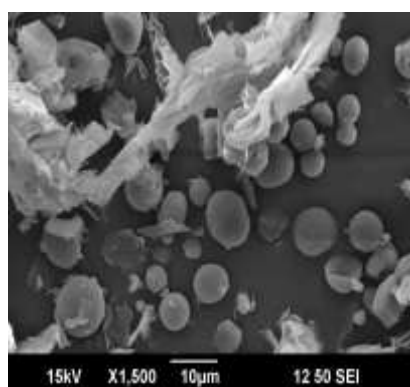
The ultrastructural changes brought about in the three biomass residues due to MW-assisted alkali pretreatment as well as after enzymatic saccharification were compared with the native (untreated) biomass using Scanning electron microscopy. It was found that the native samples of stem and peel had many starch granules with a preponderance of them in peel. Fiber particles could also be seen in the stem samples, which might have got fragmented during the powdering operation. However, intact and rigid cellulose fibrous structure was visible in the cassava leaf (Fig. 4 a, d and g). During MW-assisted

alkali pretreatment, most of the starch granules disappeared from the stem and peel samples and in the case of stem, broken or defibrillated structures could be seen (Fig. 4 b). The surface morphology of the peel samples indicated gelatinized starch coating over fibrous particles due to the preferential hydrolysis of hemicellulose (Fig. 4 h). Nevertheless, in the case of leaf samples, lot of fragmented cellulose fibres were seen, indicating the efficacy of MW-assisted alkali pretreatment in deconstructing the cellulose. Zhu et al. [50] also reported large scale separation of fibers due to lignin removal by the MW-assisted alkali pretreatment. Thin and striated surface morphology was reported in MW-assisted alkali pretreated switchgrass [22]. Microwave

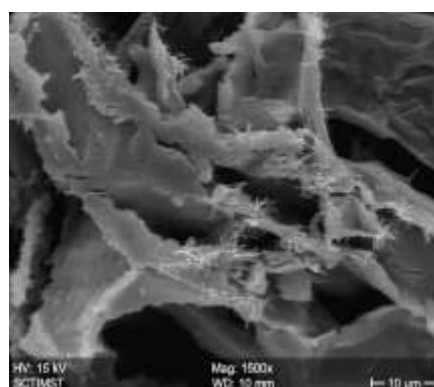
irradiation has been reported to create hot spots within the lignocelluloses matrix leading to an explosive effect on the recalcitrant structure, facilitating its faster disruption than in conventional heating [22].

The saccharified residue from the three biomasses presented a different surface morphology from the pretreated residue. In the case of stem, although starch granules disappeared totally, fragmented cellulose sheaths were visible indicating that the hydrolysis was incomplete

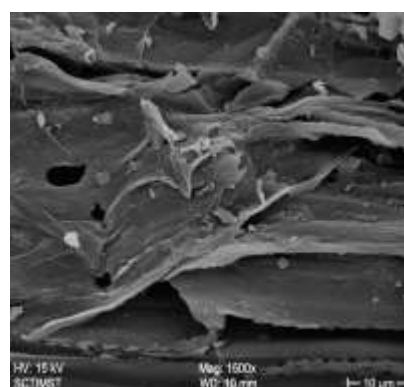
(Fig. 4 c). It was found that *ca.* 84% of potential carbohydrates were only hydrolysed from cassava stem (Table 5). Highly fragmented fiber particles were seen in leaf samples (Fig. 4 f). Although intact starch granules were not seen in saccharified residue from peel, coating of starch over fibrous particles were visible (Fig. 4 i), which stressed the need to incorporate a starch degrading enzyme also into the hydrolytic enzyme cocktail for complete saccharification.



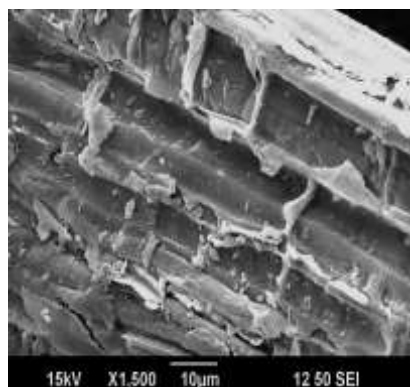
a. Native (untreated) stem



b. MW-assisted alkali pretreated stem



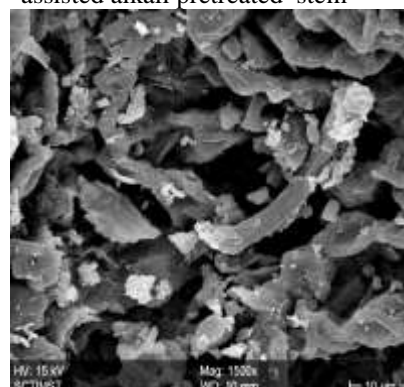
c. Saccharified residue from MW-assisted alkali pretreated stem



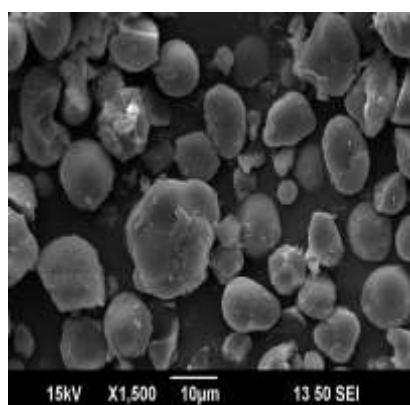
d. Native (untreated) leaf



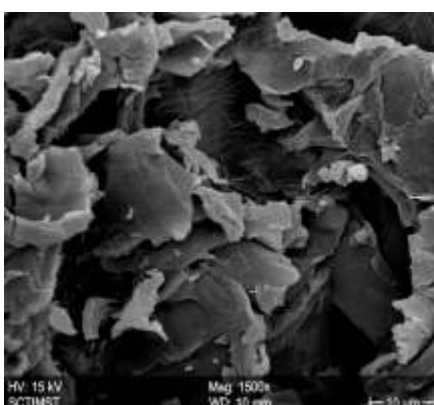
e. MW-assisted alkali pretreated leaf



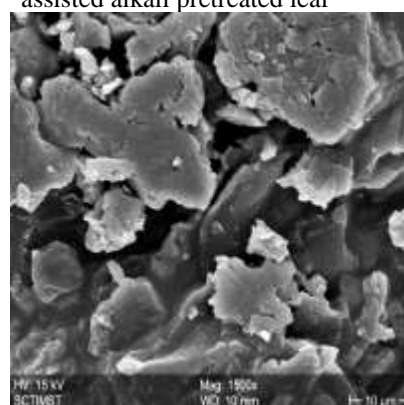
f. Saccharified residue from MW-assisted alkali pretreated leaf



g. Native (untreated) peel



h. MW-assisted alkali pretreated peel



i. Saccharified residue from MW-assisted alkali pretreated peel

Fig. 4 (a-i): SEM photographs (x 1500) of native (untreated) vs. MW-assisted alkali pretreated and enzyme saccharified cassava stem, leaf and peel.

Pooja and Padmaja [26] reported the presence of alpha-amylase existing as a co-activity along with cellulase and  $\beta$ -glucosidase in Cellic CTec 2. However, the levels may not be sufficient to bring about complete hydrolysis of starch. Previous studies on the ultrastructure of steam (30 min.) and MW (300 W)- irradiated (20 min.) samples of stem, leaves and peels showed the highly intact structure of stem with unbroken sheaths of fiber and starch-coated surface morphology for leaves and peel [26]. However, compared to those pretreatments, MW-assisted alkali pretreatment was found to efficiently deconstruct the biomasses leading to higher extent of saccharification.

#### IV. CONCLUSION

The efficacy of alkaline hydrogen peroxide treatment of steam-exposed/MW-irradiated aqueous slurries of cassava stem, leaves and peel was compared with MW-assisted alkali pretreatment in enhancing the biomass deconstruction and saccharification. While cellulose was degraded to a higher extent than hemicellulose in the saccharified residue from AHP pretreatment, both were effectively hydrolyzed in the MW-irradiated alkali slurry subjected to saccharification. Tween 20 supplementation coupled with Cellic enabled higher release of reducing sugars from the three biomasses, due to higher removal of lignin from the MW-irradiated alkali slurry. Disappearance of starch granules consequent to saccharification was evident in the ultrastructural pictures of stem and peel. Very high conversion of potential carbohydrates to reducing sugars in Tween 20 supplemented system from stem (84%), leaves (93.5%) and peel (83%) indicated that MW-assisted alkali pretreatment could effectively deconstruct the polysaccharides enhancing their hydrolysis and releasing maximum quantity of fermentable sugars.

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#### REFERENCES

[1] Wyman, C. E. 1999. "Biomass ethanol: Technical progress, opportunities and commercial challenges".

Annual Review of Energy and the Environment, 24, 189–226.

- [2] Alvira, P, Tomas-Pejo E, Ballesteros, M and Negro, M. J. 2010. "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review". *Bioresource Technology*, 101, 4851-4861.
- [3] Yang, B and Wyman, C. E. 2008. "Pretreatment: The key to unlocking low cost cellulosic ethanol". *Biofuels, Bioproducts and Biorefining*, 2, 26-40.
- [4] Mosier, M, Wyman, C, Dale, B, Elander, R, Lee, Y. Y, Holtzapple, M and Ladisch, M. 2005. "Features of promising technologies for pretreatment of lignocellulosic biomass". *Bioresource Technology*, 96, 673-686.
- [5] Lu, Y, Yang, B, Gregg, D, Saddler, J. N, Mansfield, S. D, 2002. "Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues". *Applied Biochemistry and Biotechnology*, 98–100, 641–654.
- [6] Hendriks, A.T.W.M and Zeeman, G. 2009. "Pretreatments to enhance the digestibility of lignocellulosic biomass". *Bioresource Technology*, 100, 10–18.
- [7] Zhang, B and Shahbazi, A. 2011. "Recent developments in pretreatment technologies for production of lignocellulosic biofuels". *Journal of Petroleum and Environmental Biotechnology*, 2, 2–8/108-115.
- [8] Fengel, D., Wegener, G. 1984. "Wood: Chemistry, Ultrastructure Reactions". W de Gruyter, Berlin
- [9] Gould, J. M. 1984. "Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification". *Biotechnology and Bioengineering*, 26, 46-52.
- [10] Saha, B. C., Cotta, M. A. 2006. "Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw". *Biotechnology Progress*, 22, 449-453.
- [11] Saha, B. C., Cotta, M. A. 2007. "Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to ethanol". *Enzyme and Microbial Technology*, 41, 528-532.
- [12] Saha, B. C., Cotta, M. A. 2010. "Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol". *New Biotechnology*, 27, 10-16.
- [13] Patel, M. M., Bhat, R. M. 1992. "Optimization of the alkaline peroxide pretreatment for the delignification of rice straw and its applications". *Journal of Chemical Technology and Biotechnology*, 53, 253-263.



- [14] Rabelo, S. C., Filho, R. M., Costa, A. C. 2008. "A comparison between lime and alkaline hydrogen peroxide pretreatments of sugarcane bagasse for ethanol production". *Applied Biochemistry and Biotechnology*, 144, 87-100.
- [15] Saha, B. C., Cotta, M. A. 2014. "Alkaline peroxide pretreatment of corn stover for enzymatic saccharification and ethanol production". *Industrial Biotechnology*, 10, 34-41.
- [16] S. M. Laghari. 2015. "Microwave individual and combined pretreatments on lignocellulosic biomasses" *IOSR Journal of Engineering*, 4(2), 14–28.
- [17] Tsubaki S, Sakamoto M, & Azuma J. 2009. "Application of microwave heating for utilization of agricultural biomass". *Research Advances in Agricultural and Food Chemistry* 1:1-12.
- [18] Ethaib, S, Omar R, Kamal, S. M. M, Biak, D. R. A. 2015. "Microwave-assisted pretreatment of lignocellulosic biomass: a review". *Journal of Engineering Science and Technology*, 21, 97 – 109.
- [19] Binod, P, Satyanagalakshimi, K, Sindhu, R, Janu, U, Sukumaran, R K, Pandey, A. 2012: "Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse". *Renewable Energy*, 37, 109–116.
- [20] Ooshima, H, Aso, K, Harano, Y and Yamamoto, T. 1984. "Microwave treatment of cellulosic materials for their enzymatic-hydrolysis". *Biotechnology Letters*, 6(5), 289-294.
- [21] Zhu, S, Wu, Y, Yu, Z, Chen, Q, Wu, G, Yu, F, Wang, C, and Jin, S. 2006. "Microwave assisted alkali pre-treatment of wheat straw and its enzymatic hydrolysis". *Biosystem Engineering*, 94(3), 437-442.
- [22] Hu, Z and Wen, Z. 2008. "Enhancing enzymatic digestibility of switchgrass by microwave-assisted alkali pretreatment". *Journal of Biochemical Engineering*, 38(3), 369-378.
- [23] Okahara, K, Tsubaki, S, Sakamoto, M and Azuma, J. 2008. "Refinery of woody components by microwave irradiation in the presence of hydrogen peroxide", *ASEAN COST+3: New Energy Forum for Sustainable Environment (NEFSE)*, Kyoto, Japan, May 2008.
- [24] FAOSTAT. 2014. Production statistics. <http://faostat.fao.org> Accessed 20 May, 2016.
- [25] Pooja, N. S and Padmaja, G. 2015. "Enhancing the enzymatic saccharification of agricultural and processing residues of cassava through pretreatment techniques". *Waste and Biomass Valorisation*, 6, 303–315. doi: 10.1007/s 12649-015-9345-8
- [26] Pooja, N. S and Padmaja, G. 2015. "Effect of single and sequential cellulolytic enzyme cocktail on the fermentable sugar yield from pretreated agricultural residues of cassava". *American Journal of Biomass and Bioenergy*, 4 (1), 39-53 doi: 10.7726/ajbb.2015.1004
- [27] Eriksson, T, Börjesson, J, Tjerneld, F. 2002. "Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose". *Enzyme and Microbial Technology* 31, 353–364.
- [28] Helle, S. S, Duff, S. J. B, Cooper, D. G. 1993. "Effect of surfactants on cellulose hydrolysis". *Biotechnology and Bioengineering* 42, 611–617.
- [29] Kaar, W. E and Holtzapple, M. T., 1998. "Benefits from Tween during enzymic hydrolysis of corn stover". *Biotechnology and Bioengineering* 59, 419–427.
- [30] Kurakake, M, Ooshima, H, Kato, J, Harano, Y. 1994. "Pretreatment of bagasse by non-ionic surfactant for the enzymatic hydrolysis". *Bioresource Technology*, 49: 247-251.
- [31] Anon. (2014) "Product information on Cellic" available at [www.bioenergy.novozymes.com](http://www.bioenergy.novozymes.com) (23 March, 2014)
- [32] Updegroff. D.M. 1969. "Estimation of cellulose by anthrone reagent". *Analytical Chemistry*, 32, 420-423.
- [33] Goering, H. D, Vansoest, P. J. 1975. "Forage fiber analysis". *USDA Agricultural Research Service, Washington*
- [34] AOAC Official Methods of Analysis of AOAC International, 18th Edition William Horwitz and George W Latimer (eds.). (2005)
- [35] Nelson, N. 1944. "A photometric adaptation of the Somogyi method for determination of glucose". *Journal of Biological Chemistry*, 153, 375–380.
- [36] SAS/STAT Software Version 9.3, SAS Institute Inc., Cary, NC, 2010.
- [37] Banerjee, G, Car, S, Scott-Craig, J. S, Hodge, D. B. and Walton, J. D. 2011. "Alkaline peroxide pretreatment of corn stover: effects of biomass, peroxide, and enzyme loading and composition on yields of glucose and xylose". *Biotechnology for Biofuels* 4, 1-16.
- [38] Singh, R, Tiwari, S, Srivastava, M and Mina, U. 2013. "Effect of combination of microwave and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) pretreatment on enzymatic saccharification of rice straw". *International Journal of Environmental Engineering and Management*. 4(5), 529-542.
- [39] Haven, M. O and Jørgensen, H. 2013. "Adsorption of β-glucosidases in two commercial preparations

- onto pretreated biomass and lignin". *Biotechnology for Biofuels*, 6, 165.
- [40] Karagoz, P, Rocha, I. V, Ozkan, M. and Angelidaki, I. 2012. "Alkaline peroxide pretreatment of rapeseed straw for enhancing bioethanol production by same vessel saccharification and co-fermentation". *Bioresource Technology*, 104, 349-357.
- [41] Banerjee, G, Car, S, Liu, T, Williams, D. L, Meza, S. L, Walton, J. D. and Hodge, D.B. 2012. "Scaling-up and integration of alkaline hydrogen peroxide pretreatment, enzymatic hydrolysis and ethanolic fermentation". *Biotechnology and Bioengineering*, 109, 922-931.
- [42] Ximenes, E. A, Kim, Y, Mosier, N. S, Dien, B. S and Ladisch. M. R. 2010. "Inhibition of cellulases by phenols". *Enzyme and Microbial Technology*, 46, 170-176.
- [43] Tejirian A. and Fu. F. 2011. "Inhibition of enzymatic cellulolysis by phenolic compounds". *Enzyme and Microbial Technology*, 48, 239-247.
- [44] Mithra. M. G and Padmaja. G. 2016. "Phenolic inhibitors of saccharification and fermentation in lignocellulo-starch prehydrolysates and comparative efficacy of detoxification treatments". *Journal of Biomass to Biofuel*, 3, DOI: 10.11159/jbb.2016.001
- [45] Divya Nair, M. P, Padmaja, G, Sajeev, M. S and Sheriff, J. T. 2012. "Bioconversion of cellulo-starch waste from cassava starch industries for ethanol production using pretreatment techniques and improved enzyme systems". *Industrial Biotechnology*, 8, 300-308.
- [46] Alkasrawi, M, Eriksson, T, Borjesson, J, Wingren, Galbe, M, Tjerneld, F and Zacchi, G. 2003. "The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol". *Enzyme and Microbial Technology*, 33, 71-78.
- [47] Singh, A, Tuteja, S, Singh, N and Bishnoi, N. R. 2011. "Enhanced saccharification of rice straw and hull by microwave-alkali pretreatment and lignocellulolytic enzyme production". *Bioresource Technology*, 102, 1773-1782.
- [48] Zhu, S, Wu, Y, Yu, Z, Chen, Q, Wu, G, Yu. F, Wang, C and Jin, S. 2006. "Microwave-assisted alkali pretreatment of wheat straw and its enzymatic hydrolysis". *Process Biochemistry*, 94, 437-442.
- [49] Qing, Q, Yang, B, Wyman, C. E. 2010. "Xylo-oligomers are strong inhibitors of cellulose hydrolysis by enzymes". *Bioresource Technology*, 101, 9624-9630.
- [50] Zhu, Z, Simister, R, Bird, S, McQueen-Mason, S. J, Gomez, L.D and Macquarrie, D. J. 2015. "Microwave assisted acid and alkali pretreatment of *Miscanthus* biomass for biorefineries". *AIMS Bioengineering*, 2(4), 449-468. DOI: 10.3934/bioeng.2015.4.449
- [51] Budarin, V.L, Clark, J.H, Lanigan, B. A, Shuttleworth, P and Macquarrie, D. J. 2010. "Microwave assisted decomposition of cellulose: A new thermochemical route for biomass exploitation". *Bioresource Technology*, 101, 3776-3779.
- [52] Saini, A, Aggarwal N. K, Sharma, A and Yadav, A. 2015. "Prospects for irradiation in cellulosic ethanol production". *Biotechnology Research International* ;DOI.org/10.1155/2015/157139