Effect of Single and Sequential Cellulolytic Enzyme Cocktail on the Fermentable Sugar Yield from Pretreated Agricultural Residues of Cassava

N. S. Pooja and G. Padmaja*

Received 16 October 2014; Published online 27 June 2015

© The author(s) 2015. Published with open access at www.uscip.us

Abstract

Cassava cultivation generates three types of lignocellulosic residues such as stems, leaves and peels. As a part of the investigations on their potential for second generation (2G) bioethanol production, the effect of two cellulolytic enzyme cocktails in enhancing fermentable sugar yield from pretreated (hydrothermal and microwave assisted diluted acid treated) residues was studied. Highest release of reducing sugars was observed from cassava peels, followed by stems, during saccharification for 120 h with Cellic, a multicellulolytic enzyme. Cassava leaves were highly recalcitrant to enzymatic hydrolysis and microwave pretreatment was ineffective. Standardization of enzyme levels showed that 500 mg enzyme protein was adequate for saccharification. Further, sequential saccharification of the pretreated residues using another cellulolytic enzyme, Accellerase for 72 h followed by Cellic for 48 h (at half the optimal dose of both the enzymes) was not advantageous in enhancing the fermentable sugar yield. Two stage process in which the pretreated residues were first saccharified with Accellerase, followed by further hydrolysis by Cellic was also inferior to the single enzyme (Cellic) saccharification. Ultrastructural studies on Cellic treated residues using scanning electron microscopy gave evidence for the hydrolysis of starch almost completely, while fragmented sheaths of cellulose were evident in cassava stems and leaves. The study showed that whilst the carbohydrate in cassava peels could be almost totally saccharified to fermentable sugars, the yield from the other two substrates was not optimal. The presence of higher quantities of lignin in the latter two biomasses compared to peels could be attributed to the lower extent of saccharification.

Keywords: Cassava; Agricultural residues; Pretreatment; Cellulolysis; Fermentable sugars; Ultrastructure

Abbreviations

HT- Hydrothermally treated
MW- Microwave treated

*Corresponding e-mail: padmajabn@yahoo.com
1 Division of Crop Utilization, Central Tuber Crops Research Institute, Thiruvananthapuram-695017, Kerala, India
1. Introduction

Lignocelluloses have widely been recognized as the most suited raw materials for 2G ethanol production due to the large scale availability, low raw material cost and environmentally benign production (Brodeur et al. 2011). Nevertheless, the efficient conversion to ethanol involves three key steps such as pretreatment, enzymatic saccharification and fermentation. Approximately 18% of the total cost of ethanol production from lignocellulosic biomass is contributed by the pretreatment procedures and hence development of low cost technology for overcoming the technological barriers such as recalcitrance of substrates, low extent of bioconversion to fermentable sugars and longer process duration is essential (Mosier et al., 2005; Yang and Wyman, 2008). Structurally, lignocelluloses contain cellulose, hemicelluloses, lignin, extractives and many inorganic materials (Brodeur et al. 2011). The cellulose microfibrils are attached to hemicelluloses, which are amorphous polymers containing pentose and hexose sugars and finally this structure is sealed by lignin, which provides rigidity to plant cell wall. Deconstruction of cellulose from its crystalline nature is essential for maximum fermentable sugar yield and this necessitates the removal of hemicelluloses and lignin through pretreatment, which could then expose the cellulose to the action of cellulases (Chang and Holtzapple, 2000).

Cassava (Manihot esculenta Crantz) is cultivated in approximately 102 countries of the world for its starchy tubers which finds extensive use for food and industrial purposes. Approximately, 50% of the plant biomass is compressed of stems (44%) and leaves (6%), which go as a waste during harvest (Johnson and Raymond, 1965). Cassava is cultivated in India in 0.28 millions hectares, producing 9.62 million tones of tubers (FAOSTAT, 2012) and the dry annual yields of the primary agricultural residues such as stems and leaves have been computed as 2.24 million tonnes, while 0.48 million tonnes of dry cassava peels are also generated. Earlier studies in our laboratory showed that the dry samples of cassava stem had starch (15%), cellulose (22.80%), hemicelluloses (28.8%), total sugars (2.06%), lignin (22.10%), crude protein (3.68%) and ash (1.90%), while the dry leaf powder had starch (2.43%), cellulose (17.30%), hemicelluloses (27.65%), total sugars (2.05%), lignin (20.10%), crude protein (19.96%) and ash (2.50%). The dry powder of cassava peel had starch (29.84%), cellulose (14.17%), hemicelluloses (23.4%), total sugars (4.66%), lignin (10.88%), crude protein (5.29%) and ash (3.70%) (Pooja and Padmaja, 2014). Out of the various pretreatments attempted for these biomasses, the hydrothermal treatment of the powders (40% moisture conditioned) for 30 min or microwave assisted dilute acid pretreatment of 10% (w/v) slurry for 20 min were the most effective in releasing maximum reducing sugars during enzymatic saccharification with Accellerase™ 1000 (a multienzyme cellulolytic complex from M/s Genencor USA) (Pooja and Padmaja, 2014). However, the reducing sugar yields were not optimum with this enzyme system. Hence, in the present study, only these two pretreated biomasses (cassava stems, leaves and peels) were subjected to enzymatic saccharification with a potent cellulolytic enzyme such as Cellic CTec 2 (M/s Novozymes, Bagsvaerd, Denmark), to study the hydrolytic potential of this new enzyme, which is hitherto not reported. Further, the effect of lower concentrations of binary enzyme system such as Accellerase + Cellic in enhancing the enzymatic saccharification rate...
was also studied. Ultrastructural studies using scanning electron microscopy on the residue after enzyme saccharification were made to correlate the extent of biodegradability with enzyme action.

2. 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. Stems chopped to small pieces (Ca. 5.0 cm long) were separately dried in the sun for 36-48 h. Dry stems and wilted leaves were powdered in a hammer mill to particles of size of Ca. 850µm. These were used without further sieving for the study. Peels (skin+ 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 was stored in airtight bottles until use.

2.2. Enzyme Source

Accellerase™ 1000, which is a multienzyme complex with an endoglucanase activity of 2500 CMC U (carboxymethyl cellulose units) per g and β-glucosidase activity of 400 pNPG (p-nitrophenyl β-D-glucopyranoside) units/g was supplied by M/s Genencor International (Palo Alto, CA) (Anon., 2011). Accellerase was also found to possess alpha-amylase activity (890 Units/g) in our earlier studies (Divya Nair et al., 2012). The optimum pH and temperature were found to be 4.5 and 60°C for Accellerase. Cellic® CTec 2 is an improved cellulose enzyme cocktail from M/s Novozymes, Bagsvaerd, Denmark, containing beta-glucosidase as well as xylanase, with reportedly high tolerance to product inhibition (Anon., 2014). The optimum temperature and pH of Cellic were standardized at our laboratory on these biomasses and was found to be 50°C and 5.5 respectively.

2.3. Pretreatment and Enzymatic Saccharification of Biomass

Based on our previous study, two types of pretreatment were selected such as hydrothermal treatment of moisture conditioned biomasses (40% MC) for 30 min and microwave (300 W) assisted dilute sulfuric acid treatment (Pooja and Padmaja, 2014). The moist samples of the three biomasses were spread on a steamer tray with holes and exposed to steam for 30 min in a closed vegetable steamer. After cooling, 10% (w/v) slurry of the HT30 (Hydrothermally treated for 30 min) samples was prepared and subjected to enzymatic saccharification. Parallel samples were used to determine the dry weight of the pretreated samples by the oven drying method, in order to express the results on dry basis (AOAC, 2005). Ten percent slurry of the dry powders of the three biomasses each was prepared in dilute sulfuric acid (0.75% v/v) and subjected to microwave exposure (300W) for 20 min. Four replicates were maintained for each sample. Untreated (native) slurry of the biomasses (control) was also kept to compare the enzymatic saccharification. The pH of the slurries was adjusted to 5.5 and kept in a thermostatic water bath at 50°C (SW 22, M/s Julabo Industries, Germany) for equilibration for 10 min. Five hundred milligram enzyme protein of Cellic and 20 mg sodium azide (antibacterial agent) were added to each sample and after thorough mixing, the samples were incubated at 50°C for 120 h (5 days) with sampling for reducing sugar
determination at every 24 h. The reducing sugars were quantified using arsenomolybdate method (Nelson, 1944).

2.4. Standardizing the Level of Cellic using Pretreated Biomass

Hydrothermally treated cassava stem, leaves and peels as well as the 20 min microwave (300 W) exposed residues were used for the optimization studies. The pH of 10% (w/v) slurry was adjusted to 5.5 and after adding 20.0 mg sodium azide equilibrated for 10 min. Two protein concentrations (lower and higher to 500 mg already reported above) of Cellic such as 400 mg and 600 mg were added and the mixed slurries were incubated for 72 h at 50°C, with sampling for reducing sugar determination at 24, 48 and 72 h. In the case of the HT30 samples, the change in dry weight due to hydration with steam was accounted by determining the dry weight of a parallel HT30 sample, in order to facilitate the expression of the results on dry weight basis.

2.5. Effect of Sequential Enzyme Cocktails in Enhancing Fermentable Sugar Yield

Previous studies showed that Accellerase possessed endo-glucanase and β-glucosidase activities along with alpha-amylase activity (Divya Nair et al., 2012). Nevertheless, the enzyme was not very effective in hydrolyzing the pretreated residues of cassava stems, leaves and peels (Pooja and Padmaja, 2014). Cellic contained both β-glucosidase and xylanase activities [Anon., 2014] and was found to release higher amounts of sugars in the present study at same enzyme protein levels of 500 mg/10% (w/v) slurry (Results and Discussion Section). Hence, it was thought worthwhile to investigate the possibility of further maximizing the reducing sugar yield using these enzyme cocktails at half the protein loading (250 mg enzyme protein each).

Ten percent slurry (w/v) of HT30 and MW20 as well as non treated (control) samples of the three biomasses was prepared in distilled water and after adjusting the pH to 4.5, 20 mg sodium azide was added, mixed and equilibrated in a thermostatic water bath at 60°C for 10 min. Accellerase (250 mg enzyme protein) was added and incubated at 60°C for 72 h. Reducing sugars were quantified as described earlier (Nelson, 1944).

The pH of the slurries was then raised to 5.5 and temperature brought down to 50°C. Cellic (250 mg enzyme protein) added, mixed and incubated further for 48 h. Final reducing sugar yield was quantified and the clear supernatant was subjected to HPLC analysis. The dry residue yield after the combined enzyme saccharification was also determined.

In a second experiment, the slurry after the Accellerase action was centrifuged at 8000 rpm for 20 min and the clear supernatant was kept aside for reducing sugar quantification. The residue was reconstituted with the same amount of water and after adjusting the pH to 5.5, the temperature was brought to 50°C in a thermostatic water bath. Cellic (250 mg protein) was added and incubation continued for 48 h. The final reducing sugar and residue yields were quantified as described earlier.

2.6. Ultrastructural Studies

The changes in the reorientation/structural alterations of molecules such as cellulose, hemicelluloses and lignin due to enzymatic saccharification by Cellic were studied using scanning
electron microscopy (SEM). The powdered samples were mounted on to brass stubs using double-sided carbon conductive adhesive tape. A gold coating (10-15 nm thick) was then applied using a JFC 1600 magnetron sputtering unit (JEOL, Tokyo, Japan) with 10 mA current for 80 sec. Bulk samples were examined at 10 kV and 1 Pa vacuum using a JSM 6390 LV SEM (JEOL, Tokyo, Japan). Residue from the single and binary enzyme saccharification systems was powdered and the fine powder was subjected to scanning electron microscopy (SEM). In the case of the sequential enzyme cocktails, only the control and the most effective combinations (HT samples) were subjected to SEM studies.

2.7. Statistical Analysis

The data reported were the mean of four replicates and were analyzed using the statistical package SAS 9.3 to perform ANOVA (SAS, 2010). The treatments were considered statistically significant at 5% level (p ≤ 0.05). The mean comparisons were made by the Duncan’s Multiple Range Test (DMRT).

3. Results and Discussion

Pretreatment of lignocellulosic biomass is designed to deconstruct the recalcitrant molecules which facilitate the reduction in crystallinity of cellulose, increase in solubility of hemicelluloses and lignin and increase in the pore size of the substrate to enhance the rate of enzymes and thereby increase the fermentable sugar yield from them (Galbe and Zacchi, 2007). Earlier studies in our laboratory showed that hydrothermal treatment (30 min) of moisture conditioned agricultural residues of cassava such as stem, leaves and peels as well as microwave assisted (300 W., 20 min) dilute acid treatment of the residues were highly effective in enhancing the fermentable sugar yield from them during saccharification with Accellerase (Pooja and Padmaja, 2014). Nevertheless, complete conversion of carbohydrate to sugars could not be achieved with this enzyme. Hence, in the present study a new commercial enzyme cocktail, Cellic C Tec 2 from M/s Novozymes was tried on the pretreated biomasses. The optimum pH and temperature of the enzyme were found to be 5.5 and 50°C.

3.1. Effect of Cellic on Pretreated Biomass

The comparative production of reducing sugars from stem leaves and peels at time intervals from 24 to 120 h is presented in Table 1. It was found that at each sampling period, significantly higher quantities of reducing sugars were released from HT30 and MW20 samples of cassava stem and peels. However, significantly higher reducing sugar release was observed in the case of HT30 leaf sample only, while MW20 leaf did not significantly differ from the control at all sampling periods. Further, the hydrolysis proceeded slowly in the case of pretreated cassava stems and approximately 3 units of sugar only were additionally released during 24-120 h. In the case of control (non-treated) stems 8.3 g reducing sugars were additionally released during the enzymatic saccharification period of 24-120 h. This is contrary to the results obtained earlier on cassava stems using Accellerase, where higher reducing release was noticed during 24-120 h for pretreated cassava stems (Pooja and Padmaja, 2014). Nevertheless, the amount of reducing sugars was much higher during enzymatic saccharification of control as well as pretreated cassava stems by Cellic, compared to Accellerase. This indicated that Cellic is capable of bringing about appreciable
hydrolysis at the initial periods of saccharification of cassava stems. The total carbohydrate content (starch + cellulose + hemicelluloses + sugars) of cassava stems was found to be 68.60% (Pooja and Padmaja, 2014), of which only 38.12% reducing sugars were released after 120 h saccharification of HT30 stems with Cellic. This indicated that there existed further scope for enhancing the fermentable sugar yield from cassava using other pretreatment stems using other pretreatment techniques as well as through the use of hybrid enzyme saccharification steps involving various enzymes. Significantly lower yields of reducing sugars were obtained from MW20 cassava stems (Table 1).

Microwave irradiation of dilute acid slurry (10% w/v) of pretreated cassava leaves could not produce any significant effect compared to the non-treated control (Table 1). This indicated the highly recalcitrant nature of the leaf samples, which was reported earlier using Accellerase as well (Pooja and Padmaja, 2014).

Table 1 Release of reducing sugars from pretreated biomasses by Cellic CTec2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>96h</th>
<th>120h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cassava stems</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.51&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.93&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT30</td>
<td>35.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW20</td>
<td>30.86&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cassava leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.64&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT30</td>
<td>24.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW20</td>
<td>18.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.97&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.92&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cassava peels</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.46&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT30</td>
<td>45.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW20</td>
<td>37.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Statistical comparison was made between treatments for each sample; values with different superscripts in each column for each sample are significantly different.

Hydrothermal treatment for 30 min could however significantly enhance the saccharification rate, although only 27 g reducing sugars were released after 120 h. It was reported earlier that the reducing sugars release was only 17.4% after 120 h from HT30 cassava leaves using Accellerase (Pooja and Padmaja, 2014). Nevertheless, compared to the total carbohydrate content of 49% in dry cassava leaves, the reducing sugar yield after saccharification with Cellic was also low, indicating the need for further modifications in the pretreatment/saccharification steps.

As compared to cassava stems and leaves, the peels were found to be hydrolysed to a very high extent during saccharification with Cellic. The final reducing sugar yield after 120 h was 72.7% (on dry basis) for HT30 sample, while it was 66.6% for MW20 samples. This was significantly higher than the earlier reported values of 57.6% (HT30) and 47.36% (MW20) samples using Accellerase.
Total carbohydrate content of dry cassava peels was 71.77% (Pooja and Padmaja, 2014) and hence almost complete degradation of carbohydrate to sugars could be achieved in HT30 cassava peels. Since cassava peels also contained approximately 30% starch, this meant that the Cellic enzyme cocktail might also possess α-amylase as a co-activity, as reported earlier in the case of Accellerase (Divya Nair et al., 2012). When this activity was tested using pure potato starch as substrate, it was found that the enzyme preparation possessed approximately 1757 Units of alpha-amylase activity as a co-activity in the multienzyme complex (1 unit of activity = mg glucose released per 100 ml enzyme preparation under the conditions of assay), besides β-glucosidase and xylanase as reported by the manufacturers. Hence, a part of the starch in the pretreated cassava peels also might be getting hydrolyzed along with the cellulose and this has contributed to the high glucose release during enzymatic saccharification. MW20 samples of cassava peels were degraded to a less extent that HT30 samples and only 66.6% reducing sugars were released.

Several workers have used hot compressed water to enhance the removal of hemicelluloses and facilitate higher extent of cellulolysis. (Cara et al., 2007; Laser et al., 2002; Zhang et al., 2010). Lei et al. (2013) used hydrothermal treatment of prairie cord grass at 170-210°C under pressure for varying periods and reported that the most efficient pretreatment condition was 210°C for 10 min. Water at high temperatures has been reported to lead to the autohydrolysis of lignocellulosic linkages in presence of H+ ions (Akiya and Savage, 2002). Whilst Duff and Murray [1996] reported optimal solubilization of hemicelluloses at 270°C for 1 min in steam explosion, Lei et al. (2013) observed that formation of fermentation inhibitors such as furfural and hydroxymethyl furfural (HMF) from prairie cord grass was more when temperature was 218°C for 10 min. We have used only steam exposure (120°C) of the powdered moisture conditioned (MC 40%) biomasses for 30 min and found that this technique was most effective in the case of cassava peels resulting in optimum enzymatic saccharifiation with Cellic. Thompson et al. (1992) observed that the pore size of the substrate compared to the size of the enzyme is one of the decisive factors for maximum saccharification. Removal of hemicelluloses during hydrothermal treatment could alter the structural features of the substrates, thereby increasing the pore size (Gregg and Saddler, 1996; Grethlein, 1985; Palonen et al., 2004) Drying of the pretreated biomass could cause a collapse in the pore structure leading to a low enzymatic saccharification rate (Grous et al., 1986) Hence, we had used the hydrothermally treated samples directly without drying and this might have resulted in a very high reducing sugar yield from HT30 samples of cassava peels. However, the pretreatment cassava stems and leaves could not be saccharified to the optimal extent in this study.

Compositional analysis of the three biomass residues showed that whilst cassava stems and leaves contained 22 and 20% respectively of lignin, the peels contained only 10.9% (Pooja and Padmaja, 2014). The presence of high lignin in the former two biomasses and its consequent solubilization and presence in the liquid slurry might also have led to the lower enzymatic saccharification of these biomasses. Loss of lignin during pretreatment is reported as one of the important indicators of the efficiency of pretreatment as lignin leads to the non-productive binding of cellulases to it, resulting in only lower quantity of enzyme for saccharification (Mosier et al., 2005; Sun and Cheng, 2002). Alkasrawi et al. (2003) and Eriksson et al. (2002) reported that the addition of non-ionic surfactant Tween 20 to saccharification medium could prevent the non-productive binding of lignin to cellulase, by preferentially adsorbing to lignin surface. Saccharification of cassava fibrous residues, obtained after the extraction of starch, was reported to be enhanced in pressure of Tween
20 (Divya Nair et al., 2012). Biomasses such as cassava stems and leaves with very high lignin content might also be requiring such modified systems for optimal enzyme saccharification, which is presently under study in our laboratory.

3.2. Standardizing the Level of Cellic using Pretreated Biomass

The comparative release of reducing sugars during 24-72 h from the three biomass residues by the two enzyme concentrations of Cellic such as 400mg and 600mg is presented in Fig.1 (a-b). It was found as in the case of the previous experiments that cassava leaves were the most recalcitrant substrate and least release of reducing sugars was observed during saccharification. It was found that significantly higher saccharification occurred at 24 h, when 600mg enzyme protein was used for cassava stems, while the values were higher for 500 mg treatment for the other two biomasses (Fig. 1 a vs Table 1). However, as the incubation period was prolonged, significantly higher release of reducing sugars was observed in the case of 500 mg enzyme protein for cassava stems and peels, while the values did not differ significantly between 500 and 600mg protein in the case of cassava leaves. It could be therefore concluded from the study that the optimum saccharification of the biomasses was possible with the economic dose of 500 mg enzyme protein.

![Fig.1 a. Release of reducing sugars from pretreated cassava stems by two enzyme concentrations (400 mg and 600mg); C– Cellic](image-url)
3.3. Effect of Sequential Enzyme Cocktails on Enzymatic Saccharification

Use of Cellic enzyme cocktail alone for enzymatic saccharification was found to result in incomplete saccharification of pretreated cassava stems and leaves even after 120 h. Hence, the effect of sequential enzyme saccharification using the two enzyme cocktails such as Accellerase and Cellic at half their optimal protein loading rate viz., 250 mg for 10% (w/v) slurry was studied. In this study, the pretreated biomasses were first subjected to saccharification with Accellerase under its optimal operating conditions such as pH 4.5 and 60°C for 72 h. The pH was then raised to 5.5 and temperature brought down to 50°C and saccharification with Cellic was continued up to 120 h. It
was found that the sequential loading of Accellerase and Cellic was not effective in enhancing the reducing sugar yield from these biomasses.

Approximately 23% reducing sugars only were released from HT30 cassava stems, while the corresponding yield with Cellic alone was 38% (Table 1 vs Table 2) and Accellerase alone was 31.5% (Pooja and Padmaja, 2014). Much lower values were obtained in the case of HT30 leaves and HT30 peels, indicating that this mode of saccharification was not effective to enhance the hydrolysis, when compared to saccharification by Cellic alone.

### 3.4. Effect of Two Stage Process using Accellerase and Cellic on Reducing Sugar Yield

In this study, the two enzymes were loaded separately by first saccharifying the pretreated biomasses for 72 h with Accellerase (half the optimal dose). The saccharified mash was centrifuged and the reducing sugar was quantified in the supernatant. The residue was reconstituted with distilled water to 10% (w/v) slurry, and subjected to saccharification with Cellic (half the optimal dose) for another 48 h, under its optimal operating conditions of pH 5.5 and 50°C. The supernatant after 120 h saccharification was also analyzed for reducing sugar yield. It was observed that appreciable hydrolysis of cellulose and/or starch occurred during the first phase using Accellerase. During the second phase of 48 h using Cellic, only small quantities of reducing sugars were observed from the residue (Table 3).

#### Table 2 Effect of sequential enzyme loading on the release of reducing sugars from pretreated biomasses

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Reducing sugars released (% on dry matter basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 h</td>
</tr>
<tr>
<td>Stem</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.44c</td>
</tr>
<tr>
<td>HT30</td>
<td>16.09b</td>
</tr>
<tr>
<td>MW20</td>
<td>17.32a</td>
</tr>
<tr>
<td>Leaves</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.33c</td>
</tr>
<tr>
<td>HT30</td>
<td>9.60a</td>
</tr>
<tr>
<td>MW20</td>
<td>7.74b</td>
</tr>
<tr>
<td>Peels</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.07c</td>
</tr>
<tr>
<td>HT30</td>
<td>32.20a</td>
</tr>
<tr>
<td>MW20</td>
<td>29.29b</td>
</tr>
</tbody>
</table>

Statistical comparison was made between treatments for each sample; values with different superscripts in each column are significantly different.

The two stage process was better than the sequential process, in which Cellic was added to the Accellerase saccharified slurry (Table 3 vs Table 2). The total reducing sugar yield from the control as well as pretreated biomasses was significantly lower in the sequential as well as two step process when compared to the system in which Cellic alone was used. This indicated that neither
the synergistic action of Accellerase and Cellic nor the two step action of these enzymes, even though at half the loading rate was not beneficial in enhancing the sugar yield. Besides, Cellic is reported to have high tolerance to product inhibition (Anon., 2013) and this might have contributed to the very high sugar yield especially from cassava peels.

3.5. Ultrastructural Studies

Ultrastructural studies were made on the dry samples of the three types of residues as well as the Cellic treated residues from the most effective pretreatments such as hydrothermal and microwave assisted acid pretreated residue. Scanning electron microscopic pictures (x 500) of the dry samples of stem, leaves and peels indicated the presence of starch granules in cassava stems and peels, with a preponderance in the latter biomass (Fig. 2a and 2c). Most of the granules had diffused morphology in cassava peels, which might have occurred during the physical disruption during grinding. Stem also had fibrous sheaths of cellulose, besides starch granules. Cassava leaves clearly showed the presence of fibrillar cellulose matrix, with only few diffused starch granules, which might have contributed to its high recalcitrance (Fig. 2b).

**Table 3** Effect of two stage process with Accellerase and Cellic on the release of reducing sugars from pretreated cassava biomasses

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Reducing sugars released (% on dry matter basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 h*</td>
</tr>
<tr>
<td>Stem</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.63&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT30</td>
<td>15.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW20</td>
<td>15.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leaves</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT30</td>
<td>9.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW20</td>
<td>7.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peels</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.30&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT30</td>
<td>31.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW20</td>
<td>25.63&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* 72 h refers to the quantity of sugars formed in the slurry by Accellerase; 120h refers to the sugars formed from the residue when subjected to hydrolysis by Cellic; Total quantity from the two enzymes as the added value of Column 2 and 3; Other footnotes as in Table 1.

Cellic mediated saccharification of control (non-treated) cassava stems resulted in the disappearance of the starch granules and only long sheaths of cellulose fibre matrix was visible (Fig. 2d). The pattern obtained for cassava leaves after Cellic treatment was also almost similar, with diffused amorphous masses (Fig. 2e). Nevertheless, SEM pictures (x 500) of Cellic treated cassava peels showed the presence of only a few starch granules, along with amorphous mass of starch and deconstructed cellulose fibres. This indicated that appreciable hydrolysis of starch occurred in the control peels during Cellic action (Fig. 2f).
Hydrothermal and microwave irradiation of dilute acid slurry, being the most effective pretreatments, these samples after Cellic action were also subjected to SEM. Cellulose sheaths were clearly visible in the Cellic treated (120 h) cassava stems, indicating that deconstruction and hydrolysis of cellulose fibre in the stem samples was not fully effective and the increased reducing sugar observed in the HT30 and MW20 samples after Cellic action (Table 1) might have primarily resulted from the hydrolysis of starch by the amylase, existing as a coactivity in Cellic (Fig. 2g and 2j). HT30 and MW20 cassava leaves showed the presence of diffused mass along with few sheaths in the former and uniformly distributed diffused mass in the latter (Fig. 2h and 2k). Compositional analysis had earlier indicated only a low content of starch (2.43%) in cassava leaves, compared to 15 and 29.54% respectively in stem and peels (Pooja and Padmaja, 2014). Hence, it is possible that the enhanced sugar yield from HT30 and MW20 cassava leaves might have resulted from the hemicellulose breakdown during pretreatment as well as from the partial cellulolytic saccharification. Total carbohydrate comprising starch, cellulose, hemicelluloses and sugars was only 49.43% in cassava leaves and 68.66% in stems (Pooja and Padmaja, 2014). This indicated that there was further scope for the release of additional quantities of reducing sugars (∼ 25-30 g) from these biomasses, necessitating further improvement in pretreatments or enzyme action.
Fig. 2(a-l). SEM (x 500) pictures of Control and pretreated biomasses after Cellic treatment compared with the respective dry samples

SEM pictures (x 500) of Cellic treated cassava peels (HT30 and MW20) shows complete disappearance of starch granules, unlike in the control peels after Cellic action (Fig. 2i and 2l). Fragmented cellulose sheaths were visible in the Cellic treated HT30 cassava peels, which might be due to its exposure resulting from the almost complete hydrolysis of starch. Nevertheless, gelatinized starch covering probably the fibre sheaths was visible in the MW20 cassava peels, indicating that the hydrolysis of starch was not complete in this sample. The theoretically possible yield of reducing sugars from the starch was 33.12% (ie. 29.84 x 1.11) and this along with the total sugars in the cassava peels (4.66%) could make up to only 37.78%, while as high as 72.66% was obtained from HT30 cassava peels (Table 1) and this indicated effective saccharification of both cellulose and starch and this was evident from the SEM data as well.

4. Conclusions

This study showed that out of the various enzymes systems studied such as Cellic alone, sequential enzyme loading of Accellerase and Cellic as well as two step saccharification with Accellerase and Cellic, the former was the most effective in enhancing the reducing sugar yield from pretreated cassava residues. Whilst pretreated cassava peels could be saccharified to a very high extent by Cellic (72.7% on dry weight basis) reaching almost to the level of the theoretically possible yield, only 38.12% and 27.35% sugars were released respectively from stems and leaves indicating the need for further research to optimize the pretreatment and saccharification conditions for these residues. Presence of high level of lignin (20-22%) in the cassava stems and leaves, as evidenced from our earlier studies might also be an impediment in their optimum saccharification, in comparison to only 10.9% in cassava peels. Ultrastuructural studies also provided corroborative evidence for the high degree of saccharification of cassava peels.

Acknowledgements

The first author acknowledges the research fellowship granted for the study by the Kerala State Council for Science, Technology and Environment (KSCSTE), Govt. of Kerala. Authors are thankful to the Director for the facilities provided and Dr. J. Sreekumar, Senior Scientist, CTCRI for the help
extended in statistical analyses. The help extended by the Sree Chitra Institute of Medical Sciences & Technology (SCTIMST), Thiruvananthapuram, Kerala for the SEM studies is gratefully acknowledged.

References

http://dx.doi.org/10.1021/cr000668w

http://dx.doi.org/10.1016/S0141-0229(03)00087-5


http://dx.doi.org/10.1016/j.procbio.2007.03.012

http://dx.doi.org/10.1385/ABAB:84-86:1-9:5

http://dx.doi.org/10.1089/ind.2012.0007

http://dx.doi.org/10.1016/0960-8524(95)00122-0

http://dx.doi.org/10.1016/S0141-0229(02)00134-5


http://dx.doi.org/10.1007/s00253-002-1058-9

http://dx.doi.org/10.1007/BF02941753

http://dx.doi.org/10.1016/0141-0229(86)90021-9


http://dx.doi.org/10.1016/S0960-8524(01)00103-1

http://dx.doi.org/10.4236/jsbs.2013.34034

http://dx.doi.org/10.1016/j.biortech.2004.06.025


http://dx.doi.org/10.1385/ABAB:117:1:01


http://dx.doi.org/10.1016/S0960-8524(01)00212-7

http://dx.doi.org/10.1016/0960-8524(92)90135-K

http://dx.doi.org/10.1002/bbb.49

http://dx.doi.org/10.1007/s10295-010-0847-x