Journal of Root Crops, 2017, Vol. 43 No. 2, pp. 84-94
Indian Society for Root Crops
ISSN 0378-2409, ISSN 2454-9053 (online)

# Bioethanol Production from Steam-Pretreated Agricultural Residues of Cassava using Separate Hydrolysis and Fermentation (SHF)

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Received: 1 December 2017; Accepted: 28 December 2017

## Abstract

Agricultural residues of cassava (*Manihot esculenta* Crantz) such as stems and peels contain high amount of starch in addition to cellulose, hemicelluloses and lignin and hence appropriate pretreatment and enzymatic hydrolysis strategies are to be designed for optimal fermentable sugar and ethanol yields. The potential of three steam-pretreated cassava residues (stems, leaves and peels) for bioethanol production was investigated by separate hydrolysis and fermentation (SHF). It was found that out of three enzyme cocktails such as Cellic + Stargen, Cellic + Stargen + Optimash XL and Cellic + Stargen + Optimash BG (C + S + OBG), the highest reducing sugar (RS) release was obtained from the latter system for all the residues. Maximum RS yield was obtained from cassava peels (66.43 g $l^{-1}$) followed by stems (32.18 g $l^{-1}$) using the latter enzyme cocktail. Glucose was the predominant sugar present in the hydrolysates. Sugar consumption during fermentation (48 h) using *Saccharomyces cerevisiae* was also the highest for cassava peels (47.54 g $l^{-1}$). The highest ethanol yield of 21.68 g $l^{-1}$ was obtained from peels in C + S + OBG system, while the yields from stems and leaves were 12.92 and 6.17 g $l^{-1}$ respectively. Although the highest fermentation efficiency of 91.59% was obtained from C + S + OBG saccharified stems, volumetric ethanol productivity and ethanol recovery were the highest from peels (0.452 g $l^{-1}$ $h^{-1}$ and 264.36 ml $kg^{-1}$ dry biomass), while ethanol recovery was 157.52 and 75.24 ml $kg^{-1}$ respectively from stems and leaves. The study showed that among the residues, cassava peels had the highest potential as feedstock for bioethanol production.

**Key words:** Cassava residues, steam pretreatment, separate hydrolysis, fermentation, bioethanol

## Introduction

Lignocellulosic biomass (LCB) comprising woody substrates, agricultural residues and dedicated grasses such as switchgrass, Bermuda grass or *Miscanthus* sp., municipal solid wastes etc. is considered as the most advantageous feedstock for biofuel production, owing to the cheap and abundant availability as well as renewability (Wyman, 1999). However there are several technological barriers such as highly recalcitrant nature of substrate necessitating costly pretreatment procedures, formation of inhibitory products of saccharification and fermentation during pretreatment etc. that affect the cost effective production of ethanol (Alvira et al., 2010; Mosier et al., 2005; Yang and Wyman, 2008). Pretreatment aims at the reduction in cellulose crystallinity by breaking down the hemicellulose-lignin matrix so that it becomes more accessible to hydrolytic enzymes (Mosier et al., 2005). Several reviews have appeared on the pretreatment procedures for the effective degradation of lignocellulosic biomasses for biofuel production (Hendriks and Zeeman, 2009; Mosier et al., 2005; Sun and Cheng, 2002; Yang and Wyman, 2008). Apart from pretreatment, two other key processes in the production of bioethanol from LCBs are enzymatic saccharification resulting in the conversion of carbohydrates into sugars and fermentation of reducing sugars into ethanol using either single or complement of microorganisms. Integration of these processes is important for the economic production of ethanol. The

final ethanol yield depends on the sugar composition of the enzyme saccharified mash, efficiency of the fermenting organisms to ferment the various types of monomeric sugars and also on the content of fermentation inhibitors in the mash. Low fermentation efficiency results from low sugar content of the hydrolysate besides presence of toxic inhibitors which affects the enzyme saccharification and growth of microbes. Furfural and 5-hydroxymethyl furfural (HMF) could reduce ethanol yields by decreasing the activities of many yeast enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase (Modig et al., 2002). Lignin degradation products (phenolic compounds) exert greater inhibition than furfural or HMF even at very low concentrations ( $< 100$ mg $l^{-1}$) and they cause partition and loss of integrity of biological membranes in yeast, thereby affecting their capacity to act as enzyme matrices (Parajó et al., 1998).

Cassava (*Manihot esculenta* Crantz) is a popular root crop cultivated in almost 102 countries, meeting the hunger needs of approximately 500 million people. It is grown globally in an area of 23.867 million hectares, producing 268.28 million tonnes, with a productivity of 11.24 t $ha^{-1}$ (FAOSTAT, 2014) and the starchy roots are reported to meet approximately 6% of the world's dietary energy. It is cultivated in India in 0.228 million hectares with a total production of 8.14 million tonnes (FAOSTAT, 2014). The mature cassava plant contains 50% roots (comprising *ca.* 11% water, 8% peelings and 31% starch), 44% stems and 6% leaves (Johnson and Raymond, 1965). The primary agricultural residues from cassava include stems and leaves and the secondary processing waste includes cassava peels. The above-ground parts of cassava such as stems and leaves are not economically utilized and only 10-20% of stems are further needed for replanting (Ahamefule, 2005; Pattiya et al., 2007). Kosugi et al. (2009) reported that the non-food parts of cassava could play a significant role in the production of energy, because of the huge volume of biomass. Cassava processing generates peels as waste biomass accounting for 10-15% of the fresh weight of roots. These residues are poisonous due to the high content of cyanoglucosides (Guo et al., 2008) and rarely find use as feed materials and could thus be exploited for bioethanol production. Kongkiattikajorn (2012) reported that cassava peels contained 35.86% cellulose, 9.27% hemicelluloses, 12.52% lignin and 15.82% starch and hence could be used for ethanol production. Previous studies by Pooja and Padmaja (2015 a) showed that dry cassava peels contained *ca.* 30% starch, besides 14% cellulose and 23% hemicellulose, while the stems contained 15% starch, 23% cellulose and 28% hemicellulose. Cassava leaves had the least content of starch (2.4%) besides 17% cellulose and 27% hemicellulose. Steam pretreatment of moist samples or microwave-assisted dilute sulphuric acid pretreatment were earlier reported to enhance the fermentable sugar yield from cassava peels, but not much effective for the other two residues during saccharification with Accellerase (Pooja and Padmaja, 2015 a). Saccharification with another cellulolytic complex, Cellic CTec2 gave very high sugar yields from peels, while optimum hydrolysis of polysaccharides could still not be achieved for the other two biomasses using this enzyme (Pooja and Padmaja, 2015 b). Hence the present study aims at the use of enzyme cocktails containing cellulase, xylanase and starch hydrolysing enzyme, Stargen in order to enhance the fermentable sugar yield from these biomass residues.

The enzyme saccharified mash from lignocellulosic biomass generally contains a complement of sugars such as glucose, mannose, arabinose, xylose and galactose along with small amounts of unhydrolyzed oligosaccharides (Katahira et al., 2006). There are a number of fermentation strategies which are currently employed that include (i) separate hydrolysis and fermentation (SHF) (ii) simultaneous saccharification and fermentation (SSF) (iii) simultaneous saccharification and co-fermentation (SSCF), SHF using co-culture of organisms, hybrid-SSF etc. (Buruiana et al., 2013; Taherzadeh and Karimi, 2007). Although there are some reports on the production of ethanol from cassava peels using SSF method (Godson et al., 2015; Kongkiattikajorn and Sornvoraweat, 2011), the potential of stems and leaves as biomass feedstock has been scarcely studied. The objective of the present study was to compare the ethanol yields from steam-pretreated cassava stems, leaves and peels by adopting the separate hydrolysis and fermentation technique. As the biomass residues contained hemicellulose and starch in high levels, certain enzymes such as Stargen, Optimash BG and Optimash XL other than cellulase were also used in this study.

## Materials and Methods

### Samples

Stems and leaves were collected from healthy and mature 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 with the aim of reducing the cyanoglucoside levels and further dried in the sun for 24 h. Stems were chopped to small pieces (*ca.* 5.0 cm long) and separately dried in the sun for 36-48 h. Dry stems and leaves were separately powdered in a hammer mill to particles of size of *ca.* 850 µm. Peels (skin + rind) were manually separated from the roots and chopped into pieces of *ca.* 2-3 cm length. These were further dried in the sun for 36-48 h. Dry peels were powdered in a hammer mill to particles of similar size as before and were stored in airtight bottles until use. The unscreened residue samples were used for further studies in the case of all the residues.

### Pretreatment of biomass

The powdered samples (cassava stems, leaves and peels) were moistened with distilled water (*ca.* 12 ml/20 g) to raise their moisture contents (MC) to *ca.* 40%. The moisture content of the wet samples was determined by the oven drying method (AOAC, 2005). Steam-pretreated cassava stems, leaves and peels (ST30) were prepared by exposing moist (40% MC) residues (20 g) to steam at 100 ºC for 30 min in a Vegetable Steamer (M/s Prestige India Ltd., India) and directly used without drying for the study.

### Enzymes used

Cellic®CTec2 which was the major enzyme used for the study, was provided by M/s Novozymes, Bagsvaerd, Denmark and the enzyme cocktail contained cellulase, β-glucosidase as well as xylanase, with reportedly high tolerance to product inhibition (Anon., 2014). The optimum temperature and pH of Cellic were standardized on these biomasses as 50°C and 5.5 respectively. Stargen™002 contained *Aspergillus kawachi* α-amylase (E.C. 3.2.1.1) expressed in *Trichoderma reesei* and a glucoamylase (E.C. 3.2.1.3) from *Trichoderma reesei* which work synergistically to hydrolyse granular starch substrate to glucose. As per the manufacturers, it has an activity of 570 glucoamylase units (GAU) per gram, and one GAU is the amount of enzyme that will liberate 1 g of reducing sugars (as glucose) per hour from soluble starch substrate under the conditions of the assay (Anon., 2009 a). The three supplementary enzymes such as Optimash™BG, Optimash™XL and Stargen™002 were provided by M/s Genencor International Inc. USA (presently Genencor-Danisco, Beloit, WI, USA). Optimash BG is a combination of β-glucanase and xylanase and hydrolyses cellobiose and hemicelluloses respectively during saccharification. It is produced by the submerged fermentation using a genetically modified *Trichoderma reesei* and is reported to have a pH and temperature optima of 4.0-4.5 and 60-70 ºC respectively, although these could vary depending on the type of substrates (Anon., 2009 b). Optimash BG has an activity of 10300 Carboxymethyl cellulase units/g (CMC U/g) and a crude protein content of 94.6 g $l^{-1}$ (Anon., 2009 b). Optimash XL is a combination of endoxylanase and hemicellulase with xylanase activity of 1290 XAU/ml and crude protein content of 144 g $l^{-1}$ (Anon., 2009 b).

### Enzymatic saccharifcation of steam-pretreated biomass

Enzymatic saccharification was conducted under different modes such as binary or triple enzyme cocktails on steam-pretreated biomass.

#### Saccharification with sequential application of Cellic and Stargen

The steam-pretreated biomass (20 g original weight) after adjusting the pH to 5.5 and increasing the volume to 200 ml in 250 ml Erlenmeyer flasks was used for the saccharification study. The system was supplemented with sodium azide (50 mg) as antimicrobial agent in each case. The flasks after equilibration for 10 min at 50ºC were treated with cellic (1.0 g enzyme protein/200 ml) and incubated for 72 h. The pH and temperature were then brought down to 4.5 and 40ºC respectively and Stargen (0.20 ml equivalent to *ca.* 44 mg enzyme protein) was added. Incubation was continued for another 48 h making the total incubation time to 120 h. Three replicates were kept for each biomass and sampling for reducing sugar determination was done at 120 h using arsenomolybdate reagent (Nelson, 1944).

#### Saccharification using Cellic+ Optimash BG followed by Stargen

In this experiment, the pretreated slurries after pH adjustment and volume increase up to 200 ml were equilibrated for 10 min at 50ºC and treated with Cellic (1.0 g enzyme protein/200 ml) and Optimash BG (1.0 ml equivalent to 94.6 mg enzyme protein) and incubation continued up to 72 h. The pH and temperature were then brought down to 4.5 and 40ºC respectively and Stargen (0.20 ml equivalent to *ca.* 44 mg enzyme protein) was added. Incubation was continued for another 48 h making the total incubation time to 120 h.

### Saccharification using Cellic+ Optimash XL followed by Stargen

The pretreated slurries after pH adjustment and volume increased up to 200 ml were equilibrated for 10 min at 50ºC and treated with Cellic (1.0 g enzyme protein/200 ml) and Optimash XL (1.0 ml equivalent to 144 mg enzyme protein) and incubation continued up to 72 h. The pH and temperature were then brought down to 4.5 and 40ºC respectively and Stargen (0.20 ml equivalent to *ca.* 44 mg enzyme protein) was added. Incubation was continued up to 120 h.

### Reducing sugar content and characterization of the hydrolysates

The total reducing sugar content of the enzymatic hydrolysates from the three sets of experiments was determined using arsenomolybdate method (Nelson, 1944). Enzyme blanks as well as substrate blanks were kept during the assay of RS in order to nullify the interference from sugars already present in the commercial enzyme samples and original biomass respectively.

Although three different enzyme cocktails were used, the combination Cellic + Stargen + Optimash BG (CSOBG) gave the highest RS yields from all the residues and hence this alone was carried over for the characterization of sugars using HPLC. The enzyme saccharified mash was centrifuged at 8000 rpm to obtain clear hydrolysates. At the time of analysis, the clear hydrolysates were again filtered through 0.20 µm Millipore filters. Monomeric sugars were identified and quantified using HPLC (M/s Shimadzu, Kyoto, Japan) under an isocratic mode and the conditions were: Column: SUPELCOSIL LC-$NH_2$ (250 x 4.6 mm), mobile phase: acetonitrile:water (75:25), flow rate: 1.0 ml/min; column temperature: ambient (30 ± 1ºC); RID-10 A refractive index detector; sample injection volume: 20 µl and run time: 30 min.

### Ethanol fermentation using *S. cerevisiae*

Fermentation experiments were conducted using enzymatic hydrolysates in 250 ml Erlenmeyer flasks. The enzyme saccharified mash from each of the above experiments was adjusted to pH 4.5 using 1 M HCl and temperature brought down to 30 ± 1ºC and squeezed through muslin cloth to remove the unhydrolysed residue. The filtrate was again centrifuged at 8000 rpm for 10 min to obtain clear hydrolysate which was used in fermentation studies.

### Activation of yeast

20 g dry granulated Baker's yeast (*S. cerevisiae*) was suspended in 100 ml solution containing 10 g sucrose and kept in a water bath at 37ºC for 1 h. Ten millilitres of yeast suspension were used for 200 ml of saccharified mash.

### Fermentation

Steam-pretreated and saccharified hydrolysates (200 ml) were taken in 250 ml Erlenmeyer flasks and 200 mg urea were added to it as nitrogen source. A mineral mix having $MgSO_4.7H_2O$ (100 mg), $CaCl_2.7H_2O$ (20 mg) and $FeCl_3.2H_2O$ (20 mg) was added and mixed well. Each flask was inoculated with 10 ml yeast suspension and after thorough mixing, the flasks were closed with aluminium foil and allowed to ferment for 48 h at room temperature (30 ± 1 ºC). Ethanol content was determined in the fermented liquor after 24 h and 48 h of fermentation as per the spectrophotometric method of Caputi et al. (1968) using potassium dichromate reagent.

### Calculation for yield parameters

The various parameters related to ethanol fermentation were computed based on the following formulae (Barcelos et al., 2011; Pereira et al., 2015; Yadav et al., 2011).

Initial sugar concentration in the hydrolysate = S1 (g/L) [1]

Residual sugar concentration in the fermented broth = S2 (g/L) [2]

$$\text{Percentage of sugar utilization} = \frac{[S1 - S2] \times 100}{S1} \quad [3]$$

Ethanol yield ($Y_E$) is g ethanol produced /g reducing sugar consumed during fermentation

$$Y_E = \frac{\text{Ethanol concentration (g/L) in fermented broth (Ef) x 1}}{\text{Sugar consumed (g/L)}}$$

$$= \frac{\text{Ethanol concentration (g/L) in fermented broth (Ef) x 1}}{\text{[S1-S2] (g/L)}} \quad [4]$$

$$\text{Fermentation Efficiency (\%)} = \frac{\text{Ethanol yield } (Y_E) \times 100}{\text{Theoretical ethanol yield}}$$

$$= \frac{Y_E \times 100}{0.511 \text{g/g sugar}} \quad [5]$$

Volumetric ethanol productivity (g/L/h)

$$= \frac{\text{Ethanol concentration (g/L) in fermented broth}}{\text{Fermentation time (h)}} \quad [6]$$

Ethanol yield per kg biomass

Ethanol concentration (g/L) in fermented broth = Ef

Weight of dry biomass in one litre slurry = W1 g

$$\text{Yield of ethanol (g/ kg) from dry biomass} = \frac{\text{Ef x 1000g}}{\text{W1}} = \text{W2}$$

$$\text{Ethanol yield (ml) from one kg biomass} = \frac{\text{W2 x 1ml}}{0.82} \quad [7]$$

(0.82 g ethanol is equal to 1.0 ml ethanol)

### Statistical analysis

Three 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 (2010).

## Results and Discussion

Three modes of enzyme loading for the saccharification of steam-pretreated agricultural residues of cassava (stems, leaves and peels) were adopted in the studies on separate hydrolysis and fermentation. These included (i) saccharification with Cellic (72 h) followed by Stargen (48 h) (ii) saccharification with Cellic + Optimash BG (72 h) followed by Stargen (48 h) and (iii) saccharification with Cellic + Optimash XL (72 h) followed by Stargen (48 h). The enzyme hydrolysates from the three experiments were fermented uniformly for a period of 48 h using *Saccharomyces cerevisiae*.

### Changes in RS during saccharification and fermentation

The results on the initial reducing sugar content (g $l^{-1}$) in the enzyme hydrolysate and residual sugar (g $l^{-1}$) after the fermentation period are presented for each of the three biomasses in Fig. 1-3. Among the three residues, the highest saccharification yield was obtained for cassava peels saccharified with the three modes of enzyme application and among the enzyme modes, the highest RS content (66.43 g $l^{-1}$) was obtained for peels saccharified using a complement of Cellic + Stargen + Optimash BG (Fig. 3). Lowest RS release was obtained for cassava leaves where the saccharified liquor had only 13-21 g $l^{-1}$ RS in the different systems (Fig. 2).

In the case of all the three biomass residues, Cellic + Optimash BG + Stargen gave the highest saccharification yield (Fig. 1-3). The content of residual reducing sugars

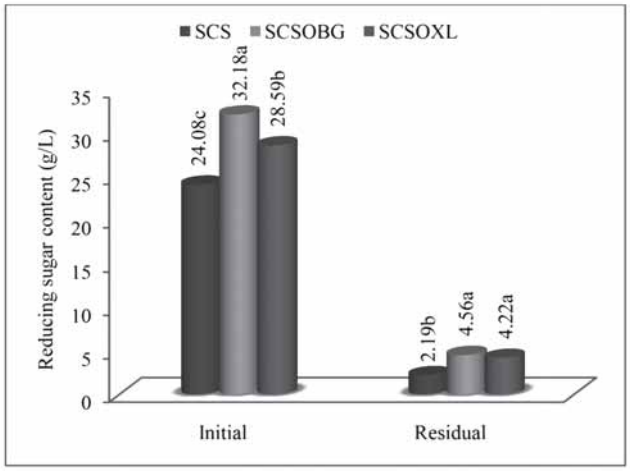


Fig. 1. Initial RS in the hydrolysate and residual RS in fermented broth (after fermentation with yeast for 48 h) from steam-pretreated cassava stems; bars with different alphabets in each set are significant at $p < 0.05$

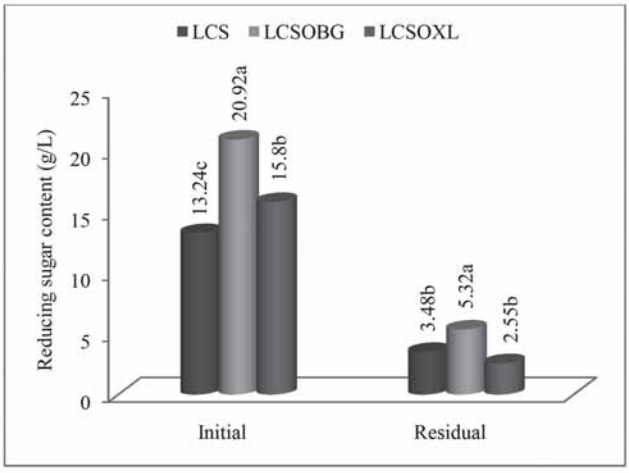


Fig. 2. Initial RS in the hydrolysate and residual RS in fermented broth (after fermentation with yeast for 48 h) from steam-pretreated cassava leaves; bars with different alphabets in each set are significant at $p < 0.05$

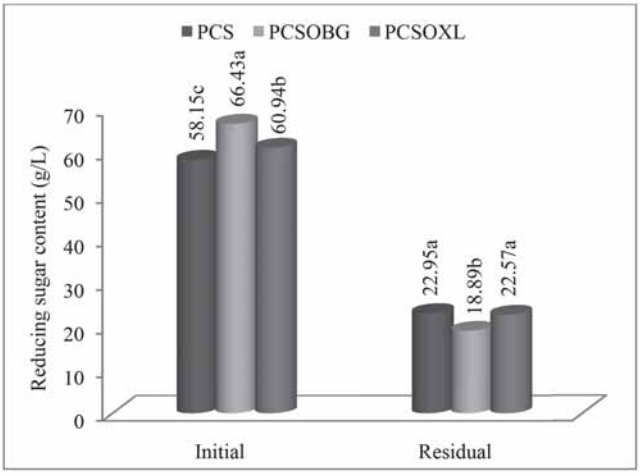


Fig. 3. Initial RS in the hydrolysate and residual RS in fermented broth (after fermentation with yeast for 48 h) from steam-pretreated cassava peels; bars with different alphabets in each set are significant at $p < 0.05$

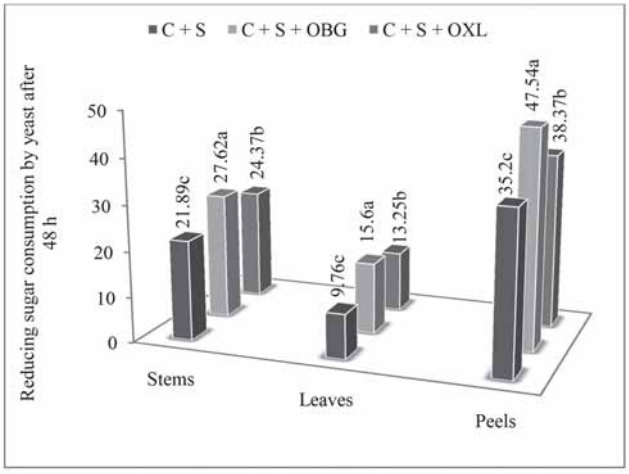


Fig. 4. Sugar consumption during fermentation of hydrolysates from steam-pretreated cassava stems, leaves and peels by yeast (48 h); C + S: Cellic + Stargen; C + S + OBG: Cellic + Stargen + Optimash BG; C + S + OXL: Cellic + Stargen + Optimash XL; bars with different alphabets in each set are significant at $p < 0.05$

(RS) was the lowest in cassava stems saccharified with Cellic + Stargen followed by cassava leaves saccharified with Cellic + Stargen + Optimash XL (Fig. 1 *vs* 2). However residual RS was the highest in cassava peels saccharified using the three enzyme modes and among these, lowest residual RS (18.89 g $l^{-1}$) was obtained in peels saccharified with C + S + OBG, indicating that this treatment (PCSOBG) was the best in the case of cassava peels. The sugar consumption by yeast from the various biomass residues during the fermentation period of 48 h are presented in Fig. 4. This also showed that 47.54 g $l^{-1}$ RS were consumed during the fermentation period of 48 h from cassava peels (Fig. 4). In the case of cassava leaves and stems also, this mode of enzyme saccharification resulted in maximum RS consumption, because of the high content of RS in the saccharified liquor (Fig. 4).

The percentage utilization of RS in the three biomass residues saccharified using the various enzyme application modes as presented in Fig. 5 a-c indicated that there was maximum sugar utilization (%) in cassava stems (85.24-90.91%), followed by cassava leaves (73.72- 83.86%). There was only 60.53-71.56% utilization of RS in cassava peels. Nevertheless, the saccharified liquor from peels contained significantly higher RS content (*ca.* twice the content in cassava stem liquor and thrice the content in cassava leaf liquor) in the C + S + OBG systems and accordingly the sugar consumption (g $l^{-1}$) was also the highest in cassava peel liquor (Fig. 4).

Saha et al. (2013) reported utilization of 16.4 g glucose during 15 h of fermentation by yeast at 37 °C. However, the total RS remaining after fermentation has not been reported in this study. It is well documented that the traditional yeast *S. cerevisiae* can only convert hexose sugars to ethanol and the pentose sugars formed from lignocellulosic substrates remain largely unutilized. Co-

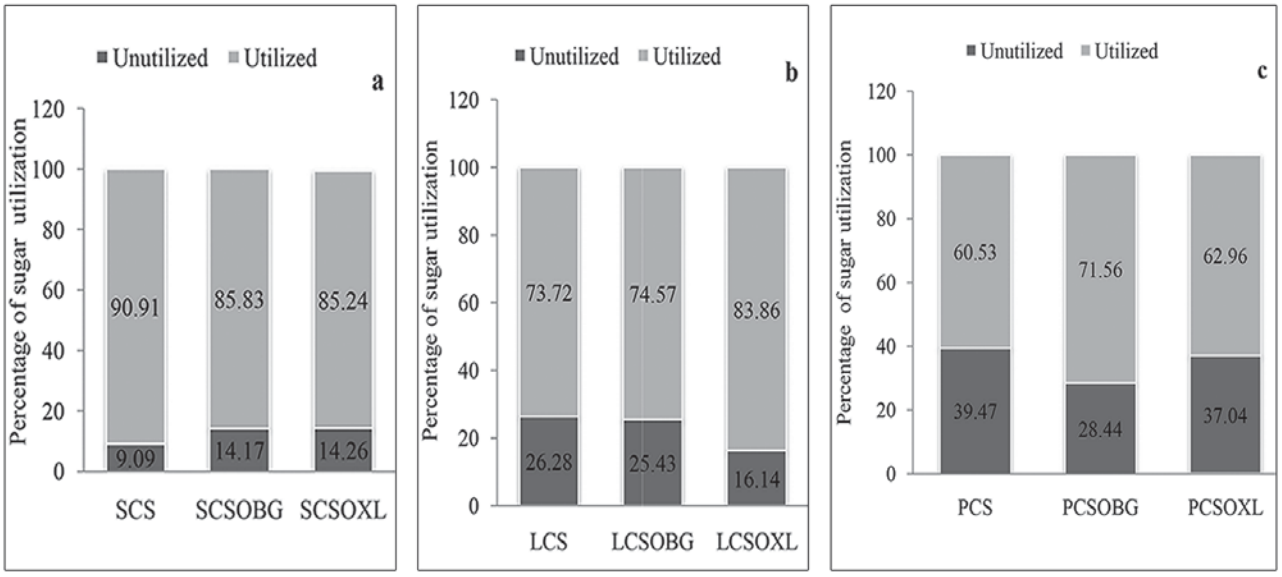


Fig. 5 (a-c). Percentage utilization of reducing sugars during fermentation of hydrolysates from steam-pretreated cassava stems (a), leaves (b) and peels (c) saccharified using binary or triple enzyme cocktails

fermentation using yeasts that could utilize both hexoses and pentoses or co-culture using hexose fermenting and pentose fermenting organisms have been reported to enhance the yield of ethanol from LCBs (Olsson and Hahn-Hägerdal, 1996; Golias et al., 2002; Olsson et al., 2006). Dahnum et al. (2015) also observed that during fermentation of alkali-pretreated empty fruit bunch of oil palm saccharified using Cellic, glucose decreased from *ca.* 9.5% to 1% within 24 h, while xylose levels remained static. The high levels of RS in the peel liquor after fermentation indicated that most of the pentoses (xylose and arabinose) were remaining unutilized. Chantawongsa and Kongkiattikajorn (2013) also reported a decrease of total RS from *ca.* 9 g $l^{-1}$ to 1 g $l^{-1}$ during 48 h fermentation of dilute sulfuric acid-pretreated banana peels saccharified using commercial cellulase.

### HPLC sugar profile

The sugar profile analysis of saccharified mash from peels, stems and leaves showed that glucose was the predominant sugar in the hydrolysates from the three residues, with a significantly higher quantity in peel hydrolysates (Table 1).

Table 1. HPLC sugar profile in the hydrolysates (120 h) from steam-pretreated cassava stems, leaves and peels saccharified using Cellic + Stargen + Optimash BG

| Type of sugars | Reducing sugar content (g $l^{-1}$) | | |
|---|---|---|---|
| | Cassava stems | Cassava leaves | Cassava peels |
| Glucose | 24.15 | 12.22 | 48.68 |
| Xylose | 1.23 | 1.86 | 2.46 |
| Mannose | 0.24 | ND | ND |
| Arabinose | 0.81 | 1.53 | 1.85 |
| Galactose | ND | ND | 0.22 |
| Total | 26.43 | 15.61 | 53.93 |

Mean from two runs; ND: nothing detected

Least quantity of glucose was present in the leaf hydrolysates due to the high recalcitrance of leaves and consequent low hydrolysis of polysaccharides including starch. Xylose content was also the highest in peel hydrolysates even though the quantity was much less than reported for most lignocellulosic substrates (Saha et al., 2013; Dien et al., 2006). Despite the high content of hemicelluloses in the residues studied (Range: 23-29% on dwb; Pooja and Padmaja, 2015 a), the contents of xylose in the hydrolysates were very low possibly because of low hydrolysis of hemicelluloses in the steam pretreated and saccharified biomass. Arabinose was uniformly present in all the hydrolysates, while galactose was present in only peel hydrolysates and mannose was present in only stem hydrolysates. This indicated that the hemicellulose composition of each part of the cassava plant might be different.

### Ethanol yield and fermentation efficiency

The ethanol content (g $l^{-1}$) during fermentation (24 h and 48 h) of the hydrolysates from the three biomass saccharified using the three modes of enzyme application is presented in Table 2. The highest ethanol yield was obtained from steam-pretreated cassava peels saccharified using (Cellic + Optimash BG + Stargen) systems and approximately 21.68 g $l^{-1}$ ethanol was obtained. Progressive increase in ethanol production was observed from 14.95 g $l^{-1}$ (24 h) to 21.68 g $l^{-1}$ (48 h). The other two systems gave ethanol yields of 15.01 g $l^{-1}$ and 16.84 g $l^{-1}$ respectively for Cellic + Stargen and Cellic + Optimash XL + Stargen. Much lower levels of ethanol (g $l^{-1}$) were obtained from both cassava stems and leaves, due to the low levels of RS in the initial hydrolysates utilized for fermentation (Fig. 1 and 2 *vs* Table 2). The potential sugar yielding carbohydrates were only 49% in cassava leaves,

Table 2. Ethanol content (g $l^{-1}$) in the fermented broth from steam-pretreated cassava stems, leaves and peels saccharified by different enzyme systems

| Treatment | Ethanol content (g $l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Cassava stems | | Cassava leaves | | Cassava peels | |
| | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h |
| C + S | $4.36^{c}$ | $9.06^{c}$ | $2.82^{a}$ | $3.73^{b}$ | $9.41^{c}$ | $15.01^{c}$ |
| C + S + OBG | $9.18^{a}$ | $12.92^{a}$ | $4.10^{a}$ | $6.17^{a}$ | $14.95^{a}$ | $21.68^{a}$ |
| C + S + OXL | $5.85^{b}$ | $10.47^{b}$ | $3.01^{a}$ | $5.21^{a}$ | $11.11^{b}$ | $16.84^{b}$ |

C + S: Cellic + Stargen; C + S + OBG: Cellic + Stargen + Optimash BG; C + S + OXL: Cellic + Stargen + Optimash XL; Statistical comparison was made within each column and values with different superscripts are significant at $p < 0.05$

Table 3. Ethanol yield ($Y_E$) and Fermentation Efficiency (FE %) from steam-pretreated cassava stems, leaves and peels saccharified by different enzyme systems

| Treatment | Cassava stems | | Cassava leaves | | Cassava peels | |
|---|---|---|---|---|---|---|
| | $Y_E$* | FE (%)** | $Y_E$* | FE (%)** | $Y_E$* | FE (%)** |
| C + S | 0.414[a] | 81.00[c] | 0.381[a] | 74.64[b] | 0.426[a] | 83.43[c] |
| C + S + OBG | 0.468[a] | 91.59[a] | 0.396[a] | 77.42[a] | 0.456[a] | 89.26[a] |
| C + S + OXL | 0.429[a] | 83.99[b] | 0.394[a] | 77.02[a] | 0.439[a] | 85.90[b] |

Treatment details as in Table 2; *g ethanol produced/g sugar consumed; **Fermentation Efficiency (as per Equation 5); Statistical comparison was made within each column and values with different superscripts are significant at $p < 0.05$

compared to 71.77% in cassava peels (Pooja and Padmaja, 2015 a) and this along with the highly recalcitrant nature of leaves accounted for the low ethanol contents of 3.73-6.17 g $l^{-1}$ in the fermented broth.

The ethanol yield ($Y_E$) computed based on gram ethanol produced/g reducing sugar consumed as per Equation 4, indicated that cassava stems saccharified using Cellic + Optimash BG + Stargen on fermentation gave the highest value 0.468 g $g^{-1}$. Fermentation of cassava peels saccharified using the same enzyme systems gave $Y_E$ of 0.456 g/g (Table 3). In both the cases, the other two modes of enzyme application gave $Y_E$ in the range of 0.414 -0.429 (stems) and 0.426-0.439 (peels). However, significantly lower ethanol yield ($Y_E$) was obtained during fermentation of the hydrolysates from cassava leaves saccharified by the three modes of enzyme application and the values ranged from 0.381-0.396 g $g^{-1}$. The low yield was evidently due to the very low RS levels in the hydrolysates (Fig. 2 *vs* Table 3).

Accordingly the fermentation efficiency (FE) computed as per the Equation 5 was also the lowest for cassava leaves. It was found that both LCSOBG and LCSOXL gave similar fermentation efficiency values of *ca*.77% (Table 3). The highest FE (%) was obtained for cassava stems saccharified using C + S + OBG (SCSOBG; 91.59%) which also had the highest sugar utilization (85.83%; Fig. 5). Cassava peels saccharified using the same systems on fermentation gave FE (%) of 89.26% while PCSOXL gave FE (%) of 85.9% (Table 3). The high FE values indicated that the saccharified liquor from peels and stems contained high quantity of glucose / fermentable hexoses in comparison to xylose, which could not be utilized by *S. cerevisiae*. Yadav et al. (2011) obtained ethanol yield of 0.40 g/g and FE of 95% from acid pretreated and concentrated rice straw hydrolysates after 36 h batch fermentation (SHF) using co-culture of *S. cerevisiae* and *Pichia stipitis*. Cheng et al. (2008) obtained ethanol content of 19 g $l^{-1}$ from pretreated sugarcane bagasse using SHF, with $Y_E$ of 0.34 g $g^{-1}$ which was significantly lower than those reported in the present study. Ferrari et al. (1992) reported ethanol content of 12.6 g $l^{-1}$ and yield of 0.35 g $g^{-1}$ during a fermentation time of 75 h from Eucalyptus wood hemicelluloses hydrolysate and as compared to this, much higher ethanol yields could be obtained from cassava stems and peels, possibly because of the low levels of xylose in the saccharified liquor. The theoretically possible yield of ethanol is 0.511 g $g^{-1}$ glucose and comparing this, the $Y_E$ from cassava peels and stems (0.456 g $g^{-1}$ and 0.468 g $g^{-1}$ in PCSOBG and SCSOBG respectively) was significantly higher. Saha et al. (2013) reported $Y_E$ of 0.49 g $g^{-1}$ from dilute acid pretreated wheat straw saccharified using three enzymes (cellulase, β-glucosidase and hemicellulase) and fermented with *S. cerevisiae*, although the ethanol yield was only 12.2 g $l^{-1}$, which is significantly lower than the levels obtained in the present study from peels, stems and leaves. Sindhu et al. (2014) adopted SHF for dilute acid pretreated Indian bamboo and reported ethanol yield of 17.6 ml $l^{-1}$ with a FE of 41.69% during fermentation for 72 h using *S. cerevisiae*. Öhgren et al. (2007) obtained ethanol content of *ca*. 17 g $l^{-1}$ and $Y_E$ of 0.46 g $g^{-1}$ glucose during fermentation of steam-pretreated corn stover saccharified with a mixture of cellulase and xylanase and the yields were comparable to those obtained for cassava peels and stems in the present study.

The volumetric ethanol productivity (g $l^{-1}$ $h^{-1}$) and ethanol yield ml $kg^{-1}$ dry biomass from the three biomasses are given in Table 4. Evidently the highest volumetric ethanol productivity (VEP) was obtained for cassava peels (Range: 0.313-0.452 in the three enzyme application modes) and

Table 4. Volumetric ethanol productivity and ethanol recovery from steam-pretreated cassava stems, leaves and peels saccharified by different enzyme systems

| Treatment | Volumetric ethanol productivity (g/L/h) | | | Ethanol recovery(ml $kg^{-1}$ dry biomass) | | |
|---|---|---|---|---|---|---|
| | Cassava stems | Cassava leaves | Cassava peels | Cassava stems | Cassava leaves | Cassava peels |
| C + S | $0.189^{a}$ | $0.078^{a}$ | $0.313^{b}$ | $110.48^{c}$ | $45.46^{c}$ | $183.05^{c}$ |
| C + S + OBG | $0.269^{a}$ | $0.129^{a}$ | $0.452^{a}$ | $157.52^{a}$ | $75.24^{a}$ | $264.36^{a}$ |
| C + S + OXL | $0.218^{a}$ | $0.109^{a}$ | $0.351^{a,b}$ | $127.62^{b}$ | $63.57^{b}$ | $205.41^{b}$ |

Treatment details as in Table 2; Statistical comparison was made within each column and values with different superscripts are significant at $p < 0.05$

PCSOBG gave the highest volumetric ethanol productivity of 0.452 g $l^{-1}$ $h^{-1}$ (Table 4). Least volumetric ethanol productivity (VEP) of 0.078- 0.129 g $l^{-1}$ $h^{-1}$ was obtained in the case of cassava leaves, indicating the poor ethanol production potential of this biomass. Enzymatic hydrolysates from cassava stems on fermentation gave VEP of 0.189-0.269 g $l^{-1}$ $h^{-1}$ and in the case of both stems and peels also, the highest VEP was obtained for the systems saccharified with C + OBG + S indicating the efficiency of this systems. Yadav et al. (2011) reported VEP of 0.33 g $l^{-1}$ $h^{-1}$ from acid hydrolysed rice straw fermented using co-culture of *S. cerevisiae* and *Pichia stipitis* and it could be observed that much higher VEP of 0.452 g $l^{-1}$ $h^{-1}$ was obtained from cassava peels (PCSOBG) in the present study using *S. cerevisiae* alone as the fermenting organism. It was also because of te high potential sugar yielding carbohydrate (71.77%) content of dry cassava peels (Pooja and Padmaja, 2015 a) and unlike in the case of rice straw, cassava peels also had high content of starch, yielding a mash rich in glucose on saccharification.

The HPLC sugar profile also confirmed that the dominant sugar in the hydrolysates from saccharification was glucose (Table 1). Pandian et al. (2016) reported ethanol yields of 26.46 g $l^{-1}$ from cassava peel hydrolysates during single step fermentation using two organisms *viz.*, *Saccharomycopsis fibuligera* and *Zymomonas mobilis* and the yield is slightly higher than that reported in the present study (21.68 g $l^{-1}$) from cassava peels fermented using *S. cerevisiae* alone. Nuwamanya et al. (2012) reported FE of 78.49%, 90.41% and 88.76% respectively from acid-alkali pretreated cassava peels, stems and leaves respectively during fermentation with *S. cerevisiae* and it could be seen that the values for stems closely resembled those reported in the present study for SCSOBG (Table 3), while higher and lower FE values were obtained for cassava peels and leaves respectively in the present study compared to those reported by Nuwamanya et al. (2012). Computation of ethanol yield (ml $kg^{-1}$ dry biomass) showed that steam-pretreated cassava residues differed widely in their ethanol production potential, with cassava peels yielding the highest and leaves, the least (Table 4). Pretreated cassava peels saccharified with the complement of Cellic + Optimash BG + Stargen (PCSOBG) yielded *ca.* 264.36 ml $kg^{-1}$ dry biomass. This was followed by the PCSOXL (205.4 ml $kg^{-1}$) and PCS (183.05 ml $kg^{-1}$). In the case of pretreated cassava stems, SCSOBG gave *ca.* 157.52 ml $kg^{-1}$ followed by SCSOXL (127.62 ml $kg^{-1}$). Least quantity of ethanol was obtained from cassava leaves (45.46-75.24 ml $kg^{-1}$) due to the low content of potential sugar yielding carbohydrates in it (49.43%; Pooja and Padmaja, 2015 a) as well as the high recalcitrance of leaves. Martin et al. (2002) reported ethanol yields of *ca.* 180 g $kg^{-1}$ (equivalent to 219.5 ml $kg^{-1}$) dry biomass from steam exploded sugarcane bagasse saccharified using commercial cellulase and detoxified with the enzyme, laccase and fermented (SHF) using recombinant xylose-utilizing *Saccharomyces cerevisiae*. The ethanol yield from steam-pretreated and saccharified hydrolysates (PCSOBG) fermented using traditional Baker's yeast gave *ca.* 264.36 ml $kg^{-1}$ biomass indicating that cassava peel could be a potential feedstock for bioethanol production. Khalil et al. (2015) obtained yield of 192 ml $kg^{-1}$ from dilute acid hydrolysed sweet sorghum fermented by SHF and the yield was slightly higher than that obtained in the present study from cassava stems (Table 4).

## Conclusion

Bioethanol production potential of steam-pretreated agricultural residues of cassava such as stems, leaves and peels was investigated using separate hydrolysis and fermentation. Out of the three enzyme cocktails used for saccharification, the highest saccharification yield was obtained from the system saccharified with triple enzyme

cocktail comprising Cellic, Optimash BG and Stargen. Among the three residues, the highest reducing sugar yield of 66.43 g $l^{-1}$ was obtained from cassava peels using this system, while the lowest was from leaves. Sugar consumption during the 48 h fermentation period was also the highest for peels, which resulted in maximum ethanol yield of 21.68 g $l^{-1}$. HPLC sugar profile indicated that glucose was the predominant sugar in the hydrolysates with the highest level in peel hydrolysates, while xylose content ranged from 1.2-2.5% only. Ethanol recovery was also the highest (264.36 ml $kg^{-1}$ dry biomass) from steam-pretreated peels saccharified with CSOBG system and fermented using *Saccharomyces cerevisiae* indicating that cassava peels had the maximum potential as biofuel feedstock.

## Acknowledgement

The financial support from the Kerala State Council for Science, Technology & Environment for the first author is gratefully acknowledged. The authors also thank the Director, ICAR- CTCRI for the facilities provided for the study. Dr. J. Sreekumar, Principal Scientist (Agricultural Statistics) for the help extended in statistical analyses and Dr. A. N. Jyothi, Principal Scientist and Mr. V. R. Vishnu, Senior Research Fellow, ICAR-CTCRI for the support extended for HPLC analyses.

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