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Comparative Sugar Yield from Lime Pretreated Lignocellulo-Starch Biomass during Enzymatic Saccharification with Binary or Triple Enzyme Cocktails

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Abstract

The comparative sugar recovery from lime pretreated lignocellulo-starch biomass (LCSBs) during saccharification with single, binary or triple enzyme systems was investigated. Reducing sugar (RS) release was the highest from lime pretreatment at room temperature (30 ± 1 ºC) for 24 h (LRT1) compared to 48 h (LRT2) or high temperature (121 ºC; 60 min.; LHT) treatments when saccharified with commercial cellulase (Ecozyme RT80) alone. Supplementation with amylolytic enzyme, Stargen after 72 h (binary system) remarkably enhanced the RS yield at 96 h, which then tapered off at 120 h. Reversal of the enzyme application sequence with Stargen+Ecozyme RT80 resulted in poor saccharification up to 48 h followed by a rapid rise in RS release after supplementation with EcozymeRT80, which was due to the preferential saccharification of starch and exposure of cellulose for hydrolysis. Supplementation of xylanase (Ecozyme XY50) along with the other two enzymes (triple enzyme system) was beneficial in the case of LCSBs such as peels of greater yam, beet root and ash gourd having high hemicelluloses content. Overall Conversion Efficiency was not significantly different for the binary and triple enzyme based saccharification for most lime pretreated residues, indicating the possibility of cost saving of enzymes depending on the type of biomass.

Key words: Lignocellulo-starch biomass, lime pretreatment, saccharification, enzyme cocktails, sugar yield

Introduction

Global warming associated climate change has been predicted as the major threat to humanity by the end of the 21st century. The main contribution towards global warming is from the burning of fossil fuels as a consequence of increased industrial activities and enhanced demand from the transportation sector. Bioethanol with its ability to reduce green house gas (GHG) emission by as high as 86% by virtue of its high oxygen content is considered as the green fuel of future (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008; Wang et al., 2007). Lignocellulosic biomass (LCBs) comprising agricultural and forest residues, woody biomass, dedicated grass such as Bermuda grass, switchgrass etc. and waste paper constitutes the major potential and sustainable feedstock for bioethanol production. Nevertheless, technological barriers exist in the cost-effective production of ethanol from LCBs and despite two decades of research, many of them remain to be fully tackled. The four processes involved in the production of ethanol from LCBs are pretreatment,enzymatic saccharification, fermentation and product recovery (Mosier et al., 2005). Typical composition of LCBs include structural polysaccharides such as cellulose and hemicellulose and the phenylpropanoid lignin which helps in binding the polysaccharides and offering structural integrity to plant cell wall through the formation of a highly ordered structure. This complexity imparts recalcitrance to the

native LCBs and pretreatment is considered as a key step to break the recalcitrance (Wyman, 1999). Pretreatment helps in the physical redistribution of components and depolymerization of hemicellulose and lignin by partial removal of the ester and glycosidic linkages, resulting in increased accessible surface area for enhanced entry of cellulolytic enzymes (Alvira et al., 2010; Yeoh et al., 2007). Several pretreatment strategies including physical, chemical, biological or combined techniques have been studied for the effective breakdown of LCBs and these have formed the basis for a number of reviews (Mosier et al., 2005; Limayem and Ricke, 2012; Yang and Wyman, 2008; Hendriks and Zeeman, 2009; Sarkar et al., 2012; Maurya et al., 2015).

As different from the typical LCBs, processing residues of agricultural crops contain starch also as a major polysaccharide, categorizing them as lignocellulo-starch biomass (LCSBs). The pretreatment and saccharification approaches for such biomasses have to target on this molecule as well. Previous studies have shown that processing residues (peels) from root crops such as sweet potato (*Ipomoea batatas*), elephant foot yam ((*Amorphophallus paeoniifolius*), tannia ((*Xanthosoma sagittifolium*), greater yam (*Dioscorea alata*) and beet root (*Beta vulgaris*) and peels from vegetables such as ash gourd (*Benincasa hispida*), pumpkin (*Cucurbita moschata*) and vegetable banana (*Musa* sp. ABB) as well as mixed vegetable waste (comprising the non-edible parts such as peels, seeds and pulp part covering them and damaged parts of common vegetables) collected from the households and restaurants offer vast potential as feedstock for bioethanol production due to their increasing load consequent to enhanced domestic and industrial activities. Owing to the high starch content, these are favourable substrates for microbial proliferation and contribute significantly to environmental pollution (Mithra and Padmaja, 2016 a). As a part of investigating the bioethanol production potential of these residues, the effect of lime pretreatment at room temperature (30 ± 1 ºC) as well as low (50 ºC) and high (121 ºC) temperatures in altering the composition and ultrastructure was studied (Mithra and Padmaja, 2016 b; 2017 c). It was found that lime pretreatment resulted in very little removal of polysaccharides, although 33-38% lignin removal was reported for lime pretreatment at room temperature for 24 h (Mithra and Padmaja, 2017 b, c). Lignin removal during lime pretreatment has been reported to improve enzyme efficiency by eliminating non-productive binding of lignin to cellulase and by increasing access to cellulose and hemicellulose to degrading enzymes (Nachiappan et al., 2011).

Although pretreatment is the costliest process operation in lignocellulosic ethanol production, it helps to reduce the cost of enzymatic saccharification by reducing the enzyme loading and time of saccharification. A complement of cellulolytic enzymes such as endoglucanase (EC 2.2.1.4), exoglucanase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21) and xylanase (EC 3.2.1.8) is necessary to bring about effective saccharification of LCBs (Sun and Cheng, 2002; Leu and Zhu, 2013; Zhang et al., 2013). However, in the case of lignocellulo-starch biomass (LCSBs) containing starch also as a major polysaccharide, amylolytic enzymes are additionally needed to complete the saccharification. Earlier studies on enzymatic saccharification of dilute sulphuric acid (DSA) and steam pretreated LCSBs showed that very high yield of fermentable sugars was possible using triple enzyme cocktail containing cellulase, xylanase and the starch degrading enzyme, Stargen (Mithra et al., 2017 a). Whilst starch and hemicellulose were hydrolyzed to a high extent in DSA pretreated LCSBs at the pretreatment step itself, swelling of starch and cellulose with little degradation were observed in lime pretreatment (Mithra and Padmaja, 2017 b, c). Hence this study had the objective of comparing the efficacy of binary and triple enzyme cocktails on the saccharification of lime pretreated LCSBs.

Materials and Methods

Root crops such as sweet potato, elephant foot yam, tannia, greater yam and beet root and vegetables such as ash gourd, pumpkin and vegetable banana were manually peeled and the peels were washed thoroughly in running tap water to remove adhering dirt and sand, drained and immediately dried in the sun for 36-48 h. Mixed vegetable wastes (comprising the non-edible parts such as peels, seeds and pulp part covering them and damaged parts of common vegetables) were collected from households and restaurants and directly dried in the sun. The dry samples were powdered in a hammer mill to particles of *ca.* 2-3 mm size and the unscreened powder was directly subjected to lime pretreatment based on an earlier study

(Mithra and Padmaja, 2017 b, c). The biochemical composition of the selected biomasses as reported earlier (Mithra and Padmaja, 2016 a) is given in Table 1.

Enzyme source

The enzymes used in the study were Ecozyme RT80 (cellulolytic enzyme complex), Ecozyme XY50 (Xylanase) and Stargen™ 002 (granular starch hydrolysing enzyme). Ecozyme RT80 and Ecozyme XY50 were supplied by M/s Ecostar Ltd., Chennai, India and Stargen was gifted by M/s Genencor International Inc; USA (presently Danisco US Inc., USA).

Ecozyme RT80 contained 22 FPU (filter paper units) of cellulase activity per ml, besides 328 units (1 unit = mg glucose released per gram cellobiose per hour under the assay conditions) of β- glucosidase activity per ml and 126 units (1 unit = mg glucose released per gram starch per hour under the assay conditions) of α-amylase activity per ml (Mithra et al., 2017 a). The crude protein contents in Ecozyme RT80, Stargen and Ecozyme XY50 were 78.8 mg, 216 mg and 5.25 mg per ml 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* that work synergistically to hydrolyze granular starch substrate to glucose. It has an activity of 570 Glucoamylase units (GAU) g^{-1} and one GAU is the amount of enzyme that will liberate one gram of reducing sugars (as glucose) per hour from soluble starch substrate under the conditions of the assay (Anon., 2009). The dosage and reaction parameters of Ecozyme RT80 were optimized earlier using Response surface methodology (RSM) and the same parameters were adopted in the present study as well (Mithra et al., 2017 a).

Enzymatic Saccharification

Single, binary or triple enzyme systems were compared for enzymatic saccharification of the lime pretreated LCSBs. The effective pretreatments from a previous study were selected and this included (i) treatment with lime (calcium hydroxide; 0.1 g g^{-1} biomass) at room temperature (30 ± 1 °C) for 24 h and 48 h (LRT1 and LRT2 respectively) (ii) treatment at high temperature (121 °C) and pressure of 0.102 MPa for 60 min. (LHT), the details of which are provided in Mithra and Padmaja (2017 b, c). Experiments were conducted in 250 ml Erlenmeyer flasks. Ten grams each of dry biomass were subjected to the three types of lime pretreatment and the pH of the pretreated biomass was adjusted to 5.0 and volume increased to 100 ml for further enzymatic saccharification. Three replicates were maintained for each biomass for the various experiments and a uniform shaking speed of 150 rpm was ensured during saccharification. Enzyme blanks and substrate blanks were kept in each case while assaying the reducing sugars (RS) to nullify the interference from sugars present in the commercial enzyme preparations and the pretreated liquor respectively.

Table 1. Compositional profile* of the selected root and vegetable processing residues (expressed as g/100 g dry basis)

Parameters	SP peel	EFY peel	Tannia peel	GY peel	BR peel	AG peel	VB peel	PK peel	MVW
Cellulose	13.31 ± 0.03	15.63 ± 0.20	17.32 ± 0.34	18.02 ± 0.58	18.94 ± 0.20	18.67 ± 0.77	22.40 ± 0.64	21.05 ± 0.79	11.71 ± 0.36
Hemicellulose	13.32 ± 0.14	14.00 ± 0.00	14.48 ± 0.35	20.02 ± 0.57	19.17 ± 0.55	18.30 ± 0.59	15.19 ± 0.56	17.74 ± 0.47	11.97 ± 0.04
Starch	32.05 ± 0.09	28.96 ± 0.42	30.46 ± 0.37	28.84 ± 0.44	27.13 ± 0.00	19.91 ± 0.39	36.56 ± 0.00	24.61 ± 0.00	28.10 ± 0.46
Lignin	8.15 ± 0.43	7.01 ± 0.13	8. 26 ± 0.11	6.72 ± 0.17	3.87 ± 0.34	10.70 ± 0.34	10.55 ± 0.33	10.66 ± 0.84	7.55 ± 0.38
Total sugars	11.21 ± 0.01	5.53 ± 0.05	2.42 ± 0.05	4.33 ± 0.00	17.07 ± 0.12	6.30 ± 0.05	2.77 ± 0.01	8.73 ± 0.06	10.45 ± 0.08
Reducing sugars	6.22 ± 0.03	2.58 ± 0.00	1.34 ± 0.00	2.17 ± 0.00	6.91 ± 0.04	5.19 ± 0.02	1.71 ± 0.00	6.50 ± 0.00	7.50 ± 0.07
Ash	3.77 ±0.15	9.67 ± 0.12	5.27 ± 0.31	3.29 ± 0.24	5.66 ± 0.10	8.47 ± 0.12	3.40 ± 0.08	4.22 ± 0.06	9.57 ± 0.12
Others**	18.19	19.20	21.79	18.78	8.16	17.65	9.13	13.00	20.65

* Mean ± SD from three replicates; ** calculated by difference (from hundred) of all the above components except reducing sugars which is part of the total sugars and include in addition to moisture (8-10%), protein, extractives, phenols, pigments, vitamins, free amino acids etc. Source: Mithra and Padmaja (2016 a; 2017 c)

Saccharification with Ecozyme RT80 alone

The pH of the pretreated slurry was adjusted to 5.0 and 0.25 % (w/v) sodium azide was added to prevent microbial growth during saccharification. The flasks were covered with aluminium foil and equilibrated in a thermostatic shaking water bath (M/s Julabo Industries, Germany) at 50 °C for 10 min. Ecozyme RT80 at a dose level of 16 FPU g^{-1} cellulose was added to each biomass slurry and incubated for 120 h. Samples were collected at 24 h intervals for RS determination and pH was maintained at 5.0 throughout the incubation period. Reducing sugars released at each sampling period was quantified using arsenomolybdate reagent (Nelson, 1944).

Saccharification using Binary Enzyme Systems

The effect of application sequence of Ecozyme RT80 and Stargen on the saccharification efficiency was compared by using Ecozyme RT80 for a period of 72 h followed by Stargen for another 48 h (making the total incubation time to 120 h) in one set of experiments for all the pretreated biomass, while in the second set, Stargen was added first and allowed to saccharify for 48 h followed by Ecozyme RT80 action for the next 72 h.

Lime pretreated slurry after pH adjustment to 5.0 and supplementation with 0.25 % (w/v) sodium azide was equilibrated in a thermostatic water bath at 50 °C for 10 min. Ecozyme RT80 equivalent to 16 FPU g^{-1} cellulose was added and incubated for 72 h. Sampling for RS determination was done at 24 h intervals and the pH was maintained at 5.0. After 72 h sampling, pH and temperature were brought down to 4.5 and 40 °C respectively. Stargen (0.25 ml or 54 mg protein) was added and incubation continued up to 120 h, with sampling at 96 h and 120 h. Reducing sugars in the saccharified mash were quantified using arsenomolybdate reagent.

In the second type of binary enzyme application mode, the pretreated slurry was adjusted to pH 4.5 and equilibrated in a thermostatic water bath at 40 °C for 10 min. Stargen (0.25 ml) was added and incubated for 48 h, with sampling for RS determination at 24 h and 48 h. Temperature and pH were then raised to 50 °C and 5.0 respectively and Ecozyme RT80 (equivalent to 16 FPU g^{-1} cellulose in the biomass) was added, incubation continued up to 120 h and reducing sugars were quantified at 72, 96 and 120 h as described earlier.

Saccharification using Triple Enzyme Systems

Ecozyme RT80 (16 FPUg^{-1} cellulose), Ecozyme XY50 (Xylanase; 3.0 mg

protein g^{-1} hemicellulose in each biomass) and Stargen (0.25 ml) were mixed and simultaneously applied to lime pretreated slurry (adjusted to pH 5.0 and supplemented with 0.25 % sodium azide). After equilibration for 10 min. at 50 °C, the enzyme mix was added, incubated at 50 °C for 120 h with sampling at every 24 h and the reducing sugar build up was quantified as described earlier.

Saccharification Efficiency (SE) and Overall Conversion Efficiency (OCE)

Saccharification Efficiency (SE %) and Overall Conversion Efficiency (OCE %) for the various enzyme systems were computed from the final reducing sugar yield at 120 h using the following equations

$$SE(\%) = \frac{[RSsm - RSp] \times 100}{[C + HC + Starch + total\ sugars]\ in\ the\ original\ biomass} \quad [1]$$

where RSsm = reducing sugar in the 120 h saccharified mash; RSp = reducing sugar in the pretreated liquor; [C + HC + Starch + total sugars] represents the potential sugar yielding carbohydrate fraction in each biomass, comprising cellulose, hemicellulose, starch and total sugars.

$$OCE(\%) = \frac{[RSsm] \times 100}{[C + HC + Starch + total\ sugars]\ in\ the\ original\ biomas} \quad [2]$$

Statistical Analysis

Experimental data were generated from three replicates for each biomass 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 (SAS, 2010).

Results and Discussion

Single and binary systems

The progressive release in reducing sugars during saccharification of lime pretreated LCSBs with either Ecozyme RT80 alone for 120 h or with Ecozyme RT80 for 72 h followed by Ecozyme RT80-Stargen combined action (binary system) from 72 to 120 h is presented in Tables 2 a and b. In the case of the entire biomass

residues, highest digestibility with Ecozyme RT80 was observed for 24 h room temperature lime pretreated residues (LRT1) compared to LRT2 or LHT. There was a steady and significant increase in RS release up to 72 h in the Ecozyme RT80 alone system and further increase during 96 h and 120 h was non-significant. It was found that the extent of hydrolysis of cellulose by Ecozyme RT80 was highly dependent on the type of biomass and the least RS release (6.53-8.63 g L^{-1}) was observed from tannia peel for all the three pretreatments (Table 2 a). Kaar and Holtzapple (2000) reported as high as 88% hydrolysis of glucan and xylan after 7 days of hydrolysis of lime pretreated (120 ºC for 4 h) corn stover using cellulase alone (25 FPU g^{-1} biomass). Besides factors such as the low exposure time (1 h) at high temperature (121 ºC) during lime pretreatment and saccharification period of 5 days in the present study, the high starch content in the residues which remained unutilized also might be responsible for the low extent of saccharification and inadequacy of single enzyme alone was clearly evident from this experiment. When Ecozyme RT80 was supplemented with Stargen from 72 h onwards there was a dramatic increase in the release of RS at 96 h for all the biomass residues (Tables 2 a and b).

Nevertheless further increase up to 120 h was non-significant. This indicated that rapid hydrolysis of starch occurred on addition of Stargen which tapered off as time increased. Slowing down of enzymatic saccharification from 48 h onwards for aqueous ammonia pretreated barley hull or lime pretreated corn stover was reported (Kim et al., 2008; Kim and Holtzapple, 2005). Saha and Cotta (2008) also reported that the cellulose and hemicelluloses saccharification rates were higher during the initial periods. The possible feedback inhibition of Ecozyme RT80 and Stargen by the RS formed might have decelerated their activity with increase in the saccharification period. The rapid hike obtained in RS release on addition of Stargen indicated that the

Table 2a.Reducing sugars released from lime pretreated root crop processing residues during saccharification with Ecozyme RT80 or Ecozyme RT80 + Stargen

Pretreatment*	Reducing sugars in the saccharified mash (g/L)						
	Ecozyme RT80 alone					Ecozyme RT80 + Stargen	
	24 h	48 h	72 h	96 h	120 h***	96 h	120 h***
(a) SP peel							
LRT1 (9.97)**	18.07^{e}	22.06^{d}	25.76^{c}	26.42^{bc}	27.41^{b}(17.44)	37.08^{a}	38.12^{a} (28.15)
LRT2 (8.25)	15.46^{e}	18.76^{d}	21.83^{c}	22.37^{bc}	23.19^{b}(14.94)	38.38^{a}	39.34^{a} (31.09)
LHT (9.20)	15.99^{e}	19.67^{d}	23.09^{c}	23.70^{bc}	24.61^{b}(15.41)	40.28^{a}	41.28^{a} (32.08)
(b) EFY peel							
LRT1 (6.69)	12.70^{e}	15.37^{d}	17.86^{c}	18.30^{bc}	18.98^{b}(12.29)	38.77^{a}	39.47^{a} (32.78)
LRT2 (4.98)	10.21^{d}	12.20^{c}	14.05^{b}	14.38^{b}	14.88^{b}(9.90)	40.09^{a}	40.81^{a} (35.83)
LHT (4.44)	8.98^{d}	10.75^{c}	12.40^{b}	12.70^{b}	13.14^{b}(8.70)	42.01^{a}	42.74^{a} (38.30)
(c)Tannia peel							
LRT1 (2.70)	6.12^{d}	7.20^{c}	8.19^{bc}	8.37^{b}	8.63^{b}(5.93)	23.98^{a}	24.29^{a} (21.59)
LRT2 (1.70)	4.94^{c}	5.63^{b}	6.25^{b}	6.36^{b}	6.53^{b}(4.83)	25.33^{a}	25.62^{a} (23.92)
LHT (2.32)	5.45^{d}	6.38^{c}	7.23^{bc}	7.39^{b}	7.61^{b}(5.29)	27.14^{a}	27.61^{a} (25.29)
(d) GY peel							
LRT1 (7.84)	13.83^{e}	16.93^{d}	19.86^{c}	20.37^{bc}	21.16^{b} (13.32)	50.63^{a}	51.47^{a} (43.63)
LRT2 (5.55)	10.65^{d}	12.85^{c}	14.92^{b}	15.28^{b}	15.74^{b} (10.29)	51.85^{a}	52.70^{a} (47.15)
LHT (4.96)	9.38^{d}	11.34^{c}	13.19^{b}	13.51^{b}	14.02^{b} (9.06)	53.84^{a}	54.63^{a} (49.67)
(e) BR peel							
LRT1 (10.52)	19.12^{e}	23.32^{d}	27.24^{c}	27.94^{c}	28.97^{b}(18.45)	38.20^{a}	39.29^{a}(28.77)
LRT2 (9.65)	17.78^{e}	21.64^{d}	25.23^{c}	25.87^{bc}	26.82^{b}(17.17)	39.50^{a}	40.40^{a}(30.75)
LHT (8.63)	15.37^{e}	18.82^{d}	22.03^{c}	22.60^{bc}	23.45^{b}(14.82)	41.43^{a}	42.32^{a}(33.69)

* LRT1 and LRT2 indicate lime pretreatment at room temperature (30 ± 1 °C) for 24 h and 48 h respectively; LHT indicates lime pretreatment at high temperature (121 °C) and pressure of 0.102 MPa for 60 min.; means with different superscripts in each row are statistically significant at $p < 0.05$; ** figures in parentheses indicate the RS content in the pretreated liquor before saccharification ; *** figures in parentheses indicate the RS released due to saccharification alone (0 h to120 h)

enzyme was free to hydrolyse the swollen starch releasing appreciable quantity of RS in to the saccharified mash before it is getting inhibited by either the RS or by the phenolic inhibitors present in the system.

Although lime pretreatment resulted in only negligible enhancement in total phenolic content of the pretreated liquor, the original extracts contained phenolics sufficient enough to inhibit cellulase as well as Stargen (Mithra and Padmaja, 2016 b). A similar pattern of sugar release was also reported in the case of steam/acid pretreated LCSBs saccharified with Ecozyme RT80 alone or in conjugation with Stargen (Mithra et al., 2017 a). There was a significant improvement in the enzyme digestibility of lime-pretreated biomass when compared to native (untreated) biomass. Although the initial saccharification rate was better for LRT1 after 120 h hydrolysis with the binary enzyme cocktail, LHT was more digestible and greater quantity of RS release was observed (28-59 g l^{-1} in LHT *vis-á-vis* 24-56 g l^{-1} in LRT1; Table 2 a and 2b) and this was because of the gelatinization of starch at 121°C and its enhanced digestibility by Stargen compared to the slow digestibility of swollen starch in the room temperature treated residues. Except in the case of greater yam peel and vegetable banana peel all other residues were digested to a much higher extent when steam/acid pretreated LCSBs were subjected to similar enzyme action (Mithra et al., 2017 a).

Further the extent of starch hydrolysis consequent to Stargen supplementation in lime pretreated LCBs was significantly higher compared to the hydrolysis reported earlier for steam/acid pretreated LCBs saccharified using similar enzyme systems (Mithra et al., 2017 a). This was because of the very low starch degradation obtained in lime pretreated biomass after the pretreatment step compared to the very high hydrolysis in steam/acid pretreatment. (Mithra and Padmaja, 2016 a; 2017 b, c).

Time course release data for RS during initial saccharification with Stargen for 48 h followed by supplementation of the system with Ecozyme RT80 as given in Tables 3a and b indicate that even though the initial RS release was slower than the Ecozyme RT80 alone system (Tables 2 a and b) there was a rapid increase from 72 h onwards due to Ecozyme RT80 addition. The final RS yield was comparatively higher than first enzyme application mode. It was earlier reported that lime pretreatment of LCBs caused only swelling of starch with very little degradation with the result that the swollen starch clogged the pores restricting free entry of cellulases (Mithra and Padmaja, 2016 a; 2017 b, c).

Table 2 b. Reducing sugars released from lime pretreated vegetable processing residues during saccharification with ecozyme RT80 or ecozyme RT80 + stargen

Pretreatment	Reducing sugars in the saccharified mash (gl^{-1})*						
	Ecozyme RT80					Ecozyme RT80 + Stargen	
	24 h	48 h	72 h	96 h	120 h	96 h	120 h
(a) AG peel							
LRT1 (9.13)	16.73^{e}	20.39^{d}	23.78^{c}	24.38^{bc}	25.28^{b} (16.15)	37.04^{a}	37.98^{a} (28.85)
LRT2 (7.82)	14.75^{e}	17.88^{d}	20.79^{c}	21.30^{bc}	22.07^{b} (14.25)	38.37^{a}	39.28^{a} (31.46)
LHT (7.72)	13.84^{e}	16.94^{d}	19.81^{c}	20.31^{bc}	21.07^{b} (13.35)	40.31^{a}	41.27^{a} (33.55)
(b) PK peel							
LRT1 (11.58)	20.52^{e}	25.15^{d}	29.46^{c}	30.23^{bc}	31.37^{b} (19.79)	43.07^{a}	44.26^{a} (32.68)
LRT2 (9.58)	17.48^{e}	21.32^{d}	24.88^{c}	25.52^{bc}	26.46^{b} (16.88)	44.38^{a}	45.58^{a} (36.00)
LHT (9.00)	15.73^{e}	19.33^{d}	22.68^{c}	23.27^{bc}	24.16^{b} (15.16)	46.32^{a}	47.52^{a} (38.52)
(c) VB peel							
LRT1 (7.3)	11.49^{e}	14.44^{d}	17.13^{c}	17.59^{bc}	18.32^{b} (11.02)	55.08^{a}	55.86^{a} (48.56)
LRT2 (4.53)	8.25^{d}	10.08^{c}	11.75^{b}	12.04^{b}	12.49^{b} (7.96)	56.20^{a}	57.16^{a} (52.63)
LHT (4.20)	7.45^{d}	9.14^{c}	10.69^{b}	10.96^{b}	11.38^{b} (7.18)	58.17^{a}	59.09^{a} (54.89)
(d) MVW							
LRT1 (10.87)	17.85^{d}	22.20^{c}	26.24^{b}	26.45^{b}	26.72^{b} (15.85)	36.32^{a}	36.64^{a} (25.77)
LRT2 (8.93)	15.19^{d}	18.76^{c}	22.09^{b}	22.25^{b}	22.48^{b} (13.55)	37.44^{a}	37.95^{a} (29.02)
LHT (9.20)	15.33^{d}	19.01^{c}	22.43^{b}	22.61^{b}	22.84^{b} (13.64)	39.38^{a}	39.92^{a} (30.72)

* Means with different superscripts in each row are statistically significant at $p < 0.05$; other footnotes as in Table 2a

Preferential hydrolysis of starch by Stargen might be facilitating faster cellulolysis by Ecozyme RT80. Besides Ecozyme RT80 was found to have α-amylase and β-glucosidase as co-activities (Mithra et al ., 2017 a) and the former might be acting on starch to enable its slow hydrolysis in the single enzyme based saccharification (Tables 2 a and b).

As in the case of the first mode of application of binary enzymes there was no further significant effect on RS release after 96 h with Stargen-Ecozyme RT80 system indicating that prolonging the saccharification time beyond 96 h was not beneficial (Tables 3 a and b).

Whereas in the case of steam/acid pretreated LCSBs, the initial (or) pretreated liquor had very high RS content due to the high degree of hydrolysis of starch and hemicelluloses the pretreated liquor from lime pretreatment had only much low quantity of RS (Mithra and Padmaja, 2016 a; 2017 b, c). Despite this, greater yam and vegetable banana peels were digested to a very high extent by the second application mode of Stargen followed by Ecozyme RT80 and this indicated the differential response of LCBs to pretreatment and saccharification strategies.

Triple enzyme cocktail

Supplementation of binary systems with xylanase (Ecozyme XY50) did not produce a remarkable impact in enhancing RS release except in the case of few residues such as greater yam or beet root peels (Tables 4 a and b). Highest RS content in the saccharified mash was obtained for VB peel followed by GY peel. As in the case of single and binary enzyme systems, saccharification yield tapered off after 96 h for all the LCSBs except pumpkin peel which indicated that prolonging saccharification beyond 96 h was not advantageous. Reducing sugar content in the saccharified mash was significantly less compared to the values reported earlier for LCSBs pretreated by steam or dilute sulfuric acid and saccharified using similar enzyme systems (Mithra et al., 2017 a).

Table 3a.Reducing sugars released from lime-pretreated root crop processing residues during saccharification with stargen + ecozyme RT80 (from 48 h)

Pretreatment	Reducing sugars in the saccharified mash (gl^{-1})				
	Stargen	Stargen + ecozyme RT80			
	24 h	48 h	72 h	96 h	120 h
(a) SP peel					
LRT1(9.97)	15.19^{d}	19.18^{c}	29.39^{b}	39.01^{a}	39.14^{a} (29.17)
LRT2(8.25)	12.78^{d}	16.08^{c}	24.97^{b}	39.52^{a}	40.56^{a} (32.31)
LHT(9.20)	13.66^{d}	17.15^{c}	26.23^{b}	41.42^{a}	42.53^{a} (33.33)
(b) EFY peel					
LRT1(6.69)	9.82^{d}	12.49^{c}	21.49^{b}	40.50^{a}	40.70^{a} (34.01)
LRT2(4.98)	7.53^{d}	9.52^{c}	17.19^{b}	41.23^{a}	42.09^{a} (37.11)
LHT(4.44)	6.83^{d}	8.20^{c}	15.54^{b}	43.18^{a}	44.00^{a} (39.56)
(c) Tannia peel					
LRT1(2.7)	3.24^{d}	4.32^{c}	11.82^{b}	25.46^{a}	25.91^{a} (23.21)
LRT2(1.7)	2.26^{c}	2.95^{c}	9.39^{b}	26.47^{a}	26.81^{a} (25.11)
LHT(2.32)	3.10^{c}	3.93^{c}	10.44^{b}	28.29^{a}	28.89^{a} (26.57)
(d) GY peel					
LRT1(7.84)	10.95^{d}	14.05^{c}	23.49^{b}	52.42^{a}	52.56^{a} (44.72)
LRT2(5.55)	7.97^{d}	10.17^{c}	18.06^{b}	52.99^{a}	53.92^{a} (48.37)
LHT(4.96)	7.05^{d}	8.75^{c}	16.37^{b}	55.02^{a}	55.90^{a} (50.94)
(e) BR peel					
LRT1(10.52)	16.24^{d}	20.44^{c}	30.87^{b}	40.13^{a}	40.38^{a} (29.86)
LRT2(9.65)	15.10^{d}	18.96^{c}	28.37^{b}	40.64^{a}	41.62^{a} (31.97)
LHT(8.63)	12.84^{d}	16.25^{c}	25.22^{b}	42.56^{a}	43.60^{a} (34.97)

Means with different superscripts in each row are statistically significant at $p < 0.05$; other footnotes as in Table 2a

Table 3b. Reducing sugars released from lime-pretreated vegetable processing residues during saccharification with stargen + ecozyme RT80 (from 48 h)

Pretreatment	Reducing sugars in the saccharified mash (gl^{-1})				
	Stargen	Stargen + ecozyme RT80			
	24 h	48 h	72 h	96 h	120 h
(a) AG peel					
LRT1(9.13)	13.85^{d}	17.51^{c}	27.41^{b}	39.87^{a}	38.97^{a} (30.64)
LRT2(7.82)	12.07^{d}	15.20^{c}	23.93^{b}	39.51^{a}	40.52^{a} (32.70)
LHT(7.72)	11.31^{d}	14.38^{c}	22.98^{b}	41.43^{a}	42.52^{a} (34.80)
(b) PK peel					
LRT1(11.58)	17.64^{d}	22.27^{c}	33.09^{b}	45.00^{a}	45.89^{a} (34.31)
LRT2(9.58)	14.80^{d}	18.64^{c}	28.02^{b}	45.52^{a}	46.85^{a} (37.27)
LHT(9.00)	13.34^{d}	16.74^{c}	25.82^{b}	47.50^{a}	48.76^{a} (39.76)
(c) VB peel					
LRT1(7.3)	8.61^{d}	11.56^{c}	20.76^{b}	56.44^{a}	57.01^{a} (49.71)
LRT2(4.53)	5.57^{d}	7.40^{c}	14.89^{b}	57.34^{a}	58.36^{a} (53.83)
LHT(4.20)	5.10^{d}	6.56^{c}	13.86^{b}	59.29^{a}	60.35^{a} (56.15)
(d) MVW					
LRT1(10.87)	14.97^{d}	19.32^{c}	29.87^{b}	37.49^{a}	38.25^{a} (27.38)
LRT2(8.93)	12.51^{d}	16.08^{c}	25.23^{b}	38.58^{a}	39.18^{a} (30.25)
LHT(9.20)	12.96^{d}	16.26^{c}	25.61^{b}	40.56^{a}	41.25^{a} (32.05)

Means with different superscripts in each row are statistically significant at $p < 0.05$;
other footnotes as in Table 2a

Lime pretreatment emerged as an inferior pretreatment strategy for LCSBs. Saha and Cotta (2008) also reported that three enzyme combinations (cellulase, â-glucosidase and hemicellulase) gave higher sugar release compared to binary enzyme-based saccharification of lime pretreated rice hulls. Addition of xylanase (Ecozyme XY50) along with the other two enzymes was found to be advantageous in the case of peels from greater yam, beet root and ash gourd which had higher hemicellulose contents of 20%, 19 % and 18 % respectively (Mithra and Padmaja, 2016 a; 2017 c). In the case of mixed vegetable waste with very low hemicellulose content of 12 % (Mithra and padmaja, 2016 a), the effect of supplementation with xylanase in enhancing RS yield was also less. The complementary role of xylanase in enhancing the saccharification yield of steam-exploded barley straw was highlighted by García-Aparicio et al. (2007). Zhang et al. (2013) reported that xylanase supplementation was more effective with acid pretreated biomass having high xylan content.

Enhanced saccharification consequent to the addition of xylanase has been reported for several biomasses (Hu et al., 2011; Moxley et al., 2012). Addition of xylanase was also beneficial to prevent the inhibition of cellulase by xylo-oligosaccharides, which are stronger inhibitors than glucose or cellobiose (Kumar and Wyman, 2009; Qing et al., 2011).

Maximum recovery of solids was reported from lime pretreated bagasse and wheat straw after saccharification with cellulase and xylanase indicating the need for both these enzymes for effective saccharification (Chang et al., 1998).Yu et al. (2003) also reported that the synergistic action of xylanase and cellulase enhanced saccharification of oat hulls by facilitating the exposure of microfibril core of cellulose.

Saccharification Efficiency and Overall Conversion Efficiency

Saccharification Efficiency (%) was significantly higher for the triple enzyme cocktail based saccharification of lime pretreated greater yam, beet root and ash gourd peels (Tables 5 a and b) and the effect was more pronounced for LHT. Nevertheless for the LCSBs such as SP peel, EFY peel and tannia peel, the binary enzyme system with Stargen (48 h) followed by Ecozyme RT80 action on LHT sample was not significantly different

Table 4a.Reducing sugars released from lime-pretreated root crop processing residues during saccharification with triple enzyme cocktail (Ecozyme RT80 + stargen + ecozyme XY50)

Pretreatment	Reducing sugars in the saccharified mash (gl^{-1})				
	24 h	48 h	72 h	96 h	120 h
(a) SP peel					
LRT1(9.97)	24.71^{d}	28.68^{c}	32.36^{b}	40.32^{a}	41.34^{a} (31.37)
LRT2(8.25)	25.10^{d}	29.07^{c}	32.75^{b}	40.71^{a}	41.73^{a} (33.48)
LHT(9.20)	26.09^{d}	30.06^{c}	33.74^{b}	41.69^{a}	42.72^{a} (33.52)
(b0 EFY peel					
LRT1(6.69)	19.68^{d}	22.33^{c}	24.80^{b}	42.35^{a}	43.03^{a} (36.34)
LRT2(4.98)	20.07^{d}	22.72^{c}	25.19^{b}	42.74^{a}	43.43^{a} (38.45)
LHT(4.44)	21.06^{d}	23.71^{c}	26.18^{b}	43.72^{a}	44.42^{a} (39.98)
(c) Tannia peel					
LRT1(2.7)	13.34^{c}	14.40^{bc}	15.37^{b}	27.80^{a}	28.09^{a} (25.39)
LRT2(1.7)	13.73^{c}	14.79^{bc}	15.76^{b}	28.19^{a}	28.42^{a} (26.72)
LHT(2.32)	14.72^{c}	15.78^{bc}	16.75^{b}	29.17^{a}	29.40^{a} (27.08)
(d) GY peel					
LRT1(7.84)	23.82^{d}	26.90^{c}	29.81^{b}	57.22^{a}	58.04^{a} (50.20)
LRT2(5.55)	24.21^{d}	27.29^{c}	30.20^{b}	57.61^{a}	58.43^{a} (52.88)
LHT(4.96)	25.20^{d}	28.28^{c}	31.19^{b}	58.59^{a}	59.42^{a} (54.46)
(e) BR peel					
LRT1(10.52)	28.69^{d}	32.87^{c}	36.77^{b}	44.37^{a}	45.44^{a} (34.92)
LRT2(9.65)	29.08^{d}	33.26^{c}	37.16^{b}	44.75^{a}	45.88^{a} (36.23)
LHT(8.63)	30.06^{d}	34.24^{c}	38.14^{b}	45.74^{a}	46.87^{a} (38.24)

Means with different superscripts in each row are statistically significant at $p < 0.05$;
other footnotes as in Table 2a

Table 4b. Reducing sugars released from lime-pretreated vegetable processing residues during saccharification with triple enzyme cocktail (Ecozyme RT80 + stargen + ecozyme XY50)

Pretreatment	Reducing sugars in the saccharified mash (gl^{-1})				
	24 h	48 h	72 h	96 h	120
(a) AG peel					
LRT1(9.13)	25.86^{d}	29.50^{c}	32.87^{b}	42.77^{a}	43.69^{a} (34.56)
LRT2(7.82)	26.25^{d}	29.89^{c}	33.26^{b}	43.16^{a}	44.08^{a} (36.26)
LHT(7.72)	27.24^{d}	30.88^{c}	34.25^{b}	44.14^{a}	45.07^{a} (37.35)
(b) PK peel					
LRT1(11.58)	29.37^{d}	33.98^{c}	38.27^{b}	48.52^{a}	49.69^{a} (38.11)
LRT2(9.58)	29.76^{e}	34.37^{d}	38.66^{c}	48.91^{b}	50.18^{a} (40.60)
LHT(9.00)	30.75^{e}	35.36^{d}	39.65^{c}	49.89^{b}	51.17^{a} (42.17)
(c) VB peel					
LRT1(7.3)	19.07^{d}	22.00^{c}	24.67^{b}	59.26^{a}	60.02^{a} (52.72)
LRT2(4.53)	19.46^{d}	22.39^{c}	25.06^{b}	59.64^{a}	60.41^{a} (55.88)
LHT(4.20)	20.44^{d}	23.37^{c}	26.04^{b}	60.63^{a}	61.39^{a} (57.19)
(d) MVW					
LRT1(10.87)	23.82^{d}	28.15^{c}	32.17^{b}	38.89^{a}	39.19^{a} (28.32)
LRT2(8.93)	24.21^{d}	28.54^{c}	32.56^{b}	39.27^{a}	39.53^{a} (30.60)
LHT(9.20)	25.19^{d}	29.52^{c}	33.54^{b}	40.26^{a}	40.51^{a} (31.31)

Means with different superscripts in each row are statistically significant at $p < 0.05$;
other footnotes as in Table 2a

from the triple enzyme cocktail indicating that Xylanase supplementation was not needed .

However in the case of lime pretreated vegetable residues except MVW, SE (%) of triple enzyme cocktail was superior to the binary systems (Table 5 b) and this was because of the high hemicellulose content in the other samples (Mithra and Padmaja, 2016 a; 2017 c), which necessitated xylanase for saccharification. The application sequence of Stargen followed by Ecozyme RT80 had a clear advantage on the SE (%) compared to the reverse mode and the preferential hydrolysis of swollen starch by Stargen facilitated the entry of cellulase and its further action on cellulose.

Significant enhancement in Overall Conversion Efficiency (%) of potential carbohydrates to RS was observed with binary and triple enzyme cocktails in the case of all the LCSBs (Fig. 1 a, b & Fig. 2 a, b). Further lime treatment at 121 °C for 60 min was more beneficial for achieving higher saccharification than the room temperature pretreatments. Overall Conversion Efficiency of LHT pretreated residues such as SP peel, EFY peel, tannia peel and MVW saccharified with Stargen + Ecozyme RT80 was not significantly different from the triple enzyme cocktail based saccharification which indicated that xylanase supplementation was unnecessary for such residues with low hemicelluloses content (11-14 %) (Mithra and Padmaja, 2016 a; 2017 c).

Table 5a. Saccharification efficiency (%) of lime pretreated root crop processing residues after saccharification (120 h) with single, binary or triple enzyme cocktails

Pre-treatments	Saccharification efficiency (%)				
	Sweet potato peel	Elephant foot yam peel	Tannia peel	Greater yam (GY) peel	Beet root (BR) peel
Ecozyme RT80					
LRT1	24.96^{f}	19.16^{g}	9.17^{e}	18.71^{i}	22.42^{i}
LRT2	21.38^{g}	15.44^{h}	7.47^{f}	14.45^{j}	20.86^{j}
LHT	22.06^{g}	13.58^{i}	8.18^{ef}	12.72^{k}	18.01^{k}
Ecozyme RT80 + stargen*					
LRT1	40.28^{e}	51.12^{f}	33.37^{d}	61.28^{h}	34.96^{h}
LRT2	44.49^{c}	55.88^{d}	36.97^{c}	66.20^{f}	37.36^{fg}
LHT	45.90^{bc}	59.74^{b}	39.09^{b}	69.75^{d}	40.93^{d}
Stargen + ecozyme RT80*					
LRT1	41.74^{d}	53.04^{e}	35.88^{c}	62.80^{g}	36.28^{g}
LRT2	46.23^{b}	57.88^{c}	38.82^{b}	67.92^{e}	38.85^{e}
LHT	47.68^{a}	61.69^{a}	41.07^{a}	71.52^{c}	42.48^{c}
Ecozyme RT80 + stargen + ecozyme XY50**					
LRT1	44.89^{c}	56.68^{d}	39.25^{b}	70.49^{cd}	42.42^{c}
LRT2	47.90^{a}	59.97^{b}	41.30^{a}	74.26^{b}	44.02^{b}
LHT	47.95^{a}	62.35^{a}	41.87^{a}	76.47^{a}	46.46^{a}

Means with different superscripts in each column are statistically significant at $p < 0.05$; other footnotes as in Table 2a; * Ecozyme followed by stargen or stargen followed by ecozyme; ** Combined application of triple enzymes

Table 5b. Saccharification efficiency (%) of lime pretreated vegetable processing residues after saccharification (120 h) with single, binary or triple enzyme cocktails

Pre-treatments	Saccharification efficiency (%)			
	Ash gourd (AG) peel	Pumpkin (PK) peel	Vegetable banana (VB) peel	Mixed vegetable waste peel
Ecozyme RT80				
LRT1	25.56^{i}	27.43^{i}	14.33^{i}	25.47^{g}
LRT2	22.56^{jk}	23.40^{j}	10.35^{j}	21.77^{h}
LHT	21.84^{k}	19.10^{k}	17.88^{h}	22.30^{h}
Ecozyme RT80 + stargen*				
LRT1	45.68^{h}	45.30^{h}	63.12^{g}	41.42^{f}
LRT2	49.81^{fg}	49.91^{f}	68.41^{e}	46.64^{c}
LHT	53.11^{d}	53.39^{d}	71.35^{c}	49.37^{b}
Stargen + ecozyme RT80*				
LRT1	48.67^{g}	47.56^{g}	64.62^{f}	44.00^{e}
LRT2	51.77^{e}	51.66^{e}	69.97^{d}	48.61^{b}
LHT	55.09^{c}	55.11^{c}	72.99^{b}	51.50^{a}
Ecozyme RT80 + stargen + ecozyme XY50**				
LRT1	54.71^{c}	52.83^{d}	68.53^{e}	45.50^{d}
LRT2	57.40^{b}	56.28^{b}	72.64^{b}	49.17^{b}
LHT	59.12^{a}	58.45^{a}	74.35^{a}	50.32^{ab}

Means with different superscripts in each column are statistically significant at $p < 0.05$; other footnotes as in Table 2a; * Ecozyme followed by Stargen or Stargen followed by Ecozyme; ** combined application of triple enzymes

Very high recovery of xylose during saccharification of lime pretreated biomass [120 ºC for 1 h using 0.1g $Ca(OH)_2$ g^{-1} biomass] with a commercial cellulase preparation was reported indicating the possibility of xylanase also existing as a co-activity in the enzyme (Chang et al., 1997; 1998). Highest OCE values were obtained for greater yam and vegetable banana peels in the present study when either binary or triple enzyme based saccharification was adopted. Among the residues, tannia peel was the least digested and even with the triple enzyme cocktail, OCE for LHT was only 45%.

However earlier studies on these LCSBs showed that very high Overall Conversion Efficiency was possible with

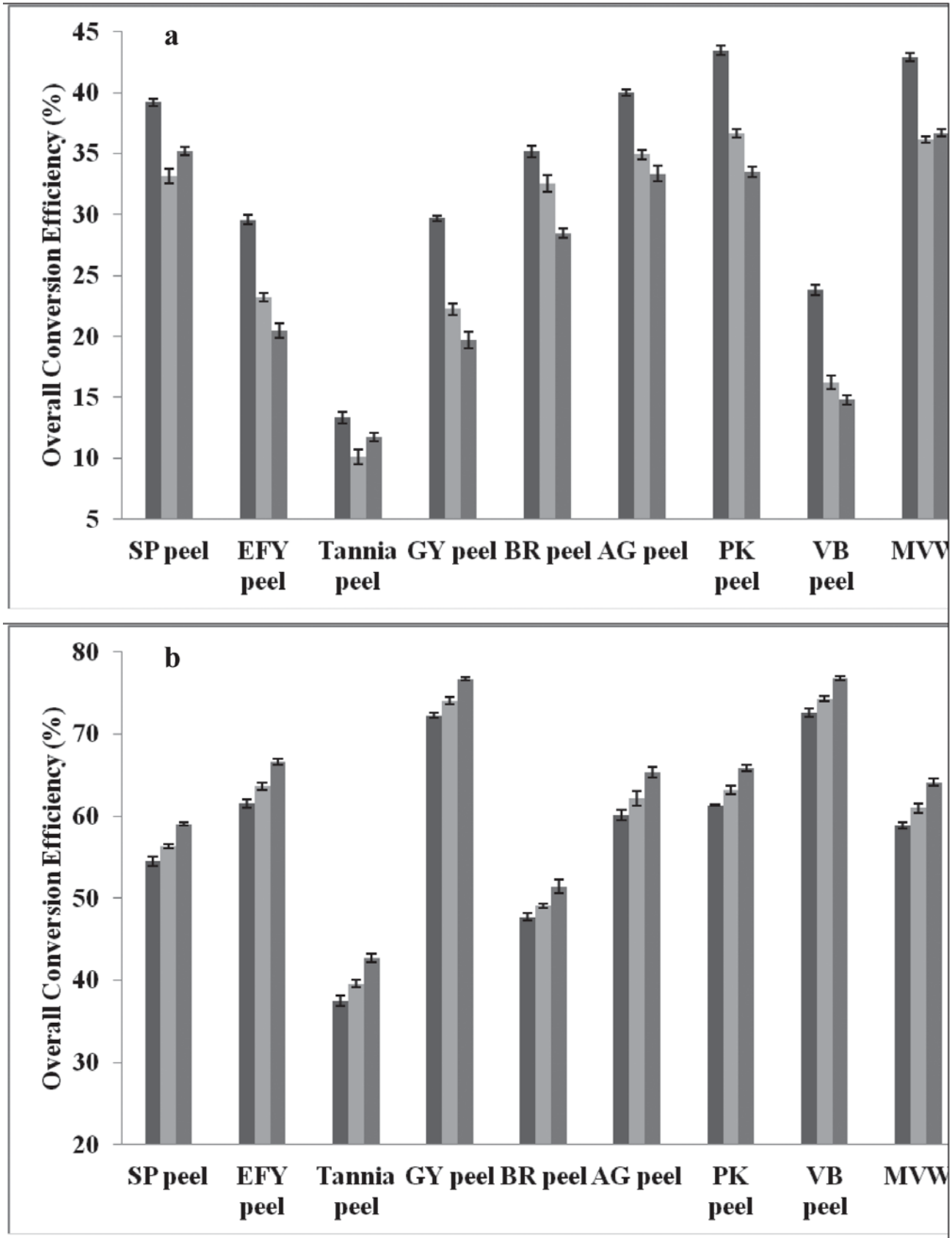

Fig. 1. Overall conversion efficiency (%) of **(a)** Ecozyme RT80 and **(b)** Ecozyme RT80 + stargen (binary) system-based saccharification of lime pretreated biomass (LRT1: first line; LRT2: second line and LHT: third line)

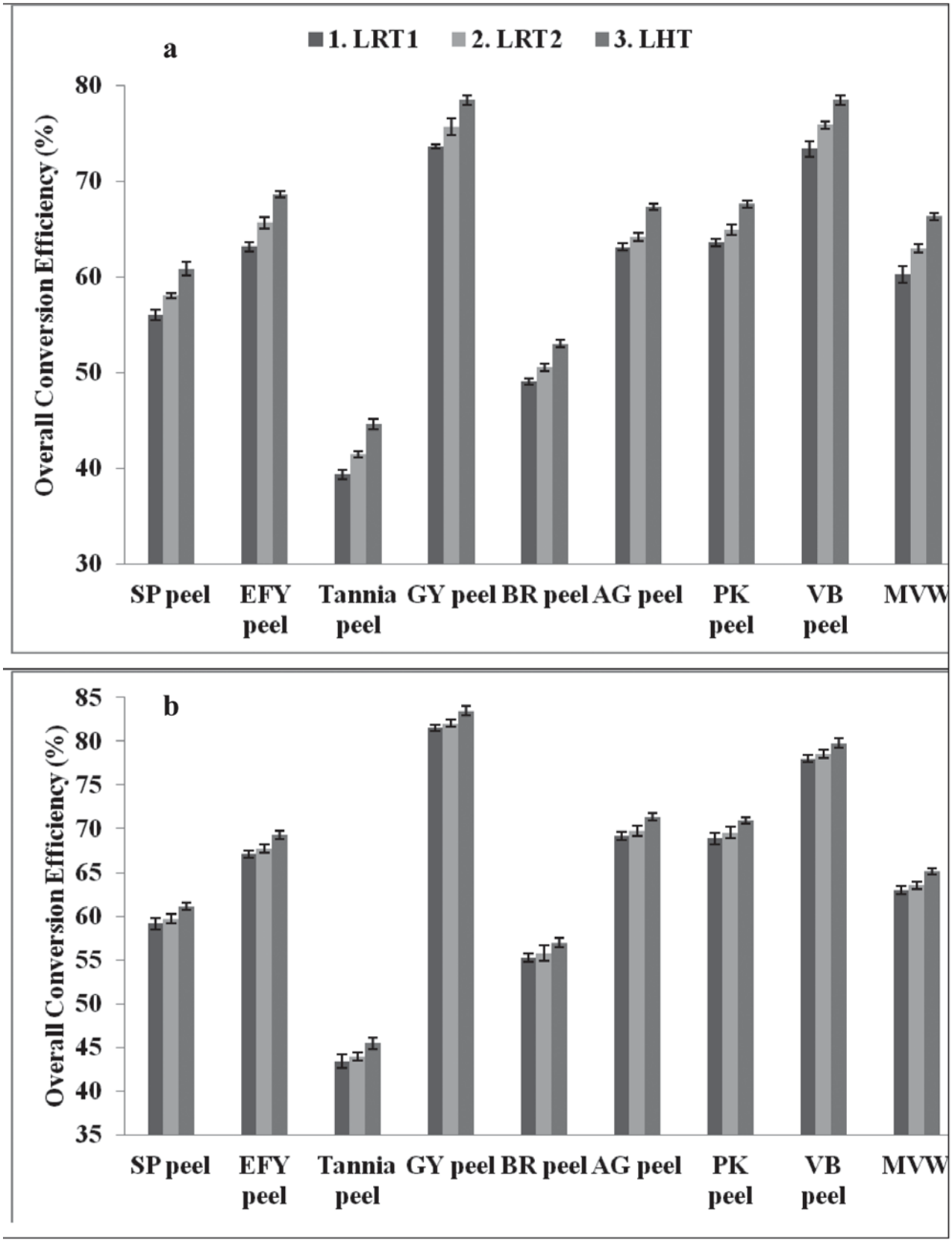

Fig. 2. Overall conversion efficiency (%) of **(a)** Stargen + ecozyme RT80 (binary) and **(b)** triple enzyme system-based saccharification of lime pretreated biomass (LRT1: first line; LRT2: second line and LHT: third line)

triple enzyme based saccharification on steam (83-95% OCE) or dilute sulphuric acid (DSA) pretreaed (88-92%) biomass (Mithra et al., 2017 a), indicating that lime pretreatment followed by saccharification was less efficient with OCE of 43-83%.

Conclusion

The fermentable sugar yield on saccharification of lime pretreated lignocellulo-starch biomass with single, binary or triple enzyme cocktails was studied and it was found that higher hydrolysis occurred with 24 h room temperature pretreated residues using Ecozyme RT80 (cellulase) alone than 48 h or high temperature (60 min.; LHT). However, when Stargen was also supplemented with Ecozyme after 72 h, there was a dramatic increase in RS yield, especially from LHT at 96 h which then slowed down. Reversing the application mode of the two enzymes with Stargen action for the initial period up to 48 h followed by Ecozyme RT80 also up to 120 h was advantageous due to preferential hydrolysis of starch and the final RS yields were higher than the first application mode. Triple enzyme cocktail was beneficial only for those residues having high hemicelluloses content in the pretreated residues. Whilst only 43-45% of carbohydrates were converted to sugars from lime pretreated tannia peel using this mode of saccharification, 83% and 80% sugar recovery was possible from peels of greater yam and vegetable banana respectively indicating the differential response of LCSBs to enzymatic saccharification.

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