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Innovative Consortia of Micro and Macro Fungal Systems: Cellulolytic Enzyme Production from Groundnut Shell Biomass and Supportive Structural Analysis

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Abstract

Renewable biomass-derived fuels are essential to meet the blend mandates and the sustainability goals. In our first-to-date study, we investigated individual and synergistic consortia of fungal cultures comprising *Pycnoporous sanguineus* (PS) in combination with *Aspergillus oryzae* (AO) and *Trichoderma harzianum* (*TH*) for production of cellulolytic enzymes using groundnut shell under solid state and submerged liquid fermentation conditions. The innovative consortia closely align with the microbial ecosystems found in nature; consequently, we anticipate a potent and effective cellulolytic enzyme system, which maximises the breakdown of biomass polymers to sugars. Under ternary combination of cultures, cellulase production was highest at 123.0 ± 1 FPU/gds; β-glucosidase production at 875.6 ± 26.4 IU/g dry substrate (gds); and CMCase at 474.95 ± 45.5 IU/gds. β-glucosidase production was highest on the 2nd day at 987.03 ± 64.2 IU/gds while CMCase peaked at 514.97 ± 21.4 IU/gds on 2nd day for PS, maximum cellulase enzyme production was observed on the 6th day at 192.2 ± 0.96 FPU/gds (AO + PS). Present work showed that synergistic combination of fungal cultures for releasing balanced enzyme activities that can efficiently saccharify biomass, such as groundnut shell to sugars, which can subsequently be fermented to various bioproducts and biomaterial monomers. Elaborate characterization studies of enzyme treated groundnut shell revealed prominent physico-chemical changes in the hydrolysate, which indicates the changes in biomass

are due to the enzymatic action and the growth effects of the consortia; thereby, leading to promising applications for the microbial fortified biomass.

Keywords

Mixed Cultures, Fermentation, Enzymatic Hydrolysis, Groundnut Shell, Biomass Characterization

1. Introduction

Large-scale substitution of petroleum based fuels by renewable alternatives is imperative to tackle climate changes and energy security [1]. The fluctuating costs of fuel over the high demand in the past decade have inspired the research to generate fuel from renewable resources, particularly ethanol from biomass [2]. Biofuels not only reduces the environmental pollution due to its high oxygen content, but also reduces our reliance on oil imports and thus alleviating the uncertainties caused by oil price fluctuations [3].

Critics have raised the potential increase in food costs [4] along with food shortages globally if utilization of food based resources is diverted to produce biofuels [5]. Biofuels derived from biomass will alleviate these concerns and are more economical [6] compared with fuels from food crops such as grain corn and sugarcane. Thus, lignocellulosic biomass feed stocks present an attractive alternative energy source for sustainable energy production [7]. Non-food based sources such as wheat straw [8], wheat bran [9], rice straw [10], corn stover [11], maize [5], sorghum, kinnow mandarin [12], and bagasse have high potential. India is the second largest groundnut producer in the world after China with production of 8.22 million tons in 2017-18 [13]. In India, Andhra Pradesh ranks second after Gujarat with 3.80 lakh tons. The crop can grow successfully with minimum rainfall and in Anantapur region, (14.16˚N, 77.80˚E) groundnut is a major cash crop. The quantity of groundnut shells (GNS) waste by-product generated after harvesting of groundnuts is substantial. GNS has created interest among researchers for application in energy production due to its composition, but cellulose is in the form of lignocellulose, hence the conversion to sugars [14].

Lignocelluloses conversion to fermentable sugars is performed by enzymatic hydrolysis of polysaccharides, preferentially with cellulases and hemicellulases resulting from biomass treatment [14]. However, the costs for these specialized enzyme systems are still significant in spite of dedicated efforts in industry and academia in the last decade. Cellulases and cellulolytic enzymes production at lower costs will substantially reduce the overall production costs of producing biofuels from lignocellulosic biomass [15]. To accomplish this, development of a highly efficient enzyme production process along with development of robust microbial strains for enzyme production is necessary [16]. Bioreactor operational modes such as solid state fermentation (SSF) and submerged fermentation (SmF) were developed for the production of enzymes at industrial scale [10]. SSF

is well known to produce cellulases while utilizing biopolymers such as cellulose and hemi-cellulose from agricultural residues; thereby offering a potentially low-cost alternative. SmF operates in free-flowing liquid substrates [15].

Filamentous fungi are one of the major sources of cellulases and hemicellulases. Among prominent fungi, *Aspergillus* [17] and *Trichoderma* have been widely exploited. *Trichoderma* strains have been reported to produce high activities of both CMCase and exo-glucanase but have poor release of β-glucosidase, which causes accumulation of cellobiose [10]. Further, cellobiose accumulation leads to feedback inhibition by repressing of cellulase enzyme activity [18]. The strains of *Aspergillus* are known to have high β-glucosidase activity release [19]. Mixed cultures are known to produce enzyme cocktails which are rich in β-glucosidase; thus diminishing the accumulated cellobiose concentration as a result of formation of glucose [20].

To increase the enzymatic digestibility of lignocelluloses, most studies focused on use of soft-cellulolytic bacteria, white and brown-rot fungi [21]. Microorganisms release cellobiose dehydrogenase that can degrade the three polymers of biomass *i.e.*, cellulose, hemicellulose, and lignin and therefore can effect complete lignocellulose degradation [22]. Combination of fungal treatments can significantly improve enzymatic digestibility and reduce the time needed for achieving high cellulolytic enzyme production. Although mono culture of endophytic fungi and/or co-culture of fungi were tried previously [23], there have been no retrievable studies found in literature which investigated the enzyme production with ternary combination of endophytic fungi, white rot fungi and filamentous fungi. Ternary combinations of fungi could possibly mimic the consortia that happen in natural degradation.

The current study was carried out to evaluate the cellulolytic enzymes production using mono and mixed-cultures, in SSF and SmF conditions on GNS with *Aspergillus oryzae* (AO, NCIM1212, micro fungi), *Pycnoporous sanguineus* (PS, wood decaying macro fungus) and *Trichoderma harzianum* (TH, endophytic fungus) independently and in combination as microbial systems. This innovative approach of using diverse consortia, nature-based, is first of its kind; the evaluation of its performance will result in significant and appropriate sustainable biomass processing of renewable resources for value added products.

2. Materials and Methods

2.1. Chemicals and Microbial Strains

Untreated GNS was purchased from local farmers in Puttaparthi, Anantapur, A.P, India. NaOH (Merck, >97%), Potato dextrose agar (Himedia), Proteose peptone (Himedia), $(NH_4)_2SO_4$ (SDFCL), KH_2PO_4 (Merck), Urea (Merck), $MgSO_4 \cdot 7H_2O$ (SDFCL), $CaCl_2 \cdot 2H_2O$, (SDFCL), $ZnSO_4 \cdot 7H_2O$ (SDFCL), $FeSO_4$ (SDFCL, 99.5%), $MnSO_4$ (SDFCL, >98%), $ZnCl_2$ (SDFCL, >98%), Dinitro-salicylic acid (Sigma Aldrich), Cellulose (Sigma ALdrich), filter paper (Whatman No.1), Cellulase (Sigma Aldrich), β-glucosidase (Sigma Aldrich),

double distilled water is used in all experiments. AO (NCIM1212) purchased from NCIM, Pune. PS from Indian Institute of Horticulture Research, Bangalore and TH, isolated from *Aegle marmelos*, courtesy department of Biosciences, SSSIHL.

2.2. Physicochemical Treatment

GNS was subjected to physicochemical treatments before being used for production of the cellulolytic enzyme system. GNS was washed thoroughly to remove dirt by repeating the steps such as soaking in water for an hour and filtering. It was then air dried and subsequently in hot air oven at 80˚C initially to remove the water. The samples were ground and sieved (BSS 25 - 16 mesh) to obtain a particle size of 600 - 1000 µm. GNS (200 g) was treated in 3000 ml of 1% (w/w) (0.25 M) Sodium hydroxide (NaOH) in boiling hot water bath. Biomass was allowed to cool followed by repeated washing in tap water to neutralize pH and rinsed in distilled water, after which it was air-dried. Pretreated GNS was stored in airtight containers at room temperature until used.

2.3. Compositional Analysis of GNS

The composition of the GNS was analysed for extractives, lignin, glucan, xylan, and arabinan content by following the NREL standard protocols. In brief, sample was extracted first with deionized water for 24 h, and then with 95% ethanol for 24 h using a Soxhlet Extraction. The sum of water-soluble extractives and acid-soluble extractives was reported as total extractives content of the biomass. The extracted sample was dried in hot-air oven at 45˚C overnight. The extraction-free sample was hydrolysed using 72% (w/w) sulphuric acid at 30˚C for one hour, followed by dilution of the slurry with DI water to make the acid concentration of 4% (w/w) and autoclaved at 121˚C for 60 minutes. The completely hydrolysed biomass slurry was filtered using a medium porosity porcelain filtering crucible. The filtrate was used to measure glucose, xylose and arabinose content using HPLC, and calculated glucan, xylan and arabinan content. The filtrate was also used to measure acid-soluble lignin content using UV-VIS spectrophotometer. The solid residue in the filtering crucible was first dried in hot-air over at 105˚C and then in muffle furnace at 575˚C to measure acid-insoluble lignin. The sum of acid-soluble and acid-insoluble lignin was reported as total lignin content of the biomass [24].

2.4. Enzyme Assay

The Mandel and Reese medium used for enzyme production had composition in g/L: Urea, 0.3; $MgSO_4$. $7H_2O$, 0.3; peptone, 0.75; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; yeast extract, 0.25; $CaCl_2 \cdot 2H_2O$, 0.4; and trace elements (mg/L): $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4 \cdot 7H_2O$, 1.6; $ZnSO_4 \cdot 7H_2O$, 1.4. The extracellular cellulases were extracted by adding 30 ml of citrate buffer (pH 5, 50 mM) to each stationary shake flask in rotary shake incubator (N-Biotek, NB-205V) at 4˚C, 60 RPM for 30 minutes.

The contents were then filtered and analysed for filter paper activity (FPU/gds), CMCase (IU/gds) and β-glucosidase (IU/gds). Enzymatic assays were carried out using standard protocols as described in Brijwani *et al.* [9]

2.5. SSF and SmF

Three cultures *Aspergillus oryzae* (AO, NCIM1212, micro fungi), *Pycnoporous sanguineus* (PS, wood decaying macro fungi) and *Trichoderma harzianum* (TH, endophytic fungi) were used in this study. Cultures were used as mono, binary (1:1) and ternary combinations (1:1:1). Pretreated GNS (2 g) was maintained at 70% moisture content (wet basis) by using Mandel and Reese media [25] of pH 5.0 and sterilization was done in vertical top load autoclave (121˚C/15 psi gauge) for 20 minutes. Cultures were added as disks with Pasteur pipette for non-sporulating fungi and 10^8 spores/ml suspension for sporulating fungi. The generation, propagation and maintenance of spore suspension are described in our earlier work. The conditions of temperature (30˚C), pH, moisture (70%), and incubation days of the SSF process used in this study were optimized in our earlier research [25]. SmF study was performed with same conditions as in SSF with respect to microorganisms used, temperature conditions and incubation period. However, the water content in SmF is at about 10 ml per 2 g of biomass along with 5 ml of Mandel and Reese media.

2.6. Biomass Characterization

The enzyme treated biomass was washed thoroughly with de-ionised water and sterilized in autoclave for 30 minutes. The residual substrate was oven dried overnight at 80˚C. Physico chemical changes in the biomass substrate were studied using thermal, spectroscopic and surface morphological studies.

2.6.1. X-Ray Diffraction Studies

Wide-angle X-ray diffraction (PANalytical, Xpert PRO) was used to estimate the crystallinity of raw and pretreated GNS. The X-rays from a Cu tube operating at 35 KV and 20 mA were collected by an energy dispersive detector that is able to resolve CuKα line. Counts were collected at a step size of 0.02˚ at a series of angles between 10˚ and 50˚. Speed of count collection was 0.6˚/minute.

2.6.2. Thermogravimetric Analysis (TGA)

The structural rigidity changes in the GNS substrate were probed using TGA of raw, alkali treated and fungal treated biomass. The biomass was tested in TGA (METTLER TOLEDO TGA/DSC1) at the rate of 0.5˚C per sec. between temperature range of 30˚C - 1000˚C. The samples were weighed to about 5 mg and loaded into alumina pan with a nitrogen flow of 10 ml per minute. DSC provides the heat flow information (mW) and TGA the percentage weight loss (mg).

2.6.3. FT-IR Analysis

The samples were dried, ground and incorporated into KBr (~3 mg in 75 mg) using hand press. The infrared spectrum measured from 4000 - 400 cm^{-1} was

obtained using THERMO SCIENTIFIC, NICOLET iS10 model by measuring 32 scans. The changes in the functional group pattern was studied by measuring the spectra and compared across all the enzyme treated substrates, reflected in the pretreated and the fungal treated biomass.

2.6.4. SEM Imaging

The morphology of GNS before and after the chemical and fungal treatment was studied with scanning electron microscope (SEM, LEO 1530 VP). The modifications of the morphology were studied by taking images at several magnifications of both chemical and fungal treated GNS. The samples were made conductive by sputtering with gold and observed using a 5 kV voltage source.

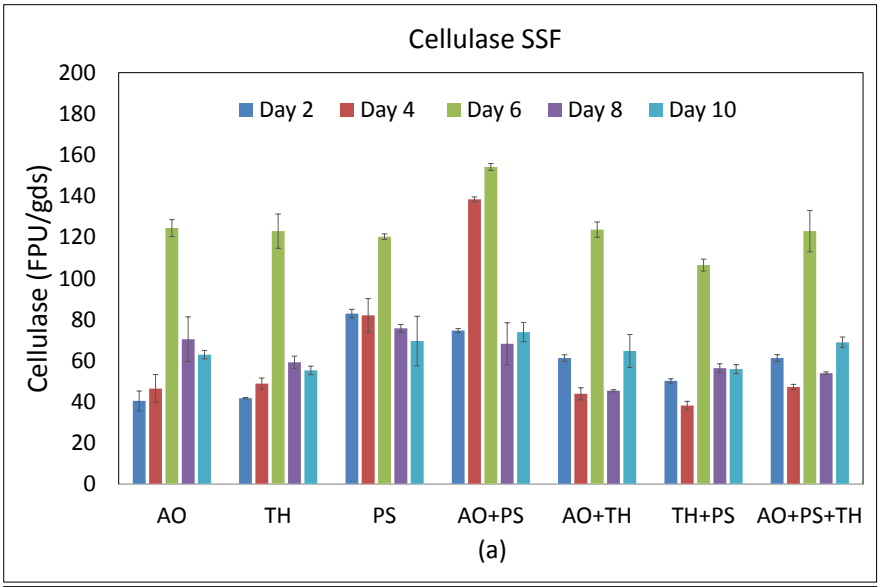
3. Results and Discussion

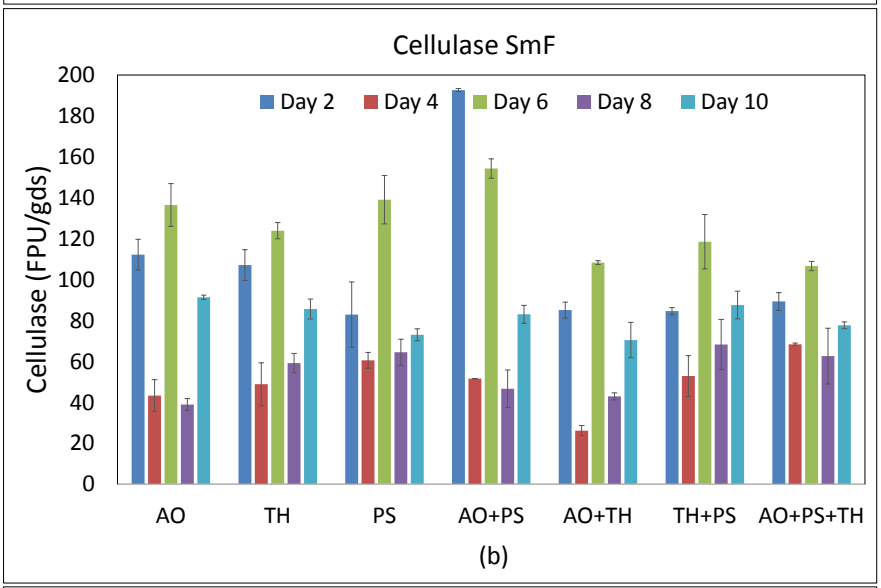
3.1. Effect of Individual and Mixed Cultures on Enzyme Production

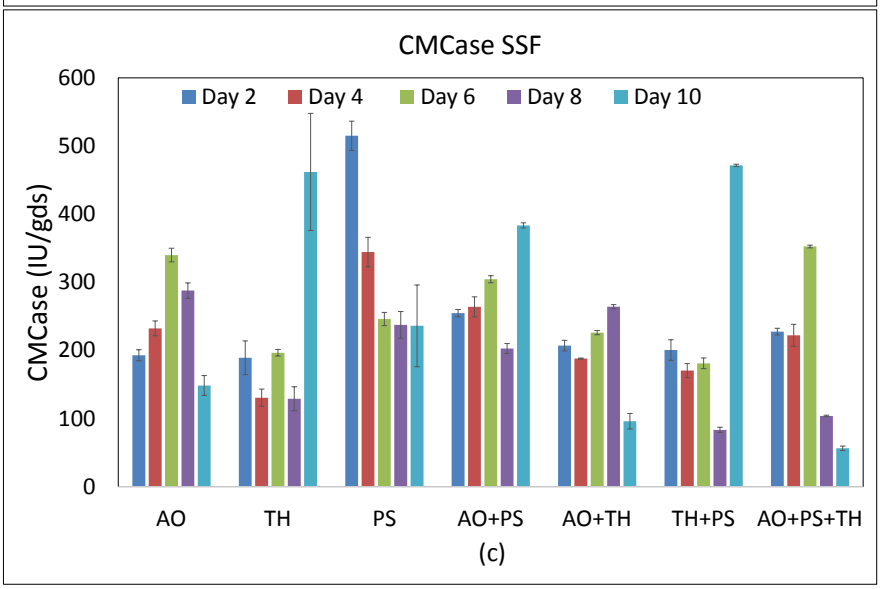
Under both SSF and SmF conditions, there is a continuous increase in FPU and CMCase until 6th day; increase in β-glucosidase until the 4th day, for all the culture combinations undertaken in this study. After the 6th and 4th day, the activity either stayed stable or declined, respectively. The authors had observed a similar trend in their previous study involving cellulase production from biomass under SSF and SmF conditions for individual and binary cultures [26]. The reason behind this trend could be attributed to the exhausting of available monosaccharide sugars, which are released by fungi during saccharification of oligosaccharides derived from cellulose. Decline of these sugars, especially glucose will signal the fungi to release more hydrolytic enzymes resulting in more sugars formation; however, the time interval at which the optimum enzyme activity occurs for these different classes of enzymes varies. This cycle would continue until all the sugars are consumed completely.

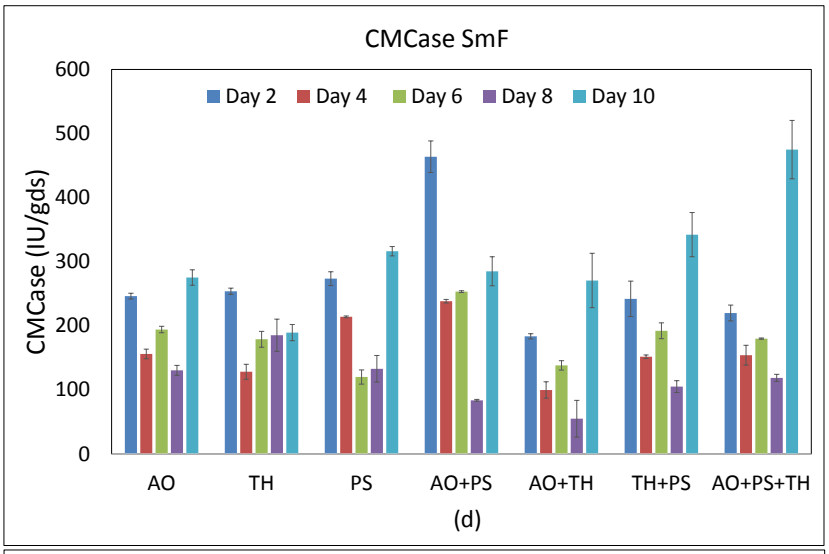
This explains why we observe rise in enzyme activity on 6th day and again on 10th day for CMCase and β-glucosidase. Cellulase (FPU/gds), CMCase (IU/gds) and β-glucosidase (IU/gds) of 40.5 ± 4.84, 192.8 ± 8.1 and 177.3 ± 6.9 are produced respectively for mono-culture (AO) on 2nd day which is higher than reported [27]. Cellulase, CMCase and β-glucosidase activity in the range 74.0 - 154.2 (FPU/gds), 180.9 - 471.5 (IU/gds), and 180.6 - 956.6 (IU/gds), respectively, for combination of cultures in 6 - 10 days were observed in SSF (**Figure 1**). With mixed fungal cultures, we observed higher productivity over individual cultures. Earlier studies on agro wastes also established that mixed cultures (*A. niger* and *T. reesei*) exhibit synergism over mono cultures in SSF conditions [28].

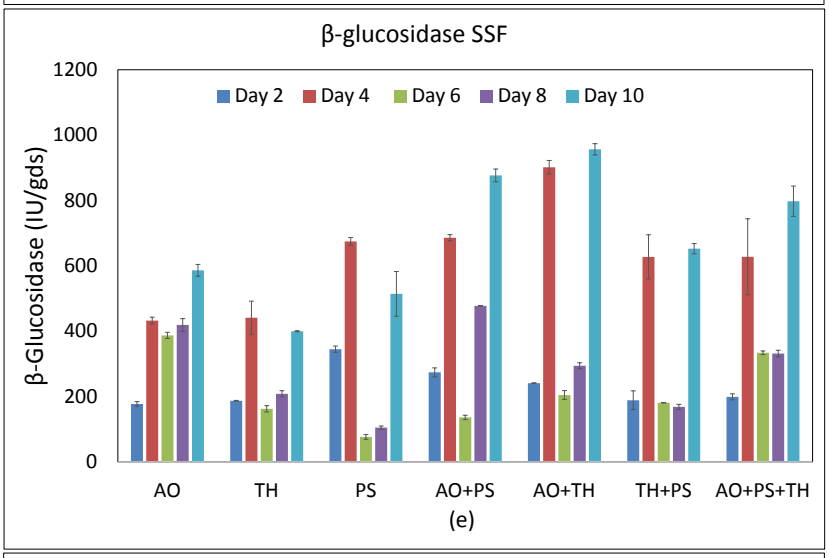
Cellulase activity: In SSF condition (**Figure 1(a)**), highest activity of 154.2 ± 1.6 FPU/gds and 123.8 ± 3.7 FPU/gds was observed on the 6th day for AO + PS and AO + PS + TH combinations respectively. In SmF conditions (**Figure 1(b)**), highest activity of 154.27 ± 4.7 FPU/gds and 139.05 ± 11.8 FPU/gds was observed on the 6th day for AO + PS combination and monoculture of PS, respectively.


Cellulase SSF
Day 2
Day 4
Day 6
Day 8
Day 10
Cellulase (FPU/gds)
200
180
160
140
120
100
80
60
40
20
0
AO
TH
PS
AO+PS
AO+TH
TH+PS
AO+PS+TH
(a)


Cellulase SmF
Day 2
Day 4
Day 6
Day 8
Day 10
Cellulase (FPU/gds)
200
180
160
140
120
100
80
60
40
20
0
AO
TH
PS
AO+PS
AO+TH
TH+PS
AO+PS+TH
(b)


CMCase SSF
Day 2
Day 4
Day 6
Day 8
Day 10
CMCase (IU/gds)
600
500
400
300
200
100
0
AO
TH
PS
AO+PS
AO+TH
TH+PS
AO+PS+TH
(c)





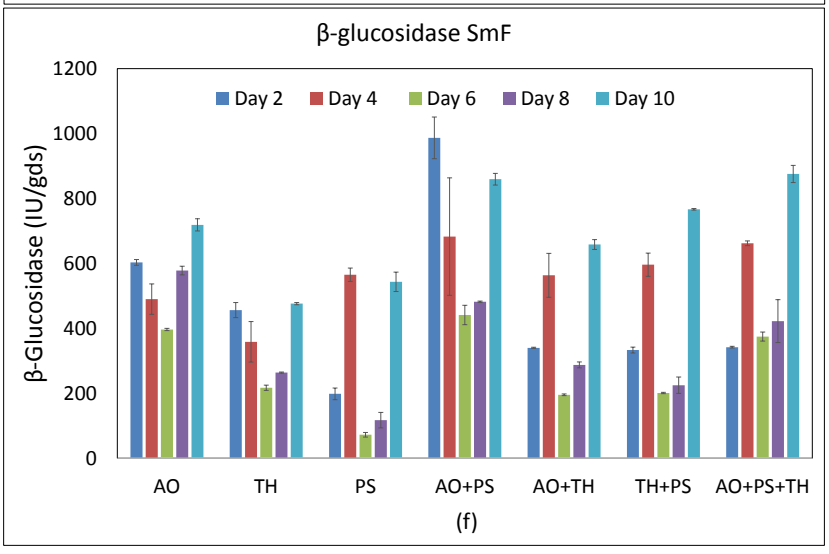


Figure 1. Quantification of enzyme activity of combinations of AO, PS and TH: Cellulase—(a) SSF; (b) SmF; CMCase—(c) SSF; (d) SmF and *β*-glucosidase—(e) SSF; (f) SmF.

CMCase activity: The increase in CMCase activity was significant on 6th day for SSF across mono and combination of cultures whereas independent PS released greater amounts of CMCase on 2nd day that decreased gradually on 10th day. The CMC activity of SSF (**Figure 1(c)**) for ternary combination of AO + PS + TH was maximum on 6th day at 352.4 ± 2.1 IU/gds; and 10th day at 471.5 ± 1.7 IU/gds for TH + PS. In SmF conditions (**Figure 1(d)**), the activity was only 179.9 ± 1.0 IU/gds (6th day) for ternary combination of AO + PS + TH and 342.12 ± 34.5 IU/gds for TH + PS suggesting that SSF conditions are more favourable over SmF conditions. The reason could be the presence of glucose in fermentation medium in higher concentration thus causing catabolic repression. Although CMCase activity declined for PS after 48 hours, an increase in CMCase was observed in combination with AO and ternary combination of AO and TH. The increase was significant for ternary combination of fungi on 6th day compared to other mono cultures.

β-glucosidase activity: *β*-glucosidase production was maximum on 4th day for all the combinations of fungi for both SSF and SmF conditions. Highest value of 797.6 ± 46.5 IU/gds and 876.7 ± 19.8 IU/gds was observed for 10th day in AO + PS + TH and AO + PS combination, respectively, in SSF condition (**Figure 1(e)**). In SmF condition (**Figure 1(f)**), 875.63 ± 26.4 IU/gds and 987.0 ± 64.2 IU/gds was observed for AO + PS + TH and AO + PS combination, respectively; suggesting that *β*-glucosidase production is promising in both SSF and SmF conditions.

From our studies (**Table 1**), we can deduce that to attain high activity of cellulase enzyme a ternary combination of AO + PS + TH cultures under SmF condition is conducive, whereas, monoculture of PO gave high activity of CMCase under SSF condition. Further, binary combination of AO + PS under SmF condition provided very high *β*-glucosidase activity. Our promising approach for targeted enzymes production is summarized in **Table 1**. Thus, we can conclude that depending on the particular class of enzyme required for production, an appropriate combination of fungal consortia and the method of fermentation will provide the desired high activity.

3.2. Characterization

3.2.1. Biomass Composition

Table 2 showed that the groundnut biomass had around 40% lignin content, which is almost equal to the total carbohydrate content in the biomass. Typically, lignin content of groundnut shell is around 26.4% as reported by Patricia *et al.* [29]. However, the plant lignin content is significantly affected by its genotype as well as the environment in which the plant is cultivated [30]. Boonmee reported 35.2% lignin content in peanut shell [31], which is in close agreement with our reported values. This clearly shows that some cultivars of GNS grown at specific agronomic environment could have higher lignin content, and hence it is important to consider the lignin content while designing fermentation procedure to generate value-added products.

Table 1. Combination of fungal cultures for efficient enzyme production on GNS.

Enzyme	Activity	Consortia	Mode
Cellulase	154.2 ± 1.63 FPU/gds	AO + PS	SmF
CMCase	514.97 ± 21.4 IU/gds	PS	SSF
β-glucosidase	875.63 ± 1.68 IU/gds	AO + PS + TH	SmF

Table 2. Composition of groundnut shell biomass.

Biomass Component	Composition of alkali extracted biomass	Composition of raw biomass
Glucan (%)	29.80 ± 0.36	24.70 ± 0.48
Xylan (%)	13.25 ± 0.75	13.47 ± 0.36
Arabinan (%)	0.91 ± 0.03	2.0 ± 0.14
Lignin (%)	39.80 ± 0.47	41.74 ± 0.49
Extractives (%)	3.80 ± 0.12	5.91 ± 0.63

In our subsequent experiments, the milder alkali pretreatment did not generate sufficient sugars with concomitant poor ethanol yield (results not included). However, the pretreated biomass was a promising feedstock to produce cellulolytic enzymes system as shown in our results. **Table 2** shows the detailed composition of GNS before and after alkali pretreatment.

3.2.2. Effect of Enzyme Treatment on Cellulose Crystallinity

Biomass constituted of amorphous and crystalline components. Hemicellulose and lignin are amorphous in nature. Cellulose contains both amorphous and crystalline allomorphs such as para-crystalline cellulose and crystalline cellulose allomorphs (cellulose I_α and cellulose I_β). Crystallinity of cellulose is an important parameter because material properties depend on crystal lattice structure, crystallite dimensions and crystallinity. Studies to track the ultrastructural changes of cellulose [32], observed depletion of allomorphs of cellulose during enzymatic hydrolysis with cellulase. Enzymatic deconstruction of cellulose follows the trend of rapid depletion of para-crystalline and amorphous cellulose, followed by cellulose I_α and cellulose I_β. This study suggests that cellulose crystallinity along with crystal lattice structure affect enzymatic hydrolysis and thus biomass recalcitrance. The powder X-ray Diffraction pattern of untreated, alkali treated and enzyme treated are presented in **Figure 2**. There are three broad peaks at 16.2˚, 22.1˚ and 34.7˚ for enzyme treated GNS which is consistent with cellulose I lattice. The fraction of cellulose crystallinity was calculated using the following equation:

$$CrI = \frac{I_{\text{total}} - I_{\text{am}}}{I_{\text{total}}} \times 100$$

where CrI is the Crystallinity Index, I_{total} is intensity of the (002) peak at 22.4˚ and I_{am} is the minimal depression of amorphous structure (intensity of the background scatter at 18.7˚) [33].

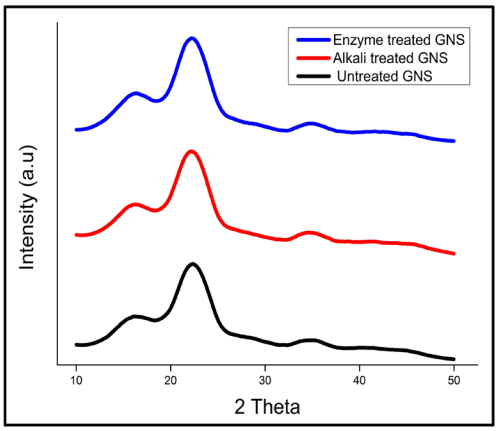


Figure 2. Powder XRD patterns of GNS: untreated, alkali treated and enzyme treated.

Treatment of GNS with alkali, influenced the crystallinity due to change in biomass composition. The raw biomass displayed lower value of 37.4% because it contains larger amounts of hemicellulose and lignin in corroboration with our biomass composition study in **Table 3**. CrI changed to 40.0% from 37.4% after alkali treatment because of removal of hemicellulose and lignin by the action of NaOH as shown in **Table 3**. The crystallinity index of fungal treated biomass turned out to be larger than the alkali treated and also varied from mono cultures to ternary cultures as shown in **Table 3**. Ganguly *et al.* observed that the fungal treatment affects amorphous cellulose more than the crystalline fragment of cellulose [34]. We conclude that the combinations of cultures have diverse effects on the composition of biomass depending on the enzymes released and treatment conditions.

3.3. FT-IR Analysis

The FT-IR spectra of GNS samples indicated chemical changes in the biomass upon pretreatment and enzyme saccharification. Variation in the absorbance values provided understanding of changes in biomass substrate composition and partial removal of lignin. Absorbance values of the analysed peaks displayed a close trend of variation, which corresponds to the cellulose, hemicellulose and lignin content as shown by a vertical line in **Figure 3** whose corresponding peaks assigned to functional groups are displayed in **Table 4**. The glycosidic linkage in hemicelluloses and lignin display a peak at 850 cm^{-1} while the 917 cm^{-1} peak was attributed to cellulose, hemicellulose and lignin's glycosidic linkage. C-O valence vibration of cellulose displays a peak at 993 cm^{-1}. Peak at 1072 cm^{-1} is due to glycosidic linkage C-O, C-C-O stretching of three polymers.

Asymmetric stretching of C-O-C in cellulose and hemicellulose could possibly result as peak at 1111 cm^{-1}. Intense peak at 1220 cm^{-1} is due to C-O stretch in lignin due to alcohol groups. Syringyl ring of lignin displays a peak at 1322 cm^{-1} due to C-O stretch clearly demonstrates decrease in lignin content from untreated GNS. Cellulose and hemicellulose in GNS showed an intense peak at

1400 cm^{-1} and 1384 cm^{-1} due to aliphatic C-H band. Dominant peak at 1590 cm^{-1} is attributed to aromatic ring C = C stretch of lignin which changed after alkali treatment due to removal of lignin. The broad absorbance peak in 3400 - 3000 cm^{-1} is due to surface hydroxyl groups stretching vibration and increased remarkably after alkali treatment indicating the exposure of cellulose and hemicellulose. Summary of functional groups present in the biomass and the corresponding absorbance values are given in Table 4. In our previous study, we observed that changes in the functional group intensities of the biomass help in comprehending the structural variation of biopolymers [35].

Table 3. Degree of crystallinity of fungal treated GNS in SSF conditions on 10th day.

Enzyme treated GNS	Degree of crystallinity
Untreated GNS	37.4
Alkali treated GNS	40.0
AO	54.0
TH	55.2
PS	49.2
AO+PS	49.7
AO+TH	51.0
PS+TH	49.1
AO+PS+TH	51.3

Table 4. FT-IR spectral analysis of the enzyme treated biomass and peak assignments.

Wave numbers (cm^{-1})	Assignment	Polymer	References
875	Glycosidic linkage	Hemicellulose	[26]
930	Glycosidic linkage	Cellulose, Hemicellulose, Lignin	
990	C-O valence vibration	Cellulose	
1035	C-O, C = C, C-C-O vibrational stretching	Cellulose, Hemicellulose, Lignin	
1160	C-O-C asymmetrical stretching	Cellulose, Hemicellulose	
1215	C-C + C-O stretching	Lignin	[33]
1327	C-O of syringyl ring	Lignin	
1335	C-H bending, O-H in-plane bending	Cellulose, Hemicellulose, Lignin	
1380	C-H bending	Cellulose, Hemicellulose, Lignin	
1440	O-H in-plane bending	Cellulose, Hemicellulose, Lignin	
1595	Aromatic ring C = C vibration	Lignin	[35]
3343	-OH stretching	Cellulose, Hemicellulose, Lignin	

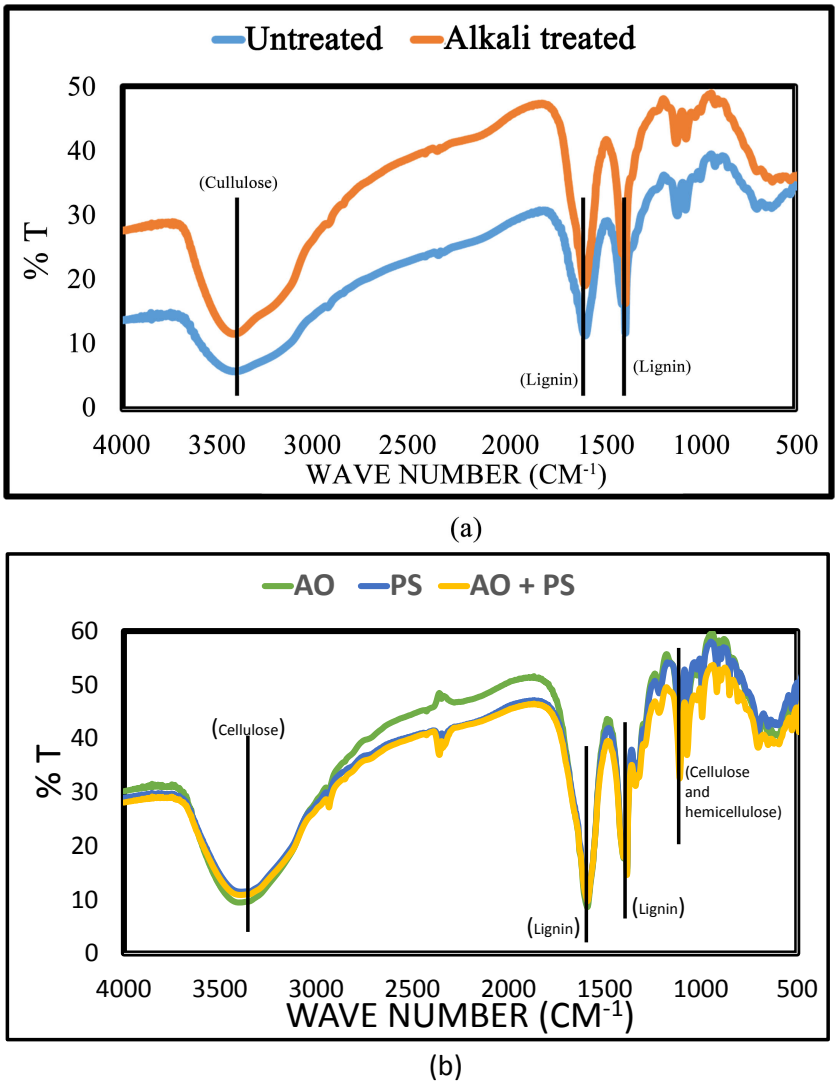


Figure 3. FT-IR of the (a) untreated GNS and alkali treated GNS; (b) AO, PS, AO + PS combinations respectively.

3.4. Thermogravimetric Analysis (TGA)

TGA analysis of the raw, alkali-treated and fungal-treated substrates was performed to test their thermal decomposition. Thermogravimetry curve which records weight change of the biomass as a function of temperature *i.e.*, the thermal behaviour of biomass on pyrolysis. First derivative of TGA known as differential thermogravimetry curve (DTG) gives the rate at which the reaction is undergoing. The DTG thermogram in **Figure 4** revealed prominently three peaks in the exothermic regions in agreement with previous studies [26].

Degradation of GNS happened in three stages. In stage one, at around 85˚C - 120˚C in which, bound water desorption occurs along with weakening of hydrogen bonds between the carbohydrates [36]. In stage two, degradation occurs around 200˚C - 350˚C where decomposition of low volatile carbohydrates such as cellulose and hemicellulose is observed. Hemicellulose starts to decompose

earlier due to its linear polymer chain structure and short side chains and at about 325˚C, relatively more stable crystalline cellulose starts to degrade between 325˚C - 400˚C.

In **Figure 4(a)**, raw GNS contain great amounts of lignin covering the cellulose and hemicellulose, whereas alkali treatment disrupts the lignin bonding with holocellulose. It is observed in **Figure 4(b)** where the exposed cellulose is resulting in a peak corresponding to cellulose degradation. In **Figure 4(c)**, the effect of fungal treatment is observed with the loss of cellulose peak due to the action of cellulases on the alkali treated GNS. In stage three, above 400˚C, reveals higher volatiles degradation such as lignin which is relatively more stable than cellulose and hemicellulose. The process displays exothermic effect till 735˚C, corresponding to the char aggregation and the heat produced is the result of the degradation of biomass [37]. This is in correlation with the data reflected in biomass composition (**Table 2**) and XRD data.

3.5. SEM Imaging

Microbe fortified biomass consisting of the various combinations of fungal cultures in both SSF and SmF conditions were analyzed for surface morphological changes using SEM. Untreated GNS display high roughness with hollow cavities and after alkali treatment the surface smoothened indicating deconstruction of lignocellulosic linkages in **Figure 5**. A & B thereby suggesting that the cellulosic fibers are more exposed.

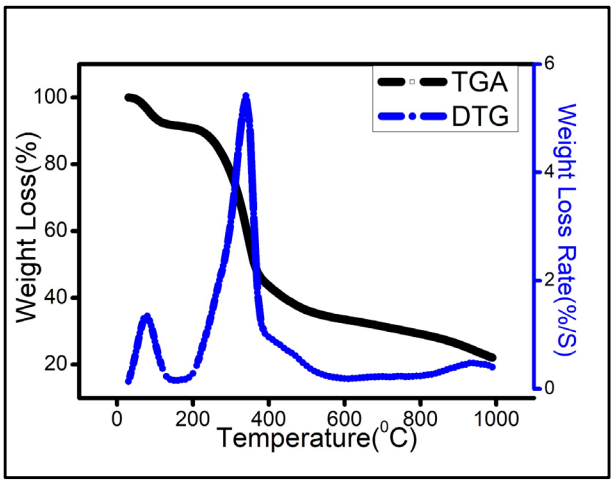
Further on enzymatic treatment with binary (AO + PS) and ternary cultures (AO + PS + TH) it was observed that the texture exhibited more porosity indicating bioconversion of cellulosic fibres as observed in **Figure 5(c)** and **Figure 5(d)**. During alkali treatment, the cell walls collapses and the fibres are detached along with formation of pores, which is evident in AO + PS + TH combination of **Figure 5(e)**. In ternary culture of GNS with AO + PS + TH **Figure 5(f)**, the result of enzymatic action on GNS disturbed the integrity of surface, forming pronounced pits on the surface; thereby indicating that the ternary consortia was more efficient in the systemic breakdown of the heteropolymers present in the biomass.

Cellulase and CMCase production improved significantly till 6th day in mixed culture fermentation whereas β-glucosidase production was produced in greater quantities. The alteration in the physico-chemical attributes in biomass composition due to alkali treatment and enzyme treatment was thoroughly studied using characterization techniques and found to be culture specific. The FT-IR spectroscopy along with X-ray crystallography studies of alkali treated and enzyme treated substrates established the changes in nature of holocelluloses. DSC and TGA revealed the changes in the structural integrity of biomass.

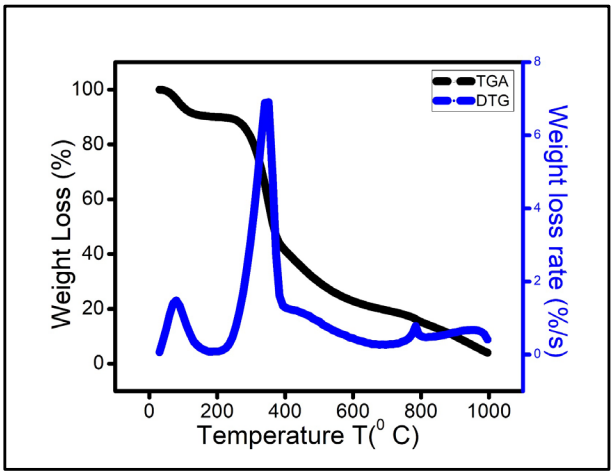
4. Conclusions

Effective hydrolysis of cellulose present in biomass requires a synergistic action

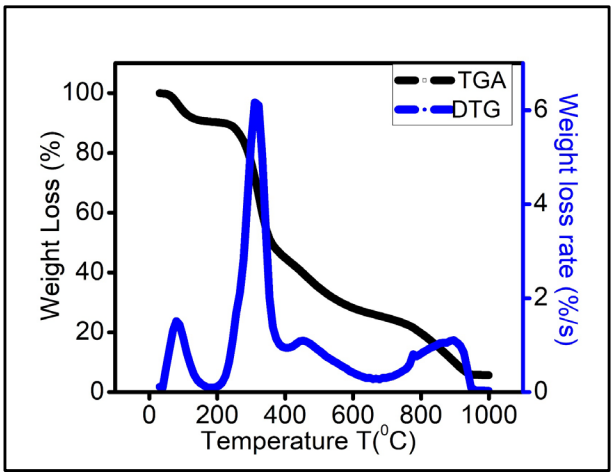
of three different classes of enzymes. Presence of microbial consortia for enzymatic hydrolysis will undoubtedly lead to the generation of these classes of enzymes.



(a)



(b)



(c)

Figure 4. TGA graphs of effect of thermal behaviour of GNS (a) raw, (b) alkali treated and (c) fungal treated.

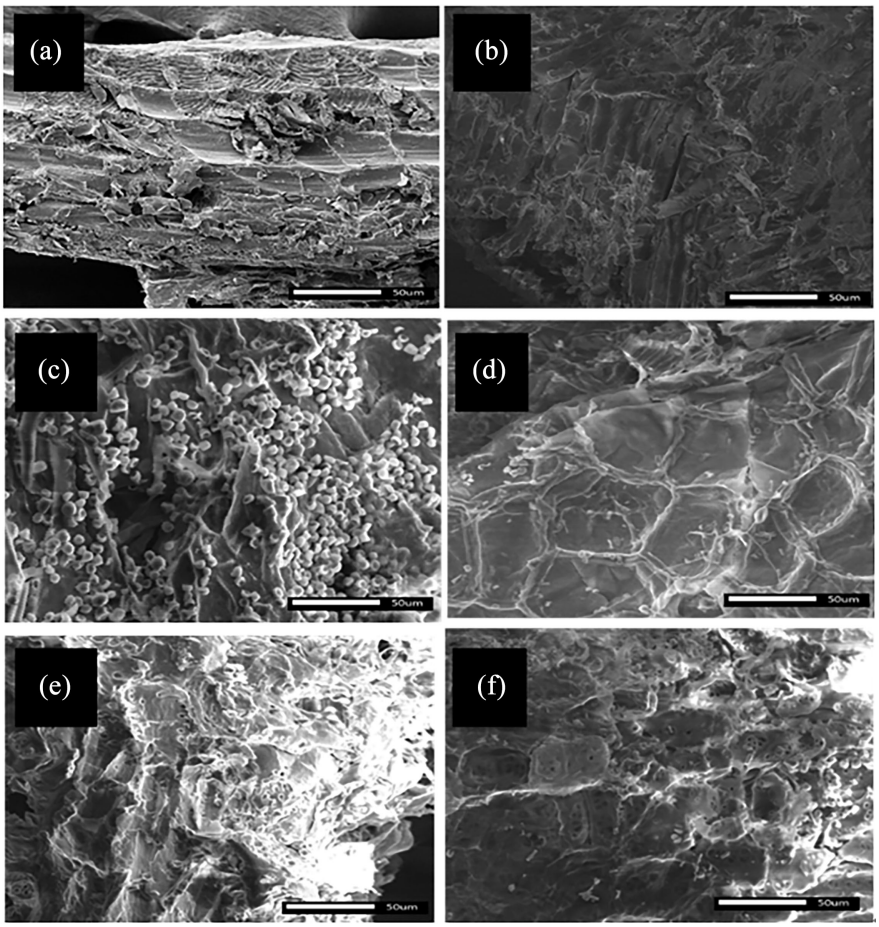


Figure 5. (a) Untreated GNS (b) Alkali treated GNS, AO + PS in (c) SSF and (d) SLF, AO + PS + TH in (e) SSF and (f) SLF.

To the best of our knowledge, current work demonstrated for the first time, the synergistic effect of enzymes production from ternary cultures of *A. oryzae* (epiphytic fungus), *T. harzianum* (endophytic fungus) and *P. sanguineus* (macro fungi) with significant amount of enzymes production from GNS. In nature, the saprophytic fungal cultures work as a synergistic consortium to efficiently decompose dead biomass; in our innovative study we carefully selected ternary cultures that will act in tandem and deliver the desired enzymes system of importance for bioconversion. We also conclude that the interplay of the combination of cultures, fermentation system and duration of the experiments has a pronounced effect on the individual class of enzyme activity, which suggests that we must pick the appropriate combination of cultures and operation mode if our interest is specific to an enzyme.

The alteration in the physico-chemical attributes in biomass composition due to alkali treatment and enzyme treatment was thoroughly studied using characterization techniques and was found to be culture specific. The FT-IR spectroscopy along with XRD studies of alkali treated and enzyme treated substrates provide molecular level understanding of delignification upon alkali and enzy-

matic treatment, respectively. DSC revealed that degradation of GNS happened in three stages namely *via* water removal followed by cellulose, hemicellulose and lignin deconstruction; crucial for understanding the structural integrity of biomass.

As per the above study, the following recommendations can be made:

1) Before the utilization of any specific biomass feedstock for value-addition, it is imperative to determine the compositional analysis of the feedstock; based on the sugars and lignin content an effective bioconversion strategy can be optimized for a targeted product.
2) The characterization of the substrate before, during, and after the bioprocessing is a rationale tool for process analysis and provides a framework for the better design of the sequential enzymatic degradation to sugars followed by fermentation process to biofuels and other products.
3) Further, the structural analysis of the biomass residue will enable its appropriate utilization for potential high-valued carbon materials such as carbon dots [38] and supercapacitors for renewable energy applications.
4) In addition to cellulase enzyme production, judicious selection of microbial consortia is required to generate hemicellulolytic and ligninolytic enzyme systems for complete utilization of all the three polymers present in biomass for sustainable value addition.
5) It makes more economic sense to pursue multiple products from the same feedstock and accordingly develop an integrated process to realize this goal.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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