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A concerted enzymatic de-structuring of lignocellulosic materials using a compost-derived microbial consortia favoring the consolidated pretreatment and bio-saccharification

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Abstract

The robustness of microbial consortia isolated from compost habitat encompasses the complementary metabolism that aids in consolidated bioprocessing (CBP) of lignocellulosic biomass (LCB) by division of labor across the symbionts. Composting of organic waste is deemed to be an efficient way of carbon recycling, where the syntrophic microbial population exerts a concerted action of lignin and polysaccharide (hemicellulose and cellulose) component of plant biomass. The potential of this interrelated microorganism could be enhanced through adaptive laboratory evolution (ALE) with LCB for its desired functional capabilities. Therefore, in this study, microbial symbionts derived from organic compost was enriched on saw dust (SD) (woody biomass), aloe vera leaf rind (AVLR) (agro-industrial waste) and commercial filter paper (FP) (pure cellulose) through ALE under different conditions. Later, the efficacy of enriched microflora on consolidated pretreatment and bio-saccharification were determined based on substrate degradation, endo-enzymes profiling and fermentable sugar yield. Among the treatment sets, AVLR biomass treated with enriched consortium (EC-5) has resulted in the higher degradation rate of lignin (47.01 ± 0.66%, w/w) and polysaccharides (45.87 ± 1.82%, w/w) with a total sugar yield of about 60.01 ± 4.24 mg/g. In addition, the extent of structural disintegration of substrate after EC-treatment was clearly deciphered by FTIR and XRD analysis. And the factors of Pearson correlation matrix reinforces the potency of EC-5 by exhibiting a strong positive correlation between the AVLR degradation and sugar release. Thus, a consortium based CBP could promote the feasibility of establishing a sustainable second generation biorefinery framework.

Keywords: Adaptive laboratory evolution; Aloe vera leaf rind; Bio-saccharification; Consolidated pretreatment; Micro-symbionts; Fermentable sugars

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1. Introduction

Lignocellulosic biomass (LCB) is the most abundant renewable carbon resource generated from forestry, agricultural and industrial sector accounts to 200 billion tons per annum, where only 3% of LCB are efficiently used in the circular bioeconomy [1–4]. The heterogeneity and the complex nature might be the major obstacle in surpassing the LCB valorization into biofuels by limiting the conversion efficiency and the techno-economic feasibility. In addition, initially biorefineries are focused only on the valorization of cellulose into a single product (biofuel) by overlooking the hemicellulose and lignin content of LCB [5,6]. Therefore, choice of appropriate conversion strategy and integration of the process for complete valorization of biomass into multiple products (i.e., biofuels and value-added chemicals) could aid in intensifying the LCB-based biorefinery. In this regard, microbial valorization of LCB through consolidated bioprocessing (CBP) is being considered as a highly specific, economical and sustainable strategy in biomass-based biorefineries [7]. CBP integrates the conversion process like hydrolytic enzymes production, saccharification and fermentation which provides a cutting-edge option to revolutionize the entire biorefinery framework [8]. In recent times, consolidated bio-saccharification (CBS) integrating the cocktail enzymes (cellulase and hemicellulase) production and saccharification seems to be a promising alternative for the CBP strategy where CBS separates the fermentation from the integrated process. This on-site saccharification approach of combining lignocellulosic enzyme production and hydrolysis enhances the conversion efficiency, simplifies the operation process, thereby reducing the overall capital and operating cost as compared to the conventional off-site strategies such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) [3,9]. Further to maximize the industrial feasibility of CBS, a subtle and energy extensive pretreatment method has to be intended owing to the complexity of lignin that encases the holocellulose (cellulose and hemicellulose) [2]. An efficient pretreatment

process should exhibit effective depolymerization of lignin, high polysaccharides recovery, and compatible with the subsequent hydrolysis step without any associated inhibitory by-products [3,10]. Notably, in several ecological niches such as peat, termite guts, cattle rumens and compost piles which harbor the microbial symbionts have been found to be evolved to tackle the recalcitrance of the LCB for complete degradation. These natural systems could serve as an ideal paradigm for consolidated pretreatment and bio-saccharification by mimicking the innate microbial degradation of LCB in a bioreactor mediated by the enzymes such as ligninases and carbohydrate active enzymes (CAZymes) respectively [11–15]. Exploration of thermophilic group of microbes in industrial bioprocessing of LCB is proven to be advantageous owing to rapid hydrolysis, enhanced productivity and lower risk of microbial contamination [16,17]. During composting of organic waste there is a prevalence of different temperature regimes in the pile such as mesophilic (surface) and thermophilic (sub-surface) that harbors diverse group of microbial symbionts that can be tapped for industrial bio-processing [18]. Further, enrichment of microbial symbionts through adaptive laboratory evolution (ALE) strategy could aid in establishing a microbial consortium with desired functional characteristics. A considerable adaptation process was generally performed for a definite time period (short-term and long term) to accustom the microbes with various biomolecules of LCB under different conditions [19]. Several studies have reported the isolation and characterization of the cellulolytic bacterial community from compost through conventional techniques [20–23]. Wang et al. [24] have reported the metagenomic analysis of compost derived microbial consortium enriched with rice straw in a nylon bag which revealed the role of distinct microbial community associated with a repertoire of CAZyme profiles in substrate degradation. However, these studies never explored the efficacy of compost-derived microbial consortium on varied substrates such as woody biomass (high lignin content- 25-30% lignin) and agro-industrial residues (moderate lignin content-15-20%) which can be

expected as substrates in an industrial biorefinery scenario [25]. Therefore, the present study focuses on establishing a microbial consortium for consolidating the pretreatment and bio-saccharification of LCB with model substrates such as saw dust (SD) (woody biomass) and aloe vera leaf rind (AVLR) (agro-industrial residue). To the best of authors' knowledge, this is the first comprehensive study on microbial community from compost piles with industrial substrates. An ALE strategy was adopted for enriching the microbial community and probing the key players involved in LCB degradation that could pave a way for establishing a consortium-based biomass processing in an integrated biorefinery framework. In addition, the efficacy of compost-derived consortia under different operating temperatures such as mesophilic and thermophilic conditions were also studied to elucidate their influence on lignin degradation and bio-saccharification by probing the modulations in the extracellular enzymes (Lignin peroxidase (LiP), Manganese peroxidase (MnP), laccase and CAZyme) and products (fermentable monomeric sugars) released, as represented in Fig. 1. Overall, this study has explored on feasibility of CBP and development of consortia from compost for LCB based biorefineries.

2. Materials and methods

2.1. Microbial inoculum

The compost sample derived from organic waste was collected from the local composting yard located at Kattankulathur, Chennai, India. The compost sample was randomly taken from the different sites of the compost pile and mixed to be used as a source of inoculum for the enrichment studies. The collected sample was immediately transported to the laboratory and utilized for the further studies.

About 5 g of organic compost sample was used to inoculate 48 mL of mineral salt media (MSM) containing the following constituents (g/L): $NaNO_3$- 2; KCl - 0.5; $MgCl_2$- 0.5;

KH_2PO_4- 1.5 and 1% (w/v) of glucose as a carbon source. The MSM was supplemented with vitamin solution (1 mL) sterilized using syringe filter (0.2 μm) and 1 mL of trace elements solution. The trace elements solution has the following components (g/L); H_3BO_3- 0.3; $FeSO_4.7H_2O$- 0.1; $CoCl_2.6H2O$- 0.19; $MnCl_2$- 0.05; $ZnCl_2$- 0.042; $NiCl_2$- 0.024; Na_2MnO_4- 0.018 and $CuCl_2$- 0.002. The culture was maintained at different conditions like mesophilic (37 ± 2 ˚C) and thermophilic temperature (45 ± 2 ˚C) at pH- 7.0 with a constant shaking of 150 rpm under aerobic (48 h) and anaerobic (72 h) conditions. The anaerobic condition was maintained by adding a reducing agent i.e., 1% (w/v) of cysteine hydrochloride to the medium, flushed with nitrogen gas followed by sealing with rubber stopper and aluminum crimp. The experimental design encompasses four different conditions i.e., mesophilic-aerobic (M1), mesophilic- anaerobic (M2), thermophilic-aerobic (T1) and thermophilic-anaerobic (T2) and henceforth referred as seed inoculum for further enrichment studies.

2.2. Lignocellulosic feedstock

Different LCB such as woody biomass- saw dust (SD) and an agro-industrial waste- aloe vera leaf rind (AVLR) were chosen as the carbon source for the enrichment of compost-derived microflora. In addition, commercial filter paper (FP) is used as a pure cellulosic substrate for enrichment to study the potential of microbial consortia towards bio-saccharification. The substrate selection was based on the lignin and holocellulosic (hemicellulose and cellulose) content i.e., substrate rich in lignin (SD) to pure cellulose (FP). The SD and AVLR biomass were procured from the local wood processing and aloe gel processing unit respectively. The AVLR was sun-dried and subjected to the pulverization by mixer. The dried biomass was passed through a sieve mesh (2 mm) to obtain a constant particle size and stored in an air-tight container for later use.

2.3. Adaptive laboratory evolution (ALE) strategy for enrichment of compost-derived microbial community

ALE strategy was adapted to progressively enrich the lignocellulose degrading microbial consortia derived from organic compost. ALE was performed by the sequential sub-culturing of seed inoculum (M1, M2, T1 and T2) at a regular time interval of 96 h for 5 enrichment cycles containing 'C' source of 0.5% (w/v); 1% (w/v); 1.5% (w/v); 2% (w/v) and 2.5% (w/v) with different substrates (SD, AVLR and FP) (Fig. S1). The microbial adaptation was carried out in a 100 mL serum vial containing enrichment media which has the following constituents namely (g/L): $NaNO_3$- 2; KCl - 0.5; $MgCl_2$- 0.5 and KH_2PO_4- 1.5; supplemented with trace elements solution (1 mL) and vitamin solution (1 mL). A working volume of 50 mL was inoculated with 5 mL of seed inoculum (10%, v/v) and aseptically maintained at four different conditions (M1, M2, T1 and T2) for a period of 96 h. The enriched consortium (EC) obtained after 5^{th} cycle were thereafter named as EC-1 to EC-12 as show in the supplementary figure (Fig. S1).

2.4. Consolidated pretreatment and bio-saccharification

The enriched consortium (EC-1 to EC-12) were used as an inoculum to characterize the dynamics of microbial consortia towards consolidated pretreatment and bio-saccharification. About 5 g of substrate (SD, AVLR and FP) was inoculated with 5 mL of EC (10%, v/v) (i.e., one obtained after 5^{th} enrichment cycles) in a serum vial containing MSM (total working volume-50 mL) supplemented with vitamin solution (1 mL) and trace elements solution (1 mL). All the vials were incubated for a period of 7 days under different conditions (M1, M2, T1 and T2).

The experimental design encompasses 12 sets with different substrate are named as follows: EC-1(SD-M1), EC-2(SD-M2), EC-3(SD-T1), EC-4(SD-T2), EC-5(AVLR-M1), EC-6(AVLR-M2), EC-7(AVLR-T1), EC-8(AVLR-T2), EC-9(FP-M1), EC-10(FP-M2), EC-

11(FP-T1) and EC-12(FP-T2). The same microbial source from enrichment sets are used for the treatment studies. In addition, a control set namely without inoculum (WOI) for all the chosen substrate was placed under the similar conditions. The degradation percentage (lignin, hemicellulose, cellulose and total substrate) with different treatment conditions were calculated as the relative degradation with the control (without inoculation).

2.4.1. Analysis of LCB degradation

To evaluate the potential of microbial consortia towards substrate degradation, the structural components (lignin, hemicellulose and cellulose) analysis of substrate before and after microbial treatment (on 7^{th} day) was performed. The solid residues obtained after centrifugation (5000 rpm for about 10 mins at 4 ˚C) was oven-dried at 50 ˚C until constant weight attained. The lignin [26], hemicellulose [27] and cellulose [28] content of the substrate were determined based on the standard National Renewable Energy Laboratory (NREL) and Anthrone method, respectively. Total degradation was determined based on the volatile solid content, where 1 g of dried sample was taken in crucible and kept in a muffle furnace at 550 ˚C for about 4 h for complete incineration and the final weight was noted [29].

2.4.2. Secretome analysis of enriched microbiota derived from organic compost

The distinct enzymatic degradation potential of microbial consortia was determined by the estimation of various extracellular enzyme activities related to lignin, hemicellulose and cellulose breakdown. After 7 days, the supernatant (metasecretome) was recovered by centrifugation for 10 min at 5000 rpm at 4 ˚C. The major ligninolytic enzymes (lignin modifying oxidases/peroxidases) such as multicopper phenol oxidase (laccases) and heme containing peroxidases (Lignin peroxidase- LiP and Manganese peroxidase- MnP) were measured using 2,2′ - azino-bis(3-ethyl-benzothiazoline- 6-sulfonic acid) (ABTS), veratryl alcohol and phenol red as a substrate respectively [30–32]. Whereas, hemicellulolytic and

cellulolytic enzyme activity were determined using xylan (1%, w/v), Carboxymethyl Cellulose (CMC) (1%, w/v) and FP as substrate respectively based on the standard assay protocol reported in the previous studies [33,34]. Total protein content (for specific activity of the enzyme) of the supernatant was estimated by Bradford method [35] using bovine serum albumin (Sigma-Aldrich, USA) as standard and the graph was plotted with R^2 value of 0.9805 (Fig. S2). Ligninolytic (LiP, MnP and laccase) (Eq. 1), hemicellulolytic (xylanase) and cellulolytic (CMCase and Total cellulolytic) (Eq. 2) enzyme activities has been calculated using the formula given below;

$$Liginolytic\ enzyme\ activity\ (LiP, MnP\ and\ laccase)\left(\frac{IU}{mL}\right) = \frac{Optical\ density \times 10^6}{\varepsilon \times Time\ (min) \times Volume\ of\ enzyme\ (mL)} \quad (1)$$

Where,

Molar extinction coefficient (ε) = 9300 $M^{-1}cm^{-1}$ (LiP), ε = 22000 $M^{-1}cm^{-1}$ (MnP) and ε = 36000 $M^{-1}cm^{-1}$ (Laccase)

$$Hemicellulolytic\ and\ cellulolytic\ enzyme\ activity\ \left(\frac{IU}{mL}\right) = \frac{Optical\ density \times slope^{-1} \times Dilution\ factor}{Molecular\ weight\ of\ xylose\ or\ glucose\left(\frac{g}{mol}\right) \times Time\ (min) \times Volume\ of\ enzyme\ (mL)} \quad (2)$$

The slope is obtained from the standard plot of xylose and glucose using dinitrosalicylic acid (DNS) method [36].

The specific enzyme activity is measured as IU/mg protein defined as units of enzyme required for catalyzing the μmole of substrates into product per min per mg of protein.

Further, the supernatant has been qualitatively analyzed for presence of various lignocellulolytic enzymes using high-performance liquid chromatography (HPLC) system equipped with LC10ADVP binary pump (Shimadzu, Japan). A mobile phase containing a mixture (70:30) of acetonitrile: water (0.1% formic acid) is passed through the C18 phenomenox column (250 × 4.6 mm × 5 μm) at a flow rate of 0.5 mL/min with isocractic

elution [37]. A sample volume of 20 µL of enzyme standards (LiP, MnP, peroxidase, xylanase and cellulase) and the experimental samples were prepared using phosphate buffer saline (pH-7). All the enzyme standards employed were prepared at a concentration of 2 mg/mL with 99.9% purity (Sigma-Aldrich, USA).

2.4.3. Estimation of fermentable sugars released

The fermentable hexose (C6 sugar -glucose) and pentose (C5 sugars- xylose and arabinose) sugar content during the microbial hydrolysis of various substrate was determined using dinitrosalicylic acid (DNS) [36] and orcinol method [38] respectively. The samples were collected at a regular time intervals of every 48 h and the supernatant separated by centrifugation at 5000 rpm for about 10 mins at 4 ˚C was used for reducing sugar analysis.

2.4.4. Structural characterization of the treated substrates by FTIR and XRD analysis

In order to elucidate the structural changes in the substrate mediated by the microbial action, FTIR and XRD were employed for the substrates (SD, AVLR and FP) that demonstrated higher degradation percentage upon treatment with EC-2, EC-5 and EC-9 respectively. After microbial hydrolysis, the substrates are separated from the culture by centrifugation at 5000 rpm for about 10 mins, followed by washing with distilled water and oven-drying at 60 °C to obtain a constant weight. To determine the functional group modification, FTIR analysis was performed for untreated and EC treated substrates using KBr pelleting technique (Agilent Technologies, Cary 600 Series, USA). FTIR spectrum was recorded within a scanning wavelength range of 4000 to 400 cm^{-1} in a transmittance mode with a spectral resolution of 0.5 cm^{-1} [39]. Further, lateral order index (LOI) and total crystalline index (TCI) were calculated from the IR absorption intensity ratio as given in equation (Eq. 3) and (Eq. 4),

$$Lateral\ order\ index\ (LOI) = \frac{A_{1437}}{A_{899}} \quad (3)$$

$$Total\ crystalline\ index\ (TCI) = \frac{A_{1378}}{A_{2900}} \quad (4)$$

The absorbance (A) was calculated from the transmittance value (T) of raw data using the

equation based on Beer's law (Eq. 5),

$$A = 2 - \log(\%T) \quad (5)$$

Crystalline nature of the treated substrate were analyzed using XRD instrument (Malvern Panalytical, AERIS—High resolution bench top XRD, UK) where the diffraction profiles were recorded using CuKα radiation at 40 kV with a scanning speed of 3°/min from 10 to 100° (2θ). Crystallinity index (CrI) of the substrates were calculated using an empirical peak intensity method as proposed by Segal et al. [40] for native cellulose.

2.5. Statistical analysis

The statistical values are average of triplicate experiments and the standard deviation is represented as (±) calculated from mean and three independent trials. The analysis was performed using MS office Excel 2019 and GraphPad Prism 8.0.1.

3. Results and discussion

Compost pile harboring symbiotic microbiome seems to be a promising model to understand the multispecies concerted degradation mechanism underlying the natural bioconversion of complex plant biomass. The endo-secretome of the compost derived microflora possess a wide variety of ligninolytic, hemicellulolytic and cellulolytic enzymes that act synergistically and specifically in plant biomass degradation. Opting symbiotic consortium for consolidated processing (pretreatment and biosaccharification) of recalcitrant LCB to achieve high degradation efficiency for fermentable sugars release serves as a vital approach for establishing a viable biorefinery framework. Several studies have explored the LCB degradation by treatment with different microbial consortium [24, 41-48]. However, the influence of different substrates and treatment conditions on enrichment of compost-derived microbial consortia remains overlooked. Therefore, in this study, the substrate type and treatment conditions are investigated as a driving factor for the enrichment of microbial

consortia, where the same microbial source serves as an inoculum for further degradation studies.

3.1. Substrate degradation efficiency and enzymatic profile of enriched symbiotic microcosms

The potential of EC (obtained after 5th cycle of enrichment) in consolidated pretreatment and bio-saccharification of various substrates under different conditions was assessed by substrate degradation capability (Fig. 2) and profiling of secreted endo-enzymes (Table1) involved in LCB degradation.

3.1.1. Lignin degradation

Lignin is the foremost non-carbohydrate cell wall component of the plant biomass where the phenolic and non-phenolic lignin moieties are targeted by the putative oxidases such as peroxidase (LiP and MnP) and laccase respectively for its breakdown. In this study, the ligninolytic potential of EC derived from organic compost was unveiled by evaluating the specific activity of ligninases associated with delignification using various substrate under M1, M2, T1 and T2 conditions.

A substantial degradation of lignin ranging from 23.21 ± 1.56% (w/w) to 47.01 ± 0.66% (w/w) was observed in AVLR and SD biomass treated with EC under different conditions as shown in Fig. 2. Among the treatment sets, the highest delignification percentage (47.01%, w/w) was observed with AVLR biomass treated with EC-5 which in turn correlated with a maximum ligninolytic activities (laccase- 120.45 ± 4.58 IU/mg, MnP- 17.24 ± 3.75 IU/mg and LiP- 1.82 ± 0.10 IU/mg) as compared to the other counterparts.

Earlier studies have reported that heme-containing peroxidases (MnP and LiP) are encompassed with higher redox potential which in turn enhances the overall degradation efficiency of lignin [49, 50]. Recently, Congfeng et al. [51] investigated the efficiency of microbial consortium in rice straw degradation where 31.18% of delignification was

observed after 7 days of incubation at 30 °C under static condition using mineral salt medium. Similar studies on microbial enrichment showed ~ 40% of lignin removal using switch grass [52] and wheat straw [53] as a sole carbon source. Further, in this study all the EC upon treatment with different substrates have shown the potency to secrete diverse ligninolytic enzymes which implies the effectiveness of the consortia towards degradation as compared to the single isolates [50]. Yang et al. [54] reported that maximum delignification of wheat straw was about 8% (7 days) and ~17% (15 days) using *Burkholderia sp. H1* isolated from rotten wood that has relatively low delignification as compared to the consortia model.

3.1.2. Holocellulose degradation

For a sugar based biorefinery platform, lignocellulosic sugars are vital intermediates for the biofuels and value added chemicals production where the polysaccharide (hemicellulose and cellulose) hydrolysis requires a series of cocktail endo-enzymes like xylanase and cellulases that could act synergistically [55-57]. Given the complexity of polysaccharide-degrading enzymes, the potential of EC in hydrolysis of the chosen substrate (AVLR, SD and FP) was investigated. Analysis of supernatant (meta-secretome) for EC upon treatment with various substrates have exemplified the presence of xylanase and cellulases (endoglucanase and total cellulolytic activity) to degrade hemicellulose and cellulose content respectively. Each enriched microbial consortia, on all the chosen substrates, across all treatment conditions, has showed the polysaccharide degradation with values ranging from 5.22 ± 0.34% (w/w) – 17.38 ± 0.67% (w/w) (hemicellulose) and 6.23 ± 1.35% (w/w) - 34.57 ± 2.74% (w/w) (cellulose). Among the LCB utilized, AVLR turned out to be the most efficiently degraded biomass (holocellulose – 45.87 ± 1.82%, w/w) on treating with EC-5 with maximum xylanase (1.94 ± 0.01 IU/mg), endoglucanase (12.55 ± 1.15 IU/mg) and total cellulolytic (3.35 ± 0.05 IU/mg) activities. Wongwilaiwalin et al. [58] investigated the LCB degradation

capability of aero-tolerant microbial consortia isolated from sugarcane bagasse pile, where the endoglucanase and xylanase activity has reached a maximum value of 1.31 (IU/mg) and 1.02 (IU/mg) respectively after 10 days of incubation. On considering the degradation of pure cellulosic substrate (FP), the maximum degradation of about 34.57 ± 2.74% (w/w) was obtained after 7 days of treatment with EC-9 indicating the increased cellulose availability of FP than the lignocellulosic substrate with lignin barrier. Studies on enrichment of lignocellulolytic microbial consortia from soil habitat has showed cellulose degradation of about 20.5% (switchgrass), 31.7% (wheat straw), 37.4% (corn stover) and 51.92% (rice straw) under mesophilic-aerobic condition [51,52, 57].

In addition, the total degradation of the various substrates were determined based on the volatile solid content before and after the treatment as shown in Fig. 2. Based on the observed results, highest substrate degradation of about 93.51 ± 1.13% (w/w) was obtained for AVLR biomass upon treating with the EC-5 consortium.

3.1.3. HPLC analysis of lignocellulolytic enzymes

The cocktail lignocellulolytic enzymes secreted by EC after treatment with various substrates were qualitatively determined by HPLC analysis. From the chromatogram (Fig. S3) of pure enzyme standards, the retention time of LiP (4.517 min), MnP (5.319 min), peroxidase (5.523 min), xylanase (5.429 min) and cellulase (5.576 min) were correlated with the peaks obtained for the extracellular enzymes secreted by the microbial consortia using different substrates (Fig. S4 and S5). A cumulative table representing the retention time of the peaks obtained in the sample that correspond to the respective pure enzyme standards are given in supplementary data (Table S1). The specific enzyme activities obtained with different substrates (Table1) were found to be correlated with the presence of peaks corresponding to the various lignocellulolytic enzymes (MnP, xylanase and cellulase) except LiP (Table S1). The possibility of failure in LiP assay of culture supernatant might be due

to the possible influence of non-enzyme molecules having some activity against the substrate (veratryl alcohol) used for LiP activity assay [59]. For instance, while performing the LiP assay for the culture supernatant supplemented with agricultural residues (AVLR and SD), other compounds like lignin, aromatics and quinonics are also absorbed strongly at 310 nm. On the other hand, certain fungi would secrete non-peroxidases like veratryl alcohol oxidases that are capable of veratryl alcohol oxidation, which could probably interfere with the enzyme assay [60]. Altogether from the analysis, the presence of secretome with multi-lignocellulolytic enzyme complex system could pave a way for establishing a sustainable consolidated pretreatment and bio-saccharification for LCB based biorefinery.

3.2. Fermentable sugar yield

The dynamic process of substrate degradation by EC is associated with the production of various lignocellulolytic enzymes that aid in the breakdown of holocellulose into various fermentable C5 (pentose) and C6 (hexose) sugars. In this study, fermentable sugar monomers were estimated for every 48 h (2 days) of incubation period using different substrates as a sole carbon source (Fig. 3a, b, and c). After two days of incubation, the various sugars yields were found to reach the highest value in all the treatment sets with the EC. However, further increase in the incubation time has resulted in a decrease of sugar yield in all the consortia sets. Probably, this could be a result of microbial metabolism involving the simultaneous production and consumption of sugar monomers [41]. The hydrolytic capability of EC in sugar release was varied for each substrate owing to its compositional, structural and treatment condition difference [45]. Among the treatment sets with LCB, EC-5 consortium showed efficient hydrolysis of AVLR with a maximum sugar yield of 40.51 ± 2.33 mg/g (glucose), 25.94 ± 0.66 mg/g (xylose) and 6.65 ± 0.25 mg/g (arabinose). Moreover, the hydrolytic efficiency of EC with pure cellulosic substrate showed a maximum glucose yield ranging from 26.77 ± 0.88 mg/g to 45.96 ± 2.17 mg/g and 24.70

± 0.86 mg/g to 27.14 ± 0.84 mg/g under mesophilic (EC-9 and EC-10) and thermophilic (EC-11 and EC-12) conditions respectively. Similar studies have reported that MCHCA and XDC-2 consortia was capable for efficient degradation of several LCB with a maximum glucose yield of about 2.13 g/L (maize silage), 1.3 g/L (rice straw) and 2.4 g/L (corn stalk) respectively [41, 61] and was comparable with the AVLR (40.51 mg/g or 4.03 g/L) and SD (16.40 mg/g or 1.64 g/L). Recently, Srivastava et al. [45] evaluated the hydrolytic efficiency of SNH-1 consortium for the degradation of various agricultural wastes, where the optimization by response surface methodology of wheat straw hydrolysis has resulted in 27 g/L of glucose (138 mg/g) at 40 °C for 72 h that was 23% higher than the un-optimized one. Thus, the synergism of microbial consortium enhances the LCB degradation abilities that could be efficient than the one using single isolates.

3.3. FTIR and XRD characterization of treated substrates

From the FTIR analysis (Fig. 4a, Table S2), the spectral bands observed around the regions of 3441- 3406 cm^{-1}, 1384-1319 cm^{-1} and 1161-1103 cm^{-1} corresponds to the strong deformation of O-H, C-H and C-O bond stretching vibrations respectively [62]. The occurrence of band stretching vibrations at wavenumbers 1280 cm^{-1} and 1033 cm^{-1} typically attributes to the lignin fractions whereas, the bands at 1437 cm^{-1}, 1378 cm^{-1}, 1110 cm^{-1} and 1635 cm^{-1} are the characteristic peaks of hemicellulose and cellulose [63]. A shift or broadening of the band at 3441-3406 cm^{-1} is observed in treated SD and AVLR substrate which attributes to the stretching vibrations of O-H group in aliphatic lignin, hemicellulose and cellulose [64]. The elimination of the bands around 1280 cm^{-1} and 1033 cm^{-1} in EC-5 treated AVLR sample are related to the aromatic C-O stretching vibration in guaiacyl skeleton and ester or β-O-4 linkages [65,66]. A characteristic peak around 1384 cm^{-1} in SD and AVLR samples attribute to the C-H and $C\text{-}H_3$ stretching in O-H, methyl and acetyl group of cellulose and hemicellulose [67]. The appearance of new peak or increase in transmittance percentage at 1635 cm^{-1} (aromatic

C=O stretching) in all treated samples are associated with the hemicellulose and cellulose derived decomposition products (carboxylic acids, alcohols, aldehydes and ketones) [68]. Further, the occurrence of signature peak around 1103-1111 cm^{-1} in all treated samples corresponds to the C-O-C, C-O and C-C stretching vibrations in cellulose, side chain and monomeric units of polysaccharides [69,70]. The degradation of cellulose content of SD (17.31%), AVLR (28.49%) and FP (34.57%) after treatment with EC-2, EC-5 and EC-9 (Fig. 2) was found to be correlated with the decrease in the crystallinity percentage to 44.99%, 19.39% and 78.64% respectively (Fig. 4b).

On the other hand, lateral order index (LOI) and total crystalline index (TCI) were determined from the FTIR spectra as a quantitative indices which gives insight into the overall and crystallinity degree of order in cellulose respectively [71] (Table 2). The absorbance ratio at 1431 cm^{-1}, 897 cm^{-1}, 2900 cm^{-1} and 1378 cm^{-1} are assigned to the C-H_2-scissoring (symmetric bending), C-H deformation of β-(1-4)-glycosidic linkage (stretching vibration), C-H_2 group (asymmetric stretching vibration) and C-H deformation (asymmetric vibration) respectively [63]. From the Table 2, it is evident that both LOI and TCI were found to be significantly decreased after microbial hydrolysis of SD, AVLR and FP. In lignocellulosic substrates, LOI decreased from 1.67 to 1.33 and TOI from 0.97 to 0.89 in EC-5 treated AVLR sample, whereas, EC-2 treated SD had a slight decrease from 1.54 to 1.48 (LOI) and 0.80 to 0.76 (TOI). Similarly, FP showed a decrease in the LOI and TCI values after treatment with EC-9. Lower LOI and TCI values correlate to the lower crystalline cellulose in the samples. Therefore, the results indicate the degradation of highly ordered structure of crystalline cellulose in all the substrates after the treatment with microbial consortia. Similarly, a decrease in the LOI and TCI values was reported in bagasse, switch grass and avicel after ionic liquid treatment [72-74].

Cellulose crystallinity determined based on crystallinity index (CrI) is considered as a predominant structural property which greatly influences the rate of microbial hydrolysis and bioconversion of LCB [75]. In this study, XRD analysis was performed to investigate the effect of consolidated pretreatment and bio-saccharification on the crystallinity of SD, AVLR and FP using enriched microbial consortia. From the Fig. 4b, it is evident that crystallinity of all treated samples was found to be decreased as compared to the untreated one. The reduction in crystallinity percentage might be owing to the synergistic action of various aforementioned delignifying (laccase, LiP and MnP) and saccharifying enzymes (xylanase and endoglucanase) in degrading the amorphous (lignin and hemicellulose) and crystalline region (cellulose) of substrates. Similarly, a significant reduction in the biomass crystallinity was observed in consolidated bioprocessing of Kans grass using a mixture of laccase and cellulase-xylanase concoction [76]. Yoshida et al. [77] reported that reduction in the crystallinity of *M. sinensis* by cellulase hydrolysis has resulted in the increased yield of sugar monomers. These results are coherent with the FTIR and compositional analysis such as functional group modification, degradation of the substrate and sugar yield upon EC treatment.

3.4. Elucidation of potent consortium by principle component analysis

In order to identify the significant patterns or trends by scaling the multi-dimensional datasets of observed parameters, principle component analysis (PCA) was performed to correlate and analyze the similarities between variable like lignocellulolytic enzyme activities and the degradation potential of 12 ECs. The PCA biplot (Fig. 5a) ordinated with enzyme activities, lignin, hemicellulose and cellulose degradation percentage upon treatment of EC with SD, AVLR and FP under different conditions (M1, M2, T1 and T2). The experiment sets (EC-1 to EC-12) mentioned in section 2.4 has been denoted as 1 to 12 in PCA plot. The PCA biplot of consortia treatment with different substrates demonstrates that, PCA accounted 89.4% of the observed variance (71.6% and 17.8%). The first two

principle components (PC1) of biplot showed that 8 out of 12 treatment sets have significantly contributes to the ligninases and hemicellulolytic activities that mainly involves the treatment sets associated with LCB (SD and AVLR) degradation. In detail, treatment sets 2, 5 and 8 were highly active with various endo-enzymes like laccase, MnP and xylanase activities that corroborates with the lignin and hemicellulose degradation of AVLR biomass, whereas, LiP activity is found be clustered for 1, 4, 7, 10 and 11. Similar clustering in PC2 showed treatment sets with pure cellulosic substrate (3, 6, 9, 12) contributed significantly to the cellulose degradation with unique potential of endoglucanase and total cellulolytic activities. This could be correlated with the highest degradation percentage (34.57 ± 2.74%, w/w) of FP cellulose (EC-9) than the other LCBs used for the consortia-based treatment. However, EC-5 has resulted in 28.49±1.15% (w/w) of cellulose removal from AVLR (closer to the FP cellulose degradation). Furthermore, PCA biplots clustering strongly indicates the positive relationship of using EC-5 for treating AVLR based on the secretion of multi-ligninolytic enzymes under mesophilic condition in turn leads to the effective degradation of structural cell wall components. Recently, Sadalage et al. [78] has evaluated the efficiency of 10 synthetic bacterial consortia for degradation of various lignocellulosic substrates. The PCA biplot analysis for various lignocellulolytic enzyme activities, revealed that CM10 consortium was found to be the most promising candidate for effective degradation of grass straw.

3.5. Correlation analysis

As EC-5 has showed the potency in effective degradation of AVLR biomass (93.51 ± 1.13%, w/w) with high endo-enzymes activities, the association between the cell wall components breakdown, fermentable sugar monomers and residual biomass were studied using a Pearson correlation matrix (Fig. 5b). A positive correlation between the AVLR cell wall components (lignin and holocellulose) removal and fermentable C5 and C6 sugars yield were observed

as a result of hydrolysis. The degradation rate of lignin and hemicellulose content showed a strong positive correlation with the C5 (0.99) and C6 (0.47) sugars yield. Whereas, glucose primarily derived from the cellulose content showed a positive correlation (0.93) between each other. However, the residual biomass obtained after 7 days of treatment showed a negative correlation with the biomass components removal rate and the sugar yield. Overall, the factors shown to unveil a strong positive correlation reinforces the significance of effective hydrolysis of AVLR biomass by EC-5. Similarly, Puentes-Téllez and Salles [79] studied the correlation between the structural components degradation and the functional diversity of minimal active microbial consortium upon LCB (sugarcane bagasse and straw) treatment. Herein, the enriched microbial community revealed a positive correlation between the lignin degradation and functional attribute diversity, whereas, hemicellulose and cellulose degradation was either not affected or negatively correlated with functional diversity. As a result, bacterial community revealed a higher degradation rate of cell wall components owing to its higher functional diversity indicating the metabolic complementarity between species. A comprehensive mass flow of the EC-based hydrolysis of various substrates has been postulated as Alluvial diagram based on substrate loss (lignin, hemicellulose and cellulose) with culmination of fermentable products (C5 and C6 sugars) under different conditions (Fig. 6).

Based on our observation, mesophilic EC-5 in consolidated pretreatment and bio-saccharification under aerobic condition could gain attention in industrial application as it would be less energy intensive and require short time duration for lignocellulose degradation than thermophilic and anaerobic condition. The concerted action of microbial consortia in lignocellulose degradation, AVLR (agro-industrial residues) was found to be more easily degraded by microbial community owing to its moderate content of lignin (lignin- 19.12 ± 0.54 % (w/w),) and holocellulose (53.96 ± 1.48%, w/w) than the SD (woody biomass) with

high lignin content (24.11 ± 0.29 %, w/w) and holocellulose (74.39 ± 1.32%, w/w). Since, the compost sample was randomly taken from the 5 different sites of the compost pile with the different temperature regimes (mesophilic- surface and thermophilic-subsurface) it would harbor a diverse group of microbial symbionts showing degradation in different conditions. However, highest degradation of EC-5 under aerobic condition could possibly due to the predominance of aerobes in the collected sample. Meanwhile, the anaerobic condition provided in the study could make the aerobic organism difficult to survive.

4. Conclusion

This study affords insight into the potency of EC isolated from compost habitat towards consolidated bioprocessing of various lignocellulosic biomass. Among the isolates, EC-5 serves as a promising candidate for the effective degradation of AVLR biomass with a strong positive correlation towards cocktail lignocellulolytic enzymes and fermentable sugar monomers production. In addition, the use of mesophilic EC-5 in consolidated pretreatment and bio-saccharification could gain attention in industrial application as it would be less energy intensive and environment friendly than any other conventional process. Further, microbial characterization of EC-5 isolates through high throughput molecular approaches could provide a new perception of consortium based consolidated bioprocessing in a biorefinery framework.

Author Contributions

GR has contributed in execution of the experiment, data generation, data analysis, and drafting the manuscript.

SJ and VK have provided the inputs towards the conceptualization, methodology, critical suggestion and technical evaluation of the manuscript.

Declaration of competing interest

The authors declare that there is no competing interest associated w/ith this manuscript.

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Table 1 Lignocellulolytic enzyme activity of enriched microbial consortia derived from organic compost using different substrates

Lignocellulolytic Enzymes	EC-1	EC-2	EC-3	EC-4	EC-5	EC-6	EC-7	EC-8	EC-9	EC-10	EC-11	EC-12
	SD				AVLR				FP			
Laccase	46.25 ± 2.12	30.83 ± 2.77	32.31 ± 1.45	23.04 ± 1.78	120.45 ± 4.58	77.63 ± 3.95	73.60 ± 5.22	60.52 ± 3.89	N.D.			
LiP	1.44 ± 0.07	0.49 ± 0.07	2.35 ± 0.18	1.91 ± 0.05	1.82 ± 0.10	1.22 ± 0.08	1.33 ± 0.06	1.15 ± 0.04	N.D.			
MnP	13.45 ± 1.44	9.11 ± 0.54	11.16 ± 1.65	3.60 ± 0.45	17.24 ± 3.75	11.44 ± 1.56	7.25 ± 0.45	3.49 ± 0.89	N.D.			
Xylanase	0.76 ± 0.05	0.10 ± 0.07	0.72 ± 0.07	0.65 ± 0.02	1.94 ± 0.01	1.69 ± 0.02	1.09 ± 0.01	0.64 ± 0.06	N.D.			
Endoglucanase	5.89 ± 0.78	5.22 ± 0.21	5.17 ± 1.17	5.58 ± 2.11	12.55 ± 1.15	9.73 ± 0.54	6.19 ± 1.00	6.21 ± 0.50	21.64 ± 1.11	32.67 ± 1.33	15.15 ± 1.00	13.90 ± 2.33
Total cellulolytic activity	1.16 ± 0.07	0.89 ± 0.05	1.35 ± 0.03	0.37 ± 0.04	3.35 ± 0.05	2.82 ± 0.05	4.14 ± 0.15	2.54 ± 0.12	7.91 ± 0.87	12.02 ± 0.57	9.71 ± 1.24	8.48 ± 0.75

The values are average of triplicate experiments and the standard deviation is represented as (±) calculated from mean and three independent trials.

N.D. - Not Detected, EC- Enriched consortia, SD-Saw dust, AVLR-Aloe vera leaf rind and FP-Filter paper

Table 2 Lateral order index (LOI) and Total crystalline index (TCI) obtained from the FTIR analysis of untreated and EC-treated samples

Sample	Lateral order index (LOI)	Total crystalline index (TCI)
Untreated SD	1.54	0.80
EC-2 treated SD	1.48	0.76
Untreated AVLR	1.67	0.97
EC-5 treated AVLR	1.33	0.89
Untreated FP	1.60	0.82
EC-9 treated FP	1.31	0.70

Figure legends

Fig. 1. A schematic representation of flow of process opted in elucidating the efficacy of EC towards consolidated pretreatment and bio-saccharification of various substrates (SD-Saw dust, AVLR-Aloe vera leaf rind and FP-Filter paper)

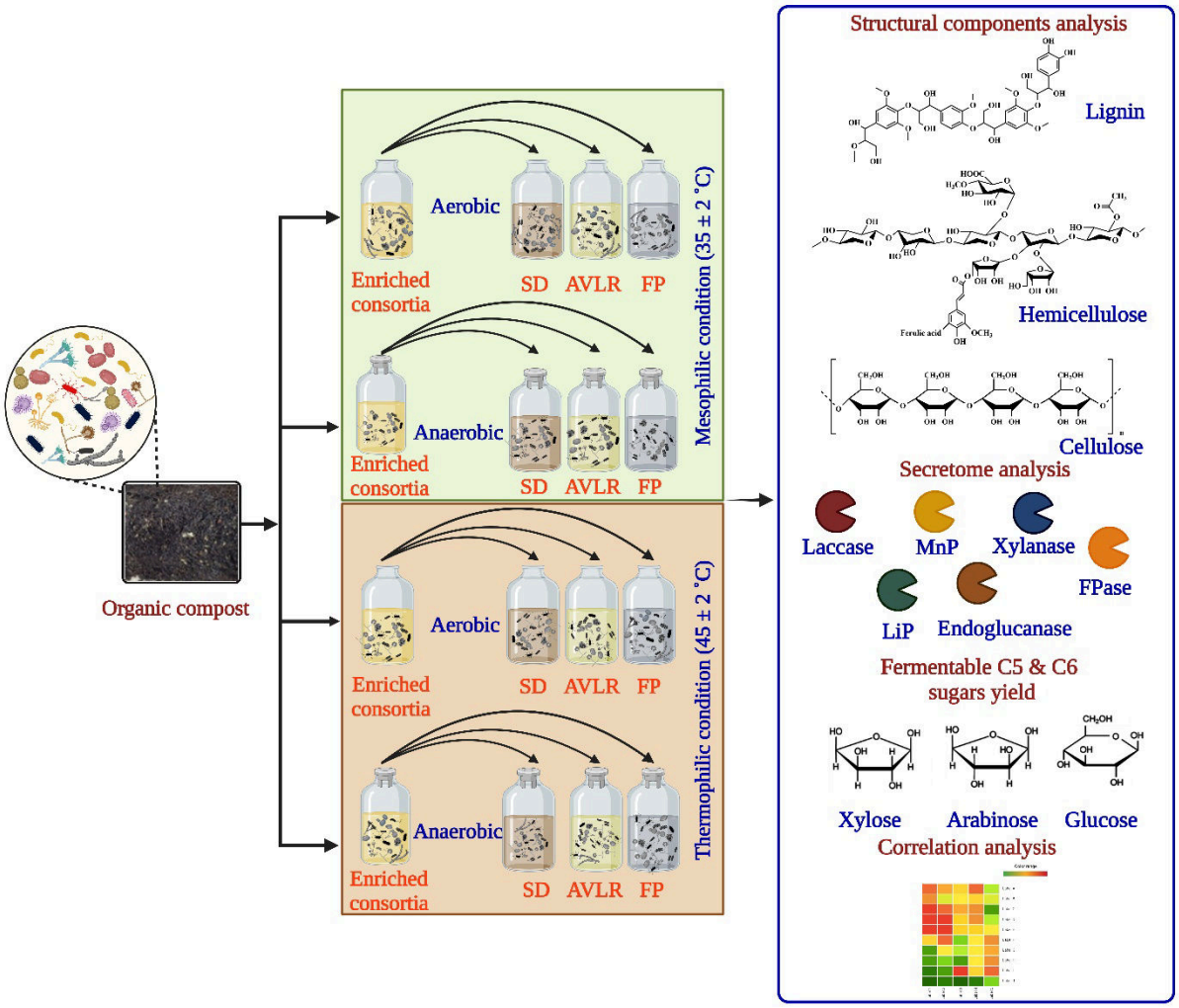
Fig. 2. Degradation of structural components of different substrates treated with EC under different conditions (Avg ± S.D.). The represented degradation percentage (lignin, hemicellulose, cellulose and total substrate) with different treatment conditions were calculated as the relative degradation with the control (without inoculation). (EC- Enriched consortia, SD-Saw dust, AVLR-Aloe vera leaf rind and FP-Filter paper)

Fig. 3. Fermentable sugars yield obtained after EC treatment using different substrates such as **(a)** SD, **(b)** AVLR and **(c)** FP (Avg ± S.D.). (EC- Enriched consortia, SD-Saw dust and AVLR-Aloe vera leaf rind, FP- Filter Paper)

Fig. 4. (a) FTIR and **(b)** XRD spectra of untreated and EC treated substrates under M1 and M2 condition (EC- Enriched consortia, SD-Saw dust and AVLR-Aloe vera leaf rind, FP- Filter Paper)

Fig. 5. (a) PCA plot to correlate and analysis the variables like lignocellulolytic enzyme activities and the degradation potential of enriched consortia (EC). The experimental sets (EC-1 to EC-12) has been denoted as 1 to 12 in PCA plot and **(b)** Correlation plot representing the influence of AVLR cell wall components towards consolidated pretreatment and bio-saccharification upon treatment with EC-5.

Fig. 6. Alluvial diagram representing the overall mass flow of the EC-based consolidated pretreatment and bio-saccharification of various substrates with culmination of fermentable products under different conditions (EC- Enriched consortia, SD-Saw dust and AVLR-Aloe vera leaf rind, FP- Filter Paper)



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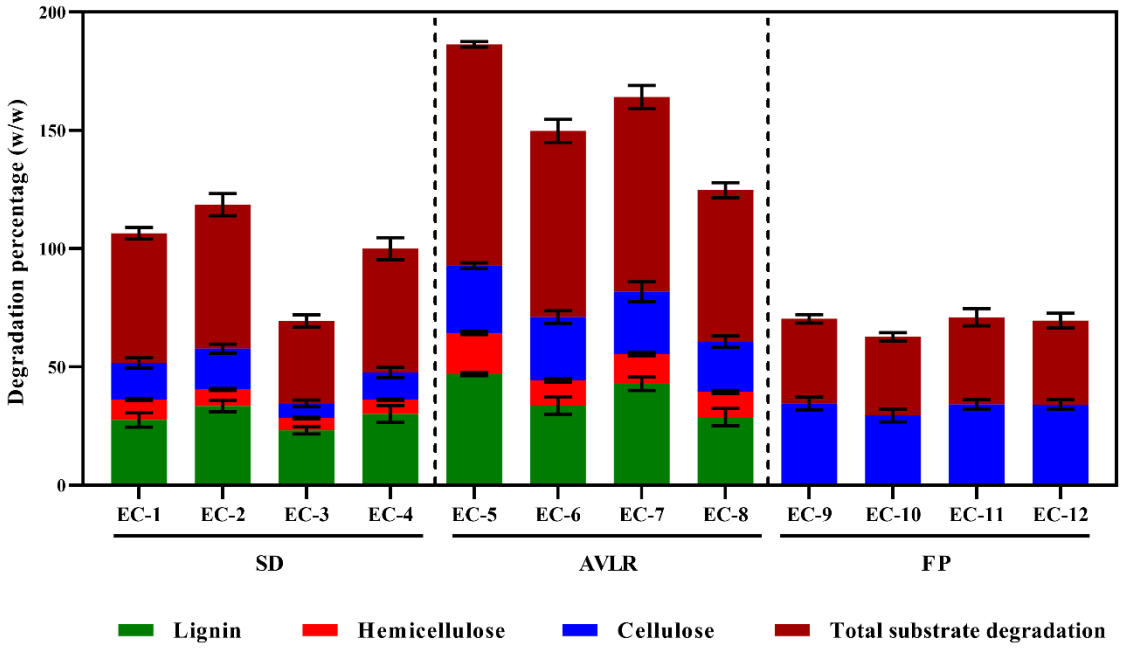

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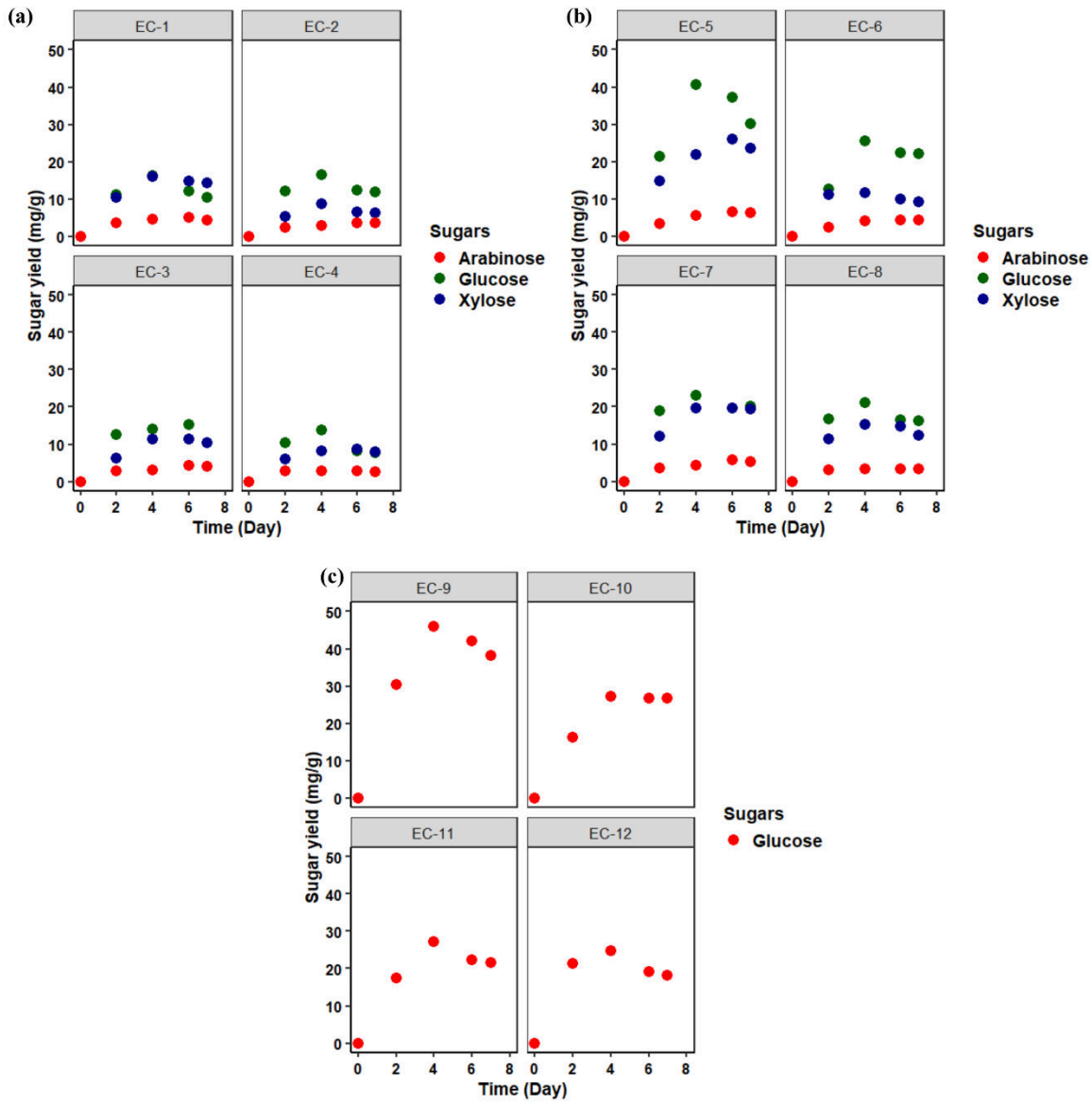

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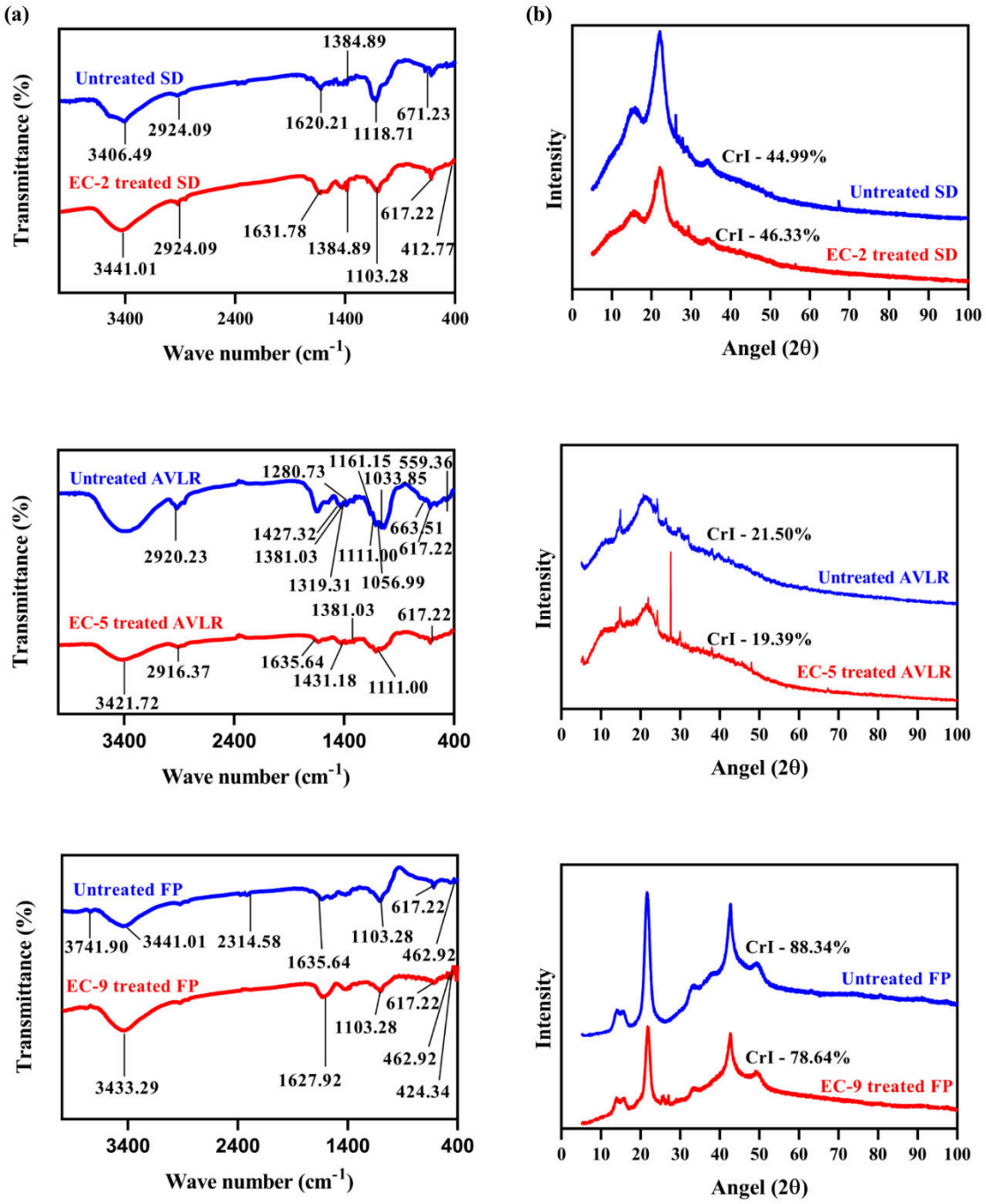

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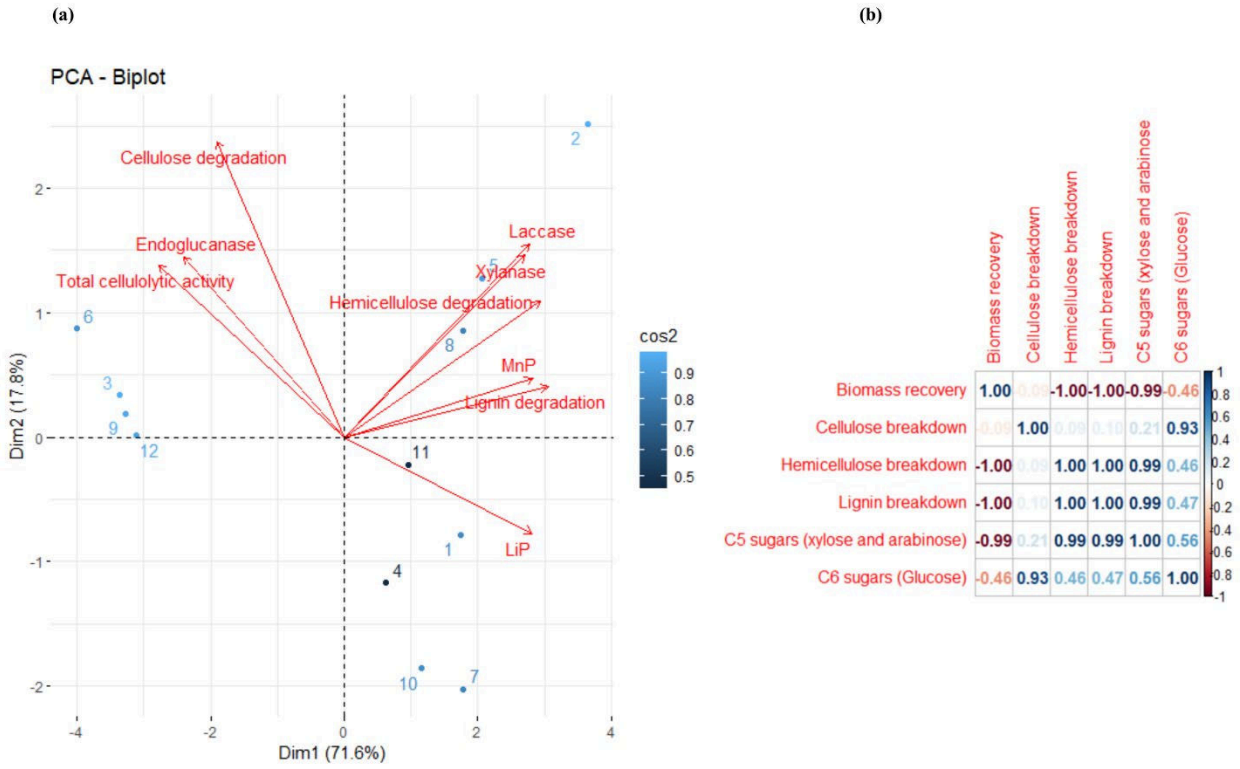

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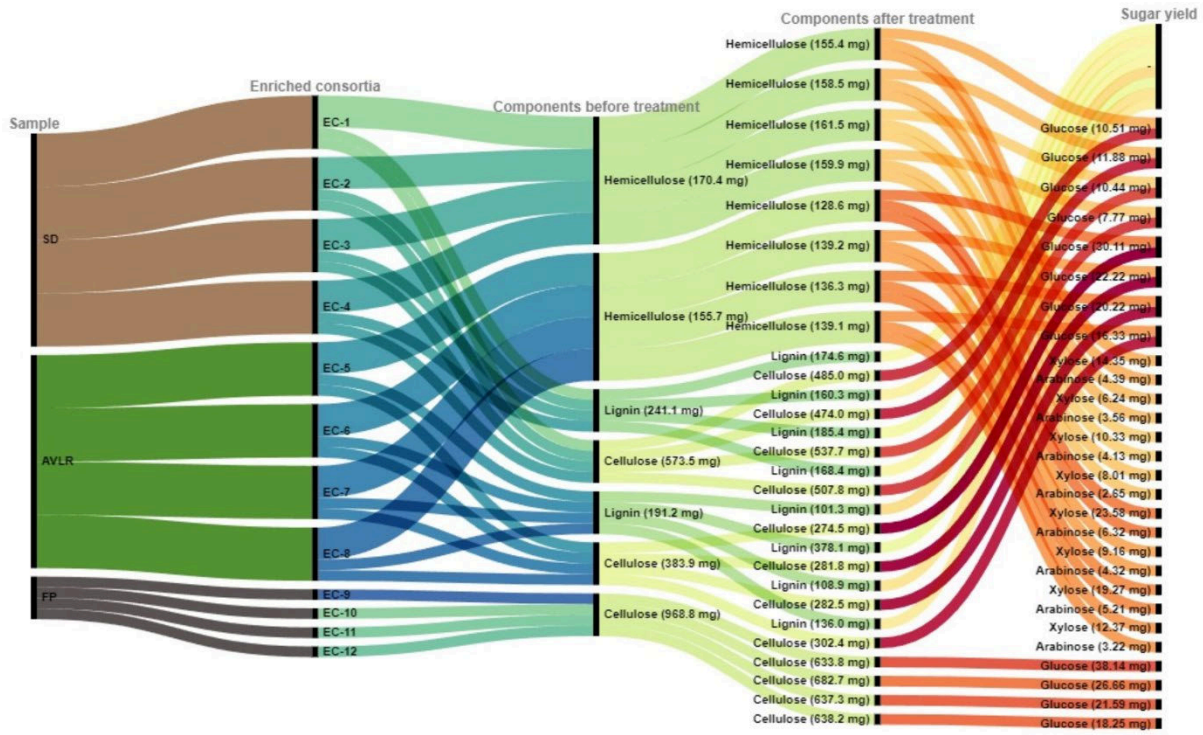

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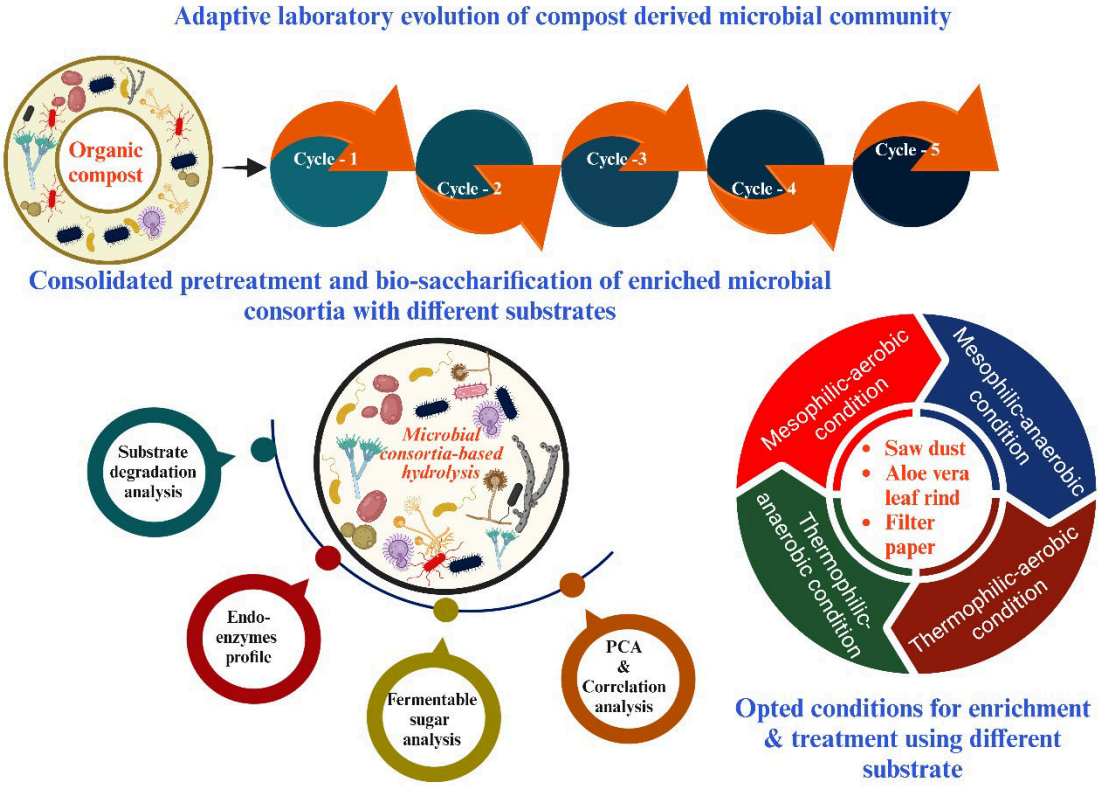

Graphical abstract