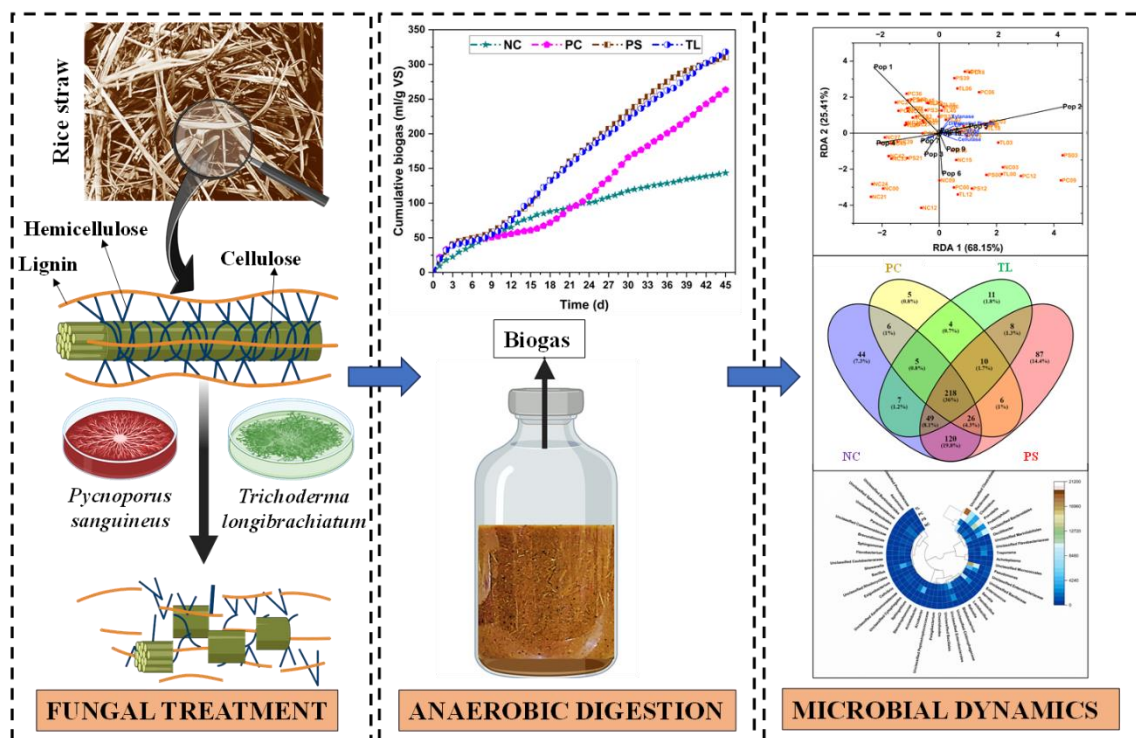


Chapter 6

Effect of fungal pretreatment by *Pycnoporus sanguineus* and *Trichoderma longibrachiatum* on the anaerobic digestion of rice straw



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6.1. Background

Rice straw is a widely used agricultural residue for anaerobic digestion (AD) due to its abundance and low cost [168]. However, its high lignin content and low biodegradability impede its effective decomposition and limit its methane yield [29]. Several pre-treatment methods have been developed to increase the biodegradability of rice straw and improve biogas production. Thermal and chemical treatment methods have received considerable attention as they effectively break down the resistant components of rice straw [197]. Thermal treatments, including steam explosion, hydrothermal carbonization, and microwave irradiation, improve the accessibility of lignocellulose to microorganisms [198]. Chemical treatments such as alkali, acid, and ozone treatments can modify the structure of lignin and hemicellulose, making them more susceptible to enzymatic hydrolysis [199]. Despite the potential benefits of thermal and chemical treatments in increasing the biodegradability of rice straw for anaerobic digestion (AD), these methods also present some drawbacks. The high energy demand associated with thermal treatments and the potential formation of inhibitory compounds may limit their feasibility and sustainability [179]. On the other hand, chemical treatments can be expensive and pose risks to both human health and the environment due to the use of hazardous substances [180]. Furthermore, the alteration of rice straw's composition and properties through chemical treatments may hinder its utilization as a valuable resource for other applications such as animal feed or soil amendment [200].

Considering low input costs and higher perceived benefits, fungal treatment of rice straw has been proposed as a cost-effective and eco-friendly way to harness bioenergy by anaerobic digestion [201]. Fungi secrete various lignocellulose-degrading enzymes that can effectively degrade the recalcitrant components of rice straw and increase its methane yield during AD [202]. Mustafa et al., 2017 found that pretreatment of rice straw using

P. ostreatus and *T. reesei* had a significant positive impact, increasing methane yield by 120% higher and 78.3% higher, respectively, compared to untreated rice straw. 1.64-2.22-fold increase in methane production was observed when rice straw was treated using *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, and *Ganoderma lucidum*. *Trichoderma longibrachiatum* is a fungus commonly found in soil, decaying wood, and plant debris [203]. It produces cellulases, xylanases, and proteases to efficiently degrade lignocellulosic biomass and grows rapidly [204]. *Pycnoporus sanguineus*, also known as the Blood-Red Bracket fungus, is an edible mushroom that grows on decaying hardwood trees and produces enzymes such as laccase and manganese peroxidase, which are crucial for the breakdown of lignin and other complex organic compounds in wood [205]. Purified enzymes from these fungi have been used for various applications related to lignocellulose degradation. However, recovery and purification of enzymes are not economically feasible for use in big digesters. That is why fungal mycelia from *T. longibrachiatum* and *P. sanguineus* were used for pretreatment.

The aim of this study was to evaluate the ability of *T. longibrachiatum* and *P. sanguineus* for the treatment of rice straw to improve the hydrolysis rate, which is the rate-limiting step in anaerobic digestion. Structural and elemental modifications were examined using SEM, EDS, and FTIR spectroscopy. The dynamics of the microbial community were evaluated through 16S rRNA sequencing and flow cytometry analysis. The results revealed a 20.79% and 17.85% increase in biogas production from PS and TL-treated rice straw, respectively, indicating improved hydrolysis and potential efficiency enhancement when integrated into biogas plants.

6.2. Results and discussion

6.2.1. Effect of fungal treatment on the anaerobic digestion of rice straw

6.2.1.1. Biogas production and sCOD

The effect of fungal treatment using *T. longibrachiatum* and *P. sanguineus* on cumulative and biogas production recorded every three days has been illustrated in Figure 6.1. A pretreatment period of 10 days was chosen as per the literature and according to the results of a separate experiment in which pretreatment for different time durations was tested, and longer treatment periods had a smaller effect on the biogas production potential. Extended pretreatment time leads to increased consumption of cellulose and hemicellulose, resulting in lower methane production [141,206]. Shorter treatment times allow for quicker turnover of materials, which can be advantageous when dealing with large volumes of rice straw. TL-treated rice straw exhibited a significant increase of 20.79% (318.10 mL/g VS, $p < 0.05$), while PS-treated rice straw showed a 17.85% (310.36 mL/g VS, $p < 0.05$) higher cumulative biogas yield, compared PC, which produced only 263.34 mL/g VS biogas as shown in Figure 6.1(a). However, the difference between PS and TL distributions is not statistically significant as $p > 0.05$. From the biogas production graph presented in Figure 6.1(b), it can be observed that the highest biogas production of 33.74 mL/g VS and 29.26 mL/g VS was achieved on day 18 in PS and TL, respectively, while PC achieved the highest production of 30.94 mL/g VS on day 30. The delay in achieving peak production in PC may be attributed to the slower hydrolysis of untreated lignocellulosic biomass. A study by Kainthola et al., 2019b showed that peak biogas production was observed on days 13 and 14 from *Phanerochaete chrysosporium* and *Ganoderma lucidum* treated rice straw as compared to untreated rice straw in which peak production was observed on day 21. The biogas yield started

decreasing post-day 18 in PS and TL and post-day 30 in PC. This can be attributed to the fact that these fungi secrete extracellular enzymes that have the ability to degrade lignocellulosic feedstocks resulting in better degradation and higher biogas production [185,207].

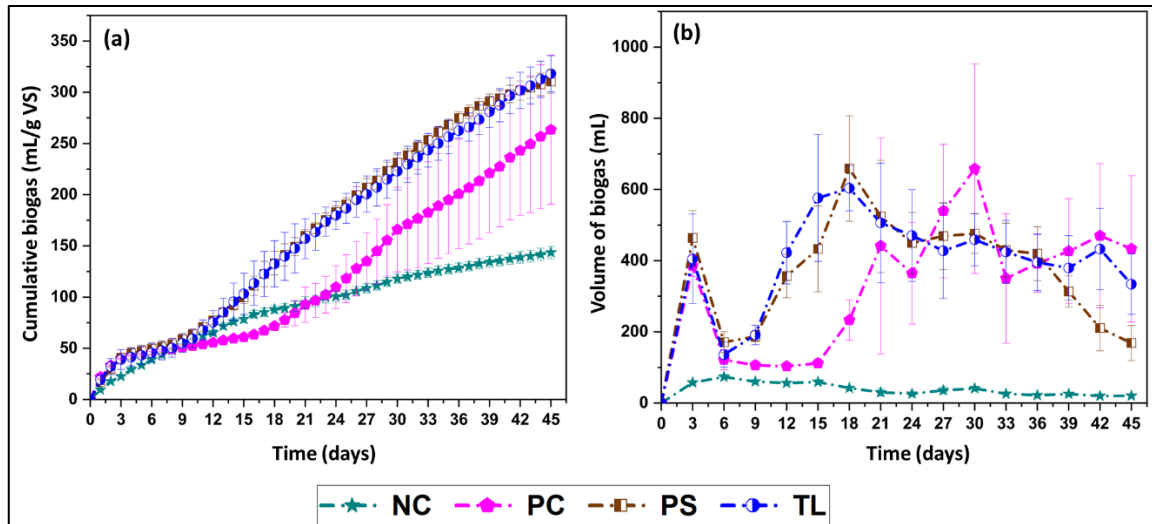


Figure 6.1. (a) Cumulative biogas and (b) biogas production recorded every three days in NC, PC, PS, and TL-treated rice straw during anaerobic digestion

6.2.1.2. Changes in VFAs, pH and sCOD

Figure 6.2(a) and Figure 6.2(b) demonstrate the profile of total VFA production in terms of acetic acid equivalents and pH values from day 0 to day 45. The production of VFAs determines the proper functioning of the anaerobic digester, as VFAs directly affect pH, which governs the activity of methanogens [208]. Higher accumulation of volatile fatty acids results in a pH drop and may compromise the functioning of the digester. The VFA production was as high as 5811.11 mg/L on day 3 and 5300 mg/L on day 9 in PC, corresponding to pH values of 5.20 and 5.84, respectively. Despite higher VFA production in PC as compared to fungal-treated samples, the biogas volume was low, representing the lesser conversion of VFA to biogas, due to which pH dropped. This may

be attributed to inefficient substrate conversion and slow adaptation of methanogens to VFA utilization. However, PS and TL showed maximum VFA production of 4222.22 and 3422.22 mg/L corresponding to pH values of 6.46 and 6.52, respectively. Fungal-treated samples exhibited reduced VFA accumulation and pH drop. Total VFA concentrations were initially high, indicating the conversion of soluble organics to organic acids. VFA levels decreased over time, reaching zero for the negative control with no substrate, which maintained a near-neutral pH throughout the 45-day digestion period.

Figure 6.2(c) presents the changes in sCOD concentrations during the anaerobic digestion of rice straw. sCOD indicates the availability of soluble organic compounds in the anaerobic digestion system, influencing the rate of hydrolysis for biogas production. Initially, at the start of the batch anaerobic digestion process, sCOD concentrations were highest due to the release of soluble organic compounds from the feedstock [208]. Subsequently, microorganisms within the digester actively metabolized these compounds, converting organic matter into biogas (methane and carbon dioxide) [209]. As days progressed, sCOD values consistently decreased. TL exhibited a 71.43% sCOD removal, while PS showed a 64.71% removal. PC demonstrated a decrease of 56.67% in sCOD.

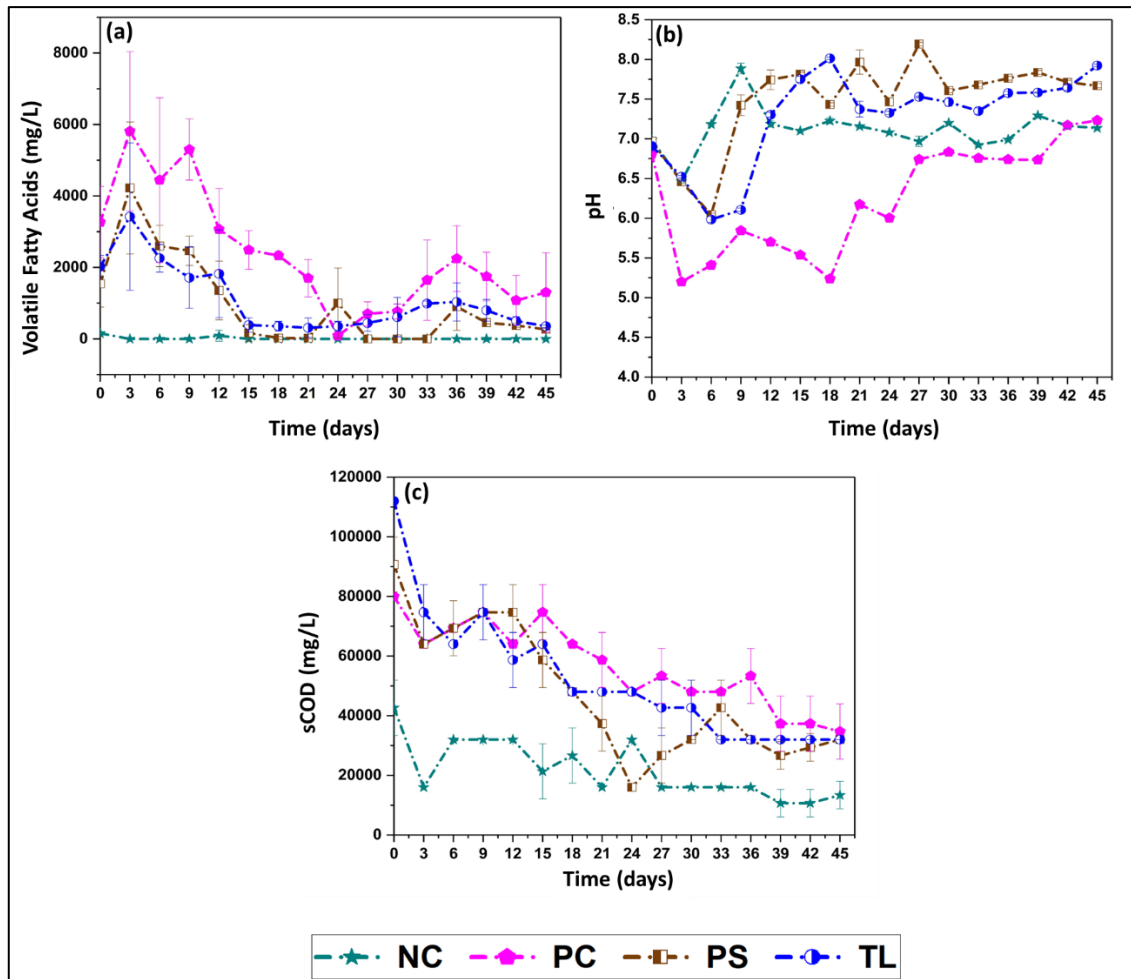


Figure 6.2. (a) Volatile fatty acids (VFAs), b) pH and (c) soluble chemical oxygen demand (sCOD) in anaerobic digestion of untreated, PS and TL-treated rice straw

6.2.1.3. Role of cellulase, xylanase, and lignin peroxidase enzyme

The trend of enzymes cellulase, xylanase, and lignin peroxidase activities have been shown in *Figure 6.3*. Microorganisms in AD produce lignocellulolytic enzymes which help in the hydrolysis of cellulose and hemicellulose to simple sugars [76]. The cellulase and xylanase activities are indicative of cellulose and hemicellulose degradation. Interestingly the cellulase activity showed a decreasing trend till day 15, was nearly stagnant till day 33, and then showed an increase in both PC and NC (*Figure 6.3(a)*). As digestion progresses, easily accessible organic matter is depleted, resulting in a rise in cellulase activity as microorganisms adjust to limited substrates and increase cellulase

production to break down remaining cellulose [210]. A sudden increase in biogas is a result of increased cellulase activity. The cellulase activity ranged between 0.02-2.62 U/mL in PC, 0.46-3.44 U/mL in PS, and 0.46-3.38 U/mL in TL-treated rice straw AD samples, respectively. AD samples containing TL-treated rice straw had the highest cellulase activities on all days, followed by PS-treated samples. Cellulase activity in PC containing untreated rice straw was lower in comparison to AD samples containing PS and TL-treated rice straw [96].

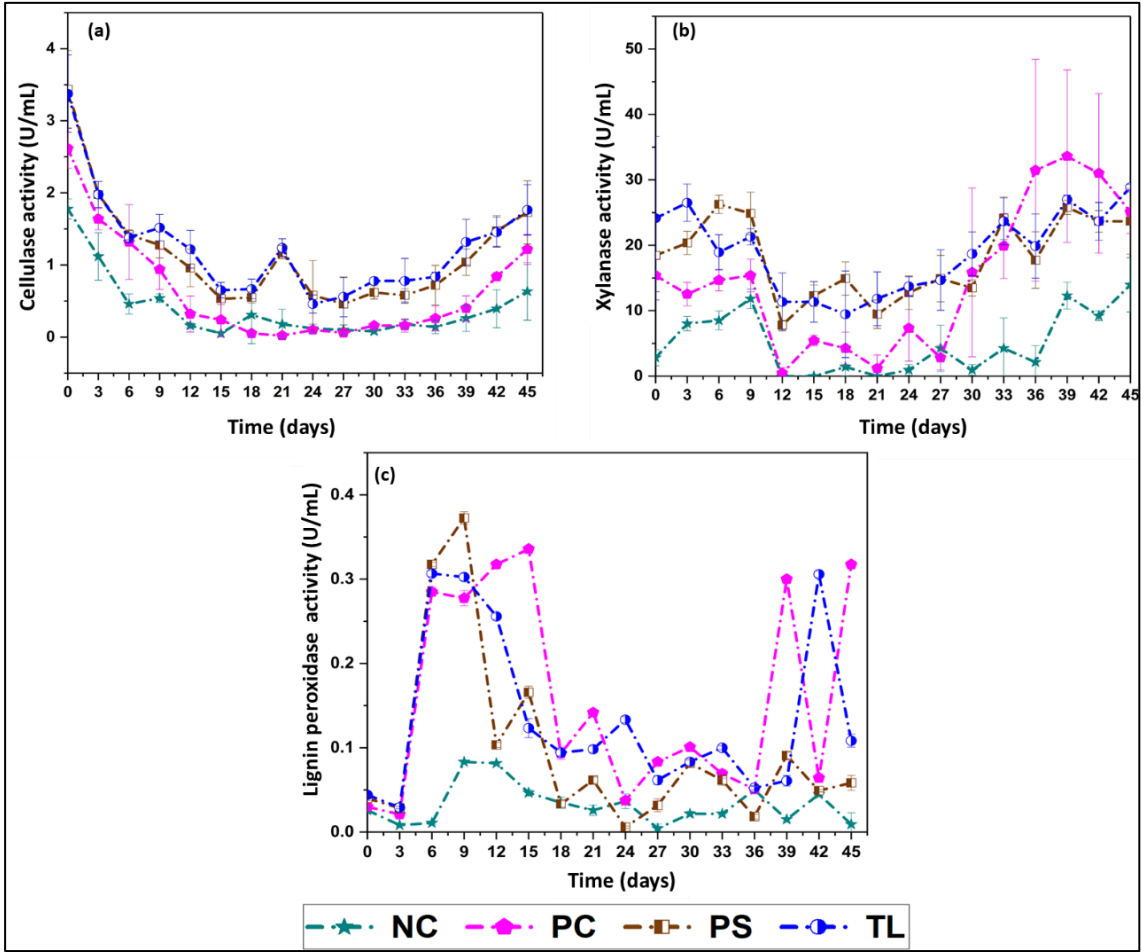


Figure 6.3. (a) Cellulase, (b) xylanase, and (c) lignin peroxidase activity in anaerobic digestion of untreated, PS and TL-treated rice straw

Figure 6.3(b) shows the trend of xylanase enzyme activity during the 45 days of anaerobic digestion of rice straw. Xylanase activity followed a similar profile as cellulase enzyme

activity, as has been observed by Gu et al., 2015. The xylanase activity dropped to 0 from day 12-21 in AD samples containing untreated rice straw. However, the least xylanase activities were as high as 7.81 U/mL on day 12 in PS-treated and 9.47 U/mL in TL-treated rice straw. The xylanase activity was high till day 9 and then decreased significantly on day 12. The decline in activity can be attributed to factors such as depletion of digestible components, inhibitory byproduct accumulation, and shifts in the microbial community. Xylanase activity increased after day 21. Fungal-treated samples showed consistently high xylanase activity, while untreated straw samples exhibited a sudden spike after day 27, possibly due to microbial acclimatization to the complex lignocellulosic biomass.

Figure 6.3(c) represents the activities of LiP enzyme in AD samples containing untreated and fungal-treated rice straw. LiP was chosen because it is a heme-containing enzyme produced by white-rot fungi, which are highly efficient lignin degraders. LiP has a higher redox potential than laccases and manganese peroxidase (MnP), and it can oxidize both phenolic and non-phenolic substructures of lignin even in the absence of mediators [211]. LiP exposes the cellulose and hemicellulose layers to bacterial attack, which are then utilised to produce biogas [212]. The LiP activity increased from 0.03 U/mL and 0.03 U/mL on day 3 to 0.37 U/mL and 0.31 U/mL on day 9 in AD samples containing PS and TL-treated rice straw, respectively. These activities started decreasing post-day 9. However, an increase in LiP activity was observed post-day 9 in PC. The LiP activity further decreased after day 9 in PS and TL-treated anaerobic samples and after day 15 in PC. The LiP activity further increased post-day 36. The LiP activity followed a somewhat reverse trend compared to cellulase and xylanase activity. This may be attributed to the fact that higher lignin was degraded during the fungal treatment, and cellulose and hemicellulose were relatively easily available for the microorganisms. However, in the case of untreated rice straw, the anaerobic microbial community had to break down lignin

to access cellulose and hemicellulose during the digestion validating the higher LiP activity in control.

6.2.2. Morphological and compositional changes in rice straw

Figure 6.4 shows the SEM micrographs of raw untreated rice straw with the anaerobically digested rice straw obtained from PC, PS, and TL for the microstructure investigations. The raw rice straw showed an intact crystalline structure with lumps and papillae marked by arrows. On the other hand, the rice straw from PC showed deformities in the structure, showing a porous and penetrable surface. The rice straw samples from PS and TL showed disintegrated fibrils that exposed the cellulose fibers for AD microbes to access them [213]. The higher cellulase and xylanase activity in PS and TL is a result of the same. Higher solubilization from fungal treatment resulted in higher biogas production and sCOD reduction.

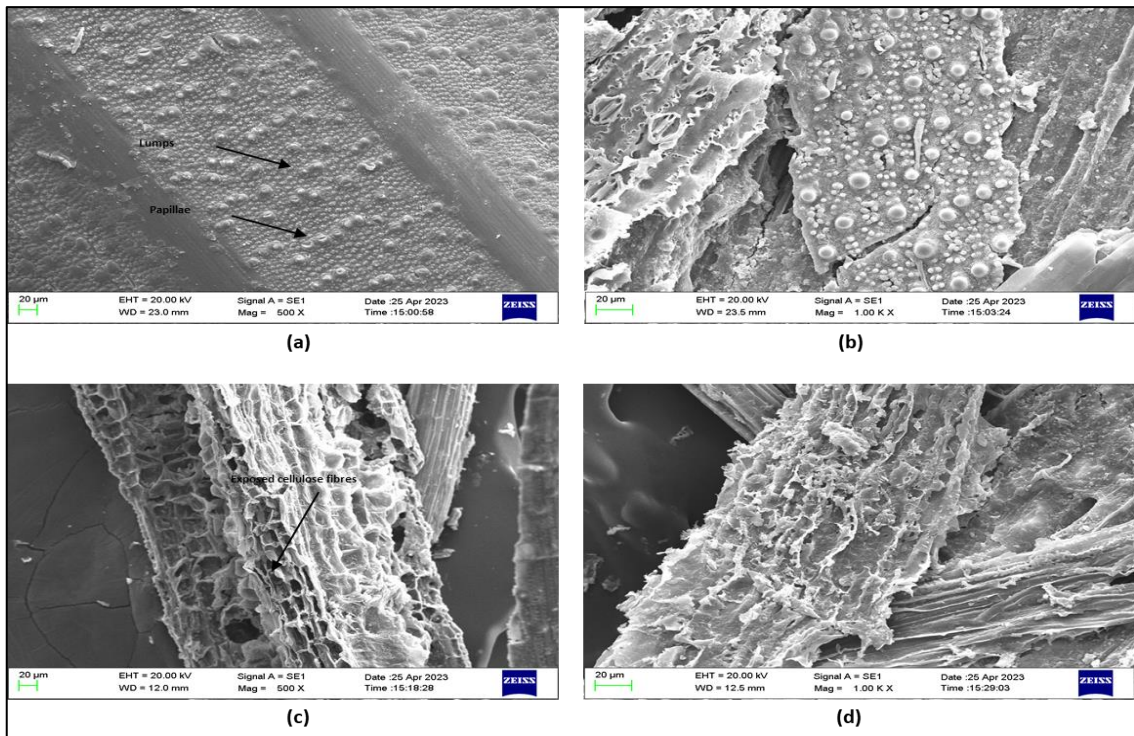


Figure 6.4. Scanning electron microscopy of (a) untreated raw rice straw showing intact structure with lumps and papillae, (b) anaerobically digested untreated, (c) PS (d) TL-treated rice straw showing exposed cellulose fibers

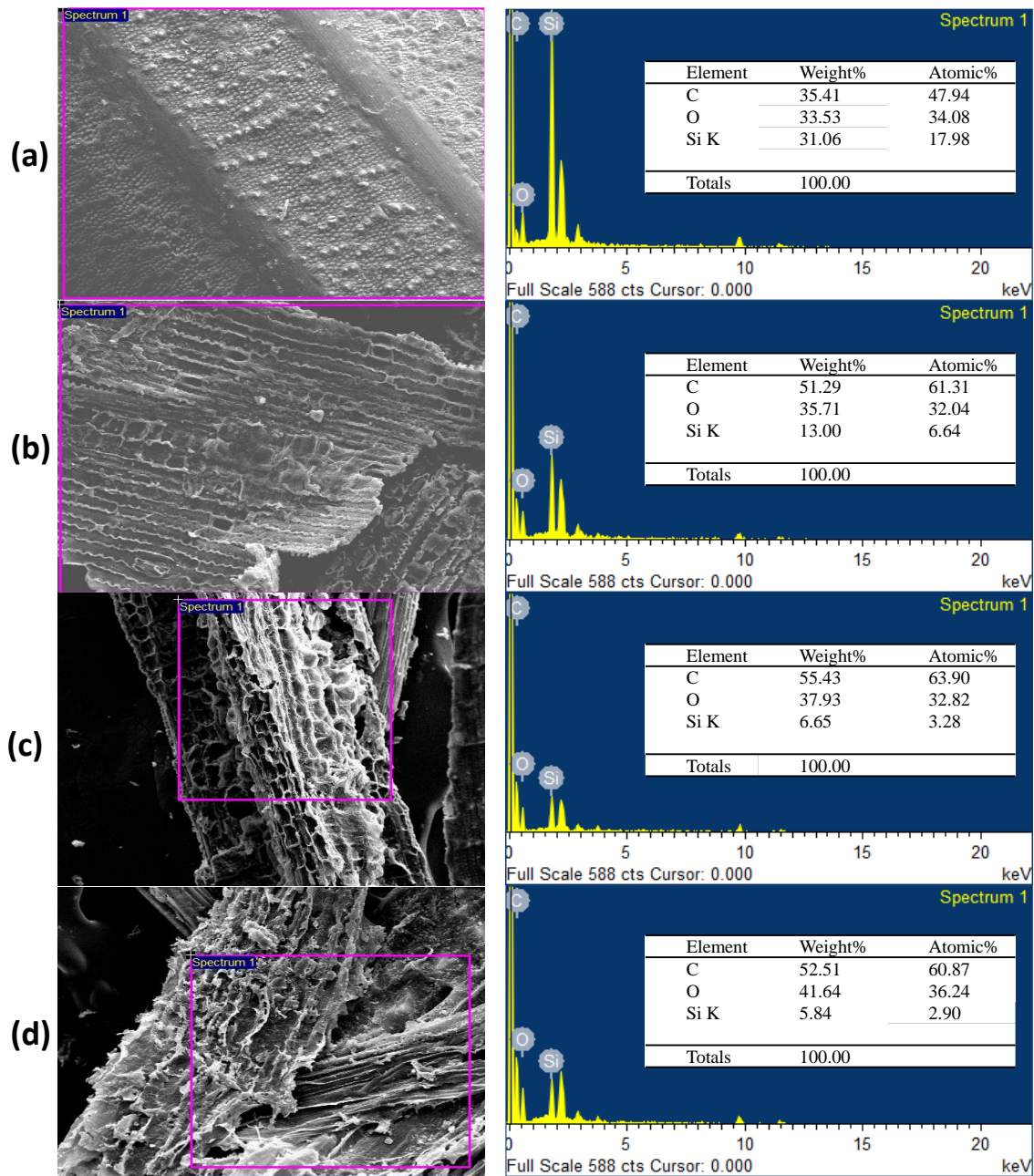


Figure 6.5. The SEM-EDS micrographs scanned to evaluate elemental composition of (a) untreated raw rice straw, (b) anaerobically digested untreated, (c) PS (d) TL-treated rice straw

To know about the elemental composition of the rice straw, SEM micrographs were also scanned by energy dispersive spectroscopy (EDS) as shown in Figure 6.5. The results of EDS showed the presence of elements carbon (C), oxygen (O), and silicon (Si) in the atomic percentage of 47.94%, 34.08%, and 31.06%, respectively, in raw rice straw. Post

anaerobic digestion, the percent of C increased to 61.31%, and O and Si decreased to 32.04% and 6.64%, respectively, in PC. The 13.37% increase in C in PC, 15.96% increase in PS, and 12.93% increase in TL indicates the exposure of cellulose and hemicellulose layer after the disintegration of outer lignin cross-links that exposed the carbon element [213]. Also, the disintegrated lignin may get re-deposited in the form of lignin droplets on the surface and pores of the rice straw, which might also be the reason for the increased carbon percent [215]. The atomic percent of Si reduced significantly to 3.28% and 2.90% in PS and TL-treated rice straw, respectively.

FTIR analysis was conducted to assess the impact of fungal pretreatment on the chemical composition and structure of rice straw after anaerobic digestion (*Figure 6.6*). FTIR spectroscopy is recommended by researchers for its simplicity and ability to provide rapid information compared to traditional chemical analysis techniques (Mahajan et al., 2020). The structural changes observed during FTIR analysis provide strong evidence for the deformation and compression of fibers within the overall structure of rice straw biomass. As compared to raw rice straw represented by RS, there was a significant decrease in intensity at 3444 cm^{-1} in PC and PS, indicating modifications in the H bond of the OH group of cellulose. On the contrary, there was an increase in intensity in TL-treated rice straw, indicating higher cellulolytic activity during AD, which can also be correlated with higher cellulase activities in AD samples containing TL-treated rice straw. The peak at 2920 cm^{-1} corresponds to the C-H stretching vibration of lignin or cellulose. As compared to raw rice straw, a decrease in intensity was observed in PC and fungal-treated rice straw. The peak at 1738 cm^{-1} reduced significantly for PS and increased for TL and untreated rice straw compared to non-digested raw rice straw. This peak represents the ester bond link between lignin and hemicellulose [218]. The peaks at 1514 and 1382 cm^{-1} indicate structural modifications in the structure of lignin post-treatment and AD.

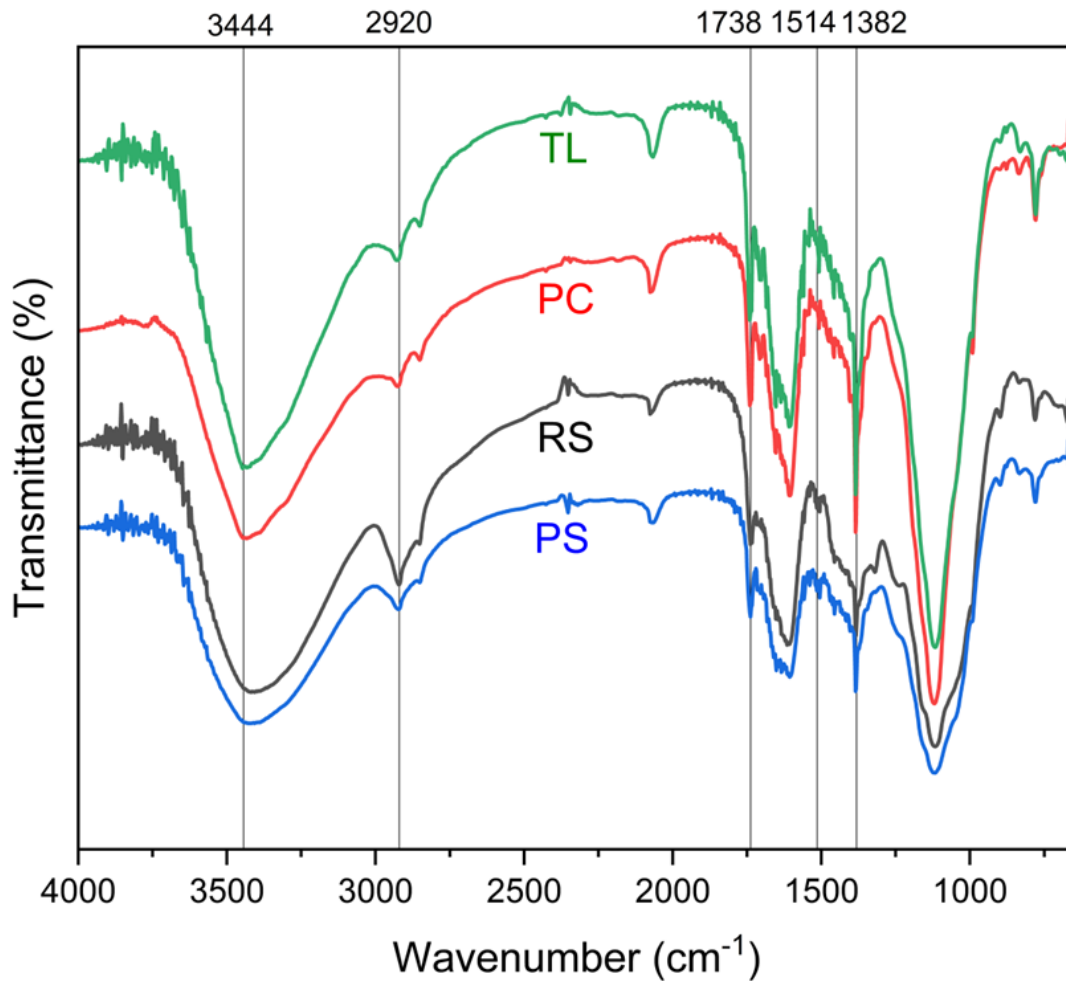


Figure 6.6. FTIR spectra of raw rice straw (RS), untreated digested rice straw (PC), digested PS and TL-treated rice straw

6.2.3. Effect of fungal treatment on microbial community

6.2.3.1. Bacterial community composition

The composition of the bacterial community on day zero in NC, on day 30 in PC, and on day 18 in PS and TL, respectively, is shown in *Figure 6.7*. These sampling days were selected based on initial microbial flora on day 0 and the days when the highest biogas production was observed in the samples. Proteobacteria, Firmicutes, and Bacteroidetes were the dominant phyla in all the samples as shown in *Figure 6.7(a)*. In the positive control, the dominant phyla included Firmicutes (44.91%), Bacteroidetes (44.05%),

Proteobacteria (4.53%), Spirochaetes (3.88%), and Chloroflexi (1.46%). The abundance of Proteobacteria and Bacteroidetes increased to 5.12% and 55.62%, respectively, and Firmicutes decreased to 35.55% in AD samples containing TL-treated rice straw as compared to PC. The abundance of Proteobacteria increased to 65.35%, and Firmicutes and Bacteroidetes decreased to 14.08% and 11.85%, respectively, in AD samples containing PS-treated rice straw. The negative control having only anaerobic inoculum, contained Proteobacteria (55.17%), Firmicutes (21.57%), Bacteroidetes (16.02%), and Actinobacteria (3.74%), respectively. Other phyla were present at sub-dominant levels. It was observed that the relative abundance of Proteobacteria in PS-treated rice straw and Bacteroidetes in TL-treated rice straw was the highest which might be the reason behind higher biogas production and higher enzyme activities in PS and TL. Proteobacteria are known for their metabolic versatility and can perform functions such as fermenting complex organic compounds, metabolizing fatty acids, and participating in syntrophic interactions. Firmicutes are known for their ability to ferment complex carbohydrates and produce VFAs as metabolic byproducts [219]. Bacteroidetes are involved in the breakdown of hemicellulose and other carbohydrates present in rice straw to organic acids [220].

The changes in the bacterial community at the genus level are represented in *Figure 6.7(b)*. The predominant genera present in the anaerobic digestion samples of TL and PS were Unclassified *Clostridiales* (24.36%, 2.95%), *Pseudomonas* (0.92%, 13.43%), *Prevotella* (26.01%, 1.47%), *Acinetobacter* (0.26%, 6.84%), *Bacillus* (0.89%, 5.59%), *Serratia* (0.30%, 3.70%) and *Bacteroides* (10.70%, 0.67%) respectively. The majority of the bacterial genera identified belonged either to Firmicutes or Bacteroidetes phyla, which are major hydrolyzers in AD. A higher abundance of hydrolyzers in TL explains the higher enzyme activities, sCOD utilization, and biogas production. An interesting

observation was that the relative abundance of *Prevotella* was highest (26.01%) in TL, followed by 16.37% in PC. This may be because organic carbon was readily available as a result of fungal treatment, while in PC, microbes had to acclimatize to break down complex carbohydrates. The lowest percent of *Prevotella* was reported in PS. *Prevotella* releases a diverse set of enzymes like cellulases and xylanases that help in the breakdown of complex lignocellulosic biomass. However, PS reported a higher diversity of microbes like *Acinetobacter*, *Bacillus*, *Serratia*, etc., which together performed the hydrolysis. *Serratia* plays an important role in hydrolysis and acidogenesis. Its high number in PS improved the AD process.

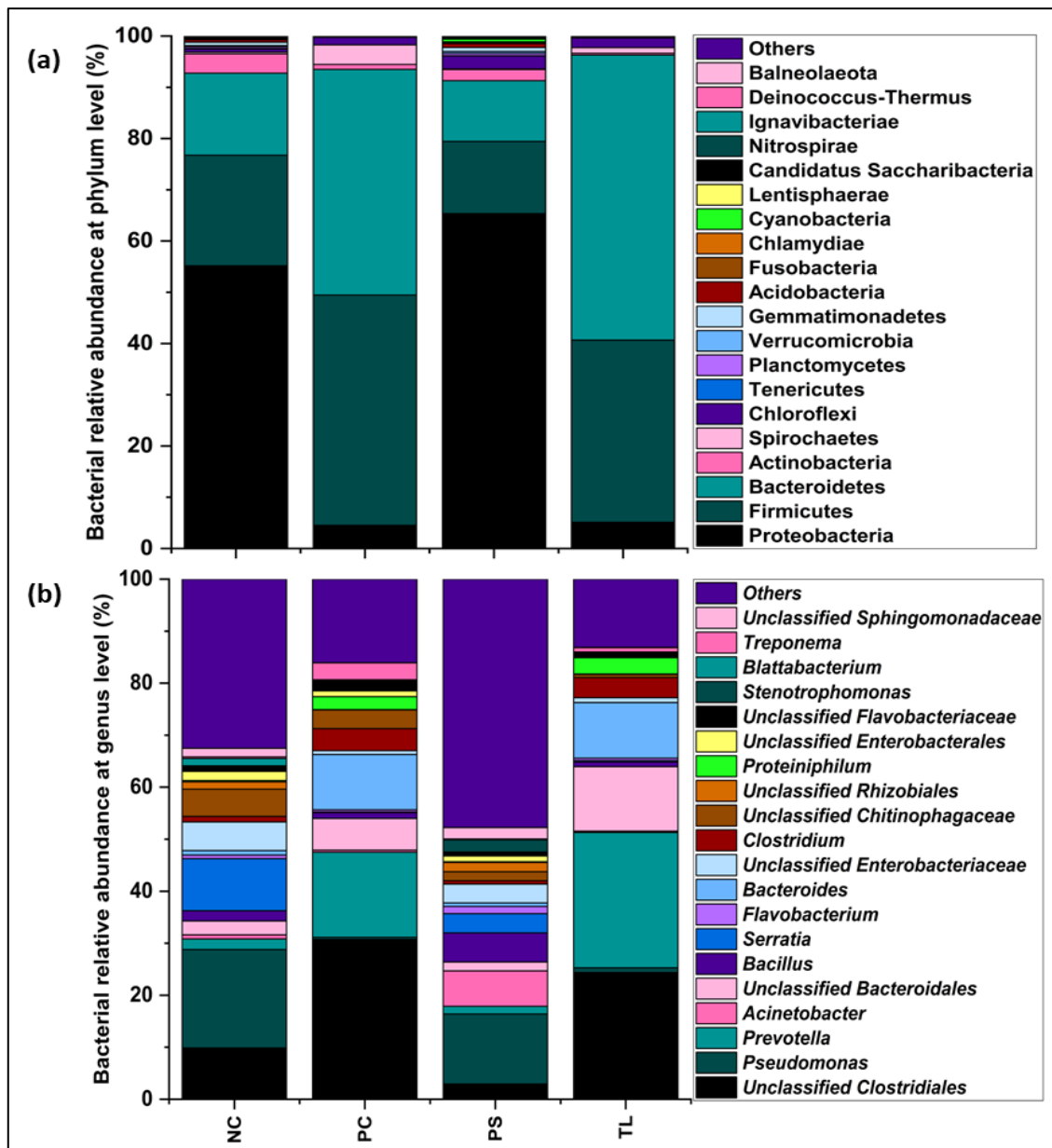


Figure 6.7. The analysis of the composition of bacterial community at (a) phylum, and (b) genus level in anaerobic digestion of untreated, PS and TL-treated rice straw

6.2.3.2. Archaea community composition

The relative abundance of the archaeal community at the genus level is presented in Figure 6.8. The major genera detected were *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter*, and *Methanolinea*. The relative abundance of *Methanosarcina* in PS and TL was 3.99% and 59.47%, respectively. *Methanosarcina* is an acetate utilizing

6.2.3.3. Comparative analysis of bacterial community diversity

Table 6.1 shows the alpha diversity indices of the bacterial community in different samples. The Chao1 index estimates species count, higher values indicating increased richness. The Shannon index considers species evenness and richness. Simpson index quantifies species diversity. Fisher's alpha quantifies species and individuals within a community.

Table 6.1. The distribution of alpha diversity indexes of bacterial communities

Sample	Chao1	Fisher	Shannon	Simpson	Number of reads
NC	387.46	53.64	3.82	0.93	88249.00
PC	258.20	30.57	2.75	0.86	68950.00
PS	390.25	53.80	4.31	0.96	71271.00
TL	262.00	32.51	2.54	0.84	77645.00

Compared to the negative control (NC), the Chao 1 index increased in AD samples containing PS and significantly decreased in TL-treated rice straw, although it remained higher than PC. *P. sanguineus* is an edible fungus, while *T. longibrachiatum* may produce mycotoxins, volatile organic compounds, and bioactive compounds, potentially affecting microbial diversity and richness [222]. It was observed from the metagenomic data that number of different species was less in TL as compared to other samples. Fungal treatment significantly increased microbial diversity, indicating positive correlations with functional versatility compared to the control. Similar observations were made for other alpha diversity indices. Alpha diversity showed significant correlations with production parameters, suggesting that increased microbial richness may contribute to enhanced

biogas production, enzymatic activities, pH resilience, and reduced accumulation of volatile fatty acids (VFAs).

The shared and unique bacterial operational taxonomic units (OTUs) among the samples have been presented in the form of a Venn diagram, as shown in *Figure 6.9*. A total of 5 unique bacterial genera were found in the positive control, 44 in the negative control, 11 in AD samples containing TL-treated rice straw, and a maximum number of 87 unique entities in AD samples containing PS-treated rice straw. It was observed that the microbiome in PS evolved the most as it had the maximum unique microbes. A total of 218 common bacterial genera were reported in all four samples. A Venn diagram visually displays the overlap and unique components among datasets, revealing shared and distinct features.

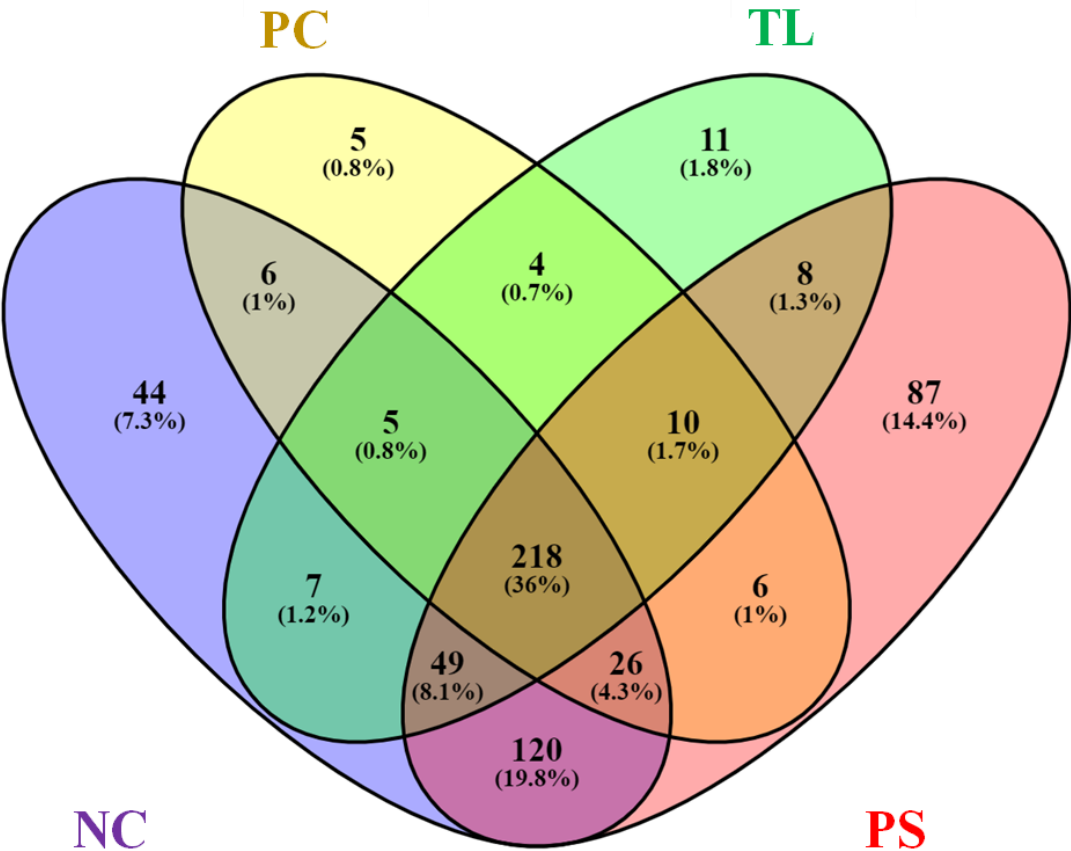


Figure 6.9. Venn diagram for the distribution of shared and unique bacterial OTUs

6.2.4. Redundancy analysis (RDA) of flow cytometry populations and physicochemical parameters

Figure 6.10 represents the redundancy analysis (RDA) of flow cytometric data of microbial population recorded every three days with the physicochemical parameters of AD like three-day cumulative biogas, pH, VFAs, cellulase, xylanase, and lignin peroxidase enzymes. RDA is used to summarize the variation in response variables by a set of computed explanatory variables [223]. Flow cytometry is a cost-effective, rapid, automated, and high-throughput method with easy sample preparation and online integration. It provides unique microbial fingerprints for analyzing population dynamics, enabling quick monitoring of anaerobic digestion community health. Microbial populations obtained in flow cytometry fingerprints were divided into gates on the basis of their scattering properties where each gate might represent a specific phenotypic population whose number has been monitored for the entire duration of the experiment. The population number represents the sequential number allotted to each population. The RDA ordination explained 68.15% (RDA 1) and 25.41% (RDA 2) of the variability, respectively, of the total distribution. The main explanatory variables affecting the microbial population were VFAs and sCOD. A positive correlation was found between population 8 and population 10 in flow cytometry data. Population 1 and Population 9 were negatively correlated, indicating significant differences between the two populations. Significant difference can be seen between population 1 and population 2. Population 3 and population 7 shared similarities.

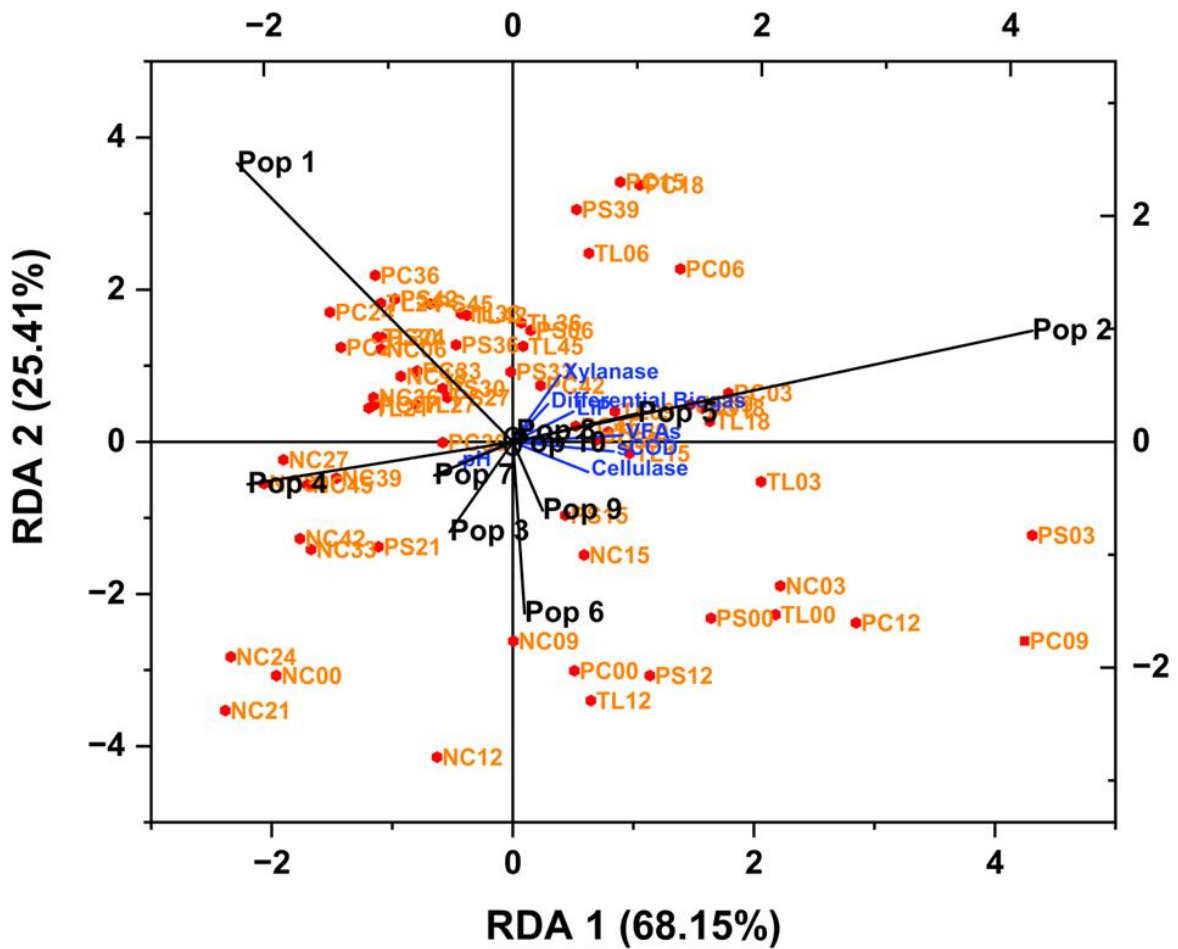


Figure 6.10. RDA analysis for studying correlations between physicochemical parameters and microbial populations analysed by flow cytometry

6.3. Conclusion

The fungal pretreatment of rice straw with *P. sanguineus* and *T. longibrachiatum* increased biogas yield by 20.79% and 17.85% respectively. Higher activities of cellulase and xylanase enzymes in AD systems with fungal treated rice straw are indicative of improved hydrolysis. The integration of fungal pretreatment into existing agricultural biogas plants can lead to enhanced efficiency, reduced environmental impact, and a more sustainable development pathway. Further research and development efforts should focus on optimizing the scale-up and commercial application of fungal pretreatment techniques.