Chapter 2

Review of Literature

2.1. Background

The disposal of rice straw (RS) through open dumping and burning is a widespread agricultural practice, particularly in South and Southeast Asia [25]. Despite the damaging effects, the practice of open dumping and burning of rice straw continues due to its low cost and perceived convenience [26]. Biogas, which can be derived from organic wastes like rice straw, stands out as a crucial and prominent renewable energy source [27]. Anaerobic digestion (AD) has emerged as a potential technological solution for the treatment of organic waste, concurrently generating biogas as a valuable and energy-generating byproduct [28]. Agricultural residues, such as rice straw, constitute a substantial proportion of the organic waste stream and possess considerable potential for anaerobic digestion owing to their abundant availability and high organic content [21].

Extensive research and development efforts have been dedicated to the study and advancement of pretreatment procedures to address the difficulties presented by the resistant characteristics of rice straw. The objective of pretreatment methods is to modify the physical, chemical, and structural properties of biomass to enhance its susceptibility to the microbial consortia that are responsible for anaerobic digestion [29]. Furthermore, this practice plays a significant role in the reduction of environmental pollution and the advancement of a circular economy through the c\onversion of organic waste into valuable bioproducts [30]. By effectively tackling these obstacles and advancing scientific investigations in this domain, the optimal use of rice straw as a renewable energy source may be streamlined, making a significant contribution towards a sustainable and environmentally friendly future [31].

This chapter summarises in detail the process and current state-of-art of anaerobic digestion of rice straw, the role of inoculum microbial dynamics, the importance of pretreatment techniques and operational parameters, the role of lignolytic enzymes, and potential value-added uses of biogas residue.

2.2. Rice straw as a valuable feedstock for anaerobic digestion

Rice straw, an abundant crop residue, is an excellent feedstock for anaerobic digestion, mainly because it contains high amounts of carbon-rich cellulose, hemicellulose, and lignin [32]. Although lignin contributes to its recalcitrance to decomposition, the composition of rice straw as a whole makes it a highly energy-rich substrate that is ideal for anaerobic digestion [33]. Rice straw, due to its high carbon-to-nitrogen ratio, promotes microbial activity, which meets the requirements for achieving optimal biogas generation that is rich in methane [12]. This biogas can be used as a sustainable energy source for generating power and providing heat. This application of rice straw aligns with the ideas of a circular economy, as it transforms agricultural waste into a valuable resource while also reducing environmental impacts [34]. In addition, anaerobic digestion decreases the release of methane, which is released due to the decomposition of rice straw in flooded fields, thus aiding in the mitigation of climate change [35]. The AD of rice straw produces a digestate that is rich in nutrients and can be used as a biofertilizer, completing the nutrient recycling cycle in agriculture [36]. Novel preprocessing techniques significantly augment the microbial degradation of rice straw, effectively tackling the obstacles associated with its recalcitrance to decomposition [37].

2.3. Recalcitrance of lignocellulosic biomass and its structure

Lignocellulosic biomass (LCB) is a renewable resource present in abundance in nature. It is made of three important components comprising of cellulose, hemicellulose, and

lignin, and this composition may vary depending upon the plant species and the geographical conditions a plant grows in [38]. LCB has the potential to be used for the production of biofuels and biochemicals in place of conventional fossil fuels [39]. However, LCB is inherently insoluble in water and resistant to degradation because of its recalcitrance to microbial attack and enzymatic breakdown, which limits its usage as an ideal feedstock for AD for biofuel production [40]. The structure of LCB is composed of complex carbohydrates which can serve as a suitable carbon source for AD for biogas production if the challenge of its recalcitrance can be addressed [41]. For an effective breakdown of LCB, understanding the structural and chemical factors associated with the plant cell wall is an important milestone to achieve [42]. The structure of LCB has been described in Figure *2.1* and explained in detail below:

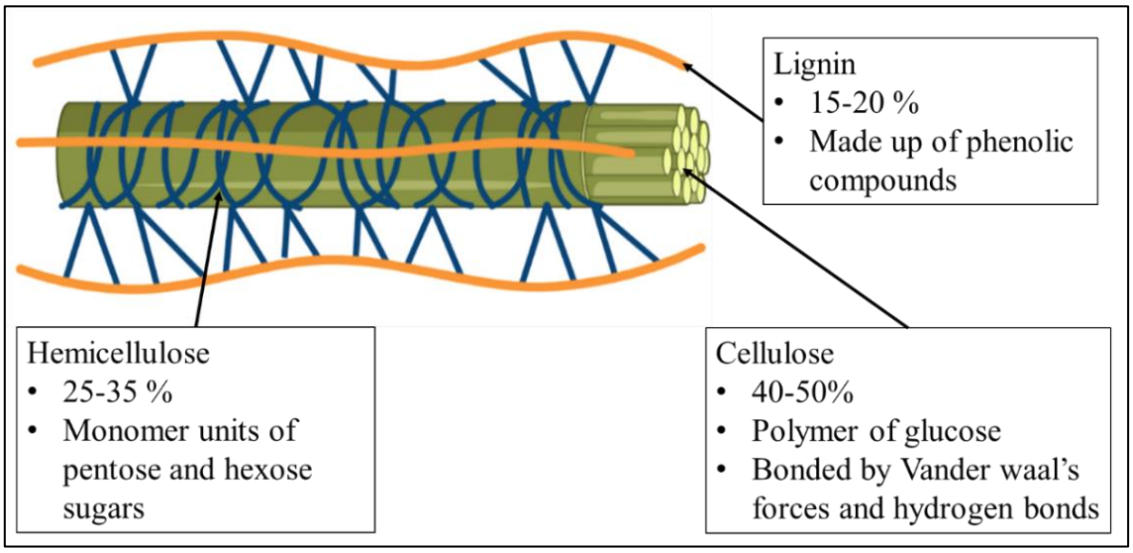

Figure 2.1. Structure of lignocellulosic biomass

2.3.1. Cellulose

Cellulose is a key component and the most abundantly present polymer in LCB. Cellulose makes up to 40-50% of the LCB in weight [43]. Cellobiose, a disaccharide reducing sugar serves as the primary repeating unit in cellulose [44]. The recalcitrance of LCB depends

upon the number of D-glucose units present in the cellulose microfibrils, also known as the degree of polymerization [40,45]. The interconnection of cellulose chains by hydrogen bonding and Van der Waals interactions forms high-tensile strength microfibrils [46]. The irregular arrangement/orientation of microfibrils creates zones of varying crystallinity in the structure of LCB generating crystalline and amorphous regions [47]. Cellulose is responsible for providing strength and rigidity to plant cell walls and is an important constituent of plant fibres.

2.3.2. Hemicellulose

Hemicellulose constitutes 25-35% of the LCB biomass weight. It is composed of heterogenous and branched biopolymers mainly comprising of pentose sugars like xylose and arabinose, hexose sugars like rhamnose, glucose, and mannose, and acids like galacturonic acid and glucuronic acid [48]. Hemicellulose has a heterogenous, amorphous and branched structure that binds non-covalently to the microfibrils of cellulose to form a matrix that provides flexibility to the plant cell wall [45]. The presence of acetyl groups in hemicellulose in LCB may limit the accessibility of cellulose to microbial enzyme attack. Pretreatment of LCB reduces the degree of acetylation and improves the accessibility of enzymes for microbial attack [49].

2.3.3. Lignin

After cellulose, lignin is the second most abundant biopolymer and makes up 15-20% of the LCB biomass [50]. It is a complex and cross-linked polymer that forms a rigid three-dimensional matrix with cellulose and hemicellulose creating a hydrophobic impermeable barrier that protects the plant cell wall from microbial attack and environmental stress [51]. Lignin is composed of three basic phenyl propanoid derivatives including ρ-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, also

known as monolignols [52]. Lignin is synthesized by monolignol dehydrogenation mediated by peroxidase enzymes that yields a heterogenous structure where basic units are interlinked by C-C, and aryl-ether bonds with aryl-glycerol and β-aryl ether. In addition to lignin, these monolignols can also bond with other biopolymers like polysaccharides and proteins present in the cell wall to form an intricate three-dimensional structure [53]. Lignin is the main component of LCB that limits its utilization as a renewable resource for energy production.

2.4. Process of anaerobic digestion

Anaerobic digestion is a process carried out by bacteria and archaea that convert complex organic substrates to biogas in an oxygen-free environment. Biogas is mainly composed of 50-75% methane (CH_4), 25-50% carbon dioxide (CO_2), and traces of hydrogen sulphide (H2S), nitrogen (N2), hydrogen (H2), ammonia (NH3) and water vapours [54]. The process of anaerobic digestion takes place in four stages which have been explained in *Figure 2.2*.

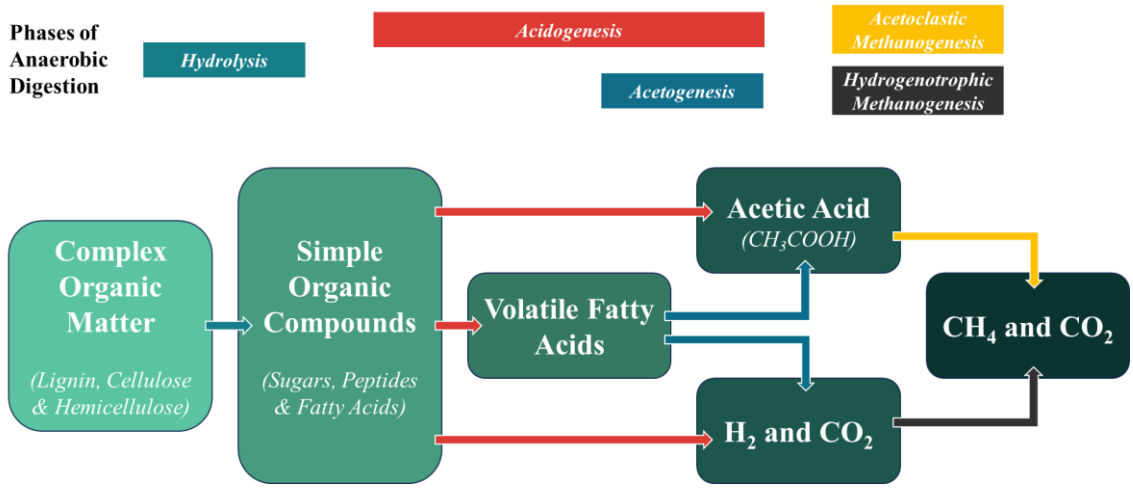

Figure 2.2. Stages of anaerobic digestion of organic matter

2.4.1. Hydrolysis

The first step or the initial stage in the AD process is hydrolysis, where complex organic matter constituted of carbohydrates, proteins, and lipids is converted to simpler soluble compounds such as monomeric sugars, amino acids, and fatty acids [55]. The bacteria that carry out hydrolysis are known as hydrolysers, which secrete hydrolytic enzymes like cellulases, amylases, proteases, and lipases that target specific types of organic molecules. The hydrolysis step increases the solubility of complex organic matter in the liquid phase, thereby increasing the accessibility of the microorganisms to metabolize the organic compounds [56]. The products of hydrolysis serve as the substrates for subsequent stages of AD.

2.4.2. Acidogenesis

The second step in the AD process is known as acidogenesis. In this stage, the fermentative acid-forming bacteria metabolizes the simple sugars, fatty acids, and amino acids produced during the hydrolysis step. The primary metabolic product of acidogenesis are the organic acids commonly called as volatile fatty acids (VFAs) such as acetic acid, propionic acid, butyric acid etc [57]. The production of VFAs may lead to a decrease in the pH inside the digester, thus, its regulation is very important for the proper functioning of the digester. These organic acids have a comparatively lower carbon-to-hydrogen ratio (C/H ratio) than the initial feedstock and the products of hydrolysis. The VFAs and the other intermediate compounds produced during acidogenesis serve as the precursors for further stages of AD [58]. Certain intermediate compounds like CO_2, H_2, and some alcohols are also produced during this stage, and their production depends on the dynamics of the microbial community and the type of organic matter in the AD feedstock [59].

2.4.3. Acetogenesis

Acetogenesis is a step in AD that follows acidogenesis and precedes methanogenesis. During this stage, homoacetogens transform VFAs from the previous step into acetate, which serves as a substrate for the methanogenesis step. Acetogenic bacteria are responsible for the metabolization of VFAs through a series of different metabolic pathways. Acetate is an important intermediate in this stage which acts as a precursor to produce methane [60]. Along with acetate, the acetogens also produce CO_2 and H_2 as additional metabolic products which also serve as substrates for methanogenesis. Acetogenic bacteria exhibit syntrophic relationships with the methanogenic archaea where the products of acetogens serve as substrates for methanogens. Acetogens are responsible for maintaining the redox balance inside an anaerobic digester [61].

2.4.4. Methanogenesis

Methanogenesis is the final stage of AD, and the intermediate products of previous stages, like acetic acid, carbon dioxide, and hydrogen, are converted to the final product, i.e., methane [62]. The key microorganisms taking part in this stage are known as methanogens and mainly composed of the archaea which produce methane as the final metabolic product. Methanogenesis may occur via two metabolic pathways, namely, acetoclastic methanogenesis and hydrogenotrophic methanogenesis. The acetoclastic pathway directly converts acetic acid to methane and carbon dioxide, while the hydrogenotrophic pathway utilizes hydrogen and carbon dioxide to produce methane [57]. The methane produced during the final step also contains trace amounts of other gases like nitrogen, hydrogen sulphide, and water vapour [63]. Methanogenesis is regarded as the rate-limiting step in AD because of the slow growth of methanogens but in the AD of LCB, hydrolysis is the rate-limiting step.

2.5. Effect of source of inoculum on AD of rice straw

The microbial community structure greatly influences the performance of an anaerobic digestion system [64]. Thus, the source and quality of inoculum play a vital role in governing the substrate degradation to fermentable sugars [65]. The inoculum acts as a seed microbial consortium composed of hydrolytic, acidogenic, acetogenic bacteria and methanogenic archaea. Cellulolytic enzymes called cellulases are responsible for the deconstruction of lignocellulose to reducing sugars like disaccharides and oligosaccharides, which are consumed by anaerobic microorganisms to produce methane through a series of complex reactions carried out by bacteria and archaea [66]. Understanding the complex dynamics of microbial interactions in AD is very important to monitor the stability of the anaerobic digestion process. A suitable inoculum should contain active microbial community that can enhance the degradation rate, improve biogas generation, and reduce the lag phase that allows the anaerobic digester to achieve stable operation more rapidly [67]. An inoculum consists of important syntrophic microorganisms whose efficient cooperation ensures effective degradation of LCB. LCB may often contain some inhibitory compounds, but an acclimatized inoculum shows higher tolerance and prevents AD disruption [68]. Inoculum also provides micro- and macro-nutrients and regulates the buffering capacity of an AD system [69]. Most studies have reported that the effluent from anaerobic digesters used as an inoculum performs better than raw manures, rumen fluid, and activated sludge [70]. The effluent from anaerobic digesters provides more total solids (TS) percent and is rich in active and acclimatised microbial community comprising of acetogens and methanogens [71]. It was reported that corn stover produced 9.8-15.5% higher biogas and 8.0-10.8% higher methane when digested with swine manure as compared to digestion with dairy manure and municipal sludge [72]. In a study comparing rumen fluid and anaerobic sludge as

inoculum for anaerobic digestion of crop residues, it was reported that rumen microbes produced four times higher VFAs while anaerobic sludge reactors produced higher biogas [73]. Different inoculums have been tested and variations in the production of VFAs and CH_4 yields have been seen in various studies [74]. *Table 2.1* compares the effectiveness of different inoculum sources for the anaerobic digestion of rice straw.

Table 2.1. Comparison of different inoculum sources for the anaerobic digestion of rice straw

Substrate	**Co-substrate**	**Inoculum**	**F/M ratio (VS basis)**	**CH_4 yield (mL/g VS added)**	**Reference**
Rice straw	Kitchen waste and pig manure	Anaerobic sludge	2	350	[75]
Rice straw	-	Digested dairy manure	2	178.30	[76]
Rice straw	-	Rumen fluid	1.25	287	[77]
Rice straw	-	Anaerobic sludge	0.5	355.30	[78]
Rice straw	-	Waste activated sludge	4	285.20	[79]
Rice straw	-	Digested cow dung	3	176.47	[80]
Rice straw	-	Anaerobic sludge	2	197	[64]

Rice straw	Food waste	Digested cow dung	1.87	323.78	[81]
Rice straw	*Hydrilla verticillata*	Digested cow dung	2.15	287.60	[82]
Rice straw	-	Liquid consortium	1	267.70	[83]
Rice straw	-	Cow manure	1	182.00	[83]
Rice straw	-	Sheep dung	1	193.50	[83]
Rice straw	-	Biogas slurry	1	203.00	[83]
Rice straw	-	Straw-decomposing consortia	1	200.80	[83]

2.6. Strategies for the pre-treatment of rice straw

Rice straw being a complex lignocellulosic material is difficult to digest anaerobically and poses certain challenges like low biogas yield, production of inhibitory intermediate products, and poor buffering capacity [54]. These challenges can be accredited to the recalcitrant structure of rice straw that needs to be pretreated before anaerobic digestion. Hydrolysis is the rate-limiting step in the AD of LCB as it is insoluble in water and its structure is resistant to microbial enzyme attack [84]. The choice of the pretreatment method depends upon several factors like crystallinity, accessible surface area, and content of cellulose, hemicellulose, and lignin in LCB. There are a few pre-requisites that need to be taken care of before selecting a pretreatment strategy that include 1) access cellulose fibres to allow microbial attack, 2) absence of possible inhibitors of hydrolytic and fermentative microorganisms, 3) low energy requirement, 4) low cost of operation,

and 5) minimal or no use of harmful chemicals [84]. Several researchers have contributed to enhancing the AD efficiency of LCB by exploring different pretreatment methods which can be broadly classified into following categories:

Table 2.2.Comparison of efficiency of different pretreatments of rice straw in biogas productions

Pretreatment type	Method	Pretreatment conditions	Methane production in untreated sample	Methane production in treated sample	Percent improvement in methane production (%)	Reference
Physical	Steam explosion	200 °C for 120 seconds	100.58 mL/g TS	197.22 mL/g TS	51.00	[85]
	Extrusion	120 rpm, driven by a 55-kW monitor	132 L/kg VS	227.30 L/kg VS	72.20%	[86]
	Microwave	900 W for 5 min	218.90 mL/g VS	196.22 mL/g VS	-10.36%	[87]
	Hydrothermal	100 °C	98.50 mL/g TS	127.6 mL/g TS	22.80	[88]
	Cold isostatic pressure	400MPa for 9 minutes	136.32 mL/g VS	239.86 mL/g VS	76	[89]
	Electrohydrolysis	25V	224.01 mL/g VS	319 mL/g VS	42.4	[17]
Chemical	Alkali	1% NH_3	268.35 mL/ g TC	831.90 mL/ g TC	210	[90]
	Alkali	3N NaOH	249.95 mL/g VS	318 mL/g VS	21.40	[91]
	Alkali	10% NaOH for 75 min	218.90 mL/g VS	267.74 mL/g VS	20.94	[87]

	Ammoniation	4% ammonia	325.61 mL/g VS	396.92 mL/g VS	21.90	[92]
Biological	Fungal	*Phanerochaete chrysosposrium* for 5 weeks	60.47 mL/g VS	339.31 mL/g VS	461	[93]
		Ganoderma lucidum for 5 weeks	60.47 mL/g VS	295.91 mL/g VS	389.35	[93]
		Pleurotus ostreatus	60.47 mL/g VS	269.99 mL/g VS	346.48	[93]
		Pleurotus ostreatus for 20 days at 75% moisture	119.54 mL/g VS	263 mL/g VS	120	[94]
		Trichoderma reesei	120.02 mL/g VS	214 mL/g VS	78.30	[94]
Combined	Alkali + Liquid Fraction of digestate (LFD)	LFD, 6% CaO	174.31 mL/g VS	274.65 mL/g VS	57.56	[95]
	Physical + Alkali	Extrusion + 10% $Ca(OH)_2$	420.26 mL/g VS	574.5 mL/g VS	36.70	[96]

Physical + Alkali	900 W for 5 min, 10% NaOH for 75 min	218.90 mL/g VS	260.87 mL/g VS	19	[87]
Physical + ammoniation	660 W for 6 min, 3.94% ammonia for 18 hours	224.47 mL/g VS	281.56 mL/g VS	25.43	[97]
Alkaline electrohydrolysis	-	111.73 mL/g VS	167.4 mL/gVS	49.82	
Fungal + Milling	Milling (≤ 2mm) + *Pleurotus ostreatus* for 30 days	97.35 mL/g VS	258 mL/g VS	165	[98]

2.6.1. Physical pre-treatment

Physical pretreatment methods usually employ thermal and mechanical methods to change the physical and structural properties of rice straw for efficient anaerobic digestion. Physical methods of pretreatment expose the bound cellulose and hemicellulose fibres for microbial attack, thus, increasing the availability of accessible carbon in LCB. This improves and enhances the AD efficiency by increasing the biogas generation. The methods of physical pretreatment include milling or grinding, palletization, microwave irradiation or radio frequency heating, steam explosion, extrusion, hydrothermal pretreatment, etc. These methods change the structure of LCB by breaking down the plant cell wall and favour the accessibility of anaerobic microbes to the organic matter. This helps in the easy digestion of LCB, which promotes higher energy generation by improving methane yield [99].

Milling and grinding pretreatment works by reducing the particle size which affects the crystallinity of the LCB. The reduction in particle size increases the available surface area for microbial attack, reduces the startup time/lag phase, and improves the buffering capacity of anaerobic digestion [98]. In extrusion pretreatment, biomass is forced to pass through a narrow passage at high temperatures and pressures that change the physical structure of biomass. Extrusion can be carried out at moderate pH and temperature, thus minimizing the production of toxic intermediates and reducing harmful environmental impact [100]. Despite its advantages, extrusion pretreatment has its limitations as it is less economical and requires high energy limiting its application at an industrial level [101].

The application of microwave irradiation and ultrasonic pretreatment induces modifications in the internal microstructure of straw, resulting in advantageous effects on anaerobic digestion reactions, as well as enhancements in fermentation and gas

production [47]. Although microwave irradiation has demonstrated potential, its industrial usefulness is constrained by the significant expense associated with its installation [102]. The utilization of steam explosion pretreatment, which encompasses the application of elevated temperatures and subsequent pressure reduction, has been demonstrated as a highly efficient and ecologically sustainable approach. This method has been found to result in a notable enhancement in the production of biogas derived from rice straw [103]. The application of hydrothermal pretreatment, performed under high temperature and pressure conditions without the use of chemicals, has been shown to improve the accessibility of cellulose and induce modifications in lignin and hemicellulose [104]. The pretreatment methodologies exhibit promising potential; however, it is imperative to address the economic implications and energy efficiency aspects in order to facilitate their wider use within industrial contexts.

2.6.2. Chemical pre-treatment

The chemical pretreatment of rice straw plays a vital role in the process of converting this lignocellulosic biomass into bioenergy via anaerobic digestion. The primary aim of chemical pretreatment is to modify the physical and chemical composition of rice straw, hence increasing its susceptibility to microbial breakdown and improving the overall efficiency of biogas generation [105].

Alkali pretreatment encompasses the utilization of alkaline agents, such as sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$), liquid ammonia or urea for soaking or spraying on LCB that opens up the ester bonds present in the LCB allowing its dissolution and accessibility to microbial enzymes [106]. Further, it was observed that a higher concentration of alkali for pretreatment breaks down the LCB structure more effectively, but after a certain concentration limit, it becomes detrimental

to the AD process [107]. Alkali treatment helps in the delignification and reduction in the crystallinity of rice straw. The output of biogas from 6% NaOH-treated rice straw rose by 27.3−64.5%. NaOH pretreatment improved rice straw biodegradability, increasing biogas output [108]. Pretreatment with NaOH may impede the AD process, particularly methanogenesis, caused by inhibition by Na^+ ions. Additionally, disposing of Na^+-containing effluent from AD systems may cause environmental issues such as soil salinization and water contamination [105].

Hydrogen peroxide (H_2O_2) is a powerful oxidant, utilized in biomass pretreatment for ethanol and biogas production and as a bleaching agent in the paper and cellulose industries. Its advantage is that it degrades into oxygen and water, minimizing residues and hazardous intermediates in biomass [109]. However, an excess of hydroxyl ions may inhibit methanogenesis. A study examined how pretreatment with H_2O_2 influenced methane production. The lignin, cellulose, and hemicellulose components of rice straw experienced substantial degradation as the concentration of H_2O_2 increased. The best conditions for anaerobic digestion of pretreated rice straw were 6.18 days, and 1.08 substrate-to-inoculum ratio. These conditions produced 288 mL/g VS of methane [110].

When pretreatment of lignocellulose biomass is concerned, dilute acid is preferred over concentrated acid. Depending on the pretreatment circumstances, dilute acid pretreatment largely hydrolyzes up to 100% of the hemicellulose into its component sugars (e.g., xylose, arabinose, and galactose) [105]. Certain research groups have also tried pretreatment of rice straw with a less toxic, thermally stable, and eco-friendly organic solvent, N-methylmorpholine-N-oxide (NMMO), which has been seen to decrease the crystallinity of rice straw [111]. Rice straw treated with 50% (w/w) NMMO at 130 °C for 3 hours with 15-minute mixing intervals produced 328 NmL CH_4/g VS [112].

2.6.3. Biological pre-treatment

Biological pretreatment of rice straw for anaerobic digestion entails employing microbes, specifically fungi, to enzymatically degrade intricate lignocellulosic structures prior to exposing the biomass to anaerobic digestion [95]. The main goal is to increase the accessibility of cellulose and hemicellulose, which will improve the efficiency of microbial breakdown and the production of biogas. White-rot fungi, such as *Phanerochaete chrysosporium* and *Trametes versicolor*, are frequently used because they have the capacity to release ligninolytic enzymes, such as lignin peroxidase, manganese peroxidase, and laccase [113]. These enzymes have a vital function in specifically breaking down lignin, which is frequently a constraining factor in the anaerobic digestion of lignocellulosic materials [114]. The biological pretreatment technique is commonly regarded as ecologically sustainable, runs at moderate settings, and has the capacity to decrease the production of inhibitory compounds [115]. Nevertheless, it is crucial to tackle obstacles such as extended treatment time and potential substrate specificity, in order to ensure the optimal conditions for achieving maximum enzymatic activity [116]. Research in this topic concentrates on improving biological pretreatment techniques, investigating various microbial communities, and incorporating these approaches into scalable and sustainable processes for efficiently converting rice straw into bioenergy. The pre-treatment of rice straw with *P. ostreatus* and *T. reesei* had a significant favorable influence. It resulted in a methane yield of 263 L/kg VS, which is a 120% increase compared to untreated rice straw. Additionally, the pre-treatment with *T. reesei* yielded 214 L/kg VS, which is a 78.3% increase compared to untreated rice straw. The methane yield achieved with *P. ostreatus* was higher than the methane yield obtained through most physical and three-chemical pre-treatment processes [98].

2.6.4. Combined pre-treatment

Combining rice straw pretreatment methods in anaerobic digestion is a novel way to boost efficiency. Combinations of physical, chemical, and biological pretreatments have been tested to overcome method limitations [117]. Thermal and alkali pretreatment improves substrate digestibility by disrupting biomass crystalline structures and breaking down hemicellulose and lignin [118]. Biochemical pretreatments using lignocellulolytic enzymes from fungi and chemical agents selectively break down lignin and hemicellulose are another option. Combined techniques increase cellulose accessibility, reduce recalcitrance, and boost biogas yields by using each method's strengths and minimizing their weaknesses [119]. Optimizing combination pretreatments requires careful consideration of temperature, time, and concentrations to create a synergistic effect without sacrificing rice straw biomass integrity [120]. In a study, rice straw treated calcium oxide (CaO) and liquid fraction from digestate (LFD) generated 274.65 mL/g VS methane, which was 57.56% higher than the untreated sample [95].

2.7. Role of microbial community dynamics in anaerobic digestion of rice straw

The effectiveness and stability of rice straw anaerobic digestion depend on microbial community dynamics [121]. Microbial communities are essential to the intricate microbial-driven conversion of organic matter into biogas, mostly methane, in anaerobic digestion [122]. Rice straw cellulose, hemicellulose, and lignin are hydrolyzed by microbial populations into simpler molecules. Hydrolyzed organic matter is metabolized by acidogenic bacteria to produce organic and VFAs [123]. Methanogens employ intermediates produced by acetogenic bacteria, which convert organic acids into acetate and other precursors. Methanogens like *Methanobacterium* and *Methanosarcina* produce methane from acetate, hydrogen, and carbon dioxide [124]. Effective methane generation requires syntrophic interactions between acetogenic bacteria and methanogens [125]. High microbial variety ensures functional redundancy and anaerobic digestion robustness

against environmental changes and inhibitory chemicals. Microorganisms must collaborate syntrophically to break down complex organic molecules and convert intermediates into methane. Microbial communities exhibit dynamic responses to variations in temperature, pH, substrate composition, and loading rates, impacting the stability and performance of anaerobic digestion [126].

Optimizing anaerobic digestion requires understanding and regulating microbial community dynamics. Metagenomics, metatranscriptomics, and cytomics provide in-depth microbial community structure and function studies. Isolating pure cultures from microbial communities has traditionally been the most critical step in identifying microbial communities in sludge [127]. A very small percentage of environmental bacteria can grow under laboratory conditions; hence, culturing and susceptibility testing have limitations for ecological bacteria. One of the main reasons is the unavailability of a quick, ubiquitous, and accurate approach for analyzing diversity in microbiomes from environmental samples [122]. Molecular biology approaches have substantially increased detection accuracy, paving the door for better interpretation and analysis. RT-qPCR offers multiple advantages over traditional methods as it offers high specificity because of uniquely designed primers, short handling time, and detection of very low copy numbers [128]. The main disadvantage of RT-qPCR is the requirement of knowledge to construct the primers for the bacterial species to be targeted. This reduces the likelihood of detecting unknown or unexpected species [129].

Metagenomics is a high-throughput method that confirms the presence or absence of specific organisms or genes in a microbiome [130]. Metagenomics has been used to identify the diversity of microbial communities in a variety of environments [131]. Metagenomic libraries are screened either using a sequence-driven method or by screening expressed phenotypes [132]. The use of NGS data in metagenomic analysis

has helped identify microbial strains associated with different stages of digestion [133]. Shotgun metagenome sequencing, which reads all of the DNA sequences included in the meta-community DNA retrieved from environmental samples, is one example of a metagenome-based technique that can reveal the overall roles of bacteria in the sample [134]. The metabolic function of microbial communities in AD has also been investigated using this method. Metagenome-related approaches like shotgun high-throughput sequencing are the most widely used sequencing platforms for finding microbial phyla in various settings, including sludge [135,136]. The Illumina high-throughput sequencing technology uses shotgun sequencing, a newly discovered molecular platform, to precisely and efficiently characterize the diversity and abundance of diverse diseases at the species level [137].

Metatranscriptomics and metaproteomics are less developed techniques as compared to metagenomics and need extensive research to establish a clearly defined methodology. In both these methods, the relative expression levels of genes of interest are estimated by mapping peptide sequences or short c-DNA reads against a selected set of genomes [138]. Metatranscriptomics focuses on the genes that are expressed by the complete microbiome and identifies the active functional profile and metaproteomics talks about the expression of proteins.

Flow cytometry is a technique used for the characterization of individual cells based on their morphology, intracellular complexity, and other physical and chemical characteristics. The cell population in the anaerobic digesters is very dynamic and diverse. In the last decade, the innovation of a unique approach that allows single-cell measurements of cytomics traits through flow cytometry has allowed for a new level of microbiome monitoring through a so-called "cytometric fingerprint," which can be presumed a morphologic identification at the single-cell level. Flow cytometry is a very

economical, robust and quick tool to monitor changes in microbial population on a day-to-day basis and can analyze small sample volumes with minimum efforts in sample preparation [136]. Flow cytometry, by virtue of its high throughput nature and ability to analyze multidimensional parameters, has the capability to outdo the most prevalent metagenomic approaches. In various studies, flow cytometry was utilized to characterize the viability of microbes in suspended sludge [139,140]. Apart from this, flow cytometry has previously been used in AD to evaluate archaea using the F420 co-factor, to assess microbial community dynamics and the genesis of sub-communities, along with directly monitoring phenotypic changes in the microbial population [140].

2.8. Research gaps

The following research gaps were identified during the literature review:

- Rice straw anaerobic digestion is impacted by factors like microbial enzyme activity, sCOD, pH, VFA production etc., other than temperature, retention period, and substrate-to-inoculum ratio that need additional study.
- Understanding the microbial communities engaged in rice straw anaerobic digestion might reveal key players and their responsibilities.
- Explore the potential of biological treatment of rice straw for AD owing to its low cost and environmental safety.
- Assess the viability of expanding rice straw anaerobic digestion systems for real-world implementation.