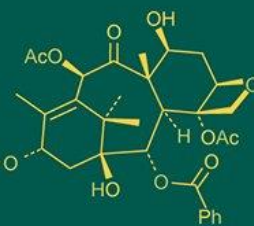
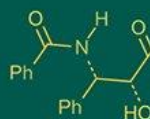


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Rahul Singh Chandel
Ph.D. Scholar, Animal
Nutrition, ICAR-NDRI,
Karnal, Haryana, India

Dr. Vibhor Agrawal
Ph.D. Scholar, Animal
Nutrition ICAR-NDRI, ERS,
Kalyani, West Bengal, India

Meetu
Ph.D. Scholar, Animal
Nutrition, LUVAS, Hisar,
Haryana, India

Rajesh S Khant
Ph.D. Scholar, Animal
Nutrition, ICAR-NDRI, ERS,
Kalyani, West Bengal, India

Lignocellulosic bond disruption in crop residues: plant cell-wall chemistry, pre-treatment markers and ruminant feed utilization

Rahul Singh Chandel, Vibhor Agrawal, Meetu and Rajesh S Khant

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Abstract

Crop residues (straws, stalks, bagasse, etc.) are rich in lignocellulosic cell walls, mostly constituted of cellulose, hemicellulose and lignin in different proportions. For example wheat straw is ~33-40% cellulose, 20-25% hemicellulose and 15-20% lignin; rice straw ~33-47% cellulose, 19-27% hemicellulose, 5-24% lignin; maize stover ~35-40% cellulose, 20-30% hemicellulose, 15-25% lignin. These strong cell walls are difficult for microbes to enter the rumen and must be broken down by pre-treatments (physical, chemical, biological) to improve digestibility. These techniques (e.g. steam explosion, alkali, ammonia, organosolv, enzymes, fungus, etc.) and the impact on cell-wall bonding have been described in important scientific work throughout the decades. In turn, a number of analytical indicators have been established to evaluate bond breakage (e.g. Klason lignin, FTIR band shifts, NMR signatures, enzymatic hydrolysis yields). Pre-treatments have been shown to increase fibre digestibility by several tens of percentage points *in vitro* and *in vivo* and to increase feed intake and milk/meat yield (e.g. ammonia fiber expansion (AFEX) of wheat straw improved digestibility by ~5-13 percentage points and increased cattle intake and milk yield by ~40-80%). Results however vary significantly with feedstock and approach. The chemistry of plant cell walls, pre-treatment techniques and their mechanistic basis and known impacts on ruminant eating are reviewed. We review the main analytical markers (chemical, spectroscopic, chromatographic, enzymatic, microscopic) used for the measurement of bond disruption and provide tables of residue composition, pre-treatment procedures, markers, animal reactions and future suggestions.

Keywords: Lignocellulosic cell walls, digestibility, meat, wheat straw

Introduction

Crop residues (cereal straws, maize stover, sugarcane bagasse, etc.) are lignocellulosic biomasses, which are mainly constituted of three biopolymers: cellulose (crystalline glucose polymer), hemicellulose (amorphous hetero polysaccharides) and lignin (heterogeneous aromatic polymer) [1, 2]. These are embedded in a matrix: cellulose microfibrils provide a fibrous framework (~25-50% of the dry mass) bound by hemicelluloses (~20-35%) and cemented by lignin (~5-30%). The proportions vary across species (see Table 1 below) [3, 4]. Lignin and hemicellulose attach to cellulose microfibrils via ether and ester bonds, producing a resistant network. The lignin normally present in grasses is rich in p-coumaryl, coniferyl and sinapyl alcohol units (H/G/S units) [5]. The hemicellulose is usually arabinoxylan (in cereals) or glucomannan (in other grasses). This composite construction, schematically shown in Fig.1, provides mechanical strength but limits the access to the enzyme [6]. Pre-treatments aim at breaking these connections. Multiple types of covalent connections must be broken. For example, ferulate cross-links between lignin and polysaccharides in grass cell walls and β -O-4 linkages that polymerize lignin [7]. Recalcitrance is also due to non-covalent interactions (hydrogen bonds between cellulose chains, hydrophobic contacts in lignin). To direct pre-treatments, it is important to understand the chemistry, e.g. acids preferentially break hemicellulose backbones (hydrolyzing acetylated xylans), bases saponify ester linkages between lignin and polysaccharides and oxidants (peroxides, ozone) attack aromatic rings and side chains [8, 9].

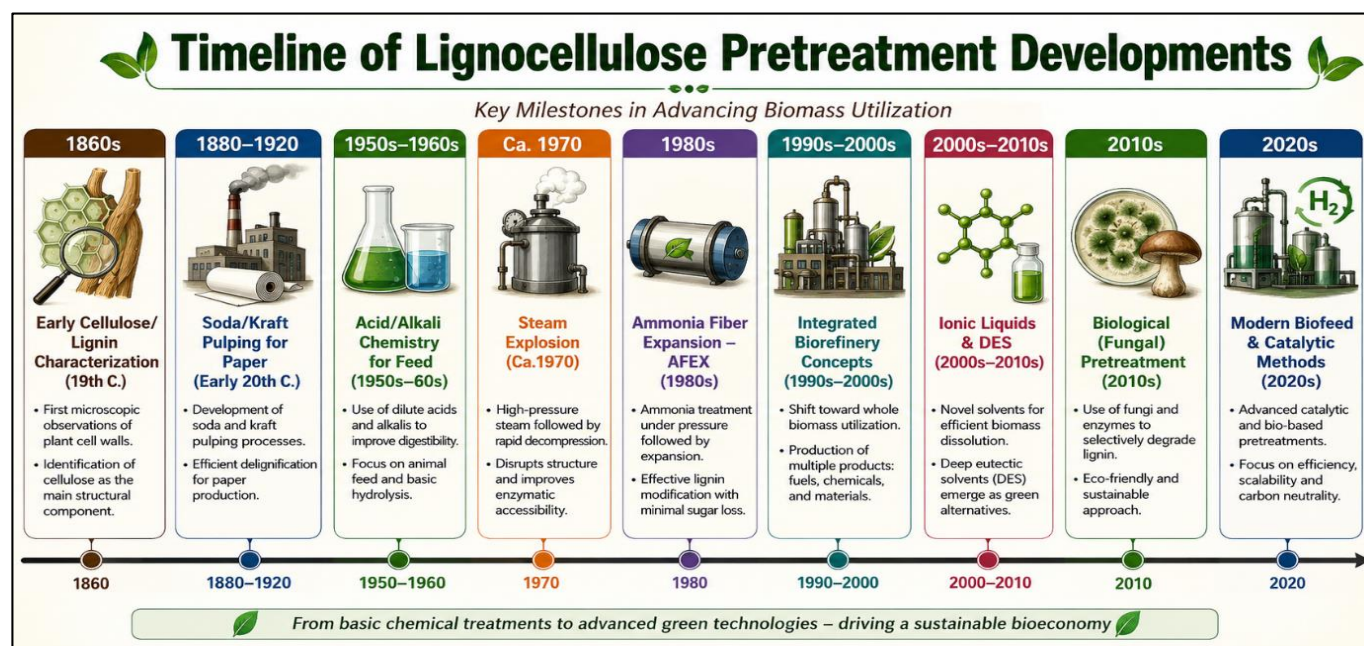
Corresponding Author:
Dr. Vibhor Agrawal
Ph.D. Scholar, Animal
Nutrition ICAR-NDRI, ERS,
Kalyani, West Bengal, India

Table 1: Major crop residues with typical cellulose, hemicellulose and lignin contents (% of dry mass) [10, 11, 12, 13, 14, 15, 16].

Crop Residue	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Wheat straw	33-40	20-25	15-20
Rice straw	33-47	19-27	5-24
Corn stover (whole plant)	35-40	20-30	15-25
Corn cobs	28	55	7
Sugarcane bagasse	35-49	16-33	11-25
Barley straw	35-40	20-30	15-25
Soybean straw	38	16	16
Rice husk (parenchyma)	35	25	20
Cotton stalk	32-46	20-28	15-30
Peanut shell	36.4	15.6	25.0
Rapeseed (canola) straw	~40-46	~22-23	~17-21
Sorghum stover	approx 35-40	approx 25-30	approx 15-20
Oat straw (approximate)	approx 30-35	approx 30-35	approx 10-15
Rye straw (approximate)	approx 30-40	approx 25-35	approx 10-15
Various lignocellulosic biomass (typical)	40-50	20-35	15-20

The lignocellulose of the plant cell-wall has been long studied. In the early 19th century, cellulose and lignin were recognized as existing. By the middle of the 20th century the pulping industries (soda and kraft processes) were using chemical delignification (e.g. NaOH, sulfite) to realize the advantages of dissolving the lignocellulose linkages [17, 18]. Dedicated pre-treatment studies were performed in the 1970s and 1980s in biofuels and biofeed research. Key advances include the development of steam explosion (explosive decompression with high pressure steam) and

alkaline ammonia (AFEX) pre-treatments to open up the biomass structure [19]. In the last decades, more sophisticated techniques were developed including ionic liquid and organosolv fractionation. Early ruminant feed pioneers (Owen and Klopfenstein, 1980s) investigated ammonia and urea for straw treatment and reported considerable increases in digestibility. Modern developments include genetic and enzymatic solutions and integrated bio refinery techniques [20].



Pre-treatment Methods: Mechanisms and Markers

Pre-treatment methods aim to “unlock” the cell-wall matrix, removing or modifying lignin and hemicellulose to expose cellulose.

1. Comminution (Milling/Grinding)

Comminution is a physical pre-treatment process to decrease the size of lignocellulose biomass particles via mechanical equipment, e.g. ball mills, hammer mills, knife mills or grinders. The major aim of this process is to break down the physical structure of the biomass, thereby increasing its surface area and, to some degree, decreasing the crystallinity of the cellulose [21]. This renders the cellulose more susceptible to enzymatic and rumen

microbial attack. It is an environment friendly pre-treatment approach since there are no chemicals involved [22]. It is often used as a first step before chemical or biological treatments. But comminution itself is not effective to remove lignin and needs a great amount of energy, particularly for extremely tiny particles. The efficiency of this pre-treatment is often measured by the crystallinity index using X-ray diffraction (XRD), BET surface area analysis and enzymatic digestibility tests [23].

2. Steam Explosion

Steam explosion is one of the most used physicochemical pre-treatment procedures for lignocellulose biomass. In this process, biomass is treated with saturated steam at high

pressure and temperatures of around 180-240 °C for 10-20 minutes, then rapidly depressurized. The abrupt release of pressure causes fibers to break ^[24]. This produces increased porosity and disruption of the matrix of lignin-carbohydrate. Hemicellulose is partly hydrolysed throughout the process, while lignin is redistributed or partially removed, resulting in better enzymatic hydrolysis. Steam explosion has little chemical additions, which is ecologically friendly ^[25]. However, extreme treatment conditions may produce inhibitory chemicals like as furfural and hydroxymethylfurfural (HMF) that may limit fermentation efficiency. The efficacy of this pre-treatment is often evaluated by HPLC soluble sugar analysis, lignin determination, scanning electron microscopy (SEM) and FTIR spectroscopy ^[26].

3. Liquid Hot Water (LHW)

The LHW pre-treatment is a process in which the biomass is treated with pressurized hot water at temperatures between 160 and 240 °C without adding chemical catalysts. In such case water works as a solvent and a catalyst, hydrolysing the hemicellulose and partly depolymerizing the lignin, while conserving the bulk of the cellulose fraction ^[27]. The technique improves the accessibility of cellulose to hydrolytic enzymes and, under regulated circumstances, decreases the generation of hazardous degradation products. LHW is regarded as an environment-friendly pre-treatment technology since no chemicals are used. However, it demands considerable energy input to sustain high temperature and high pressure and might lead to re-polymerization of lignin or partial delignification. Its efficacy is often assessed by quantifying xylan removal, residual lignin content and structural alterations using HPLC and FTIR analysis ^[28].

4. Ammonia Fiber Explosion (AFEX)

Ammonia Fiber Explosion (AFEX) is a physicochemical pre-treatment technology in which lignocellulosic biomass is immersed in liquid ammonia at moderate temperatures and high pressures, followed by a quick pressure release. Ammonia swelling the cellulose fibers, breaking the lignin-carbohydrate complexes and increasing the pore development in the biomass makes the enzyme accessible ^[29]. AFEX also shows a significant improvement in digestibility, creating relatively few fermentation inhibitors in comparison to acid-based pre-treatments. The technique is also more sustainable in that ammonia can be collected and regenerated. However, specific pressure resistant reactors and ammonia handling systems add operating expense. Lignin solubilisation, sugar yield following enzymatic hydrolysis and structural alterations by SEM are widely used to evaluate the pre-treatment effectiveness ^[30, 31].

5. Dilute Acid Pre-treatment

Dilute acid pre-treatment use modest concentrations of mineral acids, most often sulfuric acid (0.5-2.5%), to hydrolyze hemicellulose into fermentable sugars, leaving most of the cellulose untouched. The treatment is commonly performed at temperatures from 130 to 210 °C for brief periods of time and leads to increased accessibility of cellulose to hydrolytic enzymes. This approach is frequently utilized because of its fast processing, excellent hemicellulose sugar recovery and industrial application ^[32].

It nevertheless may create inhibitory chemicals such as furfural, HMF and acetic acid which are detrimental to microbial fermentation. The other is acid corrosion which needs specific equipment and effective acid recovery systems. The efficiency of dilute acid pre-treatment was assessed by HPLC measurement of monosaccharide release, inhibitor production and cellulose crystallinity by XRD ^[33].

6. Concentrated Acid Pre-treatment

Concentrated acid pre-treatment 70-90% sulfuric acid is used to enter the biomass structure, hydrolyzing cellulose and hemicellulose into monosaccharides. The approach is very effective in sugar recovery and also lowers the crystallinity of cellulose to a large extent to obtain high digestibility ^[34]. However, concentrated acid pre-treatment suffers from various drawbacks, such as excessive corrosiveness, high operating expense, safety problems and expensive acid recovery and detoxification systems. Its commercial use is thus still restricted. The quality of this pre-treatment is often tested by total sugar yield, residual crystallinity (XRD) and colorimetric sugar tests ^[35].

7. Alkaline Pre-treatment (NaOH/KOH)

In the alkaline pre-treatment, saponification processes break ester and ether linkages in the lignin-carbohydrate complex, removing the lignin by using sodium hydroxide (NaOH) or potassium hydroxide (KOH). This treatment improves significantly biomass porosity, exposes cellulose fibers and enhances enzymatic hydrolysis ^[36]. Alkaline processing of various agricultural leftovers may remove 60-70% of lignin with very few inhibitory chemicals produced. However, this procedure creates alkaline wastewater and needs a lot of washing to remove remaining chemicals before further use. Too much alkali, however, may also breakdown cellulose. The efficacy of pre-treatment is usually measured by Klason lignin analysis, acid-soluble lignin determination, microscopic inspections and NMR study of lignin structure ^[37].

8. Lime Pre-treatment (Ca(OH)₂/CaO)

Lime pre-treatment employs calcium hydroxide or calcium oxide as mild alkali to expand the plant fibers and partly solubilize the lignin. Typically the therapy is carried out at ambient or slightly increased temperatures for many days to weeks. Lime is a cheap, accessible, less corrosive and safe to handle material that may be used on the farm and to enhance animal feed ^[38]. But its comparatively sluggish reaction rate and build-up of residual calcium carbonate restricts its efficiency compared to stronger alkalis. The efficiency of lime pre-treatment is often evaluated by fiber fraction analysis (NDF and ADF), Klason lignin estimate and pH measurements following treatment ^[39].

9. Ammoniation (Urea/NH₃ Soaking)

Ammoniation is a method of treating agricultural leftovers with urea or anhydrous ammonia to enhance their digestibility and nutritional value. During treatment, ammonia breaks hydrogen bonds and weakens lignin-carbohydrate connections. The microbial breakdown of urea elevates pH and enables fiber swelling. It is a low-cost approach often employed at the farm level to upgrade cereal straw for use as animal feed ^[40]. Moreover ammoniation raises the nitrogen content of the treated biomass so as to boost its feeding value. However, the approach takes a

lengthy incubation time and unequal ammonia penetration and volatilization losses may decrease the efficacy of the treatment. Evaluations are often based on pH, Kjeldahl nitrogen concentration and increases in neutral detergent fiber (NDF) digestibility [41].

10. Oxidative Pre-treatment (H_2O_2/O_3)

Oxidative pre-treatment uses oxidizing chemicals like hydrogen peroxide or ozone to break down lignin by targeting its aromatic rings and side chains. Hydrogen peroxide under alkaline state is an excellent lignin removal agent and generates ecologically benign by-products such as water and oxygen [42]. Ozone also selectively oxidizes lignin under ambient circumstances with the production of no major hazardous residues. Such treatments greatly increase the accessibility of cellulose but must be handled with caution owing to safety issues and the potential for cellulose oxidation under harsh circumstances. The efficiency of lignin removal is generally evaluated by lignin monomer analysis (GC-MS), detection of phenolic compounds (UV-Vis spectroscopy) and calculation of residual lignin [43].

11. Organosolv Pre-treatment

Organosolv pre-treatment involves the use of organic solvents, such as ethanol, methanol or acetic acid, frequently in the presence of catalysts, to selectively dissolve and remove lignin from lignocellulosic biomass [44]. This approach has good efficiency for delignification and gives high purity lignin which may be used for value added applications in industry. Furthermore, the availability of cellulose for enzymatic hydrolysis increases the synthesis of sugar. Most solvents are recoverable and may be recycled. However, solvent recovery systems add to the operating cost and residual solvent removal is a must before biomass usage. Pre-treatment performance is often assessed based on lignin recovery and purity, fermentable sugar production and FTIR characterisation of the extracted lignin [45].

12. Ionic Liquid (IL) Pre-treatment

Ionic liquid pre-treatment employs the organic salts, notably the ionic liquids based on imidazolium, to break the hydrogen-bonding network of the cellulose and dissolve the lignocellulosic biomass. This technique reduces the crystallinity of cellulose significantly and improves the

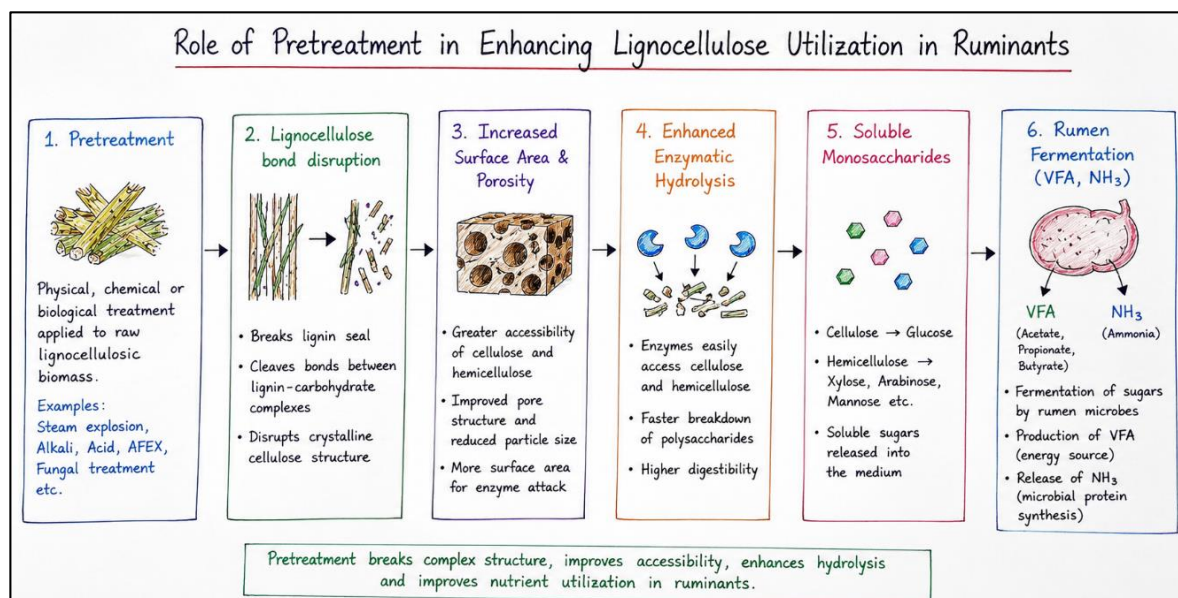
efficiency of enzymatic hydrolysis [46]. Many ionic liquids are recyclable, which makes them interesting for advanced biorefinery applications. Their high cost, possible toxicity and susceptibility to water are nonetheless still important impediments to large-scale commercialization. The efficiency of pre-treatment is often measured by measuring the decrease in crystallinity by XRD analysis, characterisation of cellulose and lignin by ^{13}C NMR and sugar yield following enzymatic hydrolysis [47].

13. Deep Eutectic Solvent (DES) Pre-treatment

Deep eutectic solvents (DESs) are mixtures of hydrogen bond donors and hydrogen bond acceptors, for example, choline chloride and lactic acid and they may generate low-melting solvent systems that can selectively dissolve lignin and hemicellulose. DES pre-treatment is performed under moderate circumstances using cheap, biodegradable and ecologically beneficial components [48]. DES is a very flexible pre-treatment process since the solvent content may be tuned for various biomass kinds. However, primary problems include high solvent viscosity, cleaning needs and little expertise at industrial scale. The effectiveness of DES pre-treatment is often evaluated using NMR or GC analysis of extracted compounds, lignin fluorescence and FTIR spectroscopy [49].

14. Biological Pre-treatment (White-Rot Fungi)

Biological pre-treatment uses lignin-degrading microorganisms, especially white-rot fungi, which preferentially destroy lignin by means of extracellular enzymes such as lignin peroxidase, manganese peroxidase and laccase [50]. This green technique is carried out at gentle settings, without harsh chemicals or excessive energy and is one of the most sustainable pre-treatment procedures. Biological pre-treatment greatly increases cellulose accessibility and digestibility, but is much slower than physical or chemical treatments, frequently needing several weeks. Moreover, extensive incubation time might lead to partial cellulose consumption and it is still difficult to maintain sterile conditions in big scale use [51]. Biological pre-treatment efficiency is often assessed by determining the decrease in Klason lignin content, enzyme activity, breakdown of phenolic compounds and microscopic analysis of cell wall structural alterations [52].



Analytical Markers and Assays for Bond Disruption

Several analytical assays and markers are required to characterize pre-treatment efficacy. Chemical compositional assays, e.g. Klason lignin and standard NDF/ADF/ADL fiber analysis, are used to quantify changes in bulk component levels^[52]. Spectroscopic methods (FTIR, NIR, Raman, solid state NMR) indicate changes in chemical structure (bond vibrations, functional groups). The released sugars or the lignin breakdown products (monomeric aromatics) are identified and quantified by chromatographic techniques such as HPLC, GC/MS and pyrolysis-MS. Visual evaluation of morphological changes (cellulose fibrillation, lignin relocation) is done by microscopy (SEM, TEM, confocal). Increased functional digestibility by enzymatic saccharification or *in vitro* fermentation tests^[53].

1. Klason Lignin (Acid-Insoluble Lignin)

The Klason lignin method is the most accepted and standardized method for determination of the acid insoluble lignin content of lignocellulosic biomass. The method involves treating the biomass with 72% sulfuric acid which dissolves and hydrolyzes the cellulose and hemicellulose, leaving the lignin as an insoluble residue^[54]. The amount of lignin is determined gravimetrically by drying and weighing the residue. The method is used for the determination of lignin with an accuracy of about $\pm 1\%$ and in general, 0.5-2.0 g of dried biomass are used for the analysis. The Klason method is widely considered the standard method for lignin quantification as it measures structural lignin remaining after acid hydrolysis directly. It is often used in conjunction with acid soluble lignin analysis to determine the total lignin content of the biomass^[55].

2. Acid-Soluble Lignin (ASL)

The amount of lignin dissolved during the sulfuric acid hydrolysis step of the Klason lignin procedure is quantified by the acid-soluble lignin (ASL) assay. Analysis of hydrolysate is generally carried out using UV-visible spectrophotometer after acid digestion. This is because lignin is known to strongly absorb ultraviolet light at the wavelength range 205-280 nm^[56]. The method is highly sensitive and can detect lignin at the microgram level. The ASL improves the Klason lignin assay by including the soluble lignin fraction that would otherwise be underestimated, thus providing a more accurate estimate of the total lignin content of biomasses^[57].

3. Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL)

The van Soest detergent fiber analysis is a common method used to analyze the digestibility and fiber content of crop residues and forage. Neutral detergent fiber (NDF) is used to measure the total cell wall fraction (cellulose, hemicellulose and lignin) and acid detergent fiber (ADF) is used to measure the cellulose and lignin. After removal of cellulose lignin is quantified as acid detergent lignin (ADL)^[58]. Approximately 0.5-1.0 g of dried biomass is detergent extracted and subsequently gravimetrically determined. The cellulose and the hemicellulose contents are estimated from the difference of the fractions. This method is widely applied in animal nutrition research as it gives valuable information on the efficacy of pre-treatment methods for improving feed value, forage quality and fiber digestibility^[59].

4. High-Performance Liquid Chromatography (HPLC) for Sugar Analysis

High-performance liquid chromatography (HPLC) is widely used for the determination of monomeric sugars from lignocellulosic biomass after acid or enzymatic hydrolysis. The filtered biomass hydrolysate is analyzed with an HPLC system equipped with either a refractive index (RID) or pulsed amperometric (HPAEC-PAD) detector^[60]. The method allows for an accurate determination of sugars such as glucose, xylose, arabinose, mannose and galactose with detection limits in the milligram per liter range. HPLC is essential for evaluation of pre-treatment methods effectiveness by measuring the extent of cellulose and hemicellulose conversion to fermentable sugars^[61].

5. HPLC or Gas Chromatography (GC) for Phenolic Compounds

Gas chromatography and HPLC are used for the identification and quantification of phenolic compounds derived from lignin that are released after oxidative or enzymatic degradation of lignin. In general, biomass samples are oxidized with nitrobenzene or cupric oxide (CuO) before chromatographic analysis^[62]. With these methods it is possible to detect vanillin, syringaldehyde, ferulic acid and p-coumaric acid with sensitivity in the microgram range. The analysis provides useful information on the composition of lignin, e.g. the syringyl/guaiacyl (S/G) ratios and can be used to evaluate the extent of lignin depolymerization that can be observed after the pre-treatment^[63].

6. Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS)

Pyrolysis-GC/MS is a complex analytical method used to investigate the chemical composition of lignocellulosic biomass. Small samples (0.1-1.0 mg) are rapidly heated to around 500 °C in the absence of oxygen. Thermal decomposition forms volatile compounds. These are identified by mass spectrometry and separated by gas chromatography^[64]. The method simultaneously detects compounds derived from carbohydrates and aromatic molecules derived from lignin with a parts-per-million sensitivity. Py-GC/MS is especially helpful when comparing untreated and pretreated materials since it gives a complete chemical fingerprint of biomass^[65].

7. Fourier Transform Infrared (FTIR) Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is a rapid non-destructive analytical method for identification of functional groups in lignocellulosic biomass. Small amounts of finely ground samples are analyzed with potassium bromide (KBr) pellets or an attenuated total reflectance (ATR) accessory. FTIR identifies specific absorption bands related to cellulose, hemicellulose and lignin^[66]. Among these bands are the C-O stretching band at about 1030 cm^{-1} , the aromatic lignin band at about 1510 cm^{-1} and the carbonyl band of hemicellulose at about 1730 cm^{-1} . The changes in the absorption peaks give qualitative and semi-quantitative information about the chemical changes caused by the pre-treatment, in particular the removal of lignin and the degradation of hemicellulose^[67].

8. Raman Spectroscopy

Another vibrational spectroscopic technique employed for the molecular shape characterization of lignocellulosic biomass is Raman spectroscopy. The approach quantifies the scattering of laser light triggered by molecular vibrations and offers data on the occurrence of cellulose, lignin and hemicellulose with little sample preparation [68]. Raman spectroscopy is less sensitive to water than FTIR spectroscopy and is thus particularly well suited for the determination of cellulose crystallinity and molecular orientation. Although less frequently used than FTIR, Raman spectroscopy is a complementary method that is utilized to investigate structural changes during biomass pre-treatment [69].

9. Solid-State ^{13}C Nuclear Magnetic Resonance (^{13}C NMR)

The molecular makeup of lignocellulosic biomass is studied by high performance analytical technique, solid-state ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy. The spectra were recorded on ~50-100 mg of dry biomass under magic-angle spinning [70]. They correspond to the carbon atoms of cellulose, hemicellulose and lignin. The method provides direct structural information on crystalline and amorphous cellulose and the guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H) units of lignin. Solid-state NMR is widely used in research to understand the chemical modifications taking place in various pre-treatment processes due to its high structural resolution [71].

10. X-ray Diffraction (XRD)

The crystallinity index (CrI) of cellulose in lignocellulosic biomass is often determined by X-ray diffraction (XRD). The Cu K α X-rays are exposed to a finely powdered sample and the diffraction patterns are noted at different diffraction angles (2θ) [72]. The ratio of crystalline to amorphous cellulose is derived from the intensity of the characteristic cellulose peaks, particularly around 22.5° . In general, pre-treatment methods that disrupt the crystallinity of cellulose lead to a lower crystallinity index, which is related to improved enzymatic digestibility. Hence, XRD is an important tool for the evaluation of the structural changes of pretreated biomass [73].

11. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is used to visualize the surface morphology of lignocellulosic biomass before and after pre-treatment. Dried samples are coated with a thin layer of conductive material, usually gold and then examined under high vacuum. SEM high-resolution images show the pre-treatment-induced surface roughness, fiber disruption, pore formation, fibril separation and loosening of structure. These images provide direct evidence for biomass deconstruction and also reveal improvements in enzyme accessibility that complement chemical analyses [74].

12. Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is used to study the internal ultrastructure of lignocellulosic biomass at nanometer scale. Heavy metals are used for staining ultrathin sections of biomass before imaging [75]. TEM after pre-treatment gives detailed information about cell wall architecture, microfibrils organization, redistribution of lignin and delamination of cell wall layers. The TEM is the

best instrument for studying the structure as well as the changes in the biomass at the nanoscale level. However, the sample preparation for the TEM is more complicated than the SEM [76].

13. Light Microscopy with Histochemical Staining

Histochemical staining and light microscopy provide a simple and rapid method to visualize the distribution of lignin in plant tissues. Biomass thin sections are stained with Phloroglucinol-hydrochloric acid (Phloroglucinol-HCl). Phloroglucinol reacts specifically with lignin to give a characteristic red coloration. The intensity of staining provides a qualitative indication of the abundance of lignin and its removal after pre-treatment. This low-cost method is commonly used to obtain a preliminary assessment of delignification before using more sophisticated analytical techniques [77].

14. Enzymatic Saccharification Assay

The enzymatic saccharification assay is a functional assay to assess digestibility of pretreated lignocellulosic biomass. The pre-treated samples were incubated with enzyme mixtures containing cellulases and hemicellulases under controlled conditions. The sugars released by enzymatic hydrolysis are quantified by HPLC or by colorimetric assays [78]. The higher sugar yields indicate a better accessibility of the cellulose thus better pre-treatment. Enzymatic saccharification is considered one of the most important assays to evaluate the performance of pre-treatment because it directly measures how efficiently biomass can be converted [79].

15. *In vitro* Rumen Fermentation

In vitro rumen fermentation is a biological assay mainly used for the evaluation of digestibility and feeding value of pre-treated lignocellulosic biomass for ruminant animals. Small quantities of substrate (approximately 0.2-0.5 g) are incubated with buffered rumen fluid under anaerobic conditions to simulate the natural rumen environment [80]. The degradation of the substrate, the concentration of ammonia, the production of gas, volatile fatty acids (VFA) and methane during the fermentation were quantified by gas analyzers, gas chromatography or NMR techniques. This method is less costly, quicker and far more reproducible than *in vivo* feeding trials. Increased gas production, increased VFA yields and improved fibre degradation are indicators of successful pre-treatment and improved nutritional quality of the biomass [81].

Effects of Pre-treatments on Ruminant Feed Utilization

Pre-treatments aim to improve ruminant utilization of residues by boosting digestibility, intake and production.

1. Ammonia Fiber Explosion (AFEX)

Ammonia Fiber Explosion (AFEX) is one of the most efficient pre-treatment methods to improve the nutritive value of lignocellulosic biomass used for ruminant feeding. Anhydrous ammonia treatments degrade lignin-carbohydrate complexes and increase cellulose and hemicellulose susceptibility to rumen microorganisms. Studies have reported improvements in *in vitro* dry matter (DM) degradability of 5 to 13 percentage points and increases in dry matter intake of up to 42% in cattle [82]. AFEX-treated feeds have also been linked with about 18%

higher milk yield due to enhanced energy availability. AFEX in the rumen improves neutral detergent fiber (NDF) digestibility, slightly increases ruminal ammonia ($\text{NH}_3\text{-N}$) concentration and shifts volatile fatty acid (VFA) production towards greater propionate formation, thereby improving feed efficiency^[83]. Despite these advantages, AFEX is limited by the requirement for specialized equipment to handle ammonia, relatively high operational costs and the need for supplementation with small quantities of urea to optimize microbial protein synthesis^[84].

2. Urea Treatment (5-6%)

One of the most commonly used chemical pre-treatment methods for improving the feeding value of cereal straws and other low-quality roughages is urea treatment. The pH increases and the lignin-carbohydrate complex is disrupted by the microbial urease activity which hydrolyzes urea to ammonia during treatment^[85]. This process increases organic matter (OM) and dry matter digestibility by about 10-17% and greatly increases dry matter intake by livestock. Urea treatment also increases the crude protein content of crop residues by providing non-protein nitrogen that supports rumen microbial growth^[86]. Studies in animals have demonstrated slight increases in the yield of milk fat, milk protein and fat-corrected milk (FCM). In the rumen, the treatment increased ammonia nitrogen concentration and pH and stimulated propionate production and reduced the acetate to propionate ratio. However, effective treatment requires an incubation period of 1-2 weeks and involves extra costs of urea application^[87].

3. Urea (2.2%) + Lime (2.2%) Treatment

The combination application of urea and lime has been developed to improve the digestibility of straw and to reduce the costs of treatment compared with the use of high-dose urea alone. Lime induces swelling and partial solubilization of lignin, whereas urea supplies ammonia for hydrogen bond cleavage and augmented nitrogen availability^[88]. The combination generally provides digestibility improvements similar to those obtained with higher urea concentrations and markedly increases dry matter intake. Animals fed straw treated with the combination of urea and lime have been reported to have higher percentages of milk fat and milk protein than animals fed with either treatment alone^[89]. Characteristics of rumen fermentation include a higher ammonia concentration, elevated pH and a shift in VFA production towards greater propionate formation without causing ammonia toxicity. This method improves digestibility and adds calcium to the diet. It is particularly helpful for the treatment of poor quality cereal straws^[90].

4. Steam Explosion

Steam explosion pre-treatment greatly enhances the digestibility of lignocellulosic biomass by disrupting the plant cell wall structure through rapid depressurization after steam treatment. *in vitro* dry matter, neutral detergent fiber (NDF) and acid detergent fiber (ADF) disappearance show consistent significant increases in laboratory studies^[91]. The structural disruption also changes the patterns of rumen fermentation by decreasing the ratio of acetate to propionate, suggesting a greater production of the energy efficient propionate. However, steam exploded feeds have seldom been tested under *in vivo* feeding conditions and

direct information on improvements in milk or meat production is still limited. Moreover, too severe treatment may cause heat damage and formation of inhibitory compounds, thus processing conditions should be carefully optimized^[92].

5. Enzymatic Pre-treatment (Xylanase)

Enzymatic pre-treatment is the use of fiber degrading enzymes such as xylanase to increase the degradation of hemicellulose and increase fiber digestibility. Xylanase typically improves digestibility by an additional 2-5% when applied to pre-treated biomass, especially AFEX-treated feed. However, the increases in dry matter intake and milk production are generally small or inconsistent under practical feeding conditions^[93]. The primary advantage of enzyme addition is enhanced fermentability in the rumen, particularly when the substrate has experienced a structural modification due to pre-treatment. Commercial enzymes are safe to the environment but their relatively high cost limits their wide use in livestock production systems^[94].

6. Sodium Hydroxide (NaOH) Pre-treatment

Alkaline sodium hydroxide pre-treatment is good at removing lignin and improving the digestibility of crop residues fiber. NaOH treatment generally improves digestibility of organic matter by 10-30% and dry matter intake by approximately 10-20%^[95]. More nutrients may result in a small increase in milk production of 5 to 15 percent. NaOH-treated biomass increases rumen pH and ammonia concentration and increases microbial degradation of structural carbohydrates. This method is very effective for delignification but the treated biomass should be thoroughly washed or neutralized before feeding to avoid an excess of sodium intake that may have an adverse effect on animal health^[96].

7. Calcium Hydroxide [$\text{Ca}(\text{OH})_2$] Pre-treatment

The lime pre-treatment is a less severe alkaline pre-treatment method using calcium hydroxide and increases the digestibility of fibrous crop residues by swelling of fiber and partial removal of lignin. Generally digestibility increases 5-15% with about 10% more dry matter intake. Minor improvements in milk fat and milk protein content have also been reported^[97]. Lime treated biomass increases the pH in the rumen, but causes a less marked increase in the ammonia concentration than does urea treatment. Lime pre-treatment is thought to be safer, less corrosive and more economical than sodium hydroxide treatment although longer reaction times are needed and residual calcium carbonate contributes little nutritional value^[98].

8. Ozone (O_3) Pre-treatment

Ozone pre-treatment takes advantage of the strong oxidative properties of ozone to selectively degrade lignin and improve the accessibility of the fibers. Experiments have shown that ozone can improve delignification and may improve the fermentability of lignocellulosic biomass^[99]. However, little information is available on improvements in digestibility, feed intake or animal performance under practical feeding conditions. Feeding livestock with ozone has not been widely used due to the need for equipment, high operating costs and safety concerns of handling highly reactive ozone gas^[101].

9. Hydrogen Peroxide (H_2O_2) Pre-treatment

Hydrogen peroxide pre-treatment, especially under alkaline conditions, oxidizes lignin and improves enzymatic hydrolysis of plant cell walls. Laboratory studies indicate increased fiber degradation and enhanced microbial growth but information on its effects on feed intake and animal productivity is limited ^[102]. This process maintains an alkaline environment conducive to microbial digestion but is challenging to deploy at the farm scale because of the requirement to deal with chemicals and the residual peroxide. Therefore, hydrogen peroxide pre-treatment is limited to research purposes ^[103].

10. Ensiling with Additives

Ensiling is a biological preservation process whereby forage is stored under controlled anaerobic fermentation, often with microbial inoculants or chemical additives. The process improves feed stability and can improve digestibility by about 5-10% due to partial breakdown of structural carbohydrates during fermentation ^[104]. The response to the supplement in relation to feed intake may vary among animals and some studies have reported only a modest increase in milk production. Fermentation results in the formation of more organic acids, mainly acetate, while the original lignocellulosic structure is mostly retained. Thus ensiling is mainly viewed as a preservation method and not as a true lignocellulosic pre-treatment method ^[105].

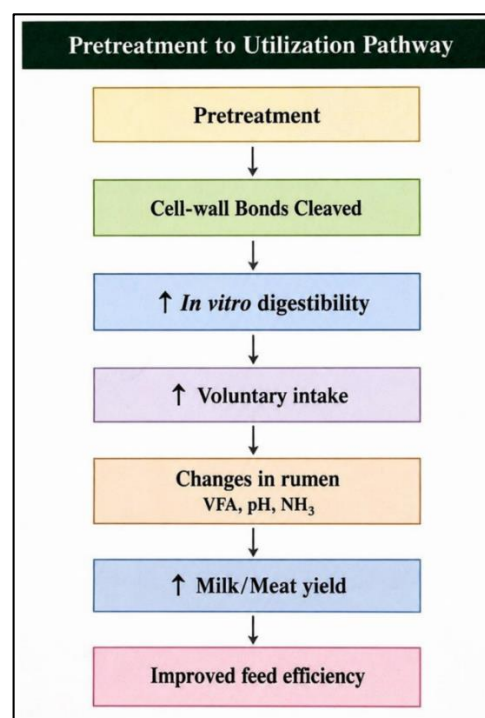
11. Biological Pre-treatment (White-Rot Fungi)

Biological pre-treatment with white-rot fungi selectively removes lignin using extracellular enzymes, such as laccases and peroxidases. Laboratory studies have shown improvements in digestibility of 10-20% and an increased release of soluble phenolic compounds ^[106]. Despite environmental benefits, the method has not been adopted for commercial livestock production, as the treatment takes several weeks, has inconsistent results and long incubation times can result in spoilage or loss of valuable nutrients. Therefore, biological pre-treatment is still largely experimental for feed applications ^[107].

Ammoniation/AFEX significantly improve digestibility and intake. For example, ammonia fiber expansion (AFEX) of wheat straw increased *in situ* rumen degradability by + 28-126%, with lactating cows and buffalo showing 5-13 percentage point higher digestibility and cattle + 42% intake and + 18% milk energy on high-AFEX diets ^[108]. Alkali (NaOH) and liming similarly improve forage digestibility (often 10-30% gains in NDF digestibility) and animal performance. In a study of dairy cows, dry-matter intake and total digestibility were significantly increased with the addition of 5.5% urea alone or urea + 2.2% Ca (OH)₂ in rice straw ^[109]. These treatments increased ruminal pH and ammonia levels (which favour fiber digestion), changed VFA profiles towards propionate (lower A:P ratio) and increased milk fat and protein concentrations. Steam explosion has been shown *in vitro* to greatly increase corn stover rumen degradability (DM, NDF, ADF disappearance) and to shift fermentation toward more propionate ^[110].

Some pre-treatments, however, offer modest or mixed benefits: Enzymatic feed additives (e.g. xylanases) may improve digestibility slightly when used with pre-treated feeds, but will give small *in vivo* gains unless combined with delignification. Biological (fungal) pre-treatment may enhance digestibility but is slow and not feasible on a farm scale ^[111]. The overall magnitude of improvement is also

dependent on context of animal and diet. Entries under “effect size” in Table 4 summarize typical reported changes (e.g. “Digestibility + 10-30%”, “Intake + 20-40%”, “Milk + 10-20%”), based on multiple studies. Caveats include the risk of secondary effects (e.g. Maillard reaction in over-treated forage, NH₃ toxicity or feed palatability issues) and the need to balance energy/protein ^[112].



Conclusions

Progress in the use of crop residues as ruminant feeds requires a holistic understanding of cell-wall chemistry, pre-treatment science and animal nutrition. This review integrates a large amount of evidence highlighting the way that specific pre-treatments (physical, chemical, biological) break down lignocellulosic bonds and which markers are used to follow these changes. We demonstrate that many pre-treatments can substantially improve fibre digestibility and animal performance, but highlight that such benefits are dependent on the standardisation of methods and markers. Future work should address scalable pre-treatments (e.g. low cost alkali, enzymatic cocktails or integrated biorefinery approaches) and link molecular bond disruption to actual feed utilization. The tables presented are a summary of current knowledge (from composition of residues to protocol recommendations) and should serve as a guide for researchers. Adoption of common protocols and markers will allow the community to move forward in a reproducible manner in the conversion of low-quality crop residues into valuable ruminant feed at a faster pace.

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