Saccharification of Wheat and Rice Straw for Lactic acid Production

DISSERTATION

Submitted by

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In partial fulfillment for the award of the degree of

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Under the Guidance of

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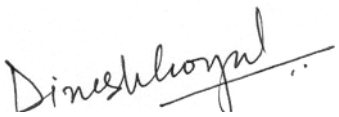
Patiala, Punjab

July, 2025

CERTIFICATE

This is to certify that the dissertation report entitled " **Saccharification of wheat and rice straw for lactic acid production"** submitted by Simran Kaur Raniyal (302301028) in the partial fulfilment of the requirement for the award of the degree of the "Master of science in Biotechnology" Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is an authentic record of student's own work carried out during the period of six months, i.e., from January to June 2025 under my supervision and guidance. This work has not been submitted for the award of any other degree or certificate in this or any other university or institute.



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DECLARATION

I hereby declare that the work presented in the dissertation entitled "**Saccharification of wheat and rice straw for lactic acid production**" in partial fulfilment of requirement for the award of degree of Masters of Science in Biotechnology at Thapar Institute of Engineering & Technology (TIET), Deemed to be University, Patiala is an authentic record of my own work during the period from January 2025 to June 2025, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, TIET. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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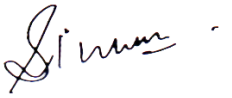

Simran Kaur Raniyal

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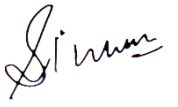

Simran Kaur Raniyal

In dedication to my parents for

making me who I am and my siblings

who supported me all the way!

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LIST OF ABBREVIATIONS

DNS	3,5- dinitro salicylic acid
et al	And others
etc	And other things
FDA	Food Drug and Administration
g/l	Gram Per Liter
GRAS	Generally Regarded As Safe
H_2SO_4	Sulfuric acid
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
LA	Lactic acid
LAB	Lactic acid bacteria
mg/mL	Milligram per millilitre
mL	Millilitre
MR	Methyl red
MT	Metric ton
NaOH	Sodium hydroxide
NA	Nutrient Agar
PLA	Polylactic acid
psi	Pounds per square inch
rpm	Revolutions per minute

LIST OF SYMBOLS

%	Percent
µl	Microliter
B	Bacillus
β	Beta
Ca	Calcium
D	Dextrorotatory
g	Gram
K	Potassium
Kg	Kilogram
L	Laevorotatory
Lb.	Lactobacillus
ml	Milliliter
N	Normal
Na	Sodium
nm	Nanometer
P	Para
s	Second

ABSTRACT

The present study explores production of lactic acid (LA) from rice straw (RS) and wheat straw (WS), through a three-stage pretreatment process involving alkali hydrolysis and acid hydrolysis for fermentable sugars and acid-chlorite technique for cellulose separation. Dilute acid pretreatment of WS and RS biomass yielded significant amounts of reducing sugars (glucose and xylose) which were subsequently fermented to LA by *Bacillus licheniformis* (DGB), *Bacillus sonorenesis* (DGS15) and *Lactobacillus reuteri*. DGS15 demonstrated superior performance, achieving LA production of 7.47 mg/ml (0.183 g/g) from RS hydrolysate and 9.03 mg/ml (0.182 g/g) from WS hydrolysate, outperforming both *Bacillus licheniformis* (DGB) and *Lactobacillus reuteri* in both yield and productivity. Delignification and acid pretreated residues for cellulose separation achieved recovery rates of 57.63% and 67% from RS and WS, respectively. These findings suggest that *Bacillus sonorenesis* DGS15 is a favorable candidate for lactic acid production from lignocellulosic residues.

CHAPTER-1 INTRODUCTION

Lignocellulosic biomass such as WS, RS, corn stover and sugarcane bagasse, which is produced globally as a by-product of agricultural practices, is rich in carbohydrates composed of cellulose, hemicellulose, and lignin. Essential energy input accounting for 10-14 % of worldwide energy production (Alisaraei et al., 2023). It is estimated that for every 6 tons of wheat harvested, around 4 tons of straw remain unused, much of which is burned in open fields, particularly in northern states of india like Punjab, Haryana, and Delhi**,** contributing significantly to air pollution and smog formation (Gupta et al., 2004). These residues holds potential to be utilized in a sustainable manner for the generation of high value products, including biofuels, biogas, organic acids (like lactic acid),ethanol, phenolic compounds, also platform sugars (Singh et al., 2020). By reducing reliance on fossil fuels, bio-derived fuels can help avoid serious pollution caused by their burning.

LA (2-hydroxypropanoic acid) exists in nature as an organic acid that appears as a white solid and forms a clear solution in water. Discovered by the Swedish chemist Carl Wilhelm Scheele in 1780, it can be produced either through chemical synthesis or more sustainably via microbial fermentation. The demand for lactic acid has been growing rapidly due to its widespread utility across several sectors including food industry, pharmaceutical field, cosmetic industry, chemical sector, bio plastics industry. Significantly, it plays a crucial role in the bio plastics industry as a monomer for polylactic acid (PLA), a biodegradable polymer with growing demands in packaging and medical devices (Datta et al., 1995; Chawla & Goyal, 2022). Chemically synthesized lactic acid is an expensive process due to utilization of costly substrates and energy-intensive processes. Agro-residues provides a more sustainable alternative for lactic acid production. The crystalline and rigid structure of cellulose, the high lignin content, the generation of inhibitory compounds during pretreatment, and the feedstock's particle size are the main causes of lignocellulosic biomass limited its digestibility. These resistant traits have prompted extensive research into a range of pretreatment techniques, which can be broadly divided into physical, chemical, and biological approaches. All of these techniques seek to enhance the accessibility of cellulose and the effectiveness of enzymatic hydrolysis (Kumar et al., 2017).

The different sugars present in LCB hydrolysates, such as glucose and xylose, are effectively utilized by microorganisms (Yue et al., 2023). Different bacteria may effectively

convert different carbohydrates into lactic acid, they are frequently utilized to produce lactic acid. The main byproduct of glucose production by lactic acid bacteria is lactic acid. Bacillus strains grow and produce l-LA in large quantities in a basic mineral salt medium with a very small amount of yeast extract (König et al., 2017). Majority of research on Bacillus strains has used pricey refined sugars, like d-glucose, as the only carbon source. However, alternatives to these pricey refined sugars are becoming more and more important for the production of LA.15. These alternatives include inexpensive and widely available biomass resources like lignocellulose (found in wood and crop residues), sucrose (found in sugarcane and beet molasses), and starches (Poudel et al., 2016). Lactobacillus strains efficiently produce lactic acid by fermenting hexoses through the Embden–Meyerhof–Parnas (EMP) pathway, producing two ATP for every glucose molecule(Ou et al., 2011). The facultative hetero-fermenters *Lactobacillus alimentarius, Lacticaseibacillus rhamnosus, Lactococcus lactis, Lactiplantibacillus plantarum, Lacticaseibacillus casei, Lactobacillus pentosus,* and *Lactobacillus xylosus* are among the LABs (Martinez et al., 2013). Several Lactobacillus and related lactic acid bacteria can use pentoses through the pentose phosphate (PP) or phosphoketolase (heterofermentative) pathway, despite the fact that pentose yields of lactic acid are generally lower than those of hexoses. (Yankov et al., 2022). The LA-producing Bacillus strains produce LA as a major end product through the homofermentative metabolism of pentose and hexose sugars via the pentose phosphate pathway (PPP) and the Embden-Meyerhof-Parnas pathway (EMP), respectively (Poudel et al., 2016). These species work best at 50 to 55 °C and pH 5.5, which is within the range where fungal cellulase enzymes operate. This allows saccharification and fermentation to occur simultaneously and lowers the risk of contamination (Aulitto et al., 2017). Thermotolerant and inhibitor-tolerant *B. sonorensis* DGS15 is well-suited for converting lignocellulosic biomass and is particularly good at co-fermenting glucose and xylose to produce lactic acid in difficult circumstances (Chawla & Goyal, 2022). *Bacillus licheniformis* DGB is perfect for lactic acid production because it can grow in minimal media and withstand high temperatures. Its capacity to utilize hexose and pentose sugars from lignocellulosic biomass has been studied, and it exhibits efficient metabolism of carbohydrates. Because its metabolic pathways can be altered to increase sugar absorption and fermentation efficiency, it is a feasible choice for sustainable bioconversion processes (Zhou et al., 2017), *L. reuteri* is known for its effective hexose fermentation via the Embden–Meyerhof–Parnas pathway, as well as probiotic qualities like reuterin production, which enhance product safety and appeal (Kralj et al., 2004). It also performs best in mesophilic environments.

Present study focuses on the scale-up of saccharification of rice and wheat straw followed by microbial fermentation to produce lactic acid, where raw biomass was pretreated chemically to remove lignin and enhance the hydrolysis of holocellulose into reducing sugars.

In order to support waste valorization and sustainable biotechnology, a method for turning agricultural waste into an industrially valuable compound that is economical, scalable, and environmentally friendly must be established.

CHAPTER 2-REVIEW OF LITERATURE

2.1 Lignocellulosic biomass

Among the renewable resources derived from plants, it is the most abundant. The pulp and paper industry, forestry, and agriculture all produce significant amounts of plant biomass, which accounts for over 60% of the total production of plant biomass. Lignin, hemicellulose and cellulose are the three main polymers as visualized in figure 1 types that comprise lignocellulosic material in different compositions as given in Table 1. Carbohydrates are tightly packed within the lignin, making it a robust mesh-like structure and protecting these carbohydrates (Saxena & Hussain, 2023). Hence, it is necessary to degrade complex forms into simpler forms to make them available for fermentation to produce value-added products.

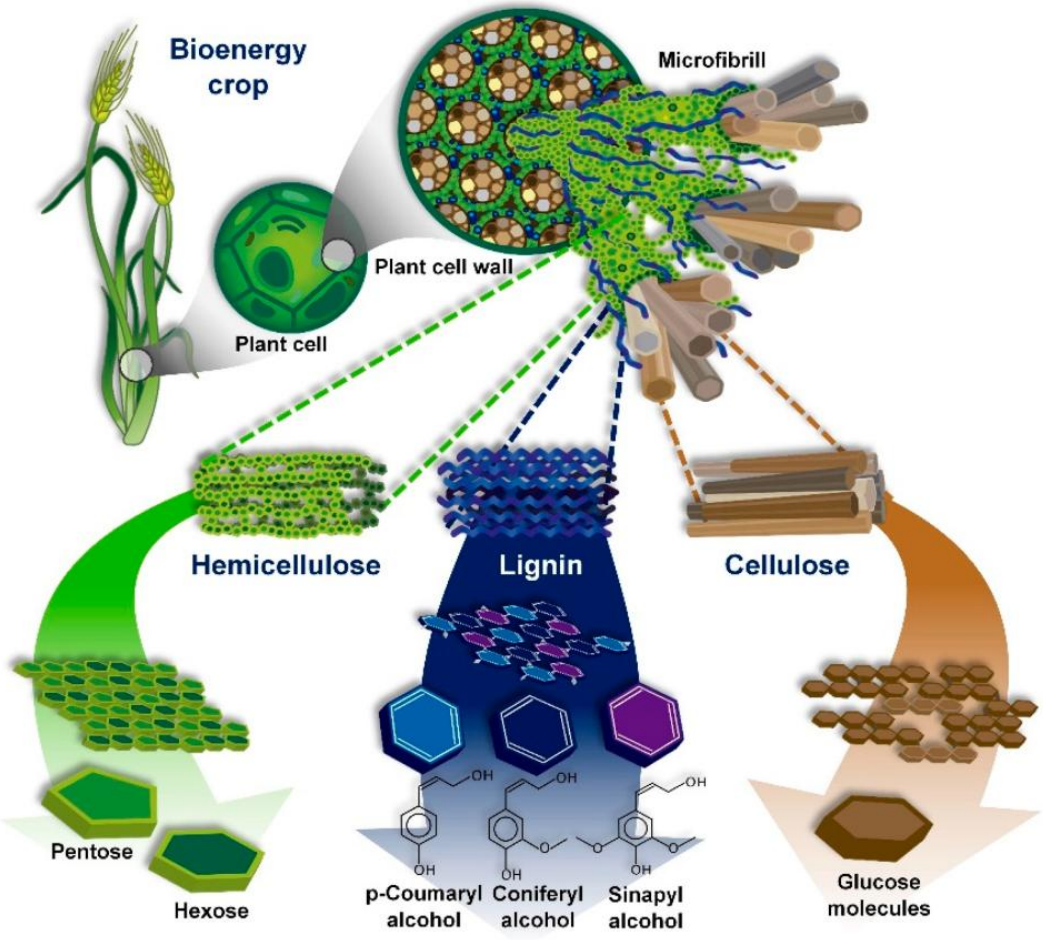

Figure 1: Structure of plant biomass and its main polymers: cellulose, hemicellulose, and lignin (Beltrán *et al*., 2019)

2.1.1 Cellulose

The two forms of plant cellulose that are present in plant cell walls are crystalline and amorphous. Van der Waals forces and hydrogen bonds preserve the crystalline structure (shown in figure 1), while torsions and twists change the ordered arrangement in the amorphous structure.. Consists of ß-D-glucopyranose joined by β-1,4 glycosidic linkages, creating long and straight polymer chains (Zhang et al., 2004). Microfibrils are formed when cellulose chains, which are made up of 500–1400 D-glucose units each, align. In the plant cell wall, these long, fibrous components are arranged in a highly crystalline and structured way. Because of their rigid lignocellulosic framework, the cellulose fibrils are resistant to enzymatic degradation (Zoghlami et al., 2019).

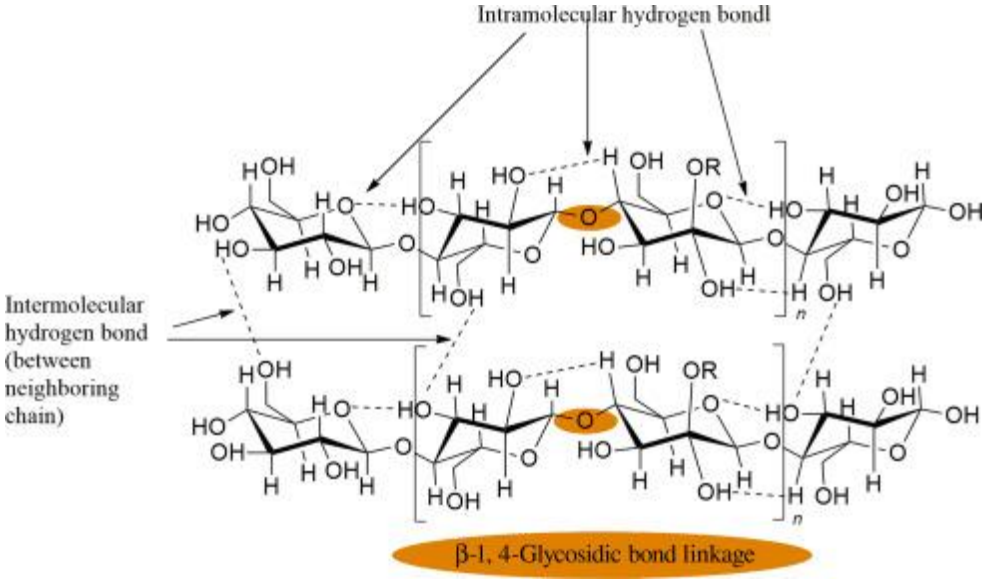

Figure 2: Chemical structure of cellulose chains (Deshavath et al., 2019)

2.1.2 Hemicellulose

Hemicelluloses are heterogeneous groups of biopolymers, representing 20–35% of the biomass weight Hemicellulose is the mixed structure of pentose sugars, such as D-xylose and L-arabinose, and hexoses, such as β-D-mannose, β-D-glucose, α-D-galactose and/ or hexuronic & penturonic acids which are primarily found in terrestrial plants (Saxena & Hussain et al., 2023). The primary constituents of the hemicellulosic fractions were (1 → 4)-linked α-d-glucan from amylose and (1 → 4)-linked β-d-xylan from trace amounts of branched sugars. It is expected to be widely utilized in the food, packaging, pharmaceutical, biomedical, skincare, textile, and paper production industries. (Rao *et al*., 2023).

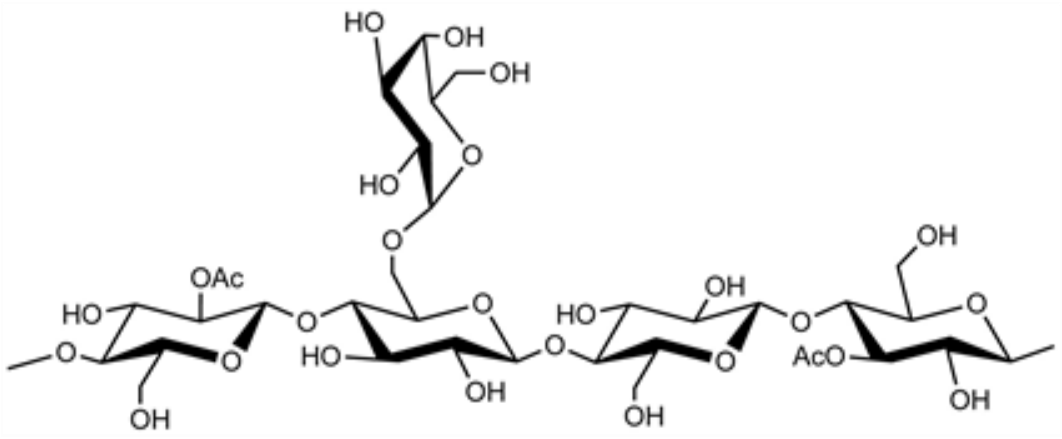

Figure 3: Structure of hemicellulose (Hu et al., 2020).

2.1.3 Lignin

Lignin is a complex, three-dimensional natural polymer made mainly of phenylpropane units connected by ester bonds (Ganewatta et al., 2019). It forms strong bonds with hemicellulose, surrounding the cellulose in plant cell walls (Akhtar, 2014). Found in the secondary cell walls of plants (Kaur, 2014), lignin strengthens and binds cells together, boosting their development and protection against microbes (Harmsen et al., 2010). It's highly resistant to both enzymatic and chemical degradation, making its removal essential during pretreatment to enhance the breakdown of holocellulose. Lignin is the largest aromatic, non-carbohydrate, water-insoluble component in lignocellulosic biomass, accounting for 15–40% of it (Yoo et al., 2020). It's formed from the oxidative polymerisation of hydroxycinnamyl alcohols into a complex, amorphous polymer that gives structural strength. This stable aromatic structure poses a major challenge for enzymatic hydrolysis, so pretreatment is vital for making cellulose more accessible.

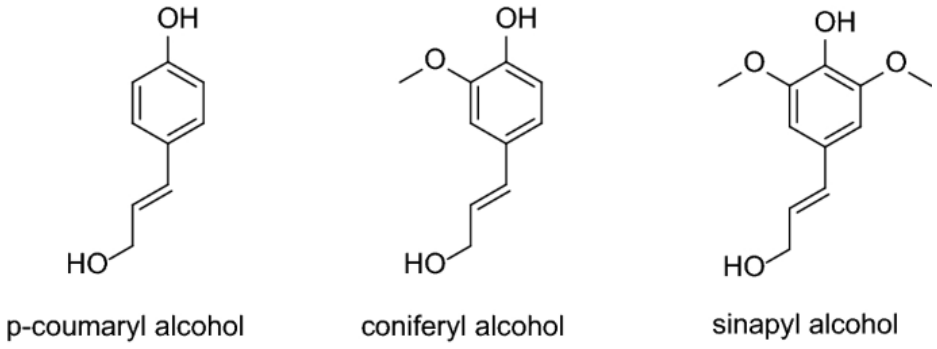

Figure 4: Three main components of lignin (Jazi *et al*.,2017)

Table 1: Percentage composition of lignocellulosic biomass

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Sugarcane bagasse	45	32	32	Kumar et al., 2021
Rice straw	50	35	25	Chen et al., 2020
Wheat straw	45	30	20	Neudecker et al., 2023
Rice husk	31	21	35	Ludueña et al., 2011
Esparto grass	32.17	19.63	32.2	May et al., 2018
Diss grass	28.13	26.26	24.95	May et al., 2018
Barley straw	19	38	45	Saini et al., 2015
Oat straw	19	38	37	Sánchez, 2009

2.2 Physiochemical properties of lignocellulosic biomass

Physicochemical characteristics including volatile matter, ash content, and moisture content serves as key indicator for evasluating and using lignocellulosic biomass for biochemical and bioenergy uses (Cai et al., 2017). Handling, microbiological stability during storage, and the effectiveness of thermochemical or biological conversion processes are all strongly impacted by moisture content, which is the quantity of water in biomass. According to Sluiter et al. (2008), excessive moisture content might result in microbial activity-induced deterioration, higher transportation and drying expenses, and decreased energy efficiency. Maintaining ideal moisture levels, on the other hand, improves feedstock flow characteristics and increases enzyme accessibility during hydrolysis. The amount of ash, which is the inorganic residue that remains after full combustion. Moisture, ash content, calorific value of various biomass shown in Table 2, varies depending on the source and influences reactor efficiency in thermal processes. Problems including slagging, fouling, and catalyst deactivation in gasification and combustion systems might result from a high ash concentration (Demirbaş, 2004). In addition, the existence of silica, potassium, and calcium in ash can hurt equipment longevity and process efficiency. Upon heating of lignocellulosic material without oxygen, the fraction that vaporises is known as volatile matter, and it offers information about the biomass's reactivity and combustion properties. According to Kumar et al. (2009), biomass

with a high volatile matter concentration is more suited for combustion-based energy systems since it typically has stronger reactivity and better flame stability. All of these factors work together to assess if biomass is suitable for a certain pretreatment and conversion technology, allowing for the most efficient process design for the recovery of energy and products.

Table 2: Moisture Content, Ash Content, and Calorific Value of Various Lignocellulosic biomasses

Biomass Type	Moisture (%)	Ash (%)	Calorific Value (MJ/kg)	Reference
Wheat Straw	8.5	4.3	17.5	Naik et al. (2010)
Rice Straw	9.0	5.0	16.8	Chandra et al. (2012)
Corn Stover	7.5	3.8	18.2	Zeng et al. (2011)
Sugarcane Bagasse	6.0	2.4	17.9	Santos et al. (2012)
Cotton Stalk	7.8	3.6	17.3	Yadav et al. (2013)
Bamboo	8.2	1.5	19.4	Nuntiya et al. (2011)
Sawdust (Pine)	10.0	1.0	19.2	Demirbaş (2001)

2.3 Lactic acid

Lactic acid (2-hydroxypropanoic acid) is an organic acid widely distributed in nature. Its existence in the form of two stereoisomers (as depicted in figure 5). LA is a crucial industrial material that acts as a starting point for the production of both small molecules like propylene glycol and larger compounds such as acrylic polymers (San-Martín, Pazos, & Coca, 1992). Its use as a precursor to polylactic acid (PLA), a biodegradable plastic with prospective uses in environmentally friendly packaging, has attracted a lot of attention to its manufacturing.(Auras et al., 2004).

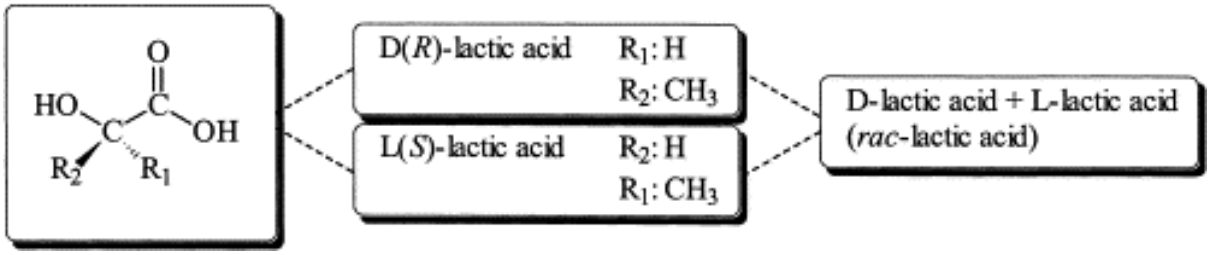

Figure 5: Stereoforms of lactic acid (Södergård et al., 2002).

2.4 Physio-chemical properties of lactic acid

The physicochemical properties of the compound play a major role in determining its chemical behavior: (a) it exhibits acidic properties when dissolved in water; (b) it has bifunctional reactivity because it contains both a carboxyl (-COOH) and a hydroxyl (-OH) group, which allows it to participate in a variety of chemical transformations; and (c) the chiral carbon atom at the C2 position gives the molecule optical activity (Martinez et al., 2013).

2.5 Applications of Lactic acid

It has moisturising, antimicrobial and rejuvenating effects on the skin, as well as on oral hygiene products. Approximately 70% of lactic acid produced is used in the food industry because of its role in the production of yoghurt and cheese, Beverages (e.g. beer, wine, soft drinks). Lactic acid also serves as a building block for making biodegradable plastic known as PLA (Polylactic Acid). From figure 6 we can see the various applications of lactic acid.

2.5.1 Cosmetic industry

In the cosmetics sector, lactic acid is incorporated into a variety of skincare and personal hygiene products because it hydrates, combats bacteria, and enhances skin renewal. Its utilisation includes dental care items as well(Martinez et al., 2013). Additionally, lactic acid derivatives like lactate esters find widespread use due to their ability to attract and retain moisture, as well as their excellent emulsifying capabilities. It is also used as an anti-acne, humectant and anti-tartar agent (Wee et al., 2006).

2.5.2 Lactic Acid as a Monomer in Biodegradable PLA Production

Lactic acid (LA) is converted to lactide (LT) via ring-opening polymerisation to create PLA. Typically, lactide is polymerised by ring-opening to create polylactic acid. Lactic acid is also converted to lactide via polycondensation and depolymerisation. Polyester, an eco-friendly thermoplastic, may break down into LA oligomers or carbon dioxide and water (Balla et al., 2021).

PLA's exceptional performance and advantages in manufacturing technique, yield, biocompatibility, and green origin, when compared to other bioplastics, make it the greatest alternative to petro-plastic and maintain its potential for use in a variety of industries, including food packaging, agriculture (J Yu et al., 2023).

2.5.3 Chemical industry

The use of lactic acid in the chemical industry has expanded considerably in recent years, owing to its versatility as a descaling agent, pH adjuster, neutraliser, and cleaning agent. It also serves as a slow-release acid source. Thanks to its exceptional solvency and solubility characteristics, lactic acid proves particularly effective for removing resins and polymers from surfaces. Its descaling ability finds application in products like bathroom cleaners and coffee machine descalers (Wee et al., 2006; Abdel-Rahman et al., 2013). Lactic acid contains both hydroxyl and carboxyl groups, allowing it to undergo self-esterification and produce poly(lactic acid) (PLA), a type of biodegradable polymer (Datta et al., 1995). PLA has garnered significant interest across industries due to its role as a sustainable packaging material, as a feedstock for fibers and textiles, and as a biocompatible polymer in the medical sector (Jamshidian et al., 2010; Auras et al., 2004).

The physical characteristics of PLA are strongly influenced by the optical purity of lactic acid. When polymerised from pure L- or D-lactic acid, PLA exhibits semi-crystalline behaviour, which imparts high tensile strength—ideal for rigid applications (Tsuji & Ikada, 1997a). Conversely, PLA derived from a racemic mixture of L- and D-lactic acid typically results in an amorphous structure, making it well-suited for controlled drug delivery systems, where uniform dispersion of active ingredients within the polymer matrix is crucial (Garlotta, 2001; Lim et al., 2008).

2.5.4 Pharmaceutical industry

Many parenteral/IV (intravenous) solutions used in pharmaceutical manufacturing contain lactic acid as an electrolyte to restore electrolytes or body fluids. Lactic acid has emerged as a key ingredient in several pharmaceutical solutions, including dialysis and Lactated Ringer's or Hartmann's solutions. The mixture of lactic acid and ammonium hydroxide serves in the production of ammonium lactate. It is considered as a crucial substance that is used in several medicinal applications. Pharmaceutical operations that depend on natural product chiral building blocks—lactic acid being one of the most significant—will continue to use chiral chemistry. The isomers (R) and (S) are now accessible. At optical purity levels close to 100 per cent. Because they are cheap, safe, synthetically flexible, and produce compounds with any required stereochemistry, pharmaceutical makers like chiral synthases like lactic acid (Alsaheb et al., 2015)

2.5.5 Food industry

Lactic acid's significance in the food sector appears to be connected to the microbial activity of specific organisms. According to the GSFA, the group of "lactic and fatty acid esters of glycerol" is unquestionably the most significant category of lactic acid derivatives with potential food uses.Lactic esters are widely used as surface actives and emulsifying agents. Lactic acid-esterified mono- and diglycerides are effective emulsifiers. Stearyl-2-lactylate is an excellent illustration (Ameen et al., 2017).

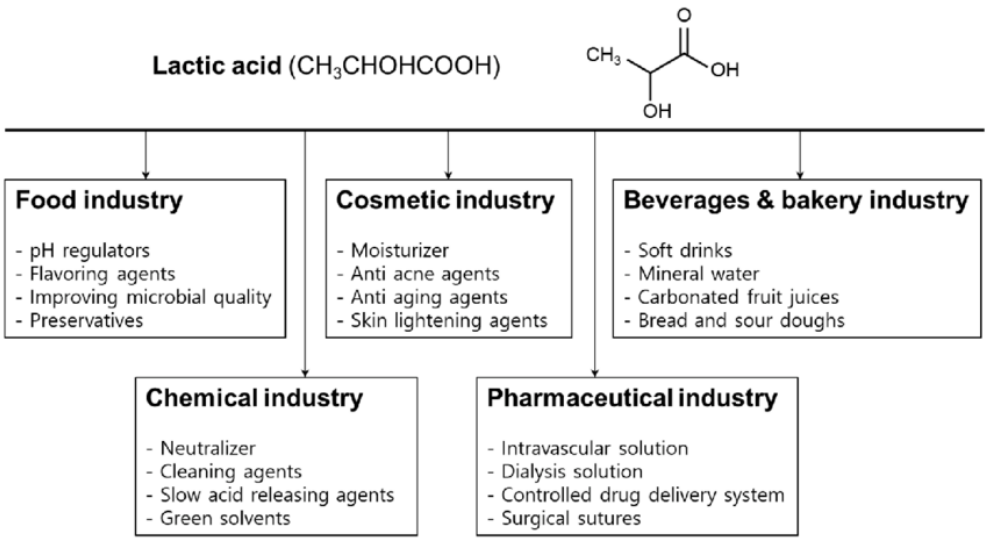

Figure 6: Applications of lactic acid. (Kim et al., 2022)

2.6 Biomass processing and size reduction

The primary goal of processing biomass is to decrease its particle size since this makes material handling easier and greatly increases the surface area, which makes subsequent processing processes easier (Harmsen et al., 2010; Akhtar et al., 2014). A variety of mechanical techniques, including chipping, grinding, milling, or a combination of these techniques, can be used to accomplish this size reduction (Mussatto et al., 2021). To produce finer particle sizes and increase process efficiency, sophisticated methods like ultrasound treatment and microwave irradiation are also used (Sadh et al., 2018). These structural changes are necessary to improve the efficiency of later processes like fermentation and enzymatic hydrolysis (Sun & Cheng, 2002).

2.7 Pretreatment of biomass

The pretreatment process is a very critical stage in lignocellulose bioconversion..Pretreatment strategies used to process lignocellulosic biomass can be broadly classified into mechanical (such as crushing and pulverising), chemical (involving bases, mild acids, oxidants, or organic reagents), physicochemical (including steam explosion, thermal hydrolysis, and wet oxidation), and biological methods. Reducing the size of biomass particles is important as it helps to preserve the pentose (hemicellulose) fractions, limits the formation of degradation products that can inhibit the growth of fermentative microorganisms, lowers energy requirement, and helps control overall costs (Mosier et al., 2005). Some of the approaches work by breaking down the lignin-carbohydrate complex, while others alter the rigid, crystalline arrangement of cellulose, thereby enhancing its accessibility for downstream conversion. To maximise the use of LCB in the production of bioethanol, it is very important to develop efficient pre-treatment techniques. Different pretreatment methods have been extensively developed; including ammonia fibre explosion and ammonia recycle percolation, lime, organosolv, liquid hot water ionic liquid, and alkaline pretreatment. Reducing Sugar Yields (Glucose and Xylose) from Various Pretreated Lignocellulosic Biomass Sources as detailed in Table 3. Dilute acid and steam explosion and enzymatic treatment.

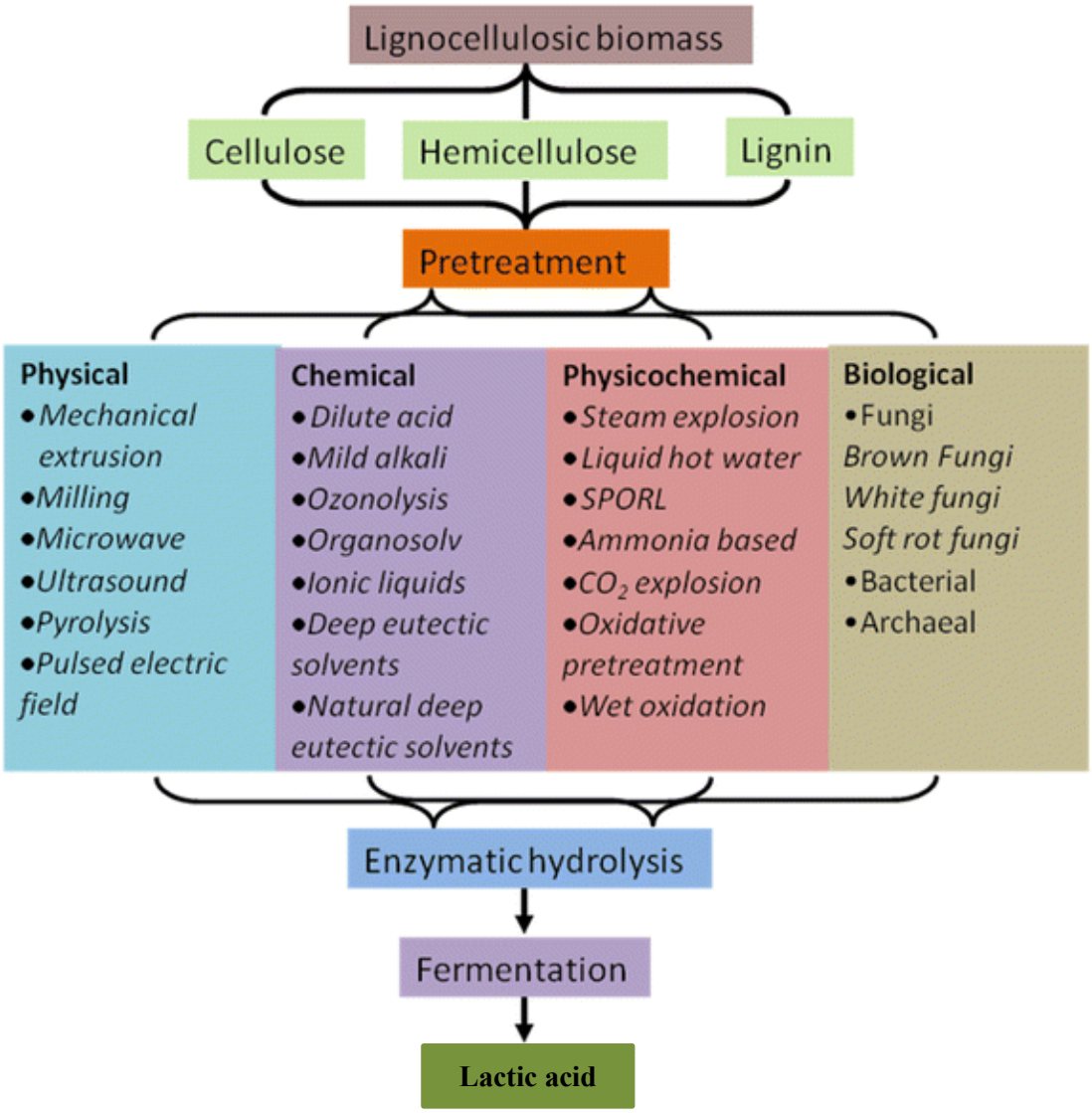

Figure 7: Overview of different pretreatment processes. (Kumar et al., 2017)

2.7.1 Mechanical pretreatment

A key factor in the transformation of feedstock into energy and polymer biomaterials is mechanical size reduction. It increases the calorific value of biomass and simplifies density operations by reducing the size of the material. The raw materials supply chain is also made more efficient, and storage conditions are improved. The breakdown of the biomass's constituents and the conversion of saccharides during hydrolysis are facilitated by this process, which also considerably expands the biomass's accessible surface area. The overall process is more efficient since it also reduces the mass and heat transfer constraints that arise during hydrolysis processes (Barakat et al., 2014).

2.7.1.1 Milling

Prolonged milling effectively reduces particle size by breaking the chemical connections between lignin and hemicelluloses (Barakat et al., 2014). Reducing the particle size increases the surface area relative to volume, making the material easier to handle and more accessible for further processing. Grinding, milling, or chipping is used to accomplish this. Several factors, including equipment cost, operational expenditures, scale-up potential, and depreciation, affect mechanical pre-treatment, which is completed prior to additional processing (Harmsen et al., 2013).

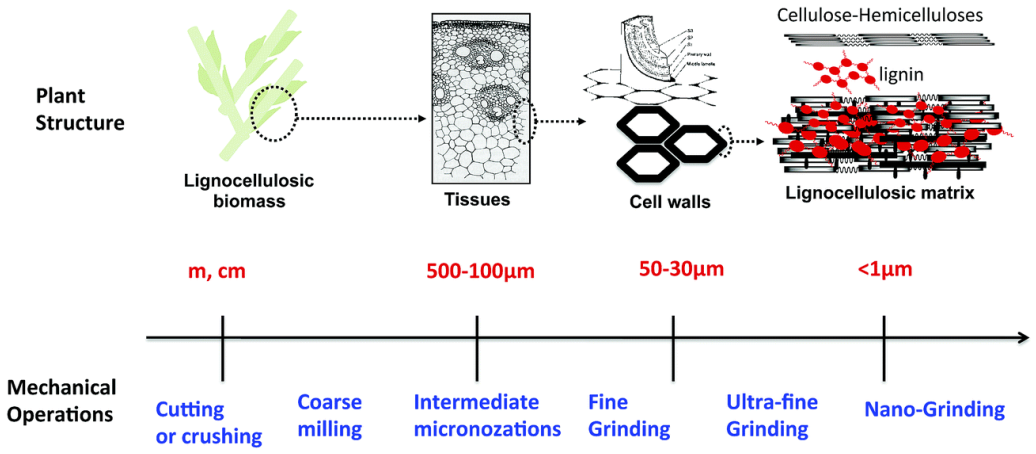

Figure 8: Mechanical Procedures for Lignocellulose Biomass Component Size Reduction (Barakat et al., 2014)

2.7.1.2 Ultrasound

Sonication is a relatively recent and promising method for pretreating biomass made of lignocellulosic materials. In laboratory experiments, small cavitation bubbles created by ultrasonic waves have been shown to degrade cellulose and hemicellulose and improve enzyme accessibility (Kumar et al., 2017) Sonicating alkaline-pretreated WS for 20 to 35 minutes increased delignification by 7.6 to 8.4% when compared to untreated samples (Sun and Tomkinson 2002).

2.7.2 Chemical pretreatment

2.7.2.1 Acid treatment

Hemicellulose's glycosidic bonds and lignin's ether linkages are hydrolyzed by mild acids. (Fengel and Wegener 1989), in which the organic acids formed by the breakdown of hemicellulose by catalyzing the cleavage of labile ester groups. The inner surface is enlarged to accomplish fractionation. Phosphoric, acetic, and sulfuric acids are the most frequently utilized acids for pretreatment.

2.7.2.2 Alkali treatments

Following pretreatments , complex plant fibres like cellulose and hemicellulose are broken down to simple sugars that can be easily fermented. The NaOH is considered one of the strong bases that solubilises lignin and hemicellulose significantly under definite conditions. It is confirmed that, in comparison to other alkaline pretreatment methods, a higher level of saccharification efficiency was attained by employing the NaOH pretreatment process. By comparing the NaOH to other alkaline pretreatment methods, it was confirmed that higher saccharification efficiency was attained. In lignin-carbohrate complexes, NaOH effectively breaks the bonds between lignin and hemicellulose , in particular ester and ether linkages in the lignin-carbohydrate complex are broken. Sodium hrydroxide is also used to breakdown the ester and carbon-to-carbon bonds in lignin molecules. By cleaving hydrogen and covalent bonds, alkali(NaOH) can deform cell wall polymers and effectively remove lignin. The surface area of wheat straw biomass is also increased by the removal of lignin and hemicellulose. Through saponification and salvation reactions, bonds (ester, aryl-ether, and alkyl-aryl), or the lignin carbohydrate complex (LCC), are broken, and acetyl and uronic acid groups in the hemicellulose are subsequently removed.

2.7.2.3 EDA aqueous solution

Ethylenediamine (EDA) pretreatment possessed various advantages in LCB deconstruction. EDA pretreatment can be conducted under mild conditions to perform efficient fractionation. It is a “dry-to-dry” process as high-solid loading was employed in pretreatment, which improves the utilisation efficiency of pretreatment reactors. EDA pretreatment introduces

nitrogen-containing groups in lignin to improve the water solubility of lignin and minimise its condensation, potentially facilitating the downstream upgrading of lignin.

2.7.2.4 Wet oxidation

By using water and oxygen (or air) at high temperatures and pressures to break down hemicellulose and degrade lignin, wet oxidation is a chemical pretreatment technique that improves the breakdown efficiency of lignocellulosic material. This process enhances the enzyme-assisted breakdown of cellulose by promoting the hydrolytic solubilization of hemicellulose along with the oxidative cleavage of lignin structures. For example, wet oxidation of common reed (*Phragmites australis*) resulted in a threefold increase in cellulose digestibility by cellulase when compared to untreated samples; during enzymatic hydrolysis, 82.4% of the cellulose was converted, while 51.7% of hemicellulose and 58.3% of lignin were solubilized (Szijártó et al., 2009). Similarly, optimisation of wet oxidation conditions for rice husk at 0.5 MPa and 185°C for 15 minutes led to 67% cellulose recovery, 89% lignin removal, and 70% hemicellulose solubilization (Banerjee et al., 2009). In their study on sugarcane bagasse, wet oxidation for 15 minutes at 195°C solubilised 93–94% hemicellulose and 40–50% lignin, significantly improving enzymatic convertibility (Martin et al., 2007). In another example, wet oxidation pretreatment of wheat straw resulted in glucose and xylose yields of 400 and 200 g/kg dry matter, respectively, following enzymatic hydrolysis (Pedersen and Meyer, 2011). Compared to acid-based methods, wet oxidation also has the advantage of generating fewer fermentation inhibitors like furfural and hydroxymethylfurfural (Hendriks and Zeeman, 2009). However, the process requires specialised high-pressure equipment, which increases operational costs, and may still result in the formation of some degradation byproducts such as carboxylic acids and phenolic compounds (Sun and Cheng, 2002; Mosier et al., 2005). Despite these limitations, wet oxidation remains a promising technique, especially for biomass with high lignin content, due to its efficiency in lignin removal and enhancement of enzymatic hydrolysis.

2.7.2.5 Organosolv

Organosolv pretreatment is a chemical process that breaks down the lignocellulosic structure using organic solvents. Its main goal is to remove lignin and hemicellulose, which increases the accessibility of cellulose for enzymatic hydrolysis (Zhao et al., 2009). Short-chain

aliphatic alcohols like methanol and ethanol, polyols like glycerol and ethylene glycol (EG), organic acids, acetone, dioxane, and phenol are examples of common solvents utilised in this process (Smit & Huijgen 2021). This technology is consistent with sustainable biorefinery principles since it uses renewable solvents including ethanol, methanol, acetone, acetic acid, and glycerol, which may be obtained from biomass (Borand & Karaosmanoğlu 2018).
The phrase "organosolv" has also been used to indicate advanced systems employing that use solvents that have demonstrated successful lignin removal and structural breakdown, such as alkylene carbonates (ACs), N-methylmorpholine N-oxide (NMMO), methyl isobutyl ketone (MIBK), and 2-methyltetrahydrofuran (2-MTHF) (Zhao et al., 2009; Smit & Huijgen, 2021). Furthermore, as they are capable of degrading lignocellulose without forming noticeable fermentation inhibitors, ionic liquids (ILs), including cholinium-amino acid-based ILs, are being utilised more and more in pretreatment (Xu et al., 2016; Sun et al., 2009). By solubilising the bonds between hemicellulose and lignin, these ILs increase the surface area of cellulose that is accessible to enzymatic assault (Sun et al., 2009). Organosolv and IL-based pretreatments provide cleaner fractionation and are frequently less hazardous or corrosive than traditional acid or alkaline treatments (Zhao et al., 2009; Xu et al., 2016). Even though organosolv has been a successful pretreatment method for many years, it has certain drawbacks, such as high cost, volatility, flammability, and recovery difficulties, which makes the entire process expensive and energy-intensive (Baruah et al., 2018).

2.7.2.6 Ionic liquids pretreatment

Ionic liquids are frequently used for pretreatment. These solvents contain special ions that melt easily, have low vapour pressure, stay stable at high temperatures (below 100 °C), and are highly polar, making them great at dissolving substances (Wasserscheid and keim, 2000). Ionic liquids dissociate the lignocellulose complex by competing with the hydrogen bond present in the complex. Ionic liquids degrade up to 80% lignin and hemicellulose (Li et al., 2011). According to Li et al. (2011), lignin and hemicellulose can be broken down by ionic liquids by up to 80%. Originally developed to break down cellulose, ionic 100 liquids have since been used to process more complex plant materials, such as lignocellulose. Numerous investigations have demonstrated the effective dissolution of lignocellulosic biomass by specific ionic liquids, particularly those possessing a strong hydrogen-bonding ability. Nevertheless, these liquids' propensity to readily absorb moisture from the air is a disadvantage that may impair their functionality (Brandt et al., 2011).

2.8 Challenges in lignocellulosic biomass conversion

2.8.1 Structural complexity of lignocellulosic biomass

The cellulose, hemicellulose, and lignin that make up lignocellulosic biomas(LCB) are firmly bound together, giving it a robust and complex structure. This strong bond serves as a natural barrier that is difficult for chemicals or enzymes to efficiently break down (Himmel et al., 2007). LCB is extremely resistant to enzymatic or chemical hydrolysis due to the crystalline nature of cellulose, the aromatic, three-dimensional network of lignin, and the heterogeneous polymeric structure of hemicellulose (Alvira et al., 2010). This resistance severely impairs enzyme accessibility, which lowers the effectiveness of fermentation and saccharification processes. Therefore, to improve the enzymatic digestibility of carbohydrates, effective pretreatment techniques are required to break the lignin-carbohydrate complex, decrease cellulose crystallinity, and raise the biomass's overall porosity (Mosier et al., 2005).

2.8.2 Generation of inhibitory compounds

When lignocellulosic biomass is processed, especially in harsh chemical or thermal conditions, it breaks down into a number of different compounds that make it much harder for enzymes to break it down and for microbes to proliferate. By making chemicals that stop fermentative microbes from doing their jobs (Almeida et al., 2007; Kim et al., 2018). Furfural and HMF, in particular, are known to disrupt glycolytic enzymes and make cells less viable, even at low doses (Jönsson et al., 2013). Phenolic chemicals are especially difficult for microbial fermentation and enzymatic saccharification since they come in many different shapes and can pile up to dangerous amounts (Aparicio et al., 2006).

2.8.3 Limited fermentation of pentose sugars

A lot of industrial microbial hosts, such Saccharomyces cerevisiae, have trouble digesting pentose sugars like xylose, which are found in lignocellulosic hydrolysates. (Jeffries et al., 2004) say that "wild-type strains of S. cerevisiae do not metabolize xylose," hence metabolic engineering must be utilized to create pathways for xylose absorption. Cofactor mismatches between the NADPH-dependent reductase and NAD+-dependent dehydrogenase pathways can make fermentation less effective. Because the native glucose transporters (Hxt family) don't like pentoses very well and glucose stops them from working, xylose absorption is even more difficult in mixed sugar situations (Nijland et al., 2020). To attain effective pentose

utilization, S. cerevisiae still needs extra adjustments, such as pentose transporter engineering and global regulatory reprogramming, despite decades of engineering efforts (Gopinarayanan et al., 2019).

Table 3: Reducing Sugar Yields (Glucose and Xylose) from Various Pretreated Lignocellulosic Biomass Sources

Lignocellulosic Biomass	Pretreatment	Glucose Yield (%)	Xylose Yield (%)	Reference
Rice Straw	NaOH + Hydrothermal	38	25	Chandra et al., 2012
Corn Cobs	Acid Treatment	35	18	Moldes et al., 2006
Sugarcane Bagasse	Acid Treatment + Steam Explosion	40	24	Van der Pol et al., 2016
Wheat Straw	Lime (Calcium hydroxide)	42	21	Maas et al., 2008
Bagasse	Ionic Liquid Pretreatment	48	22	Sun et al., 2015
Corn Stover	Dry Sulfuric Acid	43	22	Zhao et al., 2013
Sorghum Stalk	Sulfuric Acid	36	18.5	Dahunsi et al., 2019
Bamboo	Steam Explosion	30	15	Lee et al., 2017
Groundnut Shell	High-pressure Homogenization (4 mm)	40	20	Jekayinfa et al., 2020
Corn Straw	Ammonia (10%)	44	23	Song et al., 2014
Barley Straw	High-pressure Homogenization (5 mm)	38	16	Menardo et al., 2012
Rice Husk	Wet Oxidation	37	22	Banerjee et al., 2009
Pine Sawdust	Acid Hydrolysis	28	12	Kumar et al., 2010

2.9 Bacterial fermentation

Lactic acid is mainly produced in two ways- one using microbes through the fermentation process, and the other through chemical reactions in a lab. When optically pure lactic acid is required, microbial fermentation is a better option because chemical processes usually result in racemic mixtures (Iyer et al., 2000). This biological process turns carbohydrates—often from inexpensive substrates—into lactic acid employing different microbial strains. Anaerobic glycolysis is often used to metabolise glucose, whereas the pentose phosphate route is used to digest pentose carbohydrates. Then, using NADPH as an electron donor, the resultant pyruvate is converted to lactic acid. Fermentation efficiency is greatly impacted by several variables, such as pH, temperature, substrate concentration, media composition, and the presence of inhibitors (Rawoof et al., 2021; Yankov, 2022).

2.9.1 Homolactic fermentation

A single glucose molecule is converted into two molecules of lactic acid, primarily through the pentose phosphate pathway as shown in Figure 10.. This process yields nearly pure lactic acid and is carried out by bacteria such as *Lactobacillus lactis*, *Lactobacillus casei*, *Streptococcus* species, and *Bacillus coagulans*. Additionally, *Bacillus subtilis* is known to produce lactic acid from cellobiose via homofermentation (Kaur 2017; Abedi et al., 2020).

2.9.2 Heterolactic fermentation

Involves the phosphoketolase pathway, which converts glucose into one molecule of lactic acid along with other by-products like carbon dioxide, acetic acid, or formic acid. Bacteria including *Leuconostoc*, *Weissella*, *Oenococcus*, and *Lactobacillus delbrueckii* typically follow this fermentation route. Besides lactic acid, heterolactic fermentation also produces alcohols that are useful for bioethanol production (Hu et al., 2016; Wang et al., 2021).

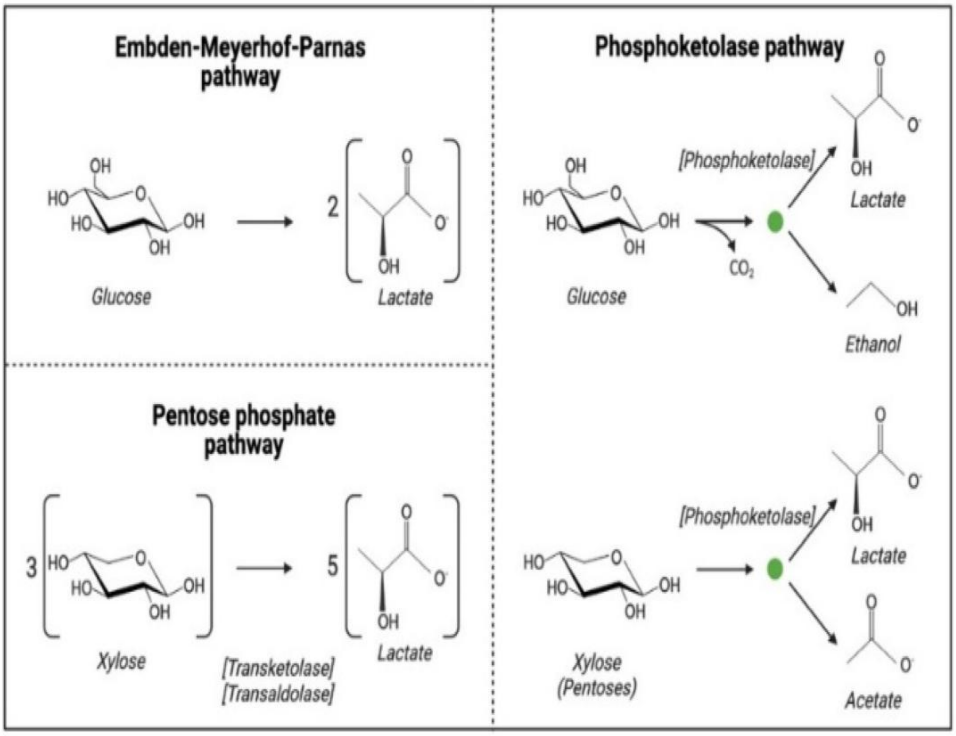

Figure 9: Different metabolic pathways (Becerra et al., 2022).

Bacillus species are increasingly favoured over traditional lactic acid bacteria (LAB) owing to several substantial benefits (Chawla & Goyal, 2022; Singh et al., 2020). These bacteria may proliferate in cost-effective media formulations, frequently employing inexpensive nitrogen sources such as minimum mineral salt medium augmented with corn steep liquor or ammonium sulfate, so substantially lowering production expenses. Because of their thermophilic characteristics, they can withstand and function effectively at temperatures higher than 50°C, negating the need for costly cooling procedures after sterilization. (Singh et al., 2020). *Bacillus* strains generate optically pure L-(+)-lactic acid with high productivity and exhibit significant resistance to prevalent fermentation inhibitors such as furfural, hydroxymethylfurfural (HMF), and acetate, typically formed throughout the pretreatment of lignocellulosic biomass (Chawla & Goyal, 2022; Kumar et al., 2019). This inhibitor tolerance improves their appropriateness for industrial-scale fermentations utilising lignocellulosic hydrolysates.After pretreatment, the sugar-rich hydrolysates, which include hexoses and pentoses, are enriched with vital nutrients before inoculating with the chosen Bacillus strains for fermentation. Differential lactic acid production across microbial strains, as tabulated in Table 4, demonstrates distinct metabolic efficiencies. These microorganisms effectively transform these sugars into desired products, including lactic acid and bioethanol, hence enhancing sustainable bioprocesses (Singh et al., 2020; Kumar et al., 2019).

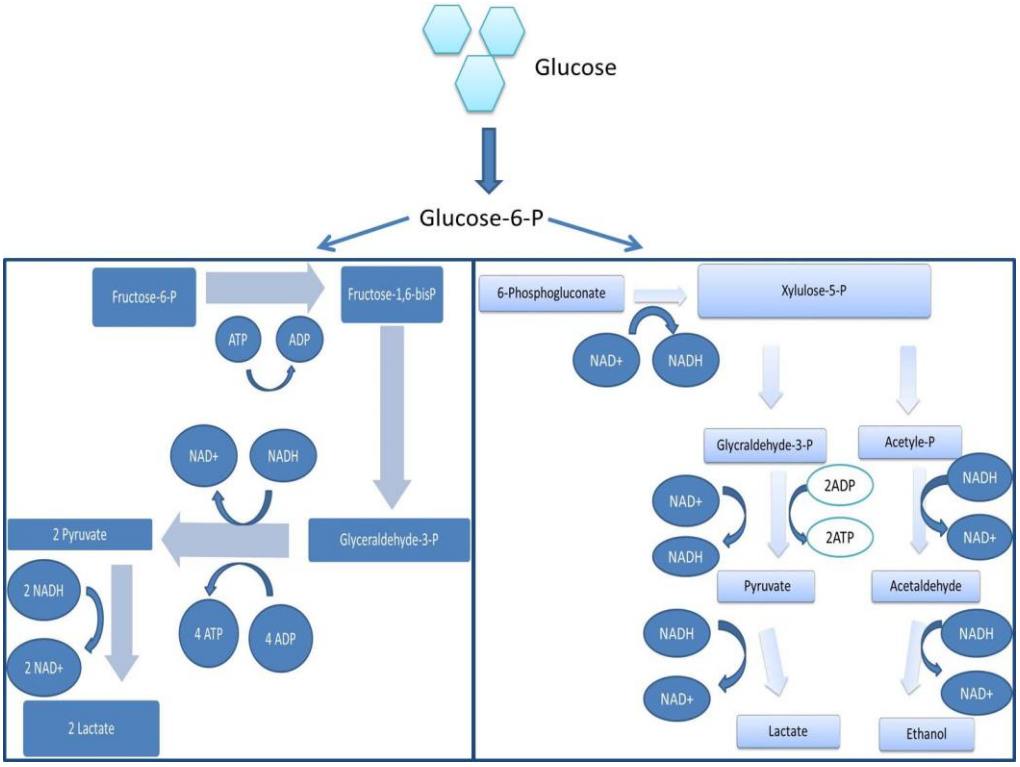

Figure 10: Homo-fermentative and hetero-fermentative pathways of lactic acid bacteria.

Table 4: Comparative analysis of Lactic acid production across microbial Strains.

Bacteria	**Lactic Acid Produced(g/L)**	**Reference**
Lactobacillus lactis	161.2	Rahman et al., 2011
Lactobacillus delbrueckii	28.0	Rahman et al., 2011
Lactobacillus amylophilus	29	Nampoothiri & Pandey et al., 2007
Lactobacillus plantarum	72.9	Nampoothiri & Pandey et al., 2007
Lactobacillus rossiae M2	1.54	Cizeikiene et al., 2018
Lactobacillus crustorum W19	2.94	Cizeikiene et al., 2018
Streptococcus inulinus	93.4	Zhang et al., 2018

CHAPTER 3-MATERIALS AND METHODS

3.1 Materials

This study involved the application of specific bacterial cultures, namely *Bacillus licheniformis* DGB and *Bacillus sonorensis* DGS15, as previously isolated by Akhtar and Goyal (2014) and Chawla and Goyal (2022). For fermentation experiments, xylose (10 g/L) and yeast extract (5 g/L) from HiMedia were used, set to a pH of 7. Shake flask fermentation was done in 500 mL Erlenmeyer flasks. The basal medium consisted of Bushnell Haas medium (3.27 g/L, HiMedia), and MRS medium (HiMedia) was also used for routine bacterial maintenance. crop residues such as wheat husks and rice straw served as lignocellulosic substrates. Various chemicals used for pretreatment and analysis included sodium hydroxide (HiMedia), hydrochloric acid, glacial acetic acid, sulphuric acetic acid, sodium chlorite, calcium hydroxide, DNSA reagent, orcinol reagent, ferric chloride, and copper sulphate. Every chemical used in this investigation was of analytical grade and came from Loba Chemie.

3.2 Processing and preparation of RS and WS

Rice straw and wheat straw were collected from neighbouring villages in Patiala, Punjab, then oven-dried for at 50°C to remove moisture. The straw was grounded to a fine 0.5 mm particle size after drying and kept for future research in airtight containers.

3.3 Three stage Pre-treatment of biomass for fermentable sugars and cellulose recovery

3.3.1 Alkali hydrolysis for delignification of rice and wheat straw

In order to delignify the dried and acid-pre-treated biomass, a 1.5% (w/v) sodium hydroxide solution was added in a 1:10 biomass-to-NaOH ratio. The mixture was autoclaved for 45 minutes at 15 psi and 121 °C. like acid pretreatment, mixture was filtered and the black liquor obtained was discarded, the solid residue was washed, dried and used further.

3.3.2 Hydrolytic processing using diluted sulfuric acid

50 g of powdered rice and wheat straw were weighed and dissolved in 1% (v/v) sulfuric acid in a 1 L reagent bottle. For 60 minutes at 15 psi, the solutions were autoclaved at 121 °C. The mixture was allowed to cool to room temperature and then passed through a muslin cloth for

separation. The hydrolysate was reserved for fermentation experiments, while the solid residue was rinsed with tap water, oven-dried, and preserved for subsequent pretreatment.

3.3.3 Extraction of cellulose using sodium chlorite and glacial acetic acid

For the extraction of pure cellulose, 4% sodium chlorite and 0.5% glacial acetic acid were used. These solutions were used to treat biomass that had been acid-pretreated in a 1:20 ratio (biomass: solution) and was incubated in a water bath at 60 °C for 60 minutes. This treatment facilitated the isolation of purified cellulose from the pre-treated biomass.

3.4 Neutralization and Detoxification of Pretreated hydrolysate

To neutralize the acid hydrolysate from section 3.3.2, 35% (w/v) calcium hydroxide was added to precipitate pretreatment derived inhibitors like HMF and furfural. Centrifugation of the samples was carried out at 8000 rpm for 5 minutes, and the resulting supernatant was used for the next set of experiments.

3.5 Bacterial strains

Rice and wheat straw hydrolysates were fermented using previously isolated strains, *Bacillus sonorensis* DGS15 and *Bacillus licheniformis* DGB, both thermotolerant and inhibitor-resistant strains isolated from the red soil of a brick kiln (Chawla & Goyal, 2022). In an enrichment broth with 10 g/L xylose and 5 g/L yeast extract, these two strains were brought back to life at 50°C and pH 6.5 (Lidan et al., 2013). Separately, *Lactobacillus reuteri* was revived in MRS medium at pH 6.0, 120 rpm for 16 h at 37°C.

3.6 Estimation of reducing sugars and lactic acid

3.6.1 Estimation of glucose by DNSA method (Miller, 1959)

DNSA reagent- in a beaker added 1 gm of 3, 5-dinitrosalicyclic acid which was dispersed in 20 ml of water, 30 ml of sodium potassium tartarate solution and 20 ml of (2N) NaOH solution were added to this 20 ml dns solution, followed by continuous stirring to obtain clear solution. After that, the mixture was filtered, and distilled water was added to bring the volume up to 100 ml. 10 µl sample of rice straw and wheat straw was added with 990 µl of

distilled water and 3 mL of DNSA reagent was added to this diluted sample. The resulting mixture was placed in a hot water bath and incubated for five minutes. A UV-vis spectrophotometer was used to measure absorbance at 540 nm after cooling.

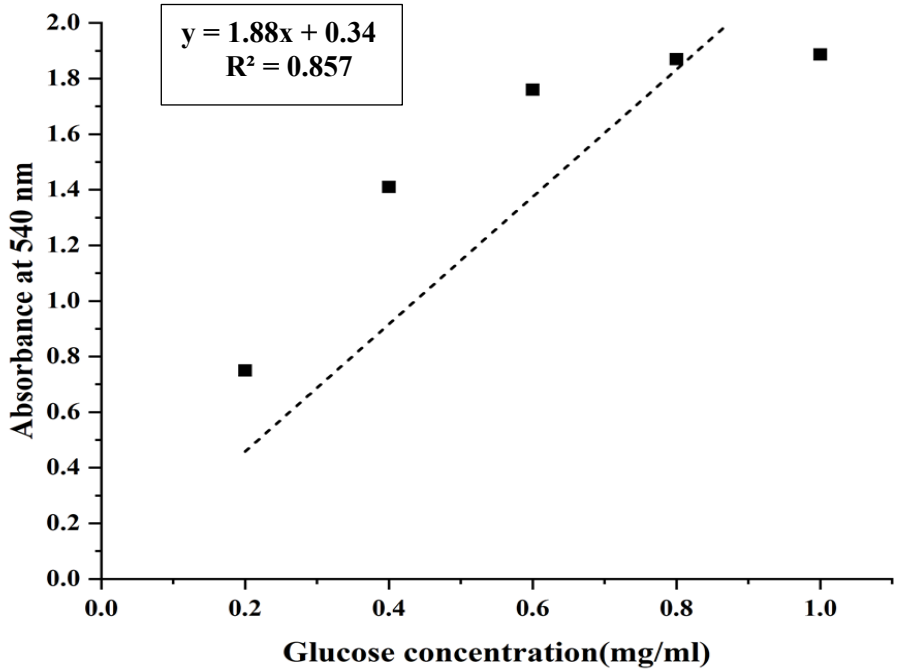

Figure 11: Standard graph of glucose

3.6.2 Estimation of xylose

Orcinol reagent- 0.3 g of FeCl3 and 0.15 g of orcinol reagent was dissolved in fifty milliliters of HCl that had been concentrated. 10 µl sample (made upto volume 1 ml) was added with 1 ml of orcinol reagent. Then prepared mixture was held at particular temperature to allow incubation for five minutes in order to develop the colour. Absorbance at 671 nm was recorded using a spectrophotometer. Following, incubation. D-xylose concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL were used to create a standard curve.

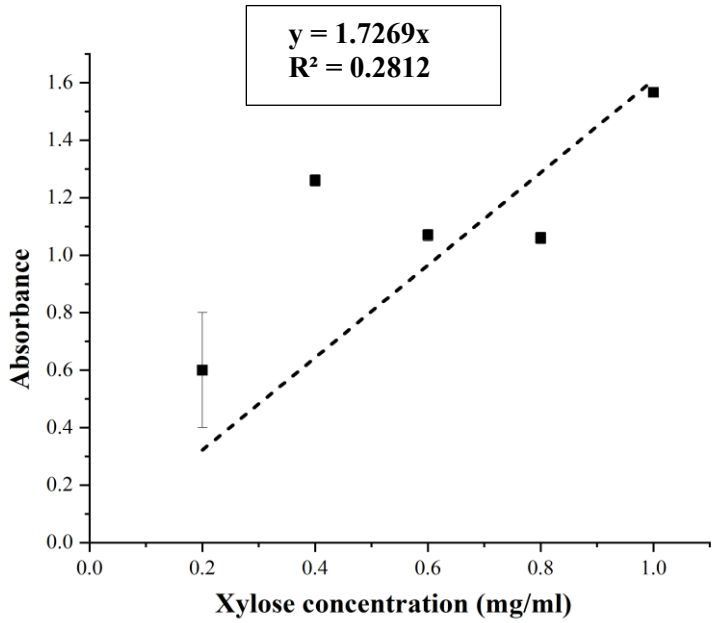

Figure 12: Standard graph of xylose

3.6.3 Lactic acid eastimation by Barker-Summerson method (Barker & Summerson, 1941).

- Took 10µl of LA sample and added 200µl of trichloroacetic acid, 200µl 20% $CuSO_4.5H_2O$, 500µl distilled water and 0.2g $Ca(OH)_2$. Centrifugation was done at 10,000rpm for 10mins; collecting the supernatant.
- After adding 3 milliliters of concentrated H_2SO_4 to 100 microliters of supernatant, the mixture was incubated for five minutes in a boiling water bath.
- After cooling the solution, the addition of 300µl p-phenyl phenol (1.5% pp in 1.5% NaOH solution)
- The 10min room temp incubation was followed by 90sec water bath boiling
- The absorbance at 560 nm was noted.
- The standard curve equation Y=0.59X was employed to calculate the lactic acid concentration.

3.7 Lactic Acid production *via* fermentation of hydrolysates

Rice and wheat straw hydrolysates, both adjusted to pH 6.5, were used in the fermentation process to produce lactic acid (LA). Both kinds of hydrolysates were fermented using the microorganisms *Bacillus licheniformis* (DGB) and *Bacillus sonorensis* (DGS15) and *L.reuteri*. The hydrolysates were enhanced with Bushnell Haas medium (3.27 g/L) and yeast extract (5 g/L) for the fermentation with DGB and DGS15 and also with MRS components for fermentation with *L.reuteri*. The concentrations of glucose, xylose, and lactic acid were measured at 0 hours, 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours at 50°C and 37°C while being shaken at 120 rpm.

The total yield was calculated as grams of lactic acid produced per gram of sugar consumed, and the total lactic acid productivity was calculated as grams of lactic acid generated per liter per hour (g L^{-1} h^{-1}).

Lacic acid yield and productivity were calculated using:

Lactic Acid Productivity (g/L/h) = actic acid produced (g/L)/ Fermentation time (h)

Lactic Acid Yield (g/g) = Lactic acid produced (g)/ Sugar consumed (g)

CHAPTER-4 RESULTS AND DISCUSSION

4.1 Processing of wheat straw and rice straw

To enhance the efficacy of the subsequent pre-treatment processes, the dried wheat straw (WS) was ground into roughly 0.5 mm-sized particles. After being finely powdered, the biomass was pre-treated to help break down the intricate lignocellulosic structure and increase the accessibility of cellulose and hemicellulose for subsequent processing. The surface area available for chemical interactions was increased by particle size reduction and encouraged consistent pre-treatment, which are both necessary to maximize the quantity of fermentable sugars generated in the following stages (Nargotra et al., 2018).

Figure 13: a. Non grinded WS, **b.** Non grinded RS, **c.** Grinded & sieved wheat straw (0.5 mm) **d.** Grinded & sieved rice straw (0.5 mm)

4.2 Delignification of RS and WS

Since sodium hydroxide (NaOH) effectively delignifies the material and enhances enzymatic accessibility, it was utilized as an alkali in the pretreatment of lignocellulosic biomass, such as rice and wheat straw. The primary objective of employing NaOH is to decompose the intricate structure of lignin and to sever the ester and ether bonds that connect lignin to hemicellulose and cellulose. In order to delignify 50 gms wheat straw (WS) and 50 gms of rice straw, 1.5% NaOH was used.This disruption leads to the partial solubilisation of hemicellulose and a substantial removal of lignin, so exposing the cellulose fibers for subsequent enzymatic hydrolysis (He et al., 2008; Bahiru et al., 2017). After the alkali treatment, the biomass slurry was filtered using muslin cloth to separate the solid biomass residue that was 57.35% of WS and 54.68% of RS from the liquid containing dissolved lignin

and other soluble components. The recovered solid biomass, now substantially free of lignin, was then utilized for additional processing to obtain pure cellulose after giving acid pretreatment. A comparison investigation revealed that NaOH pre-treatment of wheat straw achieved up to 81% delignification, whereas rice straw exhibited a delignification efficiency of around 73.6% (Bahiru et al., 2017; Samar et al., 2021)

Figure 14: NaOH Treatment of Biomass for Delignification

4.3 Acid hyrolysis and Cellulose separation in delignified and acid pretreated biomass

After delignification, RS and WS were acid-pre-treated with diluted sulfuric acid, 100 ml of double-distilled water was added, and the mixture was autoclaved at 121°C for 60 minutes. It uses fewer fermentative inhibitors and increases hemicellulose hydrolysis to yield a lot of pentose sugars (Deshavath et al., 2017). In acidic factors, the glycosidic linkages in hemicellulose get protonated, resulting in unstable intermediates that easily break, releasing monosaccharaides (Zhu & Pan, 2022; Loow et al., 2016). After pre-treatment solid recovery was 53.4% WS and 43.6% rice straw respectively and hysrolysate volume was 380ml and 340 ml. Sulfuric acid pretreatment of lignocellulosic biomass is a common technique because of its low cost (Kumar et al., 2017).

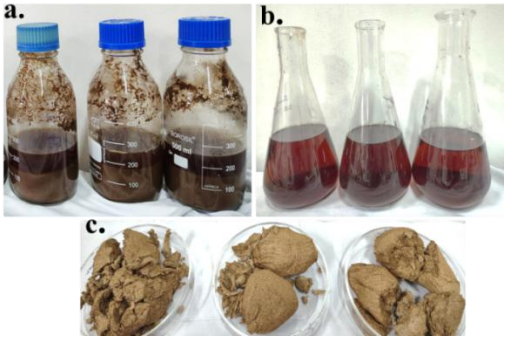

Figure 15: a. Acid pre-treatment of biomass, **b.** Hydrolysate, **c.** acid pre-treated biomass

Acid-chlorite technique, which effectively eliminates lignin while retaining cellulose and hemicellulose to isolate holocellulose. In this procedure, glacial acetic acid buffers the media to a moderately acidic pH (≈ 3.5–4), perfect for producing chlorine dioxide from sodium chlorite and permitting targeted lignin oxidation without considerable hydrolysis of polysaccharides (Wise et al., 1946; Ahlgren & Goring, 1971).The percentage of cellulose, hemicellulose has been reported earlier; where wheat straw consists of 29% hemicellulose, 36% cellulose, 19% lignin and rice straw has 24% hemicellulose, 43% cellulose and 9% lignin (Akhtar and Goyal, 2014).

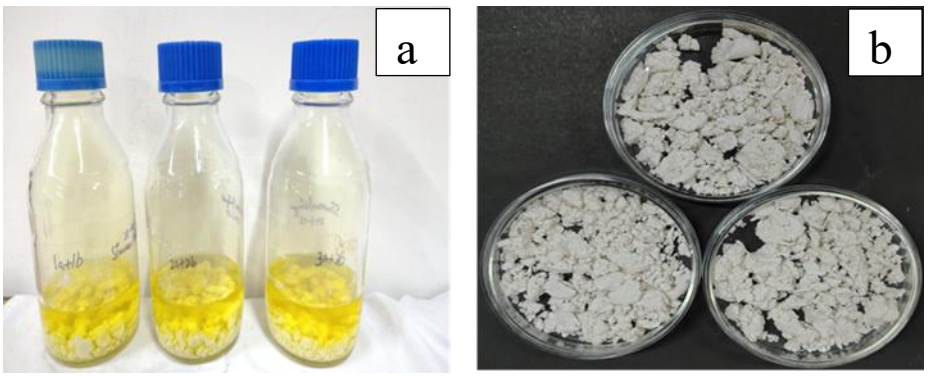

Figure 16: a. Cellulose separated biomass before filtration, **b**. Cellulose separated residue

4.4 Conversion of rice straw hydrolysate to lactic acid by *Lactobacillus reuteri*

Section 4.1.2 reports that 340 milliliters of detoxified rice straw hydrolysate were inoculated with a 5% *Lactobacillus reuteri* inoculum, and incubated at 37°C with continous shaking at 120 rpm for duration of 120 hours. The concentrations of glucose, xylose, and lactic acid were measured at intervals of 24 hours to monitor the fermentation process. During the process, *Lactobacillus reuteri* largely consumed glucose through the glycolytic route under anaerobic circumstances, creating lactic acid as the major metabolite. Simultaneously, it metabolized xylose via the pentose phosphate pathway, creating NADPH and other intermediates essential for the conversion of pyruvate into lactic acid (Yankov, 2022). This dual sugar metabolism enables the steady build-up of lactic acid during fermentation and demonstrates the organism's versatility in using both hexose and pentose carbohydrates. The rice straw hydrolysate, prepared through acid hydrolysis, was enriched with MRS medium and maintained at pH 6.5. Fermentation was conducted at 37°C with agitation at 120 rpm

over a period of 120 hours. Initially, the concentrations of glucose and xylose were 16.66 ± 0.0001 mg/mL and 10.32 ± 0.0001 mg/mL, respectively, while lactic acid was undetectable (0 ± 0 mg/mL). By 48 hours, glucose had dropped to 12.18 ± 0.0002 mg/mL and xylose to 6.95 ± 0.00012 mg/mL, while lactic acid reached 0.52 ± 0.0005 mg/mL, indicating active substrate consumption and product formation. The trend continued over time as the glucose concentration further declined to 2.19 ± 0.0001 mg/mL at 96 hours, and finally to 0.86 ± 0.0001 mg/mL at 120 hours. Similarly, xylose decreased to 4.83 ± 0.0002 mg/mL at 96 hours and 1.22 ± 0.0002 mg/mL by the end of the fermentation. Correspondingly, lactic acid production steadily increased, rising to 1.13 ± 0.0003 mg/mL at 96 hours and reaching a final concentration of 1.42 ± 0.0002 mg/mL at 120 hours. Research on other Lactobacillus species, including Lactobacillus brevis, has revealed similar results, which showed that glucose and xylose could be effectively used together in MRS medium to produce lactic acid (Kim et al., 2010).

Table 5: Rice straw hydrolysate, fortified with MRS media, served as the fermentation substrate for *Lactobacillus reuteri* to produce lactic acid at 37°C and 120 rpm

Time(hrs)	Glucose (mg/ml)	Xylose (mg/ml)	LA (mg/ml)
0	16.66±0.0001	10.32 ±0.0001	-
24	13.57±0.0023	7.18±0.0017	0.35±0.0017
48	12.18±0.0002	6.95±0.00012	0.52±0.0005
72	6.05±0.0001	6.41±0.0002	0.83±0.0003
96	2.19±0.0001	4.83±0.0002	1.13±0.0003
120	0.86±0.0001	1.22±0.0002	1.42±0.0002

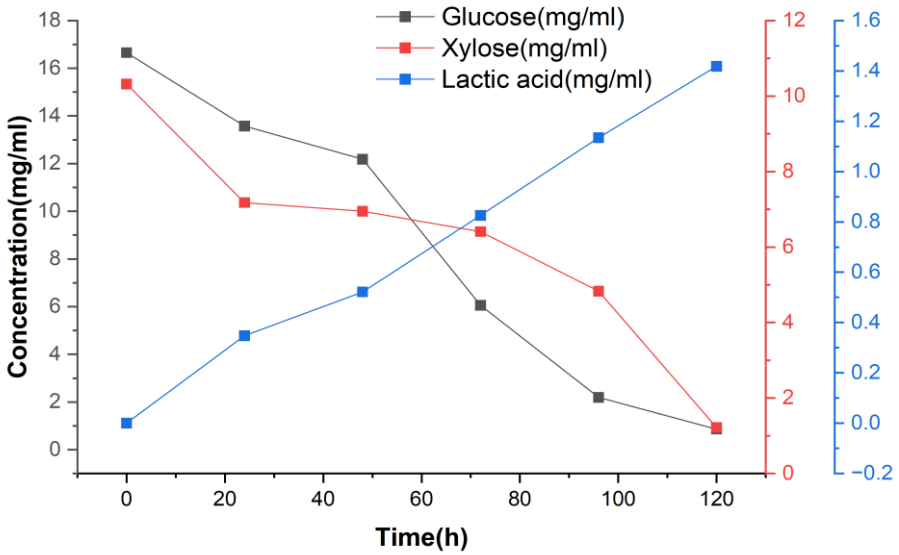

Figure 17: Lactic acid production by *Lactobacillus reuteri* in rice hydrolysate obtained by acid hydrolysis of separated cellulose, supplemented with MRS media at pH 6.5, 120 rpm, 37°C.

4.5 Lactic acid production from wheat straw hydrolysate by *Lactobacillus reuteri*

Wheat straw hydrolysate, obtained through pretreatment and detoxification, was employed as the fermentation medium to produce lactic acid. The hydrolysate was enriched with essential nutrients and inoculated with 5% Lactobacillus reuteri culture. As stated in section 4.2, the fermentation was conducted under carefully monitored circumstances. Ensuring an environment suitable for optimal microbial growth and metabolic activity. The process was monitored for 120 hours, The concentrations of lactic acid, xylose, and glucose were measured through periodic sampling.. Initially, xylose and glucose were present at concentrations of 13.70 ± 0.0002 mg/mL and 18.70 ± 0.0001 mg/mL, respectively. Sugar levels gradually decreased through fermentation, reflecting their active consumption by the bacterial strains. By 48 hours, glucose had dropped to 10.62 ± 0.0002 mg/mL and xylose to 12.57 ± 0.0001 mg/mL. This trend continued through 96 hours, where glucose reduced to 4.37 ± 0.0002 mg/mL and xylose to 7.48 ± 0.0001 mg/mL. By the end of 120 hours, only 1.72 ± 0.0002 mg/mL glucose and 6.48 ± 0.0003 mg/mL xylose remained, confirming efficient sugar uptake. Simultaneously, lactic acid production showed a progressive increase, reaching 0.138 ± 0.0005 mg/mL at 48 hours, 1.011 ± 0.0003 mg/mL at 96 hours, and finally

peaking at 1.468 ± 0.0006 mg/mL after 120 hours. This increment reflects the metabolic efficiency of L. reuteri in converting lignocellulosic sugars into lactic acid. The overall yield and productivity, recorded at 0.014 g/g and 0.0073 g/L/h respectively, and yield and productivity at 120 h recorded as 0.0606 g/g and 0.0122 g/L/h show that wheat straw hydrolysate is a viable feedstock for the sustainable bioproduction of lactic acid.

Table 6: Lactic acid production by *Lactobacillus reuteri* in wheat straw hydrolysate obtained by acid hydrolysis, supplemented with MRS media at pH 6.5, 120 rpm, 37°C

Time(hrs)	Glucose (mg/ml)	Xylose (mg/ml)	LA (mg/ml)
0	13.70±0.0002	18.70±0.0001	-
24	13.57±0.0002	13.18±0.0001	0.062±0.0005
48	10.62±0.0002	12.57±0.0001	0.138±0.0005
72	7.17±0.0023	10.60±0.0001	0.733±0.0003
96	4.37±0.0002	7.48±0.0001	1.011±0.0003
120	1.72±0.0002	6.48±0.0003	1.468±0.0006

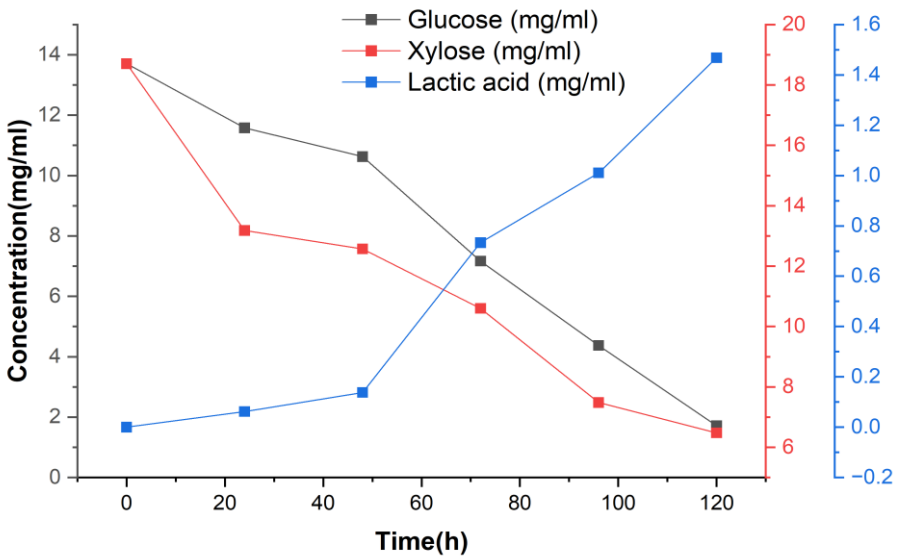

Figure 18: Lactic acid production by *Lactobacillus reuteri* in wheat straw hydrolysate.

4.6 Lactic Acid Production from rice straw by *Bacillus licheniformis*

The bacterial isolate *Bacillus licheniformis* DGB demonstrated a strong ability to ferment sugars derived from rice straw hydrolysate into lactic acid (LA) under thermotolerant conditions. The strain consumed both hexose (glucose) and pentose (xylose) sugars during a 120-hour period, indicating an active metabolism. Initially, the concentrations of xylose and glucose were 20.76 ±0.00029 mg/mL and 16.99 ± 0.0002 mg/mL, respectively. At 0 hours, with no detectable lactic acid at the beginning of fermentation. By 24 hours, glucose and xylose decreased to 15.85 ± 0.0002 mg/mL and 15.03 ± 0.0020 mg/mL, while LA began to accumulate, reaching 0.41 ± 0.0059 mg/mL. Continued bioconversion was evident as the sugars were progressively consumed and LA accumulation increased (refer to table). The trend of sugar utilization continued steadily; at 48 hours, glucose was 12.51 ± 0.0002 mg/mL, xylose 9.28 ± 0.00025 mg/mL, and LA increased to 0.92 ± 0.0059 mg/mL. At 72 hours, LA production crossed the 1 mg/mL mark, reaching 1.19 ± 0.0061 mg/mL, while glucose and xylose further dropped to 9.21 ± 0.0002 mg/mL and 6.72 ± 0.00023 mg/mL, respectively. A sharp rise in LA was observed between 72 and 96 hours, where LA reached 3.20 ± 0.0073 mg/mL, corresponding with further sugar depletion (glucose: 5.32 ± 0.0002 mg/mL, xylose: 4.16 ± 0.00020 mg/mL). As shown in figure 19, by the end of 120 hours, the maximum amount of LA was produced at 6.00 ± 0.0059 mg/mL, while the amounts of glucose and xylose were decreased to 1.60 ± 0.0002 mg/mL and 2.06 ± 0.00026 mg/mL, respectively.

The production of lactic acid (LA) by the Bacillus licheniformis DGB strain, which was cultivated on rice straw hydrolysate, was examined over a 120-hour period in order to assess the productivity and yield of fermentation. With a productivity of 0.050 g/L/h, the highest lactic acid yield of 0.176 g/g was recorded at 120 hours. With a total lactic acid concentration, this peak value shows effective use of both glucose and xylose as carbon sources.

Table 7: Lactic acid production by *Bacillus licheniformis* (DGB) in rice straw hydrolysate supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50◦C

Time (h)	Glucose (mg/ml)	Xylose (mg/ml)	LA (mg/ml)
0	16.99±0.0002	20.76±0.0029	-
24	15.85±0.0002	15.03±0.0020	0.41±0.0059
48	12.51±0.0002	9.28±0.00025	0.92±0.0059
72	9.21±0.0002	6.72±0.00023	1.19±0.0061
96	5.32±0.0002	4.16±0.00020	3.20±0.0073
120	1.60±0.0002	2.06±0.00026	6.00±0.0059

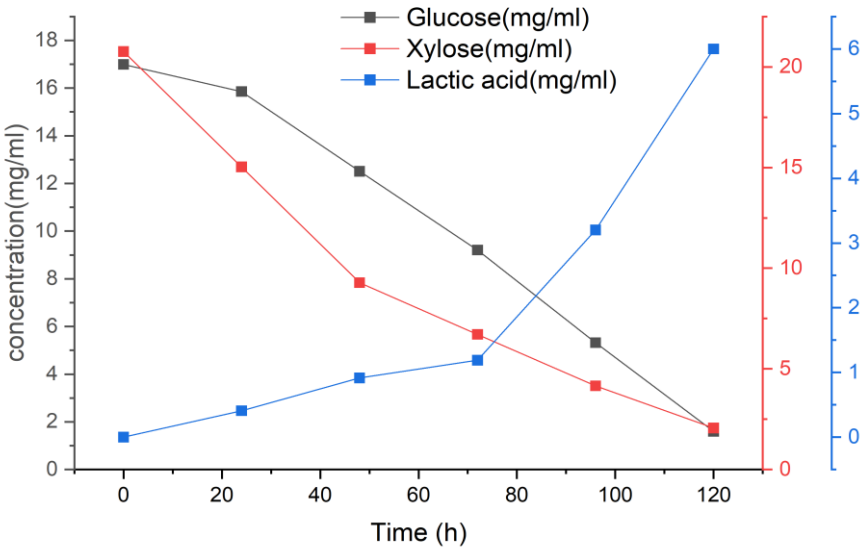

Figure 19: Lactic acid production by *Bacillus licheniformis* DGB in rice straw hydrolysate obtained by acid hydrolysis of separated cellulose, supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50◦C

4.7 Lactic acid production from rice straw by *Bacillus sonorenesis*

The thermophilic strain *Bacillus sonorensis* DGS15 exhibited impressive fermentative metabolic potential in converting rice straw hydrolysate sugars into LA under high temperature conditions. At the initiation of fermentation (0 hours), initially, the amount of

glucose and xylose in the medium was recorded as 25.42 ± 0.0001 mg/mL and 19.34 ± 0.0002 mg/mL, respectively, with no detectable traces of lactic acid. After 24 hours of incubation, these sugar levels declined to 18.51 ± 0.0002 mg/mL (glucose) and 15.02 ± 0.0001 mg/mL (xylose), while LA begin to accumulate, reaching 0.47 ± 0.0080 mg/mL. This trend of sugar utilization and acid production continued with time: by 48 hours, glucose and xylose decreased to 15.86 ± 0.0002 and 7.70 ± 0.0002 mg/mL, and lactic acid concentrations rose to 1.10 ± 0.0059 mg/mL. At 72 hour point, active metabolism was eveident as the lactic acid concentration reached 1.57 ± 0.0061 mg/mL, and sugar levels further declined (glucose: 10.37 ± 0.0002 mg/mL; xylose: 5.44 ± 0.0001 mg/mL), indicating active metabolism. A notable surge in synthesis of LA was observed between 72 and 96 hours, where LA peaked to 4.20 ± 0.0070 mg/mL, while glucose and xylose dropped to 7.67 ± 0.0001 mg/mL and 3.16 ± 0.0002 mg/mL, respectively. Finally, by 120 hours, Lactic acid production peaked at 7.47 ± 0.0076 mg/mL, with remaining glucose and xylose at 2.93 ± 0.0002 and 0.96 ± 0.0002 mg/ml. these detailed trends are clearly summarized in table 8 and illustrated in figure 20, showcasing the efficiency of these thermophlic bacterial strains in valorizing lignocellulosic biomass. Overall, *Bacillus sonorensis* DGS15 demonstrated exceptional efficiency in channeling fermentable sugars towards lactic acid production under thermotolerant conditions. The highest LA yield (0.183 g/g) and maximum productivity (0.0622 g/L/h) were obtained at end of fermentation (120 hours), while the lowest yield (0.0418 g/g) and productivity (0.0196 g/L/h) were observed at 24 hours. This trend indicates a gradual and efficient conversion of sugars.

Table 8: Lactic acid production by *Bacillus sonorenesis* (DGS15) in rice straw hydrolysate, supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50ºC

Time (h)	Glucose (mg/ml)	Xylose (mg/ml)	LA (mg/ml)
0	25.42±0.0001	19.34±0.0002	-
24	18.51±0.0002	15.02±0.0001	0.47±0.0080
48	15.86±0.0002	7.70±0.0002	1.10±0.0059
72	10.37±0.0002	5.44±0.0001	1.57±0.0061
96	7.67±0.0001	3.16±0.0002	4.20±0.0070
120	2.93±00002	0.96±0.0002	7.47±0.0076

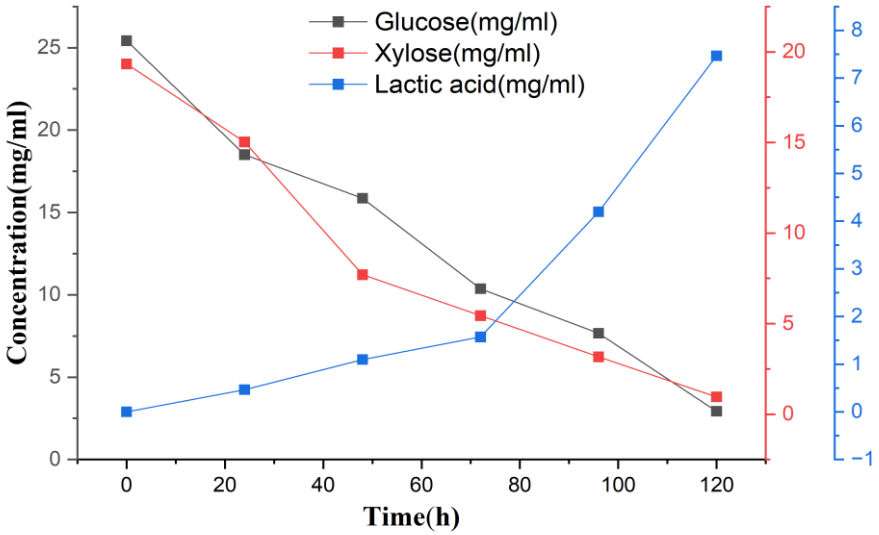

Figure 20: Lactic acid production by *Bacillus sonorenesis* (DGS15) in rice straw hydrolysate, supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50ºC.

4.8 Lactic acid production from wheat straw by (DGB) in Bushnell Haas Minimal Medium with yeast extract

Wheat straw (WS), a plentiful lignocellulosic agricultural residue, acts as a valuable feedstock owing to its high content of fermentable sugars like glucose (derived from cellulose) and xylose (sourced from hemicellulose). The fermentation experiment showed a gradual depletion of glucose and xylose over the 120-hour period, with a steady in LA concentration, indicating efficient sugar assimilation and bioconversion. Initially, the culture medium comprised 18.59 g/L (±0.0010) of glucose and 24.89 g/L (±0.0003) of xylose. By 24 hours, glucose dropped to 16.07 g/L (±0.0002), xylose to 18.73 g/L (±0.0002), and LA accumulated to 0.85 g/L (±0.0068). A significant increase was recorded at the 48 hours mark, where LA rose to 2.03 g/L (±0.0071) with glucose and xylose concentrations reduced to 14.68 g/L (±0.0002) and 12.62 g/L (±0.0003), respectively. By 72 hours, LA production further increased to 3.39 g/L (±0.0061), while glucose and xylose reduced to 11.49 g/L (±0.0002) and 9.60 g/L (±0.0003). At 96 hours, glucose and xylose decreased significantly to 6.17 g/L (±0.0003) and 6.52 g/L (±0.0002), respectively, while LA reached 4.03 g/L (±0.0068). Finally, at 120 hours, the residual sugar levels were minimal, with glucose at 0.17 g/L (±0.0002) and xylose at 3.89 g/L (±0.0001), while the highest LA concentration of 5.05

g/L (±0.0068) was attained. These trends clearly illustrated in Table 9 and Figure 21, underscore the efficient and sustained fermentative performance of *Bacillus licheniformis* DGB throughout the process. The fermentation performance of *Bacillus licheniformis* DGB on wheat straw hydrolysate exhibited the maximum lactic acid output (0.128 g/g) and productivity (0.0421 g/L/h) at 120 hours, corresponding with near-complete fermentable sugar consumption. In contrast, the minimum values were noted at 24 hours, with a yield of 0.0979 g/g and a productivity of 0.0354 g/L/h, indicating that the strain commences acid production promptly and sustains effective conversion for the whole process.

Table 9: Lactic acid production by *Bacillus licheniformis* DGB in wheat straw hydrolysate, supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50ºC

Time (h)	Glucose (mg/ml)	Xylose (mg/ml)	LA (mg/ml)
0	18.59±0.0010	24.89±0.0002	-
24	16.07±0.0002	18.73±0.0002	0.85±0.0067
48	14.68±0.0002	12.62±0.0002	2.03±0.0070
72	11.49±0.0002	9.60±0.0002	3.39±0.0061
96	6.17±0.0002	6.52±0.0002	4.03±0.00685
120	0.17±0.0002	3.89±0.0001	5.05±0.0067

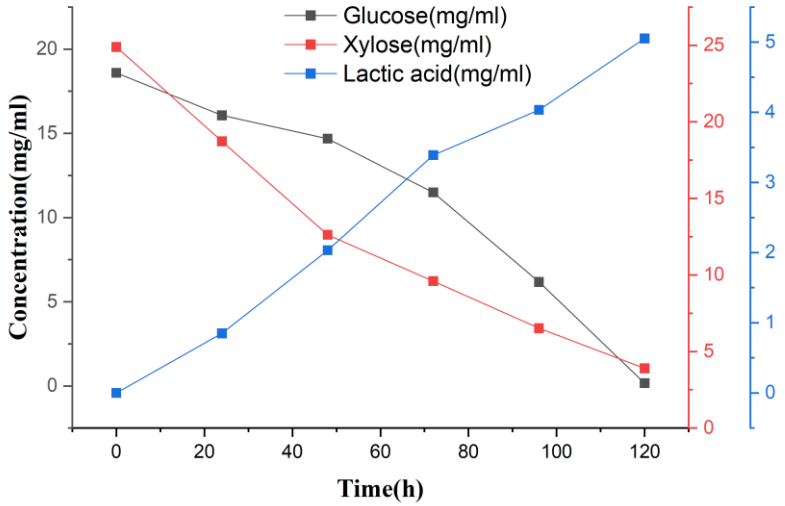

Figure 21: Lactic acid was produced by *Bacillus licheniformis* DGB when cultivated on wheat straw hydrolysate, enriched with Bushnell Haas medium, under controlled conditions of pH 6.5, 120 rpm shaking, and a temperature of 50°C.

4.9 Lactic acid generation by *Bacillus sonorensis* (DGS15) using wheat straw as a carbon source in Bushnell Haas Minimal Medium.

Table 10 highlights the initial concentrations of glucose and xylose when fermentation began were 28.05 g/L (±0.0019) and 23.44 g/L (±0.0002), respectively, with no detection of lactic acid. It was observed that at 24 hours, the glucose levels were dropped to 19.69 g/L (±0.0002) and xylose to 17.22 g/L (±0.0002), while LA production began at 0.57 g/L (±0.0008). The trend of rising LA levels remained consistent, growing to 2.30 g/L (±0.0068) by 48 hours, as glucose and xylose were consumed down to 13.46 g/L and 11.05 g/L. By 72 hours, LA inclined further to 4.99 g/L (±0.0068), with sugar concentrations falling to 8.62 g/L for glucose and 8.15 g/L for xylose. At 96 hours, a substantial yield of 5.34 g/L (±0.0054) LA was recorded, and the residual sugars dropped to 5.23 g/L (glucose) and 5.18 g/L (xylose Ultimately, after 120 hours, DGS15 produced the most lactic acid that is 9.03 g/L (±0.0060), with minimal leftover sugars—glucose at 0.70 g/L (±0.0002) and xylose at 2.56 g/L (±0.0003). Bacillus sonorensis DGS15 fermented wheat straw hydrolysate at 50°C, giving rise to highest yield of lactic acid that is 0.1872 g/g and a productivity of 0.0753 g/L/h at 120 hours, demonstrating a highly effective sugar-to-acid conversion process. The minimum values were observed at 24 hours**,** with a yield of 0.0391 g/g and productivity of 0.0238 g/L/h, depicting the initial metabolic adaptation phase. The trend observed was plotted in a concentration vs time graph as illustrated in figure 22. The recorded metabolic performance indicates that the organism can take in and convert sugar well even when it's heated up, which shows that it could be used to make lignocellulosic products. Adding important cofactors and precursors for active biosynthesis and redox equilibrium greatly increased the productivity of yeast extract. DGS15's ability to keep making lactic acid in a minimum medium that mimics cost-sensitive commercial biorefinery settings makes it a good biocatalyst for creating greener, second-generation bioprocesses that use agricultural waste.

Table 10: The bioconversion of wheat straw hydrolysate to lactic acid was performed by *Bacillus sonorensis* (DGS15) under optimized conditions in Bushnell Haas medium (pH 6.5, 50°C, and 120 rpm).

Time (h)	Glucose (mg/ml)	Xylose (mg/ml)	LA (mg/ml)
0	28.05±0.0019	23.44±0.0002	-
24	19.69±0.0002	17.22±0.0002	0.57±0.0008
48	13.46±0.0002	11.05±0.0002	2.30±0.0068
72	8.62±0.0002	8.15±0.0002	4.99±0.0068
96	5.23±0.0001	5.18±0.0002	5.34±0.0054
120	0.70±0.0002	2.56±0.0003	9.03±0.0060

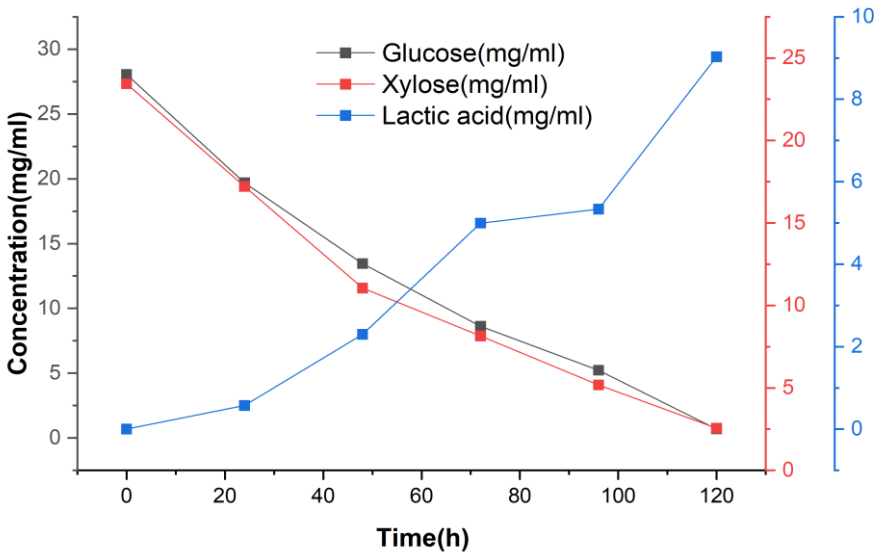

Figure 22: The bioconversion of wheat straw hydrolysate to lactic acid was performed by *Bacillus sonorensis* (DGS15) under optimized conditions in Bushnell Haas medium (pH 6.5, 50°C, and 120 rpm).

CONCLUSION

1. Acid-pretreated rice and wheat straw hydrolysates were fermented using *Lactobacillus reuteri*, resulting in lactic acid production of 1.42 mg/ml and 1.468 mg/ml, respectively. The corresponding yields were 0.057 g/g for rice straw and 0.0606 g/g for wheat straw, with productivities of 0.0118 g/L/h and 0.0122 g/L/h, indicating efficient conversion of xylose and glucose into lactic acid.

2. The highest efficiency in releasing glucose and xylose from rice and wheat straw hydrolysates was demonstrated by *Bacillus sonorensis* DGS15, suggesting that it has a great potential for converting lignocellulosic biomass.

3. Using rice straw hydrolysate, *Bacillus licheniformis (DGB)* produced 6.00 mg/ml lactic acid with a yield of 0.176 g/g and productivity of 0.05 g/L/h, while *Bacillus sonorensis* (DGS15) produced 7.47 mg/ml with a yield of 0.183 g/g and productivity of 0.0622 g/L/h. In *wheat straw*, *DGB* produced a maximum of 5.05 mg/ml lactic acid (yield 0.128 g/g, productivity 0.0421 g/L/h), whereas DGS15 showed the highest value of 9.03 mg/ml (yield 0.182 g/g, productivity 0.0753 g/L/h). These results highlight the superior fermentation performance of *Bacillus sonorensis* in both the substrates.

4. After applying the three-stage pretreatment process, cellulose recovery was achieved at 57.36% in rice straw and 67% in wheat straw, it shows that non-cellulosic fractions were largely eliminated, making it easier for enzymes to saccharification of cellulose.

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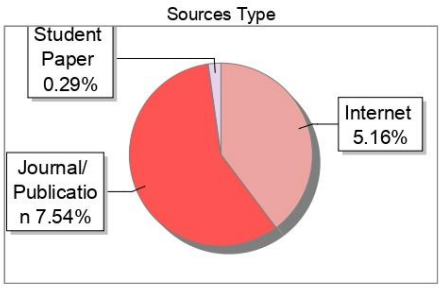

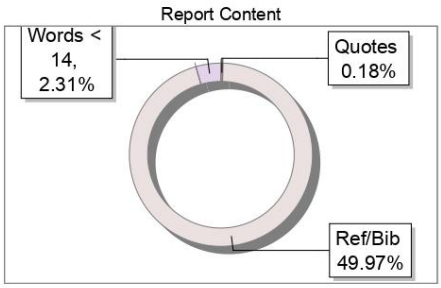

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