Production of lactic acid from lignocellulosic hydrolysates by thermotolerant bacteria

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CERTIFICATE

Certified that the thesis "Production of lactic acid from lignocellulosic hydrolysates by thermotolerant bacteria" which is submitted by Ms. Simarpreet Kaur Chawla, Registration No- 901500001, in fulfillment of the requirement for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering and Technology, Deemed to be University, Patiala, Punjab, India, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. 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.

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DECLARATION

I hereby declare that the work which is being presented in this thesis "Production of lactic acid from lignocellulosic hydrolysates by thermotolerant bacteria" submitted by me for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering and Technology, Deemed to be University, Patiala, Punjab, India, is a true and original record of my own independent and original research work carried out under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Deemed to be University, Patiala, Punjab, India. 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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Simarpreet Kaur Chawla
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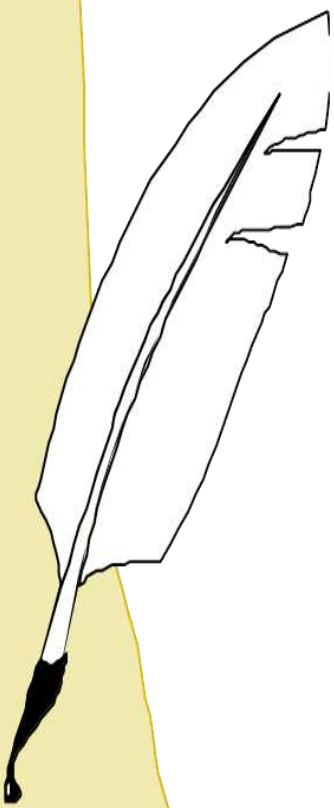
Dedicated to

my Parents

&

my dear

Husband



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ABSTRACT

Lactic acid (LA), is an industrially important organic acid with extensive applications in pharmaceuticals and food industries as well as biodegradable plastics. It has garnered significant attention for sustainable production from renewable lignocellulosic biomass resources. This study was aimed at lactic acid production by isolating thermotolerant and inhibitor-tolerant bacterial strains, optimizing lignocellulosic biomass utilization and employing co-cultivation strategies for enhancing lactic acid yields.

Among 45 bacterial isolates obtained from compost, soil, and fermented food two strains B*acillus licheniformis* DGB and *Bacillus sonorensis* DGS15 were identified as high lactic acid producers which were thermotolerant and inhibitor-tolerant. They demonstrated robust growth in Bushnell Haas medium and efficiently utilized glucose and xylose at elevated temperatures (45°C–50°C), while tolerating inhibitory compounds (furfural and hydroxymethyl furfural) commonly derived from lignocellulosic biomass pretreatment. Initial fermentation trials yielded 13.2 g/L and 12.8 g/L of lactic acid for DGB and DGS15, respectively. Morphological, biochemical and molecular analyses confirmed their identity, and their thermotolerance and inhibitor resistance ensured suitability for industrial applications. Furthermore, both strains exhibited efficient carbon catabolite repression (CCR) bypass features, enabling simultaneous glucose and xylose metabolism.

Rice straw and wheat straw were selected as lignocellulosic feedstocks due to their abundance and high cellulose content. Pretreatment processes involving acid hydrolysis and enzymatic saccharification were optimized using Response Surface Methodology (RSM) to maximize sugar release. Pretreated rice straw yielded 50.8 g/L of total reducing sugars (TRS) from 100 g of biomass, while wheat straw yielded 48.6 g/L. Structural analysis using FTIR confirmed effective delignification, with the disappearance of lignin peaks at 1670 cm^{-1}, and SEM analysis revealed significant disruption of biomass structure. The crystallinity index (*CrI*) of pretreated rice straw increased by 15.5% compared to untreated material, highlighting enhanced accessibility for enzymatic digestion. In fermentation trials, DGB and DGS15 achieved lactic acid yields of 46.5 g/L and 44.7 g/L from rice straw and wheat straw hydrolysates, with yield efficiencies of 96.8% and 94.1%, respectively. Among the two biomass

sources, rice straw emerged as the most suitable substrate due to its higher sugar release and conversion efficiency.

To enhance productivity, a co-cultivation strategy was employed, leveraging the complementary metabolic pathways of DGB and DGS15. The co-culture system effectively utilized glucose and xylose, achieving 70% sugar consumption within 15 hours and complete substrate utilization within 48 hours. Optimized fermentation conditions (10% (w/v) mixed hydrolysate substrate (rice straw and wheat straw), 1:1 inoculum ratio, 50°C, pH 6.0) resulted in a maximum concentration of 64.3 g/L lactic acid, with a yield of 0.98 g/g and productivity of 1.036 g/L/h. Compared to monoculture fermentation, co-cultivation increased yields by 28% and reduced fermentation time by 12%. The Separate Hydrolysis and Co-Fermentation (SHCF) process addressed key challenges in lignocellulosic biorefining, demonstrating scalability and process efficiency. Co-cultivation proved to be the most suitable approach for lactic acid production, offering higher yields and better process efficiency compared to monoculture systems.

Present study successfully demonstrated a comprehensive sustainable approach to lactic acid production by integrating thermotolerant and inhibitor-tolerant microbial strains, optimized lignocellulosic biomass utilization, and co-cultivation strategies. Among the strains, *Bacillus licheniformis* DGB was identified as the best performer due to its higher lactic acid yield and adaptability. Rice straw was identified as the most suitable biomass source for lactic acid production and co-cultivation emerged as the most efficient production strategy, offering enhanced yields, reduced fermentation times and robust process stability. Future studies could focus on refining biomass pretreatment, exploring genetic engineering of strains and incorporating integrated biorefinery models to enhance sustainability and economic feasibility.

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LIST OF ABBREVATIONS

dNTP	2’-deoxynucleoside-5’-triphosphate
C_3H6O_3	2-hydroxypropanoic acid
DNS	3, 5-dinitrosalicylic acid
HMF	5-hydroxymethyl furfural
ARP	Ammonia recycling percolation
APS	Ammonium persulfate
ANOVA	Analysis of variance
etc	And other things
et al	And others
viz.	As follows
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BBD	Box behnken design
$Ca[OH]_2$	Calcium hydroxide
CO_2	Carbon dioxide
CO	Carbon monoxide
CMC	Carboxy methyl cellulose
CBU	Cellobiose unit
CFU	Colony forming units
Conc.	Concentrated
CAPD	Continuous Ambulatory Peritoneal Dialysis
CDD	Control drug delivery
CrI	Crystallinity index
DNA	Deoxyribonucleic acid
K2HPO4	Di potassium hydrogen phosphate
Dil.	Dilute
EMP	Embden-Meyerhof-Parnas
Eq.	Equation
EDTA	Ethylenediamine tetraacetic acid
FeSO4.7H2O	Ferrous sulphate heptahydrate
Fig.	Figure
FPU	Filter paper unit
FDA	Food Drug and Administration
FTIR	Fourier transform infrared spectroscopy
GRAS	Generally Regarded As Safe
g	Gram
g/g	Gram per gram
g/L	Gram per liter
g/L/h	Gram per litre per hour

GHGs	Green house gases
Hz	Hertz
h	Hour
HCl	Hydrochloric acid
H2O2	Hydrogen peroxide
J/mole/ºC	Joule per mole per degree Celsius
kb	Kilo base
KV/cm	Kilo volt per centimeter
kGy	Kilo-gray
KHz	Kilohertz
KJ/mole	Kilojoule per mole
LA	Lactic acid
LAB	Lactic acid bacteria
LDH	lactic acid dehydrogenase
LCB	Lignocellulosic biomass
L	Litre
MgSO4.5H2O	Magnesium sulphate pentahydrate
MHz	Mega hertz
Mj/kg	Mega joules per kilogram
MPa	Mega Pascal
MR	Methyl red
MT	Metric ton
MT	Metric ton
MTCC	Microbial type culture collection
µg	Microgram
µL	Microlitre
mA	Milliampere
mg	Milligram
mg/ml	Milligram per millilitre
ml	Millilitre
mm	Millimetre
mM	Millimolar
MMT	Million metric ton
min	Minute
M	Molar
mol/g	Mole per gram
ng	Nanogram
nm	Nanometre
NCBI	National centre for biotechnology information
NCIM	National collection of industrial microorganisms
HNO_3	Nitric acid
NOx	Nitrogen Oxides

N	Normal
NA	Nutrient Agar
OD	Optical Density
O2	Oxygen
ppm	Parts per million
PP	pentose phosphate
PK	phosphoketolase
H_3PO_4	Phosphoric acid
PLA	Poly lactic acid
PAGE	Polyacrylamide gel electrophoresis
PCA	polycaprolactone
PGA	polyglycolide
PCR	Polymerase chain reaction
KOH	Potassium hydroxide
Psi	Pounds per square inch
RSM	Response surface methodology
rpm	Revolution per minute
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscopy
s	Second
SHCF	Separate hydrolysis and Co-fermentation
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
SDS	Sodium dodecyl sulfate
NaOH	Sodium hydroxide
H_2SO_4	Sulfuric acid
i.e.	that is
TGA	Thermo gravimetric analysis
TFA	Triflouroacetic acid
US	United States
VP	Voges- proskauer
W	Watt
w/w	Weight by weight
w/v	Weight per volume
XRD	X-ray diffraction
YE	Yeast extract

LIST OF SYMBOLS

%	Percentage
µ	Micron
C	Carbon
Da	Dalton
H	Hydrogen
h	Hours
kDa	Kilo Dalton
L	Litre
M	Molar
Mg	Magnesium
N	Nitrogen
S	Sulfur
U	Unit
V	Volt
v/v	Volume by volume
w/v	Weight by volume
α	Alpha
β	Beta
%	Percent
°C	Degree Celsius
®	Registered
□	Angle
TM	Trademark

CHAPTER I: INTRODUCTION

Biomass is by far the largest source of energy, accounting for 1,150 million tons of oil equivalent and 79% of total energy supply. Researchers have been committed to exploring the synthesis of value-added chemicals and bio fuels derived from lignocellulosic biomass due to the depletion of fossil fuels and environmental contamination (Benti *et al*., 2021).

The focus on developing sustainable and renewable resources has led to a significant increase in the need for improving fermentative products, such as food and chemical additives, acidifying agents etc. (John *et al*., 2007). Numerous intermediate building components have been synthesized from biomass using biotechnological methods (Gao *et al*., 2011). According to predictions, the usage of sustainable resources might account for over half of global resource consumption by 2050 (Chen *et al*., 2017). The most promising sources of alternative energy at present are those that include the production of valued molecules such as ethanol, xylitol, saccharides, phenols, aldehydes, acids, and biofuels like biodiesel and bio gasoline lignocellulosic biomass (Baruah *et al*., 2018; Chen *et al*., 2017; Singh *et al*., 2018). The lactic acid (LA) is one of the most demanded value-added products. Additionally, the estimated yearly total consumption was between 130000 and 150000 metric tons (Sikder *et al*., 2012). It is anticipated that lactate will be used more widely in the coming years (Ghaffar *et al*., 2014). Recently, LA has become more and more popular as a foundation for the synthesis of polylactate (Kunasundari *et al*., 2017), providing environmentally friendly and sustainable alternatives to petroleum-based polymers (Abdel-Rahman & Sonomoto, 2016). Renewable poly lactones such as polyglycolide (PGA), polylactide (PLA), and polycaprolactone (PCL) along with their co-polymers are being used in medical devices (Lasprilla *et al*., 2012). Commercial LA production can be achieved by two methods: chemical or microbiological. Compared to the usual synthetic technique, the microbiological pathway for LA synthesis has several advantages, such as reduced consumption of energy and preliminary expenses, reduced parameters for the process, and products of excellent quality (John *et al*., 2007) Pure LA can be produced by choosing the right microbial species, but a chemical procedure will always result in a racemic mixture, which lowers the cost of distilling and separating the finished product (Farooq *et al*., 2012; Karnaouri *et al*., 2020).

LA is a common chemical used in both food and non-food industries, such as cosmetics, pharmaceuticals, and chemical manufacturing. However, it is expected that its use in bio-based plastic would outpace its other applications (Wang *et al*., 2013). Polylactic acid (PLA) is a sustainable polymer that is both renewable and biodegradable and is growing rapidly as an alternative to petroleum-based plastics, owing to growing concerns about the limited supply of fossil fuels and the environmental impact of greenhouse gases discharged into the atmosphere (Li and Wilkins, 2020). Chemical synthesis can be used to produce racemic mixture of LA, while microbial fermentation can yield an optically pure LA using the desirable organisms. Lignocellulosic biomass being composed of cellulose (C6-sugars), hemicellulose (mainly C5-sugars) and lignin has been used for the large-scale production of several bio-based products (Hendriks & Zeeman, 2009). However, due to complex and difficult to degrade nature of cell wall, pretreatment of biomass becomes a critical step in its conversion into reducing sugars, for which acid or alkali pretreatment is employed to separate cellulose from amorphous lignin and hemicellulose (Jiang *et al*., 2016). Plant cell walls, particularly their hemicellulose component, are hydrolyzed by acid pretreatment. In dilute and acidic states, H_2SO_4, HNO_3, and HCl are commonly employed for acid pretreatment (Ou *et al*., 2016). In acidic conditions, solubilized hemicellulose can be transformed to xylose, α-monomer, and then over degraded in a severely acidic environment. Though glucose and xylose can be biologically transformed to a variety of biochemical building blocks, they can also be over degraded to by-products such as furfural and hydroxymethylfurfural (HMF) (Tong *et al*., 2010). These inhibitors pose a serious threat to the fermentative utilization of lignocellulosic sugars and efficient of biochemicals. Therefore, adequate acid concentration, reaction temperature, and other key variables must be determined experimentally in order to achieve selective hydrolysis (Tong *et al*., 2010).

Nowadays, fermentation is the primary method (95%) for producing LA from plant based raw materials. LA bacteria (LAB) are the main type of microorganisms that may produce LA (Waśko *et al*., 2012). They are the main producers of LA that is utilised in industry. LA bacteria (LAB) are largely anaerobic, non-spore-forming, catalase-negative, and evolutionarily diversified group of non-motile microorganisms including bacteria, fungi, yeasts, and algae, are capable of producing LA by utilising various lignocellulosic substrates (Kim *et al*., 2012). LAB either homofermentative and

heterofermentative strains operate through different routes/ pathways such as Embden-Meyerhof-Parnas (EMP) route, pentose phosphate (PP) pathway, phosphoketolase (PK) pathway for the production of LA (Cubas-Cano *et al*., 2018; Grewal *et al*., 2020). Commercially, homofermentative strains producing pure LA are preferred due to their optimum productivity and ease of further processing after fermentation. Other bacteria that may produce LA from lignocellulosic biomass includes *Bacillus subtilis, Bacillus coagulans, Bacillus sonorensis, Bacillus stearothermophilus, Bacillus licheniformis, Corynebacterium glutamicum, and Escherichia coli* (Juturu & Wu, 2016; Kumar *et al*., 2014).

Bacillus sp. offer several advantages over LAB as they can thrive and ferment in nutrient limiting conditions utilizing inexpensive nitrogen components at high temperatures, which can lower the cooling costs associated with medium sterilisation (Juturu & Wu, 2016). They have potential industrial uses because, at higher temperatures, they increase starch solubility, reduce viscosity, inhibit microbiological contamination, and shorten reaction time (Kumar *et al*., 2014). Also, they are resistant to inhibitors produced during pretreatment which reduces the cost and time used for detoxification of hydrolysate. The characteristic feature of the *Bacillus* species is that they produce optically pure L-LA for use in secondary metabolite production, they are generally recognized as safe (GRAS) (Juturu & Wu, 2016; Shetty *et al*., 2017). Lactic acid bacteria (LAB) also produce proteolytic enzymes upon the fermentation of sugars to LA. These proteolytic enzymes provide nitrogen compounds to the cells, are crucial for the growth of LAB and sustaining their growth in the broth (Tong *et al*., 2010). Due to their capacity to effectively ferment hydrolysate of various biomass at comparatively higher temperatures (50- 65 °C), thermotolerant LA producing bacteria have drawn the attention of numerous researchers. This allows for efficient carbon dioxide production and mass LA production without the need for cooling (Zhang *et al*., 2014).

Production of LA using microorganisms involves consumption of both glucose and xylose in sequence which leads to less productivity than those that consume the sugars concurrently (Zhang & Vadlani, 2015). An organism's rate of sugar consumption may fall into a fairly narrow range if it were able to take both sugars at the same time. Fundamentally, the rate of consumption of two substrates by a single microbe might not be able to adapt to changing sugar concentrations (Sahoo & Jayaraman, 2019). Therefore, fermenting the hydrolysate by two distinct organisms for the simultaneous

consumption of both types of sugar is a desirable feature of a method to handle sugar mixtures. Additionally, through functional modularity, microbial consortium synergies can lead to more productive substrate utilization and higher product production (Wang *et al*., 2016).

The aim of the present study was to isolate inhibitor tolerant; pentose sugar utilising and thermotolerant strain for efficient production of LA with complete utilization of all sugars such as pentoses and hexoses derived from lignocellulosic biomass (rice and wheat straw) after hydrolysis with following objectives:

1. Isolation, screening and characterization of thermotolerant LA producing bacteria
2. Production of LA from different lignocellulosic biomass
3. Optimization of LA production from lignocellulosic hydrolysates

CHAPTER II: REVIEW OF LITERATURE

2.1 LA

LA, a naturally existing acid, also referred to as 2-hydroxypropanoic acid ($CH_3CHOHCOOH$; C_3H6O_3). It is an essential biochemical intermediate in most living beings, including bacterial cells and animals. LA may also be chemically synthesized (Datta & Henry, 2006). Due to its substantial synthesis through a range of organic substrates, it is a significant bio-product that has garnered a lot of attention (Karnaouri *et al*., 2020)

LA was first discovered in sour milk by Swedish chemist Scheele in 1780. Since then, it has been utilised for the purpose of fermentation and food preservation (Alsaheb *et al*., 2015; Martinez *et al*., 2013), and the earthy-colored substance was given the term "*Mjo¨lksyra*." (Ghaffar *et al*., 2014, Rodrigues *et al*., 2016). The Latin word "*lact*," which means "milk," is whence the substance LA derives its name. After nine years, in 1789, Lavoisier coined the word "*acide lactique*" to describe that milk component (Ajala *et al*., 2020). This turned into the most likely origin of the current phrases with LA. In 1839, Fremy fermented a variety of carbohydrates (lactose, starch, sucrose, etc.) to produce LA for the first time (Vijayakumar *et al*., 2008). Louis Pasteur found in 1857 that LA was not a component of milk, but rather a metabolite produced by a specific microbial strain during fermentation (Wee *et al*., 2006). In 1881, Littleton, Massachusetts resident CE Avery became the first to commercialise LA (Jem & Tan, 2020; John *et al*., 2007; Krishna *et al*., 2018). The renowned chemical business, Boehringer Ingelheim, manufactured organic LA for the first time in 1895 (Alsaheb *et al*., 2015). LA was assessed in 2010 U.S. Department of Energy assessed as substances that could be used as building blocks in future (Martinez *et al*., 2013).

2.2 Properties of LA

It is the least complex and prevalent hydroxycarboxylic acid (Martinez *et al*., 2013; Rodrigues *et al*., 2016), with three carbon atoms: one at the end that is necessary for the carboxylic acid portion, one in the middle that is a part of a hydrocarbon cluster, and one in the centre that is present and contains the alcohol category (Narayanan *et al*., 2004). It exists in two distinct isomeric structures due to chirality: the levorotatory structure known as D (-) or R LA and the dextrorotatory structure named L (+) or S LA

(Cubas-Cano *et al*., 2018). The marks (+) and (-) represent the directional way in which a chemical rotates planar polarised light (Jem & Tan, 2020) (Fig.2.1). However, the racemic combination of both is less beneficial than pure versions like L-LA and D-LA (Abdel-Rahman & Sonomoto, 2016).

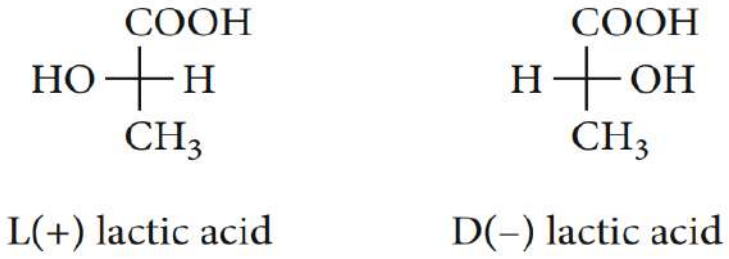

Figure 2.1: Isomers of LA (Pohanka, 2020)

Due to the faster metabolic processes of LA in the human body, L-LA is more frequently found in living forms than D-LA due to the presence of isomer-specific lactate dehydrogenase enzyme in mammals (Martinez *et al*., 2013; Jem & Tan, 2020). D-LA production result in metabolic issues such as D-lactate poisoning, lactic acidosis, and decalcification (Martinez *et al*., 2013; Pohanka, 2020).

Table 2.1: Physical and thermodynamic properties of LA (Abiola *et al*., 2022; Arshad *et al*., 2022; Song *et al*., 2022)

Properties	**Values**
Compound name	LA
IUPAC name	2-Hydroxypropanoic acid
Chemical formula	$C_3H_6O_3$
Molecular weight	90.08 $molg^{-1}$
Melting point	16.8ºC
Boiling point	82ºC at 0.5 mm Hg, 122ºC at 14 mm Hg
Dissociation constant, pKa at 25ºC	1.37x 10^{-4}
Heat of combustion, ΔH_c	1361 KJ/mole
Specific Heat, Cp at 20ºC	190 J/mole/ºC
Heat of fusion	11.33 kJ/mol
Density at 20ºC	1.249 g/l

Pure LA is either a solid white crystalline substance or a high-quality syrup that is almost colourless and odourless, but it can catch fire when coupled with steam that has been heated to a high temperature. While LA is readily soluble in alcohol and water, it is practically insoluble in chloroform (Yadav *et al*., 2011). It exists as a weak acid (lactate) because of its pKA value of 3.86 (Nwamba *et al*., 2021) (Table 2.1).

2.3 Applications of LA

Food, cosmetic, medicinal, and chemical industries have huge demand for LA and its derivatives (Abdel-Rahman & Sonomoto, 2016) (Fig. 2.2).

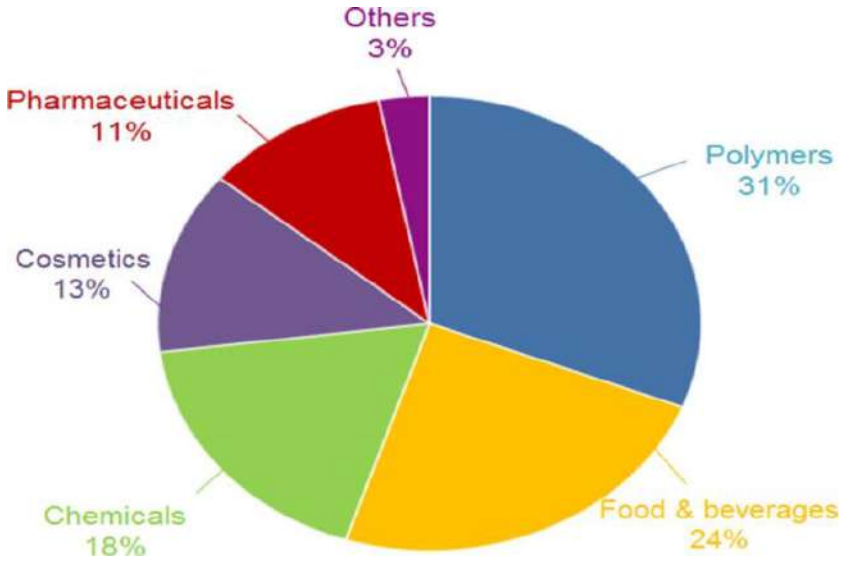

Figure 2.2: LA demand across multiple sectors (Rawoof *et al*., 2021)

2.3.1 Food sector

The food and drug administration (FDA) have classified it as GRAS (generally recognized as safe) in the USA (Alsaheb *et al*., 2015; Narayanan *et al*., 2004). Many different types of food products, such as drinks, ice creams, bakery goods, dairy products, salad dressings, sauces, and prepared meals, include LA (Ameen & Caruso, 2017). In a variety of supplemented products, it serves as an acidifying, flavouring agent, preservative, and pH balancing ingredient to lengthen shelf life and prevent microbiological deterioration (Datta & Henry, 2006; Wee *et al*., 2006). The sodium or potassium salts of LA are used to increase the shelf life of beef, poultry, and seafood (Krishna *et al*., 2018). The fatty esters of LA can be used for the three major process parameters- stabilisation, sequestration, and emulsification (Ameen & Caruso, 2017).

The main producers of LA are LAB, which are a diverse group of probiotic-friendly microorganisms (Alsaheb *et al*., 2015). More than 70 percent of the LA delivered to the food sector because of its importance in the production of cheese and yoghurt (Martinez *et al*., 2013).

2.3.2 Pharmaceutical sector

The LA and its derivatives serve as the main components for the production of skin compounds, medications for dermatology and osteoporosis, pH regulation, metal sequestration, and parenteral preparations that replace the body's water and electrolytes all need LA, and particularly its salts, as a precursor (Alsaheb *et al*., 2015). For example, dialysis solutions (e.g., sodium citrate) and Ringer's Lactate solution (RL) for (CAPD) utilising synthetic renal apparatus (Alsaheb *et al*., 2015). LA is present in mineral formulations, tablets, prosthesis, operative stitches, controlled medicine administration systems, and orthopedic implants (Wee *et al*., 2006). For this purpose, lactide-co-glycoside is the ideal material (Alsaheb *et al*., 2015). Mineral LA formulations have been utilised for a variety of purposes, including magnesium salt (hypertension, muscle weakness), manganese lactate (dysglycemia), zinc lactate (dermatosis), ferrous lactate (anemia), and zinc lactate (hypocalcemia therapy and anti-dental caries agent) (Jodłowski & Strzelec, 2021; Zhang *et al*., 2022).

2.3.3 Chemical sector

In a range of chemical-based products and processes, LA reactions—which produce salts as a byproduct—are employed as solvents, humectants, chiral precursors, neutralizers, cleaners, pH regulators, delayed acid discharge agents, and antibacterial agents. Because of its descaling qualities, it is used in many decalcification products, including toilets, coffee makers and lavatory cleaners (Wee *et al*., 2006). Because lactate has the hydroxyl and the carboxyl group, it can be converted into intermediate chemicals like pyruvic acid (dehydrogenation), 1,2-propanediol (hydrogenation), ethanal (decarboxylation), 2,3-pentanedione (condensation), and dilactide (self-esterification) (Martinez *et al*., 2013; Dey *et al*., 2019).

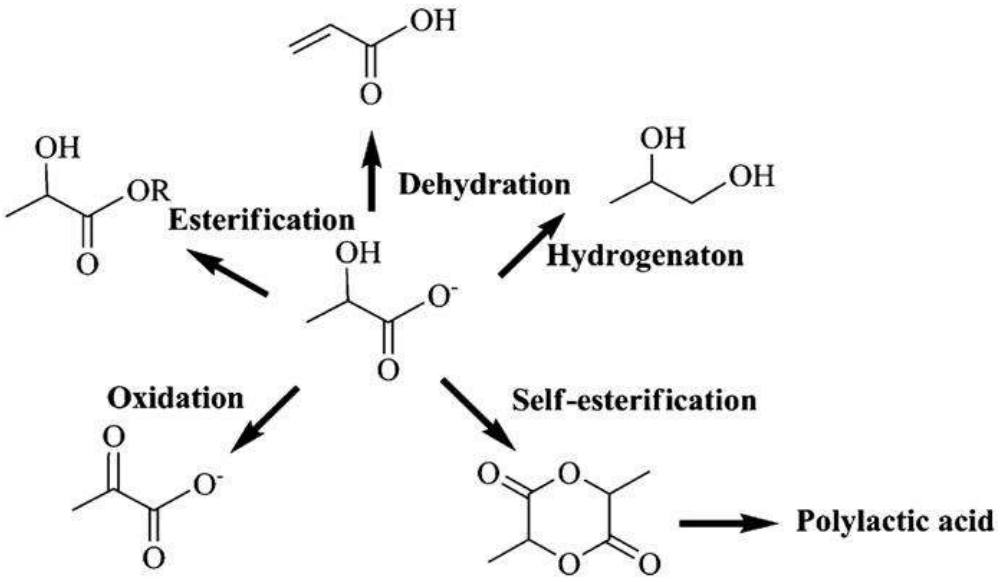

Figure 2.3: Lactate-derived chemicals (Hilares *et al.*, 2018)

Pure L and D optical isomers are used to create highly organised poly (L-(+)-LA) (PLLA) or poly (D-(-)-LA) (PDLA) (Cubas-Cano *et al*., 2018). Because of its mechanical robustness, PLA is a desirable material because it is comparable to typical plastic polymers like styrofoam, polypropylene (PP), and polythene (Kunasundari *et al*., 2017) (Fig. 2.3). It is essential for the production of a wide range of laminated materials, coverings, PLA films, containers (bottles and cups), disposables, natural absorbing stents, screws, and implants, as well as surgical stitches (Jamshidian *et al*., 2010; Lasprilla *et al*., 2012). Conversely, D-LA-produced polymers are generally amorphous and are less marketable (Pessione *et al*., 2014). Relatively low-quality LA is utilised in several textile polishing processes, such as acidic dying of wool, to deal with cheaper mineral acids (Datta & Henry, 2006).

2.3.4 Skin care and cosmetics sector

Lactate is used as a pH control, skin brightening, moisturising, anti-pimple, anti-tartar, cleanser, and skin restoration substance. As a moisturizer, LA does have the capacity to retain water, and its ability to block the synthesis of tyrosinase is what causes the skin-lightening effect (Wee *et al*., 2006). In addition to preventing wrinkles and blemishes on the face and body, LA acts as an anti-pimple agent by promoting the synthesis of collagen (Krishna *et al*., 2018). Because of their deliquescence and emulsification properties, LA esters are widely utilised (Martinez *et al*., 2013). Since

LAs are natural substances, they are typically preferred over polyalcohol as moisturising ingredients in cosmetics (Martinez *et al*., 2013; Datta & Henry, 2006). The removal of skin discolorations can be achieved by "straightforwardly repressing melanin deposition," which involves the use of LA in removal of pigment from skin and re-genomic organisation of pigment (typically melanin) (Alsaheb *et al*., 2015).

2.4 LA synthesis

Currently, 90% of the LA produced worldwide comes from microbial fermentation; the remaining 10% is produced artificially and is supplied by companies such as NatureWorks LLC, Cargill Dow's enterprise (USA), Galactic (Belgium), Purac (the Netherlands), and a number of Chinese enterprises (John *et al*., 2009). There have been several attempts to produce LA with higher cell density, efficiency, and final percentage (Wee *et al*., 2006) (Fig. 2.4).

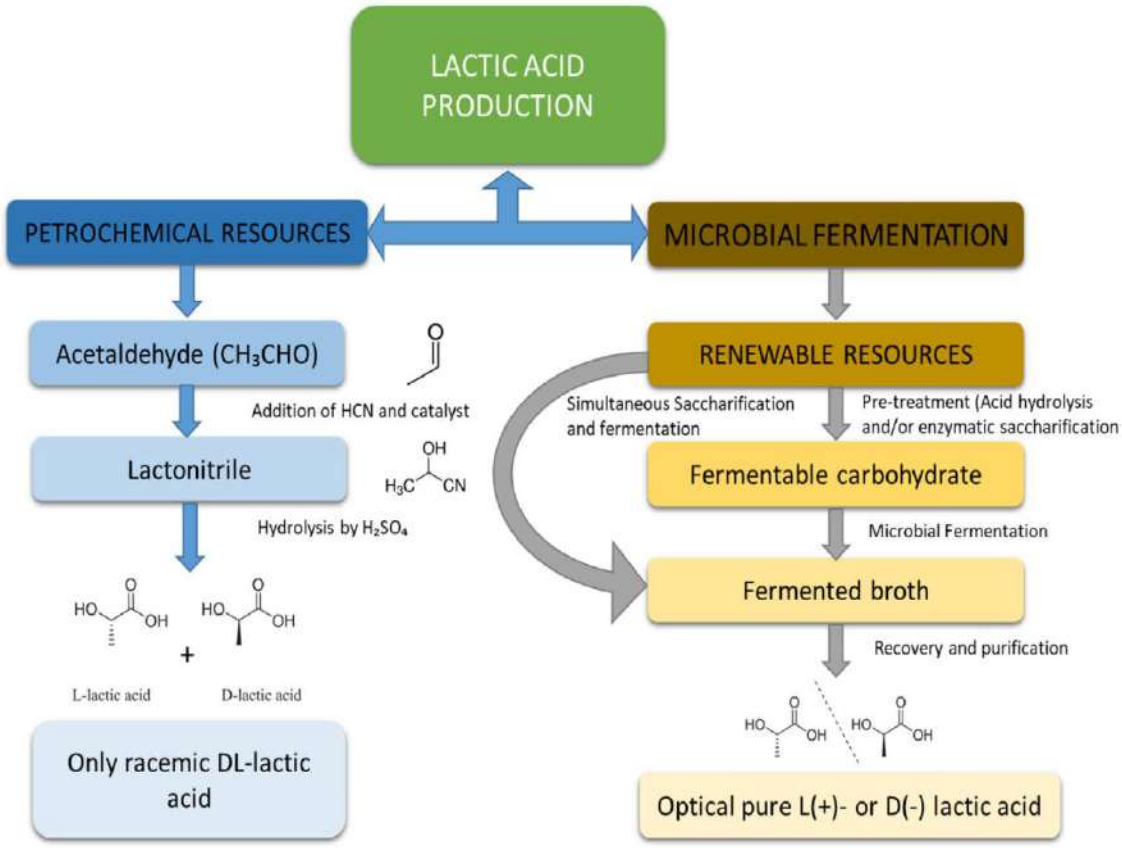

Figure 2.4: Methods of production of LA – (a) Chemical Synthesis (b) Microbial Fermentation (Wee *et al*., 2006)

2.4.1 Chemical synthesis

Chemical synthesis or microbial degradation of polysaccharides and agro-industrial feedstocks are the two methods used to create LA (Pessione *et al*., 2014). Starting mass transfer, nitrogen concentration, and the process characteristics (pH and temperature) are the primary factors influencing the efficiency of fermentation (Martinez *et al*., 2013). A significant drawback of chemical synthesis is the equal production of D-LA and L-LA; in contrast, carbohydrate fermentation technology produces a desired stereoisomer (almost 99% of L- LA and 1% of D-LA) (Jem & Tan, 2020).

Among the other chemical processes for producing LA are base-catalyzed sugar degradation, propane-1,2-diol combustion, and chloropropanoic hydrolysis (Martinez *et al*., 2013; Lasprilla *et al*., 2012). Despite the fact that there are several chemical synthesis routes for producing lactate, neither of them appears to be technically or financially feasible (Gao *et al*., 2011; John *et al*., 2009).

[1] Addition of Hydrogen cyanide and catalyst

CH_3CHO + HCN → $CH_3CHOHCN$

Acetaldehyde Hydrogen cyanide catalyst Lactonitrile

[2] Hydrolysis by H_2SO_4

$CH_3CHOHCN + 2H2O + ½\ H_2SO_4 \rightarrow CH_3CHOHCOOH + ½\ (NH_4)_2SO_4$

Lactonitrile Sulphuric acid LA Ammonium salt

[3] Esterification with methanol

$CH_3CHOHCOOH + CH_3OH \rightarrow CH_3CHOHCOOCH_3 + H_2O$

LA Methanol Methyl lactate

[4] Hydrolysis by water

$CH_3CHOHCOOCH_3 + H_2O \rightarrow CH_3CHOHCOOH + CH_3OH$

Methyl lactate LA Methanol

2.4.2 Fermentation by microbes

Essentially, fermentation is the process by which a microbial population uses sugar as a feedstock to naturally break down other molecules like lactate, citrate, and alcohol (Rodrigues *et al*., 2016). Enzymes produced by microbes, such as the enzyme LA dehydrogenase (LDH), convert pyruvate to lactate. Supplemental additions cause fermentation and a well-established microbial infestation in carbohydrate combinations (Fig 2.5). Consideration must be given to a number of factors, including pH, temperature, aeration, agitation, and the microorganisms being employed (Martinez *et al*., 2013). Both homofermentative and heterofermentative LAB are possible (Reddy *et al*., 2008).

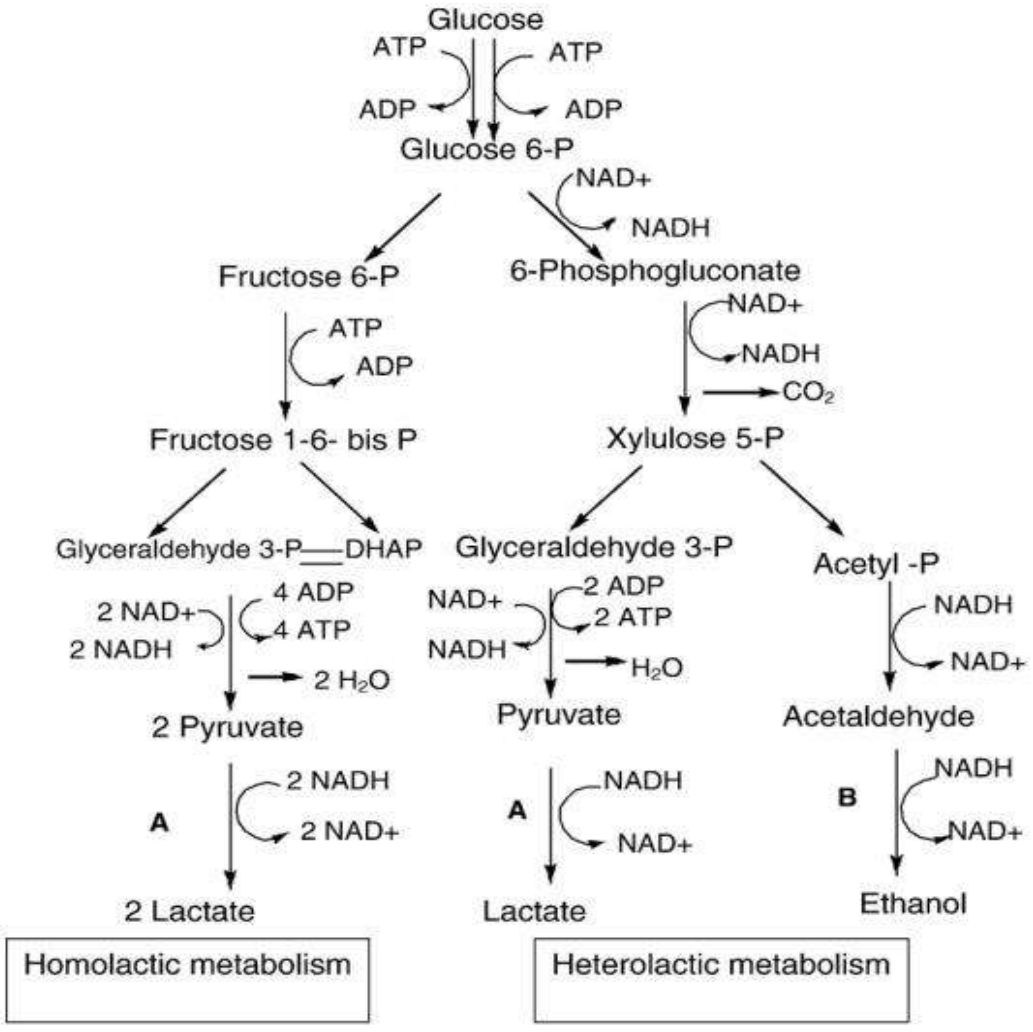

Figure 2.5: LA bacteria's homofermentative and heterofermentative metabolic processes (Reddy *et al*., 2008)

2.4.2.1 Homolactic fermentation

There are two steps to the technique. Homofermentative LA microbes ferment glucose to produce pyruvate in the first step of the anaerobic glycolysis process. Pyruvate is subsequently converted to lactate by the reducing agent, NADH, using the enzyme lactate dehydrogenase (L-LDH or D-LDH) (Mayo *et al*., 2010). Using the EMP route, homofermentative LAB metabolizes glucose to create solely LA (Ajala *et al*., 2020).

Glucose = 2 ATP + 2 LA

Therefore, homo fermentative LAB is used in the commercial manufacture of LA. This includes *Lactobacillus* species including *sakei, plantarum, casei,* and *bulgaricus*, as well as *delbrueckii, acidophilus,* and *helveticus* (Cui *et al*., 2011), *salivarius, amylophilus, casei, lactis,* and *bulgaricus* (Martinez *et al*., 2013). Through a homolactic fermentation process, genetically engineered *Lactobacillus plantarum*, also known as *Lactiplantibacillus* (Okano *et al*., 2009) and *Enterococcus mundtii* (Abdel-Rahman *et al*., 2011), may convert pentose carbs to LA.

2.4.2.2 Heterolactic Fermentation

In addition to LA, the arrangement of coproducts from fermentation, such as carbon dioxide, alcohol, and acetate, characterizes heterolactic fermentation (Martinez *et al*., 2013).

LA + CO_2 + Ethanol + ATP = Glucose

LA + CO_2 + Acetic acid + 2ATP + 2NADH = Glucose

Low yields of LA are produced by obligatory heterofermentative microbes, which include *L. pentosus, L. bifermentans* and other LAB species such *reuteri, brevis, parabuchneri,* and *fermentum* (Cui *et al*., 2011; Rodrigues *et al*., 2016). *Lactobacillus* species pentosus can use a heterofermentative pathway to convert xylose to lactate and acetate and a homofermentative fermentation to convert glucose to lactate (Bustos *et al*., 2004).

2.5 Microorganisms producing LA

Microbes producing LA includes bacteria, fungi, yeasts and microalgae (Abdel-Rahman *et al*., 2013).

2.5.1 LA bacteria

The majority of LA is mostly produced industrially by use of microorganisms that produce LA (Krishna *et al*., 2018). These gram-positive bacteria, known as LA bacteria (LAB), are commonly used as starter cultures (Indiarto *et al*., 2021; Rakhmanova *et al*., 2018). Throughout the ages, it has been associated with the production of fermented edibles (Akbar *et al*., 2016). Co-cultures or mixed cultures of microorganisms that produce LA have the potential to contribute to the creation of more energy-efficient and sustainable systems (Cui *et al*., 2011; Rombouts *et al*., 2020). Date juice was used to examine the LA fermentation processes of *L. casei* and *L. lactis* single and mixed cultures (Nancib *et al*., 2009). LA is produced by these microaerophilic organic entities fermenting hexose carbohydrates (Kahraman-Ilıkkan, 2024; Makarova *et al*., 2006). Selecting the right strain is essential, especially when it comes to lower nutrient requirements, superior optical purity in LA production, and promoting high yields and efficiency. LAB typically has a number of drawbacks, such as the production of lactate form combinations, low yield from other chemical synthesis, and a high risk of virus contamination that can cause cell rupture (Rodrigues *et al*., 2016). A microbe's choice is mostly determined by the kind of sugar that is employed. Certain bacterial species (like *L. delbrueckii*) are better at using sucrose sugar, whereas other species (like *L. bulgaricus*) do better with lactose. Some individuals possess the ability to efficiently ferment a blend of carbs. Starch can be used by *L. amylophilus* and *L. amylovorus* (Narayanan *et al*., 2004). The use of bioprocess is reduced by certain bacteria that are unable to metabolize pentoses and favor the use of glucose over other sugars known as carbon catabolite repression (CCR) (Dey *et al*., 2019).

By using multi-pulse fed batch fermentation, an alkaliphilic strain of *Bacillus* species WL-S20 synthesized L-LA, with a concentration of 225 g/L and a yield of 99.3% (Meng *et al*., 2012). In addition to being able to use xylose and arabinose, strains *of L. pentosus, P. acidilactici,* and *P. pentosaceus* are also resistant to a wide range of inhibitors, even when exposed to concentrations greater than those found in the biomass (Boguta *et al*., 2014). A mutant strain of *L. lactis*, strain RM2-24, produced 81g of LA per litre, which is more than three times the amount produced by the native strains (Joshi *et al*., 2010). Genetically modified *E. faecalis* N4 was used to cultivate *L. pentosus* microorganisms in various attempts at synthesizing LA. After 12 h of

incubation, the highest synthesis was 3.68 g/L of LA in an h (Ahmad *et al*., 2020; Eş *et al*., 2018).

2.5.2 Fungi

In an aerobic environment, glucose is utilized by certain thread-like fungi, such as *Rhizopus sp.*, to produce LA. *Rhizopus arrhizus* and *R. oryzae,* are known for their α-amylase enzymes, which enable them to convert starch directly into L-LA (Wee *et al*., 2006). With just a basic medium and the ability to use starch as feedstock, *R. oryzae* can produce biofilms that are easy to collect from the fermenter, making downstream operations convenient and affordable (Zhang *et al*., 2007). However, vigorous agitation is necessary for the process since it takes place in an oxygen-carrying environment, and mass transfer limitation can result in a low production rate (Krishna *et al*., 2018). The preferred microorganism for producing L-LA through semi-continuous fermentations of fungal pellets is the restricted to aerobic *R. oryzae* (Wu *et al*., 2011). Nevertheless, as *Rhizopus* species only produce one isomeric form—L-lactate—they are more frequently used in the manufacture of L-LA (Taskin *et al*., 2012).

2.5.3 Yeasts

Although most natural yeast strains produce little lactate as a main metabolic byproduct, a lot of effort has gone into creating engineered yeasts that can create LA in good quantities. A high yield of LA is produced by specific genetically modified yeast species, including *Kluyveromyces* (Bae *et al*., 2018), *Saccharomyces* (Watcharawipas *et al*., 2021), *Pichia* (Ilmén *et al*., 2007), *Candida* (Osawa *et al*., 2009), and *Zygosaccharomyces* (Abdel-Rahman *et al*., 2013; Ortiz-Merino *et al*., 2018). Yeast's ability to multiply in nutrient medium and resistance to pH values (1.5) make it an excellent choice for nutrition sources because they stop precipitated calcium lactate from regenerating and lower the need for expensive neutralizing chemicals (Krishna *et al*., 2018). The β-glucosidase 1 (bgl1) gene of *Aspergillus aculeatus* was amplified within a strain of *Saccharomyces cerevisiae*, a yeast that produces LA, to facilitate the synthesis of the LA using cellobiose. The resulting modified yeast form produced approximately 80 g/L of LA using approximately 100 g/L of cellobiose (Tokuhiro *et al*., 2008).

2.5.4 Cyanobacteria and microalgae

Photosynthetic microorganisms, such as microalgae and blue green algae, offer a novel approach to LA synthesis by removing the need for expensive carbohydrate substrate (Abdel-Rahman *et al*., 2013; Ahmad *et al*., 2020). Algal biomass can also be used as a substrate for the production of LA. While they do have high protein and carbohydrate contents, they do not have lignin like LCB does (Martinez *et al*., 2013). The starch that microalgae consume can be converted by them into biological materials such formate, lactate, acetate, and alcohol in an anaerobic environment. Sakito Island is home to *Nannochlorum* sp. 26A4, which has an amylum content of forty percent (dry weight) and an optical purity of about 99.8%. Under anaerobic conditions, the bacteria convert starch to D-LA (26 g/L) at a rate of 70 percent in the dark (Silva *et al*., 2012; Haldar & Purkait, 2020).

2.6 Raw material for production of LA

Microbial, biotech, and genome editing advances are contributing to a very a novel idea towards transforming LCB (200 billion tonnes annually), the largest global sustainable organic feedstock. Value-added items include meals, medications, biological substances, organic acids, biofuels, and bioactive peptides (Silva *et al*., 2012). These materials are considered environmentally acceptable due to their high sugar content (Abdel-Rahman & Sonomoto, 2016), which makes them an organic, abundant, renewable, and affordable source of carbohydrates for bacteria to convert into biomolecules and energy (Asgher *et al*., 2013; Patel & Shah, 2021). Lignocellulosic biomass is mainly made up of three primary polymers: cellulose, hemicellulose, and lignin (Fig. 2.6). These polymers are involved in a non-uniform three-dimensional framework to varying degrees (Wang *et al*., 2021). It also includes other materials like ash-like material, extracts (soluble nonstructural materials like proteins), pectins, and other soluble nonstructural materials (Kumar *et al*., 2009). Among the bioactive components included in biomass are antioxidants, lignans, flavonoids, and carotenoids (Srivastava *et al*., 2021).

Examples of lignocellulosic feedstocks include industrial waste (sawdust, pulp and paper waste, food industry residues, etc.); residues from agriculture (corn stovers, bagasse from sugarcane, straws, rice and wheat biowaste, peelings, cobs, stalks, straw); forestry residues (hard and soft wood, grasses, etc.); municipal solid waste (Balat *et al*.,

2011; Behera *et al*., 2014); and the energy agricultural (grasslands of prairie, miscanthus, switchgrass, and small cycle annual crops like oak trees and poplar) (Akhtar, Gupta, *et al*., 2016) (Table 2.2).

In general, 15-20% lignin, 40–50% cellulose, and 25–30% hemi-cellulose makes up the majority of agricultural biomass (Chen *et al*., 2017; Tayyab *et al*., 2018). Softwoods comprise more lignin than hardwoods, whereas hardwoods include more hemicellulose and cellulose (Balat *et al*., 2011). In addition to the three basic elements, there are also inorganic, such as calcium, sodium, potassium, silicon, magnesium, and aluminium, and secondary components like extractives, proteins, and water. These insignificant elements have a negligible impact on the biomass's overall structure (Tayyab *et al*., 2018).

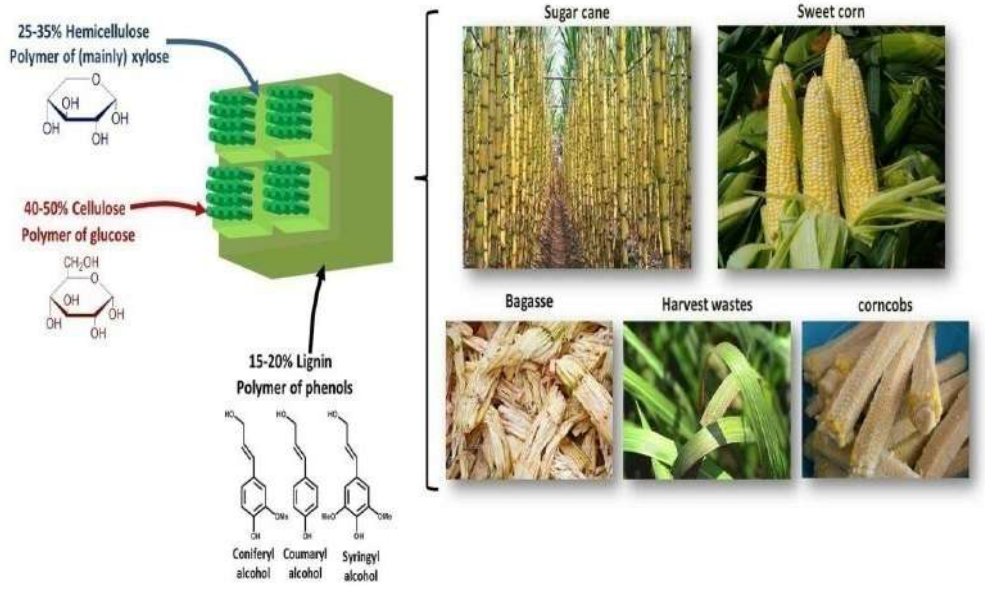

Figure 2.6: Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin (Gallo & Trapp, 2017)

2.6.1 Cellulose

Cellulose $(C_6H_{10}O_5)n$ is a sugar hexose with 6 carbons that is present in agricultural fodder and wood (Ferdeş *et al*., 2020). This is the prevalent polysaccharide on our planet, and it even has a lot of good qualities, like tactility, solubility, biodegradability, and active hydrogen atoms (Chen *et al*., 2017; Tayyab *et al*., 2018). Microbes, fungi, and phytoplankton can all include cellulose, which is the primary structural polymer that gives the cell wall support, chemical stability, and mechanical strength (Agbor *et*

al., 2011; Srivastava *et al*., 2023). It is also the most widely used feedstock for fermentation. Repeating D-glucopyranose units joined by β-1,4-glycosidic linkages form the linear polymer cellulose (Cheah *et al*., 2020; Den *et al*., 2018). Geometry can be either well-organised solid form or poorly-ordered amorphous, depending on the degree to which linkages between polymer bonds are arranged (Pasin *et al*., 2020). Long chains of hydrogen bonding, primarily in the type of primary and micro-fibrils, make up cellulose, which is most frequently observed in bundles or macrofibrils (Yadav *et al*., 2020). Cellobiose, which is created by the recurrent unit formed through this linkage, forms the cellulose chains (Kumar *et al*., 2009). Plant biomass is made up of 40–50% cellulose molecules. Cellulose fibers are joined by a plethora of intra- and intermolecular hydrogen bonds Deconstruction of lignocellulosic biomass with ionic liquids (Brandt *et al*., 2013; Mood *et al*., 2013). Consequently, it remains insoluble in water and other carbon-containing solvents, despite the solubility of monomers and short oligomers. At high temperatures, the power applied is enough to break the gas connections responsible for its crystalline structure, making it soluble (Yadav *et al*., 2020).

2.6.2 Hemicellulose

It is not structurally homogeneous like cellulose (Agbor *et al*., 2011; Anwar *et al*., 2014), and it is the second most frequent heterogeneous polymer (Anwar *et al*., 2014). Hexoses (glucose, galactose, and mannose), the pentoses (xylose and L-arabinose), and urgonic acids (primarily galacturonic, glucuronic, and methyl galacturonic acids) are among the several polymers that make up hemicellulose polymer ($C_5H_8O_4$). L-fucose and L-rhamnose are two other sugars that may be present in very modest amounts (Avanthi *et al*., 2017). Mannose is the principal sugar in softwoods, whereas xylose predominates in hardwoods and agricultural wastes (Balat *et al*., 2011; Yadav *et al*., 2020). β-(1-4) linkages bind xylopyranose, the polymer's backbone. Biomass has a molecular mass of about 30,000, up to 200 degrees of polymerization, and a 25–35% hemicellulose concentration (Anwar *et al*., 2014). The hemicellulosic components of the lignocellulosic complex engage covalently with lignin and hydrogenally interact with the cellulose monomer to form a physical link (Akhtar *et al*., 2016). The most soluble hemicellulose molecules are galactose, arabinose, glucose, xylose, and mannose, in increasing order of solubility (Roy *et al*., 2020). Because of its irregular, branching, and amorphous form, hemicellulose is readily degraded with acid into its

monomer units, exhibiting low resistance to acid hydrolysis and increased sensitivity to depolymerization (Brandt *et al*., 2013; Kumar *et al*., 2019). However, the degree of polymerization stays below 200 units and can only reach a maximum of 150 monomers. At lower temperatures than cellulose, hemicellulose usually becomes insoluble in water; but, at higher temperatures, it starts to hydrolyze and becomes soluble (Harmsen *et al*., 2010).

2.6.3 Lignin

However, lignin is a large, intricate molecular structure that shields cellulose and hemicellulose from invasive microbes and activities (Srivastava *et al*., 2021). It is made up of phenol monomers that have been linked together into polymers (Abdel-Rahman & Sonomoto, 2016). Its intricate hydrophobic and cross-linked characteristics hinder the hydrolysis process. It has a polyphenolic structure and is a three-dimensional heterogeneous polycrystalline (Cheah *et al*., 2020; Tayyab *et al*., 2018). Three phenol alcohol monomer units, namely trans-p-coniferylic, trans-p-sinapylic, and trans-p-cumarylic, combine to form lignin (Kucharska *et al*., 2018). Plants possess robustness, rigidity, and resistance to oxidative damage and microbial invasion due to lignin, an intricate and uneven phenolic polymer (Senatore *et al*., 2021). Because the amorphous polymer is also water insoluble and optically inactive, lignin breaks down very quickly. Lignin's solubility in neutral, acidic, or alkaline environments depends on its precursor (Bhukya & Keshav, 2022; Datta & Henry, 2006). The soft woods (fir, cedar, pine, poplars, balsam) contain the highest amounts of lignin, which has a molecular weight of less than 20,000. Hardwoods (20–40% dry weight) and herbaceous plants (grasses, bagasse, rice hulls, straws) follow (Balat, 2011). The breakdown of both hemicellulose and cellulose (commonly referred to as holocellulose) to produce fermentable carbohydrates is more difficult in biomass that contains lignin. Moreover, during the breakdown process, it might produce furan molecules, which could prevent fermentation (Akhtar *et al*., 2016). It has been discovered that lignin can be dissolved by low-molecular-weight ethanol, which is dimethyl sulfoxide, an ether, acetone, and azine (Harmsen *et al*., 2010). Lignin provides advantageous chances to obtain valuable goods such as aromatics, emulsifiers, surfactants, sequestrants, binders, and carbon filaments or fibres (Yusuf & Inambao, 2019).

Table 2.2: Lignocellulosic Biomass composition as a percentage of total biomass in several sources

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Rice straw	26.5	32.6	13.5	(Ruan *et al*., 2016)
Wheat straw	28.6	20.5	15.4	(Ruan *et al*., 2016)
Rice husk	37.1	29.4	24.1	(Baruah *et al*., 2018)
Waste papers	65	13	1.0	(Baruah *et al*., 2018)
Almond shell	27	30	36	(Hassan *et al*., 2018)
Japanese cedar	52.7	13.8	33.5	(Hassan *et al*., 2018)
Rye straw	31	22	25	(Tian *et al*., 2018)
Oat straw	39	27	18	(Tian *et al*., 2018)
Rice husk	49.09±0.15	3.28±0.07	31.28±0.28	(Montipó *et al*., 2019)
Eucalyptus	44.9	28.9	26.2	(Montipó *et al*., 2019)
Maize straw	38.33	29.76	3.82	(Montipó *et al*., 2019)
Micanthus	45.31	23.37	12.97	(Montipó *et al*., 2019)
Pinewood	43.74	16.2	29.14	(Montipó *et al*., 2019)
Corn straw	42.60	21.30	15.10	(Srivastava *et al*., 2021)
Barley straw	33.25	20.36	17.13	(Montipó *et al*., 2019)

2.6.4 Pectin

Pectins contain heterogeneous polysaccharide that have axial linkages with α-1,4-D galacturonic acid components and side chains made up of xylose, galactose, L-rhamnose, and arabinose (Pasin *et al*., 2020). Acidic cell walls contain a polysaccharide called pectin, which functions as a sol-like network for a variety of purposes, including the retention of ions and moisture, the support and facilitation of enzymes that modify

cell walls, cell signalling, permeability of cell membranes, physiological regulation, cellular adhesion, growth, and defence (Bazoti *et al*., 2023).

2.7 Lignocellulosic biomass pretreatment: procedure of processing the biomass to fermentable sugars

The complexity of lignin and hemicellulose structure allows just 20% of the original cellulose (not altered or broken by any pretreatment techniques) to be converted to fermentable sugars (Fig. 2.7) Therefore, the key to increasing bioproduction is to break down the hemicellulose and lignin barrier prior to any bioconversion process (Tian *et al*., 2018). The production of the LA along with additional bioproducts requires the disruption of this refractory structure in order to eliminate lignin and perform a pretreatment technique that separates cellulose from hemicellulose (Ajala *et al*., 2020). A mere twenty percent of cellulose hydrolysis happens when hydrolysis (directly with enzymes) is not employed, however it is more than 90 percent when pretreatment is undergone (Srivastava *et al*., 2015). Pretreatment aims to modify the chemical composition, macrostructure, and microstructure of lignocellulose (Tayyab *et al*., 2018). Pretreatment procedures that are straightforward, inexpensive, ecologically friendly, and economical should be employed (Hassan *et al*., 2018).

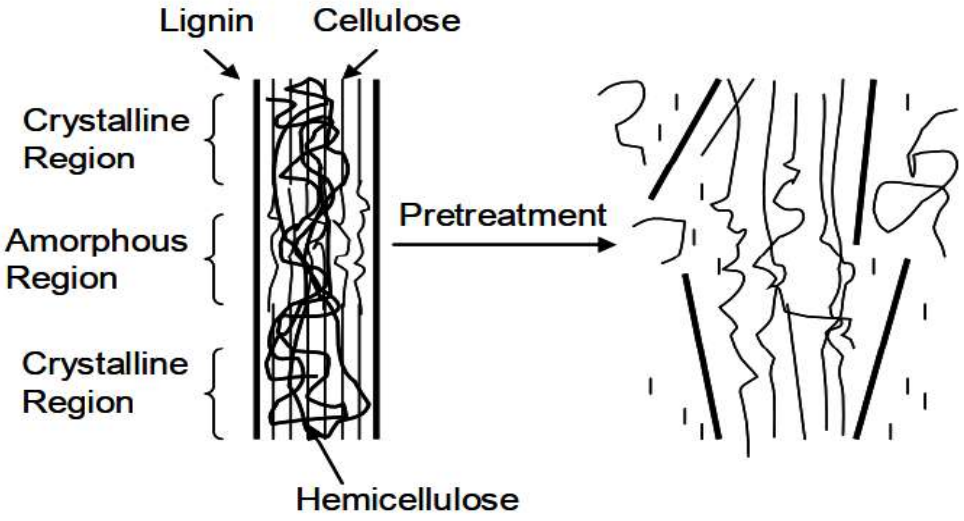

Figure 2.7: Pretreatment of lignocellulosic biomass (Mosier *et al*., 2005)

Four categories of pretreatment technology exist: Physico-chemical (1) Strategies that are (2) physical, (3) chemical, and (4) biological (Yusuf & Inambao, 2019). Physical

pretreatment should be used as a primary method to enhance surface area. This is often followed by an alkali or weakly acidic pretreatment phase, and finally, catalytic enzymes or maybe microorganisms (Bayrakci & Koçar, 2014).

2.7.1 Physical pretreatment

Physical techniques including grinding, cutting, shearing, and milling reduce particle size and increase the contact area of the substrates (Eş *et al*., 2018), improving flow properties and enhancing porosity and evident density (Mood *et al*., 2013) . The maximum size of the pretreated particles determines the total energy used, and the crystallinity of the lignocellulosic material decreases (Brodeur *et al*., 2011). Pretreatment biomass size reduction is an expensive and energy-intensive step, but it is thought to be cost-effective (Arora *et al*., 2018).

2.7.1.1 Grinding

The process of mechanical comminution involves the combination of cutting, crushing, and grinding of lignocellulosic materials to decrease their crystalline character (Cheng & Timilsina, 2011), increase their accessible surfaces, and facilitate hydrolysis (Abo *et al*., 2019; Pereira *et al*., 2021). Because of the physical pretreatment technique, the reduction in particle size is observed to be 10 to 30 mm and 0.2 to 2.0 mm, respectively, after crushing, grinding, or milling (Kumari & Singh, 2018; Srivastava *et al*., 2023). Pinball, colloid milling, knife, vibratory, hammer, disc, centrifugal, two- roll are a few of the various frequently utilised milling techniques (Eş *et al*., 2018; M. Yadav *et al*., 2020). The preprocessing of maize stover ball-milling increased the yields of glucose and xylose when any of the weak chemicals listed below were added to the preprocessing process: $Ca(OH)_2$, H_3PO_4, NaOH, KOH, NH_3H_2O, CH_3COOH, H_2SO_4, HCOOH, HCl, HNO_3 (Lin *et al*., 2010). Research on the disintegration of cells using yeast, microalgae and cyanobacteria has made extensive use of bead milling (Cheah *et al*., 2020).

2.7.1.2 Radiation

Alternative energy input techniques include pretreatment processes such as electron beam irradiation, microwave and gamma ray (Den *et al*., 2018). Refractory structures are more easily disrupted by microwave irradiation because it creates explosions inside the material's particles (Srivastava *et al*., 2023). Because MW irradiation processing

only modifies the cellulose's ultrastructure and eliminates or partially destroys hemicelluloses and lignin, it appears to be a more popular option than heating (Kumari & Singh, 2018). This is widely used due to its ease of use, low energy requirements, quick heating capacity, low inhibitor production (Kumar *et al*., 2019), and ability to degrade the cellulose portion using γ-radiations, microwaves, and electron beams to speed up the hydrolysis reaction (Cheah *et al*., 2020; Tayyab *et al*., 2018). Compared to standard heating processing, microwave pretreatment of sugarcane bagasse prepared with NaOH and H_2SO4 was significantly more successful, yielding four times more reducing sugar production with 5.7 times shorter pretreatment period (Devi *et al*., 2022). Following microwave processing of lignocellulose feedstock, microwave activation causes hydrogen bonds to break and intermolecular water to evaporate. The stability of the lignocellulose molecules declines as a result (Zhu *et al*., 2016). The particular surface of lignocellulosic biomass is increased while its degree of crystallinity decreases due to the disruption of 1,4-glycosidic connections brought about by electron radiation (Kucharska *et al*., 2018).

2.7.1.3 Sonication

Using the principles of surface erosion and delignification, ultrasound-assisted pretreatment is an additional option for lignocellulosic biomass pretreatment. It runs at a temperature that is lower, requires fewer chemicals, and ensures a shorter process period (Cheah *et al*., 2020). Ultrasonication with a periodicity of 20 to 40 kHz typically disrupts the inner hydrogenous bonding of the bio polymer (Kucharska *et al*., 2018). The acoustic cavitation produced by ultrasonic waves in any medium results in the spontaneous formation of microbubbles, which in turn causes the holocellulose fragments to rupture (Devi *et al*., 2022). Thus, rupturing the cellulosic and hemicellulosic fractions and causing the deteriorating enzymes to be more efficient in converting cellulose to simple sugars. At 50ºC, the maximum cavitation produced. Long-term ultrasound has not been shown to have any more effects (A. K. Kumar & Sharma, 2017). Additionally, the α-O-4 and β-O-4 connections of lignin can be disrupted by ultrasound, resulting in the separation of cellulosic and lignin fractions (Hassan *et al*., 2018). Much better results were obtained by ultrasonication in alkaline (KOH) prepared wheat straw, wherein over 50% of the lignin was solubilized for a 5- to 35-minute sonication period (Sun & Tomkinson, 2002)

2.7.1.4 Condensation using pyrolysis

Pyrolysis is employed to pre-treat lignocellulosic materials because feedstock can serve as a platform for a quick pyrolysis that converts structural polysaccharides to fermentable sugars, usually with higher yield (Balat, 2011). Exposure of biomass to high heating temperature exceeding 300°C is known as pyrolysis. At that temperature, the hemicelluloses and a portion of lignin and cellulose will decompose into gas (H_2 and CO) and dark substances (char), while the tight structure of the lignocelluloses will be destorted (Kumari & Singh, 2018; Yadav *et al*., 2020). The pyrolysis process is accelerated when oxygen is present and can be performed using 1 N H_2SO_4 at 97°C for 2.5 h (Yadav *et al*., 2020). Packed bed reactors, fluidized bed reactors and pyrolysis reactor, circulation, and void have all been well studied for practical pyrolysis research (Wang *et al*., 2017).

2.7.1.5 Electric field pulses

Pulse electric field exposes the cellulose in the to break into basic sugars by creating holes in the membrane's layers. During this process, the biomass is exposed to a brief pulse of voltage lasting a few milliseconds, with a level of 5 to 20 kV/cm (Kumar & Sharma, 2017). Based on this tendency, numerous applications in bioscience and medicine have explored the use of high voltage pressures to facilitate conformational changes in a range of natural systems (Kumar *et al*., 2011). The PEF system was utilised to treat samples of wood chips and switchgrass up to pulse beam intensity of 10.0 kV/cm (Kumar *et al*., 2011).

2.7.2 Physico chemical pretreatment

2.7.2.1 Explosion of steam

Because it uses few chemicals and little energy, steam-explosion is the most popular physico-chemical waste treatment method and it has been extensively studied (Den *et al*., 2018). The process of biomass is conducted using high-pressure saturated steam at temperatures ranging from 160°C to 240°C and pressure range from 7 to 48 bars, which leads to the digestion of lignocellulosic biomass (Kumar & Sharma, 2017). The dissolution of structural elements occurs by mechanical tearing, steam heating (thermal), and auto-hydrolysis including glycosidic connections (synthetic) (Kumari & Singh, 2018). The industrial application of steam processing has been established at the

Masonite facility. Once the temperature reaches a specific level, the successful release of hemicellulose sugars is possible. But as the temperature rises, more sugars are lost, which causes the total amount of sugar returned to decrease (Taherzadeh & Karimi, 2008). When H_2SO_4 (or SO_2) or CO_2 [typically 0.3–3% (w/w)] is combined with steam explosion, reaction time and temperature are lowered, hydration efficiency is increased, interfering chemical production is minimized, and hemicelluloses are totally eliminated (Behera *et al*., 2014).

2.7.2.2 Pre-treatment based on ammonia

The three primary ways of processing ammonia are Ammonia Fibre Explosion (AFEX), Ammonia Recycle Percolation (ARP), and Soaking in Aqueous Ammonia (SAA) (Chaturvedi & Verma, 2013). AFEX treats lignocellulosic biomass with ammonia for a brief period of time at high pressures (250–300 psi) and a specific range of temperatures (60ºC to 100°C) (Tayyab *et al*., 2018). After a brief period of time, the pressure is released and the ammonia evaporates, resulting in a rapid temperature shift, the structural breakdown of cellulose, an increase in surface area, and improved enzyme accessibility (Chen *et al*., 2017). The AFEX treatment is affected by a number of variables, including moisture content, treatment duration, temperature, pressure, water and ammonia loading (Balat, 2011; Mood *et al*., 2013). This high-pressure, high-temperature ammonia treatment that produces swelling reduces cellulose crystallization of lignocellulosic materials and breaks the sugar-lignin connections (Yusuf & Inambao, 2019). This means that while this method is effective for biomasses like lucerne, wheat chaff and straw, it is less effective for woody lignin-based biomass (Cheah *et al*., 2020). A technique involving ammonia pretreatment and processing was developed to turn sweet-stemmed sorghum grain and forage into alcohol (Li *et al*., 2010).

2.7.2.3 Pretreatment of supercritical fluids

Supercritical fluids can exhibit gas-liquid co-existence, in which the gas functions as a solvent (Tayyab *et al*., 2018) at temperatures and pressures that exceed the minimum necessary to decompose the complex crystalline constitution of biomass (Kumar & Sharma, 2017). Enzymes have more surface area available when structural biomass is disturbed by the digester's pressurized gas discharge (Mood *et al*., 2013). CO_2 is a supercritical liquid that is used extensively due to its low critical temperature and pressure (31.1ºC and 7.36 MPa, respectively) and its non-toxic, recyclable, affordable,

and environmentally advantageous properties (Cheah *et al*., 2020; Daza Serna *et al*., 2016). There is a rise in open area as a result of the disruption of the substrate structure by compressed gas (Harmsen *et al*., 2010). Co-solvents such as ethanolic acid–water and alcohol–water could efficiently speed the removal of lignin by promoting depolymerization under severe pressures (Serna *et al*., 2016). It is common practice to employ supercritical CO_2 (73.0 Bars and 35ºC), which increases sorghum's sugar output by 50–70% (70 percent aspen and 14 percent yellow pines) (Datta & Henry, 2006).

2.7.2.4 Disintegration

When lignin is treated to decomposition with an oxidant such as oxygen, ozone, or hydrogen peroxide (Yadav *et al*., 2020), as well as acid, the hemicelluloses may break down excessively and fermentation may not be possible (Kumar & Sharma, 2017). Strong hydrolytic oxidant per-oxyacetic acid preferentially solubilizes lignin while minimizing losses of carbohydrates. The productivity and accessibility of cellulose hydrolysis can increase by approximately 98% when peracetic acid is used in conjunction with NaOH during biomass processing (Yadav *et al*., 2020). At 30°C, 2% H_2O_2 solubilized 50% of the lignin and nearly all of the hemicellulose in 8 h (Kumar & Sharma, 2017). Tri-oxygen, often known as O3, is a potent water-soluble oxidizing agent that can break down lignin to produce soluble low molecular mass compounds like acetate and formate, while also somewhat breaking down hemicellulose and cellulose (Mujtaba *et al*., 2023).

2.7.2.5 Oxidation in wet conditions

The interaction of air/oxygen (O_2) with moisture at relatively high pressure and temperature is known as wet oxidation (Chaturvedi & Verma, 2013). At pressures between 0.5 and 2.0 MPa and temperatures over 120 °C, this process takes around 30 minutes (Mujtaba *et al*., 2023). Wet oxidation has less impact on celluloses, but it oxidizes lignin and breaks down hemicelluloses into monomers such as pentoses (Kumar & Sharma, 2017). Less inhibitory chemicals are produced during wet oxidation (Kumar & Sharma, 2017). It has been discovered that keeping Na_2CO_3 within a normal to basic pH range inhibits the formation of inhibitory compounds (Alvira *et al*., 2010). A rich waxy covering made of proteins or silicon was eliminated by wet oxidation from lignocellulosic biowastes such as grasses, hay, flax, and other cereal crops (Brodeur *et al*., 2011).

2.7.3 Chemical pre-treatment: This technique uses a mild combination of basic and acidic substitutes. This process hydrolyzes the material and yields sugars.

2.7.3.1 Pretreatment using dilute acid

Natural and mineral acids, such as sulfuric, nitric, hydrochloric, maleic, and acetic acids, have been used in acid pre-treatment (Den *et al*., 2018). The primary goal of chemical processing that uses acids is to solubilize biomass's hemicellulose component and increase cellulose's susceptibility to enzymatic breakdown (Alvira *et al*., 2010). Sulphuric acid (H_2SO_4) is the acid that is most frequently used in this process because it is very successful at separating the components of cell walls and produces lignin, cellulose, and hydrolyzed hemicellulose (Yusuf & Inambao, 2019). There are two primary categories of pretreatment with acid: diluted acidic treatment at elevated temperatures (>200 °C) and concentrated acidic treatment (30–70 percent) at a lower temperature (50 °C) (Singh *et al*., 2022). Chemical pretreatment generally entails introducing concentrated or strong or diluted acids (often between 0.2 and 2.5 percent w/w) into the biomass, followed by continuous stirring at temperatures between 130 and 210ºC (Menon & Rao, 2012). Poplar wood treated with diluted acid produced increased sugar yields of 82.8% and a cellulase concentration of 15 FPU/g, according to research conducted by US CAFI laboratories (Zhu & Pan, 2010). Five chemicals—TFA, H3PO4, HCl, H2SO4, and HNO3—were evaluated on *Pinus taeda* in a reaction chamber (Marzialetti *et al*., 2008). When the *Eulaliopsis binata* was treated with an acidic solution before to the dissolving pulp production over 160 °C over 30 min, it was found that 21.02 percent of total reducing sugars were produced (Tang *et al*., 2013). The composition of intricate lignocellulosic frameworks is altered by the pretreatment of water hyacinth biomass with 1, 3, and 5% dilute sulphuric acid, thereby enabling their rapid hydrolysis into single sugars (Ganguly *et al*., 2012).

2.7.3.2 Pretreatment with alkaline

Alkaline pretreatment causes expansion, resulting in an increase in intracellular surface area, dissolution of the lignin network, permiting enzymes to break down cellulose and hemicellulose. This results in separation of bonds between sugars and lignin, and reduction of polymerization or crystallinity (Canilha *et al*., 2012). A variety of alkali compounds are used in this treatment, such as aqueous ammonia (NH_3), sodium hydroxyl derivatives (NaOH), potassium hydroxyl derivatives (KOH), calcium

hydroxyl derivatives ($Ca(OH)_2$), and ammonium hydroxyl derivatives (NH_4OH) (Behera *et al*., 2014; Tayyab *et al*., 2018). Conversely, it has been demonstrated that calcium hydroxide, often known as slaked lime, is the most economical and successful pretreatment agent (Cheah *et al*., 2020; Yadav *et al*., 2020). Alkali degradation of polysaccharides and ester side chains results in structural changes in lignin, swelling and decrystallization of celluloid, and partial solubilization of polyose (Cheah *et al*., 2020; Yadav *et al*., 2020). When 2% NaOH was applied to rice straw beforehand, the amount of sugar increased by roughly 54.83%, the amount of hemicellulose decreased by 61.07%, and the amount of lignin decreased by 36.24%. All of these effects can aid in the process of enzyme hydration (Zhang & Cai, 2008). Weak dilute hydroxide was the most widely recognized technique for increasing cellulose availability, followed by soaking of ammonia and lime ($Ca(OH)_2$) (Bali *et al*., 2015). Following pretreatment with NaOH solution, the composites exhibited optimal stiffness, and following modification with KH560, they displayed optimal ductility (Lu *et al*., 2014). The division of the three main components in rice straw was mostly affected by the synergistic effects of applying mildly acidic or basic solutions successively (Sun *et al*., 2016).

2.7.3.3 Pretreatment with Organosolv

Chemical solutions such as alcohol and methyl alcohol, ethan-1,2-diol, tetrahydrofurfuryl alcohol, etc. can be used in the organosolv method with or without the presence of a catalyst support (Mood *et al*., 2013). As catalysts, certain bases (NaOH, NH_3, and $CaCO_3$) and acids (HCl and H_2SO_4) from the mineral and organic categories may be used (Kumari & Singh, 2018). Solvents need to be kept apart since they can interfere with fermentative bacteria and enzymatic hydrolysis (Alvira *et al*., 2010). Reaction temperature, duration, and solution concentration are the main factors influencing pretreatment processing (Ichwan & Won Son, 2011; Tian *et al*., 2018). Yields of 50.1%, 48.1%, and 41.7%, respectively, were observed after the effects of various solvents, including ethylene glycol, alcohol, and an ethanoic acid with water combination, on cellulose extraction out of palm oil pulp were investigated (Ichwan & Won Son, 2011).

2.7.3.4 Liquid ions (ILs)

The covalent structural bonding of the biomass's constituents is disrupted by the designer fluids' potent hydrogen bond acceptors (Ferdeş *et al*., 2020; Tayyab *et al*., 2018), which causes the breakdown of a three-dimensional network. Ionic liquids consist of anions and cations and have the following characteristics: low vapour tension, non-flammability, low melting point (below 100°C), greater polarity, low wettability, low toxicity, improved electrical properties, and higher reactivity rates (Ferdeş *et al*., 2020). Important ionic liquids (ILs) are classified into four categories based on the individual metal ions they contain: quats, N-alkylpyridinium, N-alkyl isoquinolinium, and 1-alkyl-3-methylimidazolium ionic liquids (Ma *et al*., 2016). Using a simple synthetic technique, many pyrrolidonium-based ILs were investigated for digesting maize stalks over 90°C for 30 minutes (Liu *et al*., 2012).

2.7.4 Pretreatment with biological agents

Bacterial treatment of biomass is thought to be an economical, low-energy, and environmentally benign solution. During incubation, a small number of microorganisms can release the enzymes cellulase and hemicellulase, which exclusively break down lignin (Yusuf & Inambao, 2019). This produces a feedstock material that, in mild conditions, can be quickly broken down into simple sugars. Typically, filamentous fungi are utilised as microbes and are typically found in soil, native plants, or LCB matter (Yusuf & Inambao, 2019). During biological processing, white, brown, and soft rot fungal types are frequently used to liberate the cellulosic fiber from the lignin-hemicellulose framework. Pretreatment breaks down the components of lignin and hemicellulose but not the cellulose (Akhtar *et al*., 2016; Okano *et al*., 2009). While white rot and soft rot fungi equally target lignin and cellulose, brown fungus are specialized to break cellulosic framework (Cheng & Timilsina, 2011).

The most advantageous organisms are a few species of white rot fungus related to the Basidiomycetes group (Kumari & Singh, 2018; M. Yadav *et al*., 2020). Several fungal species, such as *Ceriporiopsis subvermispora* (Wan & Li, 2011), *Pycnoporus cinnabarinus* (Meza *et al*., 2006), *Streptomyces viridosporus*, *Polyporus versicolor*, *Trichoderma viride*, *Flammulina velutipes*, *Ceriporia lacerata*, *Phanerochaete chrysosporium*, *Cyathus stercolerus*, and oyster mushroom, have been tested on a range of agricultural residues or feedstocks and have shown a significant delignification

efficaciousness (Kucharska *et al*., 2018; Tian *et al*., 2018). Following pretreatment with *C. subvermispora*, sugarcane bagasse yields increased and 47 percent of the prospective glucose was recovered (Machado & Ferraz, 2017). Brown rot fungi preferentially break down pectin and cellulose over hemicellulose and lignin, while lignin is only altered during this process, in contrast to white rot fungus (Tian *et al*., 2018). A few of the species that have been employed in biodegradation investigations are *Serpula lacrymans*, *Gloeophyllum trabeum*, *Laetiporus sulphureus*, *Coniophora puteana*, and *Meruliporia incrassata* (Akhtar *et al*., 2016). It is possible to use other species, like worms, insects, and gastropods (Cheah *et al*., 2020). Major disadvantage of this process is that many microorganisms used for lignin degradation or hemicellulose solubilization, including fungi (e.g., white-rot fungi) and bacteria (e.g., ligninolytic bacteria), may metabolize sugars from the biomass as an energy source. This results in reduced fermentable sugar availability for subsequent processes like biofuel or biochemical production, thereby decreasing overall process efficiency. The extent of sugar loss depends on factors such as microbial strain selection, incubation time, and nutrient availability. Additionally, slow processing times and limited substrate specificity can make biological pretreatment less competitive compared to chemical or physicochemical methods (Sharma *et al*., 2019).

2.8 Hydrolysis by enzymes

Enzyme breakdown of polymers is the biochemical process by which cellulose and hemicelluloses are converted into digestible sugars by enzymes produced by microorganisms (fungi and bacteria) (Abo *et al*., 2019). Cellulases and hemicellulases are the two main types required for this task (Ajala *et al*., 2020). Three groups of cellulases have been identified: (i) endo-glucanases (EG) catalyse the hydrolysis of glycosidic chains; (ii) glucosidases degrade cellulosic components into glucose monomers; and (iii) exo-glucanases, also known as cellobiohydrolases (CBH), which break down two cellulose components out of free end chains (Balat, 2011; S. Wang *et al*., 2017). Enzyme breakdown usually occurs in a moderate pH range of approximately 4.8 and a temperature range of 45–50ºC (Cheng & Timilsina, 2011). Most cellulase enzymes are produced by fungal species like *Aspergillus/Trichoderma* sp. under suitable conditions of growth (Da Silva *et al*., 2012). The well-known fungus *Trichoderma reesei* produces cellulolytic enzymes, which jointly break down cellulose into simple monomer units. Typically, these enzymes consist of two cellobiohydrolases

(CBH), five endo-glucanases, and two glucosidases (Zhang & Cai, 2008). Using either C5 or C6 isolates of the yeast *S. cerevisiae*, the physical pre-treatments of hay and bagasse were investigated, as well as the application of enzyme hydrolysis, feedstock sap characterization, and fermentation (Silva *et al*., 2012). *Thermoascus aurantiacus* RCKK has been used to optimise the synthesis of xylanases and thermostable cellulases (Jain *et al*., 2015). On the other hand, the complex hemicellulase system comprises mannosidases, endo-mannanases, xylosidase, which aids in the liberation of xylan, and endo-xylanases, which hydrolyze the intrinsic links in a hemicellulosic polymer in their corresponding monomer units (Adsul *et* al., 2020; Jørgensen *et al*., 2007).

2.9 Inhibitor formation and detoxification

Pretreatment has the unintended consequence of generating lignocellulose-derived byproducts from lignocellulosic feedstocks that inhibit bacterial and enzymatic biocatalysts (Jönsson & Martín, 2016). By lowering the development rate of fermenting microbes, extending the lag phase, inducing cell density loss, and producing unfavorable conditions for fermentative microorganisms, inhibitors lower yield (Kumar *et al*., 2020). The inhibitors fall into three categories: a) weak and feeble acids, such as phenolics (mainly from the breakdown of lignin); b) phenolics (through the breakdown of furfural), and c) furan derivatives, such as 5-(hydroxymethyl) furfural (HMF) and furfural (through the breakdown of sugar) (Cubas-Cano *et al*., 2020). As was already established, phenolics—in particular, tiny molecular mass phenol compounds—have a stronger inhibitory effect than furfural and HMF, making them far more dangerous to microorganisms (Wu *et al*., 2011). Using yeast and bacteria, the detrimental effects of several chemicals have been studied during the conversion of biomass the hydrolysate into alcohol (Jungmeier, 2017). In *B. coagulans* GKN316, the ARTP variation boosts inhibitor resistance and facilitates aldehyde reductions that aid in the breakdown of inhibitors vanillin, furfural, syringaldehyde, and hydroxymethyl furfural (HMF) during in situ cultivation (Wu *et al*., 2011). Perhaps due to the quantity of 5.0 g/L inhibitory chemicals, *B. coagulans* strain A162 was identified and produced the highest amount of L-LA (almost 2.4 g/l/h) (Cubas-Cano *et al*., 2020). Depending on the metabolic type, furfural and HMF have varying effects on the rate of growth of LAB; in the presence of these substances, growth kinetics are reduced for homofermentative LA bacteria (LAB) but enhanced for heterofermentative LAB (Giacon *et al*., 2021).

2.10 The process of detoxifying hydrolysates

One common strategy to reduce the inhibitory effect on LA producers and boost production rates is to detoxify pretreated lignocellulosic biomass (Abdel-Rahman, Hassan *et al*., 2021). A range of mechanical, chemical, and microbiological detoxification techniques can be applied to lower inhibitor concentrations prior to enzymatic processing and breakdown (Jungmeier, 2017). This technology includes steps like enzymatic treatment, heating and vaporisation, liquid–solid extraction, liquid–liquid extraction, and overliming with calcium carbonate or calcium hydroxide. It also includes the use of chemical additions like alkali, reducing agents, and polymers. Ion exchange and activated carbon treatment are two examples of techniques covered by liquid-solid extraction (Jönsson & Martín, 2016).

2.10.1 The biological approach

Researchers have looked into using microorganisms as a possible safe and eco-friendly way to lessen the harmful impacts of inhibitory materials such as lignocellulosic hydrolysates (Kumar *et al*., 2020). The popularity of biological detoxification techniques can be attributed to their ease of use, low side effects, softer working conditions that do not require additional chemicals, and lower energy consumption (Giacon *et al*., 2021). During fermentation, *L. plantarum* detoxifies the hemicellulosic hydrolysate obtained from sugarcane. At the conclusion of the fermentation process, 98% furfural and 86% HMF were lost, resulting in a final lactate content of 34.50 g/L (Bazoti *et al*., 2023). Furan inhibitors (furfural and HMF) up to 5.0 g/L could be detoxified by a novel *B. coagulans* Az-10, which could also withstand larger concentrations of most lignocellulose-derived major inhibitors, such as furans, weak acids, and phenolic compounds (Abdel-Rahman *et al*., 2013).

2.10.2 The Overliming technique

Due to its low cost, overliming—the addition of an alkali, such as calcium hydroxide—is one of the most popular purification techniques for adsorbing or precipitating inhibitors from the the hydrolysate. Using an acid-base combination to adjust pH is a low-cost treatment that produces excellent results (Avanthi *et al*., 2017). When S. cerevisiae is used to produce bioethanol from lignocellulose, alkaline detoxification enhances the hydrolysates' fermentability (Alriksson *et al*., 2006). Following

overliming, solid calcium hydroxide was used to adjust the reaction mixture's pH to about 10.0. After around 30 minutes of heating the contents to 50°C, the pH was then brought to 7. Filter paper was used to screen the mixture (Kucera *et al*., 2017).

2.10.3 Using activated charcoal

Activated charcoal is an affordable inhibitor removal method that is easy to use and has strong adsorption capabilities (Kaur *et al*., 2020). Activated carbon is frequently used to remove particles from liquid states via adsorption in order to sanitise, purify, or recover synthetic substances or compounds (Abdel-Rahman, Hassan, Fouda, *et al*., 2021). Sap (100 mL) and 2.5 weight percent activated carbon were combined in a 250 mL conical flask, and the mixture was kept at 4°C for approximately five h. For fifteen minutes, the resultant solution was spun down at 7000×g. After that, the supernatant was extracted and put in a mixing bowl along with 30 g of calcium carbonate and 10 g of yeast extract (pre-treated sap liquid) (Kunasundari *et al*., 2017). Adsorbents include chitin, peats, zeolites, clays, chitosan, and siliceous materials; only activated charcoal and resins have industrial uses (Costa-Trigo *et al*., 2020).

2.10.4 Resins with ion exchange

Since chelating resins may be recycled and regenerate, they are one of the most efficient and economical methods of eliminating inhibitory compounds [158]. More phenols, aldehydes, furans, and alkyl acids were removed from the liquid fraction by anion exchangers, which also produced 1.71 g/L/h and 0.46 g/g of ethanol, respectively. Although cationic resin productivity was low (0.65 g/L/h), the yield was comparable (0.45 g/g). Unprocessed hydrolyzates were less profitable and productive (Abdel-Rahman *et al*., 2021). For 30 minutes at room temperature in a batch system with stirring, concentrated and neutralised hydrolysate was mixed with the cation exchanger, for example, Amberlite IR-120 in hydrogen ion state, at a rate of 10 g of ion - cation exchanger per 1 g of acetic acid. in the the hydrolysate (Domínguez *et al*., 2021).

2.11 Fermentation of LA

Following the discovery of *Lactobacillus* species by scientist Louis Pasteur in 1856, fermentation was extensively used to manufacture LA (Ameen & Caruso, 2017). The hydrolysis products, such as C- 6 sugar glucose from cellulose and C-5 sugars such as pentose and arabinose from hemicellulose, are transformed into LA by LAB.

Nutrients are supplied by the fermentation broth, and the fermentation process starts when cultured LAB is added to the sugar solution (Ajala *et al*., 2020). pH, temperature, nutrition, inoculum size, sugar, the final product, by-product concentration, and sensitivity to hazardous chemicals are factors that affect LA fermentation (Rawoof *et al*., 2021). Using batch, repeated-batch, fed-batch, partially continuous fermentation (Vijayakumar *et al*., 2008), SSF, and SHF (Wang *et al*., 2017), lactate production was investigated. Hydrolysis and fermentation are two of the many processes that are carried out in different compartments during the treatment of biomass hydrolysate. This approach is called SHF, or separate hydrolysis & fermentation. Simultaneous saccharification and fermentation, or SSF, is the term for fermentation that occurs in a single unit (Srivastava *et al*., 2021).

Due to high cellular density and cell regeneration, the first technique for D-LA production to be reported was continuous fermentation, which resulted in an enhanced performance of 18 $gL^{-1}h^{-1}$ at a dissolution ratio of 0.87 per h (Abdel-Rahman *et al*., 2011). Fermentation in a closed batch method yields less volumetrically but has the highest LA bioconversion ratio when compared to continuous processes (Ahmad *et al*., 2020). A synchronous transformation of sugars under fed batch processing with naturally occurring E. mundtii produced 87.9 g/L enantiomerically pure L-LA, with maximal efficiency of 3.25 $g\ L^{-1}h^{-1}$ and yield above 1 $g\ g^{-1}$ in the feed intake stage using HDSS as a carbon source (Hoheneder *et al*., 2021).

B. licheniformis BL1 was reported to ferment glucose to optically pure L-lactate at 50 ◦C with fermentation productivity of 25.4 g/L in Luria– Bertani broth (Wang *et al*., 2011). *B. coagulans* C106 was found to convert both pentoses and hexoses to LA at pH 5, 50 °C (Ye *et al*., 2013). *Bacillus coagulans* strain IPE22 was found to produce 38.73 g/L LA from pretreated wheat straw with dilute sulphuric acid 2% (w/v) at 40 °C after 60 h of incubation, whereas using DGS15 upon pretreatment with 1% sulphuric acid, 32.5 g/L LA was produced after 60 h at 50 °C, pH 7, 120 rpm. 0.59 g/g LA was produced by *L. brevis* whereas *L. pentosus* produced 0.88 g/g LA from wheat straw hydrolysates pretreated with 4% sulphuric acid (Garde *et al*., 2002). Wheat straw pretreated with lime produced 40.71 g/L LA by using *B.* coagulans DSM 2314 after 60 h of incubation at 50 °C (pH 6.0) by using cellulase enzyme (Maas *et al*., 2008). 56.37 g/L L-LA was recovered from lignocellulosic hydrolysates with mesophilic *L. delbrueckii* and *L. plantarum* by simultaneous saccharification and fermentation (SSF)

(Sreenath *et al*., 2001). Alkali treatment of wheat straw hydrolysate removes lignin and fermentation of sugars become efficient to produce LA. High L-lactate production (56.37 g/L) by thermophilic *Bacillus* sp. NL01 was obtained from hydrolysate of lignocellulosic biomass, which contained the solid residue produced upon enzymatic saccharification (Ouyang *et al*., 2013). *Bacillus* strain is the most common genera for producing L-LA from lignocellulosic biomass (Table 2.3). *B. coagulans* MA-13 produced 29.7 g/L LA from lignocellulosic hydrolysate at 55 °C and pH 5.5 (Aulitto *et al*., 2017). *B. licheniformis* TY7 was isolated as a thermotolerant L-LA-producing bacteria, growing best at pH 6.5 and 30 °C, with normal growth at 65 °C. The strain produced 40 g/L L-LA with 97% optical activity and 2.5 g/L productivity using raw kitchen waste at 50 °C (Sakai & Yamanami, 2006). The *Bacillus* sp. strain XZL9 isolated by (Wang *et al*., 2011) produced 74.7 g/L LA from a variety of carbohydrates present in corncob molasses such as xylose, arabinose and glucose. The mixed cultures of *L. rhamnosus* and *L. brevis* were studied, in order to maximise the utilisation of C6 (cellulosic sugars) and C5 (hemicellulosic sugars) for the efficient production of LA (Cui *et al*., 2011). It was found that the mixed cultures upon simultaneous saccharification and fermentation (SSF) of maize stover treated with NaOH produced a LA yield of 0.70 g/g, that was estimated to be 18.6% and 29.6% higher than that of *L. rhamnosus* and *L. brevis* single cultures, respectively (Garde *et al*., 2002) . A hemicellulose hydrolysate (HH) of wet-oxidized wheat straw was used to test the LA generation capabilities of *L. brevis* and *L. pentosus*. Wide range of sugars produced by the acid and enzyme treatments could not be fully utilized by either of the two strains on its own, but by co-inoculating the two strains, the LA production was boosted to 95% of the theoretical maximum output (Garde *et al*., 2002). Co-cultivation involves using two compatible microorganisms that do not exhibit antagonistic effects toward one another (Gupta *et al.*, 2015).

2.12 Comparison of LA production from lignocellulosic biomass with other studies

In order to produce LA from lignocellulosic biomass, a significant amount of research has been conducted till date (Table 2.3). Historically, a single cultured microorganism has been employed in the microbial production of chemicals to facilitate the fermentation of substrate to finished product. More and more studies are now focusing

on utilizing a flexible co-culture technique, which involves co-growing microbes to enable a divide-and-conquer strategy for biochemical production (Jones & Wang, 2018). Liquefied cassava bagasse supernatant was used as a substrate with the combined culture of *L. rhamnosus* and *B. coagulans* to produce 31.0 g/L LA (Chen *et al*., 2020). *L. brevis* and *L. pentosus* were co cultivated on enzymatic hydrolyzed wheat hemicellulosic hydrolysate resulting in 95% yield by utilizing all the sugars (Y. Zhang & Vadlani, 2015) . Similarly, using poplar hydrolysate as substrate and sequential fermentation using *L. brevis* and *L. plantarum*, the initial concentrations of glucose and xylose were 35.4 g/L and 14.3 g/L, respectively, the glucose was entirely metabolised in 26 h. After 96 h of fermentation, xylose (2.2 g/L) was still present. There was no ethanol found, and 38.0 g/L LA content was observed (Zhang & Vadlani, 2015) . Higher bioethanol and LA titers were obtained by co-inoculation of *Kluyveromyces marxianus* CECT 10875 and *B. coagulans* A20 than by sequential inoculation (Demiray *et al*., 2024). Thus, the favorable effect of co-inoculation was explained by the results pointing to synergistic interactions between microorganisms. In a study led by (Zou *et al*., 2021), engineered *Pseudomonas putida* KT2440 and *B. coagulans* NL01 together contributed towards high yield of LA 0.8 g/g. It was noticed that the process of fermentation was improved significantly due to the co-culture system as the *P. putida* KT2440 exhibited tolerance to inhibitors while *B. coagulans* utilised sugars present in the hydrolysate (Zou *et al*., 2021). In 2018, Shahab *et al* showed that the combination of *L. pentosus and T. reesei* could co-ferment hexoses and pentoses from beech wood that had been processed without detoxification, without the need for carbon catabolite suppression. The findings demonstrated the possibility of creating microbial consortia to facilitate the conversion of lignocellulosic biomass into high-value biochemicals (Shahab *et al*., 2018). Using whey permeate (lactose), a mixed batch technique involving *L. delbrueckii and L. lactis* TSG3 exhibited an improved balanced yield that was 0.90 g/g and approximately 45 g/ L- D-LA (Sahoo & Jayaraman, 2019). Using a pre-hydrolysis and simultaneous saccharification and co-fermentation (SScF) procedure, the isolated strains of *Lactiplantibacillus plantarum* TSKKU P-8 and *Levilactobacillus brevis* CHKKU N-6 were utilised to convert cellulose and xylan in sugarcane bagasse (SB) into LA (LA) (Haokok *et al*., 2023). The process produced 91.9 g/L of LA, resulting in a volumetric productivity of 0.85 g/L/h. Through mixed fermentation of *B. coagulans* and *L. rhamnosus* in non-sterile conditions, the generation of l-LA from sweet sorghum juice was optimized. This resulted in a LA concentration

of 121 g/L, yielding 0.94 g/g and an overall productivity of 2.18 g/L/h (Wang *et al*., 2016).

There are three primary steps for producing LA from lignocellulosic biomass: hydrolysis, pretreatment and fermentation. Microbial fermentation and hydrolysis can be done together with simultaneous saccharification and fermentation (SSF) or separately with separate hydrolysis and fermentation (SHF) (Yankov, 2022).

Since fermentation can be done in the same vessel as hydrolysis to prevent end-product inhibition of the enzymes, SSF has emerged as the favored method during the last ten years (Zou *et al*., 2021). Because fewer tanks are needed, the plant's capital investment cost is therefore decreased (Li *et al*., 2021). A drawback of SSF is that, unlike SHF, where these parameters can be adjusted independently, the operating temperature of SSF must be a compromise between the ideal temperatures for fermentation and hydrolysis (Baral *et al*., 2021).

In general, hemicellulose content is high in rice straw and other agricultural leftovers (Li *et al*., 2021). Thus, for these lignocellulosic raw materials, xylose fermentation is crucial for efficiently converting all of the sugars into LA and raising the concentration in the fermentation broth. Through the homo fermentative pathway, bacteria are able to ferment both xylose and glucose into LA at the same time. First, bacteria convert xylose to xylulose-5-phosphate (X5P), which is then processed via the pentose phosphate pathway (PPP) and the phosphoketolase (PKP) pathway to produce acetic acid and LA (Huang *et al*., 2021). Also, the efficiency of the bacteria for usage in large-scale production because they are resistant to inhibitors like furfural and HMF (Karcher *et al*., 2015).

Table 2.3: LA production, yield and productivity from various substrates by distinct microorganisms and using different methods of fermentation

S.No	Substrate	Fermentation Method	Cultivation of Microorganism	LA (g/L)	LA yield (g/g)	LA productivity (g/l/h)	Reference
1	Rice straw hydrolysate	Separate Hydrolysis and Co culture	*B. licheniformis and B. sonorensis*	49.75	0.97	1.036	Our study
2	Cassava bagasse	Simultaneous saccharification and co-fermentation	*L. rhamnosus and B. coagulans*	112.5	0.88	2.74	(Abdel-Rahman *et al*., 2021)
3	Wheat straw hydrolysate	SSF	*L. brevis and L. pentosus*	19	-	-	(Tu *et al*., 1992)
4	Poplar Hydrolysate	SSF	*L. brevis and L. plantarum*	38	0.8	0.4	(Kshirsagar *et al*., 2015)
5	Pomegranate peel	SSF	*K. marxianus CECT 108755 and B. coagulans A20*	-	0.98	-	(Demiray *et al*., 2024)
6	corn stover hydrolysate	Sequential Hydrolysis and Co culture	*P. putida KT2440 and B. coagulans NL01*	35.8	0.8	0.37	(Shahab *et al*., 2018)
7	Microcrystalline cellulose	Consolidated Bioprocessing (CBP)	*T. reesei and L. pentosus*	34.7	0.62	0.16	(Zou *et al*., 2021)
8	Beech wood	Consolidated Bioprocessing (CBP)	*T. reesei and L. pentosus*	19.8	-	0.1	(Zou *et al*., 2021)

9	Whey permeate	Batch	*L. delbrueckii and engineered L. lactis*	45	0.9	-	(Shahab *et al*., 2018)
10	Sugarcane Bagasse	Simultaneous saccharification and Co fermentation	*LactiplantiBacillus plantarum and LeviLactobacillus brevis*	91.9	-	0.85	(Li *et al*., 2021)
11	Sweet sorghum juice	Batch	*B. coagulans LA1507 and L. rhamnosus LA-04-1 (Mixed culture)*	121	0.94	2.18	(Wang *et al*., 2016)
12	Corn stover hydrolysate	SSF	*L. rhamnosus and L. brevis*	14.8	0.7	0.58	(Cui *et al*., 2011)
13	Cassava bagasse hydolysate	Simultaneous saccharification and co-fermentation	*L. rhamnosus and B. coagulans*	31	0.91	1.5	(Chen *et al*., 2020)
14	Wheat straw hydrolysate	Batch	*L. brevis and L. plantarum*	38	0.8	0.4	(Zhang & Vadlani, 2015)
15	Kitchen waste	Batch	*B. coagulans and B. thermoamylovorans*	39.2	-	1.09	(Tashiro *et al*., 2016)
16	Wheat straw hydrolysate	SHF	*L. pentosus CECT 4023 T*	12.58	0.55	0.22	(Cubas-Cano *et al*., 2020)
17	Rice straw hydrolysate	SHF	*L. sp. TERI-D3*	11.16	0.67	-	(Verma & Subudhi, 2021)
18	Wheat straw hydrolysate	SHF	*B. coagulans CC17A*	26.30	0.71	0.25	(Ouyang *et al*., 2013)
19	Corn stover diluted acid hydrolysate	SSF	*B. coagulans GKN316*	35.37	0.83	0.91	(Sun *et al*., 2016)

20	Acid-catalyzed sulfite hydrolysate	SHF	*B. coagulans GKN316*	18.71	-	-	(Sun *et al*., 2016)
21	Lignocellulosic hydrolysate	SHF	*B. coagulans MA-13*	29.7–33.7	0.92	0.55	(Aulitto *et al*., 2017)
22	Pretreated wheat straw	Co-fermentation	*B. coagulans strain IPE22*	38.73	0.813	0.39	(Ouyang *et al*., 2013)
23	Corn stover hydrolysate	SHF	*B. coagulans*	32.8	0.94	0.33	(Garrett *et al*., 2015)
24	Kitchen refuse	SSF	*B. licheniformis TY7*	24-40	1.29-1.35	1.72	(Wang *et al*., 2019)
25	Hydrolysate of wheat flour	SHF	*L. delbrueckii subsp. bulgaricus ATCC 11842*	18–26	0.11–0.18	-	(Abedi & Hashemi, 2020)
26	Wheat straw	SHF	*L. brevis CHCC 2097 and L. pentosus CHCC 2355*	7.1	0.95	-	(Garde *et al*., 2002)

-: Data not provided

CHAPTER III: MATERIAL AND METHODS

Objective 1: Isolation, screening and characterization of thermotolerant LA producing bacteria

3.1 Collection of samples to isolate LA-producing microorganisms

Decaying wood samples from the barks of trees, soil and degraded wood samples from brick kilns, rotten samples from municipal corporation waste, soiled food waste samples, samples from food manufacturing units were collected from Patiala, Punjab. Samples collected from various places were processed for screening and characterization of microorganisms that produce LA. Samples were assessed for their physio-chemical characteristics, and bacterial isolates were examined for their capacity to produce LA. Additionally, morphological and biochemical characterization of the screened bacterial isolates were also employed.

3.2 Isolation of LA producing bacteria

In order to isolate bacterial strains, about 1 g of the collected sample was weighed, inoculated in 50 ml of nutrient broth (Appendix 1), and then incubated at 37ºC for 24 h under shaking condition at 120 rpm. All samples were serially diluted in saline (0.85%) and plated on NA plates in order to isolate pure cultures. Further, these plates were incubated at 37ºC.

3.3 Screening thermotolerant LA-producing microorganisms (Ye *et al*., 2013)

Colonies on nutrient agar were selected, inoculated into enrichment broth (Appendix I), and incubated at 50ºC without agitation. These isolates were serially diluted and then plated on agar plates containing 0.2 g/l bromocresol green, 10 g/l agar, 5 g/l yeast extract, and 10 g/l xylose. The plates were then incubated at 50ºC for 24-48 h until the appearance of colonies with clear colour change from green to yellow. A sterile loop was used to pick up the isolated colony, which was then grown in modified nutritional media (Appendix 1) and incubated at 50ºC until the absorbance reached 600 nm. The process was repeated using bacterial strains that had already been isolated.

3.4 Morphological and biochemical characterisation of bacterial isolates

All bacterial strains were biochemically and morphologically characterised using standard biochemical and physiological procedures as outlined in Microbiology: a Laboratory Manual, (Cappuccino & Welsh, 2018).

3.4.1 Gram staining

The glass slide was washed and thoroughly cleaned using distilled water and was air dried. The water droplet was placed at the centre of the slide and the bacterial smear was prepared by picking a loop of bacteria from plate and transferring it to the slide and spreading it properly. The bacterial smear thus formed was air dried and then smear was fixed by heating it slightly. Heating is advantageous as it results in the binding of the cellular proteins because of which the cells sticks to the slides surface. Further, crystal violet which is the primary stain is added to the slide for 1 min and the slide was gently washed with water to remove the excess stain. Next, gram iodine was added to the slide for 1 min and was washed gently with water. Further, decolorizing agent was added to the slide for 5s and the slide was quickly washed under tap water. Next, the counter stain, safranin, was added for 1 min and washed under tap water to remove excessive stain. The slide was air dried and observed under microscope at different magnifications.

3.4.2 Catalase test

The glass slide was washed and thoroughly cleaned using distilled water and was air dried. At the centre of the slide, a drop of 3% hydrogen peroxide was applied. Using a sterile loop, a loop full of bacterial culture was taken aseptically in a laminar flow chamber and placed onto the hydrogen peroxide drop onto the slide. The outcomes were then observed.

Observations:

1. If there is bubbling or foaming in the drop- Catalase positive
2. If there is no bubbling or foaming in the drop- Catalase negative

3.4.3 Nitrate reduction test

Since bacteria may produce the enzyme nitrate reductase, the ability of bacteria to convert nitrates to nitrites was tested using nitrate broth medium (Appendix I). Nitrate is the primary ingredient in nitrate broth medium. After weighing and dissolving the components of the nitrate broth medium in 100 millilitres of distilled water, the pH was brought to 7.2. Test tubes containing approximately 10 millilitres of broth were filled with the mixture and autoclaved for 15 minutes at 121ºC and 15 psi pressure. Room temperature cooling was allowed for the tubes. In broth tubes, the bacteria are aseptically introduced, and they were then cultured for 48 h at 37°C. Subsequently, each test tube was filled with 0.5 ml of nitrate reagent solution A (Appendix II) and nitrate reagent solution B (Appendix II), shaken thoroughly, and after 15 minutes, a colour change was noticed.

Observation:

1. Red colouration: Positive nitrate reduction
2. No red colouration: Negative nitrate reduction

3.4.4 Methyl red test

The MR test was performed to determine whether bacteria could use any monosaccharide sugar to produce acids such as LA, acetic acid, or formic acid. The components used for the MR broth medium (Appendix I) were weighed, dissolved, and the pH was adjusted to 6.9 in 100 mL of distilled water. The test tubes containing the broth were filled with 10 ml each with broth and autoclaved for 15 minutes at 121ºC and 15 psi of pressure. Using a sterile loop, the test bacteria were aseptically injected into broth tubes, and the tubes were then incubated at 37ºC for 24 to 48 h. Methyl red acetate solution (Appendix II) was added to each test tube.

Observations:

1. Red colouration observed: positive MR
2. No production of red colour: MR negative

3.4.5 Urease test

A urease test was conducted to determine whether bacteria could use the enzyme urease to hydrolyse urea into ammonia. Except for urea, all of the components of the urea agar medium solution (Appendix I) were weighed, diluted in 90 mL of distilled water, and the pH was adjusted to 6.9. The test tubes and urea agar medium, with the exception of urea, were sterilised for 15 minutes at 121ºC. Since heat cannot sterilise urea since it degrades when heated, a 20% solution of urea was prepared and sterilised using a membrane filtering device. 90 millilitres of urea agar medium were divided evenly across test tubes and mixed with 10 millilitres of sterile urea solution. In order to obtain urea agar slants, the test tubes were maintained in an inclined posture. Using a flamed needle, the bacterium was aseptically injected into the slants in a laminar flow chamber, and the samples were then incubated for 24 h at 37ºC.

Observations:

1. Pink colour production observed: positive for urease
2. No production of pink colour: Urease negative

3.4.6 Voges-Proskauer test

To determine if bacteria could transform glucose into acetoin (acetylmethylcarbinol), the Voges-Proskauer test was used. After weighing and dissolving the components of the VP broth medium (Appendix I) in 100 mL of distilled water, the pH was brought to 6.9. Test tubes containing approximately 10 mL of broth are filled with it and sterilized for 15 minutes at 121ºC (15 psi pressure). Using a sterile loop, the test microorganisms were aseptically inoculated. The broth tubes that were infected were kept in an incubator at 37ºC for a duration of 72 h. One mL, or VP solution 1 (Appendix II), was added to each test tube. Additionally, 0.6 ml of VP solution 2 (Appendix II) was added to each test tube, gently mixed, and left to stand for a duration ranging from 30 to 2 h.

Observations:

1. The colour rose pink was produced: VP positive
2. No production of rose pink colour: VP negative

3.4.7 Carbohydrate fermentation test

The purpose of the test was to evaluate the bacteria's capacity to ferment carbohydrates, namely sugars. The components of the carbohydrate broth medium (Appendix I) were weighed out to be 100 mL, dissolved in 100 mL of distilled water, and then the pH was brought down to 6.8 using either 0.1 N HCl or 0.1 N NaOH. Test tubes containing roughly 10 mL of broth were filled. Each test tube's broth was filled with the Durham tube inverted. The test tubes were sterilised for 15 minutes at 121°C and 15 psi of pressure. Using a flame-sterilized loop, the bacteria were introduced into the soup. For a whole day, the infected broths were incubated at 37ºC.

Observations:

1. If the broth turns yellow and gas builds up in the Durham tube, the bacteria are aerogenic and fermentative
2. If the broth becomes yellow in colour but no gas builds up in the Durham tube, it is anaerogenic and fermentative.
3. If the broth's colour does not change, the bacteria are not fermentative when it comes to carbohydrates.

3.4.8 Starch hydrolysis test

The purpose of the starch hydrolysis test was to determine whether bacteria could create the enzyme "α-amylase" and whether they could hydrolyse starch. After weighing and dissolving the components of the starch agar medium (Appendix I) in 100 millilitres of distilled water, the pH was adjusted to 7.2. For fifteen minutes, the flask holding the starch agar medium was sterilised at 121ºC and 15 psi of pressure. Starch agar was put aseptically onto petri dishes while it was still warm and molten, and it was cooled for approximately one h at 37ºC. The test bacteria were inoculated onto plates aseptically in a laminar flow chamber. The inoculation plates were incubated for 24 to 48 h at 37ºC, or until the bacterial colonies were visible. Lugol's iodine solution was poured over the plates (Appendix II).

Observations:

1. Clear, transparent zones developed surrounding bacterial colonies: positive for starch hydrolysis

2. Transparent, transparent areas that do not form around bacterial colonies: negative starch hydrolysis

3.5 LA production by bacterial isolates at different temperature

The isolated bacterial strains were revived in enrichment broth(Ye *et al*., 2013) that consists of 10 g/L xylose and 5g/L yeast extract and effect of temperature was checked by incubating at different temperatures (25°C, 37°C and 50°C). Bacterial growth was observed at absorbance of 600nm after 48 h followed by LA estimation.

3.6 LA production by bacterial isolates in minimal media supplemented with xylose

Bacterial isolates were inoculated in Bushnell Haas minimal media (HiMedia) at pH 7 supplemented with carbon source xylose (10 g/L) and 5g/L yeast extract and incubated at 50°C with agitation at 120 rpm for 48 h thereafter LA production was estimated and most competitive strain was selected.

3.7 Estimation of LA

3.7.1 Treatment of fermentation broth (Barker, 1957)

The Summerson and Barker, 1957 approach was employed to estimate LA. 1ml from the fermentation broth was taken and was centrifuged at 10,000 rpm for 5 minutes. Upon centrifugation, the supernatant was taken and trichloroacetic acid (100 µl) was added to it. Further, centrifugation at 10,000 rpm was done for 5 minutes for the precipitation of the proteins. Further, the supernatant was treated with 1ml of 20% copper sulphate, pentahydrate ($CuSO_4.5H_2O$). Further, calcium hydroxide (1g) was added to the suspension. Distilled water was used to get the final volume down to 10 ml. After thoroughly mixing the components with a vortex, the solution was centrifuged for 10 minutes at 10,000 rpm. Then, the supernatant was taken apart to estimate the amount of LA. For the standard LA solution, no such process was carried out.

3.7.2 Spectrophotometric analysis of LA (Barker, 1957)

For the purpose of the spectrophotometric analysis of LA estimation, 100µL of the sample was diluted with distilled water. 50 µL of pentahydrate (4% copper sulphate) was added. Following each step, the liquid was vortexed and 6 mL of concentrated sulphuric acid was added. After five minutes at 95–100˚C, the sample was cooled to room temperature. Following a vortex and a 30-minute rest at room temperature, 100µL of 1.5% pPP reagent was added. To stabilise the colour, the mixture was placed in a boiling water bath for ninety seconds. At 560 nm, the absorbance in comparison to the blank was measured. To determine the amount of LA in the samples, 1.2g/L of an 81% LA stock solution was used to plot the standard curve (Appendix 2)

3.8 Tolerance to inhibitors

The effect of inhibitors such as furfural and 5- hydroxymethylfurfural (HMF) on cell growth and LA production of *B. sonorensis* DGS15 and *B. licheniformis* DGB was evaluated by using a modified method (Liu *et al*., 2018). Cells were grown in Bushnell Haas medium (10 g/L xylose and 5 g/L yeast extract (pH 7.0) with different concentrations of furfural and HMF ranging between 0 and 2.4 g/L at 50 °C for 48 h (Liu *et al*., 2018). Broth samples were collected at specific time interval and optical density at 600 nm was measured to describe the inhibition of cell growth. The value of relative optical density (ROD) at 620 nm was used to describe cell growth inhibition. The percent OD620 of the experimental flasks as compared to the control flasks (with no inhibitor) was used to calculate ROD (Zhang *et al*., 2014) .

3.9 ESTIMATION OF INHIBITORS (Tu *et al*., 1992)

3.9.1 5-Hydroxymethylfurfural

The standard curve was constructed using a 0.01 g/ml HMF stock solution.

700µL of 8×10^{-3} M thiobarbituric acid and 850µL of 4M hydrochloric acid were added to 100µL of HMF using a pipette. The mixture was incubated in a hot water bath at 40˚C. At 436 nm, the absorbance was measured. The concentration of HMF in enrichment broth, rice straw, and wheat straw was determined using the standard curve equation (Appendix 2).

3.9.2 Furfural A stock solution containing 0.002 g/ml was prepared using the same protocol as HMF. At 436 nm, the absorbance was measured. The concentration of

Furfural in enrichment broth, rice straw, and wheat straw was determined using the standard curve equation (Appendix 2).

3.10 MOLECULAR CHARACTERIZATION OF STRAIN DGS15 BY 16S rRNA ANALYSIS

Genomic DNA was isolated from bacterial strain DGS15 showing highest LA production using DNA Extraction Solution (Cat No. 2120600021730) and 16S rRNA gene was amplified by polymerase chain reaction (PCR) by using primers 27 F (5 - AGAGTTTGATCCTGGCTCAG - 3) & 1392 R (5 - GGTTACCTTGTTACGACTT - 3). 16S rRNA gene sequencing of isolate DGS15 was done by Genei Laboratories Pvt Ltd and found to be 1466 bp long gene sequence. In order to identify the strain, sequences which were closely related to the 16S rRNA gene of DGS15 were downloaded from the NCBI website (16S ribosomal RNA sequences (Bacteria and Archaea) database) using an algorithm Basic Local Alignment Search Tool (BLAST). Identification of 16S rRNA sequence of DGS15 was confirmed using available DNA data in EzTaxon-e database. Closely related sequences obtained were undergone CLUSTALW (pairwise alignment) and phylogenetic tree was constructed by MEGA X software, using neighbor-joining method with Kimura two-parameter distance model for calculating evolutionary distances (Kumar *et al*., 2018). Bootstrap analysis was performed with 1000 replications to assess the statistical support for the phylogenetic tree.

Objective 2: Production of LA from different lignocellulosic biomass

3.11 Collection and processing of agricultural feedstock

Rice straw and wheat straw were procured from the nearby villages of Patiala and were washed, cleaned, and dried at 50°C. Dry biomass was pulverised using a grinder, and the powder was sieved using a 0.5mm pore-size test sieve and was stored in air tight containers at room temperature.

3.11.1 Physico- chemical characterization of rice and wheat straw biomass

3.11.1.1 Moisture content

The oven drying test method described in ASTM 3173-87 was used to evaluate the moisture content of the biomass. A carefully blended biomass sample weighing 5.0 g

was dried in a hot air oven at 105 ± 5°C until a consistent weight was noted. The sample was dried and then placed in a desiccator to cool down without absorbing moisture. The percent moisture was calculated as follows:

Moisture (%) = $[(W_2-W_f)/ (W_f-W_1)] \times 100$

Where W_1 denotes the weight of the empty crucible, the weight of the crucible and sample is denoted by W_2, and W_f denotes the constant weight of crucible and sample after drying.

3.11.1.2 Ash Content

The methodology outlined in ASTMD 3174-04 was followed in order to determine the biomass's ash content. A 1.0 g oven-dried biomass sample was placed in a crucible, and the mass of the crucible was calculated in addition to the sample, in order to determine the ash content. The crucible was kept at 575 ± 10°C for four h in a muffle furnace. After being taken out of the furnace, the crucible was set into a desiccator. Until a steady weight was reached, the heating and cooling procedure described above was repeated. Unburned carbon and volatiles can be eliminated by this procedure.

Ash (%) = $[(W_f-W_1) / (W_2-W_1)] \times 100$

The weight of the silica crucible was denoted by W_1, the weight of the sample dried in oven and the weight of the crucible was denoted by W_2, and the consistent weight of the crucible and the sample after combustion was denoted by W_f.

3.11.1.3 Volatile matter

The method outlined in ASTM D 3175-07 was used to determine the biomass's volatile matter content. A 1.0 g sample of biomass was placed in a covered crucible, and the initial weight was recorded. To achieve quick heating, the covered crucible was put in a muffle furnace set to 950 10°C for 7 minutes. Following its removal from the furnace and cooling to room temperature in a desiccator, the covered crucible was weighed and documented. The percent volatile matter was taken to be equal to the percentage of weight loss.

Volatile matter (%) = $[(W_f-W_1) / (W_2- W_1)] \times 100$

The weight of the silica crucible was denoted by W_1, the weight of the sample dried in oven and the weight of the crucible was denoted by W_2, and the consistent weight of the crucible and the sample after combustion was denoted by W_f.

3.11.1.4 Fixed carbon

The total of the percentages of moisture, ash, and volatile matter deducted from 100 yielded fixed carbon. Every percentage was based on the comparable moisture standard.

Fixed carbon (%) = 100- (Moisture % + ash % + volatile matter %)

3.11.1.5 Estimation of cellulose

Anthrone Assay was done to estimate the amount of cellulose in samples of biomass (Updegraff, 1969). In order to do this, 0.1 g of biomass was added with 10 mL of acetic -nitric acid reagent (150 mL of eighty percent acetic acid combined to 15 mL of concentrated nitric acid), and the combination was then heated to 100 °C for 30 minutes. Following cooling, the material was centrifuged for 10 minutes at 8,000 rpm, and the supernatant was eliminated. Following two rounds of distilled water washing, 10 millilitres of 67% v/v sulphuric acid were added to the residue, and the suspension was left to stand for an h. The suspension was diluted to 5 times (50 mL volume), and 4 mL of Anthrone reagent was added to 1 mL of diluted mixture. After diluting the solution five times (to a volume of 50 mL), 1 mL of the diluted mixture was mixed with 4 mL of Anthrone reagent. To develop the colour, the entire mixture was incubated for 15 minutes in a bath of boiling water. Following tube cooling, the OD was measured at 620 nm using the reagent blank (1 mL Anthrone reagent and 4 mL distilled water). Using 0, 40, 80, 120, 160, and 200 µg/L of cellulose, a standard graph was drawn to determine the quantity of cellulose in the sample. The concentration of cellulose in native and pretreated rice straw, and wheat straw was determined using the standard curve equation (Appendix 2).

3.11.1.6. Estimation of hemicellulose

The methodology described by (Goering & Van, 1975) was used to estimate the amount of hemicellulose in the biomass samples.

Neutral detergent fiber (NDF) determination:

Biomass (1.0 g) was combined with 100 mL of cold neutral detergent solution (solution A) (18.61g of disodium ethylenediamine tetraacetate and 6.81g of sodium borate decahydrate) in 200 mL of distilled water and dissolved with intermittent heating. 30 g of sodium lauryl sulphate and 10 mL of 2-ethoxy ethanol were added to solution A. Disodium hydrogen phosphate (4.5 g) was added to this, and the volume was increased to 1 L while the pH was lowered to 7.0. 0.5 g of sodium sulphite and 2 mL of decalin (decahydronaphthalene) were added to this biomass detergent solution. The above mixture was brought to a boil and refluxed for one h. Sample was cooled, then filtered, and residue was obtained. The residue was collected in a pre-weighed crucible, cleaned with hot water and acetone, and baked for eight h at 100 ± 5 °C. Crucibles were weighed after drying, and the percentage of weight difference was reported as NDF.

$$NDF\ (\%) = [(W_f - W_1) / (W_2)] \times 100$$

The weights of the biomass sample (W2), the silica crucible (W1), and the crucible and NDF (Wf) are indicated.

Acid detergent fiber (ADF) determination:

The biomass sample (1 g) was transferred to a refluxing flask, and 100 mL of cold acid detergent solution (2 g CTAB in 100 mL 1N sulphuric acid) was added. The sample was refluxed as described for neutral detergent fibre (NDF), and the weight difference was reported as ADF. The hemicellulose content of the biomass samples was calculated as the difference between NDF (%) and ADF.

$$ADF\ (\%) = [(W_f - W_1) / (W_2)] \times 100$$

Wf is the weight of the crucible and ADF, W2 is the weight of the biomass sample, and W1 is the weight of the silica crucible.

$$Hemicellulose\ (\%) = NDF\ (\%) – ADF\ (\%)$$

3.11.1.7. Estimation of lignin

(TAPPI, 2011)was used to evaluate acid insoluble lignin (AIL), and (TAPPI UM 250, 1991)was used to estimate acid soluble lignin (ASL). 5 g of extractive less biomass sample were prepared using the previously stated methods of n-hexane, ethanol, and water extraction for lignin measurement. After the sample was completely dried in an oven at 60°C until a constant weight was reached, 1 g of the dried biomass was

weighed. In a beaker, 1 g of dried biomass sample was combined with 15 mL of 72% (v/v) sulphuric acid, and the mixture was well mixed with a glass rod. Beakers were submerged in a water bath at 30 °C for one h, with a stirring rod used every five to ten minutes. The beakers were taken out of the water bath after the hydrolysis process took 60 minutes to complete. The sample was diluted with 420 mL of distilled water to achieve a concentration of 4%. After that, the preparation was autoclaved for one h at 121°C. The samples were filtered after cooling. For AIL and ASL, respectively, the residual and filtrate were utilised. To calculate AIL, the residue was weighed following filtration. To calculate ASL, the filtrate's optical density was measured at 320 nm. Dilution variables were noted after the samples were diluted to an absorbance of 0.7–1.0. Sulphuric acid (4%, v/v) was utilized as a blank and to dilute the samples.

The amount of acid insoluble lignin (AIL) was determined using the formula below:

AIL (%) = (A/W) X 100

where W is the sample's oven-dried weight and A is the lignin's weight in grams.

ASL (%) = (UVabs × filtrate volume × dilution factor) / ε × B × a] × 100

Where dilution is the volume of sample diluted with a diluting solvent per volume of sample, ε is the absorptivity of biomass at a certain wavelength (113 L/g-cm), B is the sample's oven-dried weight, and an is the route length. The volume of filtrate is 440 mL.

AIL (%) + ASL (%) = Total lignin (%)

3.12 Pre-treatment of rice straw and wheat straw

3.12.1 Delignification of rice straw and wheat straw biomass

Rice straw and wheat straw biomass (25g) respectively were dried and grinded and were alkali pretreated with 1% sodium hydroxide (w/v) at 105 °C for 60 minutes to remove lignin (Peng *et al*., 2014). The black liquor (supernatant) was filtered using Whatman paper and the delignified lignocellulosic biomass of rice was washed and neutralized using deionized water and was dried at 50°C for 24h for further pretreatment by dilute sulphuric acid.

3.12.2 Optimization of pretreatment with dilute sulphuric acid using response surface methodology (RSM)

As described by (Peng *et al*., 2014), Box-Behnken Design (BBD) which is the Response Surface Methodological (RSM) method was employed to design the optimization experiment for the recovery of high concentrations of fermentable sugars using sulphuric acid (0.1-1%) pretreatment followed by hot water treatment (100 °C, 30 min) (Table 3.1).

Table 3.1: 3-factor BBD with respect to independent variables, actual and coded values for both rice straw and wheat straw pretreatment

Independent variables	Symbols	Coded and actual values		
		-1	0	+1
H_2SO_4 (%)	X_1	0.1	0.55	1
Temperature (°C)	X_2	60	90	120
Time (h)	X_3	15	60	105

The pretreatment was done on 25g delignified rice straw biomass using the autoclave at 15psi under respective concentrations of dilute sulphuric acid, temperature and time intervals as shown in Table 3.2

After being transferred to falcon tubes, the hydrolysates were centrifuged for 10 minutes at 8000 rpm. After preserving the supernatant (I), the pellet was combined with 100 ml of distilled water and autoclaved at 100˚C for 60 minutes. It was then centrifuged for 10 minutes at 8000 rpm. After being separated by centrifugation, the supernatant (II) was combined with the supernatant (I) and used calcium carbonate for detoxification. Using spectrophotometric analysis, the levels of the inhibitor and the reducing sugar in the hydrolysate were found. Characterizing the pellet that remained after pretreatment allowed for the conservation of the residual material and the determination of changes in the lignocellulosic biomass of rice and wheat straw.

Table 3.2 BBD and their responses for 3 level, 3 factor response surface analysis for rice and wheat straw

	Factor 1	Factor 2	Factor 3
Run	**H_2SO_4 (%)**	**Temperature (°C)**	**Time (h)**
1	0.55	120	105
2	0.55	90	60
3	0.55	60	15
4	0.55	90	60
5	0.55	90	60
6	0.55	90	60
7	0.1	120	60
8	0.1	60	60
9	0.55	90	60
10	1.0	120	60
11	0.1	90	105
12	0.55	60	105
13	0.55	120	15
14	1.0	90	15
15	0.55	90	60
16	0.1	90	150
17	1.0	60	60

3.12.2.1 Design of Experiment

To maximize the effective release of total reducing sugars in the hydolysate from delignified rice and wheat straw. Design Expert statistical software (StatEase version 13®) was utilized to design a mathematical model and determine the best parameters for maximal reducing sugar recovery in the hydrolysate. Box–Behnken experimental design (BBD) was used to test and evaluate the sugar recovery process factors The entire Box–Behnken experimental design (BBD) was made up of 17 possible trials (Table 1) showing optimization of different parameters such as sulphuric acid (H_2SO_4) concentration (A), hydrolysis temperature (°C) (B), and hydrolysis time (h) (C) for release of reducing sugar in the hydrolysate. A second-degree polynomial equation was obtained which is a desirable fit of the model with a quadratic response.

The coded and the actual values can be correlated using the (2,3) equations:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \quad (2)$$

The coded value is represented by xi.

The variable's value is represented by Xi.

The variable's value is encoded as X_0 at the centre.

The representation of the difference between X_i and X_0 is denoted by ΔX_i

The following equation and Design Expert v13 is used to determine the response:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (3)$$

Y denotes the response; A, B and C denote the variables which are independent; β_0 is the intercept; β_1, β_2, and β_3 are the linear coefficient; β_{11}, β_{22} and β_{33} are the square coefficient and β_{12}, β_{13}, and β_{23} are the interaction coefficient.

The main effects of each pretreatment parameter and the regression coefficients were estimated using a variance test (ANOVA). The level of significance of all factors in the quadratic model was determined by computing the F-value at the probability of 0.05. The estimated coefficients were utilised to perform statistical computations in establishing three-dimensional contour lines from the regression analysis.

Using an ultraviolet-visible spectrophotometer (U-2900, HITACHI), the reducing sugar at 540 nm in rice straw hydrolysate after pretreatment was estimated following the Dinitro salicylic (DNS) assay (Miller, 1959). All the experiments were performed in triplicates.

3.13 Estimation of reducing sugar (Miller, 1959)

1ml of DNSA reagent was added to each 1 ml of sample (DNSA composition: 1g DNSA reagent, 30g of sodium potassium tartarate,1.6 gm NaOH, 100 ml distilled water). The tubes were placed in boiling water bath for 10 minutes. Absorbance was taken at 540 nm. The concentration of reducing sugar in enrichment broth, rice straw, and wheat straw was determined using the standard curve equation (Appendix 2).

3.14 Estimation of xylose in biomass sample (Fernell & King, 1953)

To 1ml of sample, 3mL of orcinol reagent was added and incubated at 100^0C for 20 min and absorbance was recorded at 660 nm. Standard curve for O.D v/s xylose

concentration (g/L) (Fig. 8) was plotted using 0.2, 0.4, 0.6, 0.8 and 1.0 g/L xylose, and estimating xylose as above.

The concentration of xylose in enrichment broth, rice straw, and wheat straw was determined using the standard curve equation (Appendix 2).

3.15 Morphological and structural characterization of the biomass

3.15.1 Scanning Electron Microscopy (SEM) of biomass samples

The SEM analysis was done to check the physical appearance of the dried native rice straw and wheat straw biomass. Furthermore, the changes in the appearance of the processed biomass pellet were studied using SEM-JEOL JSM 6510-LV, USA, where the samples were coated with gold to give conductivity using gold sputtering at a voltage of 10-15 kV.

3.15.2 X Ray Diffraction (XRD): XRD analysis was done to assess the changes in the crystallinity of the native biomass following pretreatment. The PANalytical X'Pert Pro X-ray diffractometer was put into practice for the examination of the crystallinity index of the materials. The samples were subjected to monochromatized copper potassium alpha radiation (1.5406) and assessed at a potential of 45 kV and an applied current of 40 mA within the scan range of 2theta, or 5° to 50°. (Segal *et al*., 1959) computed the Crystallinity index (*CrI*) as follows:

$$CrI = \frac{(I_{002} - I_{am})}{I_{002}} \times 100 \quad (1)$$

The peak for the crystalline nature (cellulose) of rice straw was determined by I002 at 2θ value of 22.5°, while the amorphous portion (cellulose and hemicellulose) was determined using the peak at I_{am} at 2θ value of 18° [40].

3.15.3 FTIR: To examine the structural and chemical characteristics of biomass, a Perkin Elmer-Spectrum 400 FT-IR/FIR spectrometer with a resolution of 1 cm^{-1} was employed. Using the KBr disc approach, the spectrogram was recorded in the spectral IR range spanning from 4000 to 400 cm^{-1}. Each spectrum was the average of 64 scans, with a total scan time of 15 seconds.

Objective 3: Optimization of LA production from lignocellulosic hydrolysates

3.16 Microbial culture and growth conditions

B. licheniformis DGB (Akhtar *et al*., 2016) and *B. sonorensis* DGS15 were revived in 5 mL of enrichment media consisting of 10 g/L of xylose and 5 g/L of yeast extract of pH 7 in 25mL culture tubes separately and kept at 50°C in an incubator for 24 h at 120 rpm.

3.16.1. Shake flask fermentation in minimal media

Shake flask fermentation was performed in 500mL Erlenmeyer flasks at 120 rpm containing 3.27 g/L of Bushnell Haas medium (HiMedia) supplemented with 10g/L glucose and xylose in the ratio 1:1 and 5 g/L of yeast extract, pH 7.0 which was maintained using Ca $(OH)_2$, 35 % (w/v) at regular intervals. The temperature was maintained at 50°C separately for the mono-culture of both DGB as well as for DGS15.

Similarly, in a co cultivation experiment, both DGB and DGS15 were inoculated with inoculum (1% w/v) at the same time in the beginning of the fermentation and for each strain.

3.16.2. LA production from hydrolyzed rice straw and wheat straw by co-cultivation of *Bacillus* strains

50g rice straw was delignified using 1% sodium hydroxide (w/v) at 105 °C for 60 minutes to remove lignin (Peng *et al*., 2014). Further the delignified biomass was pretreated using optimized pretreatment of dilute sulfuric acid under ideal conditions (0.55 % (v/v) H_2SO_4 concentration, 120°C pretreatment temperature and 105 minutes of hydrolysis time) followed by hot water treatment. The pH was set at 7 using Ca $(OH)_2$ (35 % w/v) and the hydrolysate thus obtained was supplemented with Bushnell Hass medium (3.27 g/L) and yeast extract (5 g/L). Separate experimentation was conducted as a monoculture of DGS15 (5 % v/v) and DGB (5 % v/v) in three replicates and also the co- cultivation of DGS15 and DGB (5 % v/v) was done separately and kept at 50°C in an incubator at 120 rpm in triplicates. A sample of fermenting broth was taken at regular intervals to determine the concentration of LA, left over glucose, and xylose. The pH of the broth was maintained using Ca $(OH)_2$ (35 % (w/v) at regular intervals.

3.16.3 Analytical method

Bacterial growth was measured 600 nm using UV–Vis spectrophotometer (U-2900, HITACHI. DNS assay and Orcinol assay method was used to measure reducing sugars glucose and xylose in the hydrolysate at 540nm and 660 nm respectively. The inhibitors furfural and HMF present in the hydrolysate upon pretreatment were determined using the Tu *et al*. spectrophotometric method at 414nm and 436nm, respectively. The spectrophotometric analysis for LA estimation was done using Barker-Summerson method at 560 nm. LA productivity (g/L per h) was calculated using the formula i.e. LA concentration (g/L)/ incubation time (h) and the yield was calculated using the formula gram equivalent LA (g)/ gram equivalent sugar utilized (g).

CHAPTER IV: RESULTS AND DISCUSSION

Objective 1: Isolation, screening and characterization of thermotolerant LA producing bacteria

4.1 ISOLATION AND SCREENING OF LA PRODUCING BACTERIA

LA producing bacteria were isolated from various locations of Patiala, Punjab such as food manufacturing units, paper mills, brick kilns, municipal compost pits, degraded bark of mango tree, degraded and decomposed wood (Table 4.1).

The screening was done on the basis that the isolated microorganism could efficiently utilize pentose as well as glucose sugar. Out of 58 isolates, 12 bacterial isolates were identified for the production of LA. Seven LA-producing bacteria were recovered from dairy waste and decaying wood samples. These bacteria were chosen based on how well they could grow on a medium that contained xylose as a carbon source. Growth was tracked using a UV-VIS spectrophotometer (Table 4.1) as xylose is transformed in the pentose phosphate pathway to pyruvate, which is created as an intermediary and gets converted to LA (Reddy *et al*., 2008) at a thermotolerant temperature of 50ºC (Payot *et al*., 1999). DGS15 was found to produce maximum LA after 48 h of incubation followed by DGN2 > DGS21 > DGB > DGS18 > NA9 > DGS20 > DGN1 > DGS3 > DGS14 > DGS1> DGS17 > DGS19 > NA15 > T8 > DGS16.

The experiment used bromocresol green media to examine the ability of various bacterial isolates (DGB, DGN1, DGN2, DGS3, NA9, NA15, T8, DGS1, DGS15) to produce LA. The figure shows the change in colour of the bromocresol medium where the presence of LA (LA) is shown by the pH indicator bromocresol green, which changes from green to yellow in response to acid generation.

However, the same concept—a colour shift from green to yellow in conditions containing bromocresol green—is used in the experiments conducted by (Ye *et al*., 2013) that verify that suggests that the bacteria being studied are producing LA. This is in line with accepted LA detection procedures, where the colour shift serves as an obvious visual cue that fermentation is producing acid (Fig. 4.2).

Table 4.1: Screening and production of LA by different bacterial isolates on xylose containing media

Source	Bacterial isolates	Location	Yield of LA (g/g)
Previously isolated	*B. licheniformis* (DGB)	Akhtar., 2014	0.415
Previously isolated	*B. licheniformis* (NA9)	Akhtar., 2014	0.403
Previously isolated	*B.* subtilis (NA15)	Akhtar., 2014	0.17
Municipal corporation waste	DGS18	30°18'41.7"N 76°25'30.5“E	0.405
Compost	T8	30°18'41.7"N 76°25'30.5“E	0.17
Bakery Waste	DGS3	30°19'24.2"N 76°24'02.0“E	0.228
Bakery Waste	DGS1	30°19'24.2"N 76°24'02.0“E	0.203
Soil sample (Red)	DGS15	30°19'42.0"N 76°27'26.9"E	0.452
Soil sample (Brick kiln)	DGS21	30°19'42.0"N 76°27'26.9"E	0.418
Wood sample (Brick kiln)	DGS14	30°19'42.0"N 76°27'26.9"E	0.214
Stone sample (Brick kiln)	DGS19	30°19'42.0"N 76°27'26.9"E	0.174
Wood sample (Brick kiln)	DGS16	30°19'42.0"N 76°27'26.9"E	0.152
Decaying Wood	DGN2	30°21'20.1"N 76°22'04.0"E	0.432
Decaying Wood	DGN1	30°21'20.1"N 76°22'04.0"E	0.315
Lignocellulosic sample (RS+WS)	DGS20	30°23'47.5"N 76°24'09.4"E	0.319
Water sample (Manikaran Sahib, H.P)	DGS17	32°01'37.6"N 77°20'42.3"E	0.178

Differential LA generation is indicated by the varying degrees of colour change that are displayed across the isolates in the figure. Comparing isolates like T8, which exhibit little to no change, to isolates like DGS15, DGB, NA15, and DGN2, which exhibit a noticeable change, could indicate increased acid production.

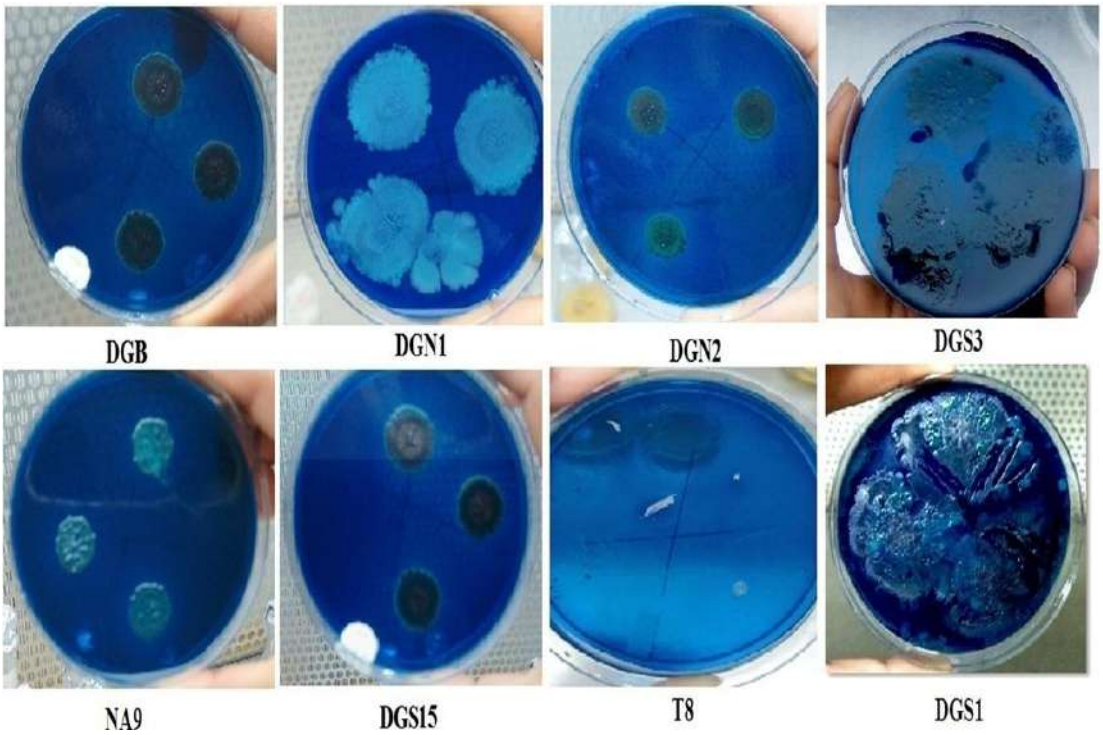

Figure 4.1: Colorimetric detection of LA production by bacteria-color shift to yellow observed after incubation with bacteria, indicating the production of LA

The color change in these plates supports the conclusions of the work that use the same principle of bromocresol green as a LA indicator, indicating that some isolates (e.g., DGB, DGS15) have a higher propensity for producing LA (Fig. 4.1).

Thus, the microorganism can efficiently bio transform sugars produced from lignocellulose which is a cocktail of C-5 and C-6 into LA upon pretreatment. Also, development of a low-cost method and selection of microorganisms that ferments beyond inhibitor barriers is important for lignocellulosic biorefineries scale up. Further, the microorganism is required which can effectively ferment sugars at comparatively higher temperatures (50 °C) (Zhang *et al*., 2014). Isolates producing more LA may be distinguished based on the intensity of the color change in the medium is supported by the agreement of these results with (Ye *et al*., 2013) 's experiment.

4.2 Production of LA in Bushnell Haas minimal media with xylose supplemented at various temperatures

The graphs represent the LA (LA) production by various bacterial isolates that were cultured in Bushnell Haas minimal media (3.27 g/L) supplemented with xylose (10 g/L) at varying temperatures (50°C, 37°C, and 28°C) after 48 (Fig 4.2) and 72 h (Fig 4.3). The bacterial strain and incubation conditions both influence the yield of LA, which is a critical indicator of bacterial fermentation activity. The information provides insights into the thermophilic and mesophilic properties of the bacterial strains under investigation by demonstrating notable trends in LA generation across isolates and temperatures.

The efficiency of fermentation and bacterial metabolism are strongly influenced by temperature. The LA production of the bacterial isolates in this investigation varied significantly depending on the temperature, with the maximum output across almost all isolates being observed at 50°C as compared with 37°C and 28°C. The result is especially important when using these bacteria in industrial settings because higher fermentation temperatures may encourage more effective fermentation (Yankov, 2022).

After 48 and 72 h, the majority of bacterial strains showed higher amounts of LA at 50°C. For example, strains DGS15, DGS1, and DGS16 produced more than 6 g/L of LA after 48 h, and this amount increased to nearly 8 g/L after 72 h. The fact that these isolates performed consistently over the course of two time points raises the possibility that they are thermophilic in nature, with higher metabolic activity at higher temperatures. Because mesophilic organisms can be inhibited by higher working temperatures, which lowers the requirement for sterilisation and operational expenses, thermophilic bacteria are frequently beneficial in industrial fermentation operations.

Conversely, nearly all isolates showed a significant decrease in LA generation at 28°C. Strains like as DGS18 and DGN2, for instance, produced less than 3 g/L after 48 h at this temperature, highlighting the isolates' sensitivity to temperature. Since these bacteria are probably less active in colder climates, using them in industrial settings at lower temperatures may not be feasible. The majority of isolates prefer higher temperatures, as evidenced by the trends at 37°C, which are higher than those at 28°C but still lower than those at 50°C.

As compared to 48 h, the LA production levels after 72 h increased, indicating that prolonging the fermentation period increases LA yield. The strains that demonstrated the most notable increases were those that were observed at 50°C, where several strains (such DGS1 and DGS19) increased by more than 2 g/L in 48 and 72 h. This delayed LA accumulation suggests that these bacteria can efficiently metabolize xylose for a prolonged amount of time, which is a trait that is desired in long-term fermentation operations (Zhang *et al*., 2014).

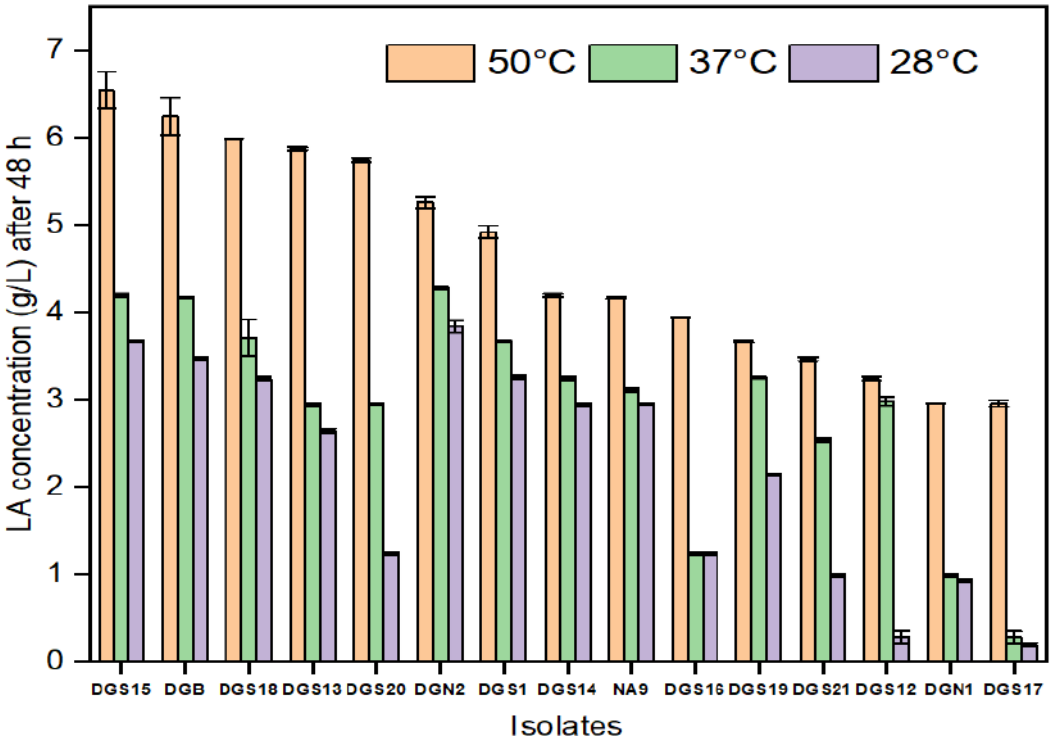

Figure 4.2: LA production in Bushnell Haas minimal media (3.27g/L) supplemented with carbon source xylose (10 g/L) at different temperature (50, 37, 28°C) after 48 h

However, even with longer fermentation durations, the generation of LA did not increase as much at 28°C as it did at higher temperatures. This could indicate that the LA production-related metabolic pathways are less active at lower temperatures, or that the bacteria entered a stationary phase earlier.

There are several benefits to doing bacterial fermentation at 50°C, particularly when LA generation is involved. This temperature is important since it is close to the sterilisation threshold, which is normally between 50 and 55°C. The growth of undesired mesophilic pollutants is reduced when fermentation is carried out at

temperatures close to sterilisation limits, creating a more regulated and sterile fermentation environment. This lessens the requirement for additional sterilisation processes or chemical preservatives, which can affect the quality of the product and are expensive. Furthermore, thermophilic bacteria are more resilient and able to endure harsher environmental conditions, which makes them appropriate for industrial fermentations on a large scale when sterility is difficult to maintain (Zhang *et al*., 2014).

In addition to exhibiting increased LA production, the isolates DGS1, DGS16, and DGS19 that flourished at 50°C in this investigation also showed promise for application in thermophilic fermentation systems. This is especially important for biotechnological applications where yield, cost-effectiveness, and efficiency are crucial. When fermentation is carried out at high temperatures, such as 50°C, the pace at which LA is produced can be increased while cooling costs and contamination hazards are decreased.

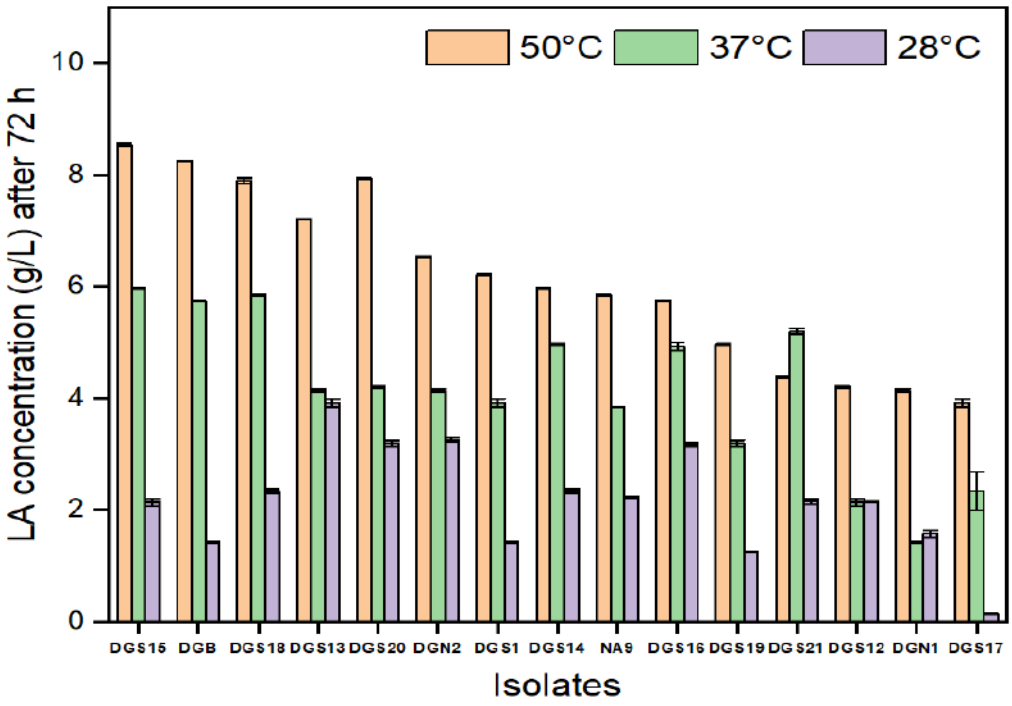

Figure 4.3: LA production in Bushnell Haas minimal media (3.27g/L) supplemented with carbon source xylose (10 g/L) at different temperature (50, 37, 28°C) after 72 h

In summary, this study's findings show that a variety of bacterial isolates' ability to produce LA is significantly influenced by temperature. The enhanced efficiency seen

at 50°C implies that thermophilic fermentation could be a feasible approach for the generation of LA in an industrial setting. Furthermore, 50°C is a highly desirable temperature for lowering contamination and raising the fermentation process's general efficiency because it may be used close to sterilisation temperatures without significantly diminishing yield (Yankov, 2022). The results of the study also highlight the significance of choosing bacterial strains that can continue to be highly productive at high temperatures. Three strains—DGS15, DGS16, and DGS19—consistently outperformed other isolates in thermophilic environments.

4.3 Producing LA at 50 °C in Bushnell Hass minimal medium by bacteria

LA (LA) is an important organic acid that finds extensive application in the culinary, pharmaceutical, and cosmetic sectors, in addition to biodegradable plastics. An environmentally friendly substitute for chemical synthesis is the microbial synthesis of LA, particularly when it is produced by fermentation. Both aerobic and anaerobic circumstances can effectively convert carbohydrates into LA by a number of bacterial strains, most notably LA bacteria (LAB). This work examines the ability of several bacterial isolates to produce LA when grown on Bushnell Hass minimal media supplemented with xylose and yeast extract at a thermotolerant temperature that is 50°C (Fig 4.4).

The bacterial strains that were isolated were cultivated in Bushnell Hass minimal media (3.27 g/L), which offers fundamental nutrients. In addition, xylose (10 g/L) was added as a carbon source and yeast extract (5 g/L) as a nitrogen source. The pentose sugar xylose, which is frequently present in hemicellulose, was selected to mimic the breakdown of lignocellulosic biomass, a crucial step in the synthesis of bio-based LA. Yeast extract is used to supply vital vitamins, peptides, and growth factors that improve bacterial metabolism. The capacity of the isolates to create LA at elevated temperatures—which might be favorable for industrial fermentation by lowering contamination risks and energy costs—was evaluated by incubating the media at 50°C, a thermotolerant condition.

The LA generation by the bacterial isolates over 48 and 72 h of incubation is shown in Fig 4.5. The findings show that all isolates produced considerably more LA between

48 and 72 h. DGS15, DGB, DGS18, DGS13, and DGS20 were the isolates that produced the most LA, with concentrations of 8 to 9 g/L after 72 h. After 72 h, isolates such as DGN2, DGS14, NA9, and DGS16 generated moderate levels of LA, ranging from 6 to 7 g/L. Conversely, isolates DGN1 and DGS17 showed minimal or nonexistent LA production.

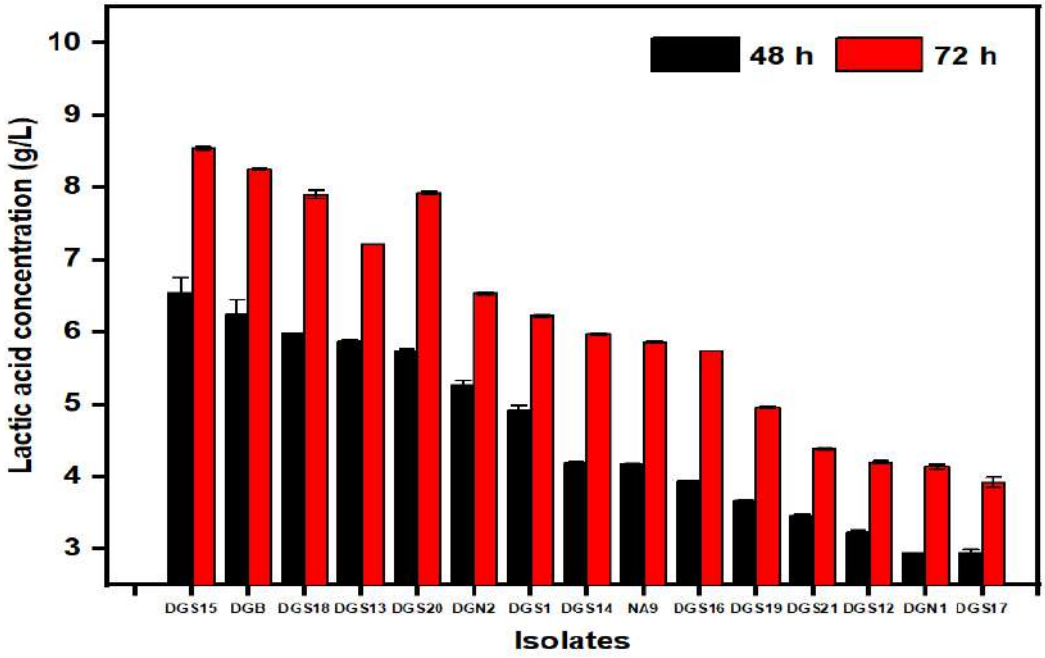

Figure 4.4: LA production by isolated bacteria in Bushnell Hass minimal media (3.27g/L) supplemented with carbon source xylose (10g/L) and yeast extract (5 g/L) producing LA at a thermotolerant temperature of 50° C

The fact that there was a 72-h rise in LA synthesis indicates that the bacterial strains were able to effectively use xylose as a carbon source over time. Because xylose is frequently present in lignocellulosic biomass, these bacterial strains have the potential to produce large amount of LA giving the bacteria the nutrients they needed to speed up the production of LA, the addition of yeast extract increased the bacterial metabolism even more. These strains' capacity to generate LA at 50°C further demonstrates their thermotolerant nature, which is very advantageous in bioprocessing applications. High fermentation temperatures make the process more energy-efficient by lowering cooling expenses and lowering the risk of contamination.

It's interesting to note that in earlier tests, the high LA producers in this study (DGS15, DGB, and DGS18) displayed a significant colour shift in the bromocresol green (BCG)

medium, indicating the formation of acid. The quantity of LA generated is directly correlated with the pH indicator BCG medium's colour change from green to yellow. The quantitative information derived from the LA assays is supported by this visual confirmation.

Significant LA generation was seen by the bacterial isolates evaluated in this investigation when they were grown at 50°C in Bushnell Hass minimum media supplemented with xylose and yeast extract. The most fruitful strains might make excellent candidates for industrial production of LA, particularly in lignocellulosic biomass processes. These findings demonstrate the possibility of improving fermentation parameters, such as temperature and nutrient addition, to increase LA yields, so rendering microbial synthesis of LA an economically feasible substitute for conventional chemical synthesis. To further boost LA synthesis in these bacterial strains, more investigation may be conducted into genetic or metabolic engineering techniques.

4.4 Biochemical characteristics of bacterial isolates

The biochemical properties of different bacterial isolates are displayed in the Table 4.2, with particular attention paid to their capacities for starch hydrolysis, carbohydrate fermentation, and the synthesis of enzymes such as nitrate reductase, catalase, and urease. The isolates' diverse environmental origins, including deteriorated wood, red soil, bakery trash, and municipal garbage, demonstrate their ecological variety. The observed biochemical properties are interpreted in detail as follows (Table 4.2).

4.4.1 Carbohydrate fermentation

Fermentation of carbohydrates is a crucial characteristic in determining the metabolic activity of bacteria, particularly in the formation of LA. When the isolates' capacity to ferment carbohydrates was examined, the results either indicated NF (No Fermentation) or A&F (Acid and Gas Production):

Fermenters that are positive: A variety of isolates, including DGB, DGN2, NA9, DGS15, DGS16, DGS18, and DGS21, showed signs of fermentation of carbohydrates. These strains are able to ferment glucose into byproducts like LA, which has several industrial uses, as evidenced by the formation of both gas and acid.

Those who do not ferment: DGS1, DGN1, DGS19, and DGS20 strains were devoid of any fermentation activity. This suggests that these isolates might produce energy through different metabolic pathways, like respiration rather than fermentation.

4.4.2 Urease Activity

The enzyme urease hydrolyses urea to produce carbon dioxide and ammonia. By using urea as a nitrogen source, urease-positive organisms can thrive in settings with low nitrogen levels:

Positive Urease Activity: The urease activity tests for isolates DGS1, DGN1, DGS18, DGS19, and DGS20 were positive, indicating that these organisms are capable of metabolizing urea. This characteristic could be helpful in areas where organic nitrogen is scarce.

Negative Urease Activity: The absence of urease activity in isolates like DGB, DGS15, DGS16, and NA9 suggests that these isolates' nitrogen metabolism is probably dependent on external nitrogen sources.

4.4.3 The Activity of Catalase

The enzyme catalase converts hydrogen peroxide into oxygen and water, shielding microorganisms from oxidative damage. It indicates either facultative anaerobic respiration or aerobic respiration:

Positive Catalase Activity: By detoxifying reactive oxygen species, isolates NA9, DGN1, DGS15, DGS19, and DGS20 demonstrated positive catalase activity, indicating they may be able to withstand and even flourish in oxygen-rich environments.

Catalase activity was negative in isolates like DGB, DGS1, DGN2, and DGS16. This could mean that they are obligate anaerobes or that they have different defense mechanisms against oxidative stress.

4.4.4 Starch hydrolysis

Since amylase, an enzyme that breaks down starch into simpler sugars, is absent from these strains, none of the isolates tested positive for starch hydrolysis. This could imply that the principal carbon source for these bacteria is not complex polysaccharides like starch, or that they need particular environmental cues to display this function.

4.4.5 Gram staining

Bacteria are distinguished by the structure of their cell walls via gram staining. With the exception of DGN1, DGS19, and DGS20, most of the isolates were Gram-positive. Because of the thick peptidoglycan layer in their cell walls, gram-positive bacteria are able to withstand harsh conditions better than other types of bacteria, such as soil or decomposing debris. On the other hand, the outer membrane of the Gram-negative isolates may resist specific environmental pressures, but it may also make them more vulnerable to osmotic pressure.

4.4.6 Capsule Staining

The lack of a noticeable polysaccharide capsule was shown by the negative capsule staining tests performed on each isolate. The lack of capsules in these strains implies that they are probably non-pathogenic and may be well suited for industrial or environmental applications where biofilm formation is not necessary for survival. Capsules are frequently linked to pathogenicity and resistance to phagocytosis.

4.4.7 Test of Voges-Proskauer (VP)

Acetoin is a neutral metabolic byproduct that can be detected using the VP test:

Positive VP Activity: Isolates DGN1, DGN2, DGS18, DGS19, DGS20, and DGS21 all showed positive results, demonstrating their ability to use the butanediol fermentation pathway to convert glucose to acetoin. This might be helpful in fermentations involving mixed acids.

Negative VP Activity: The absence of VP activity in strains NA9, DGB, DGS1, DGS15, and DGS16 indicates that they create more acidic end products during fermentation, such as lactic or acetic acid, which is consistent with the emphasis on the formation of LA.

4.4.8 Activity of Nitrate Reductase

During anaerobic respiration, the enzyme nitrate reductase allows bacteria to use nitrate as a terminal electron acceptor:

Positive Nitrate Reductase Activity: The ability of the strains NA9, DGB, DGN2, DGS15, DGS16, and DGS21 to convert nitrate to nitrite or even nitrogen gas is

indicated by their positive nitrate reductase activity. This may be beneficial in nitrogen-limited settings such as anaerobic soil or decomposing organic waste.

Negative Nitrate Reductase Activity: The absence of nitrate reductase activity in strains DGS1, DGN1, DGS18, and DGS19 suggests that they obtain their energy from fermentation or other sources of respiration.

4.4.9 Methyl Red Test

Mixed acid fermentation byproducts are identified using the methyl red test:

The methyl red test result for each isolate was negative, meaning that none of them produced any appreciable levels of stable acid through the fermentation of glucose. This shows that rather than producing a complicated mixture of acids, the bacteria either create LA as the principal acid produced, as suggested by the VP-positive results, or they produce neutral byproducts.

The biochemical traits of the isolated bacteria show a variety of metabolic capacities, indicating their adaptability to different environmental circumstances. The isolates NA9, DGB, DGS15, and DGS18 have favorable nitrate reductase findings, catalase activity, and carbohydrate fermentation capabilities that make them suitable for use in industrial LA production. These isolates are ideal for procedures that call for strong fermentation, thermotolerance, and the capacity to adjust to low nutrition environments.

Table 4.2: Morphological and biochemical characterisation of bacterial isolates

Location	**Bacterial strains isolated from**										
30° ′ 19 ″ 42.0 ″ N, 76 ° 27 ′ 26.9 ″ E	**Previously isolates (*Bacillus licheniformis* strains)**		**Bakery waste**	**Degraded wood sample from bark of mango tree**		**Red soil of brick kiln**	**Degraded wood of brick kiln**	**Decaying Municipal corporation waste**	**Stone sample from brick kiln**	**Degraded lignocellulosic biomass**	**Red soil of brick kiln**
Test	NA9	DGB	DGS1	DGN1	DGN2	DGS15	DGS16	DGS18	DGS19	DGS20	DGS21
Carbohydrate fermentation	A&F	A&F	NF	NF	A&F	A&F	A&F	NF	A&F	NF	A&F
Urease	-	-	+	+	-	-	-	+	+	+	-
Catalase	+	-	-	+	-	+	-	-	+	+	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-
Gram staining	+	+	+	-	+	+	+	+	-	-	+
Capsule staining	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	+	+	-	-	+	+	+	+
Nitrate reductase	+	+	-	-	+	+	+	-	-	-	+
Methyl red	-	-	-	-	-	-	-	-	-	-	-

*** NF: Non- Fermentative, A & F: Anaerogenic & Fermentative, +: Positive reaction, -: Negative reaction**

4.6 Phylogenetic analysis

Based on sequence similarities, the neighbor-joining phylogenetic tree created with MEGA 10.1.8 shows evolutionary relationships. Based on 1000 repeats, bootstrap values more than 50% substantiate the resilience of the phylogenetic clusters. The close clustering of *B. sonorensis* strains DGS15 and DGN2 indicates their genetic closeness. *B. licheniformis* DGB, on the other hand, forms a separate branch, suggesting a different evolutionary route (Fig. 4.5).

High bootstrap values indicate a closer evolutionary link between *B. sonorensis* DGS15 and DGN2. Common biochemical characteristics such as nitrate reductase activity and Gram-positive staining are correlated with their genetic closeness. Their variations in catalase and urease activity, however, are indicative of niche-specific adaptations.

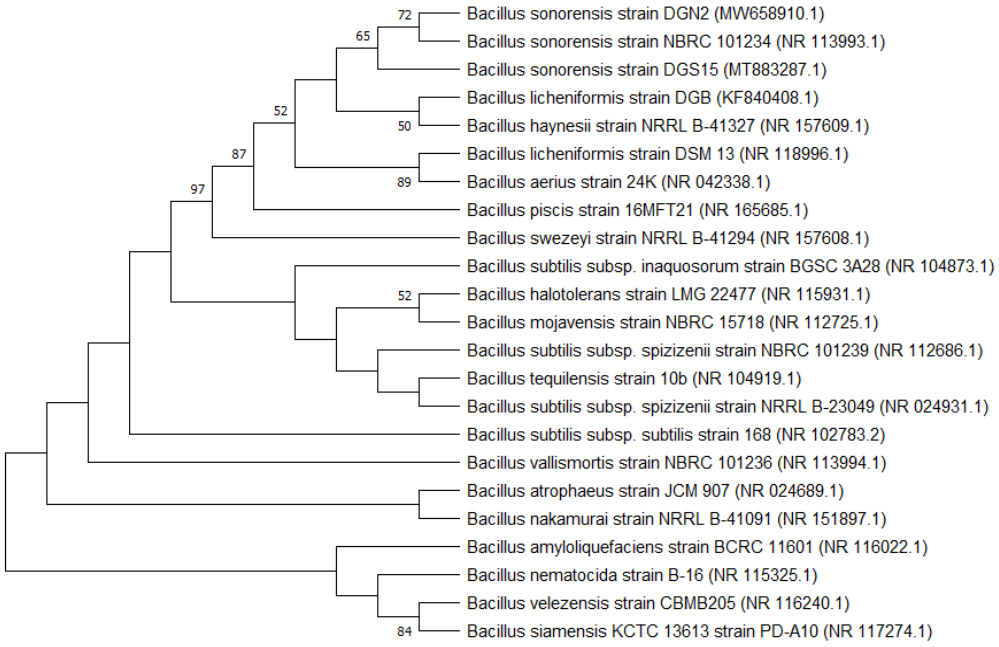

Figure 4.5: Neighbor-joining phylogenetic tree of *Bacillus sonorensis* DGS15, *Bacillus sonorensis* bootstrap values of more than 50% (based on 1000 replicates) are given in the nodes of the tree along with NCBI accession numbers.

B. licheniformis DGB is genetically different from DGS15 and DGN2, although it functions similarly in terms of carbohydrate fermentation and nitrate reductase activity. Given their evolutionary separation, *B. licheniformis* and *B. sonorensis* may

have evolved to distinct ecological niches. In conclusion, the comparative study (Table 4.3) shows that although *B. sonorensis* and *B. licheniformis* have a lot of biochemical characteristics in common, their urease, catalase, and fermentation pathways differ significantly. These variations might be a result of how each has adapted to different environmental factors, like temperature, oxygen availability, and nitrogen supply. The evolutionary divergence between *B. sonorensis* and *B. licheniformis* is further highlighted by the phylogenetic analysis, which shows that *B. sonorensis* DGS15 and DGN2 are more closely linked to one another than they are to *B. licheniformis* DGB. These results demonstrate the strains' adaptability in terms of metabolism, which makes them attractive options for industrial uses such as the generation of LA and bioremediation.

Table 4.3: Bacterial isolates and their closest relative species inferred from 16S rRNA gene sequences of NCBI database

Bacterial Isolate	Accession No	Nearest Match	Length (bp)	Phylum	Query coverage	E value	% Identity to closest related bacterial sp.
DGS15	MT883287.1|	*B. sonorensis* strain NBRC 101234	1466	Firmicutes	100	0	98.47
DGN2	MW658910.1	*B. sonorensis* strain NBRC 101234	1618	Firmicutes	100	0	98
DGB	KF840408	*B. licheniformis* strain BAB-1836	1511	Firmicutes	100	0	99

4.6 Impact of inhibitors on *B. sonorensis* DGS15 and B. *licheniformis* DGB on bacterial growth

The resistance of the isolate Bacillus sonorensis DGS15 to furfural and 5-hydroxymethylfurfural (HMF), two common inhibitors found in hydrolysates, was investigated. These inhibitors can severely restrict microbial development, which has an impact on fermentation processes. They are generally generated during the hydrothermal breakdown of sugars in lignocellulosic biomass. By evaluating the inhibition of cell growth via Relative Optical Density (ROD) at 620 nm, these inhibitors' effects on *B. sonorensis* DGS15 are assessed.

4.6.1 Impact of furfural on growth of *B. sonorensis* DGS15

Furfural was tested at different concentrations ranging from 0.3 g/L to 1.2 g/L to see how it affected DGS15 growth (Fig 4.6). An obvious tendency of inhibition is seen as the concentration of furfural rises: the ROD is 75% at 0.3 g/L of furfural, suggesting a moderate suppression of cell growth. The cells still show a comparatively excellent resistance to furfural at this dose. The ROD decreases to 66.6% at 0.6 g/L, indicating a more severe inhibition. Cell growth is significantly suppressed at 0.9 g/L, and ROD sharply drops to 25%. high indicates that high quantity of furfural seriously compromises the metabolic machinery of DGS15. The ROD drops to 3.3% at 1.2 g/L, indicating that furfural is almost entirely inhibitory of DGS15 at this dose (Table 4.4).

Table 4.4: Effect of furfural and HMF on relative optical density of *B. sonorenesis* DGS15 after 24 h of growth at 50 °C and 120 rpm. The value of relative optical density (ROD) at 620 nm was used to describe cell growth inhibition. The percent OD620 of the experimental flasks as compared to the control flasks (with no inhibitor) was used to calculate ROD

Furfural inhibitor concentration (g/L)	Relative Optical Density (ROD) %	HMF inhibitor concentration (g/L)	Relative Optical Density (ROD) %
0.3	75 ± 0.03	0.6	85.7 ± 0.04
0.6	66.6 ± 0.02	1.2	42.8 ± 0.02
0.9	25 ± 0.08	1.8	14.3 ± 0.04
1.2	3.3 ± 0.04	2.4	4.2 ± 0.05

According to this findings, *B. sonorensis* DGS15 can tolerate furfural up to a concentration of 0.3 g/L, but greater concentrations significantly impede its growth. The inhibitory effect seen is probably due to furfural's known ability to impede important metabolic processes, like glycolysis, by impairing enzyme performance and raising oxidative stress in microbial cells.

4.6.2 Impact of HMF on growth of *B. sonorensis* DGS15

Hydroxymethyl furfiural (HMF) is another significant degradation product generated during the breakdown of hexose sugars in biomass, was studied for its inhibitory effect on growth. The ROD is 85.7% at 0.6 g/L of HMF, suggesting that DGS15 is more resistant to HMF than furfural. The ROD dramatically drops to 42.8% at 1.2 g/L, indicating that HMF starts to exert a severe inhibitory impact at this dose. The ROD further decreases to 14.3% at 1.8 g/L, showing a significant suppression of cell growth. The ROD is only 4.2% at 2.4 g/L, indicating that HMF completely inhibits DGS15 at this high dose (Table 4.4).

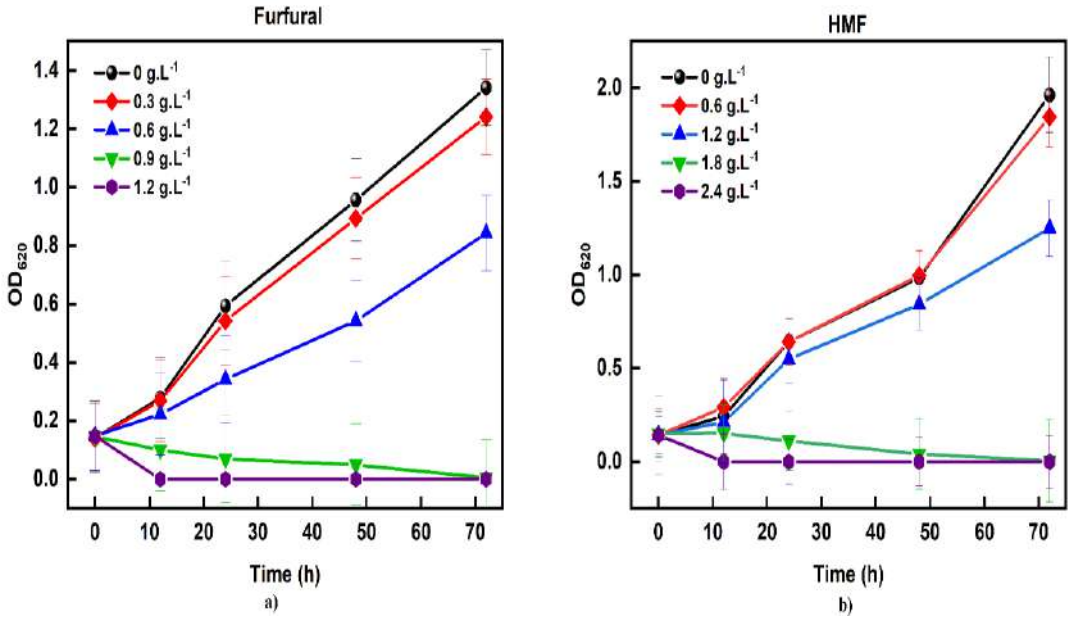

Figure 4.6: Effect of inhibitors (a)furfural and (b) 5-hydroxymethylfurfural (HMF) on growth of *B. sonorensis* DGS15

Like furfural, HMF blocks cellular metabolism by inhibiting important enzymes, producing reactive oxygen species (ROS), and preventing DNA replication, among other mechanisms. At equal concentrations, *B. sonorensis* DGS15 seems to have a

marginally greater tolerance to HMF than furfural. For example, at 0.6 g/L, the ROD for furfural was 66.6%, but it was 85.7% for HMF, suggesting that the presence of HMF improves the growth profile. Nevertheless, both substances significantly reduce bacterial growth as their concentration rises, reaching nearly total growth suppression at 1.2 g/L of furfural and 2.4 g/L of HMF (Figure 4.6).

These results hold significance for industrial bioprocesses that employ *B. sonorensis* DGS15 in hydrolysates of lignocellulosic biomass. Adaptive evolution or improved detoxification techniques may increase the strain's resistance to these inhibitors, enabling more effective biomass conversion for biotechnological uses.

The study's findings show that *B. sonorensis* DGS15 has varying levels of tolerance to HMF and furfural. Although the bacterium is greatly inhibited by both inhibitors, it is somewhat more resistant to HMF at lower concentrations. These observations lay the groundwork for enhancing *B. sonorensis* DGS15's bioprocessing resilience in lignocellulosic biomass hydrolysates, which are frequently abundant in these inhibitory substances (Ghosh *et al*., 2012).

4.6.3 Impact of Furfural on growth of B. *licheniformis* DGB

Similarly, *Bacillus licheniformis* DGB's resistance to inhibitors that are present in lignocellulosic hydrolysates, including 5-hydroxymethylfurfural (HMF) and furfural was assessed. After 24 h of growth at 50°C and 120 rpm, the Relative Optical Density (ROD) at 620 nm was measured to determine the level of inhibition on *B. licheniformis* DGB.

The impact of furfural concentrations on *B. licheniformis* DGB growth was investigated, with concentrations ranging from 0.3 g/L to 1.2 g/L (Fig. 4.7). The ROD is 86.13% at 0.3 g/L, indicating that DGB exhibits a comparatively high tolerance to low furfural concentrations with little suppression of cell growth. The ROD drops to 55.47% at 0.6 g/L, indicating a considerable slowdown in cell proliferation. It is possible that this quantity begins to moderately impair the cells' metabolic functions. The ROD quickly decreases to 20.8% at 0.9 g/L, showing a severe growth inhibition. Furfural probably interferes with a number of cellular processes at this level, such as enzyme activity and energy production routes (Table 4.5).

Table 4.5: Effect of furfural and HMF on relative optical density of *B. licheniformis* DGB after 24 h of growth at 50 °C and 120 rpm

Furfural inhibitor concentration (g/L)	Relative Optical Density (ROD) %	HMF inhibitor concentration (g/L)	Relative Optical Density (ROD) %
0.3	86.13 ± 0.21	0.6	99.8 ± 0.25
0.6	55.47 ± 0.034	1.2	95.7 ± 0.032
0.9	20.8± 0.047	1.8	63.1 ± 0.054
1.2	5.5 ± 0.028	2.4	6.1 ± 0.041

Furfural is strongly inhibitory at this dose, effectively stopping cell growth in DGB, as seen by the ROD reaching 5.5% at 1.2 g/L. This indicates that furfural is harmful for the bacterial cells at this concentration, which include disruption of glycolysis and production of reactive oxygen species (ROS) (Liu *et al*., 2018).

4.6.4 Impact of HMF on growth of B. *licheniformis* DGB

Likewise, HMF's impact on *B. licheniformis* DGB was investigated at doses between 0.6 and 2.4 g/L (Fig.4.7). The ROD is 99.8% at 0.6 g/L, suggesting that DGB exhibits remarkable resistance to low HMF doses with very minimal growth inhibition. This implies that DGB's cellular metabolism is extremely resilient to HMF's harmful effects at this dose. The ROD is still high at 95.7% at 1.2 g/L, indicating that the cells can still proliferate with little inhibition even at this higher dosage. The ROD sharply decreases to 63.1% at 1.8 g/L, indicating a more potent inhibitory action of HMF on cell growth. Growth is reduced at this dose because HMF starts to obstruct important metabolic pathways. The ROD drastically drops to 6.1% at 2.4 g/L, indicating that HMF becomes extremely toxic at this concentration and severely inhibits growth (Table 4.5).

The tolerance of *B. licheniformis* DGB to furfural and HMF inhibitors differs noticeably from one another. At lower doses, furfural is more inhibitory of DGB. The ROD is 55.47% even at 0.6 g/L, indicating significant inhibition; at 0.9 g/L and above, the inhibition gets severe. HMF, however, exhibits substantially reduced inhibitory activity at equivalent doses. Up to 1.2 g/L (ROD of 95.7%), DGB demonstrates a high resistance to HMF; substantial inhibition only happens at higher doses (1.8 g/L and above).

This suggests that *B. licheniformis* DGB is more resistant to furfural than to HMF, which is in line with the inhibitors' established harmful actions. HMF's inhibitory effects are frequently less noticeable at low concentrations, whereas furfural tends to form adducts with important enzymes, leading to quicker inhibition. Nevertheless, HMF can damage DNA and interfere with cellular respiration at greater quantities, which explains why cell growth is reduced at 1.8 and 2.4 g/L concentrations.

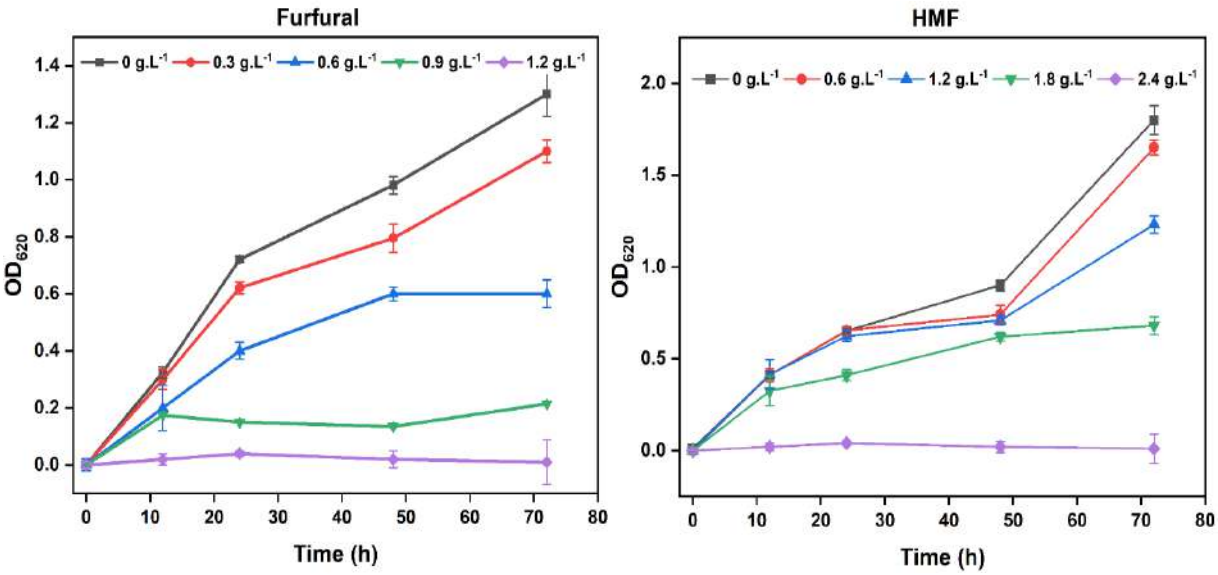

Figure 4.7 Effect of inhibitors (a)furfural and (b) 5-hydroxymethylfurfural (HMF) on cell growth of *B. licheniformis* DGB

4.7 Comparative consequences for industrial bioprocessing: B. *licheniformis* DGB and *B. sonorensis* DGS15

A number of significant ramifications for industrial bioprocessing become apparent when contrasting the tolerance of *B. sonorensis* DGS15 and *B. licheniformis* DGB to furfural and 5-hydroxymethylfurfural (HMF). The selection or engineering of the most effective microbial strains for use in the conversion of lignocellulosic biomass into biofuels and biochemicals depends on these implications.

1. Tolerance to Furfural: *B. sonorensis* DGS15 displays a moderate level of tolerance to furfural up to 0.3 g/L (ROD of 75%), but as concentrations rise above 0.6 g/L, there is a marked suppression of growth, which reaches an almost total stop at 1.2 g/L (ROD of 3.3%). On the other hand, B. *licheniformis* DGB

exhibits a greater resistance to furfural at 0.3 g/L (ROD of 86.13%), but at higher doses, it also shows a notable inhibition, with ROD falling to 5.5% at 1.2 g/L.

Both strains are prone to inhibition at higher concentrations of furfural, whereas DGB has a marginally greater tolerance at lower values. Unless the hydrolysate is further detoxified or further adapted, neither strain would be suitable for use in industrial procedures requiring high amounts of furfural. It might be required to use pretreatment techniques to lower the concentration of furfural or to create genetically engineered strains with increased resistance to furfural.

2. Resistance to HMF: *B. sonorensis* DGS15 has a modest level of resistance to HMF, achieving a low ROD of 4.2% at 2.4 g/L, although growth rapidly decreases beyond this concentration, peaking at 85.7% ROD at 0.6 g/L. On the other hand, *B. licheniformis* DGB exhibits remarkable resistance to HMF, preserving a high ROD of 99.8% at 0.6 g/L and 95.7% at 1.2 g/L. Notably, substantial inhibition happens alone at the maximum dose of 2.4 g/L (ROD of 6.1%).

   B. licheniformis DGB is a better option for bioprocesses utilising hydrolysates with high HMF content since it is significantly more tolerant of HMF than *B. sonorensis* DGS15. This implies that DGB would function better in fermentations where lowering or controlling HMF levels is challenging.

3. Suitability for Lignocellulosic Biomass Hydrolysates: *B. licheniformis* DGB is more appropriate for bioprocessing hydrolysates derived from lignocellulosic biomass, which frequently contain both inhibitors, due to its increased tolerance to HMF and comparatively better performance at lower furfural concentrations. If HMF and furfural concentrations are high, *B. sonorensis* DGS15 could need to be adjusted or extra detoxification processes taken. *B. sonorensis* DGS15 might be better appropriate for hydrolysates with low furfural concentrations.

   B. licheniformis DGB would be a more effective strain for industrial applications where biomass pretreatment results in high amounts of HMF but modest levels of furfural. On the other hand, *B. sonorensis* DGS15 might be a good substitute in procedures where the inhibitors are limited.

4. Possibility for Genetic or Adaptive Engineering: Given that both strains are sensitive to furfural, there may be a chance to enhance furfural tolerance by genetic engineering or adaptive evolution. Both strains' effectiveness might be

raised by boosting furfural reductase activity or creating pathways for furfural detoxification. *B. licheniformis* DGB is a good candidate for further engineering to improve other features, such furfural resistance or overall fermentation efficiency, because of its high HMF tolerance.

There are chances for strain improvement due to the varying degrees of inhibitor tolerance. While *B. sonorensis* DGS15 might be modified to better withstand inhibitor stress for a wider range of applications, *B. licheniformis* DGB's innate HMF tolerance could provide the basis for the development of a resilient, multi-tolerant strain.

4.8 SUGAR UTILIZATION PROFILES OF DGS15 AND DGB IN MINIMAL MEDIA CONTAINING GLUCOSE AND XYLOSE

In Bushnell Haas minimum media supplemented with glucose, xylose, and yeast extract, the fermentation efficiency and sugar utilisation capacity of *B. sonorensis* DGS15 (Fig 4.8 a) and *B. licheniformis* DGB (Fig 4.8 b) were investigated separately under certain optimized conditions. The ability of the strains to metabolize glucose and xylose, two essential sugars found in lignocellulosic hydrolysates, was used to evaluate the formation of LA (LA).

After being grown in the medium for 72 h, the strain *B. sonorensis* DGS15 showed effective use of both xylose and glucose. DGS15 overcome carbon catabolite suppression (CCR), a common phenomenon that frequently favors glucose over other sugars, and used xylose and glucose at the same time. After 72 h, DGS15 generated 18.47 g/L of LA, yielding 0.92 g/g of sugar consumed and a productivity of 0.25 g/L/h overall. Throughout the incubation period, both glucose and xylose were efficiently used, with glucose being used initially and xylose being consumed gradually after that. The ability to withstand high temperatures is one of *B. sonorensis* DGS15's main benefits. Reducing the energy needed for cooling during the fermentation process by operating at 50°C is essential for industrial-scale applications. Additionally, DGS15 can use both C6 (glucose) and C5 (xylose) sugars, which makes it very useful for fermenting lignocellulosic hydrolysates, which frequently include high quantities of xylose (Liu *et al*., 2018).

CCR stops many microbes from using xylose and glucose at the same time. *B. sonorensis* DGS15, on the other hand, showed that it could get around CCR and convert

both carbohydrates into LA at the same time. Because it maximizes substrate utilisation without the delays imposed by sequential sugar metabolism, this feature greatly increases its potential in industrial processes that use mixed sugar feedstocks.

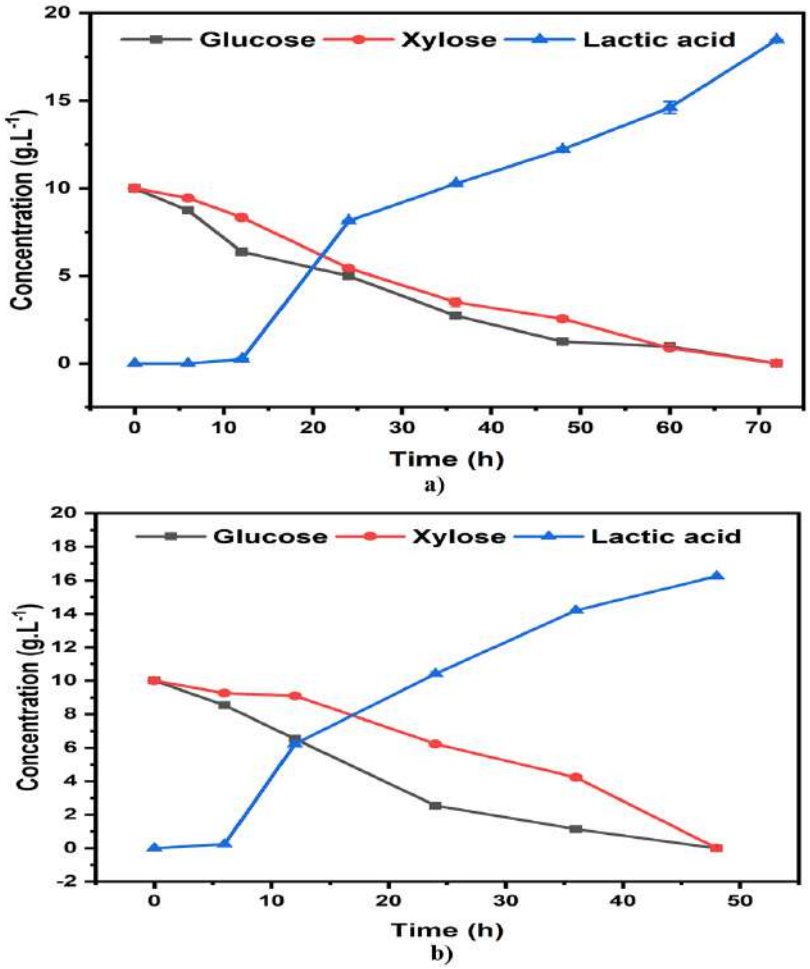

Figure 4.8: Sugar Utilization Profiles (a) DGS15 and (b) DGB were cultivated in Bushnell-Haas minimal medium containing 10 g/L glucose and 10 g/L xylose. The utilization of sugars was monitored over time. Error bars represent the standard deviation of triplicate measurements

The same conditions were used to incubate *B. licheniformis* DGB for 48 h in parallel experiments. DGB produced LA at a higher rate of conversion than DGS15, but at a slightly lower rate overall. In 48 h, DGB yielded 0.81 g/g of sugar and produced 16.23 g/L of LA, with an overall productivity of 0.33 g/L/h. Due to its quicker digestion of the available sugars, DGB demonstrated a greater rate of productivity even if its total LA production was lower than that of DGS15. One of the traits shared by many *B. licheniformis* strains is the effective utilization of xylose by DGB. It was noted that the

strain utilized glucose first, a common behavior in many *Bacillus* species, and then rapidly absorbed xylose.

The fact that DGB is a strong generator of cellulase gives it an advantage over other *B. licheniformis* strains. This characteristic improves its utilization of xylose, particularly in hydrolysates made from biomass that is lignocellulosic. A major benefit in procedures using xylose-rich hydrolysates is that DGB can effectively convert xylose into LA.

When *B. sonorensis* DGS15 and *B. licheniformis* DGB are compared, it becomes clear that both strains have unique benefits when it comes to using glucose and xylose to produce LA. *B. sonorensis* DGS15 is an extremely effective LA producer for mixed-sugar feedstocks because it can overcome carbon catabolite inhibition and produces more LA over an extended fermentation period. *B. licheniformis* DGB, on the other hand, is suited for procedures requiring quick fermentation cycles because it exhibits quicker sugar utilization, particularly xylose, and higher output in the short term.

To study the growth and sugar utilization pattern of *Bacillus* strains, they were incubated separately and were also co- cultivated at 50 °C and pH 7 in the Bushnell Haas minimal media (3.27 g/L) supplemented with 10 g/L glucose, 10 g/L xylose and 5 g/L yeast extract.

DGS15 produced an efficient 18.4713 g/L LA after 72 h of incubation yielding 0.92 g/g LA with an overall productivity of 0.25 g/L/h (Fig 4.8 a). DGS15 was found to utilize glucose as well as xylose efficiently and thus produce LA.

Separately, DGB alone was found to produce 16.23 g/L LA after 48 h of incubation at 50 °C yielding 0.81 g/g LA with an overall productivity of 0.33 g/L/h (Fig 4.8 b).

Objective 2: Production of LA from different lignocellulosic biomass

LA production from rice and wheat straw biomass offers a sustainable alternative to traditional methods, utilizing India's abundant agricultural waste. The process involves breaking down biomass into fermentable sugars, followed by microbial fermentation. Despite challenges in pretreatment, microbial strain development, and process optimization, overcoming these hurdles can make this method viable, boosting India's bio economy, promoting eco-friendly agriculture, and generating income for farmers (Yankov, 2022).

The pretreatment of rice and wheat straw biomass is a vital step in producing fermentable sugars for LA production, but it poses significant challenges. The complex structure of lignin, hemicellulose, and cellulose makes it difficult to break down the biomass, while pretreatment methods can also generate inhibitory compounds that hinder microbial fermentation (Jönsson & Martín, 2016). To address these challenges, it is crucial to develop efficient pretreatment methods that maximize the yield of reducing sugars, such as glucose and xylose, which directly impacts the overall efficiency of LA production (Kumar *et al*., 2009).

4.9 Statistical optimization of pretreatment of wheat straw

The fitted data with coded values of independent variables and total reducing sugar concentration as a response variable has the following second order polynomial model equation:

Reducing sugar = $7.56 + 0.96A - 0.36B + 0.15C + 2.75AB + 1.48AC - 0.675BC + 2.07A^2 - 1.78B^2 - 1.60C^2$ (4)

where A= H_2SO_4 concentration, B= Temperature, C= Time The results of acid hydrolysis of wheat straw for reducing sugar production are shown in (Table 4.6).

At an experimental run number of 4, temperature of 106 °C, acid concentration of 0.98% v/v, and hydrolysis period of 45 min, a maximum concentration of 11.36 g/L was obtained as in (Table 4.7). Also, at an experimental run number of 12, temperature of 60 °C, acid concentration of 0.55% v/v, hydrolysis period of 15 min, the minimum reducing sugar of 3.4 g/L was obtained.

Table 4.6: The Box Behnken design and their responses for three level, three factor response surface analysis

	Factor 1	Factor 2	Factor 3	Response 1
Run	**A:H_2SO_4 Conc**	**B:Temp**	**C:Time**	**Reducing sugars (g/L)**
1	0.55	60	105	5.3
2	0.55	120	105	3.6
3	0.55	120	15	4.4
4	0.1	90	15	8.5
5	1	120	60	11
6	0.55	90	60	7.3
7	0.55	90	60	7.5
8	1	90	15	7.5
9	0.55	90	60	7.6
10	0.55	90	60	7.9
11	0.1	90	105	5.6
12	0.55	60	15	3.4
13	0.1	60	60	10.2
14	0.1	120	60	3.6
15	1	90	105	10.5
16	1	60	60	6.58
17	0.55	90	60	7.51

Also, the findings of the maximum and lowest concentrations of reducing sugars how that the total reducing sugar (TRS) extraction was lower at lower temperatures and acid concentrations. The increase in total reducing sugar (TRS) production at higher temperatures could be owing to sufficient temperature and acid concentration to hydrolyze wheat straw, but lower temperatures were insufficient to hydrolyze wheat straw. Table 4.8 displays the results of an analysis of variance (ANOVA) for the fitted quadratic model.

The model summary for regression coefficients (R^2 99.35%, adjusted R^2 98.51%, and anticipated R^2 92.59%) reveals that the quadratic model fits the experimental data. The influence and relevance of factors in the regression equation were evaluated using ANOVA research for the quadratic model. The *p* values for all linear and interaction coefficients are 0.05 in the ANOVA results shown in Table 4.8, indicating that all variables and their interactions have a significant effect on dilute sulfuric acid

hydrolysis of wheat straw, i.e., Lack of Fit with F-value and *p* value of 2.8 and 0.1728, respectively, indicates that Lack of Fit is not significant when compared to the pure error, indicating that the model was significant.

Table 4.7: **Values of total reducing sugar (TRS) as predicted by RSM model and the experimental data upon acid pretreatment of wheat straw**

Run	Concentration (g/L)	Temperature (°C)	Time (min)	Predicted reducing sugar (g/L)	Experimental reducing sugar (g/L)
1	0.99	100.8	69.00	11.29	11.13 ± 0.05
2	0.99	97.10	95.14	11.09	10.90 ± 0.06
3	0.99	100.54	102.30	11.03	10.08 ± 0.04
4	0.98	106.00	79.50	11.36	11.21 ± 0.02
5	0.99	111.97	60.80	11.25	11.18 ± 0.06

The 3D plots (shown in Fig. 4.9) illustrate an infinite number of interactions of H_2SO_4 concentration with temperature and time of pretreatment. The interaction of independent variables was seen using the contours. The graph between the interaction of H_2SO_4 concentration with temperature (AB) (Fig. 4.9 a), dilute sulfuric acid concentration and hydrolysis time (AC) (Fig. 4.9 b), temperature and hydrolysis time (BC) (Fig. 4.9 c) on the total reducibility produces an elliptical and elliptical nature. The following were the ideal settings for dilute sulfuric acid hydrolysis of wheat straw under this model for highest total reducing sugar output of 113.3 mg/g: concentration of H_2SO_4 1% v/v, temperature 120 °C, and hydrolysis period 60 min.

To validate, the experiments of dilute sulfuric acid hydrolysis of wheat straw were conducted in triplicate under optimized conditions (H_2SO_4 concentration 1% v/v, temperature 120 °C, hydrolysis time — 60 min) to ensure reproducibility of results predicted by BBD experiments and RSM analysis, the validation experiment results under ideal conditions were in good agreement with model predictions. The most abundant sugars found in the cellulosic biomass were glucose and xylose. Pretreated wheat straw had 32% cellulose, 18.4% hemicellulose, 12.4% lignin, while native wheat straw biomass had 36% cellulose, 25% hemicellulose, 20% lignin, and 0.94 g/L (Table 4.9).

Table 4.8: Analysis of variance (ANOVA) for the response surface quadratic model of the reducing sugar content

Source	Sum of squares	df	Mean Square	F-value	P-value	
Model	89.98	9	10	118.31	<0.0001	Significant
A-H_2SO_4 Conc.	7.37	1	7.37	87.26	<0.0001	
B-Temp	1.04	1	1.04	12.27	0.01	
C- Time	0.18	1	0.18	2.13	0.1878	
AB	30.36	1	30.36	359.3	<0.0001	
AC	8.7	1	8.7	102.99	<0.0001	
BC	1.82	1	1.82	21.57	0.0024	
A^2	17.98	1	17.98	212.8	<0.0001	
B^2	13.39	1	13.39	158.5	<0.0001	
C^2	10.83	1	10.83	128.12	<0.0001	
Residual	0.5915	7	0.0845			
Lack of Fit	0.4006	3	0.1335	2.8	0.1728	Non Significant
Pure Error	0.1909	4	0.0477			
Cor Total	90.57	16				
Std. Dev.	0.2907		R^2	0.9935		
Mean	6.94		Adjusted R^2	0.9895		
C.V. %	4.19		Predicted R^2	0.9259		
			Adeq. precision	35.4797		

There was 30–36-fold increase in concentration of reducing sugars after pretreatment with acid. The dilute sulphuric acid pretreated hydrolysates and hot water treatment showed maximum amount of reducing sugars in wheat straw, i.e., 28.20 g/L. Dilute (0.4 N) sodium hydroxide treatment was given to wheat straw for delignification of the structural component lignin.

Table 4.9: The crystalline index (*CrI*) including the cellulosic, hemicellulosic, lignin, and reducing sugar content in case of native as well as pretreated rice straw

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	*CrI* (%)	Reducing sugar (g/L)
Native wheat Straw	36	25	20	35.23	0.94 ± 0.04
Pretreated wheat straw	32	18.4	12.4	44.9	28.2 ± 0.08

Delignified wheat straw hydrolysate after pretreatment had an increase in cellulose by 89%, while there was decrease in hemicellulose and lignin content by 73% and 62% respectively. Other researchers also concluded, rice straw that had not been treated included approximately 41.34% glucan and 28.46% xylan, the amount of glucan increased to 49.77% after dilute acid pretreatment (Kim *et al*., 2012). The hemicellulose percentage of pretreated rice straw was 19%, while native rice straw had a 28% hemicellulose content. It could be because of its amorphous form, which allows it to hydrolyze quickly during dilute acid pretreatment. Due to the dissolution of amorphous components from the biomass, the cellulose content of acid pretreated rice straw increased by 47% (Kshirsagar *et al*., 2015).

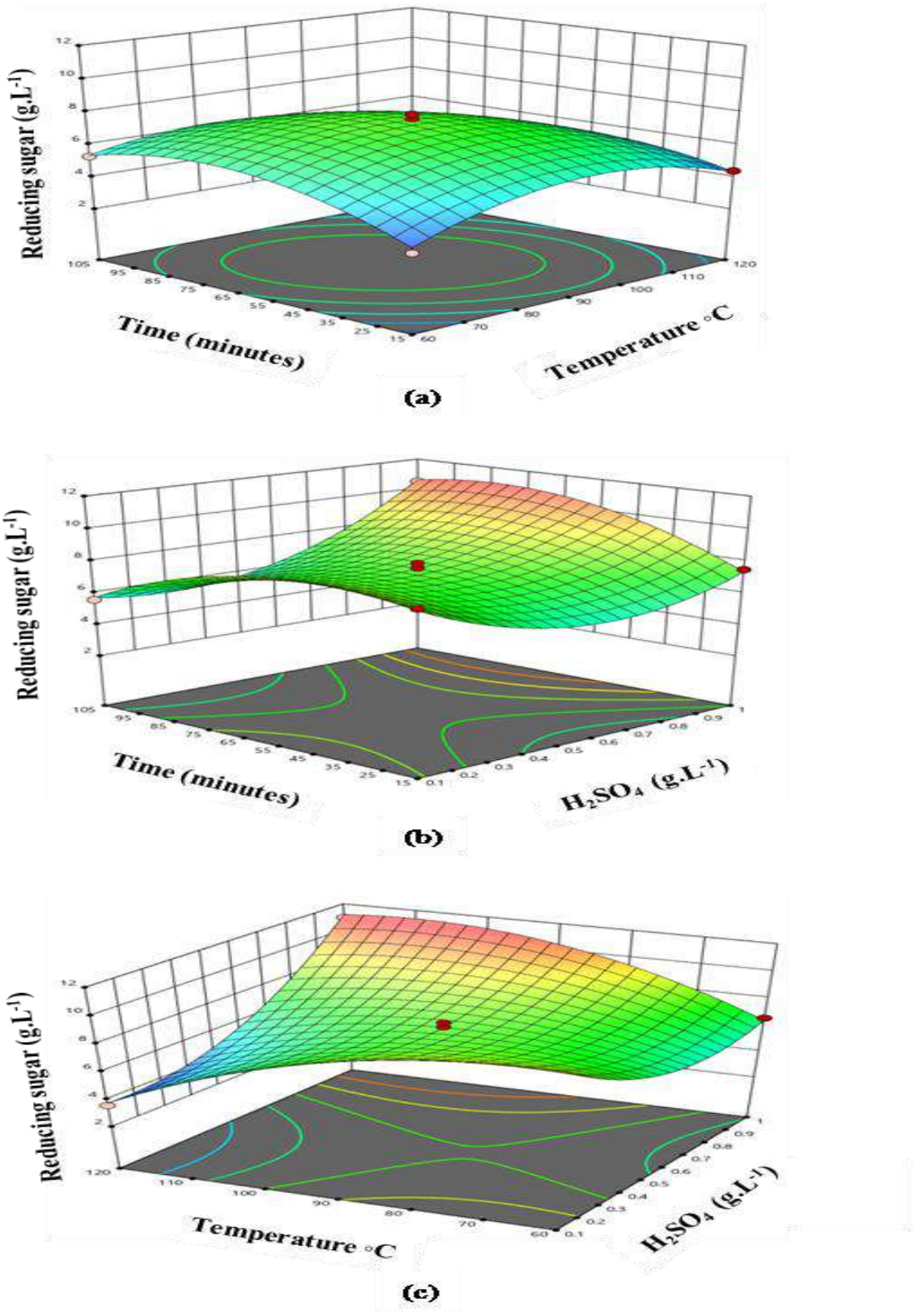

Fig 4.9. 3D surface plot demonstrating the effect of (a) temperature and hydrolysis time, (b) sulfuric acid concentration and hydrolysis time, (c) sulfuric acid concentration and temperature on total reducing sugar

4.10 Characterization of native and pretreated wheat straw

4.10.1 Scanning Electron Microscopy (SEM)

SEM micrographs of native wheat straw (Fig. 4.10 a), pretreated wheat straw (Fig. 4.10 b) showed that upon pretreatment the structure of native wheat straw was distorted and the pretreatment led to structural changes in the biomass. SEM showed that the compact, ordered structure of the native biomass was destroyed in the pretreated biomass.

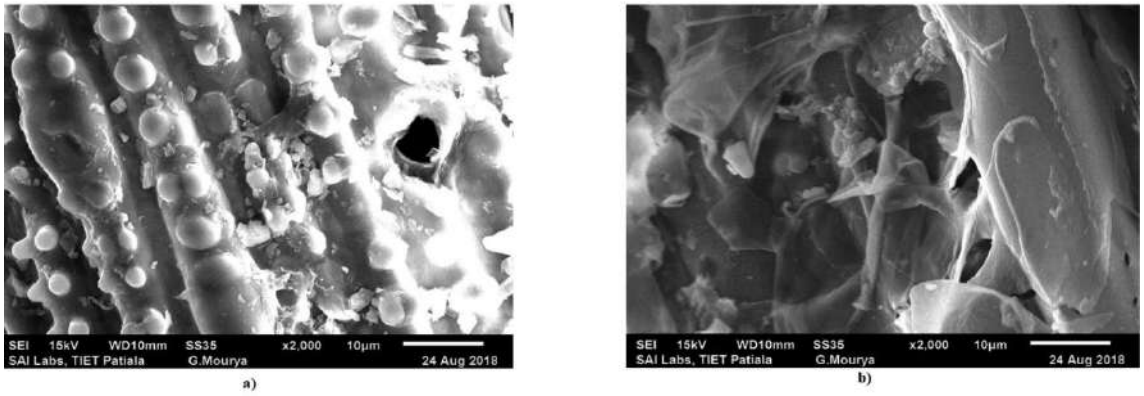

Figure 4.10: Scanning electron micrographs of (a) native wheat straw, (b) pretreated wheat straw (magnification: 1000X)

4.10.2 XRD analysis of Native and Pretreated Wheat straw

The XRD profile of native and pretreated wheat straw is presented in (Fig. 4.11). *CrI* in native and pretreated wheat straw was observed as 35.23% and 44.9% respectively. An increase in *CrI* (9.71%) was observed in pretreated wheat straw in comparison with native biomass. Other researchers have also found similar results in native rice straw (*CrI* 59.37%), dilute acid pretreatment of rice straw had a greater crystallinity degree (67.2%) (Shetty *et al*., 2017).

An increase in *CrI* by 6% was also reported for Lantana camara biomass which was enzyme treated (Kuila *et al*., 2011). The fatty components of the biomass and the molecular mass of hydrocarbons define the crystallinity of the biomass. The hydrolysis of glycosidic connections in the cellulose exposed regions could explain the increased crystallinity index (*CrI*) following pretreatment. The effect of acid pretreatment on crystallinity increased greater in the amorphous region than in the crystalline region. The decline in crystallinity was due to the acidic nature of sulphuric acid,

delignification by alkali NaOH and impulsive autoclaving leading to enhanced saccharification of wheat straw (Akhtar *et al*., 2016)

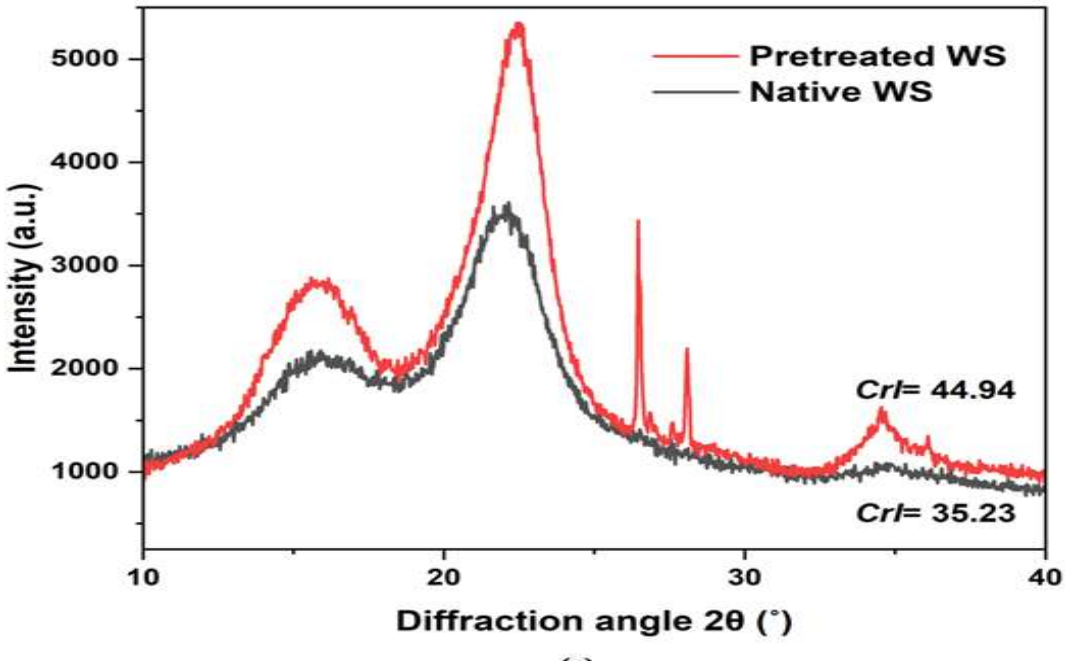

Figure 4.11: XRD analysis of Native and Pretreated Wheat straw

4.10.3 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Changes in the functional groups of native and pretreated wheat straw biomass are presented by FTIR spectra (Fig. 4.12). In native wheat straw, the broad band at 3333.1 cm^{-1} and 3334.2 cm^{-1} is related to O-H stretching vibrations caused by the presence of alcoholic and phenolic hydroxyl group involved in the hydrogen bond. The change in higher wave number and broadening of the OH stretching band was the result of pretreatment with sulphuric acid, which is an indication of weakened hydrogen bonding both intra and inter molecular and thus causes decrease in crystallinity (Kim *et al*., 2012). The band position at 2915 and 2935 cm^{-1} for native and pretreated wheat straw respectively were attributed to C-H stretching vibration in native and pretreated wheat straw showed a small variation in peak intensity, suggesting that the methylene (-CH2) and the methyl (-CH3) portions of cellulose were distorted. The highest reduction was observed at 1640 cm^{-1} band in case of native wheat straw, due to breakage in acetyl and uronic esters, ester linkage of the ferulic and p-coumaric acids and hemicellulose or lignin (Du *et al*., 2016) (Table 4.10).

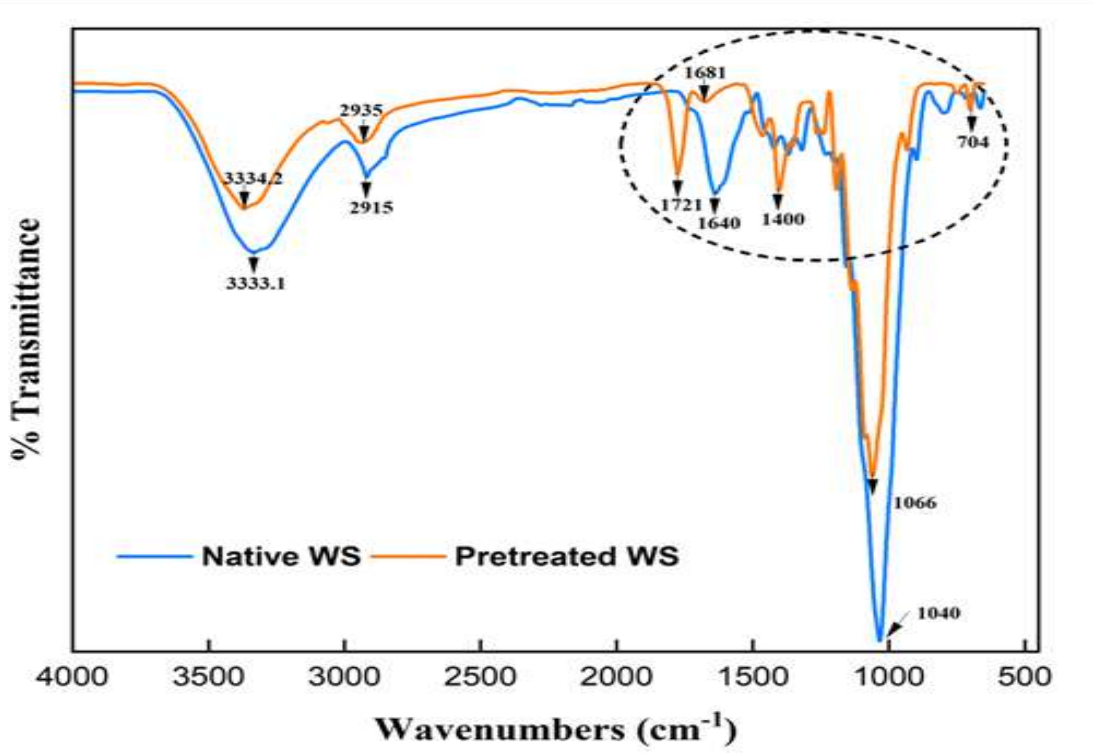

Figure 4.12: FTIR analysis of Native and Pretreated Wheat straw

Table 4.10: FTIR analysis of Native and Pretreated Wheat straw

Name of characteristic group	**Wavenumber (cm^{-1})**
O-H stretching vibration (hydrogen bonds)	3334.2
Stretching of $–CH_2$ and $-CH_3$	2935
C=O stretching of unconjugated ketone, carbonyls, and ester groups; C=O in xylan acetates (hemicelluloses)	1721
Bending mode of the absorbed water and stretching of C=O in lignin	1681
C-O stretching vibration in cellulose and hemicellulose	1400
Lignin fingerprint region	704

This glycosidic linkage of hemicellulose was absent in pretreated wheat which indicated that the pretreatment cleaved the glycosidic bond from the hemicellulose. The -CH deformation due to absorption within the methoxyl group of lignin and hemicellulose was represented by the absorption peak at 1400 cm^{-1} in pretreated wheat straw. The release of acid-soluble lignin was observed due to adsorption in pretreated biomass. The structural changes in cellulose and hemicelluloses were due to the bands in the peak range of 1000–1200 cm^{-1}. The change in the absorption peak at 1040 and

1066 cm^{-1} in native and pretreated wheat straw respectively suggested a cellulose distortion in pretreated biomass (Du *et al*., 2016).

4.11 Statistical optimization of pretreatment of rice straw

Total reducing sugar concentration as mentioned in (Table 4.11) serves for fitted data with coded values of independent variables using the 2^{nd} order polynomial model equation:

Reducing sugar = $9.26 - 0.3745A + 1.37B + 1.76C - 0.0060AB - 1.25AC + 1.25BC - 3.36A^2 + 0.1453B^2 - 1.11C^2$ (4)

where A= sulphuric acid (H_2SO_4) concentration, B= Hydrolysis temperature, C= Hydrolysis time

In order of reducing sugar production, (Table 4.12) displays the outcomes of acid hydrolysis of rice straw. During an experimental run at 120°C, 0.55% (v/v) acid concentration, and 105 minutes hydrolysis time, a maximum concentration of 12.56 g/L was achieved. During an experimental run at 90°C, and an acid concentration of 0.1% v/v a minimum reducing sugar of 2.54 g/L were also achieved. Lower acid concentrations and shorter hydrolysis periods were associated with lower total reducing sugar (TRS) concentrations, according to the results of the greatest and lowest reducing sugar concentrations. The increased TRS generation at relatively high temperature could be the result of proper acid concentration, temperature, and hydrolysis time for rice straw, as the efficiency of rice straw hydrolysis dropped at lower hydrolysis time.

The model summary for regression coefficients demonstrate that the quadratic model is consistent with the experimental data i.e. R^2 value was 98.07%, adjusted R^2 value was 95.58%, and anticipated R^2 value was 81.14%.

The ANOVA results given in Table 4.13 indicate that the breakdown of rice straw is greatly influenced by the concentration of sulphuric acid, the hydrolysis time, and the hydrolysis temperature. The p values for all linear and interaction coefficients, including B, C, AC, BC, A^2, and C^2, are less than 0.05 and thus were found to be statistically significant.

Table 4.11: BBD and their responses for 3 level, 3 factor response surface analysis

	Factor 1	Factor 2	Factor 3	Response 1
Run	**H_2SO_4 (%)**	**Temperature (°C)**	**Time (min)**	**Reducing sugar (g/L)**
1	0.55	120	105	12.56
2	0.55	90	60	8.54
3	0.55	60	15	6.54
4	0.55	90	60	9.56
5	0.55	90	60	8.95
6	0.55	90	60	7.54
7	0.1	120	60	4.56
8	0.1	60	60	4.56
9	0.55	90	60	9.54
10	1.0	120	60	7.52
11	0.1	90	105	8.54
12	0.55	60	105	7.54
13	0.55	120	15	6.54
14	1.0	90	15	3.54
15	0.55	90	60	9.65
16	0.1	90	150	2.54
17	1.0	60	60	4.56

Furthermore, a lack of fit with a F value of 1.75 indicates that the lack of fit is not important in relation to the pure error, and noise has a 29.51% chance of being the cause of a lack of fit with a large F-value. The contour plots (shown in Fig. 4.13) illustrate an infinite series of interactions between several hydrolysis parameters such as temperature, duration, and H_2SO_4 concentration.

Table 4.12: The RSM model and experimental data estimate the total reducing sugar (TRS) values following acid pretreatment of rice straw

Run	H_2SO_4 (%)	Temperature (°C)	Time (min)	Predicted Reducing sugar (g/L)	Experimental Reducing sugar (g/L)
1	0.55	60	105	7.43	8.42
2	0.43	99.85	39.87	8.26	8.51
3	0.47	71.95	98.93	8.67	8.89
4	1.0	120	60	7.03	7.58
5	0.53	102.69	54.06	9.54	10.02

The contours were used to show how independent variables interacted. Plotting the contours allowed researchers to look into how two independent factors interacted. The 3D surface plots (Fig. 4.13) depict an infinite number of combinations of the two test variables, with the third and fourth test variables retained at zero (the centre point) level. Temperature and diluted sulfuric acid concentration combine to form an ellipse and elliptical nature contour map.

The graph shows the relationship between the acid concentration and pretreatment temperature (AB) (Fig. 4.13a), acid concentration and hydrolysis time (AC) (Fig.4.13b), and pretreatment temperature and hydrolysis time (BC) (Fig. 4.13c) on the TRS concentration. The F and p values of the variables' interaction coefficients in the ANOVA analysis indicate that there is a substantial impact among the variables on the total reducing sugar yield. According to this model, the ideal rice straw hydrolysis conditions for diluted sulfuric acid produced a maximum of 12.56 g/L of reducing sugar at a 0.55 % (v/v) H_2SO_4 concentration, 120°C pretreatment temperature and 105 minutes of hydrolysis time.

Table 4.13: Reducing sugar content for the response surface quadratic model using Analysis of Variance (ANOVA). Three replicate experiments were conducted for all the mean and standard deviation values

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	107.68	9	11.96	39.45	<0.0001	Significant
A-H_2SO_4 Conc.	1.12	1	1.12	3.70	0.0958	
B-Temp	14.98	1	14.98	49.40	0.0002	
C- Time	24.67	1	24.67	81.34	<0.0001	
AB	0.0001	1	0.0001	0.0005	0.9832	
AC	6.20	1	6.20	20.44	0.0027	
BC	6.28	1	6.28	20.71	0.0026	
A^2	47.44	1	47.44	156.43	<0.0001	
B^2	0.0889	1	0.0889	0.2931	0.6050	
C^2	5.15	1	5.15	16.97	0.0045	
Residual	2.12	7	0.3033			
Lack of Fit	1.20	3	0.4016	1.75	0.2951	Non Significant
Pure Error	0.9181	4	0.2295			
Cor Total	109.81	16				
Std. Dev.	0.5507		R^2	0.9807		
Mean	7.23		Adjusted R^2	0.9558		
C.V %	7.62		Predicted R^2	0.8114		
			Adeq. precision	24.8737		

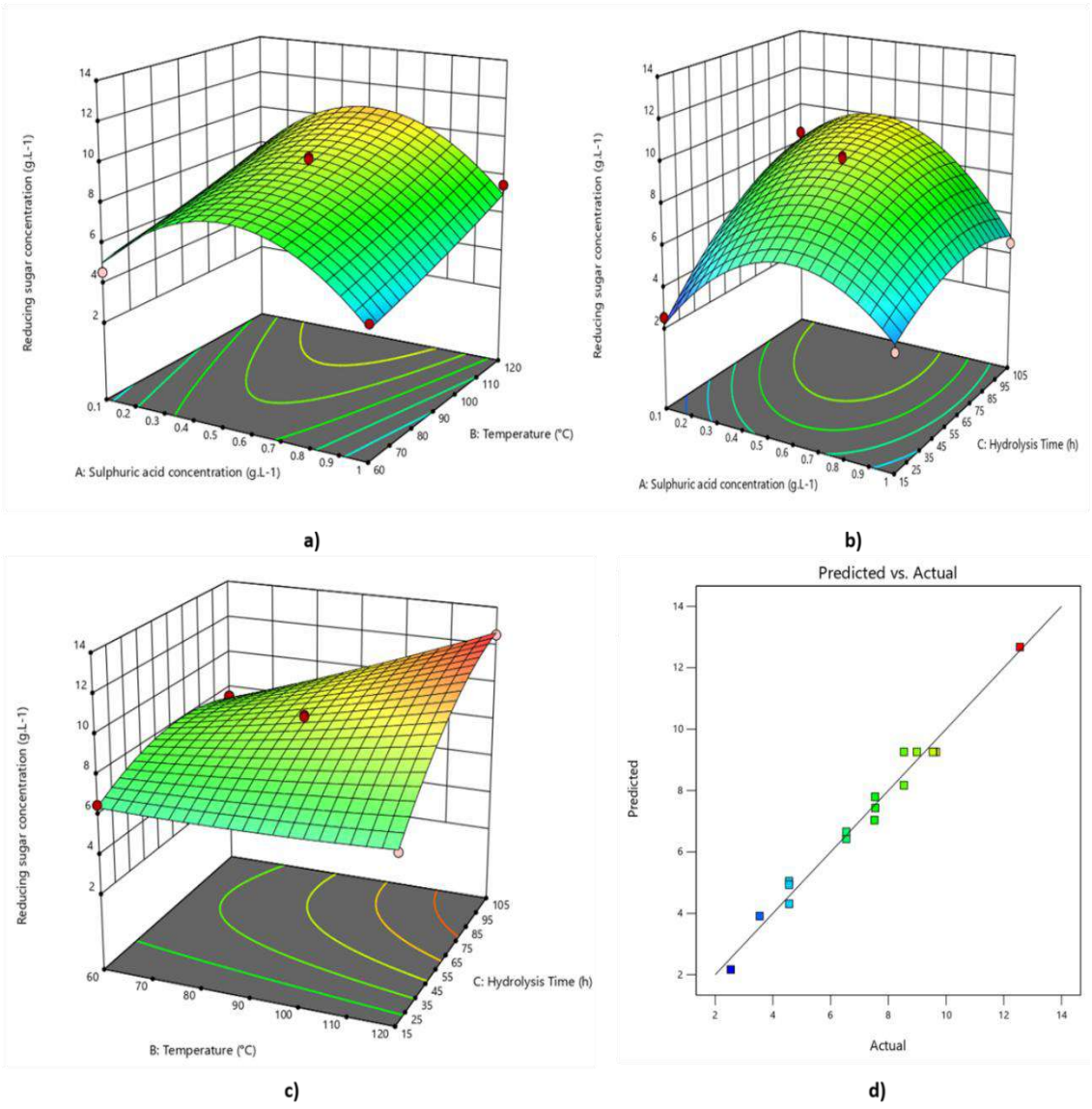

Figure 4.13: Three-dimensional surface map showing the relationship between total reducing sugar concentration and

a) sulfuric acid concentration and hydrolysis temperature (AB);
b) sulfuric acid concentration and hydrolysis time (AC); and
c) hydrolysis temperature and hydrolysis time (BC);
d) **shows how the actual and anticipated values of lowering sugar relate to one another**

To ensure reproducibility of the results predicted by BBD experiments and RSM analysis, confirmation tests of the breakdown of rice straw with diluted sulfuric acid were conducted in triplicate under ideal conditions (0.55 % (v/v) H_2SO_4 concentration, 120°C pretreatment temperature and 105 minutes of hydrolysis time). The outcomes of the confirmation tests closely matched the predictions made by the model in ideal circumstances.

Lignocellulosic biomass of rice straw had 38 % cellulosic, 24 % hemicellulosic and 8 % total lignin content. Pretreated rice straw had 28.06 g/L and 40 % cellulose, 16.5 % hemicellulose, and 4.7 % lignin (Table 4.14). After pretreatment, the cellulosic content of dignified rice straw hydrolysate increased by 5.53 %, while the contents of lignin and hemicellulose decreased by 31.25 and 41.25%, respectively.

Table 4.14: The crystalline index (*CrI*) including the cellulosic, hemicellulosic, lignin, and reducing sugar content in case of native as well as pretreated rice straw

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	*CrI* (%)	Reducing sugar (g/L)
Native Rice straw	38	34	8	31.04	0.77 ± 0.04
Pretreated Rice straw	40.1	16.5	4.7	46.58	28.06 ± 0.08

After pretreatment with acid, the concentration of reducing sugars increased 36.44 times in the hydrolysate (Table 4.14). Rice straw was treated with a dilute sodium hydroxide solution (0.4 N NaOH) for the delignification of the structural component lignin. After pretreatment, the cellulose content of dignified rice straw hydrolysate increased by 5.5%, while the amounts of hemicellulose and lignin dropped by 31.25 and 41.25 %, respectively. Other investigators found that untreated rice straw has a glucan content of around 41.34% and xylan content of about 28.46%; however, after dilute acid pretreatment, the glucan content rises to 49.77% (Kumar *et al*., 2014) . Additionally, a recent investigation on the synthesis of bio methane from rice straw found that lignin and hemicellulose are reduced but cellulose content is maintained by an alkali pretreatment of 1% NaOH for three hour at room temperature. Pretreatment increases methane emission by around 34% as compared to untreated rice straw (Akhtar *et al*., 2016).

4.12 Characterization of native and pretreated rice straw

4.12.1 Scanning Electron Microscopy (SEM)

The structural changes in rice straw caused by the pretreatment procedure were studied using SEM. The surface and physical structure of the native rice straw were both conserved (Fig. 4.14). Because the cuticle wax and silica layers were destroyed during

the dilute acid treatments, the biomass morphology changed, with more papillae and wart like features (Fig. 4b).

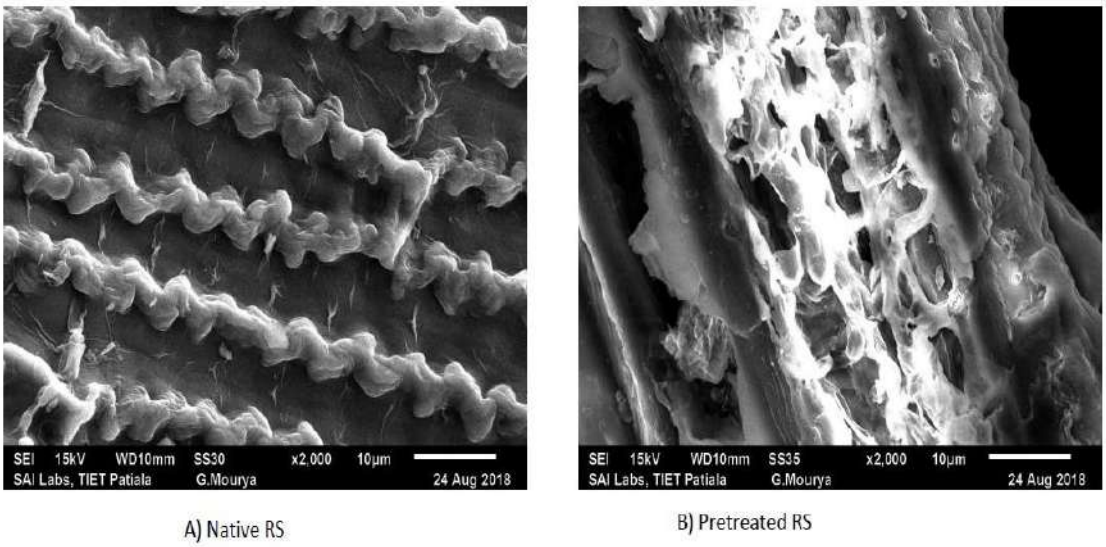

Figure 4.14: Scanning electron micrographs of (a) native rice straw, (b) pretreated rice straw (1000X)

The surface lignin in the biomass was removed by alkaline pretreatment, providing access to the inner cellulose and hemicellulose components. The combined two-step processing caused substantial damage to the biomass's silicified waxy surface as well as cell wall disintegration.

4.12.2 X Ray Diffraction (XRD) analysis of Native and Pretreated Rice straw

The native and the pretreated XRD profile of rice straw is depicted in (Fig. 4.15). According to Table 5, the crystalline index (*CrI*) of pretreated rice straw was 46.58%, while that of raw rice straw was 31.04%. The native biomass in comparison to pretreated rice straw had a higher percentage of *CrI* (15.54). Increase in *CrI* by 15.54% was observed after pretreatment. The dilute acid pretreatment of rice straw resulted in an increased crystallinity index (51.49 %), other researchers observed equivalent findings in native rice straw (*CrI* 40.84 %).

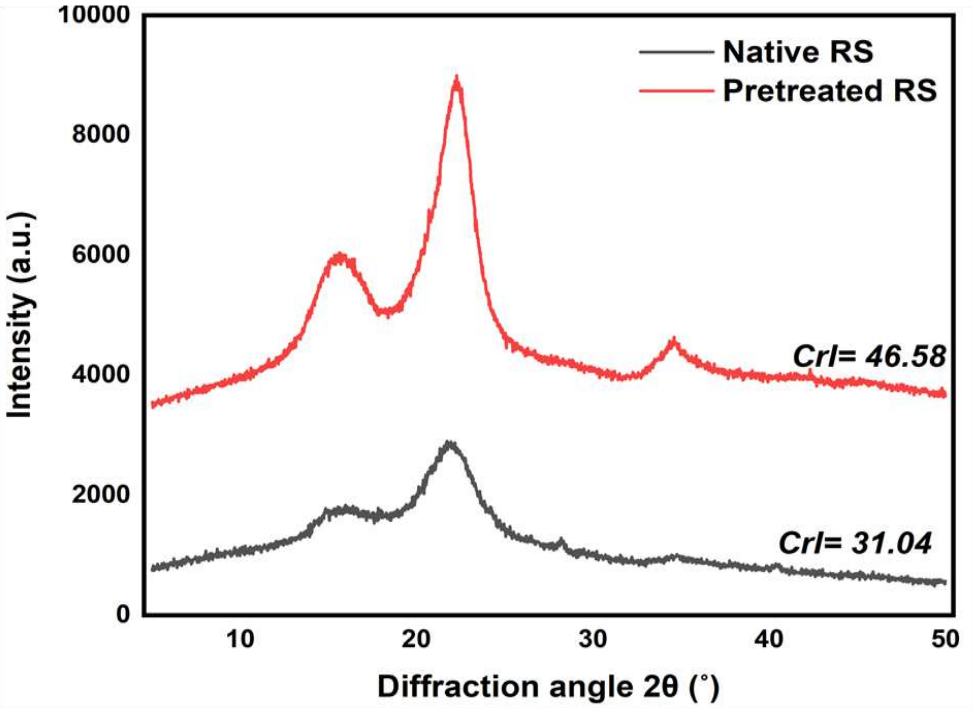

Figure 4.15: XRD analysis of Native and Pretreated Rice straw

Glycosidic bond hydrolysis caused a 10.65% increase in *CrI* in areas that were accessible to cellulose *(Grewal et al., 2020)*. The crystallinity of the material is dependent on factors such as the molecular weight of hydrocarbons and the fatty components of biomass. The hydrolysis of glycosidic bonds in the exposed areas of the cellulose maybe the cause of the increased *CrI* following pretreatment. In the amorphous zone as opposed to the crystalline region, acid pretreatment had a stronger effect on crystallinity. Increased saccharification of rice straw was caused by the sulphuric acid's acidity, delignification by the alkali NaOH and high temperature treatment (Akhtar and Goyal., 2014).

4.12.3 Fourier Transform Infrared (FTIR) Spectroscopy Analysis of Native and Pretreated Rice straw

FTIR spectra reveal differences in the altered functional groups of pretreated rice straw and untreated biomass (Fig. 4.16). O-H stretching vibrations are produced in lignocellulosic rice straw biomass at a broad spectrum at 3327 cm^{-1} by hydrogen bonding between phenolic and alcoholic hydroxyl groups, and in pretreated rice straw at 3337 cm^{-1}.

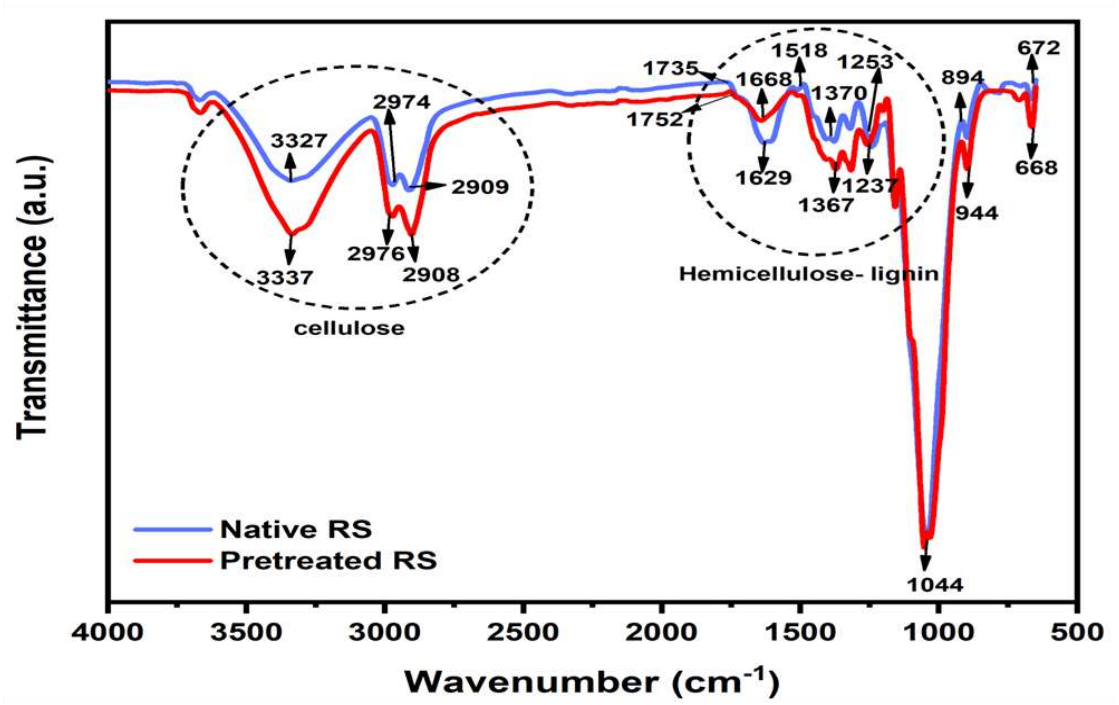

Figure 4.16: FTIR analysis of Native and Pretreated Rice straw

A change in wave number and stretching of the O- H band following sulfuric acid pretreatment suggested less hydrogen bonding between and within molecules, which led to a decrease in crystallinity (Grewal *et al*., 2020).

The band locations at 2974 cm^{-1} for native rice straw and 2976 $cm^{-1}$for pretreated rice straw suggested the C-H stretching vibration. The fact that the peak intensities of the native and processed rice straws differed slightly is an evidence for bending of cellulose's methylene ($-CH_2$) and methyl ($-CH_3$) sections (Table 4.15). The degradation of acetyl and uronic ester compounds, the ester bonding of ferulic and p-coumaric acids, and hemicellulose or lignin caused the largest drop in the 1629 cm^{-1} band in native rice straw. The absence of the hemicellulosic glycosidic connection in pretreated rice straw suggested that the glycosidic bond was damaged upon pretreatment. C-H stretching vibration was demonstrated by the band positions at 2974 cm^{-1} for native and 2976 cm^{-1} for pretreated rice straw (Du *et al*., 2016).

Table 4.15: FTIR analysis of Native and Pretreated Rice straw

Name of characteristic group	Wavenumber (cm^{-1})
O-H stretching vibration (hydrogen bonds)	3337
Stretching of $–CH_2$ and $-CH_3$	2976
C=O stretching of unconjugated ketone, carbonyls, and ester groups; C=O in xylan acetates (hemicelluloses)	1752
Bending mode of the absorbed water and stretching of C=O in lignin	1668
C-O stretching vibration in cellulose and hemicellulose	1237
Glycosidic linkage of hemicelluloses	944
Lignin fingerprint region	944,668

The significant absorption peak seen at 1370 cm^{-1} in the pretreated rice straw signifies the -CH deformation caused by absorption inside the methoxyl groups of hemicellulose and lignin. The adsorption in pretreated biomass resulted in the release of acid soluble lignin component, resulting in structural alterations in cellulose and hemicelluloses at the peak 1253 cm^{-1}, 1237 cm^{-1}, and 1044 cm^{-1}. The results showed a variation in the absorption peak at 894 cm^{-1}in native and 944 cm^{-1} in pretreated rice straw. This difference suggested that enzymatic cellulose deformation had occurred in the pretreated biomass (Du *et al*., 2016).

Objective 3: Optimization of LA production from lignocellulosic hydrolysates

4.13 Sugar utilization profile upon co- cultivation of DGS15 and DGB

LA (LA) production from lignocellulosic biomass has become a promising avenue in industrial biotechnology, particularly with the growing focus on sustainable processes. In this context, the co-cultivation of microorganisms has gained traction, offering several advantages over monocultures, including enhanced substrate utilization, improved productivity, and the ability to tolerate complex biomass feedstocks. This discussion aims to compare the co-cultivation of *Bacillus licheniformis* DGB and

Bacillus sonorensis DGS15 with other co-culture systems in terms of LA yield, productivity, substrate specificity, and scalability.

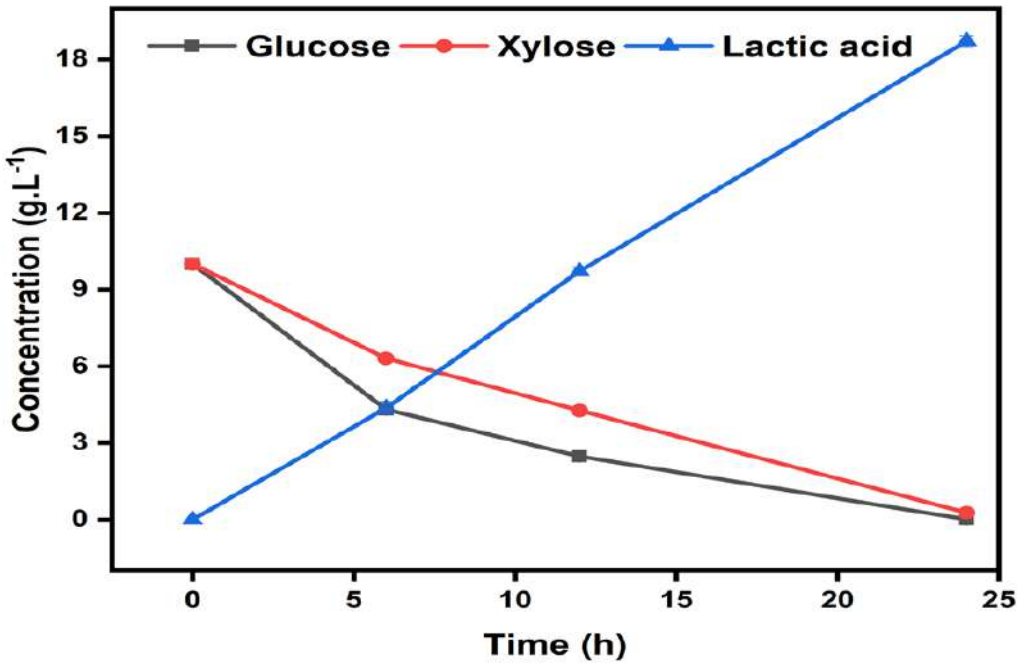

Figure 4.17: **Co- cultivation of *Bacillus licheniformis* (DGB) and *Bacillus sonorensis* (DGS15) during 24 h of complete fermentation in shake flask at pH 7.0, 120 rpm, 50 °C. Error bars represent the standard deviation of the trials with three replicates.**

The co-cultivation of both strains in the same minimal medium to determine whether the combination could result in improved sugar utilisation and LA production was a crucial component of this investigation. The outcomes showed that the two strains worked in concert:

LA Production: After 24 h of co-cultivation, 18.96 g/L of LA were produced, yielding 0.94 g/g of sugar eaten and an overall productivity of 0.79 g/L/h. With a faster utilisation of xylose and glucose than either strain showed alone, this is the highest productivity found in the study (Fig 4.17).

Synergistic Sugar Utilization: Within 6 h of incubation, glucose was fully used, and within 24 h, xylose was as well. Higher production resulted from the two strains' effective labour division, which accelerated the metabolism of both sugars.

The productivity of LA fermentation was greatly increased by the co-cultivation method. The co-culture was able to fully utilize both sugars in the minimal medium by taking advantage of each strain's special skills, such as DGB's quick xylose metabolism and GGS15's capacity to resist CCR. The increased rate of glucose utilization and the subsequent 24-h consumption of all xylose indicate that both strains made an equal contribution to the total amount of LA produced.

4.14 LA production from acid pretreated wheat straw hydrolysate

The synthesis of LA from lignocellulosic biomass, such as wheat straw hydrolysate, provides an environmentally benign and long-term industrial bioprocessing route. In order to evaluate the effectiveness of LA generation from wheat straw hydrolysate, two bacteria that are known for their capacity to ferment LA, *B. sonorensis* DGS15 and *B. licheniformis* DGB, were separately evaluated and co-cultivated in this study.

B. sonorensis DGS15: Based on the novel features of strain DGS15, a process was developed to produce LA from wheat straw biomass hydrolysate. Wheat straw (50 g dry weight) were acid pretreated and the obtained hydrolysate was adjusted to pH 7.0, then the fermentation was operated as described earlier.

Upon dilute sulphuric acid and hot water pretreatment of wheat straw, the concentration of glucose and xylose was 19.06 g/L and 9.14 g/L respectively in the WS hydrolysate. After 48 h of incubation, glucose and xylose concentration declined to 10.5 g/L and 5.02 g/L respectively with the production of 16.54 g/L LA. After 72 h further decline in glucose and xylose concentration were observed to 2.87 g/L and 4.32 g/L respectively with increase in LA concentration to 27.5 g/L (Fig. 4.18). In case of wheat straw hydrolysate, the concentration of inhibitors furfural and HMF was 0.47 g/L and 0.16 g/L respectively where furfural was consumed after 72 h of fermentation and HMF got accumulated with 3.75-fold increase in concentration in the fermentation broth.

DGS15 showed effective utilization of the two main sugars present in wheat straw hydrolysate, xylose and glucose. After 72 h of fermentation, the LA concentration rose to 27.5 g/L, yielding 0.96 g/g. DGS15's total production was 0.3819 g/L/h, which suggests that the fermentation rate was largely constant over time. Because DGS15 is thermophilic, it can function at 50°C, which increases its potential application in industrial environments by lowering the danger of contamination. For the strain to fully

use the sugars found in lignocellulosic biomass, it was necessary to overcome carbon catabolite repression (CCR), which allowed for the simultaneous absorption of xylose and glucose. LA concentration increases steadily while glucose and xylose are gradually depleted in the monoculture fermentation of DGS15 (Fig. 4.18). Whereas xylose was depleted after 72 h, glucose was finished in 48 h. This suggests that the fermentation process is steady but sluggish.

B. licheniformis DGB: This well-characterized LA producer yielded 0.86 g/g with a better productivity rate of 0.4013 g/L/h than DGS15, although producing a little lower LA concentration of 24.08 g/L after 48 h. The strain's shown ability to ferment hemicellulosic carbohydrates and generate cellulases is consistent with its ability to use xylose effectively. DGB is beneficial for procedures when time is a constraint because the LA generation process requires a shorter incubation period. In the case of DGB (Fig. 4.18), xylose was fully utilised in 48 h and glucose in 36 h. During the first 24 to 48 h, LA production increased upto 24.08 g/L.

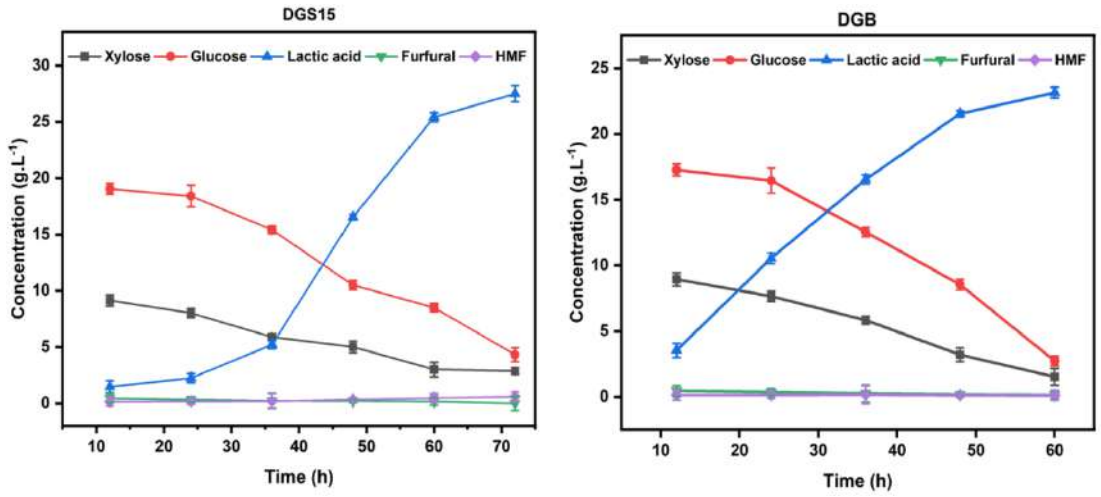

Figure 4.18. LA from wheat straw hydrolysate by *B. sonorensis* DGS15 and *B. licheniformis* DGB at pH 7.0, 120 rpm, 50°C

Co-Cultivation of DGB and DGS15

B. sonorensis DGS15 and *B.s licheniformis* DGB were co-cultivated, and after just 24 h, the LA concentration was 26.19 g/L with a yield of 0.93 g/g and a much higher productivity of 0.54 g/L/h. The synergistic interaction between the two strains is responsible for the notable increase in productivity when compared to mono cultures. In this co-culture system, DGB sped up the consumption of glucose, resulting in quick sugar depletion during the first 24 h of fermentation, whereas DGS15 helped to effectively utilize xylose.

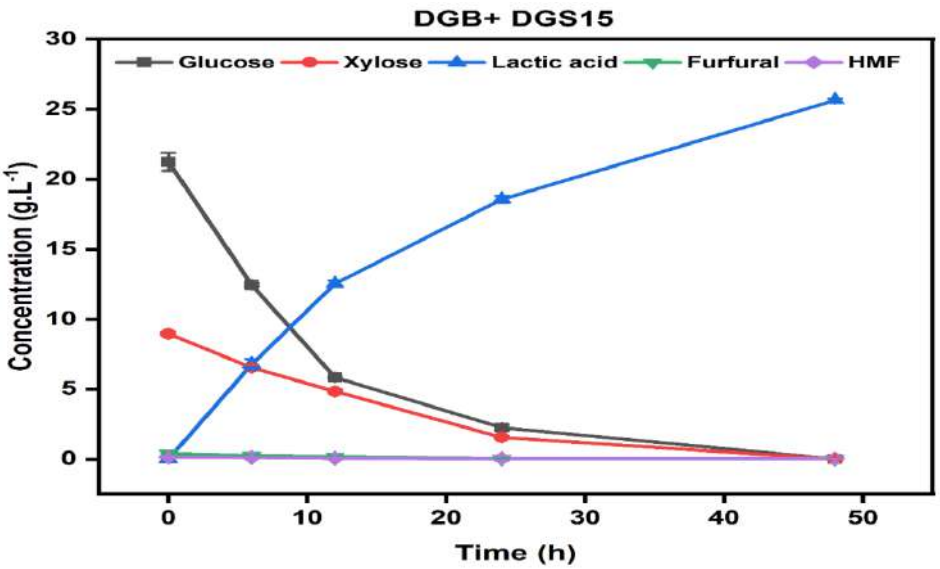

Figure 4.19: LA from wheat straw hydrolysate by co cultivation of *Bacillus sonorensis* DGS15 and *Bacillus licheniformis* DGB at pH 7.0, 120 rpm, 50°C

Both glucose and xylose were eaten far more quickly in the co-cultivation situation (Fig. 4.19), with total sugar depletion happening within 24 h. The quick uptake of sugar is consistent with the sharp increase in LA concentration, which at the conclusion of the process reached 26.19 g/L, demonstrating the effectiveness of the co-culture approach.

Table 4.16: Summary of LA production from wheat straw hydrolysate by single strain or co- cultivation of DGB and DGS15

Isolate	LA concentration (g/L)	Yield (g/g)	Productivity (g/L/h)
DGS15	27.5	0.96	0.3819
DGB	24.08	0.86	0.4013
DGB + DGS15	26.19	0.93	0.54

The co-cultivation approach made use of both strains' metabolic capacities, with DGS15's capacity to overcome CCR being particularly important, and DGB's capacity for cellulolysis increasing the total yield of LA. Remarkably, compared to DGS15 alone, the co-culture system was able to attain a similar LA concentration and a higher productivity rate in a shorter amount of time (Table 4.16).

When compared favourably to other studies on LA production from lignocellulosic biomass, the performance of DGS15, DGB, and their co-culture is demonstrated. For example, it has been demonstrated that mixed cultures of *Lactobacillus rhamnosus* and *Bacillus coagulans* can produce 31 g/L of LA from cassava bagasse, with a productivity of 1.036 g/L/h and a yield of 0.97 g/g. Similarly, 95% of the theoretical maximum LA output was obtained by co-cultivating *Lactobacillus pentosus* and *Lactobacillus brevis* on wheat hemicellulosic hydrolysate. These conclusions are supported by the co-culture of DGS15 and DGB, which produced favourable outcomes in terms of LA output and productivity, indicating the system's potential for use in industrial settings.

Producing LA from lignocellulosic biomass using the co-cultivation of *Bacillus sonorensis* DGS15 and *Bacillus licheniformis* DGB is a highly promising approach. The co-culture method holds appeal for commercial bioprocessing due to its improved sugar utilization, accelerated fermentation time, and heightened productivity. These strains' potential for large-scale uses is further strengthened by their resistance to inhibitors and thermotolerance. In order to meet industrial demands, future research should concentrate on improving the co-culture conditions and scaling up the procedure.

4.15 LA production from acid pretreated rice straw hydrolysate

Bacillus sp. of DGS15 and DGB has proved beneficial for LA production, due to their ability to produce LA utilizing both glucose as well as xylose at high incubation

temperature and overcoming inhibitor stress. Total reducing sugars produced upon the optimized pretreatment of RS could be utilized for LA production.

50 gm of rice straw (dry weight) was alkali pretreated and delignified using 0.1% NaOH, the results were confirmed using the FTIR where the largest drop in the 1629 cm^{-1} band in native rice straw suggested delignification. Further, the biomass was treated with optimized sulphuric acid conditions and hot water, the pH of the resultant hydrolysate was adjusted to 7.0 before fermentation, as previously reported. The rice straw hydrolysate thus obtained upon pretreatment consisted of 18.86 g/L of glucose and 6.566 g/L of xylose. Upon monoculture inoculation, DGS15 was inoculated and it was noticed that after 48 h the glucose concentration dropped to 5.6 g/L while the xylose concentration reduced to 1.2 g/L with the production of 18.72 g/L of LA which further increased to 21.09 g/L LA after 72h of incubation yielding 0.82 g/g LA with an overall productivity of 0.29 g/L/h (Fig. 4.20). Rice straw hydrolysate consisted of inhibitors furfural and HMF i.e. 0.4 g/L and 0.15 g/L respectively. After 72 h there was 0.1 g/L of furfural and 0.11 g/L of HMF left in the broth. It was noticed at furfural was utilized during LA production while HMF was still left in the fermentation broth.

Mono-culturing DGB in the pretreated RS hydrolysate consisting of 18.747 g/L glucose and 6.56 g/L xylose. After 60h of incubation 16.21 g/L LA was produced at 50°C, pH 7 yielding 0.64 g/g LA with an overall productivity of 0.27 g/L/h where the pH was maintained after every 12 h (Fig. 4.20).

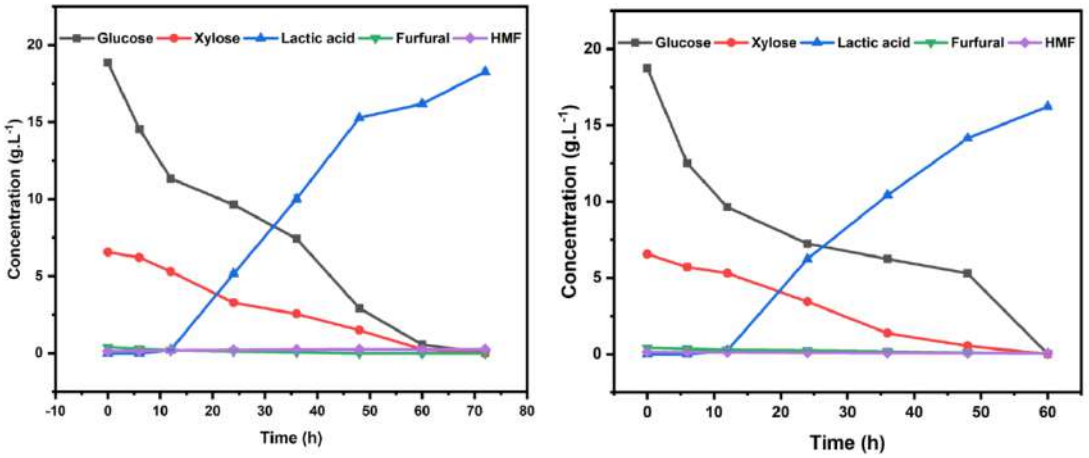

Figure 4.20: Production kinetics of LA and total sugar utilization {Rice straw Hydrolysate supplemented with Bushnell Haas medium (3.27 g/L) and yeast extract (5 g/L)} at pH 7.0, 120 rpm, 50 °C by a *Bacillus sonorensis* (DGS15) in 72 h; *Bacillus licheniformis* (DGB) in 60 h; Error bars represent the standard deviation of three independent replication

Further, co cultivation of *Bacillus* sps. DGB and DGS15 resulted in the efficient production of LA in even less time of incubation. The initial concentration of glucose was 18.197 g/L and xylose was 6.4g/L which reduced to 2.287 g/L and 2.33 g/L respectively after 12h of inoculation (Table 4.17). Glucose and xylose were completely consumed after 48h of incubation. The concentration of furfural and HMF continued to sustain in the broth which DGB and DGS15 being least affected by the stress due to inhibitor concentrations. The efficiency of co cultivation of DGB and DGS15 produced an efficient 21.15 g/L of LA after 48 h yielding 0.85 g/g LA with an overall productivity of 0.44 g/L/h. (Fig. 4.21)

Large scale production was also carried out with the optimized sulphuric acid pretreatment conditions of (H_2SO_4: 0.55 %(v/v); temperature: 120°C; hydrolysis time: 105 minutes) followed by NaOH pretreatment and treatment with hot water. 100 g of rice straw was pretreated producing 50.852 g/L reducing sugar and following separate hydrolysis and co-fermentation (SHCF), *B. sonorensis* DGS15 and *B. licheniformis* DGB produced 49.75 g/L LA after 48 h of fermentation with a yield of 0.97 g/g LA and productivity of 1.036 g/L/h.

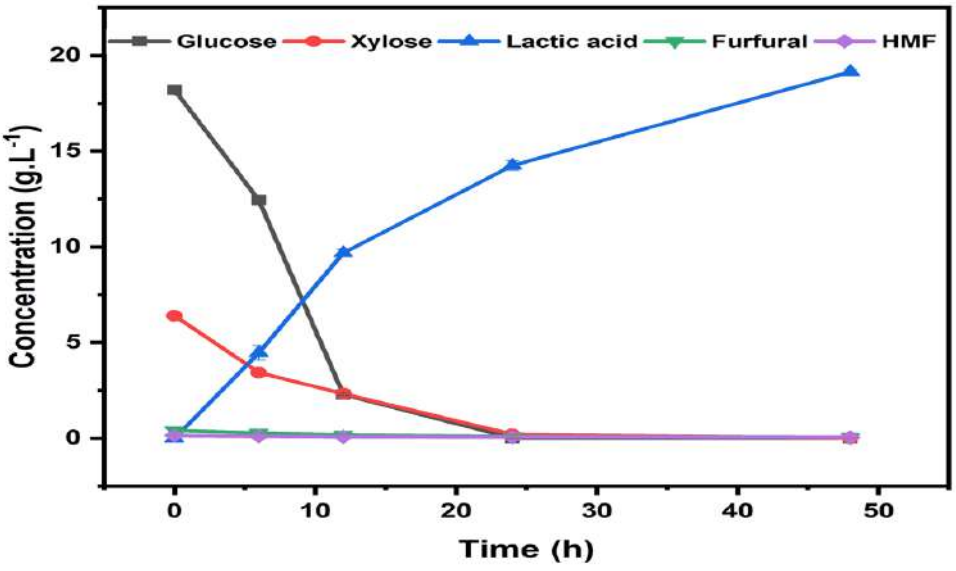

Figure 4.21: Production kinetics of LA and total sugar utilization {Rice straw Hydrolysate supplemented with Bushnell Haas medium (3.27 g/L) and yeast extract (5 g/L)} at pH 7.0, 120 rpm, 50°C by Co cultivation of *Bacillus licheniformis* (DGB) and *Bacillus sonorensis* (DGS15) in 48 h Error bars represent the standard deviation of three independent replication

Table 4.17: Summary of LA production from rice straw hydrolysate by single strain or co- cultivation of DGB and DGS15

Isolate	LA concentration (g/L)	Yield (g/g)	Productivity (g/L/h)
DGS15	21.09+/- 0.05	0.82 +/- 0.21	0.29 +/- 0.02
DGB	16.21 +/- 0.06	0.64 +/- 0.18	0.27 +/- 0.01
DGB + DGS15	21.15 +/- 0.05	0.85 +/- 0.22	0.44 +/- 0.03

.4.16 Comparative analysis of LA production from lignocellulosic biomass with other studies

In order to produce LA from lignocellulosic biomass, a significant amount of research has been conducted till date (Table 4.18). Historically, a single cultured microorganism has been employed in the microbial production of chemicals to facilitate the fermentation of substrate to finished product. More and more studies are now focusing on utilising a flexible co-culture technique, which involves co-growing microbes to enable a divide-and-conquer strategy for biochemical production (Jones & Wang, 2018). Following the trend, mixed cultures of *Bacillus licheniformis* DGB and *Bacillus sonorensis* DGS15 tremendously increased the LA yield and productivity with respect to the methods based on mono-culturing.

A maximum LA yield and productivity of 0.97 g/g and 1.036 g/L/h respectively were attained under the optimized conditions. Liquefied cassava bagasse supernatant was used as a substrate with the combined culture of *L. rhamnosus* and *B. coagulans* to produce 31.0 g/L LA (Chen *et al*., 2020). *L. brevis* and *L. pentosus* were co cultivated on enzymatic hydrolysed wheat hemicellulosic hydrolysate resulting in 95% yield by utilizing all the sugars (Zhang & Vadlani, 2015). Similarly, using poplar hydrolysate as substrate and sequential fermentation using *L. brevis* and *L. plantarum*, the initial concentrations of glucose and xylose were 35.4 g/L and 14.3 g/L, respectively, the glucose was entirely metabolized in 26 h. After 96 h of fermentation, xylose (2.2 g/L) was still present. There was no ethanol found, and 38.0 g/L LA content was observed (Zhang & Vadlani, 2015). Higher bioethanol and LA titers were obtained by co-

inoculation of *Kluyveromyces marxianus* CECT 10875 and *B. coagulans* A20 than by sequential inoculation (Demiray *et al*., 2024). Thus, the favorable effect of co-inoculation was explained by the results pointing to synergistic interactions between microorganisms. In a study led by (Wang *et al*., 2021), engineered *Pseudomonas putida* KT2440 and *Bacillus coagulans* NL01 together contributed towards high yield of LA 0.8 g/g. It was noticed that the process of fermentation was improved significantly due to the co-culture system as the *Pseudomonas putida* KT2440 exhibited tolerance to inhibitors while *Bacillus coagulans* utilized sugars present in the hydrolysate (Wang *et al*., 2021). (Shahab *et al*) IN 2018 showed that the combination of *Lactobacillus pentosus and Trichoderma reesei* could co-ferment hexoses and pentoses from beech wood that had been processed without detoxification, without the need for carbon catabolite suppression. The findings demonstrated the possibility of creating microbial consortia to facilitate the conversion of lignocellulosic biomass into high-value biochemicals (Sahoo & Jayaraman, 2019). Using whey permeate (lactose), a mixed batch technique involving *Lactobacillus delbrueckii and Lactobacillus lactis* TSG3 exhibited an improved balanced yield that was 0.90 g/g and approximately 45 g/ L- D-LA (Haokok *et al*., 2023).

Using a pre-hydrolysis and simultaneous saccharification and co-fermentation (SScF) procedure, the isolated strains of *Lactiplantibacillus plantarum* TSKKU P-8 and *Levilactobacillus brevis* CHKKU N-6 were utilised to convert cellulose and xylan in sugarcane bagasse (SB) into LA (LA) (Wang *et al*., 2016). The process produced 91.9 g/L of LA, resulting in a volumetric productivity of 0.85 g/L/h. Through mixed fermentation of *Bacillus coagulans* and *Lactobacillus rhamnosus* in non-sterile conditions, the generation of l-LA from sweet sorghum juice was optimized. This resulted in a LA concentration of 121 g/L, yielding 0.94 g/g and an overall productivity of 2.18 g/L/h (Wang *et al*., 2016).

Recent studies have explored innovative methods for lactic acid (LA) production from lignocellulosic biomass, utilizing advanced fermentation strategies and microbial consortia. (Wan *et al*., 2023) investigated the use of engineered *L. plantarum* for efficient conversion of corn stover hydrolysate, achieving an LA yield of 0.92 g/g with a final concentration of 78.3 g/L, demonstrating the potential of genetic modifications in enhancing productivity. Similarly, (Zhang *et al*., 2022) optimized simultaneous saccharification and co-fermentation (SSCF) of wheat straw using *L. brevis* and *B.*

coagulans, resulting in a 45% increase in LA productivity compared to traditional fermentation. (Yue & Zhang, 2023) conducted a bibliometric analysis of research trends in this field from 1991 to 2022, highlighting a consistent growth trajectory with a significant increase in publications since 2009. Their study emphasizes the global interest in utilizing lignocellulosic biomass for lactic acid production. (Shan *et al.*, 2023) reviewed microbial tolerance engineering as a strategy to boost lactic acid production from lignocellulose. They discussed how engineering microbial strains to withstand inhibitors derived from biomass pretreatment can enhance fermentation efficiency, thereby improving lactic acid yields. (Yankov, 2022) provided a comprehensive overview of fermentative lactic acid production from lignocellulosic feedstocks. The review covers various substrates, microorganisms, and process optimizations, offering insights into achieving economically viable lactic acid production from renewable resources. These studies collectively underscore the advancements and ongoing research efforts aimed at optimizing lactic acid production from lignocellulosic biomass, focusing on microbial engineering, process optimization, and economic feasibility.

B. licheniformis DGB *and B. sonorensis* DGS15 microbial consortia emerged in this work to effectively produce LA from lignocellulosic rice straw hydrolysate containing high inhibitor concentrations. The coculture system produced LA at a titre and yield that were similar to those previously documented. While this approach has yielded encouraging results, further work is needed to optimise the microbial co-cultivation process and regulate the process in order to maximise LA productivity and concentration, which is necessary to meet the practical requirements of lignocellulose bio refinement.

Table 4.18 LA production, yield and productivity from various substrates by distinct microorganisms and using different methods of fermentation

S.No	Substrate	Fermentation Method	Co cultivation of Microorganism	LA (g/L)	LA yield (g/g)	LA productivity (g/l/h)	Reference
1	Rice straw hydrolysate	Separate Hydrolysis and Co culture	*Bacillus licheniformis* and *Bacillus sonorensis*	49.75	0.97	1.036	Our study
2	Cassava bagasse	Simultaneous saccharification and co-fermentation	*Lactobacillus rhamnosus* and *Bacillus coagulans*	112.5	0.88	2.74	(Abdel-Rahman *et al*., 2021)
3	Wheat straw hydrolysate	SSF	*Lactobacillus brevis* and *Lactobacillus pentosus*	19	-	-	(Tu *et al*., 1992)
4	Poplar Hydrolysate	SSF	*Lactobacillus brevis and Lactobacillus plantarum*	38	0.8	0.4	(Kshirsagar *et al*., 2015)
5	Pomegranate peel	SSF	*Kluyveromyces marxianus* CECT 108755 and *Bacillus coagulans* A20	-	0.98	-	(Demiray *et al*., 2024)
6	corn stover hydrolysate	Sequential Hydrolysis and Co culture	*Pseudomonas putida KT2440 and Bacillus coagulans NL01*	35.8	0.8	0.37	(Shahab *et al*., 2018)
7	Microcrystalline cellulose	Consolidated Bioprocessing (CBP)	*Trichoderma reesei* and *Lactobacillus pentosus*	34.7	0.62	0.16	(Zou *et al*., 2021)
8	Beech wood	Consolidated Bioprocessing (CBP)	*Trichoderma reesei* and *Lactobacillus pentosus*	19.8	-	0.1	(Zou *et al*., 2021)

9	Whey permeate	Batch	*Lactobacillus delbrueckii* and engineered *Lactococcus lactis*	45	0.9	-	(Shahab *et al*., 2018; Zou *et al*., 2021)
10	Sugarcane Bagasse	Simultaneous saccharification and Co fermentation	*LactiplantiBacillus plantarum* and *LeviLactobacillus brevis*	91.9	-	0.85	(Li *et al*., 2021)
11	Sweet sorghum juice	Batch	*Bacillus coagulans* LA1507 and *Lactobacillus rhamnosus* LA-04-1 (Mixed culture)	121	0.94	2.18	(Wang *et al*., 2016)
12	Corn stover hydrolysate	SSF	*Lactobacillus rhamnosus* and *L. brevis*	14.8	0.7	0.58	(Cui *et al*., 2011)
13	Cassava bagasse hydolysate	Simultaneous saccharification and co-fermentation	*Lactobacillus rhamnosus* and *Bacillus coagulans*	31	0.91	1.5	(Chen *et al*., 2020)
14	Wheat straw hydrolysate	Batch	*Lactobacillus brevis* and *Lactobacillus plantarum*	38	0.8	0.4	(Zhang & Vadlani, 2015)
15	Kitchen waste	Batch	*Bacillus coagulans* and *Bacillus thermoamylovorans*	39.2	-	1.09	(Tashiro *et al*., 2016)

There are three primary steps for producing LA from lignocellulosic biomass: hydrolysis, pretreatment and fermentation. Microbial fermentation and hydrolysis can be done together with simultaneous saccharification and fermentation (SSF) or separately with separate hydrolysis and fermentation (SHF) (Yankov, 2022)

Since fermentation can be done in the same vessel as hydrolysis to prevent end-product inhibition of the enzymes, SSF has emerged as the favored method during the last ten years (Zou *et al*., 2021). Because fewer tanks are needed, the plant's capital investment cost is therefore decreased (Li *et al*., 2021). A drawback of SSF is that, unlike SHF, where these parameters can be adjusted independently, the operating temperature of SSF must be a compromise between the ideal temperatures for fermentation and hydrolysis (Li *et al*., 2021).

In general, hemicellulose content is high in rice straw and other agricultural leftovers (Li *et al*., 2021). Thus, for these lignocellulosic raw materials, xylose fermentation is crucial for efficiently converting all of the sugars into LA and raising the concentration in the fermentation broth. Through the homo fermentative pathway, *B. sonorensis* DGS15 and *B. licheniformis* DGB were able to ferment both xylose and glucose into LA at the same time. First, bacteria convert xylose to xylulose-5-phosphate (X5P), which is then processed via the pentose phosphate pathway (PPP) and the phosphoketolase (PKP) pathway to produce acetic acid and LA (Huang *et al*., 2021). They are also efficient candidates for usage in large-scale production because they are resistant to inhibitors like furfural and HMF (Kärcher *et al*., 2015).

The economic feasibility of scaling up this process to an industrial level was addressed and the overall viability and competitiveness of LA production from rice straw was compared to the existing methods. Pretreatment involves delignification using 0.1% NaOH (w/v) and hydrolysis of cellulose and hemicellulose to obtain C-5 and C-6 sugars in the hydrolysate using optimized 0.55% H_2SO_4 (w/v) followed by hot water treatment. The use of relatively cheap acid, less toxic alkaline and acidic substitutes for pretreatment cuts down the cost of pretreatment. The reducing sugar in the hydrolysate can directly be utilized for LA production while no further costly enzymes such as cellulase, xylanase are not required to be used. Further, co- fermentation using two *Bacillus* strains DGB and DGS15 which are closely related and are involved in utilizing both glucose as well as xylose respectively as the carbon source as compared to

monocultures thereby enhancing the yield and productivity of LA in a relatively very short time. Most of the pretreatment processes require a detoxification step as the inhibitors are produced upon acidic pretreatment but DGS15 and DGB being inhibitor tolerant contribute towards cost effectiveness. Moreover, the overall process is comparatively viable as rice straw can be effectively utilized and produce good yield of LA.

4.17 Salient findings

1. DGS15 a strain of *Bacillus sonorensis* accession No. MT883287, isolated from brick kiln near Patiala, Punjab and DGB a strain of *Bacillus licheniformis* accession No. KF840408.1, previously isolated by Akhtar and Goyal., 2014 from paper mill are found to have excellent xylose and glucose utilising capability respectively, thermotolerant and inhibitor tolerant to convert into LA.

2. Rice straw had 38 % cellulosic, 24 % hemicellulosic and 8 % total lignin content. Upon optimised pretreatment using RSM based BBD model on 50g rice straw, the resultant rice straw hydrolysate consisted of 28.06 g/L total reducing sugar. Further, the cellulosic content of dignified rice straw hydrolysate increased by 5.53 %, while the contents of lignin and hemicellulose decreased by 31.25 and 41.25%, respectively. Similarly, the delignified wheat straw hydrolysate consisted of 28.20 g/L total reducing sugar and had an increase in cellulose by 89%, while there was decrease in hemicellulose and lignin content by 73% and 62% respectively.

3. Wheat straw hydrolysate cultivated with DGS15 resulted in 27.5 g/L LA production after 72 h of incubation with decline in glucose and xylose concentration to 2.87 g/L and 4.32 g g/L respectively yielding 0.97 g/g LA and overall productivity of 0.381 g/L/h. Also, in case of rice straw hydrolysate, cultivated with DGS15 resulted in 21.09 g/L LA production after 72 h of incubation yielding 1.33 g/g LA and overall productivity of 0.29 g/L/h.

4. Both rice and wheat straw hydrolysates can be used as an efficient substrate for LA production. To enhance utilisation of C6-cellulosic sugars and C5-hemicellulosic sugars obtained from substrates and for the synthesis of LA, the mixed culture of *B. licheniformis* DGB and *B. sonorensis* DGS15 was

investigated, since efficient bacterial utilization of hexoses and pentoses is of significance for the economic conversion of lignocellulosic biomass to biochemicals.

5. Upon large scale production of LA, DGB and DGS15 individually yielded 0.64 g/g and 0.82 g/g LA respectively, where as their co-cultivation led to effective utilisation of both glucose and xylose within 15 h (70%) and complete utilisation in 48 h, producing 49.75 g/L LA with a yield of 0.98 g/g and productivity of 1.036 g/L/h, and resulting in reduction in fermentation time in case of rice straw hydrolysate.

6. Separate hydrolysis of rice straw and co-fermentation (SHCF) of hydrolysates by *Bacillus* spp. enhanced the production of LA, can circumvent challenges in biorefining of lignocellulosic biomass.

7. Further, the co-cultivation of DGB and DGS15 enhanced the productivity of LA production as the glucose and xylose were consumed within 48 h of incubation in case of both the substrates. In case of wheat straw, the efficiency of co cultivation of DGB and DGS15 produced an efficient 24.15 g/L of LA after 48 h yielding 1.16 g/g LA with an overall productivity of 0.51 g/L/h. While, in case of rice straw, an efficient 21.15 g/L of LA was produced after 48 h yielding 0.85 g/g LA with an overall productivity of 0.44 g/L/h.

8. The ability of *Bacillus licheniformis* DGB and *Bacillus sonorensis* DGS15 to utilize glucose and xylose and tolerance to inhibitors such as furfural and HMF, makes them a desirable candidate for the fermentation of rice straw hydrolysate into LA. Co-cultivation of DGS15 and DGB reduces the time of fermentation from 48 to 15 h with effective utilisation of sugars. *Bacillus* spp. being thermotolerant, therefore sterilising medium becomes energy efficient and cost effective.

4.18 Future scope of work

The proposed technique for converting pretreated lignocellulosic biomass into LA (LA) holds significant promise for future adoption in biorefineries and industrial applications. This method leverages the efficiency of specific bacterial strains, particularly the *Bacillus* strains DGB and DGS15, to address key challenges in the

bioconversion process. One of the main advantages of this approach is its ability to bypass the need for costly enzymes, such as cellulase and xylanase, typically used in biomass hydrolysis. Instead, the reducing sugars in the hydrolysate can be directly utilized for LA production, enhancing the economic feasibility of the process.

A critical aspect of the proposed method is the use of less expensive and less toxic alkaline and acidic substitutes for biomass pretreatment. This innovation helps reduce pretreatment costs significantly, which is a major hurdle in the overall process. The ability to use cheaper acid alternatives reduces the financial burden on biorefineries, contributing to a more sustainable and cost-effective operation.

Moreover, the co-fermentation process, involving the two closely related *Bacillus* strains DGB and DGS15, adds another layer of efficiency. These strains are capable of utilizing both glucose and xylose as carbon sources, as opposed to monoculture fermentation, which typically relies on glucose alone. This dual capability enhances the overall yield and productivity of LA, enabling a faster fermentation process and higher LA output in a relatively short time frame. This is a crucial factor in making the biorefining process more competitive with traditional chemical synthesis methods (Chen *et al*., 2020).

Additionally, the proposed technique benefits from the natural inhibitor tolerance of DGS15 and DGB strains. In many pretreatment processes, a detoxification step is necessary due to the production of toxic inhibitors during acidic pretreatment. However, the use of these *Bacillus* strains, which can tolerate such inhibitors, eliminates the need for this costly and energy-intensive detoxification step. This can result in significant savings and further contribute to the overall cost-effectiveness and sustainability of the process.

The feasibility of utilizing rice straw, a widely available and underutilized agricultural waste, as the feedstock for this process is another key benefit. Rice straw can be efficiently converted into LA with high yields, making it an attractive raw material for large-scale biorefinery operations. This sustainable feedstock choice also addresses environmental concerns related to agricultural waste disposal.

In summary, the proposed technique demonstrates strong potential for widespread application in biorefineries and industrial settings. By reducing the need for costly enzymes, utilizing cheaper pretreatment options, and enhancing fermentation efficiency

through co-fermentation with inhibitor-tolerant bacterial strains, this approach offers a more cost-effective, sustainable, and scalable solution for LA production from lignocellulosic biomass.

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APPENDIX I

NUTRIENT BROTH (pH- 7.0)

INGREDIENTS	QUANTITY (g/l)
Peptone	5
Beef extract	3
Sodium chloride	5
Distilled water	1000 ml

ENRICHMENT BROTH (pH-7.0)

INGREDIENTS	QUANTITY (g/l)
Xylose	10
YE	5

MODIFIED MINERAL SALT MEDIUM (pH- 7.0)

INGREDIENTS	QUANTITY (g/l)
Ammonium sulphate	2
Di potassium phosphate	2
Sodium chloride	2
Magnesium sulphate heptahydrate	0.2
Manganese sulphate heptahydrate	0.05
Ferrous sulphate heptahydrate	0.01
YE	5
Xylose	50
Calcium Carbonate	3

NITRATE BROTH MEDIUM (pH- 7.2)

INGREDIENTS	QUANTITY (g/l)
Peptone	5
Beef extract	3
Potassium nitrate	5
Sodium chloride	5
Distilled water	1000 ml

MR- VP BROTH (PH- 6.9)

INGREDIENTS	QUANTITY (g/l)
Peptone	7
Dextrose	5
Di potassium orthophosphate	5
Distilled water	1000 ml

UREA AGAR (pH- 6.9)

INGREDIENTS	QUANTITY (g/l)
Peptone	1
Sodium chloride	5
Xylose	1
Di potassium orthophosphate	2
Phenol red	0.012
Agar	15
Distilled water	900 ml
Urea solution (20 %)	100 ml

CARBOHYDRATE BROTH (pH- 6.8)

INGREDIENTS	QUANTITY (g/l)
Peptone	10
Sodium chloride	3
Xylose	10
Bromocresol purple	0.016
Distilled water	1000 ml

STARCH AGAR (pH- 7.2)

INGREDIENTS	QUANTITY (g/l)
Peptone	5
Beef extract	3
Sodium chloride	5
Starch	2
Agar	20
Distilled water	1000 ml

APPENDIX II

LUGOL'S IODINE SOLUTION

INGREDIENTS	QUANTITY (g/l)
Iodine	10
Distilled water	1000 ml

METHYL RED SOLUTION

INGREDIENTS	QUANTITY (g/l)
Methyl red	0.2
Ethyl alcohol (95%)	600 ml
Distilled water	400 ml

NITRATE REAGENT (SOLUTION A)

INGREDIENTS	QUANTITY (g/l)
Sulphanilic acid	8
5N acetic acid	1000 ml

NITRATE REAGENT (SOLUTION B)

INGREDIENTS	QUANTITY (g/l)
A-Napthylamine	5
5N acetic acid	1000 ml

VP SOLUTION I

INGREDIENTS	QUANTITY (g/l)
Naphtol	50
Ethanol (absolute)	950 ml

VP SOLUTION II

INGREDIENTS	QUANTITY (g/l)
Potassium hydroxide	400
Distilled water	950 ml

DNS REAGENT (1%)

INGREDIENTS	QUANTITY (g/l)
DNS	10
NaOH	10
Sodium sulphite	0.25
Potassium tartrate	192

Volume was made up to 1000 ml

All chemicals and reagents used for microbiological and chemical determinations were of the highest analytical grade and purchased from Sigma unless otherwise specified. Standard media components were purchased from Fisher Scientific (USA) or Sigma Aldrich (USA) and Hi-Media (Mumbai, India). Enzymes, Restriction enzymes and other chemicals of molecular biology grade were procured from New England Biolabs, UK unless otherwise specified. Sterilization of the media and chemicals were done in an autoclave at 121°C (15 lbs) for 15 min unless otherwise specified

BUFFERS

1. 50 mM Sodium Citrate buffer (pH 4.0, 5.0 and 6.0)

Stock solutions: A: 0.1 M citric acid; B: 0.1 M sodium citrate.

Use x mL A+ y mL B and dilute to 100 mL with 50 mL distilled water.

A (mL)	B (mL)	pH
37	13	3.5
33.0	17.0	4
25.5	24.5	4.5
20.5	29.5	5
13.7	36.3	5.5
9.5	41.5	6

2. 50 mM Potassium phosphate buffer (pH 7.0 and 6.0)

Stock solutions: A: 1M K_2HPO_4; B: 1 M KH_2PO_4

Use x mL A+ y mL B and dilute to 1000 mL with 995 mL distilled water.

A (mL)	B (mL)	pH
3.075	1.925	7
0.66	4.34	6

3. Glycine-NaOH buffer (pH 8.0, 9.0 and 10.0)

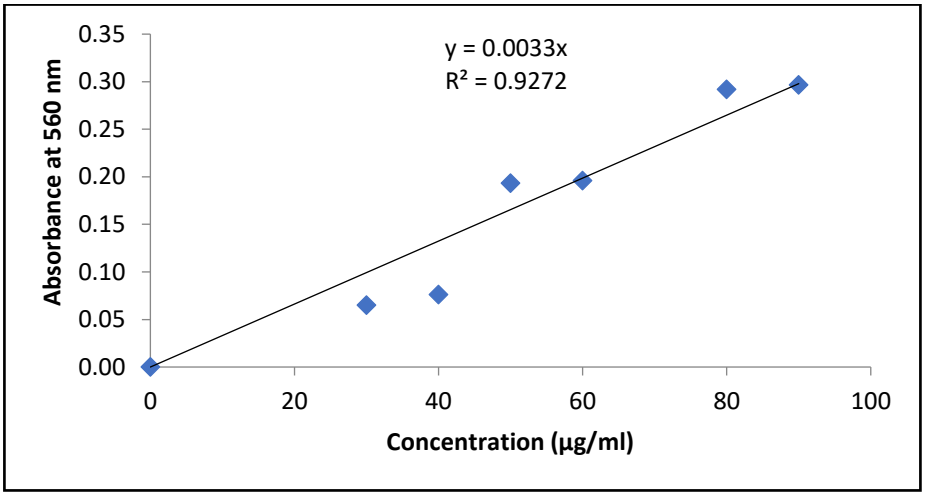
Stock solutions: 0.2 M glycine; 0.2 M NaOH. Combine 25 mL glycine stock with x mL 0.2 M NaOH and dilute with distilled water to make 100 mL solution.

0.2 M NaOH	pH
2	8
6	9
19.3	10

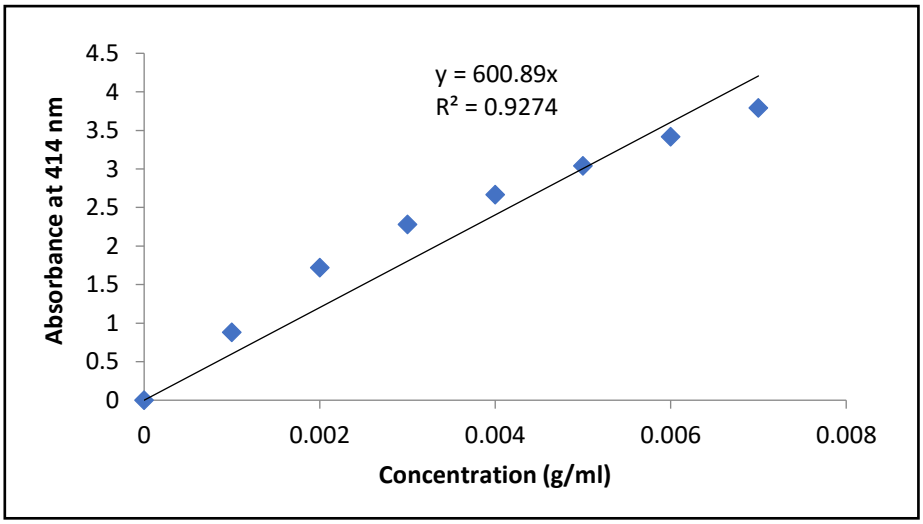
4. DNS Reagent

Distiiled Water	1415 mL
3, 5-Dinitrosalicyclic acid	10.5 g
NaOH	19.8
Rochelle salts (Na-K tartatate)	306 g
Dissolve the above and then add: Rochelle salts (Na-K tartatate)	306 g
Phenol (melt at 50°)	7.6 mL
Sodium metabisulphite	8.3 g

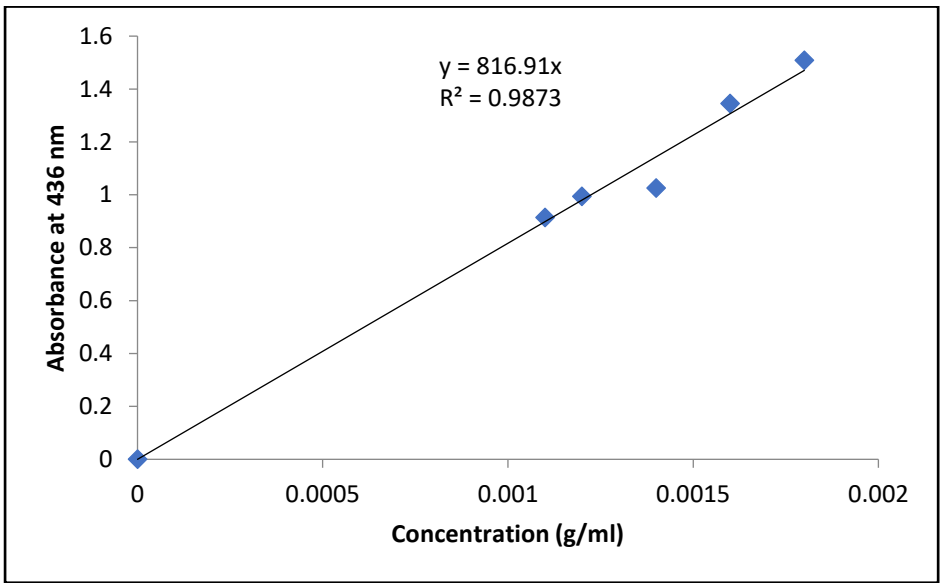
5. Standard Curves



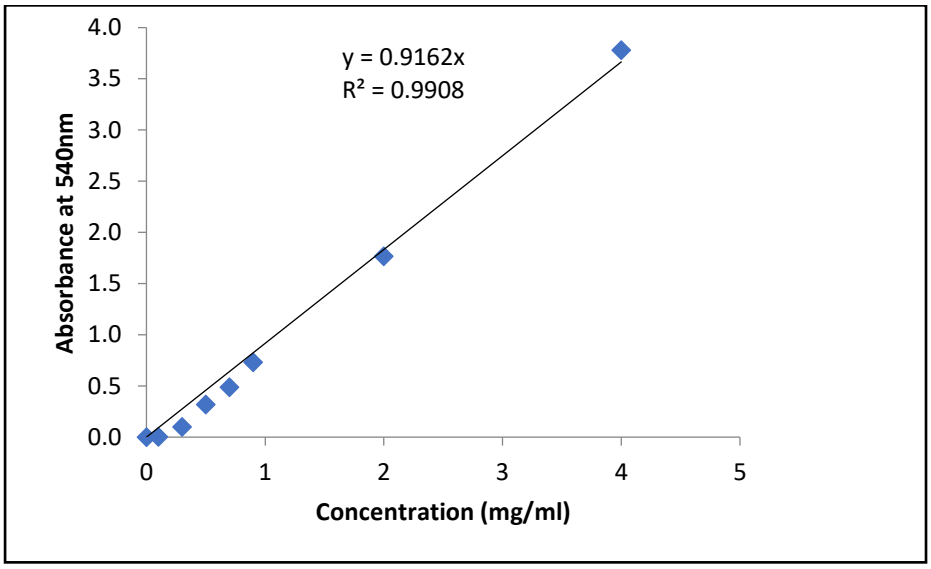
Standard curve of lactic acid



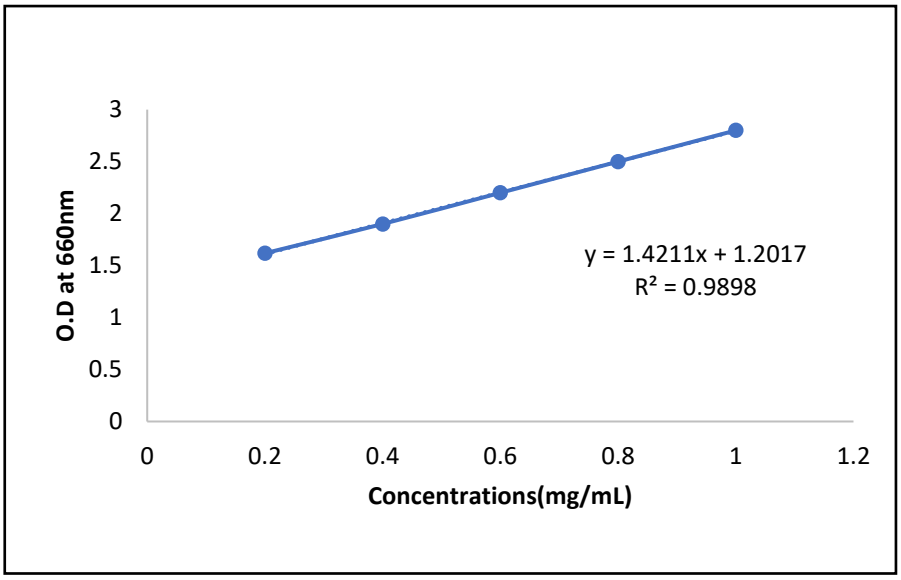
Standard curve for HMF



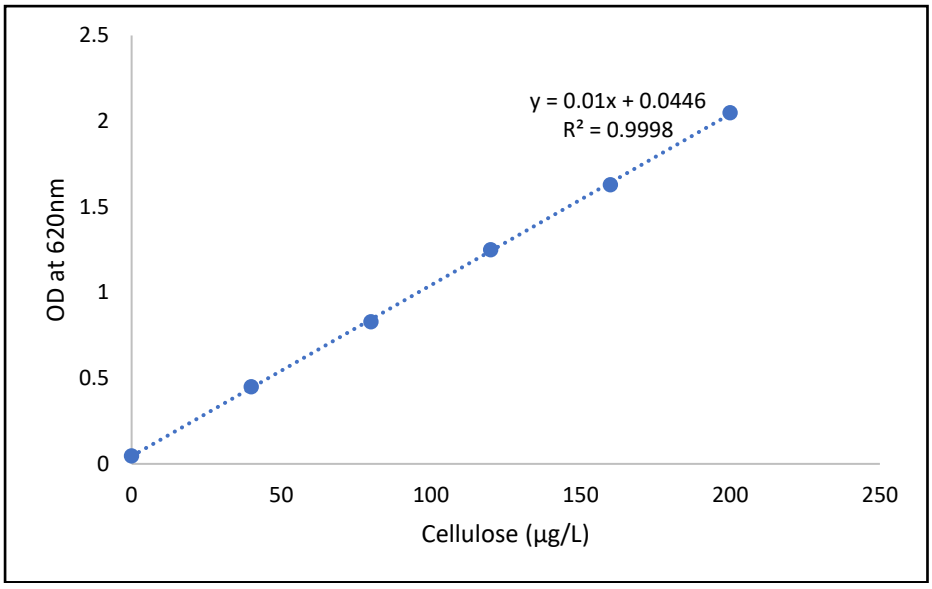
Standard curve for furfural



Standard curve for reducing sugar



Standard curve for estimation of xylose



Standard curve of cellulose

Appendix-III

NCBI GenBank details of bacterial isolates

Bacillus sonorensis strain DGS15 16S ribosomal RNA gene, partial sequence

GenBank: MT883287.1

FASTA Graphics

Go to:

LOCUS MT883287 1404 bp DNA linear BCT 31-DEC-2020
DEFINITION *Bacillus sonorensis strain* DGS15 16S ribosomal RNA gene, partial sequence.
ACCESSION MT883287
VERSION MT883287.1
KEYWORDS .
SOURCE *Bacillus sonorensis*
ORGANISM *Bacillus sonorensis*
Bacteria; *Bacillati; Bacillota; Bacilli; Bacillales; Bacillaceae;Bacillus.*
REFERENCE 1 (bases 1 to 1404)
AUTHORS Chawla,S. and Goyal,D.
TITLE Direct Submission
JOURNAL Submitted (14-AUG-2020) Biotechnology, Thapar Institute of Engineering and Technology, Bhadson Road, Patiala, Punjab 147004, India
COMMENT Sequences were screened for chimeras by the submitter using DECIPHER.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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Bacillus sonorensis strain DGN2 16S ribosomal RNA gene, partial sequence

GenBank: MW658910.1

FASTA Graphics

LOCUS MW658910 1470 bp DNA linear BCT 02-MAR-2021
DEFINITION *Bacillus sonorensis* strain DGN2 16S ribosomal RNA gene, partial sequence.
ACCESSION MW658910
VERSION MW658910.1
KEYWORDS .
SOURCE *Bacillus sonorensis*
ORGANISM *Bacillus sonorensis*
Bacteria; *Bacillati; Bacillota; Bacilli; Bacillales; Bacillaceae;* Bacillus.
REFERENCE 1 (bases 1 to 1470)
AUTHORS Chawla,S. and Goyal,D.
TITLE *Bacillus sonorensis* DGN2, 16S rRNA, partial sequence
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1470)
AUTHORS Chawla,S. and Goyal,D.
TITLE Direct Submission
JOURNAL Submitted (25-FEB-2021) Biotechnology, Thapar Institute of Engineering and Technology, Bhadson Road, Patiala, Punjab 147004, India
COMMENT Sequences were screened for chimeras by the submitter using Decipher v2.0.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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ORIGIN

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Production of lactic acid from lignocellulosic hydrolysates by thermotolerant bacteria

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