Production of lactic acid from wheat straw hydrolysates

DISSERTATION

Submitted by

HARSHITA SINGLA

302101011

In partial fulfilment for the award of the degree of

Master of Science in Biotechnology

Under the Guidance of

Prof. Dinesh Goyal

Department of Biotechnology

THAPAR INSTITUTE
OF ENGINEERING & TECHNOLOGY
(Deemed to be University)

Department of Biotechnology
Thapar Institute of Engineering & Technology, Patiala
August, 2023

CERTIFICATE

This is certified that the thesis entitled **"Production of lactic acid from wheat straw hydrolysates"** submitted by **Ms. Harshita Singla** (302101011), in partial fulfilment of requirement for the award of degree of **Master of Science** in Biotechnology at Thapar Institute of Engineering & Technology (TIET), Deemed to be University, Patiala is a record of student's bona fide work carried out under my supervision and guidance. The matter embodied in the thesis has not been submitted in part or full to any other university or institute for award of any other degree.

(Supervisor)
Dr. Dinesh Goyal
Professor
Department of Biotechnology
TIET, Patiala

DECLARATION

I hereby declare that the work presented in the dissertation entitled **“Production of lactic acid from wheat straw hydrolysates”** in partial fulfilment of requirement for the award of degree of **Masters of Science** in Biotechnology at Thapar Institute of Engineering & Technology (TIET), Deemed to be University, Patiala is an authentic record of my own work during the period from January 2023 to July 2023, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, TIET. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Date: 27-07-23
Place: Patiala

Harshita

Harshita Singla

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Date: 27-07-23

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Harshita

Harshita Singla

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List of Abbreviations

%	**Percent**
β	**Beta**
°C	**Celsius**
U	**Enzyme activity**
g	**Gram**
L	**Litre**
µl	**Microlitre**
mg	**Milligram**
ml	**Millilitre**
mm	**Millimetre**
mM	**Millimolar**
nm	**Nanometre**
h	**Hours**
TRS	**Total reducing sugars**

Abstract

Present research work is aimed at the production of lactic acid from wheat straw (WS), a lignocellulosic waste biomass. Grinded WS (0.5mm) and non-grinded WS were subjected to delignification followed by cellulose separation. Saccharification of cellulose was done by recombinant cellulases and compared with two different thermochemical treatments. The separated cellulose from grinded WS when subjected to enzymatic saccharification, using different enzyme loading ratios, which gave 2.39 mg/ml glucose (119.16±0.35 mg/g) in 48hrs. The cellulose obtained was also subjected to dilute acid hydrolysis at 121°C, with a sugar yield of 10.03 mg/ml and a yield of 0.32g/L lactic acid in 72hrs upon fermentation by *Bacillus sonorenesis* DGS15 at 50°C. The third pretreatment method was modified acid hydrolysis of delignified biomass, obtained from the first pretreatment at 60°C, 80°C and 121°C. Maximum yield of total reducing sugar and lactic acid of 0.528g/L was obtained from acid treatment of wheat straw at 121°C with overall yield of 0.014g/g and productivity of 0.0073g/L/h.

Chapter-1 Introduction

Wheat straw is a lignocellulosic biomass consisting of cellulose, hemicellulose and lignin. For every 6 tons of wheat produced in a year, 4 tons of leftovers are burned in open fields leading to air pollution in areas of Punjab, Haryana, Delhi (Gupta *et al.*,2004). Hence, with such abundant availability and high carbohydrate contents, it can be used for scale up production of various value-added products such as biogas, biofuels, organic acids, sugars, phenolic compounds, ethanol and other alcohols.

Among organic acids, lactic acid can be produced from wheat straw after chemical pretreatment in large quantities. Lactic acid (2-hydroxypropanoic acid) is an organic acid that is white in solid state and produces a colourless solution when dissolved in water. First discovered by a Swedish chemist Scheels (1780), it can be synthesized both chemically and using microorganisms. Production of lactic acid is in huge demand due to its widespread application in several industries such as

1. In food industry, it is used as a food additive, emulsifying agents, acidulant in beverages, gelling agents and inhibitors of bacterial spoilage in processed foods. It increases the shelf life of food items.
2. In pharmaceutical industry, it is used in formulation of ointments, lotions , anti-acne solutions, dialysis applications and in therapy of calcium deficiency (Wee *et al.*,2006).
3. In cosmetic industry, LA is used in moisturizers due to its high-water retaining capacity and for skin lightening and rejuvenation (Wee *et al.*, 2006)
4. LA usage has been increased in chemical industries such as acidulant in leather industry, solder flex, pH regulator, cleaning agent for decalcification, antimicrobial agent, starting material for production of herbicides and many others. Lactic acid esters can be used in green solvents (Dimerci *et al.*, 1993).
5. LA is used in production of bio-plastics and polylactic acid polymers (PLA) (Chawla & Goyal, 2022). Upon esterification, the hydroxyl and carboxyl groups form poly-LA (Datta *et al.*,1995).

The study was focussed on bacterial fermentation of wheat straw hydrolysate for production of lactic acid. Raw wheat straw was pretreated with chemicals, to remove the lignin encapsulation and facilitate the hydrolysis of holocellulose that results in breakdown of complex carbohydrates to reducing sugars followed by their bioconversion to lactic acid using thermotolerant bacteria *Bacillus sonorenesis* DGS15 (Chawla & Goyal, 2022). The hydrolysis was performed using different pretreatment methods such as using enzymes and acids to obtain maximum yield of reducing sugar yield for fermentation.

Cellulosic biomass including agricultural wastes, forestry residues, algal biomass, woody crops can be used as a sustainable source of fuels and chemicals production and can serve as feedstock for biorefineries. Despite the high availability, the complex structure of biomass restricts its hydrolysis to simpler forms. Hence, it is necessary to degrade complex forms into simpler forms in order to make them available for fermentation to produce value-added products. The general process of bioconversion is shown in Fig.1.

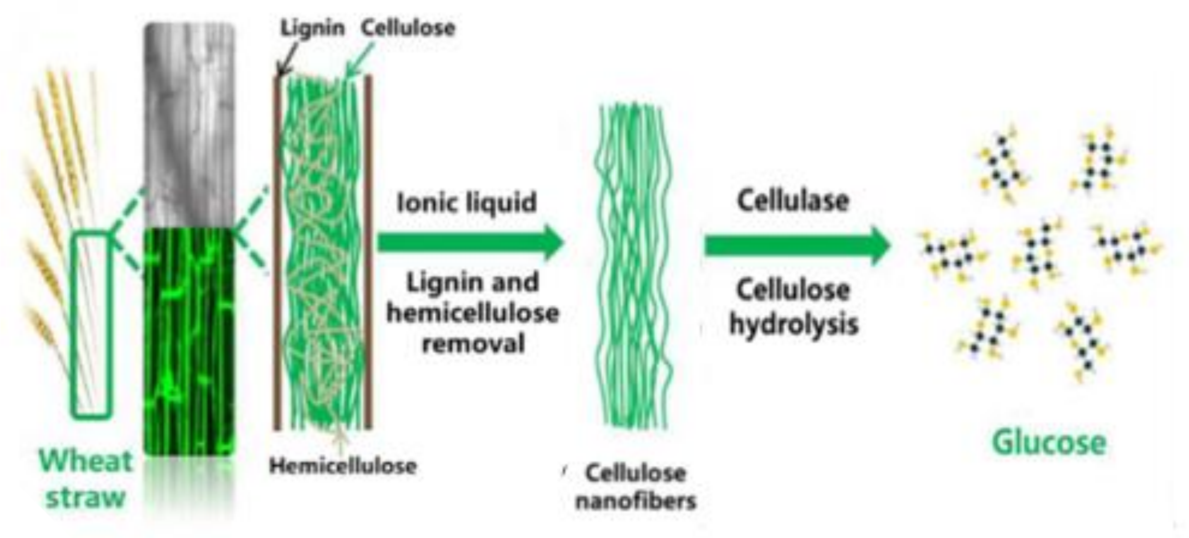


Fig.1 Process of bioconversion of wheat straw (Ying *et al.*,2022)

2.1 Plant derived lignocellulosic biomass

Plant lignocellulosic biomass is an abundant and sustainable source that can be used for making various organic products. The plant cell wall is composed of cellulose fibres held together by branched hemicellulose and complex network of lignin as shown in Fig.2. Hence, the biomass is composed of polysaccharides (cellulose and hemicelluloses), aromatic polymer (lignin), some lipids, minerals, and protein content (Yankov, 2022).

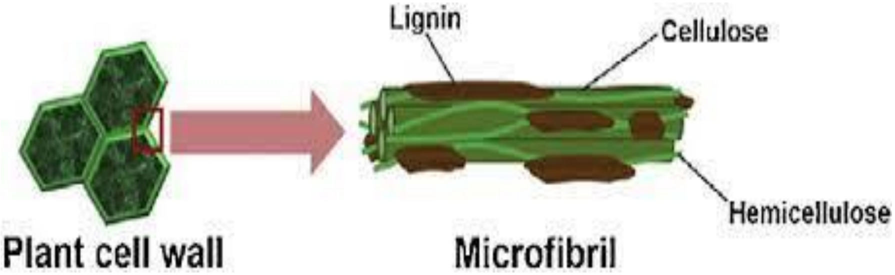


Fig.2 Structure of plant cell wall (Patchiya *et al.*,2018)

There is a significant difference in the lignin and cellulosic content of biomass based on its source. The woody plants have more lignin as compared to others, seen in Table 1.

Table 1. Chemical composition of different lignocellulosic biomasses

Plant Biomass	**Composition**			**References**
	Cellulose (%)	**Hemicellulose (%)**	**Lignin (%)**	
Wheat straw	36	25	20	Chawla & Goyal, 2022
Rice Straw	38	18	21	Tsegaye *et al.*, 2019
Oil palm fruit	40	23	21	Ilyas &Sapuan, 2018
Barley straw	14-16	27-30	31-40	Saini *et al*., 2015
Sorghum bagasse	35-40	18-27	14-21	Saini *et al*., 2015
Sugarcane bagasse	42	22	30	Saini *et al*., 2015; Kim & Day, 2011
Cornstalk	34.45	27.55	21.81	Ma *et al*. 2011
Corn stover	38-40	24-26	7-19	Qu *et al*., 2011
Bamboo stem	46.4	27.1	25	Chen *et al*., 2011
Sugarcane tops	36.1	24.2	33.3	Ferreira-Leitao *et al.*, 2010
Soy	56.4	12.5	18	Alemdar & Sain, 2008

2.1.1 Cellulose

Cellulose, the most abundant organic compound, is primarily a polymer of glucose with β-1,4 glycosidic bonds arranged in long straight chains (Zhang *et al.,* 2004). A major structural component of a primary cell wall, it also provides mechanical strength as shown in Fig.3. The structure of cellulose with hydrogen bonds makes it partially insoluble in most solvents and the solubility of the polymer is strongly related to the degree of hydrolysis (Kaur, 2014). Cellulose in lignocellulosic biomass is present in two forms i.e., crystalline and amorphous (Mussato *et al.,*2010; Kaur, 2017*).*

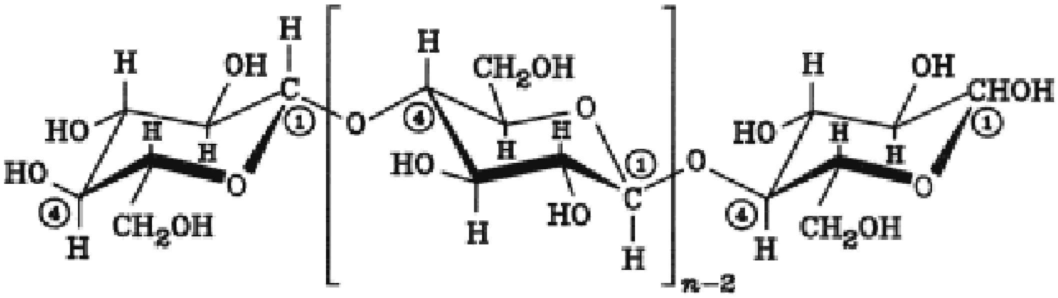


Fig.3 Structure of single cellulose molecule (Harmsen *et al.,*2010)

2.1.2 Hemicellulose

Hemicellulose is a heterogenous polymer primarily composed of pentoses (xylose, arabinose), hexoses (glucose, mannose, galactose) and other acids (Singh *et al.,*2008; Akhtar, 2014). It has a random, amorphous and branched structure (Fig.4) and is much easier to hydrolyse in acids as compared to cellulose (Taherzadeh and Karimi, 2008). Xylan is most common type of hemicellulose polysaccharide, consisting of D-xylopyranose linked by β-1,4-linkage (Mod *et al.,*1981). Hardwood hemicellulose consists of agricultural raw materials rich in pentoses while softwood hemicellulose is built from hexoses (Yankov, 2022). It reinforces the cell wall by interaction with cellulose and provides structural support.

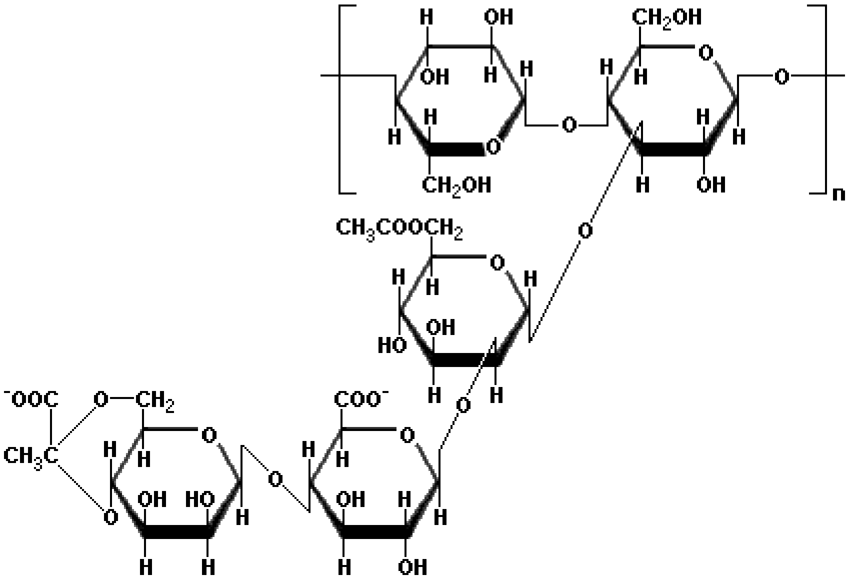


Fig.4 Structure of hemicellulose (Kulkarni *et al.*,2012)

2.1.3 Lignin

Lignin is a complex natural three-dimensional polymer (Fig.5). It is composed predominantly of phenylpropane units linked with ester bonds and forms complex with hemicellulose to encapsulate cellulose (Akhtar, 2014). Derived from wood, it is an integral part of secondary cell wall of plants (Kaur, 2014) and acts as a binding material between cells and enhances cell's endurance, development and provides resistance to microbial attack (Harmsen *et al.*,2010). Lignin is resistant to enzymatic and chemical degradation and thus, must be eliminated during pre-treatment to improve depolymerisation of holocellulose.

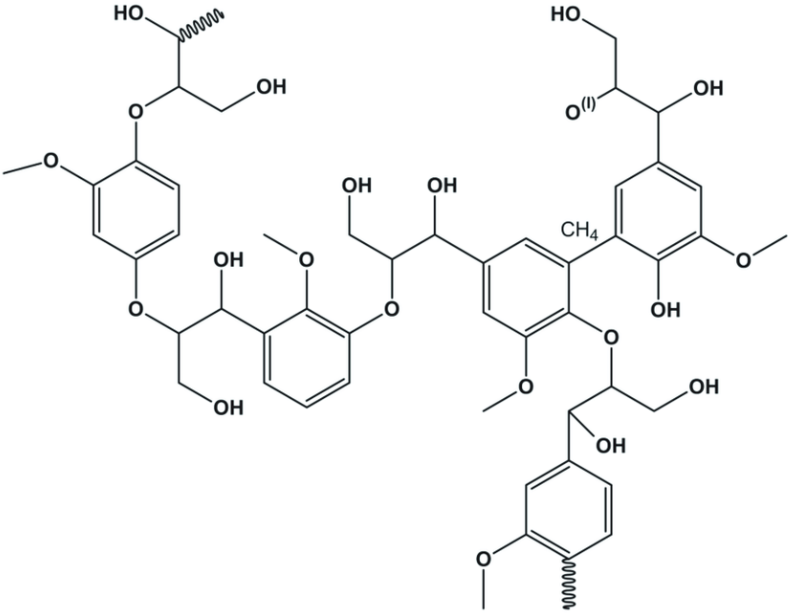


Fig.5 Phenylpropane units as building blocks of lignin (Sarika *et al.*,2020)

2.2 Processing of biomass

Reduction in particle size is the primary goal of biomass processing and is necessary to make material handling easier and to increase surface/ volume ratio. This increases efficiency of downstream processing and involves chipping, grinding, milling and their combination, microwave irradiation or ultrasound treatment (Harmsen *et al.*, 2010; Akhtar, 2014). This also decreases crystallinity of cellulose and degree of polymerisation in cellulose and hemicellulose (Yankov, 2022).

2.3 Pretreatment of lignocellulosic biomass

Lignocellulose restricts enzyme access to cellulose and hemicellulose fibres and limits effective hydrolysis due to its complex structure. Therefore, biomass is subjected to pretreatment to break open its structure for efficient saccharification by removing the lignin barrier, reducing the crystallinity of cellulose and it also contributes in cost reduction. Due to different compositions of lignocellulosic biomass, each method results in different effects; hence a universal method is not defined and the composition of hydrolysates obtained depends on the severity of treatment defined by parameters like temperature, concentration, incubation time and others (Yankon *et al.*,2022). These methods are mainly categorised into physical pretreatment, chemical pretreatment, physio-chemical pretreatment and biological pretreatment. The physical treatment includes irradiation, extrusion, pyrolysis, pulse electric field while biological treatment involves use of white, brown and soft rot fungi, bacteria to release holocellulose from lignin matrix. The combinations of these treatments can be used to enhance the digestibility of cellulose (Akhtar, 2014). Each pretreatment has a specific effect on the components of biomass (Govumoni *et al.*,2013).

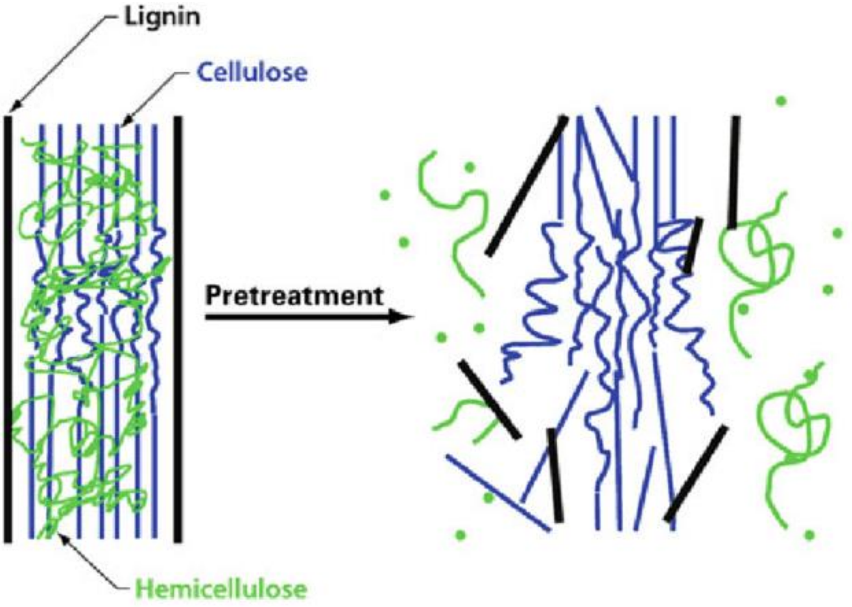


Fig.6 Representation of lignocellulosic biomass pretreatment (Xiao-Jun *et al.*,2012)

2.3.1 Chemical pretreatment

Chemical pretreatment includes reaction between biomass and various chemicals in water phase. The disruption of biomass is done using acids and alkali as oxidizing agents (Talebnia *et al.,* 2010; Harmsen *et al.,* 2010).

Acid treatment

Acidic pretreatment is used to remove hemicellulosic portion and some fraction of lignin (Govumoni *et al.*,2013). It is done using dilute or concentrated acids, reducing the cost of experiment. Mainly, sulphuric acid and hydrochloric acid are employed as they are powerful agents for hydrolysis. Holocellulose content is predominantly hydrolysed by conc. sulphuric acid treatment at low temperature and pressure (Ogier *et al.*,1999). The optimum temperature for treating with dilute acid is 100-200°C with 0.5-3% acid (Sun *et al.*,2016). The pretreated wheat straw with dilute acid produced 4.92 g/L glucose, 17.17 g/L xylose and 46.12 g of LA (Zhang *et al.*,2014; Kaur, 2017). The major drawback is sometimes, acid treatment leads to over degradation of biomass, with production of inhibitors such as furfural, hydroxymethylfurfurals (HMF).

Alkali treatment

In comparison to acids, hydroxides are less corrosive and can be applied at moderate temperatures and pressures. Treatment with hydroxides like NaOH, KOH, calcium hydroxide leads to disruption of cell wall by dissolving hemicellulose, lignin, silica; swelling and partial decrystallization of holocellulose (Cheng *et al.*,2010; Govumoni *et al.*,2013). The ester linked substituents of hemicellulose can also be cleaved by alkali (Ternrud, 1987). Lime and Na are common catalysts used at temperature 30-130℃ and low pressure, hence is a cost-effective operation (Parayo *et al.*,1996). Barley straw led to 86% increase in reducing sugars, when pretreated with aqueous NaOH (0.5-2%) at 105℃ for 10 min to remove lignin (Haque *et al.*,2012). Lime has been used to treat wheat straw at 85℃ for 3 hrs producing effective results of hydrolysis (Chang *et al.*,1998).

Different pretreatment methods were studied for different lignocellulosic (Table 2) with increase in yield of reducing sugars. Alkali and biological treatments were less effective than acid hydrolysis, since acid treatment gave higher yield of reducing sugars.

2.4 Hydrolysis of pretreated biomass

Saccharification is a critical step for lactic acid production, as it converts complex carbohydrates into simpler reducing sugars using acid, alkali and enzymes (Ferreira *et al.*,2010). Complete hydrolysis of solid residue of hemicellulose was observed at 180 °C and of cellulose at 200 °C after around 120 min reaction time. Generally, the hydrolysis of cellulose produce hexoses, glucose and hemicellulose produces pentoses, hexoses and organic acids. Due to high stability of cellulose structure, it prevents the onset of hydrolysis in milder conditions. Hence, the selection of optimum conditions is crucial for maximizing the yields of sugars (Świątek *et al*., 2020).

Table 2. Comparison of different pre-treatment methods of lignocellulosic biomasses

Lignocellulosic Biomass	**Pre- treatment**	**Yield of reducing sugars (%)**	**Reference**
Wheat straw	Acid by sulphuric acid	57	Chawla & Goyal, 2022
	Steam explosion	32	Cubas-Cano *et al*., 2019
	Physical (1.1 atm pressure for 30 min at 121 °C)	58.8	Cizeikiene *et al*., 2018
	Sulfuric acid	46	Zhang *et al*., 2014
	Autohydrolysis	50	Ertas *et al*., 2013
	Lime by Calcium hydroxide	63	Maas *et al*., 2008
	Wet oxidation	61	Garde *et al*., 2002
	Enzymatic treated hemicellulose hydrolysate	51	Garde *et al.*, 2001
Corn Straw	Sodium hydroxide 8%	62.9	Song Z *et al*., 2014
	Ammonia 10%	67.7	Song Z *et al*., 2014
Corn stover	NaOH 5%	68	Hu *et al*., 2015
	Enzymatic (laccase)	24.3	Schroyen M *et al*., 2015
	Dry sulfuric acid	65	Zhao *et al*., 2013
Corn fibre	Sulfuric acid	39	Bischoff *et a*l., 2010
Corn cobs	Acid treatment	53	Moldes *et al*., 2006
Rice Straw	Ammonia	53.9	Wang J *et al*., 2014
	High-pressure homogenization 0.3mm	10	Chandra R *et al*., 2012
	NaOH + hydrothermal	63	Chandra R *et al*., 2012
Groundnut shell	High-pressure homogenization 4mm	60	Jekayinfa *et al*., 2020
Sorghum stalk	Sulphuric acid	54.5	Dahunsi *et al*., 2019
Barley Straw	High-pressure homogenization 5mm	54.1	Menardo S *et al*., 2012
Sugarcane bagasse	Acid treatment	60	Oliveria *et al*., 2019
	Acid treatment + steam explosion	64	Van der Pol *et al*., 2016

2.4.1 Acid hydrolysis

This method employs concentrated and dilute acids, such as sulfuric and hydrochloric acid, to produce fermentable sugars (Goldstein,1983). Dilute acid treatment is effective on most materials including softwood, hardwood, agricultural crops, wastepaper and herbaceous crops. Dilute acid pretreatment (DAP) increases crystalline portion of cellulose and swelling of biomass by solubilizing xylose from biomass (Kim *et al.*,2012). Using dilute sulphuric acid as catalyst at higher temperatures led to increase in release of sugar concentration (Świątek *et al*., 2020). The use of concentrated acids may lead to severe degradation and formation of inhibitors (Alvira *et al.*,2010).

2.4.2 Enzymatic hydrolysis

Enzymatic saccharification is an essential stage to bioconvert polymeric sugars to mono-sugars prior to fermentation. It is an effective and green process to enhance acids and bioethanol production (Yadav, 2017). Cellulase and hemicellulase are the two broad categories that are used to describe enzymes. Endo-1,4-β xylanase and others are required to hydrolyse xylan of hemicellulose part (Carvalheiro *et al.*,2008). Cellulase enzymes can convert cellulose chains into glucose molecules under relatively moderate conditions. For effective degradation 3 categories of cellulolytic enzymes are used (Abedi *et al.*,2020):

(i) **Endo-β-1,4-glucanases** randomly hydrolyse accessible intramolecular β-1,4-glucosidic bonds of cellulose chains and introduce a water molecule in the -(1,4) bond, forming a new pair of reducing and non-reducing chain;

(ii) **Exo-β-1,4-glucanases** or cellobiohydrolases can hydrolyse cellulose chains at the polymer ends, resulting in the release of soluble cellobiose or glucose;

(iii) **β -Glucosidases (cellobiase)** cellobiose is further hydrolysed by cellobiase into 2 glucose molecules.

Table 3. Enzymes required to degrade lignocellulose to monomers (Van & Pletschke., 2012)

Lignin	Laccase, Manganese peroxidase, Lignin peroxidase Pectin Pectin methyl esterase, pectate lyase, polygalacturonase
Hemicellulose	Endo-xylanase, acetyl xylan esterase, β-xylosidase, endomannanase, β-mannosidase, αglucuronidase, ferulic acid esterase, α-galactosidase
Cellulose	Cellobiohydrolase, endoglucanase, β-glucosidase

Several species of bacteria (*Bacillus licheniformis, Psedomonas, Cellulomonas, Clostridium)* and fungi (*Trichoderma, Aspergillus niger*) can produce enzymes (Sun & Cheng, 2002). Many commercially pure enzymes are produced around the world such as *Celluclast*® 1.5 L (Novozyme, Denmark), Accellerase® (DuPont industrial bioscience), Cellic® CTec (Novozymes), CelluZYME® (Verenium Corporation) and others. The cost of these enzymes is high and a huge amount is required on industrial scale. The enzymes can be engineered from bacteria and fungi using recombinant DNA technology, producing recombinant cellulases with improved catalytic activity, stability and high gene modification potential. The advantages also include cost effective production with optimized process, consistency in the enzyme product, customization for specific enzyme activities enhancing biomass conversion; lower waste generation. The risk of contamination present in natural cellulase mixtures is also reduced. Recombinant cellulases including Cel6A, Cel8A, BGL1, CtCel8A, CtCBH5A, HtBH1 are effective in hydrolysis of different substrates producing efficient sugar yields.

2.5 Detoxification of hydrolysates

The pretreatment produces inhibitory compounds in the hydrolysates due to factors such as hydrolysis time, temperature and acid concentration. Therefore, detoxification is an essential step in bioconversion. Pentoses dehydrate into furfurals, whereas hexoses break down into hydroxy-methylfurfural (HMF). These compounds are toxic to fermentative microorganisms as they affect the cell integrity of membranes (Kaur, 2019). The detoxification can be done using physicochemical (adsorption, ion exchange, extraction, overliming, activated charcoal method) and biological (gene manipulation of microorganisms) methods (Yankov *et al.,*2022). Zhang *et al.,*2016 used Calcium hydroxide to neutralize acid pretreated corn stover and bio-detoxification was done using *Amorphotheca resinae*.

The overliming method used calcium hydroxide to bioconvert the principal inhibitor group; furfural using NADH (Taherzadeh *et al.,*2000). The dried calcium hydroxide is added in acidic hydrolysates that results in gypsum, used as plaster of paris on walls. Activated charcoal has high capacity to adsorb compounds without affecting sugar levels whereas ion exchange resins help in removal of lignin- derived inhibitors (Chandel *et al.,*2011).

2.6 Fermentation of monosaccharides

Lactic acid can be produced either by chemical synthesis or microbial fermentation. Unlike the racemic yield achieved through chemical synthesis, optically pure lactic acid can be produced through microbial fermentation of carbohydrates (Iyer *et al.*, 2000). The microbial fermentation can use cheap raw materials for lactic acid production, depending on the microbial strains used in the process. The bacteria generally convert glucose using glycolysis under anaerobic conditions and pentoses are metabolised under pentose-phosphate pathway which processes pyruvate in presence of NADPH to lactic acid. Other variables that affect the fermentation process include pH, temperature, media composition, substrate sugar concentration, media composition, the effects of the end product, and inhibitors (Rawoof et al., 2021; Yankov, 2022).

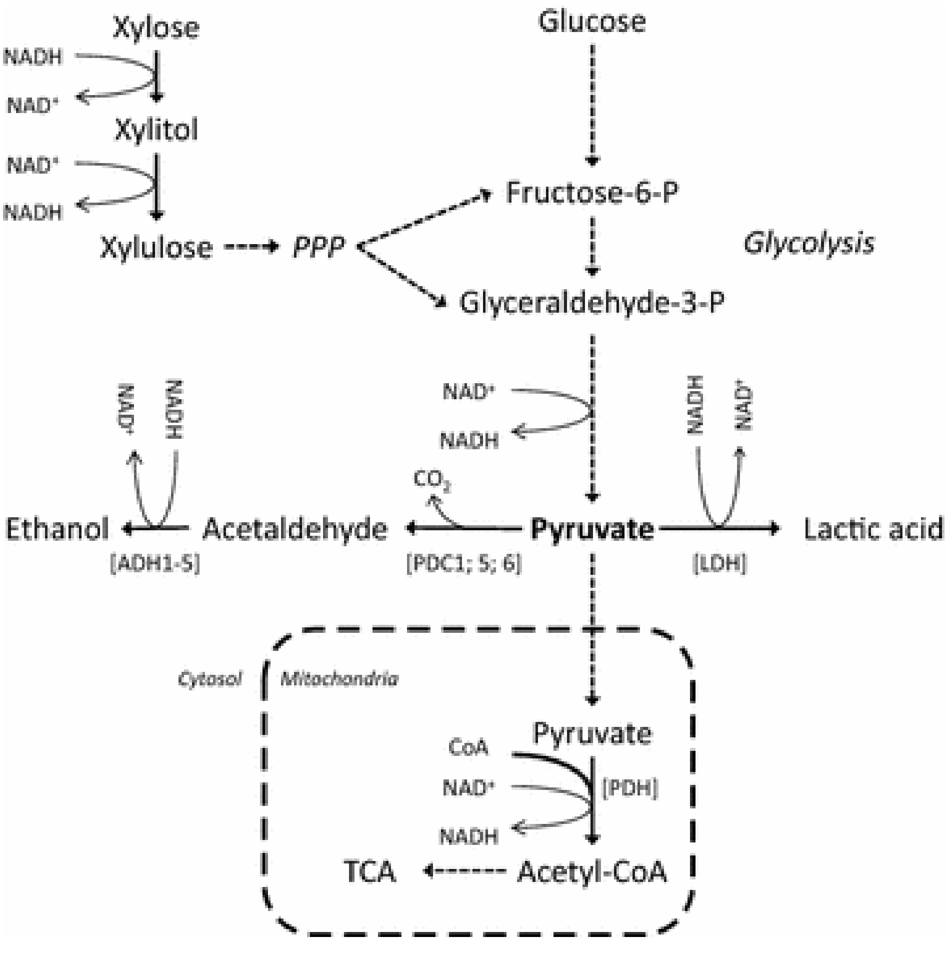


Fig.7 Fermentation pathway of glucose and xylose (Novy *et al.*,2018)

There are two methods of microbial fermentation depending upon the mode of metabolism of sugars (Gruber *et al.*,2006; Eiteman & Ramlingam, 2015).

Homolactate fermentation

In homolactate fermentation, one glucose molecule is metabolised into two lactic acid molecules using the pentose- phosphate pathway. Some examples are: *Lactobacillus lactis, Lactobacillus casei, Streptococci, Bacillus coagulans* and others. *Bacillus subtilis* produces LA from cellobiose using homofermentation. This method yields almost pure lactic acid (Kaur, 2017; Abedi *et al.*,2020).

Heterolactate fermentation

The heterolactate fermentation utilises phosphoketolase pathway for production of one molecule of lactate with one molecule of carbon dioxide or acetic acid, formic acid. Bacteria like *Leuconostoc, Weissella, Oenococcus, Lactobacillus delbrueckii* perform heterolactic fermentation (Hu *et al.*,2016; Wang *et al.*,2021). This pathway also produces alcohol, used in production of bioethanol.

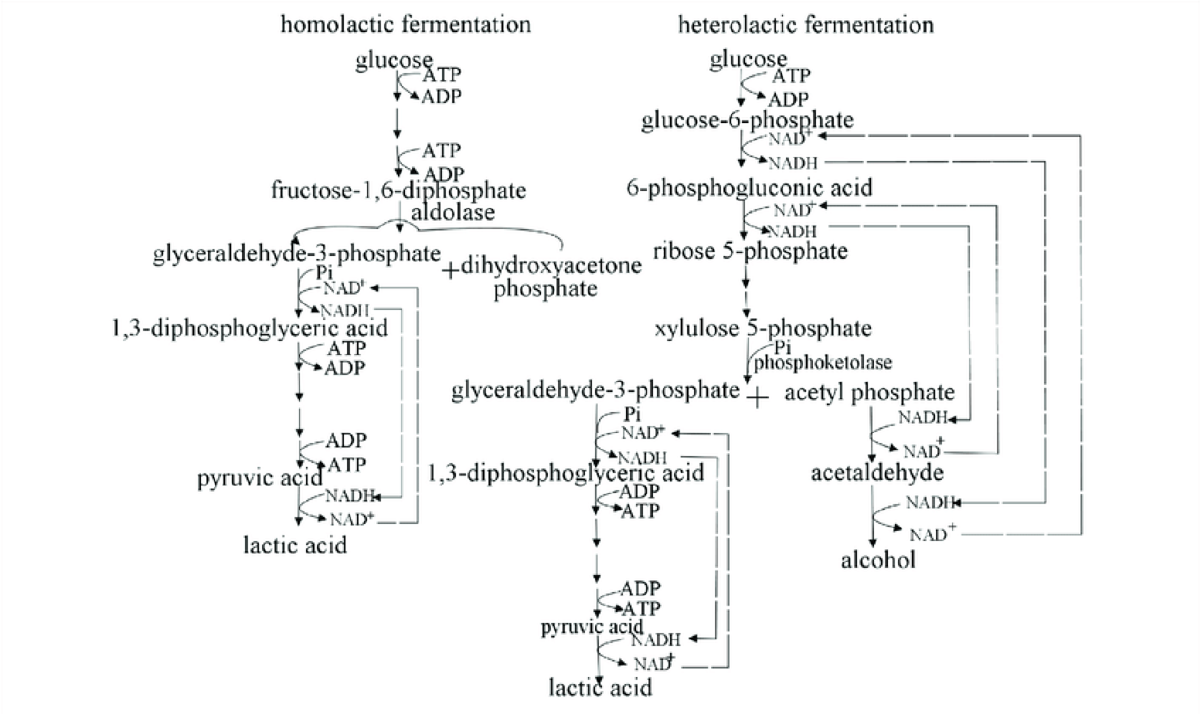


Fig. 8 Metabolic pathways for homolactate and heterolactate fermentation by bacteria (Wang *et al.*,2021)

Bacillus sp. are preferred over lactic acid bacteria (LAB) due to several advantages (Chawla & Goyal, 2022) as listed below:

- Growth media of *Bacillus* is cheap with inexpensive nitrogen source, e.g., minimal mineral salt media with corn steep liquor, ammonium sulfate
- There thermophilic nature prevents the cooling of media after sterilization, since the bacteria is stable at >50℃
- Produces optically pure lactic acid with high productivity
- Some strains are resistant to inhibitors like furfurals, HMF, acetates

The sugar solutions after pretreatment are supplemented with nutrients and inoculated with selected microorganism for fermentation of hexoses, pentoses into desired products (organic acids, ethanol).

Chapter-3 Materials and methods

The wheat straw was collected from the nearby villages of Patiala and was sun dried and grinded using a mixer grinder to a particle size of 0.5mm. The native or non-grinded biomass of wheat straw (WS) was analysed for composition of cellulose, hemicellulose and lignin. Grinded WS (0.5mm) and non-grinded WS were subjected to delignification followed by cellulose separation and saccharification. Enzymatic saccharification was compared with thermochemical treatment and compared for release of total reducing sugars. Hydrolysates containing cocktail of reducing sugars was fermented by *Bacillus sonorensis* for the production of lactic acid. The chemicals used in the study were procured from Sigma-Aldrich and Himedia.

3.1 Bacterial and recombinant strains

The thermotolerant, inhibitor tolerant strain of *Bacillus sonorenesis* DGS15 was used in fermentation of hydrolysates an isolate in the lab from red soil of brick kiln (Chawla & Goyal, 2022). The strains were revived using enrichment broth (10g/L xylose, 5 g/L yeast extract) (Lidan *et al.*,2013) at 50°C, pH-6.5.

The bacterial strains with recombinant cellulases used for saccharification were procured from IITG, Guwahati. The plasmids containing the genes CtCel8A, CtCBH5A, HtBg1 cloned from *Hungateiclostridium thermocellum* were transferred to *Escherichia coli* BL21 and the cells were used as glycerol stocks (Khaire *et al.*,2021).

3.2 Pre-treatment and delignification of wheat straw

10g of grinded biomass was mixed with 100ml of distilled water in a 250ml reagent bottle. The solution was added with 4g of sodium chlorite and 0.5 ml of glacial acetic acid. After mixing thoroughly, the solution was kept in a water bath at 60°C for 3hrs and filtered using Whatman no 1 filter paper to separate filtrate and treated biomass. The biomass was washed in tap water, followed by neutralization using distilled water. The de-lignified residue was dried at 80°C for 24hrs and later weighed for further treatment (Khaire *et al.*,2021). The delignification efficiency was measured by (Mukherjee *et al.*,2018):

Delignification efficiency(%) = $(\frac{M1-M2}{M1}) * 100$

where, M1= lignin content in native wheat straw

M2= lignin content in different forms of pretreated wheat straw

3.2.1 Cellulose separation from de-lignified residues

The cellulosic content was separated using alkaline treatment at 10% biomass loading. The treated biomass was mixed with a solution containing 10% sodium hydroxide and 1% boric acid in distilled water. The mixture was stirred on a hot plate at 60°C, 1200 rpm for 3hrs. The residue was filtered using a muslin cloth, collecting the filtrate and cellulosic residue separately. The solid residue was washed with tap water and neutralized for basic pH using distilled water. The cellulosic fibres were dried at 80℃ for 12 hrs (Khaire *et al.,*2021) and further used for compositional analysis. The hydrolysate was further estimated for xylose and glucose. The cellulose yield was measured by following equation (Khaire *et al.*,2021):

Cellulose yield (%)= [Dried wt. of cellulose/ Total cellulose present in native biomass]*100

where, Dried wt. of cellulose = mass of solid residue after treatment

Total cellulose in native biomass = 4.76g

The pretreatment efficiency is calculated using (Maibam &Goyal,2022):

$$\text{Pretreatment efficiency (\%)} = \left(1 - \frac{\left(mass\ ratio\ of \frac{lignin}{cellulose} of\ treated\ biomass\right)}{mass\ rotio\ of \frac{lignin}{cellulose} of\ wheat\ straw}\right)$$

3.2.2.a Enzymatic saccharification of cellulose obtained after delignification

The saccharification of treated residue was carried out in the reaction mixture of 5 ml at 2% (w/v) biomass loading in sodium phosphate buffer (pH 7.5) in 15ml centrifuge tubes. The optimization of saccharification was done by varying the enzyme loading units. The CtCel8A loading was varied from 200-250 U/g of biomass, CtCBH5A for 20-50 U/g and HtBG1 for 20-60 U/g of biomass with the addition of 0.03% sodium azide, kept at 50°C for 24/48/72 hrs. The reactions were stopped by boiling in a water bath for 5 min followed by the collection of supernatant after spin down at 10,000 rpm for 10 min. Using the DNSA method, released sugars were determined from the supernatant. The TRS yield was determined using the following equation (Lempiäinen *et al.*,2022):

$\text{TRS} = (C_{TRS} \times V) / m$

where C_{TRS} is the concentration of sugars in the solution,

V is the volume of the solution,

m is the mass of biomass treated

- **Production and activation of recombinant cellulases**

Culturing- Luria broth media was prepared and distributed in 3 test tubes of 10 ml each and 3 flasks of 150 ml each. In each test tube, 10ul of kanamycin (100mg/ml) along with 100 µl of culture from each glycerol stock was added separately and incubated at 37°C for 12hrs. Another inoculation of 100ul was done and incubated at 37°C for 12hrs till the OD at 600nm reached 0.6-0.8.

Expression- For sub culturing, the fully grown cultures were transferred to 150 ml LB flasks each along with 70 µl kanamycin solution. The flasks were incubated at 37°C for 24hrs till the OD at 600nm reached 0.9-1. Then added 150ul of IPTG solution (1M) for protein expression and incubated at 24°C for 16-18hrs. The flasks were centrifuged at 8000rpm for 10min and the pellet formed was stored at -20°C.

Purification- The cell pellet was resuspended in 2-5ml lysis buffer (50mM sodium phosphate buffer, pH- 7.5) and sonicated for 1hr each (9s on/ 9s off pulse). The sonicated cells were centrifuged at 8000rpm, 4°C for 10 min and the collected supernatant was stored at -20°C. The pellet was further suspended in the buffer and supernatant was collected after centrifugation. The supernatant contains the crude enzymes. The concentration of enzymes was determined by Bradford method at absorbance 595nm using the BSA standard plot.

- **Assay of enzyme activity of crude recombinant enzymes**

The enzyme activities of endo-β-1,4-glucanase (CtCel8A) and exo-β-1,4-glucanase (CtCBH5A) were determined by incubating 10ul of crude enzyme with 1% carboxymethyl cellulose (CMC) in 50mM sodium phosphate buffer (pH 7.5) in 100µl reaction mixture at 70°C for 1 min and 60°C for 2 min, respectively. The total reducing sugars released were calculated by the DNSA method (Khaire *et al.*,2021; Miller G, 1959).

Whereas a 10µl sample of β-1,4-glucosidase (HtBg1) with 1%(v/v) 4-Nitrophenyl-β-D-glucopyranoside (PNPG) in 50 mM sodium phosphate buffer (pH 7.5) in a 100µl reaction mixture for 5 min at 65°C was used to determine its activity, using Nelson-Somogyi method. The enzyme activity was calculated by following equation (Karimi A.M., 2020):

Enzyme activity (U/ml) = Concentartion of glucose (mg/ml) * 1000* reaction volume/ 180* incubation time* enzyme vol used

- **Bradford assay for protein estimation**

- Assay reagent: dissolved 100 mg of Coomassie Blue (G250) in 50 mL of 95% ethanol. This solution was mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Whatman No. 1 filter paper and then stored in dark bottle at room temperature. The dye is stable for several weeks.
- Protein standard: bovine γ-globulin at a concentration of 1mg/mL (100µg/mL for the micro-assay) in distilled water was used as a stock solution. This should be stored frozen in small aliquots at −20°C.

1. Pipetted volumes of 10,20,40,60, 80 and 100µl of 1 mg/mL γ-globulin standard solution into test tubes, and made each up to 500µl with distilled water. Pipetted 100µl of distilled water into a further tube to provide the reagent blank.
2. Add 1 mL of the assay reagent to each tube and mix well by inversion or gentle vortex. Avoid foaming, which will lead to poor reproducibility.
3. Measured the absorbance of samples at 595 nm and protein standards against BSA concentration and OD was plotted.

3.2.2.b Saccharification of cellulose by dilute acid

The saccharification of treated residue was carried out in the reaction mixture of 150 ml at 5% (w/v) biomass loading in 1% H_2SO_4 solution in 500ml reagent bottle. The solution was autoclaved at 121°C, 15psi for 30 min (Chawla & Goyal, 2022). After cooling, the solution was filtered using muslin cloth to remove solid residues and use the hydrolysate for fermentation.

3.2.3 Acid hydrolysis of delignified biomass

The delignified biomass was dissolved in 1% H_2SO_4 solution at 5% (w/v) loading in 150ml solution and heated at different temperatures. The first set was heated at 60°C for 2hrs on hot plate with constant stirring. The second set was treated at 80°C for 3hrs in water bath and the third set was autoclaved at 121°C, 15psi for 30mins. The solutions were cooled and filtered to collect the hydrolysates for detoxification and fermentation.

3.3 Detoxification of hydrolysates

The alkaline hydrolysate of step (3.3.2.) was neutralized using conc. hydrochloric acid and the solution was centrifuged at 8000 rpm for 15 min, collecting the supernatant for glucose and xylose estimations. To detoxify acidic hydrolysates of saccharification, 2-3g of calcium hydroxide was used and solution was centrifuged at 8000 rpm for 15 min, collecting the supernatant for estimations. The TRS yield was determined using the following equation (Lempiäinen *et al.*,2022):

$TRS = (C_{TRS} \times V) / m$

where C_{TRS} is the concentration of sugars in the solution,

V is the volume of the solution,

m is the mass of biomass treated

3.4 Fermentation of hydrolysates for lactic acid production

Bacterial fermentation of LA using hydrolysates (80ml, pH 6.5) was done in two sets. One set of experiments was done by combining xylose containing hydrolysate (from 3.3.2 treatment) with glucose containing hydrolysate (from 3.3.2.b acid treatment) in equal volumes. The other set was performed using hydrolysates of delignification followed by dilute acid hydrolysis at different temperatures (from 3.3.3 acid hydrolysis). The hydrolysates were supplemented with yeast extract (5g/L) , Bushnell Haas media (3.27g/L) and 5% bacterial inoculum of DGS15 strain (Chawla & Goyal, 2022) and incubated at 50°C, 120 rpm for 72hrs. Aliquots of fermentation broth were taken after every 24hrs for the estimation of lactic acid, residual xylose and glucose. The overall productivity ($g\ L^{-1}\ h^{-1}$) was expressed as concentration of LA ($g\ L^{-1}$)/ time of incubation (h) and the overall yield was expressed as grams L-lactic acid/grams sugar consumed (Chawla & Goyal, 2022).

3.5 Composition analysis of biomass

3.5.1.a Estimation of lignin

The total lignin content present in biomass was given by acid insoluble lignin (AIL) determined by TAPPI T 222 and acid soluble lignin (ASL) using TAPPI UM 250. For estimation, 1g of treated biomass was added in 10 ml of 72% (v/v) sulphuric acid; mixed using glass rod. The solution was kept in a water bath at 30°C for 2hrs while stirring at the interval of 10-15 min. Then, 170 ml of distilled water was added to dilute the solution to 4% concentration. The flask was tightly sealed and autoclaved at 121°C / 15psi for 1hr. The sample was cooled to room temperature and filtered using filter paper to collect black residue for AIL estimation and filtrate for ASL calculation.

After washing with hot water and neutralizing with distilled water, the residue was dried in an oven at 105°C. The weight was determined for AIL calculations

$\text{AIL}(\%) = (A/W) * 100$

where, A= weight of oven dried lignin (g)

W= weight of sample used

The collected filtrate was measured for volume and OD was measured at 320 nm, diluting the sample with 4% sulphuric acid.

$\text{ASL}(\%) = [\ (\text{UVabs} * \text{vol of filtrate} * \text{Dilution factor}) / \varepsilon * B * a] \times 100$

where, ε= absorptivity of biomass (113 L/g-cm)

B= weight of sample used

a= path length (1 cm)

$\text{Total lignin}(\%) = \text{AIL}(\%) + \text{ASL}(\%)$

3.5.1.b Estimation of holocellulose

Polysaccharides can be estimated using the Designer Energy Ltd (DE) method which uses holocellulose, i.e., total polysaccharides containing hemicellulose and cellulose. 0.5g of treated biomass was dissolved in 40ml distilled water, 0.5g sodium chlorite (NaClO2) and 1ml glacial acetic acid in a reagent bottle. The solution was kept in a water bath at 90°C for 45 min while stirring after 15 min intervals. Then, 0.5g sodium chlorite (NaClO2) was added to the bottle while the treatment was continued for another 45 min. After cooling for 30 min, the solution was poured in falcon tubes and centrifuged at 4500g for 10 min. The solid residue collected was washed with hot tap water and distilled water to a pH 7. The residue was dried in oven at 105°C for 8-9hr till constant weight was achieved (Ioelovich *et al.*,2015), calculating the percentage of holocellulose (HC) by:

$\text{HC}(\%) = [(P-Pt)/Ps] \times 100$

where, P = weight of dried HC with petri dish

Pt = weight of empty petri dish

Ps = weight of sample used

3.5.1.c Estimation of cellulosic content

The cellulose was separated from HC by acid treatment. The obtained holocellulose sample was added with 45ml 2% hydrochloric acid and boiled on a hot plate for 2 hrs. The sample was cooled to room temperature for 30 min and centrifuged at 4500g for 10 min. After filtering with filter paper, the white colour residue was washed with hot water (at 50℃),1% sodium bicarbonate collusion and neutralized with distilled water. The biomass was dried at 105℃ till constant weight and percentage of cellulose and hemicellulose (H) was determined (Ioelovich *et al.*,2015).

C = HC (dried weight of cellulose)/ P
H = HC - C
where, HC= holocellulose percentage
P= dry weight of holocellulose sample used
H= hemicellulose percentage
C= cellulose percentage

3.5.2 Estimation of reducing sugars

3.5.2.1 By Nelson- Somogyi method

Reagent-A

Dissolved 25g of Na_2CO_3 (anhydrous), 25g Rochelle salt, 20g sodium bicarbonate in 700ml water and added 200g sodium sulphate (anhydrous). The volume was made up to 1L and kept at room temperature.

Reagent-B

Dissolved 15g $CuSO_4.5H_2O$ in 100ml water and added 1-2 drops of conc.H_2SO_4.

Arseno-molybdate reagent

Added 25g ammonium molybdate in 450ml water and 21ml of conc.H_2SO_4. Dissolved 3g sodium arsenate in 25ml water and added to above solution. Stored in a brown bottle for 24hrs at 37°C.

Reagent-C

Mixed 25ml reagent-A in 1ml of reagent-B.

Method

- Sample of enzyme and substrate were incubated at 65°C for 5min, later halting the reaction by boiling in water for 5mins.

- Added 1ml of reagent-C, vortex and boiled the tubes in water bath for 20min.
- Cooled at room temp for 5min and added 1ml of arseno-molybdate reagent with brief vortex and incubated at 37^{o}C for 5min
- Diluted with 5ml distilled water and noted absorbance at 540nm.

The standard was plotted using 0.1, 0.2, 0.4, 0.5 mg/ml glucose, to calculate the amount of reducing sugars present in the sample.

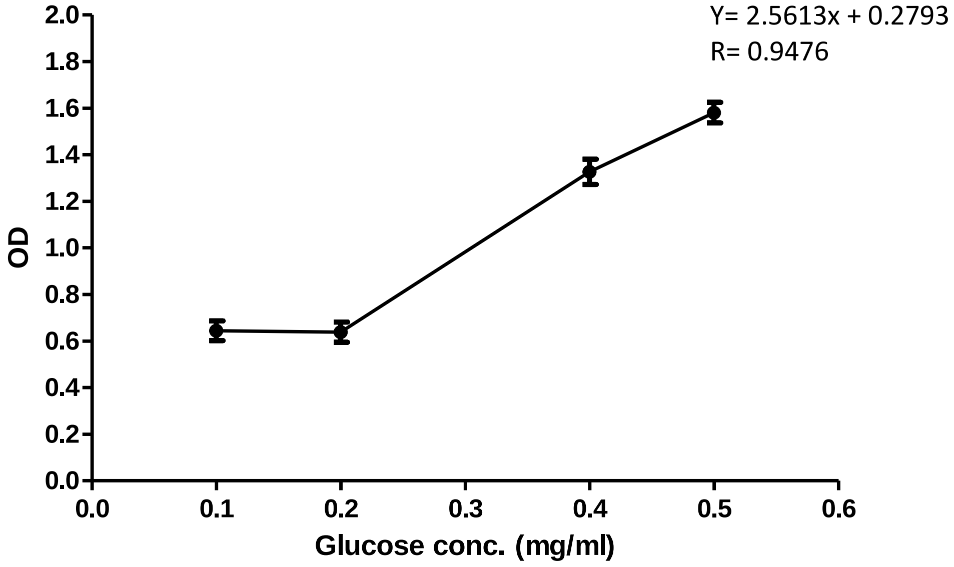


Fig.9 Standard curve of glucose by Nelson-Somogyi method

3.5.2.2 Reducing sugar estimation by DNSA method (Miller, 1959)

Sample size of 1ml was added with 1ml DNSA reagent. The solution was vortex and incubated in boiling water bath for 10min. Noted the OD at 540nm after cooling and calculated reducing sugar concentrations in samples.

3.5.3 Estimation of xylose

Orcinol Reagent- Added 33ml conc. H_2SO_4 in 17ml distilled water and 0.1g orcinol. The solution was vortexed and cooled under tap water (Fernell & King., 1953).

Method

Added 5ul, 10ul samples in distilled water (made up vol 1ml). Then, 3ml of reagent was added and incubated the solution at 100^{o}C for 15min. Noted the absorbance at 560nm (Bruckner, 1955) (Vasseur, 1948). The standard graph was plotted using 0.05,0.07,0.09, 0.1, 0.2, 0.3 mg/ml D-xylose, to calculate xylose concentrations in the samples.

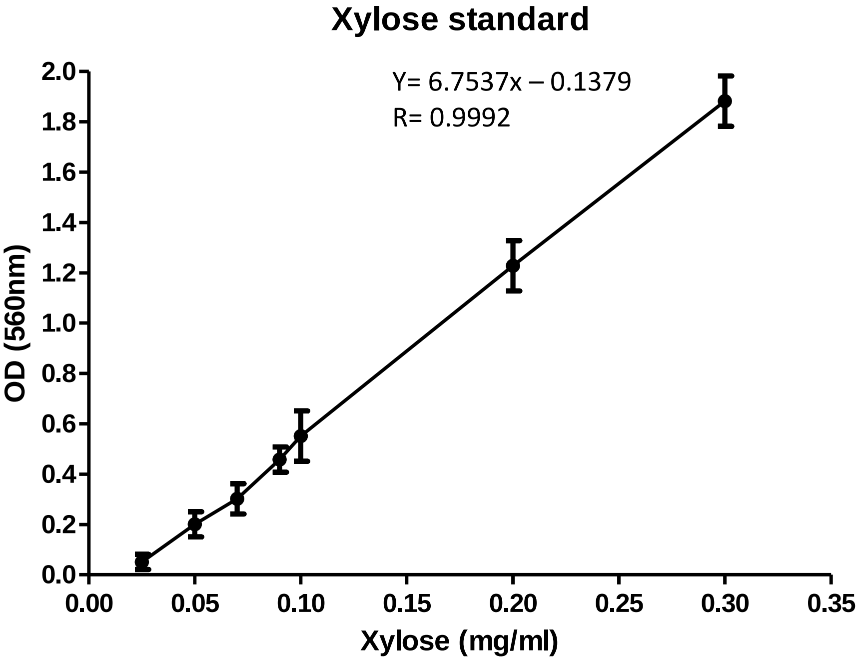


Fig.10 Standard curve of xylose conc. estimation

3.5.4 Estimation of lactic acid

- Took 10µl of Lactic acid sample and added 200µl of trichloroacetic acid, 200µl 20% $CuSO_4.5H_2O$, 500µl distilled water and 0.2g $Ca(OH)_2$. The solution was centrifuged at 10,000rpm for 10mins; collecting the supernatant.
- 100µl of supernatant was added with 3ml conc. H_2SO_4 and incubated in boiling water bath for 5min.
- The solution was cooled down with addition of 300µl p-phenyl phenol (1.5% pp in 1.5% NaOH solution)
- The 10min room temp incubation was followed by 90sec water bath boiling
- Noted the readings at 560nm (Barker & Summerson, 1941).

Chapter-4 Results and Discussion

4.1 Processing of wheat straw

The dried wheat straw (WS) was grinded to a particle size of 0.5mm for effective pretreatment (Fig.11). This biomass was further pretreated and results were compared with non-grinded WS.

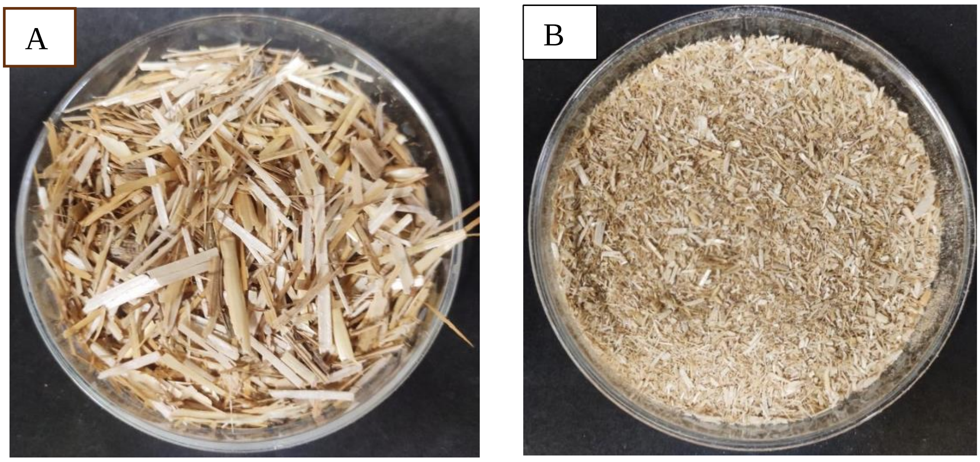


Fig.11 **A**. Non-grinded WS, **B**. Grinded WS with particle size 0.5mm

4.1.1 Delignification of wheat straw

10g of grinded and non-grinded WS were delignified using sodium chlorite and glacial acetic acid. After treatment, we were left with 7.11g of solid residue and 40ml hydrolysate and 8.83g of solid residue and 35ml hydrolysate of grinded and non-grinded WS respectively. Sodium chlorite, in water, gives off chlorine dioxide ,which reacts with lignin and dissolves it (Fig.12A) (Espinosa, 2016).

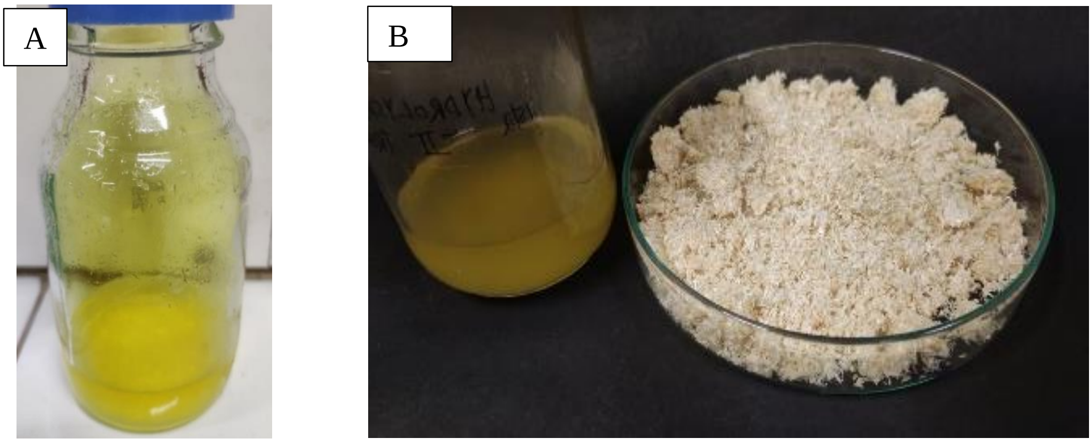


Fig.12 **A**. Delignified biomass before filtration, **B**. Delignified solid residue after filtration

4.1.2 Cellulose separation in delignified biomass

The delignified biomass was subjected to sodium hydroxide and boric acid treatment for cellulose separation (Khaire *et al.*,2020). After this treatment, 2.90 g of solid residue and 43ml hydrolysate were obtained for grinded WS (Fig 13) and 3.65g solid residue and 40ml hydrolysate for non-grinded WS respectively. The hemicellulose content got separated in the filtrate leaving the cellulose in solid residue.

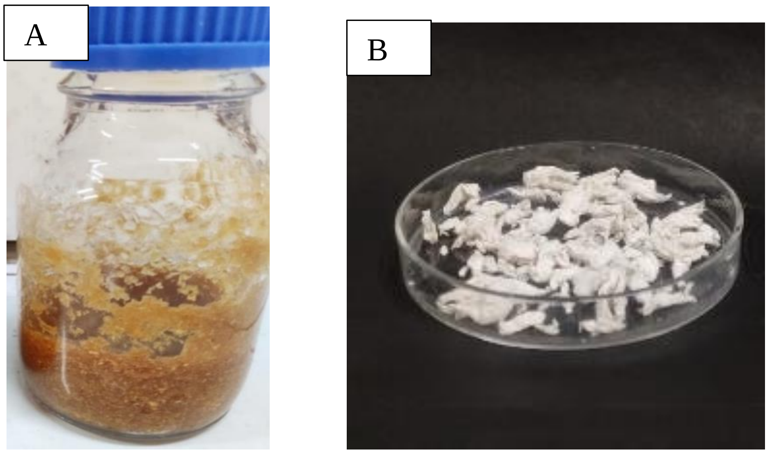


Fig.13 A. Cellulose separated biomass before filtration, **B**. Cellulose separated solid residue after filtration

4.1.3 Compositional analysis of native, pretreated grinded and non-grinded WS

The compositional analysis of grinded, non-grinded and native WS was done using TAPPI, DE methods (Table 4). Cellulose content of 56.4% was determined in grinded WS while 71.8% and 47.6% in non-grinded and native WS respectively. Maximum lignin content of 18.2% and hemicellulose content of 16.84% was seen in native WS, indicating the efficient removal of lignin and hemicellulose in pretreatment.

Table.4 Compositional analysis of native, pretreated grinded and non-grinded biomass

Types WS	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Native wheat straw	47.6	18.2	16.84
Grinded {pre-treated}	56.4	9.6	6.17
Non- grinded {pre-treated}	71.8	10.8	5.81

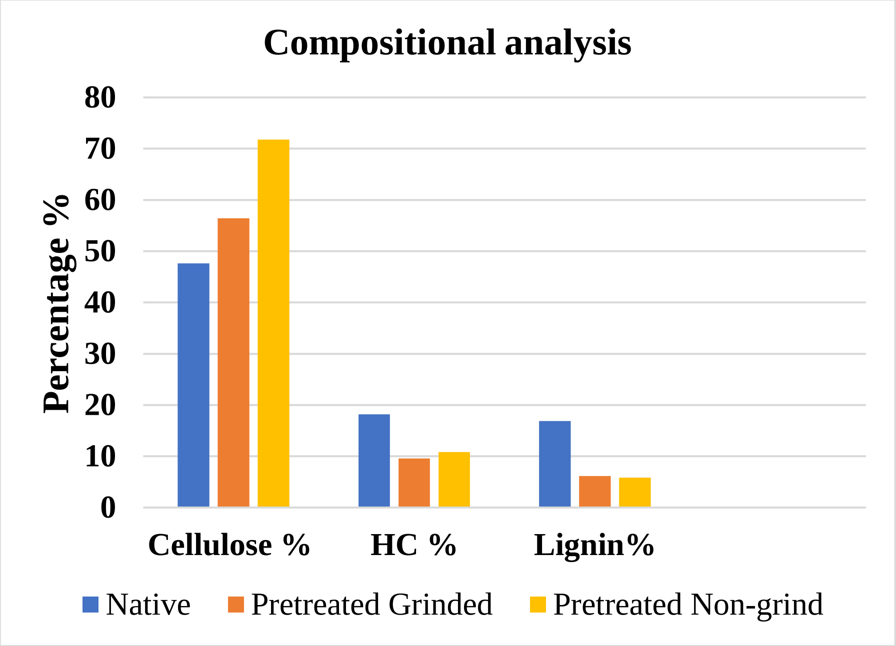


Fig.14 Compositional analysis of native, pretreated grinded and non-grinded biomass

Based on the cellulose and lignin content before and after pretreatment, delignification and pretreatment efficiencies were calculated (Table 5) and a pretreatment efficiency of 68.9% was seen in case of grinded WS as compared to 77.3% in non-grinded WS.

Table 5. Pretreatment and delignification efficiency of pretreated grinded and non-grinded

Type WS	Delignification efficiency (%)	Dried cellulose fibres (g)	Cellulose yield (%)	Pretreatment efficiency (%)
Grinded	63.3	2.87g	60.29	68.9
Non-grinded	65.46	3.66g	76.89	77.3

4.2 Enzymatic saccharification of separated cellulose

The solid residues obtained from pretreatment of grinded and non-grinded WS, were subjected to hydrolysis to break down cellulose into reducing sugars by recombinant cellulases at 50℃ 120rpm. The enzyme loading was varied according to the enzyme activity (Table 6). Different enzyme cocktail ratios (CtCel8A: CtCBH5A: HtBg1) were used and their total reducing sugars were estimated at an interval of 24hrs. 3 recombinant cellulases are used for hydrolysis: CtCel8A is a endo-β-1,4-glucanase that randomly hydrolyse accessible intramolecular β-1,4-glucosidic bonds of cellulose chains and introduce a water molecule in the bonds; CtCBH5A is exo-β-1,4-glucanase that can hydrolyse cellulose chains at the polymer ends and HtBg1, a β-glucosidase further hydrolyse cellobiase into 2 glucose molecules (Khaire *et al.*,2020).

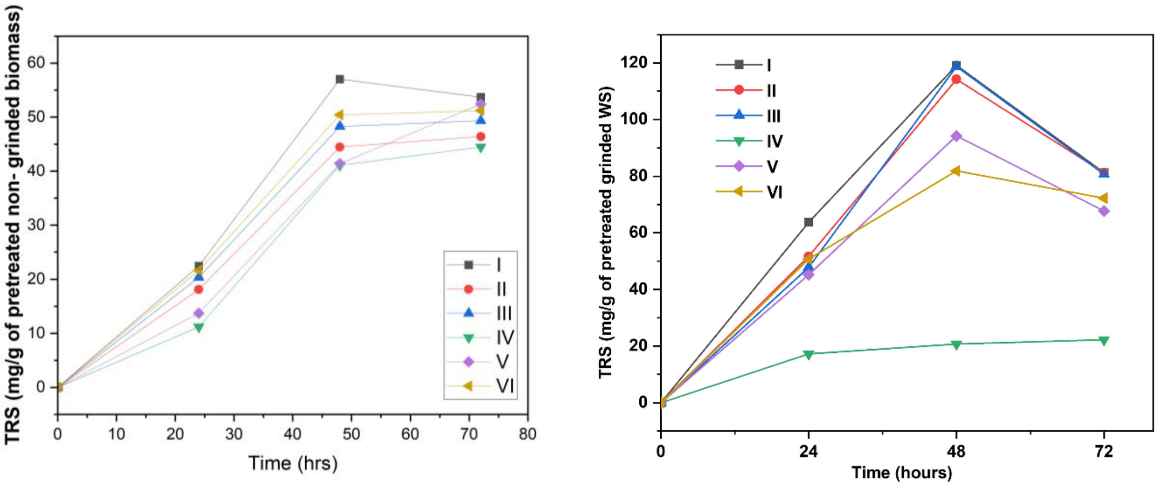
Table 6. Enzyme activity and protein concentration of recombinant cellulases

Enzymes	**Method**	**Conditions**	**OD at 540nm**	**Glucose (mg/ml)**	**Enzyme Activity (U/ml)**	**Protein (mg/ml)**
CtCel8A	DNSA	10µl, 70℃, 1min	0.55	0.477	29.1	1.78
CtCBH5A	DNSA	10µl,60℃, 2min	0.0719	0.062	1.9	1.44
HtBg1	Nelson somogyi	10µl, 65℃, 5min	1.0539	0.3024	3.69	2.18

Table 7. Total reducing sugar (mg/g) obtained after enzymatic hydrolysis at different time interval of pretreated; **A.** grinded WS, **B.** non-grinded WS at different enzyme loading ratio (CtCel8A: CtCBH5A: HtBg1)

Enzyme cocktail	**A. TRS (mg/g) in pretreated Grinded WS**			**B. TRS (mg/g) in pretreated Non-grinded WS**		
	24hrs	**48hrs**	**72hrs**	**24hrs**	**48hrs**	**72hrs**
200:50:50	63.73±0.25	119.16±0.35	81.23±0.32	22.44±0.45	57.02±0.123	53.68±0.307
200:40:60	51.7± 0.3	114.23±0.25	81.23±0.14	18.11±0.28	44.46±0.189	46.4±0.319
210:50:40	47.8±0.2	118.73±0.34	80.73±0.67	20.36±0.172	48.27±0.439	49.34±0.119
230:30:40	17.23±0.25	20.73 ±0.21	22.23±0.12	11.21±0.179	41.1±0.24	44.46±0.185
250:20:30	45.23±0.25	94.23±0.44	67.73±0.22	13.71±0.198	41.39±0.115	52.43±0.23
250:30:20	50.73±0.25	81.86±0.12	72.26±0.13	21.64±0.24	50.43±0.169	51.21±0.245

As seen in Table 7; Fig 15, with enzyme loading ratio of 200:50:50 (U/g) of grinded WS, TRS value increased from 63.73 ± 0.25 mg/g at 24 h to 119.16 ± 0.35 mg/g at 48 h after which it decreased to 81.23 ± 0.32 mg/g at 72 h. The non-grinded biomass produced less TRS yield due to less surface volume ratio, thus decreasing its accessibility to enzymes for degradation.



A **B**

Fig.15 Total reducing sugar (mg/g) obtained after enzymatic hydrolysis at different time interval of pretreated; **A.** non-grinded WS, **B**. grinded WS at different enzyme loading ratio (CtCel8A: CtCBH5A: HtBg1)

4.3 Acid hydrolysis of separated cellulose and LA production from hydrolysate

In another pretreatment method, the solid residue obtained after cellulose separation treatment (section 4.1.2) was subjected to acid hydrolysis. Among pretreatment methods, chemical treatment is the most effective method and dilute acid treatment at high temperatures hydrolyses the cellulose into reducing sugars by splitting the bonds into shorter chains via addition of water molecules (Fan *et al.*,1987). 7g of pretreated biomass was dissolved in 150ml of 1% sulphuric acid solution and was autoclaved for 30min to obtain the reducing sugars in the hydrolysate. The 90ml hydrolysate obtained from this step was combined with 45ml of cellulose separated hydrolysate in equal volumes to be used as a fermentation broth for LA production. The glucose and xylose concentration of 10.03mg/ml and 25.7mg/ml respectively.

The 90ml grinded WS hydrolysate obtained after detoxification was inoculated with DGS15 strain (5%) and incubated at 50°C, 120 rpm for 72hrs. Glucose, xylose and lactic acid was estimated of 24hrs interval. The bacteria converts glucose using glycolysis under anaerobic conditions and xylose is metabolised by pentose-phosphate pathway which process pyruvate in presence of NADPH to lactic acid (Yankov, 2022).

Table 8. Lactic acid production by *Bacillus sonorenesis* DGS15 in hydrolysate obtained by acid hydrolysis of separated cellulose, supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50◦C

Time (hrs)	**Xylose (mg/ml)**	**Glucose (mg/ml)**	**LA (g/l)**
0hrs	25.7±0.53	10.03±0.32	----
24hrs	6.17±0.5	1.21±0.22	0.12±0.01
48hrs	5.55±0.45	1.16±0.34	0.21±0.014
72hrs	4.96±0.55	0.87±0.23	0.32±0.02

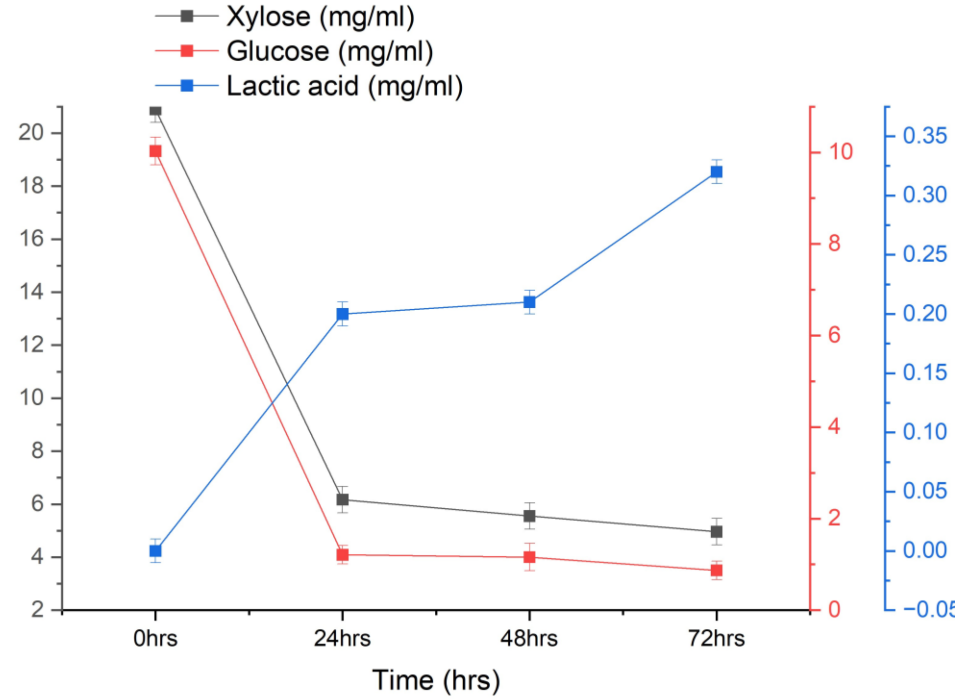


Fig.16 Lactic acid production by *Bacillus sonorenesis* DGS15 in hydrolysate obtained by acid hydrolysis of separated cellulose, supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50◦C

4.4 Acid hydrolysis of delignified WS and fermentation of hydrolysate for LA production

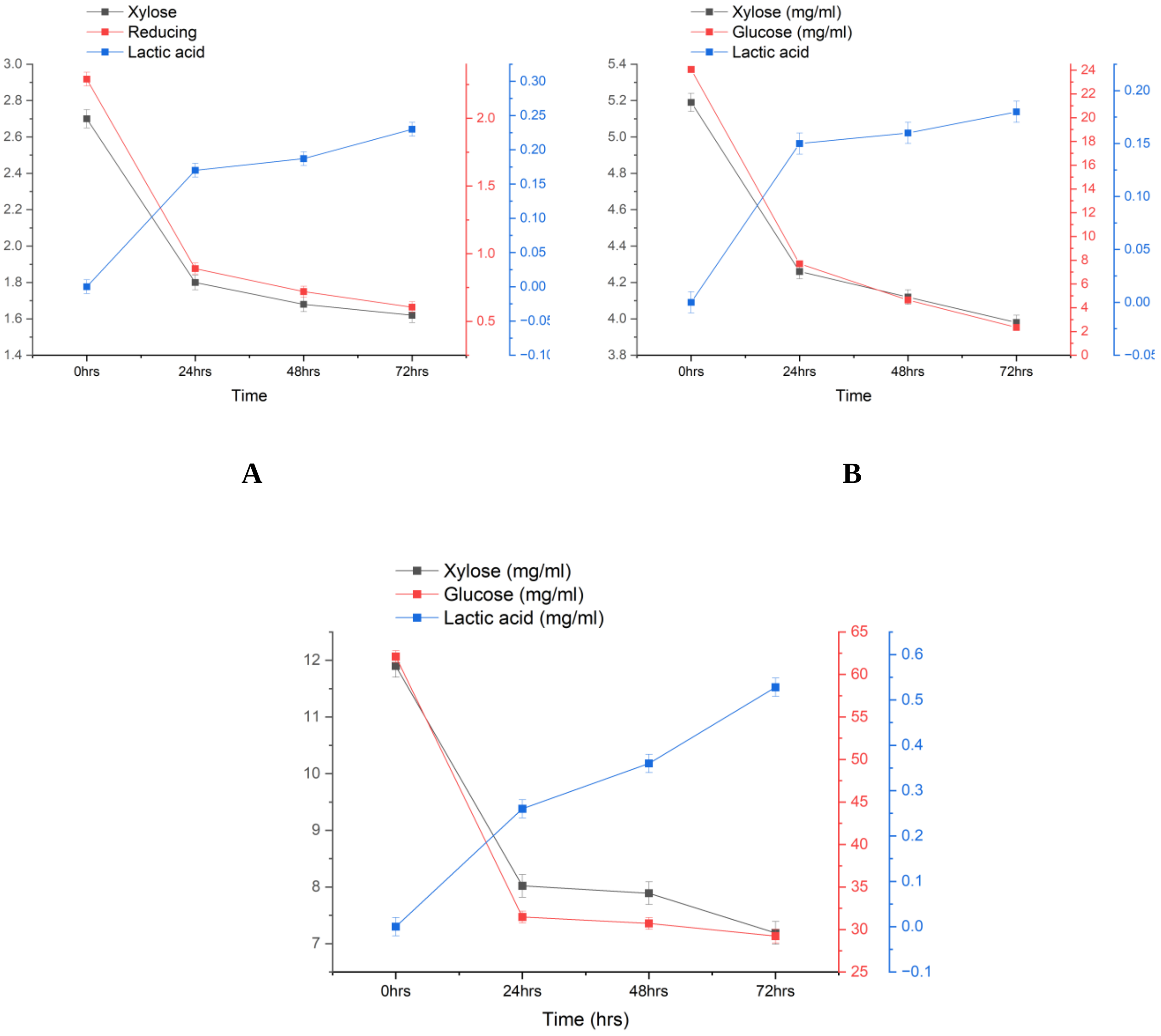
Another pretreatment method with acid hydrolysis at three different temperatures was used for breakdown of delignified biomass. 7g of delignified solid was treated with 1% sulphuric acid solution and heated on hot plate at 60°C for 2hrs, heated in water bath at 80°C for 3hrs and autoclaved at 121°C for 30 mins; collecting 90ml hydrolysate in each case. The hydrolysate were detoxified and estimated for reducing sugar. Maximum reducing sugars were obtained, when autoclaving was done at 121°C. The treatment was not effective at lower temperatures (Table 10).

The detoxified hydrolysates after centrifugation, were supplemented with yeast extract and Bushnell Hass media and autoclaved. The 80ml of media, pH- 6.5, in each case was inoculated with 5% *Bacillus sonorenesis* DGS15 inoculum in 3 flasks and incubated at 50°C, 120 rpm for 72hrs and lactic acid concentration was estimated every 24hrs.

Maximum lactic acid yield of 0.53g/L was obtained after bacterial fermentation when acid treatment was done at 121°C, which produced glucose (62.1mg/ml) and xylose (11.9mg/ml) with overall yield of 0.014g/g and productivity of 0.0073g/L/h. Yield was low when pretreatment was done at 60°C and 80°C, owing to release of much lower amount of reducing sugars (Table 10; Fig.17). The results indicates that thermos-chemical processing at higher temperatures in presence of dilute sulphuric acid leads to better hydrolysis of cellulose into monomeric units.

Table 9. Lactic acid production by *Bacillus sonorenesis* DGS15 and residual sugars in hydrolysates obtained by acid hydrolysis of delignified biomass, supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50◦C ; **A.** at 60◦C, **B**. at 80◦C, **C**. at 121◦C

Time (hrs)	**A. 60°C**			**B. 80°C**			**C. 121°C**		
	Xylose (mg/ml)	**Glucose (mg/ml)**	**LA (g/L)**	**Xylose (mg/ml)**	**Glucose (mg/ml)**	**LA (g/L)**	**Xylose (mg/ml)**	**Glucose (mg/ml)**	**LA (g/L)**
0hrs	2.7±0.05	2.29±0.04	0	5.19±0.05	24.06±0.045	0	11.9±0.2	62.1±0.73	0
24hrs	1.8±0.04	0.89±0.045	0.17±0.01	4.26±0.034	7.7±0.04	0.15±0.01	8.02±0.23	31.47±0.75	0.26±0.02
48hrs	1.68±0.043	0.72±0.05	0.187±0.012	4.12±0.04	4.65±0.034	0.16±0.012	7.89±0.19	30.71±0.69	0.36±0.023
72hrs	1.62±0.045	0.605±0.04	0.23±0.02	3.98±0.042	2.34±0.042	0.18±0.014	7.19±0.16	29.23±0.8	0.53±0.03



A

B

C

Fig.17 Lactic acid production by *Bacillus sonorenesis* DGS15 and residual sugars in hydrolysates obtained by acid hydrolysis of delignified biomass, supplemented with Bushnell Haas media at pH 6.5, 120 rpm, 50◦C ; **A.** at 60◦C, **B**. at 80◦C, **C**. at 121◦C

Conclusion

1. Dried wheat straw was grinded to reduce particle size to 0.5mm and compared with non-grinded form for different pretreatment methods to obtain maximum yield of reducing sugars and further lactic acid upon bacterial fermentation.
2. Native wheat straw was found to have 47.6% cellulose, 18.2% hemicellulose and 16.84% lignin. After pretreatment, the lignin part was removed with efficiency of 63.3% and cellulose yield of 60.29% was obtained in case of grinded WS, while 65.46% efficiency with 76.89% yield was found in non-grinded WS.
3. The enzymatic saccharification of separated cellulose of grinded and non-grinded WS yielded glucose of 2.39mg/ml and 1.14 mg/ml respectively; at 48hrs.
4. The acid hydrolysis of separated cellulose of grinded WS at 121℃ yielded glucose of 10.03 mg/ml, xylose 25.7mg/ml and their fermentation by *Bacillus sonorenesis* DGS15 produced 0.32 g/L lactic acid.
5. In another treatment method, delignified biomass of grinded WS was given dilute acid treatment at 60℃, 80℃ and 121℃. Acid pre-treatment of delignified biomass at 121℃, yielded maximum amount of 62.1 mg/ml glucose and 11.9 mg/ml xylose, which upon fermentation produced 0.53g/L lactic acid with overall yield of 0.014g/g and productivity of 0.0073g/L/h.

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Production of lactic acid from wheat straw hydrolysates

Harshita

Harshita Singla

Dr. Dinesh Goyal