Utilization of rice straw and wheat straw for production of lactic acid

DISSERTATION

Submitted by

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301501018

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 University, Patiala

JULY, 2017

CERTIFICATE

Certified that the thesis entitled '**Utilization of rice straw and wheat straw for production of lactic acid**' submitted by Ms. **Tavinder Kaur** (301501018) in partial fulfilment of the requirement for the award of the degree of **Master's of Science** in Biotechnology in the Department of Biotechnology, Thapar University, Patiala, Punjab is the record of 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 award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis "**Utiliztion of rice straw and wheat straw for production of lactic acid**" submitted by me for the award of the degree of **Masters in Science** in the Department of Biotechnology, Thapar University, Patiala, is 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 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.

Tavinder Kaur
(Tavinder Kaur)

Place: Patiala

Date: July, 10, 2017

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Last but not least, it has been a privilege to spend memorable two years in Thapar University. I wish to acknowledge all those, whose names have not been figured here, but who helped me in any form during the period of my research work.

Date: 10/07/2017

Place: Patiala

Tavinder Kaur

Tavinder Kaur

Dedicated

To

My parents

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List of Abbreviations

$Ca[OH]_2$	Calcium hydroxide
CDD	Control drug delivery
DNS	3,5- dinitro salicylic acid
et al	And others
etc	And other things
FDA	Food and drug administration
Fig.	Figure
g/L	Gram per litre
g/L/h	Gram per litre per hour
GRAS	Generally regarded as safe
H_3PO_4	Phosphoric acid
HCl	Hydrochloride acid
hr	Hour
i.e.	that is
LA	Lactic acid
LAB	Lactic acid bacteria
min	Minute
ml	Millilitre
NaOH	Sodium hydroxide
PLA	Poly lactic acid
psi	Pounds per square inch
rpm	Revolutions per minute
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
w/v	Weight per volume

List of Symbols

%	Percentage
µL	Microliter
D	Dextrorotatory
g	Gram
Kg	Kilogram
L	Levorotatory
N	Normal
nm	Nanometer
α	Alpha
β	Beta

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Abstract

Present research work was aimed at utilization of lignocellulosic biomass such as rice straw and wheat straw for production of lactic acid. Rice straw and wheat straw were collected from nearby villages of Patiala. Since lignocellulosic materials exhibit strong recalcitrance to enzymes, a suitable prior pretreatment was done to facilitate access of enzymes to the plant polysaccharides. Acid and alkali hydrolysis of rice straw and wheat straw biomass was done to obtain hydrolysates followed by detoxification using calcium hydroxide to neutralize the effect of inhibitors.

Fermentation using different strains of bacteria i.e. DGB, NA9, DGN1 and DGN2 was carried out in enrichment broth containing 10 g/L xylose and 5 g/L yeast extract (pH 7) and at different temperatures (25℃, 37℃, 45 ℃ and 50℃). The maximum growth of DGN1 was observed at 50 °C with 58.85 g/L of lactic acid production and overall productivity of 1.23g/L/h followed by DGB with lactic acid concentration of 56.65 g/L and overall productivity of 1.18 g/L/h. Using glucose+xylose as substrate in Bushnell Haas minimal medium, maximum lactic acid titer of 18.08 g/L was obtained using *Bacillus licheniformis* (DGB) with overall productivity of 0.38 g/L/h at optimum temperature of 50℃.

Bacillus licheniformis (DGB) was used to produce lactic acid by exploiting sugars from hydrolysates of rice straw and wheat straw obtained after pretreatment and detoxification at 50 °C with agitation at 120 rpm, after 72 hr incubation. The maximum lactic acid titer of 96.21g/L was reported using concentrated sulphuric acid pretreated and detoxified rice straw hydrolysates with an overall productivity of 1.59 g/L/h, whereas in case of wheat straw maximum lactic acid titer of 114.22 g/L was obtained in alkali pretreated hydrolysates with an overall productivity of 1.33 g/L/h. Results showed that *Bacillus licheniformis* (DGB) was an efficient strain for lactic acid production from pretreated lignocellulosic biomass. Utilization of rice straw pretreated with concentrated sulphuric acid and detoxification and wheat straw pretreated dilute alkali alone is an efficient method for higher lactic acid production.

Introduction

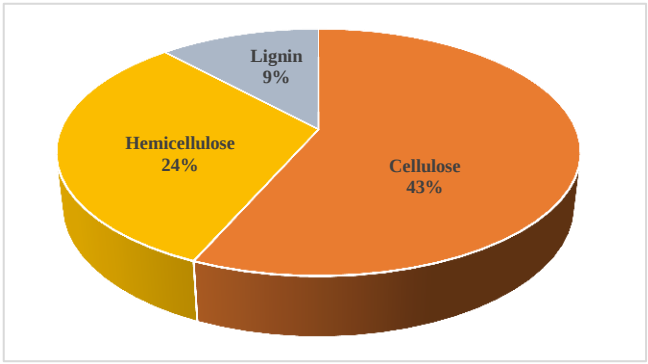
Lactic acid (LA) (2-hydroxypropionic acid) is an organic acid which was first discovered by the Swedish chemist Scheele in 1780. It is synthesized chemically and is also produced by metabolism of living organisms. Now days it has become a large asset because of its utilization in various industries such as, in food, chemical and pharmaceutical. It has many applications as feedstock for biodegradable polymers, oxygenated chemicals, plant growth regulators, as a special chemical intermediate and is a environment friendly "green solvent" (Tong *et al*., 2003). The use of poly lactic acid (PLA) for the formation of biodegradable plastics, controlled drug discovery and pesticides is leading to multimillion dollar market. Commercial production of thermostable lactic acid was prompted fifty years ago due to its huge demand.

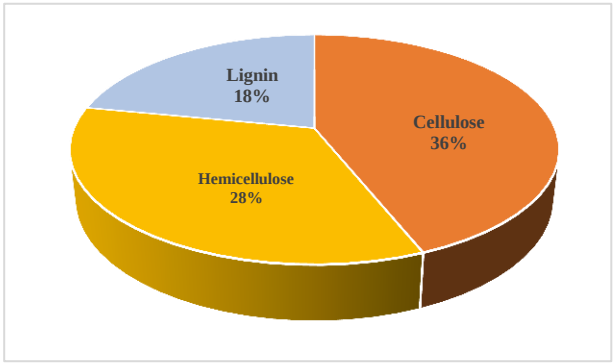
Lactic acid can be synthesized either chemically or by microbial fermentation. However, racemic mixture is obtained through chemical synthesis while pure lactic acid can be obtained through microbial fermentation (Randhawa *et al.,* 2012). Therefore, a lot of studies are being done on the production of lactic acid by microbial fermentation.

Due to exhaustion of crude oil resources and soaring of petroleum prices, the production of chemicals by biological processes has become more competitive. Agro-industrial biomass comprising of lignocellulosic waste is an inexpensive, renewable and abundant natural resource for production of industrial-scale and cost-efficient bio-energy production (Anwar *et al*., 2014).

In Punjab approximately 19-20 million tons of paddy straw and about 20 million tons of wheat straw is produced in which about 85-90 % paddy straw consisting of wheat straw and 7-8 million tons of rice straw are burnt (Kumar *et al*., 2015) leading to pollution.

Structural units of lignocellulosic biomass are cellulose, hemicellulose and lignin. In our lab previously, percentage of cellulose, hemicellulose and lignin was found in both rice straw and wheat straw (Fig.1) which after pretreatment with acid, alkali or enzyme leads to hydrolysis of the hemicellulosic and the cellulosic fractions resulting in production of pentose and hexose sugars, which can thus be bioconverted by microorganisms to produce lactic acid.





(i) Rice straw **(ii) Wheat straw**

Fig. 1 Percentage of cellulose, hemicellulose and lignin (Akhtar and Goyal, 2014)

However, there are at least two obstacles in production of lactic acid from lignocellulosic biomass. First, pretreatment and hydrolysis of lignocellulosic biomass and second is an efficient utilization of pentose sugars from the hydrolysates to form lactic acid (Zhang *et al*., 2014). Pretreatment is done to reduce the effect of biomass recalcitrance so that enzyme activity can be enhanced on cell wall polysaccharides, thus it can easily convert polysaccharides into monomers (Wyman *et al*., 2005). However, pretreatment lead to generation of aromatic compounds such as phenolics and acetyl derivatives (Toquero and Bolado, 2014), which have impact on lactic acid fermentation and downstream processing. Besides overcoming an inhibitor repression, a productive fermentation process with lignocellulosic biomass is also required for production of lactic acid. The biochemical fermentation of lignocellulosic biomass is based on two modes i.e SHF and SSF (Olofsson *et al*., 2008).

Researchers are showing more interest in thermophilic microorganisms for the production of lactic acid. The major reason behind this could be low chances of contamination, high temperature range process can be applicable at tropical levels and moreover production efficiency also increases (Bannat *et al*., 1998; Cardona *et al.*, 2007; Wingren *et al.*, 2003).

Present study focuses on pretreatment of rice and wheat straw, determination of pentose and reducing sugars and bioconversion of these sugars to lactic acid by using thermotolerant bacterial isolated strains.

Review of Literature

Lactic acid (LA) is an important chemical with wide range of its application in various industries (Yadav *et al*., 2011). The review of literature focuses on lactic acid history, its application, mode of production from lignocellulosic biomass, pretreatment and detoxification so that lignocellulosic biomass can be reduced from highly complex molecules to monosaccharides.

2.1. Lactic acid

LA is an organic acid also stated as 2-hydroxypropanoic acid (Fig. 2) was first isolated by Scheele in 1789 from sour milk (Tong *et al.,* 2004). The microorganism first reported for fermentation of the lactic acid was *Streptococcus lactis.* In 1789, this component was named as *acide lactique* by Lavoisier. In 1980, it was first produced at industrial level (Zadow, 1992).

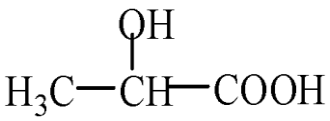


Fig. 2 Structure of LA (Zhao *et al*., 2015)

2.2. Properties and applications of lactic acid

LA with pK value 7 dissociates freely at around pH 7 resulting in formation of lactate (Ewaschuk *et al.,* 2005).It exist in the form of two stereoisomers due to presence of chiral carbon i.e. in form of L - LA and D - LA (Fig.3) (Eiteman and Ramalingam, 2015) with similar physical and chemical properties. L - LA is an important biological isomer as it is a prominent cause of muscle fatigue and lactic acidosis (Gladden *et al.,* 2004). LA is also used in food, pharmaceutical and chemical industries (Wee *et al*., 2006).

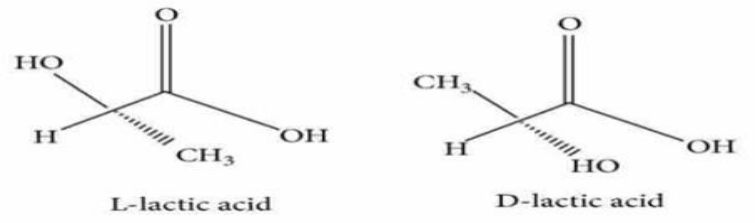


Fig. 3 Structure of L and D - LA (Hernandez *et al*., 2014)

2.2.1 Food Industry

LA is classified as GRAS by FDA for usage as food additive (Data and Henry., 1995) i.e. it is used in almost every section of food industry such as for regulating pH, fortifying minerals, improving microbial quantity and moreover commercially it is used as food additives to increase shelf life in meat and poultry. It is also used in confectionary to produce clear candies. Due to acidic nature of LA it acts as acidulants in dressings, salads, baked goods, vegetables such as pickles etc (Wee *et al.,* 2006).

2.2.2 Cosmetic Industry

LA is used in moisturisers as it has high water retaining capacity. Also used as a skin lightening agent as it suppresses formation of tyrosinase thus acting as an active ingredient in skin-lightening and rejuvenating formulations. It is also used as anti-acne, humectants and anti-tartar agent (Wee *et al.*, 2006).

2.2.3 Pharmaceutical Industry

LA is used in parental or intravenous preparations as electrolyte such as in lactated ringer's solutions, used in dialysis solution for conventional artificial kidney. They are also used for mineral preparation such as tablets, CDD, prostheses, surgical sutures (Wee *et al*., 2006).

2.2.4 Chemical Industry

LA usage have been increase in chemical industries as they can be used as desalting agent, pH regulator, neutralizer, cleaning agent, slow acid release agent. It's high solvency power and solubility is useful for removing polymers and resins. Due to its desalting property it is used in decalcification products, such as bathroom cleaners, coffee machines etc.

Hydroxyl and carboxyl functional groups are present in LA and these undergo self-esterification and forms poly-LA (PLA) and also called as biodegradable plastic (Datta *et al*., 1995). It plays major role in various industries such as in packaging, as a raw material for fibres, clothes and is a biocompatible material in medical sector (Jamshidan *et al.,* 2010). PLA physical properties depend on optical purity of LA; it can be either semi-crystalline or amorphous i.e. if backbone is L or D- LA then PLA is semi-crystalline in nature whereas if backbone of polymer is a racemic mixture of L and D-

LA then PLA is usually amorphous (Garlotta, 2001) which can be used in drug delivery so that active ingredients can uniformly disperse in carrier whereas semi-crystalline form is used where high tensile strength is required (Tsuji and Ikada, 1997a).

2.3 Lignocellulosic biomass

Lignocellulosic biomass is the waste by product that is generated through various agricultural practices (Perez *et al*., 2002) which are burnt leading to pollution. Due to its renewable nature lignocellulosic biomass has attracted many researchers (Asgher *et al.,* 2013).

Building blocks of lignocellulosic biomass are cellulose, hemicellulose and lignin (Fig. 4; Table 1 shows composition of different biomass). Lignocellulosic biomass can be forestry residue, agricultural wastes, grasses and woody substances (Anwar *et al*., 2014).

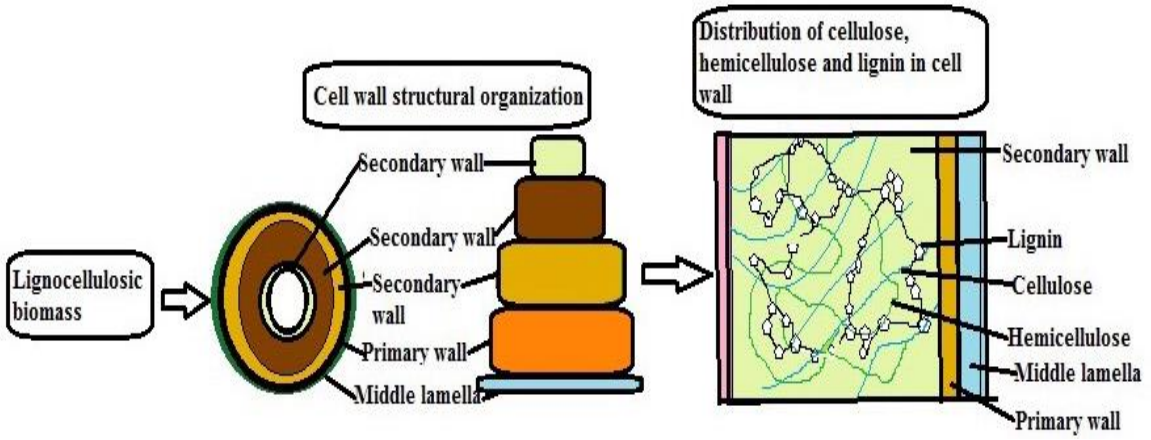


Fig. 4 Illustration of lignocellulosic biomass with its three structural units i.e. cellulose, hemicellulose and lignin (Menon and Rao, 2012)

Cellulose

It is the most plenteous and highly stable polymer found on earth (Anwar *et al.,* 2014) with structural formula $(C_6H_{10}O_5)_n$. (1,4)-$_D$-glucopyranose units linked via β-1,4 glycosidic linkages (Himmel *et al*., 2007) forms cellulose. It is a linear homopolymer with high molecular weight allied together with hydrogen and vanderwaal forces. Cellulose in lignocellulosic biomass exists in two forms i.e. crystalline and amorphous (Mussato *et al*., 2010).

Hemicellulose

It is the second most plenteous natural heterogenous polymer formed by units of glucuronoxylan, glucurnomannon and trace amounts of some polysaccharides (Anwar *et al.,* 2014). Sugars such as D-galactose, D-glucose, D-mannose, L-arabinose and D-xylose act as backbone of these polymers where L-arabinose and D-xylose are pentoses and rest three are hexoses. Xylan is most common type of polysaccharide in hemicellulosic family, formed of D-xylopyranose units which is linked together by 1,4 linkages (Musatto *et al*., 2010; Mod *et al.,* 1981; Ademark *et al.,* 1998). Hemicellulose has amorphous structure therefore it is much easier to hydrolyze as compared to cellulose (Taherzadeh and Karimi, 2008).

Lignin

It is the most complicate biopolymer. It is a heterogenous polymer with long chain of phenyl propane units which are linked by ether bonds. It acts like glue as it fills gap between and around cellulose and hemicellulose (Anwar *et al.,* 2014). Units of phenyl propane are composed of p-coumaryl alcohol, n-coniferyl alcohol and sinapyl alcohol. Due to its configuration they are resistant to chemical and enzymatic degradation therefore they can't be used for fermentation and are removed by pretreatment.

Table 1. Percentage composition of lignocellulosic biomass

Biomass	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**References**
Rice straw	24	43	9	Akhtar and Goyal, 2014
Wheat straw	28	36	18	Akhtar and Goyal, 2014
Poplar wood	44	32	21	Meng *et al.,* 2012
Sugarcane baggase	42	25	20	Kim and Day, 2011
Banana waste	13	15	14	Sanchez *et al*., 2009
Corn strover	38	26	19	Zhu *et al*., 2005
Hardwood	40	24	18	Malharbe and Cloete, 2002
Softwood	45	25	25	Malharbe and Cloete, 2002

2.4 Pretreatment for lignocellulosic biomass

Lignocellulosic biomass is a potential source for production of biofuels and organic acids. Due to complex structure of lignocellulosic biomass it can't be used directly therefore fermentation is required. Pretreatment plays an important role in separating cellulosic material from lignin fractions i.e. it breaks down the lignin barrier leading to release of cellulosic content (Anwar *et al.,* 2014) which further breaks down upon pretreatment leading to release of glucose, xylose, xylanose, arabinose which upon fermentation gets converted to product. Some of the pretreatment methods are explained below:

2.4.1 Acid based hydrolysis

Concentrated acid of dilute acid are generally two methods used in acid based hydrolysis.

Concentrated acid hydrolysis

Lignocellulosic biomass treatment with concentrated sulphuric acid (H_2SO_4) and concentrated hydrochloride acid (HCl) is conventional method of pretreatment (Anwar *et al.,* 2014). Cellulose and hemicellulose are predominately hydrolysed by concentrated sulphuric acid treatment. This entire process is conducted at low temperature and pressure (Ogier *et al.,* 1999).

The drawback of using concentrated sulphuric treatment is that it leads to severe degradation of cellulose, forming inhibitors in high concentration and also causing corrosion of equipment (Alvira *et al.,* 2010).

Dilute acid hydrolysis

Dilute acids such as H_3PO_4, HCl and H_2SO_4 are used for pretreatment of various lignocellulosic biomass (Alvira *et al.,* 2010). Optimum temperature for treating with dilute sulphuric acid is believed to be between 100℃ to 200℃ with 0.5 - 2.0 % concentrations of dilute sulphuric acid (Sun *et al*., 2016). Zang *et al* (2014) pretreated wheat straw with dilute sulphuric acid producing 4.92 g/L glucose and 17.17 g/L xylose and 46.12 g of LA from 100 g of dry wheat straw. Saha *et al* (2005) used various concentrations of dilute sulphuric acid for pretreating wheat straw at 121℃ for 1 hr and got maximum fermentable sugars at 1.00 % concentration of dilute sulphuric acid with 20 mg/g of glucose, 158 mg/g of xylose and 20 mg/g of arabinose. Kim *et al* (2010)

prepared rice straw hydrolysates giving dilute acid pretreatment and obtained 10.6 g/g glucose and 4.4 g/g of xylose.

However, acid pretreatment is not propitious for delignification, high contents of lignin are usually released after pretreatment therefore, further treatment is always required for removal of lignin which improves the breakdown of the lignocellulosic residues (Sun *et al.,* 2015).

2.4.2 Alkali based pretreatment

Bases such as sodium and ammonium hydroxides are used for pretreating lignocellulosic biomass. It leads to exhaustion of lignin barrier, swelling of cellulose and partial decrystallization and dissolution of cellulose and hemicellulose (Cheng *et al*., 2010; Ibrahim *et al.,* 2011; Sills *et al.,* 2011; Zhu *et al.,* 2010). There is decline in crystalline nature of cellulose due to swelling which results in structural hindress of cellulose of lignocellulosic biomass. Mcintosh and Vancov (2011) pretreated wheat straw with 0.5% - 2.0% NaOH at 121 ℃ for 30 min leading to 20% and 30% solubilisation of hemicellulose respectively. Zhao *et al* (2008) evaluated the enzymatic hydrolysis behaviours of spruce pretreated with aqueous NaOH/urea at different temperatures (15℃, 23 ℃ and 60 ℃). They observed higher enzymatic hydrolysis efficiency at lower temperature i.e. 15 ℃ than higher temperatures, thus inferred that the swelling of cellulose in aqueous NaOH or NaOH/urea was much higher at low temperature hence enzymes could easily accessible sugars at this temperature. Barley straw when pretreated with aqueous NaOH (0.5% -2.0%) at 105℃ for 10 min removed 84.8% lignin, 79.5% hemicellulose leading to 86.5% of reducing sugars (Haque *et al.,* 2012).

For pretreatment of various lignocellulosic materials $Ca(OH)_2$, also known as lime had been used. It confers various advantages over NaOH such as lower cost and its easy recovery by reaction with CO_2 (Alvira *et al.,* 2010). Lime pretreatment is conducted at a relatively low temperature for a long reaction time due to its low solubility at higher temperature (Kim and Holtzapple, 2005). Moreover, lime treatment leads to removal of lignin moderately, 43.6 - 48.4% of lignin was removed when corn stover was pretreated with non-oxidative $Ca(OH)_2$ at 25 ℃ for 16 weeks whereas, on oxidative lime pretreatment 57.8 - 87.5% of lignin was removed as treatment with oxidative lime results in removal of both lignin as well as acetyl groups (Kim and Holtzapple, 2005).

Oxidative delignification

Oxidative reagents such as ozone, oxygen, hydrogen peroxide, chlorine dioxide, sodium hypochlorite and chlorine gas are generally used as they release plenty of free radicals leading to excessive oxidative fragmentation and deligninification (Sun *et al*., 2015). Lignin content was reduced with little degradation of hemicelluloses on pretreatment of wheat straw and rye straw with O_3. No loss of cellulose was observed as enzymatic hydrolysis and 89% and 57% yield was obtained for O_3 pretreated wheat straw and rye straw respectively when compared with non-pretreated (García-Cubero *et al.,* 2009). It is usually performed at mild conditions with formation of negligible amount of inhibitory compounds (Sun and Cheng, 2002). Since it is an expensive technique thus it is used as secondary pretreatment, especially in case of alkali pretreatments (Qi *et al*., 2009).

Organosolv process

Organic or aqueous solvents such as ethanol, methanol, acetone, organic acid, acetone and ethylene glycol are primarily used for delignification (Sun *et al*., 2015; Zhao *et al.,* 2009b). Organosolv pretreatment of pitch pine with 2% NaOH remarkably reduced the lignin content from 26.17% to 11.72%, whereas no change in lignin content was observed in absence of NaOH pretreatment (Park *et al*., 2010).

2.4.3 Enzymatic hydrolysis

Cellulose and hemicellulose remained as such after pretreatment were depolymerised through enzymatic hydrolysis into fermentable sugars. Enzymes are classified into two categories in pretext of lignocellulosic degradation, first cellulase and second hemicellulase. To maximise sugar yield three major groups of cellulases (endo-β-1, 4-glucanases, exo-β-1, 4 glucanases and β-glucosidases) react collaberately for cellulose degradation (Zhang *et al.,* 2007). Endo- β-1, 4-glucanases randomly cut intramolecular β-1, 4-glycosidic bonds of cellulose chains whereas exo-β-1,4-glucanases and β-glucosidases produces soluble glucose and cellobiose by hydrolyzing cellulose chains at the ends of the polymer and β-glucosidases cleaves cellobiose into two glucose units (Lynd *et al.,* 2002). Endo-1,4-β xylanase, β- xylosidase, β- glucuronidase, α-L-arabinofuranosidase are required for hemicellulose hydrolysis whereas xylan is hydrolyzed by using acetyl xylan esterase enzyme (Carvalheiro *et al*., 2008). Glucomannan is cleaved by β -mannanase and α-mannosidase (Kumar *et al*., 2008).

2.5 Detoxification of hydrolysates

Pretreatment leads to deterioration of cellulose, hemicellulose and lignin, releasing inhibitory compounds such as furan derivatives, organic acids and phenolic compounds (Palmqvist *et al*., 1999). Thereafter, removal of inhibitory compounds or detoxification is an essential step. Following methods of detoxifying hydrolysates are used: water washing, overliming method [$Ca(OH)_2$], ion-exchange, activated charcoal adsorption (Li *et al.,* 2008; Cantarella *et al*., 2004; Mussatto *et al.,* 2004).

Overliming method

Furfural is the principal inhibitor groups (Taherzadeh *et al.,* 1997a) which are suggested to be bioconverted from furfuryl alcohol and furoic acid using NADH and NAD^+ respectively (Taherzadeh *et al.,* 2000a; Horvath *et al.,* 2003) wherein alcohol dehydrogenase and aldehyde dehydrogenase catalysis and oxidation reactions, leading to arrest of cell growth until all furfurals are biotransformed.

Overliming process leads to conversion of furfurals into non-toxic form (Purwadi *et al*., 2004). In this treatment dried $Ca(OH)_2$ is added in acidic hydrolysates which results in formation of gypsum hence it can be used as plaster of Paris which has large commercial value (Chandel *et al*., 2011).

Activated charcoal method

It is an economical method with high capacity to adsorb compounds without affecting the level of sugars in hydrolysate (Canilha *et al.,* 2008; Chandel *et al.,* 2007a; Mussatto and Roberto, 2004). 95.40% of phenolic compounds were removed on detoxifying oak wood with activated charcoal method (Converti *et al.,* 1999). Yucel and Aksu, 2015; sugar pulp hydrolysates were detoxified using combined activated charcoal, overliming method and fly ash treatment and it was observed that phenolics and furans were reduced by charcoal and overliming detoxification whereas fly ash removed furans and resulted in 48.2 g/L reducing sugars.

Ion-exchange resin

Ion exchange resins helps in removal of lignin-derived inhibitors, furfurals and acetic acid (Chandel *et al*., 2011). Effect of the four different ion exchange resins (cationic and anionic) for detoxification of Eucalyptus hemicellulosic hydrolysate were investigated

for xylitol production by *Candida guilliermondii* and observed that ion exchange detoxification remarkably increased xylitol production to 32.7 g/L (Villarreal *et al.,* 2006). 63.4% of furans and 75.4% of phenolics were removed from sugarcane baggase acid hydrolysate (Chandel *et al.,* 2007a). Zhuang *et al*., 2009; observed that 90.36% of furfural, 77.46% of phenolic and 96.34% of acetic acid were removed by using ion-exchange resin D 311 and overliming method of detoxification from wheat straw hydrolysates.

***In-situ* microbial detoxification**

The microorganisms utilize inhibitory compounds formed after pretreatment of lignocellulosic biomass and transform their chemical nature, leading to removal of inhibitory compounds (Lopez *et al*., 2004). Inhibitory compounds furfural, HMF, aromatic, aliphatic acids and aldehydes present in corn stover hydrolysate were metabolized by *Coniochaeta ligniaria* (NRRL 30616), a fungal isolate (Nichols *et al*., 2008). Another fungal isolate *Amorphotheca resinae* ZN1 was reported to metabolize furfural and HMF by increasing glucose yield from 21.4 g/L to 32.9 g/L in corn stover hydrolysates (Zhang *et al.,* 2010). Rice straw and olive tree pruning hydrolysates were detoxified with *Sacchromyces cerevisae* and observed that in case of untreated rice straw hydrolysates, *Pitchia stipitus* was not able to ferment whereas in case of treated rice straw hydrolysates ethanol productivity observed was 0.17g/g (Fonseca *et al*., 2013).

2.6. Lactic acid fermentation

LA production from petrochemicals results in racemic mixtures whereas some microorganisms have capability generate optically pure LA i.e. either L-LA / D-LA depending upon the expression of lactose dehydrogenase enzyme which converts LA into pyruvate (Wehrenberg, 1981; Blomqvist, 2001; Tsuji, 2002). There are two modes of fermentation for production of LA by microbial fermentation; it can be either heterolactate fermentation or hemolactate fermentation depending upon the mode of metabolism of sugars followed by microorganism (Eiteman and Ramlingam, 2015).

Homolactate fermentation

In homolactate fermentation microorganisms, yield pure LA by following the pentose-phosphate pathway (Fig. 5) in which hexose sugars are metabolised. Some of the examples are: *Lactobacillus lactis, Lactobacillus casei, Bacillus coagulans* etc. *Bacillus*

coagulans through homofermentative mode of fermentation produced LA from sweet sorghum juice (Ou *et al.*, 2015). *Bacillus subtilis* through homofermentative mode of fermentation produced LA from cellobiose.

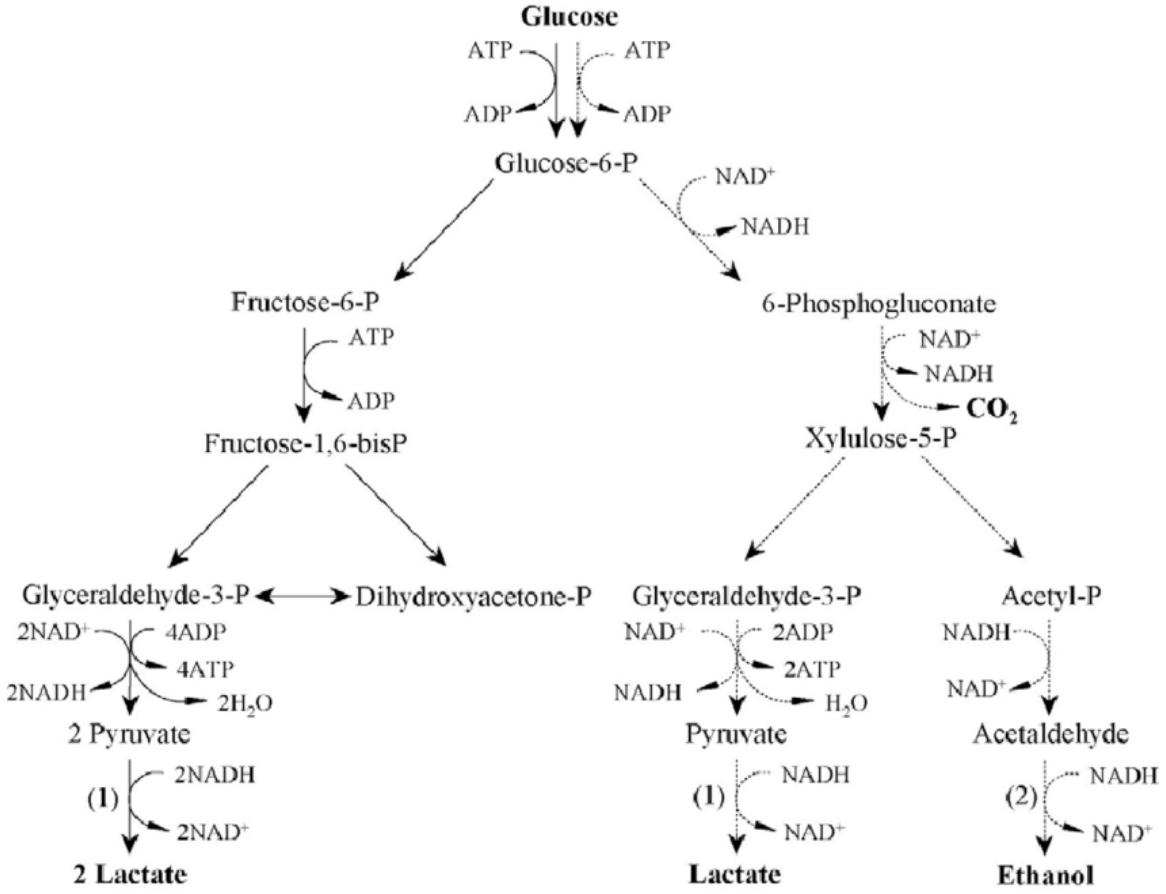


Fig. 5 Metabolic pathways of homolactate (solid line) and heterolactate fermentation (dotted lines in LA bacteria where (1) is lactate dehydrogenase and (2) alcohol dehydrogenase enzymes (Wee *et al.*, 2006)

Heterolactate fermentation

In this mode of fermentation microorganisms use phosphoketolase pathway for LA production with by products such as carbon dioxide, formic acid or acetic acid. Some of examples are: *Leuconostoc sp, Lactobacillus delbrueckii, Rhizophus oryzae* etc. Hu *et al* (2016) identified heteroferementative *Lactobacillus pentosus* FL0421 strain for production of LA. *Lactobacillus delbrueckii* through heterofermentative mode of fermentation produced LA through cellobiose (Adsul *et al.*, 2007). *Lactobacillus*

manihotivorus through heterofermentative mode of fermentation produced LA through food waste.

Sugar solutions are inoculated after supplementation of nutrients with selected microorganism where it is necessary to select the most favourable conditions such as temperature, pH, aeration etc. as per the microorganism. The substrate most frequently used can be classified into two groups, firstly monosaccharides and disaccharides and other is polymeric substances, where monosaccharides and disaccharides include byproduct of food industry such as molasses and whey and polymeric substances include lignocellulosic biomass (Martinez *et al.,* 2013).

The lignocellulosic hydrolysates are rich in sugars such as glucose, xylose, arabinose and mannose depending upon different type of lignocellulosic biomass. Glucose is smoothly metabolized by microorganism to form LA however, most microorganisms lack xylose metabolizing enzymes. Fermentation technologies for LA production should be economical so that use of biotechnology can be promoted on industrial scale (Bustos *et al.,* 2007).

Simultaneous saccharification and fermentation (SSF)

SSF is combination of enzymatic hydrolysis and fermentation in a single step. Thus it has various advantages over SHF such as it minimizes the reactor volume, processing time, enzyme loading with consequent increase in the LA productivity and rate of hydrolysis (Hofvendhal and HahnHagerdal, 2000). In SHF accumulation of sugars results in inhibition of cellulolytic enzymes whereas in SSF cellulose and xylanase enzymes shows resistance to feedback inhibition caused due to accumulation of sugars. Rate limiting step for SSF is hydrolysis (Philippidis and Smith, 1995). SHF and SSF processes by using empty fruit bunch as substrate was done simultaneously by using different concentration of enzymes to compare their efficiency, it was observe that 4.74 % of ethanol was produced through SHF whereas 6.05 % of ethanol was produced through SSF through 40 FPU of enzyme (Dahnum *et al*., 2015). LA production by SSF has been studied using corn stover, corncob, waste wood, wheat straw and alfalfa fibre (Sreenath *et al.,* 2001; Garde *et al.,* 2002; Lee *et al.,* 2004; Miura *et al.,* 2004; Cui *et al.,* 2011). SSF has certain limitations such as, difference between optimum temperature and pH required for saccharification and fermentation (Huang *et al.,* 2005), inhibition effect of LA on enzymes (Takagi, 1984).

2.6.1 Lactic acid producing microorganisms

Microorganisms widely used for LA production are fungi and bacteria. Filamentous fungi have ability to release extracellular amylases which are able to hydrolyse starchy materials (Deng *et al*., 2012; Jin *et al.,* 2005). Fungi such as *Rhizopus* are able to produce L-LA from different lignocellulosic biomass as described in Table 2. LA production commercially is done mostly by bacteria as fungi have low productivity.

Table 2. Lactic acid production from different microorganisms

Microorganisms	Substrate	LA produced (%)	Reference
Bacteria			
Bacillus coagulans	Sweet sorghum juice	80	Ou *et al*., 2016
B. coagulans strain	Coffee pulp	94	Pliensser *et al*., 2016
Lactobacillus amylophilus	*Zizyphus oenophlia*	99	Bishai *et al*., 2015
Lactobacillus paracasei	Rice straw	0.97	Kuo *et al.*, 2015
Bacillus coagulans	Wheat straw	46.12	Zhang *et al.,* 2014
Lactobacillus brevis	Cassava syrup	73	Quientero *et al*., 2012
Pediococcus acidilactici DQ2	Corn stover	77.2	Zhao *et al.*, 2012
Bacillus sps.(XZL9)	Corn corb mollases	50	Wang *et al*., 2011
Bacillus subtilis	Cellobiose	82	Garcia *et al*., 2009
Bacillus coagulans	Wheat Straw	43	Mass *et al.,* 2008
Lactobacillus rhamnosus	Paper sludge	83	Tong *et al*., 2004
Lactobacillus amylophilus	Wheat flour	90	Vishnu *et al.,* 2002
Lactobacillus pentosus&	Wheat Straw	88	Garde *et al.,* 2002
Lactobacillus brevis		61	
Fungi			
Rhizopus oryzae	Paper sludge	9.33	Takano and Hoshino., 2016
Rhizopus oryzae	Corn starch	85	Yin *et al.,* 1997

Effect of temperature

Temperature is one of the important factors that affects lactic acid production. The majority of LAB, such as, *L. delbrueckii*, are mesophilic bacteria, which grow at 17-50℃ and have optimum growth temperature between 37-43℃ (Hofvendahl *et al.,* 2000). Goksungur and Guvenc (1997) reported the optimal temperature for

L.delburueckii IFO 3202 is 45℃; when the temperature increased above 45℃, lactic acid production and yield decreased rapidly.

Commercial thermophilic strains are preferred over mesophilic strains as chances of contamination are more in mesophilic temperatures. Secondly, cost of recovery is also low (Banat *et al.*, 2010). *Bacillus* sp. is thermophilic and has been used as an efficient LA producer. *B. coagulans* IPE22 was fermented under different temperatures (46°, 49°, 52°, 55° and 58℃) and highest LA titer of 52.20 g/L was obtained at 52℃ (Zhang *et al.*, 2014). *R. oryzae* NBRC 5384 was cultured at different temperatures i.e. 28, 36, 40 and 42℃ respectively, and observed that the rate of LA production increased with rise in temperature till 36℃ and with further increase in temperature the production decreased up to 42℃ (Takano and Hoshino, 2016).

Material and methods

3.1 Bacterial strains and isolates

Previously isolated bacterial strains (NA9, DGB, DGN1 and DGN2) were revived in enrichment broth (10 g/L xylose and 5g/L yeast extract) (Lidan *et al.*, 2013) and effect of temperature was checked by incubating at different temperatures (25 ℃, 37 ℃, 45 ℃ and 50 ℃). Bacterial growth was observed at absorbance of 600 nm after 48 hr followed by LA estimation as described in section 2.1.1

3.2 Production of lactic acid by bacterial isolates

Bacterial isolates were inoculated in Bushnell Haas minimal media at pH 7 supplemented with carbon source (either glucose, xylose and glucose+xylose) 5g/L of each sugar and incubated at 50 ℃ with agitation at 120 rpm for 48 hrs thereafter LA production was estimated and most competitive strain was selected.

3.2.1 Estimation of lactic acid (Borshchevskaya *et al.*, 2016)

Spectrophotometerical analysis were done for LA estimation by taking 50 µL sample in 2mL of 0.2% solution of iron (III) chloride after vortexing, absorbance with respect to blank was taken at 390 nm. The standard curve (Fig. 6) was plotted using 0, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L stock solution of LA for calculating concentration of LA present in the sample.

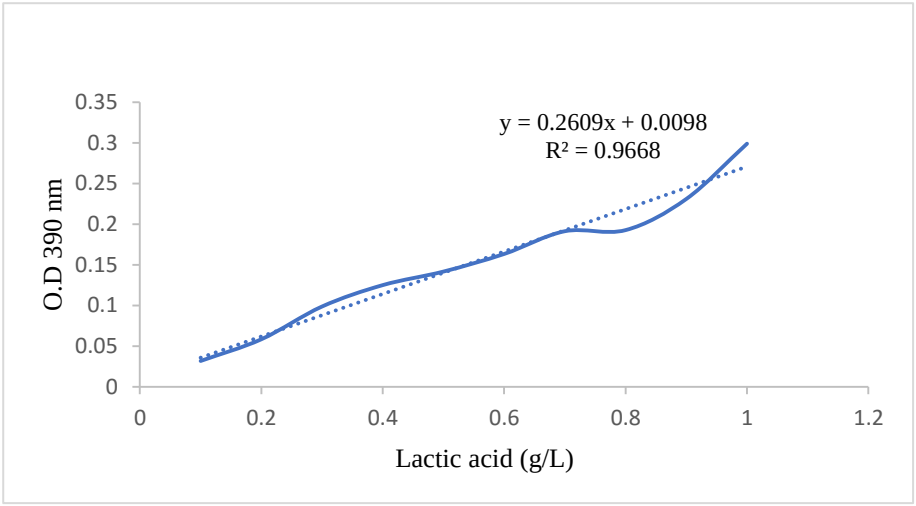


Fig. 6 Standard curve for lactic acid

Calculations

Overall productivity was calculated by using formula:

$$\text{Overall productivity (g/L/h):} \quad \frac{\text{Concentration of LA (g/L)}}{\text{Time of incubation (h)}}$$

3.2.2 Estimation of reducing sugar by DNS method (Miller, 1959)

Reducing sugars in fermentation broth were estimated after 48 hrs of bacterial incubation by taking 0.1ml of broth in test tube containing 500µL of potassium phosphate buffer (50mM) and 1ml of volume was made by using distilled water. To this solution 3mL of DNS reagent (Appendix) was added and incubated for 10 min at 100℃ in water bath. It was allowed to cool at room temperature and afterwards absorbance was recorded at 540 nm. The standard curve for O.D v/s glucose concentration (g/L) (Fig. 7) was plotted with different glucose concentration (0, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L) by using 1g/L glucose stock solution, and estimating reducing sugar as above.

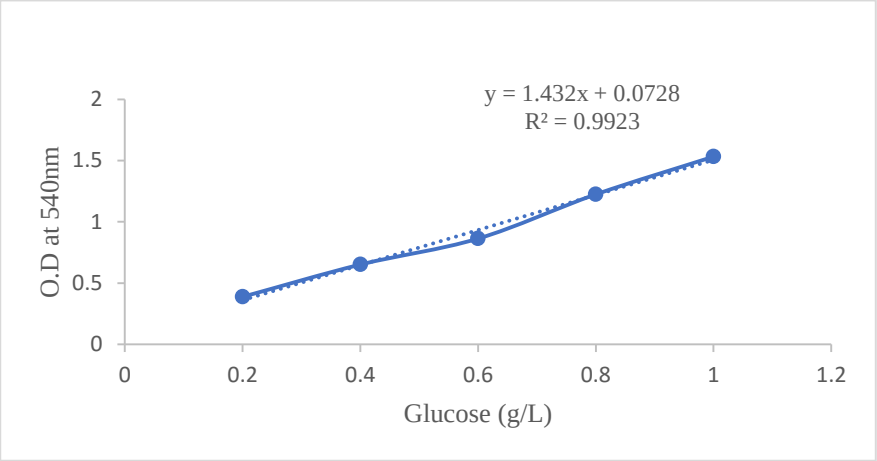


Fig. 7 Standard curve for reducing sugar

3.2.3 Estimation of xylose in biomass sample (Fernell and King, 1953)

1ml of native biomass hydrolysates, pretreated and pretreated and detoxified samples of rice straw and wheat straw before and after inoculation in 3ml of orcinol reagent (Appendix) and incubated at 100℃ 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.

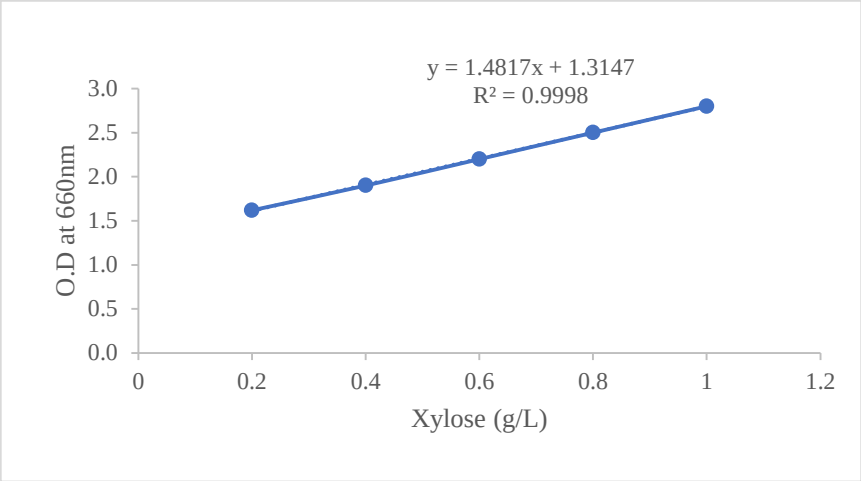


Fig. 8 Standard curve for xylose

3.3 Collection and processing of rice straw and wheat straw

Rice straw and wheat straw were collected from nearby villages of Patiala, Punjab, dried in hot air oven at 50℃ for 72 hr and pulverized to particle size of 0.5mm. These were sieved and were stored in air tight container at room temperature.

3.4 Pre-treatment of rice straw and wheat straw

3.4.1 Acid hydrolysis

3.4.1.1 Pretreatment with concentrated sulphuric acid (Nigam *et al*., 2002)

10 g of powderised biomass was taken in conical flask and 2 ml of concentrated sulphuric acid was added and volume was made upto 100 ml using double distilled water and was autoclaved at 121 ℃ for 60 min at 15 lbs. After autoclaving, the hydrolysates were filtered using muslin cloth and pH of filterate was maintained to 7 by using 0.1N NaOH.

3.4.1.2 Pretreatment with dilute (0.6 N) sulphuric acid (Peng *et al.*, 2014)

10 g of powderised biomass was taken in flask and 2 ml of 0.6 N sulphuric acid was added followed by volume made upto 100 ml using double distilled water and was

autoclaved at 121 ℃ for 60 min at 15 lbs. After autoclaving, the hydrolysates were filtered using muslin cloth and pH of filterate was maintained to 7 using 0.1N NaOH.

3.4.2 Alkali hydrolysis

3.4.2.1 Pretreatment with dilute (0.4 N) sodium hydroxide (Peng *et al.*, 2014)

10 g of powderised biomass was taken in flask and 2ml of 0.4 N sodium hydroxide was added followed by volume made upto 100 ml by using double distilled water and was autoclaved at 121 ℃ for 60 min at 15 lbs. After autoclaving, the hydrolysates were filtered using muslin cloth and pH of filterate was maintained to 7 using 0.1N NaOH.

3.5 Detoxification of pretreated hydrolysates by over liming (Purwadi *et al.*, 2004)

After acid or alkali pretreatment, hydrolysates thus obtained were equally divided into two aliquots, where one aliquot was taken for detoxification process by adding 5% $Ca(OH)_2$ to maintain pH at 10. After pH was set it was incubated at 60 ℃ in water bath shaker for 30 min to avoid deposition of $Ca(OH)_2$. After incubation pH was maintained at 7 with H_2SO_4, followed by vacuum filtration.

3.6 Production of lactic acid from rice straw and wheat straw

Bacterial production of LA from rice straw and wheat straw was done in two sets of experiments. One set of experiment was done using hydrolysates obtained after pretreatment and the other set was done by using pretreated and detoxified hydrolysates of both rice straw and wheat straw which was autoclaved at 105 ℃ for 30 min. After autoclaving pretreated as well as pretreated and detoxified media were inoculated with *Bacillus licheniformis* (DGB) and incubated at 50 ℃ with continuous agitation of 120 rpm. Spectrophotometrically, bacterial growth (600 nm), LA, reducing sugar and xylose in broth were estimated after 48 and 72 hr as described in 2.1.

Results and Discussion

Seven bacterial isolates from dairy, bakery wastes, cow dung/compost and eight previously isolated strains were screened for their LA production potential in media supplemented with xylose. After 48 hrs the order of growth was observed as DGN1 > DGB >T8 > NA15 >NA9 > DGS3 >DGN2 > DGS1 and then these were transferred to plates containing bromocresol green. Four strains NA9, DGB, DGN1 and DGN2 were selected for further study on the basis of colour change from green to yellow, significant of their lactic acid production.

4.1 Effect of temperature on lactic acid production by bacterial isolates

Fermentation using different strains of bacteria i.e. DGB, NA9, DGN1 and DGN2was carried out in enrichment broth (pH 7) containing 10 g/L xylose and 5 g/L yeast extract at different temperatures (25℃, 37℃, 45℃ and 50℃). The maximum growth of DGN1 was observed at 50℃ with 58.85 g/L (Table 3 and Fig. 9) of LA production and overall productivity of 1.23 g/L/h (Table 4) followed by DGB with LA concentration of 56.65 g/L and overall productivity of 1.18 g/L/h. Using glucose + xylose as substrate in Bushnell Haas minimal medium, maximum LA titer of 18.08 g/L was obtained using *Bacillus licheniformis* (DGB) with overall productivity of 0.38 g/L/h at 50℃. 50℃ was the optimum temperature for growth of *Bacillus* species for LA production.

Table 3. Growth of different bacterial isolates (600nm) when grown in media enriched with xylose (10 g/L) and lactic acid (g/L) production at different temperatures (25℃, 37℃, 45℃ and 50℃) after 48 hrs

Bacterial isolates	**Temperature**							
	25℃		37℃		45℃		50℃	
	O.D 600nm	LA (g/L)	O.D 600nm	LA (g/L)	O.D 600nm	LA (g/L)	O.D 600nm	LA (g/L)
NA9	0.141	3.19	0.343	10.43	0.484	20.70	1.246	40.28
DGB	0.11	2.77	0.446	18.62	0.521	21.54	1.768	56.65
DGN1	0.055	0.85	0.415	17.43	0.180	4.02	1.860	58.85
DGN2	0.059	0.90	0.165	3.77	0.16	3.70	1.072	33.22

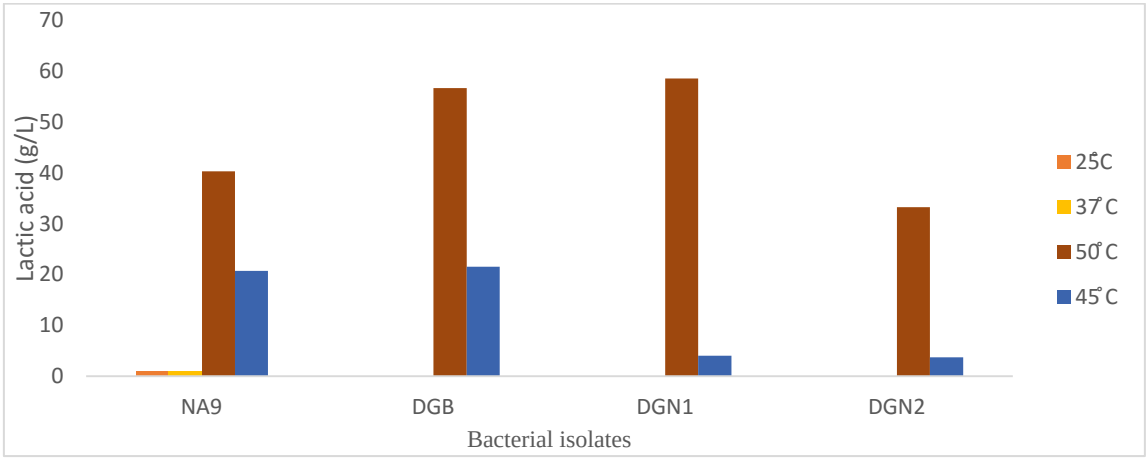


Fig. 9 Growth of different bacterial isolates (600nm) when grown in media enriched with xylose (10 g/L) and lactic acid production (g/L) at different temperatures (25℃, 37℃, 45℃ and 50℃) after 48 hrs

Table 4. Overall productivity of lactic acid (g/L/h) by bacterial isolates at different temperature

Bacterial isolates	**Temperature**			
	25℃	37℃	45℃	50℃
NA9	0.07	0.22	0.43	0.84
DGB	0.06	0.39	0.45	1.18
DGN1	0.02	0.36	0.08	1.23
DGN2	0.02	0.08	0.08	0.69

Lidan *et al*., 2013 screened thermotolerant strains of bacteria at 50 ℃ and pH 6 and observed 83.6 g/L LA titre in mineral salts medium containing 1-2% (w/v) of yeast extract with productivity of 7.5 g/L/h in batch fermentation with 8.5 g/L xylose. High fermentation temperature has many advantages such as at high temperature chances of contamination are decreased, secondly cooling cost is also decreased with increase in enzyme activity thereby decreasing the cost of enzymes (Banat *et al.*, 2010).

4.2 Production of lactic acid by bacterial isolates

Production of LA by bacterial isolates was carried out in batch in Bushnell Haas minimal media (pH 7.0) supplemented either with glucose, xylose or both glucose+xylose at a concentration of 5g/L to select the most competitive thermotolerant strain among four

bacterial strains at 50℃. All the four strains grew well in minimal media but NA9 and DGB gave maximum LA production. There was no sugar left in fermentation broth after 48 hr of incubation (Table 5). Since, no sugar was present, indicating its complete utilization by bacterial isolates in minimal media.

Carbon catabolite repression is a regulatory mechanism in which the utilisation of other carbon source is hindered due to presence of preferred substrate (Wang *et al*., 2014). Glucose is the primary metabolic regulator in most of the organisms. But according to the pentose phosphate pathway, xylose utilisation is the key aim for the production of the LA. Carbon catabolite repression was observed in case of DGN1 and DGN2 as the LA concentration decreased, when both glucose and xylose were supplemented in media, compared to when only xylose was supplemented as the sole carbon source. DGB and NA9 both strains of *Bacillus licheniformis* were seen to overcome carbon catabolite repression and produced higher amount of LA i.e. 18.08 g/L and 15.87 g/L respectively with overall productivity of 0.38 g/L/h and 0.33 g/L/h respectively in media supplemented with both glucose (5 g/L) and xylose (5 g/L) (Table 6 and Fig. 10, Fig. 10a) DGN1 showed less LA production of 9.81 g/L and overall productivity of 0.20 g/L/h respectively whereas, in DGN2, 9.25 g/L of LA production and overall productivity of 0.19 g/L/h was observed. When compared to their yield in the media supplemented with xylose only, all the four strains, NA9, DGB, DGN1 and DGN2 were productive and with similar LA production i.e. 7.38 g/L, 7.95 g/L, 7.68 g/L and 7.46 g/L and overall productivity of 0.15 g/L/h, 0.17 g/L/h, 0.16 g/L/h, 0.16 g/L/h respectively.

When media was supplemented with glucose, DGB showed maximum LA production of 7.70 g/L and overall productivity of 0.16 g/L/h followed by NA9, where the concentration of LA was 6.94g/L with overall productivity of 0.14g/L/h. DGN1 and DGN2 showed the minimum concentration and overall productivity i.e. 4.27g/L and 0.09 g/L/h in case of DGN1 and 4.95g/L and 0.10g/L/h respectively in case of DGN2. It was observed that DGB is thermotolerant that overcomes carbon catabolite repression and produced good amount of LA i.e. 18.08 g/L in glucose and xylose in Bushnell Haas minimal media at 50℃.

Table 5. Growth of bacterial strains was observed at absorbance of 600nm with residual reducing sugar content after 48 hrs where'-' no residual sugar observed.

Bacterial isolates	Carbon Source					
	Glucose+Xylose(5g/L)		Glucose(5g/L)		Xylose(5g/L)	
	O.D 600nm	Reducing sugars	O.D 600nm	Reducing sugars	O.D 600nm	Reducing sugars
NA9	1.181	-	0.631	-	0.790	-
DGB	0.871	-	0.778	-	0.791	-
DGN1	1.029	-	0.699	-	0.622	-
DGN2	0.885	-	0.793	-	0.647	-

Table 6. Lactic acid (g/L) and overall productivity (g/L/h) by bacterial strains in Bushnell Haas minimal media supplemented with either glucose+xylose, glucose or xylose

Bacterial isolates	Carbon source (5g/L)					
	Glucose + Xylose		Glucose		Xylose	
	LA (g/L)	Overall productivity (g/L/h)	LA (g/L)	Overall productivity (g/L/h)	LA (g/L)	Overall productivity (g/L/h)
NA9	15.87±0.06	0.33	6.94±0.0	0.14	7.38±0.0	0.15
DGB	18.08±0.08	0.38	7.70±0.0	0.16	7.95±0.0	0.17
DGN1	9.81±0.04	0.20	4.27±0.0	0.09	7.68±0.0	0.16
DGN2	9.25±0.05	0.19	4.95±0.0	0.10	7.46±0.0	0.16

It can be inferred that DGB utilizes carbon in Bushnell Haas minimal media with order of preference for carbon source is glucose+xylose > xylose > glucose.

Wang *et al* (2011) also observed that BL1 strain of *Bacillus licheniformis* could ferment glucose to optically pure L-lactate with maximum specific productivity of 7.8 g/L/h in LB medium at 50℃. LB medium is nutritionally rich medium while DGB was grown on minimal medium yielding efficient amount of LA which is thus cost effective.

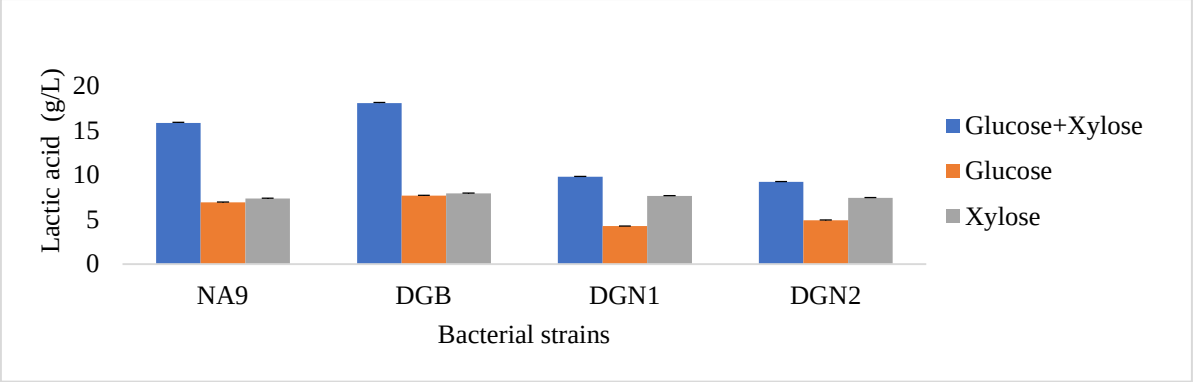


Fig. 10 Lactic acid (g/L) by bacterial strains in Bushnell Haas minimal media supplemented with either glucose+xylose, glucose or xylose

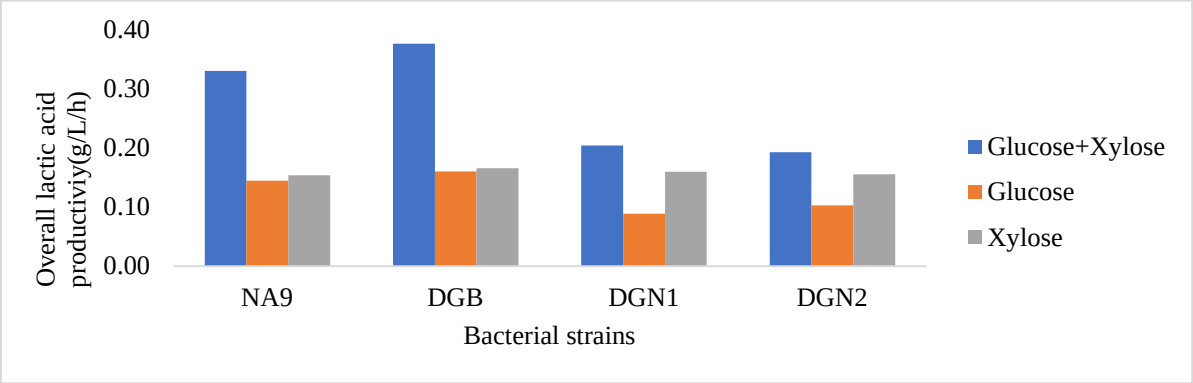


Fig.10a Overall lactic acid productivity (g/L/h) by bacterial strains in Bushnell Haas minimal media supplemented with either glucose+xylose, glucose or xylose

DGB strain of *B. licheniformis* was previously characterized as (Goyal, 2016) Gram positive. It showed positive result for methyl red test as distinct red colour complex was formed after incubation. Hence, this proved DGB's efficiency for the fermentation of LA, formic acid etc because methyl red is a pH indicator dye and formation of organic acids leads to decrease in pH due to which yellow colour of dye changes to red.

DGB was found to be fermentative but anaerogenic, since due to the fermentation of sugars, acid was produced which reduced pH, changing the colour of bromocresol purple to yellow without any production of gas and represents a potential strain for the LA production. DGB was further characterised for its efficiency for LA production using lignocellulosic biomass such as rice straw and wheat straw as substrate.

4.3 Pretreatment of rice straw and wheat straw

Rice straw and wheat straw are important agricultural wastes which are being burnt in many states of India especially in Punjab. Hence, utilisation of these agricultural wastes for production of value added products is a major concern. For wheat straw and rice straw to be completely utilized their pretreatment is necessary. Pretreatment of such lignocellulosic biomass i.e. wheat straw and rice straw leads to hydrolytic release of sugars by breakdown of cellulosic and hemicellulosic structural components to pentoses and hexoses. The percentage of cellulose, hemicellulose has been reported earlier; where wheat straw consists of 29% hemicellulose, 36% cellulose, 19% lignin and rice straw has 24% hemicellulose, 43% cellulose and 9% lignin (Akhtar and Goyal, 2014).

4.3.1 Reducing sugar

Glucose and xylose are the most abundant sugars found in the cellulosic biomass. The reducing sugar assay was done on native rice straw and wheat straw biomass i.e. without pretreatment of rice straw and wheat straw, on pretreated rice straw and wheat straw with dilute (0.6 N) sulphuric acid, concentrated sulphuric acid, dilute (0.4 N) sodium hydroxide and also on the detoxified rice straw and wheat straw.

In native biomass there has negligible concentration of reducing sugars i.e. 0.94 g/L in case of wheat straw and 0.77 g/L in case of rice straw (Table 7; Fig.11). There was 15-20 fold increased in concentration of reducing sugars after pretreatment with acid. The dilute (0.6 N) sulphuric acid pretreated hydrolysates showed maximum amount of reducing sugars in both rice straw and wheat straw i.e. 12.10 g/L and 8.32 g/L respectively. It was followed by concentrated sulphuric acid treatment of reducing sugars were more in wheat straw as compared to rice straw i.e., 12.07 g/L and 5.92 g/L respectively (Table 7; Fig. 11). Dilute (0.4 N) sodium hydroxide treatment was given to wheat straw and rice straw for delignification of the structural component lignin and reducing sugar obtained upon delignification was 4.88 g/L in case of rice straw and 4.97 g/L in case of wheat straw. Pretreatment with sulphuric acid yielded more reducing sugars as compared to alkali treatment.

Table 7. Concentration of reducing sugars and xylose (g/L) in native biomass and after pretreatment and detoxification

S.No.	Treatment	Xylose	Reducing sugars
1.	Native rice straw	0.40±0.05	0.77±0.10
	Native wheat straw	0.70±0.03	0.94±0.60
2.	Rice straw pretreated with concentrated sulphuric acid	4.55±0.04	11.40±0.09
	Wheat straw pretreated with concentrated sulphuric acid	4.58±0.04	12.07±0.06
3.	Rice straw pretreated with dilute (0.6N) sulphuric acid	4.56±0.02	12.10±0.03
	Wheat straw pretreated with dilute (0.6N) sulphuric acid	4.73±0.04	8.32±0.42
4.	Rice straw pretreated with dilute sodium hydroxide	3.58±0.02	4.88±0.03
	Wheat straw pretreated with dilute sodium hydroxide	4.56±0.03	4.97±0.17
5.	Rice straw pretreated with concentrated sulphuric acid and detoxified	5.31±0.10	12.66±0.06
	Wheat straw pretreated with concentrated sulphuric acid and detoxified	5.64±0.00	13.16±0.41
6.	Rice straw pretreated with dilute (0.6N) sulphuric acid and detoxified	4.65±0.00	13.96±0.14
	Wheat straw pretreated with dilute (0.6N) sulphuric acid and detoxified	4.73±0.00	8.96±0.30
7.	Rice straw pretreated with dilute (0.4N) sodium hydroxide and detoxified	4.00±0.03	4.94±0.01
	Wheat straw pretreated with dilute (0.4N) sodium hydroxide and detoxified	4.57±0.00	5.76±0.26

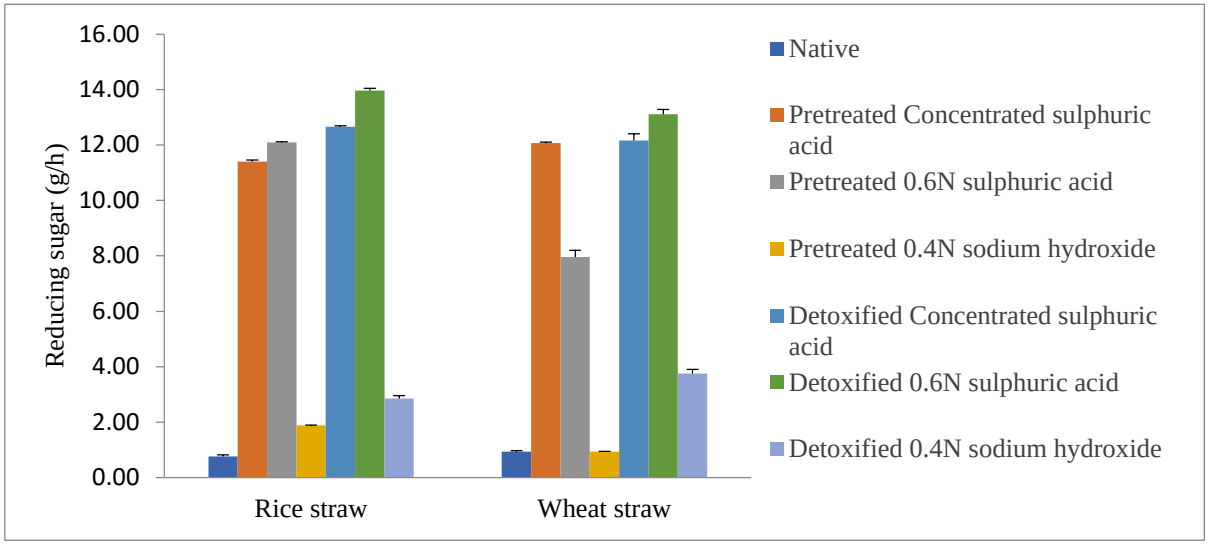


Fig. 11 Concentration of reducing sugars (g/L) in native biomass and after pretreatment and detoxification

After pretreatment inhibitory compounds such as furfural, HMF (5-hydroxymethylfurfural), acetic acid are produced which reduces the impact of LA production and affects microbial metabolism. Overliming has been considered as promising detoxification method for sulphuric acid pretreated hydrolysate of lignocellulosic biomass. This is an efficient method for the removal of fermentation inhibitory compounds. Calcium hydroxide (overliming) has been previously studied in hemicellulosic hydrolysate detoxifixation process. Upon detoxification no inhibition of ethanol production was observed by Chi *et al* (2013), also treatment was essential for consistent ethanol production. Chandel *et al* (2011a) observed spontaneous removal of furfural (41.75%), total phenolics (33.21%) by overliming method of detoxification.

Upon detoxification, there was increase in reducing sugar concentration in all the pretreated hydrolysates i.e., concentrated sulphuric acid, dilute (0.6 N) sulphuric acid as well as dilute (0.4 N) sodium hydroxide in case of both rice straw and wheat straw. In rice straw, the concentration increased from 11.40 g/L to 12.46 g/L upon detoxification when pretreated with concentrated sulphuric acid while for dilute (0.6 N) sulphuric acid pretreatment the concentration increased from 12.10 g/L to 13.96 g/L. Similarly, in dilute (0.4 N) sodium hydroxide pretreatment it's concentration increased was only from 4.88 g/L to 4.94 g/L. In wheat straw, similar increase was seen i.e. concentration increased from 12.07 g/L to 13.16 g/L when pretreated with concentrated sulphuric acid. In dilute (0.6 N) sulphuric acid pretreatment the reducing sugar concentration was increased from 7.96 g/L to 8.92 g/L and with dilute alkali pretreatment it increased from 4.97 g/L to 5.76

g/L. It can be clearly inferred that detoxification leads to increase in reducing sugar content in hydrolysates.

4.3.2 Xylose estimation in native biomass, after pretreatment and after pretreatment and detoxification of rice straw and wheat straw.

Pentose and hexose can be estimated when present together in mixture by heating them with orcinol and acid along with ferric chloride. The colour produced is measured at 660 nm as pentose sugars such as xylose, arabinose and ribose. Xylose along with glucose is the main source of carbon in hydrolysates of rice straw and wheat straw.

In native biomass there was negligible amount of xylose 0.40 g/L in rice straw and 0.70 g/L in wheat straw (Table 7; Fig. 12). The concentrated sulphuric acid pretreated hydrolysates showed maximum amount of pentose 4.55 g/L in rice straw and 4.58 g/L in wheat straw respectively. In dilute (0.6 N) sulphuric acid pretreated hydrolysates concentration of xylose was more in 4.73 g/L in wheat straw than 4.56 g/L in rice straw respectively.

Dilute (0.4 N) sodium hydroxide pretreatment concentration of xylose was 3.58 g/L in rice straw and 4.56 g/L in wheat straw. Pretreatment with sulphuric acid yielded more xylose as compared to alkali treatment as acid pretreatment leads to breakdown of glucosidic bonds of hemicellulose to partially solubilise it.

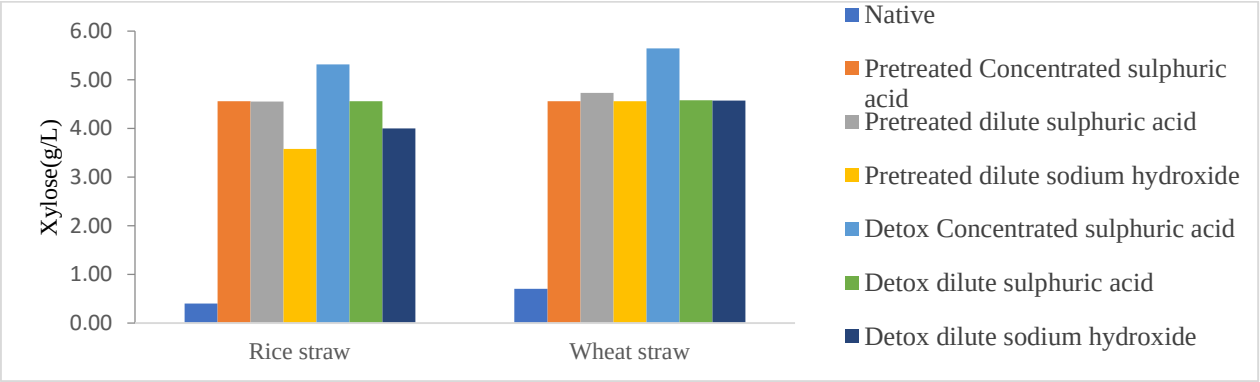


Fig. 12 Concentration of xylose in native biomass, after pretreatment and after detoxification

Detoxification lead to increase in pentose sugars as observed in pretreated hydrolysates of rice and wheat straw. In rice straw, concentration of xylose increased from 4.55 g/L to 5.31 g/L upon detoxification, when pretreated with concentrated sulphuric acid while in dilute (0.6 N) sulphuric acid pretreatment concentration of xylose increased from 4.56

g/L to 4.65 g/L and in dilute (0.4 N) alkali pretreatment it increased from 3.58 g/L to 4.00 g/L.

Similarly, in wheat straw increase in pentose concentrations was seen, i.e. the concentration increased from 4.56 g/L to 5.64 g/L in concentrated sulphuric acid pretreated hydrolysates and in dilute (0.6 N) sulphuric acid pretreatment it increased from 4.58 g/L to 4.73 g/L. In dilute (0.4 N) sodium hydroxide pretreated hydrolysates its concentration increased from 4.56 g/L to 4.57 g/L. It is clearly inferred that concentration of xylose increases after detoxification.

4.4 Production of lactic acid by *Bacillus licheniformis* (DGB) using pretreated and detoxified rice straw and wheat straw as substrate

Pretreated, pretreated and detoxified rice straw and wheat straw were used as substrate for the growth of *Bacillus licheniformis* (DGB) which is the potential LA producer among all isolates. LA along with residual sugars was estimated in fermentation broth after 48 and 72 hr at 50℃.

It was observed that in rice straw maximum growth was observed after 48 hrs in concentrated sulphuric acid pretreated and detoxified hydrolysate. In wheat straw maximum growth was observed after 48 hr in pretreated sulphuric acid hydrolysate (Table 8).

Table 8. Growth of *Bacillus licheniformis* (DGB) at 600 nm after 48 and 72 hr in both pretreated and pretreated and detoxified rice straw and wheat straw as a substrate

Treatment	Concentrated H_2SO_4		0.6 N H_2SO_4		0.4 N NaOH	
	48 hrs	72 hrs	48 hrs	72 hrs	48 hrs	72 hrs
Rice straw pretreated	3.122	2.445	3.064	2.502	2.73	2.536
Wheat straw pretreated	3.413	3.355	4.000	3.355	3.41	3.308
Rice straw pretreated and detoxified	3.415	2.693	2.481	2.510	2.36	2.312
Wheat straw pretreated and detoxified	1.365	1.311	1.762	2.002	3.32	3.114

In rice straw, pretreated with concentrated sulphuric acid, concentration of reducing sugars and xylose were 11.40 g/L and 4.56 g/L respectively. After 48 hr of incubation, reducing sugar and xylose declined to 11.11 g/L and 4.54 g/L respectively with production of 89.90 g/L of LA (Table 9; Fig. 13a). After 72 hr, reducing sugar and xylose were further declined to 10.46 g/L and 1.12 g/L, with increase in LA concentration to 90.35g/L (Table 9; Fig. 13b). Upon detoxification of rice straw pretreated with concentrated sulphuric acid, 7.66 g/L of reducing sugar was obtained. After 48 hr, it declined to 10.83 g/L with production of 84.05 g/L LA with decline in xylose concentration to 4.57g/L (Table 9; Fig. 13a). After 72 hr, reducing sugars concentration declined to 10.15 g/L with production of 96.21 g/L LA with decline in xylose concentration to 4.54 g/L (Table 9; Fig. 13b). Concentration of LA produced is more as compared to produced by concentrated sulphuric acid pretreated rice straw hydrolysates.

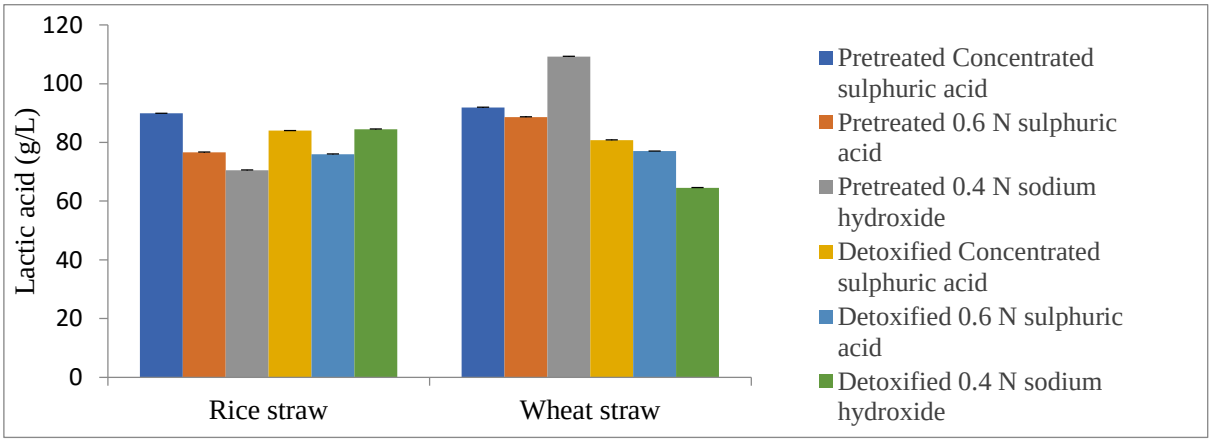
In rice straw pretreated with dilute (0.6 N) sulphuric acid, concentration of reducing sugars and xylose was 12.10 g/L and 4.55 g/L respectively. After 48 hr, concentration of reducing sugar and xylose declined to 8.82 g/L and 1.79 g/L with production of 76.58 g/L LA (Table 9; Fig. 13a). After 72 hr, reducing sugar and xylose further declined to 3.97 g/L and 1.63 g/L respectively with increase in LA concentration to 83.60 g/L (Table 9; Fig. 13b). Upon detoxification of rice straw pretreated with dilute (0.6 N) sulphuric acid concentration of reducing sugars and xylose were 13.96 g/L and 4.56 g/L respectively. After 48 hr, reducing sugars and xylose declined to 4.25 g/L and 3.94 g/L respectively due to production of 75.98 g/L LA (Table 9; Fig. 13a). After 72 hr, reducing sugars and xylose concentration declined to 4.01 g/L and 1.88 g/L due to production of 81.92 g/L LA (Table 9; Fig. 13b).

Rice straw pretreated with dilute (0.4 N) sodium hydroxide, concentration of reducing sugars and xylose was 4.88 g/L and 3.58 g/L respectively. After 48 hr, concentration of reducing sugar and xylose declined to 2.50 g/L and 1.75 g/L due to production of 70.55 g/L LA (Table 9; Fig. 13a). After 72 hr, reducing sugar and xylose were declined to 1.01 g/L and 0.99 g/L with increase in LA concentration to 79.55 g/L (Table 9; Fig. 13b). Upon detoxification of rice straw pretreated with dilute (0.4 N) sodium hydroxide, concentration of reducing sugars and xylose was 4.94 g/L and 4.00 g/L respectively. After 48 hr their concentration declined to 2.93 g/L and 1.49 g/L respectively due to production of 84.50 g/L LA (Table 9; Fig. 13a). After 72 hr, reducing sugars and xylose

concentration declined to 2.12 g/L and 1.01 g/L with accumulation of LA of concentration 94.25 g/L (Table 9; Fig. 13b).

From above it is inferred that in detoxified media LA production increased after 72 hrs and was more than pretreated hydrolysates, whereas in case of dilute (0.4 N) sodium hydroxide LA produced had less concentration than pretreated hydrolysates it may be due to loss of 10% sugars during detoxification process or treating with calcium hydroxide. Rice straw was fermented at 30℃ under anaerobic conditions using SFC – 2 consisting of microbial community (*L. plantarum*, *L. fermentum* and *L. paracase*) and produced 30.32 g/Kg of LA after 30 days of fermentation (Gao *et al*., 2007) whereas in this study production of LA (96.21 g/L) from concentrated sulphuric acid pretreated and detoxified rice straw was good enough in 72 hrs.

a. After 48 hr



b. After 72 hr

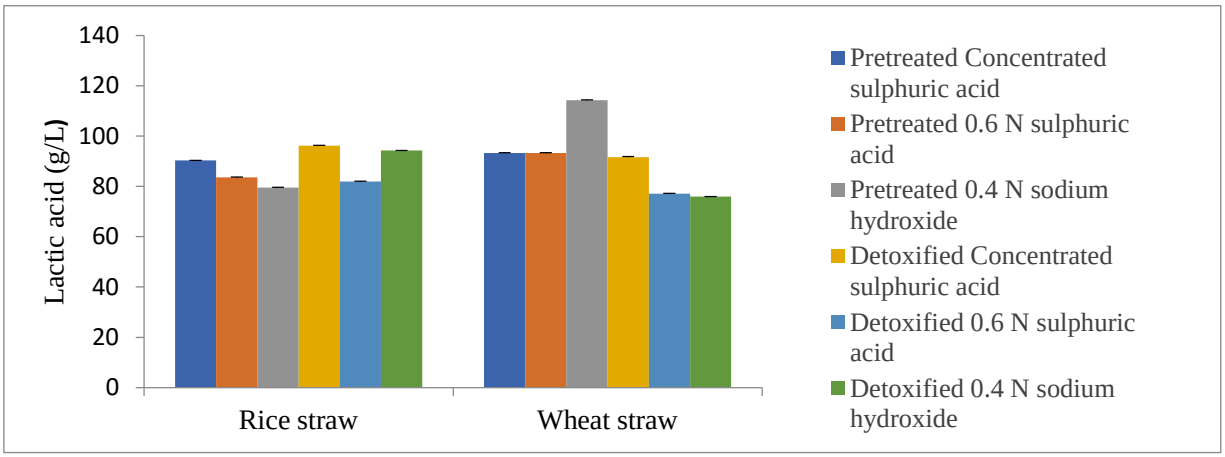


Fig. 13 Fermentation of rice straw and wheat straw by *Bacillus licheniformis* (DGB) at 50℃ (pH 7.0) and estimation of LA

Table 9. Fermentation of rice straw and wheat straw hydrolysates by Bacillus licheniformis (DGB) at 50℃ (pH 7) and estimation of lactic acid, xylose and reducing sugars after 48 and 72 hrs

Treatment	Lactic acid		Xylose		Reducing sugar	
	48 hr	72hr	48 hr	72 hr	48 hr	72 hr
Rice straw pretreated with concentrated sulphuric acid	89.90±0.07	90.35±0.01	4.54±0.0	1.12±0.0	11.11±0.02	10.46±0.15
Wheat straw pretreated with concentrated sulphuric acid	91.93±0.06	93.28±0.05	3.83±0.0	1.78±0.01	11.10±0.09	11.02±0.25
Rice straw pretreated with dilute (0.6 N) sulphuric acid	76.58±0.16	83.60±0.05	1.79±0.0	1.63±0.0	8.82±0.37	3.97±0.01
Wheat straw pretreated with dilute (0.6 N) sulphuric acid	88.66±0.04	93.28±0.04	4.20±0.0	1.09±0.06	7.96±0.62	6.08±0.29
Rice straw pretreated with dilute (0.4 N) sodium hydroxide	70.55±0.05	79.55±0.12	1.75±0.0	0.99±0.74	2.50±0.11	1.01±0.1
Wheat straw pretreated with dilute (0.4 N)sodium hydroxide	109.22±0.08	114.22±0.09	2.51±0.23	0.85±0.1	3.37±0.04	1.86±0.45
Rice straw pretreated with concentrated sulphuric acid and detoxified	84.05±0.02	96.21±0.08	4.57±0.0	4.54±0.0	11.41±0.2	10.13±0.1
Wheat straw pretreated with concentrated sulphuric acid and detoxified	80.81±0.05	91.58±0.28	4.55±0.0	3.83±0.49	10.83±0.68	10.15±0.35
Rice straw pretreated with dilute (0.6 N) sulphuric acid and detoxified	75.98±0.05	81.92±0.11	3.94±0.15	1.88±0.33	4.25±0.0	4.01±0.15
Wheat straw pretreated with dilute (0.6 N) sulphuric acid and detoxified	77.05±0.06	77.12±0.1	4.30±0.0	4.20±0.17	8.38±0.12	5.27±0.05
Rice straw pretreated with dilute (0.4 N) sodium hydroxide and detoxified	84.50±0.05	94.21±0.10	1.49±0.12	1.01±0.12	2.93±0.03	2.12±0.49
Wheat straw pretreated with dilute (0.4 N) sodium hydroxide and detoxified	64.54±0.06	75.89±0.11	3.27±0.04	2.06±0.22	3.74±0.03	3.12±0.04

In concentrated sulphuric acid pretreated wheat straw concentration of reducing sugar and xylose, 12.07g/L and 4.58 g/L respectively. After 48 hr it declined to 11.10 g/L and 3.83 g/L due to production of 91.93 g/L LA. After 72 hr, concentration of reducing sugar and xylose further declined to 11.02 g/L and 1.78 g/L with increase in LA concentration to 92.98g/L.

In sulphuric acid pretreated and detoxified wheat straw, initial concentration of reducing sugars and xylose was 12.16 g/L and 5.64 g/L. After 48 hrs it declined to 10.83 g/L and 4.55 g/L respectively due to production of 80.81g/L LA. After 72 hr, reducing sugars and xylose concentration declined to 10.15 g/L and 3.83 g/L respectively with increase in LA concentration to 91.58g/L (Table 9; Fig. 13b).

In dilute (0.6 N) sulphuric acid pretreated wheat straw, concentration of reducing sugars and xylose was 8.32 g/L and at 48 hr it declined to 7.96 g/L and 4.20 g/L due to production 88.66 g/L LA. At 72 hr further decline in reducing sugar concentration were observed to 6.08 g/L with increase in LA concentration to 93.28 g/L and decrease in concentration of xylose to 1.09 g/L.

In dilute (0.6 N) sulphuric acid pretreated and detoxified, concentration of reducing sugars and xylose was 8.96 g/L and 4.73 g/L. After 48 hr, it declined to 8.38 g/L and 4.30 g/L due to 77.05g/L LA production. After 72 hr, reducing sugars and xylose concentration declined to 5.27 g/L and 4.20 g/L respectively with increase in LA concentration to 77.12 g/L.

In dilute (0.4 N) sodium hydroxide pretreated wheat straw, concentration of reducing sugars and xylose was 4.97 g/L and 4.56 g/L respectively. After 48 hr it declined to 3.37 g/L and 2.51 g/L, due to production of 109.22 g/L LA production. After 72 hr, further decline in reducing sugar and xylose concentration was observed to 1.86 g/L and 0.85 g/L with increase in LA concentration to 114.22 g/L.

In dilute (0.4 N) sodium hydroxide pretreated and detoxified, concentration of reducing sugars and xylose was 5.76 g/L and 4.57 g/L. After 48 hr, it declined to 3.74 g/L and 3.27 g/L due to 64.54 g/L production of LA. After 72 hr, reducing sugars and xylose concentration declined to 3.12 g/L and 2.06 g/L LA concentration increased to 71.27 g/L.

From above data it was inferred that in case of wheat straw pretreated hydrolysate as substrates showed more LA production then detoxified hydrolysate which may be due to

loss of sugars and formation of inhibitory compounds during production of LA. Zhang *et al.,* 2014; by using *Bacillus coagulans* strain IPE22 produced LA from pretreated wheat straw with dilute sulphuric acid i.e., 2% (w/v) at 40℃ of concentration of 38.73 g/L at 60^{th} hr whereas DGB with pretreated dilute (0.6 N) sulphuric acid in case of wheat straw is able to produce 88.46 g/L after 48 hrs at 50℃. 0.59 g/g of LA was produced by *Lactobacillus brevis* whereas *Lactobacillus pentosus* produced 0.88 g/g of LA using wheat straw pretreated with sulphuric acid (Garde *et al*., 2002). Wheat straw pretreated with lime produced 40.71 g/L of LA by using *Bacillus coagulans* DSM 2314 after 55 hrs of incubation at 50℃ (pH 6.0) by using cellulose enzyme (Maas *et al.,* 2008) whereas in this study wheat pretreated alkaline alone without addition of lime or $Ca(OH)_2$ showed 114.22 g/L of LA production. Treatment with alkali in case of wheat straw is an efficient process for production of LA as it removes lignin and DGB ferment sugars to produce LA.

Overall Productivity

In rice straw, pretreated with concentrated sulphuric acid LA overall productivity was observed to be 1.56 g/L/h (Table 10; Fig. 14) in hydrolysate pretreated with concentrated sulphuric acid and then detoxified followed by hydrolysate pretreated with dilute alkali treatment, 1.48 g/L/h. Pretreated dilute (0.6 N) sulphuric acid hydrolysate gave 1.40 g/L/h. While in wheat straw, pretreated with concentrated sulphuric acid, dilute (0.6 N) sulphuric acid, dilute (0.4 N) sodium hydroxide overall LA productivity was calculated to be 1.60 g/L/h, 1.57 g/L/h and 1.94 g/L/h respectively.

Upon detoxification in rice straw pretreated with concentrated sulphuric acid, dilute (0.6 N) sulphuric acid and dilute (0.4 N) sodium hydroxide overall LA productivity calculated was 1.59 g/L/h, 1.36 g/L/h and 1.2 g/L/h respectively. In wheat straw pretreated with concentrated sulphuric acid, dilute (0.6 N) sulphuric acid and dilute alkali productivity was 1.48 g/L/h, 1.37 g/L/h and 1.33 g/L/h.

In rice straw maximum LA productivity was calculated in pretreated and detoxified concentrated sulphuric acid hydrolysates while in wheat straw maximum productivity was observed in dilute alkali pretreated hydrolysates.

Table 10. Overall LA productivity (g/L/h) using pretreated rice straw and wheat straw hydrolysates as substrate for *Bacillus licheniformis* (DGB)

S.No.	Treatment	Rice straw	Wheat straw
1.	Concentrated sulphuric acid pretreated	1.56	1.6
	After detoxification with over liming	1.59	1.48
2.	Dilute (0.6 N) sulphuric acid pretreated	1.4	1.57
	After detoxification with overliming	1.36	1.37
3.	Dilute (0.4 N) sodium hydroxide pretreated	1.48	1.94
	After detoxification with overliming	1.2	1.33

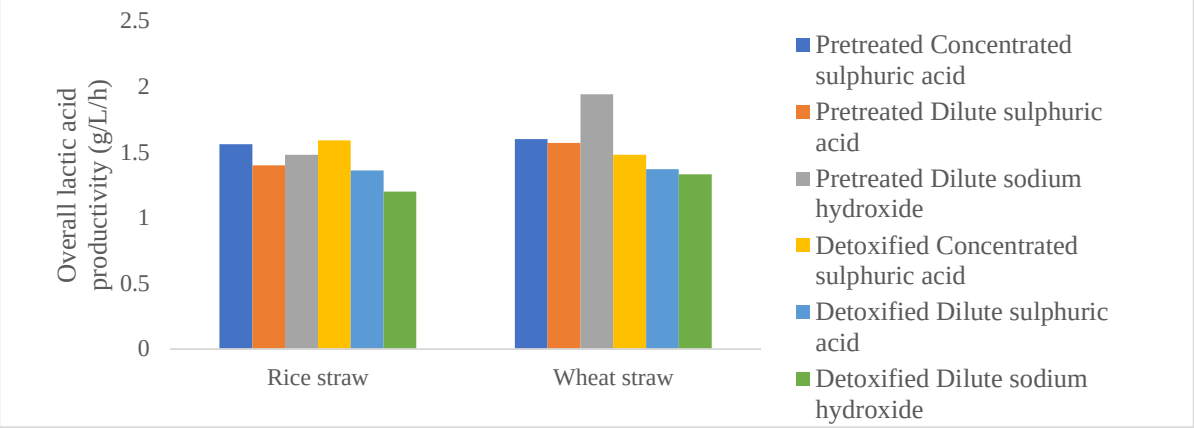


Fig. 14 Overall lactic acid productivity in g/L/h in rice straw and wheat straw after pretreatment and pretreatment and detoxification

Maximum lactic acid productivity was observed in wheat straw because of presence of increased amount of hemicellulose (Akhtar and Goyal, 2014) as compared to rice straw.

Conclusion

1. Growth of bacterial isolates was observed in the order of DGN1 > DGB > NA9 > DGN2 in enrichment broth containing xylose and yeast extract at 50℃. Hence, optimum temperature for growth of *Bacillus* sps was observed to be 50℃.
2. *Bacillus licheniformis* (DGB) produced highest amount of lactic acid (18.08 g/L) among all isolates, when grown in Bushnell Haas minimal media supplemented with carbon source (glucose+xylose).
3. Pretreatment of rice straw with sulphuric acid (0.6 N) and detoxification yielded (13.96 g/L) and in case of wheat straw pretreated with concentrated sulphuric acid and detoxified (13.16 g/L) more reducing sugars as compared to alkali treatment. However, concentration of xylose was maximum in concentrated sulphuric acid pretreated and detoxified wheat straw (5.64 g/L) and 5.31g/L in case of rice straw.
4. Fermentation of hydrolysates by *Bacillus licheniformis* (DGB) gave maximum lactic acid (114.22 g/L) using dilute sodium hydroxide (0.4 N) pretreated wheat straw with an overall productivity of 1.94 g/L/h followed by concentrated sulphuric acid pretreated and detoxified rice straw i.e. 96.21 g/L with an overall productivity of 1.59 g/L/h.

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APPENDIX

DNS reagent	
Ingredients	**Quantity (g/L)**
DNS	10
NaOH	10
Sodium sulphite	0.25
Sodium potassium tartarate	192
Distilled water	1000 ml

Orcinol reagent	
Ingredients	**Quantity (g/L)**
$FeCl_3.6H_2O$	0.15
Oricinol	0.4
Distilled water	10 ml
Make up volume upto 200 ml HCl [30% (w/v)]	