Production of lactic acid from lignocellulosic biomass using bacterial isolates

DISSERTATION

Submitted by

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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, 2016

CERTIFICATE

This is to certify that the thesis entitled '**Production of lactic acid from lignocellulosic biomass using bacterial isolates**' which is submitted by Ms. **NITIKA** (301401010), in the partial fulfilment in the requirements for the degree of **Master's of Science** in Biotechnology in the Department of Biotechnology, Thapar University, Patiala, Punjab is the record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The work embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

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DECLARATION

I hereby declare that the thesis entitled "**Production of lactic acid from lignocelluosic biomass using bacterial isolates**" is being submitted in the partial fulfilment for the degree of **Master's in Biotechnology** in Thapar University, Patiala is true and original record of my work carried out under the guidance and the supervision of Dr. Dinesh Goyal, Professor & Head, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The work embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

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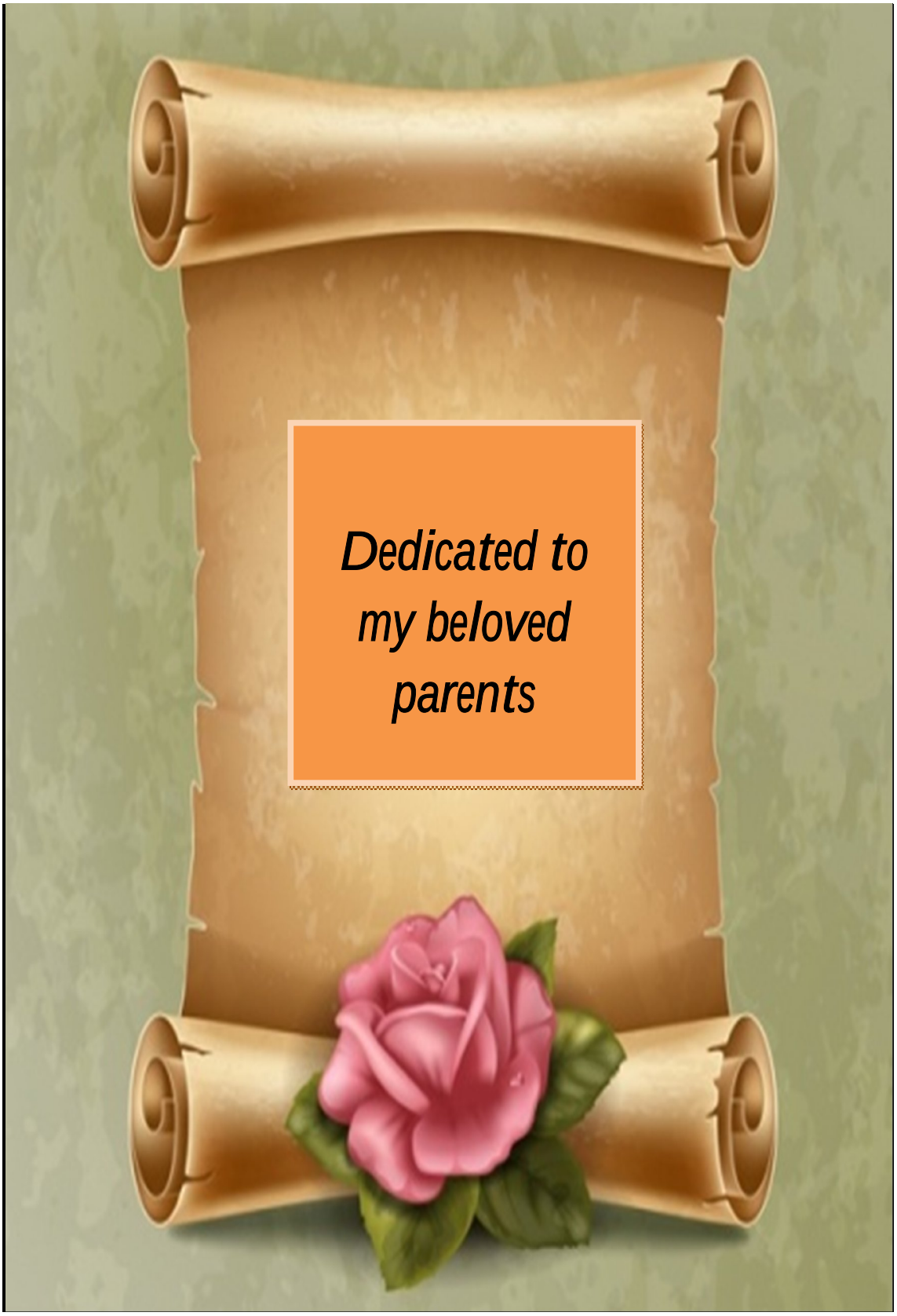
Dedicated to
my beloved
parents

ACKNOWLEDGEMENT

I would like to express my gratitude and appreciation to all those who gave me the possibility to complete this report. I would also like to acknowledge the Head of Department and my course coordinator **Dr. Dinesh Goyal**. He has always been there to help me at every single step during my project work, motivating me, guiding me and encouraging me to complete my work.

I would like to thank **Science and Technology Entrepreneur's Park (STEP)** and **Biotechnology department**, Thapar University for providing me necessary equipments and facilities to carry out work.

My acknowledgement will never be complete without the the special mention of all my lab seniors **Ms. Prerna, Ms. Jyotika** and **Ms. Ravneet Kaur** who have taught me the lab culture and willingly devoted their valuable time whenever I needed their help.

Words fail me to express my appreciation to my seniors come siblings for their moral support and motivation whenever I was low, **Mrs. Simarpreet Kaur Lamba, Ms. Ishtpreet Kaur.**

Most of work would have been incomplete without the sincere support of lab aasistants. So, I owe my word of thanks to **Mr. Ram Newal Yadav, Mr. Lallan Yadav, Mr. Chandan Bhandari, Mr. Surinder** and **Mr. Mohinder.**

Some friends are never too busy to give us a hand whenever they are needed. I want to make a special thanks to my friends who showed faith in me. I could not have asked for more than what I got from you all. I express my sincere love and affection towards all those benevolent souls and true friends **Swati, Preetika, Nitika Garg, Kashmi, Shitanshu, Shamli Thakur, Rahul and Shammi Lubana.**

I find it difficult to pen down my deepest sense of thanks towards my parents and all other family members who soulfully provided me their constant support and right impetus to undertake the challenge of this proportion like all other spheres of life, for what I cannot measure but treasure. It was my Father and Mother who gave me unconditional support during this time. They have elucidated the meaning of love, life and living. My sincere thanks to my parents who had always keep my morals high through tough time.

I highly appreciate cooperation and cheerful assistance of my loving brother **Nitin Kashyap** and my loving sisters **Sejal** and **Bhumika.** There abundant affection, unflagging love and support were the constant source of inspiration for me.

It has been great privilege to spend two years in the **Department of Biotechnology, Thapar University, Patiala** and its members will always remain dear to me.

Last but not the least, I wish to acknowledge all those, whose names have not figured here, but who helped me in any form during the period of my project work.

Date: 14 July, 2016

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LIST OF ABBREVIATIONS

ARP	Ammonia recycling percolation
CO_2	Carbon dioxide
Conc.	Concentrated
Dil.	Dilute
DNS	3,5- dinitro salicylic acid
et al	And others
Etc	And other things
FDA	Food Drug and Administration
g/l	Gram Per Liter
GRAS	Generally Regarded As Safe
H_2SO_4	Sulfuric acid
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
LA	Lactic acid
LAB	Lactic acid bacteria
mg/ml	Milligram per millilitre
Ml	Millilitre
MR	Methyl red
MT	Metric ton
NaOH	Sodium hydroxide
NA	Nutrient Agar
PLA	Poly lactic acid
Psi	Pounds per square inch
Rpm	Revolutions per minute
TFA	Triflouroacetic acid
US	United States
VP	Voges- proskauer
w/w	Weight by weight
YE	Yeast extract

LIST OF SYMBOLS

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

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Abstract

Present research work was aimed at utilization of lignocellulosic biomass i.e. rice straw, wheat straw and eucalyptus leaf litter for production of value added product i.e. lactic acid. Rice straw, wheat straw and eucalyptus leaf litter were collected from and nearby villages of Thapar University, Patiala. Using xylose as substrate for lactic acid production from environmentally isolated bacterial strains, maximum lactic acid yield obtained is 0.432g/l using DGN2 bacterial isolate by utilizing 50g/l xylose.

Biomass pre-treatment plays a important role in a lignocellulosic feed-stock based biorefinery for processing of cellulose, hemicelluloses and lignin into chemicals and biofuels. Acid and alkali hydrolysis of rice straw, wheat straw and eucalyptus leaf litter biomass was done to get hydrolysates. Reduction in residual concentration of sugar after inoculation with NA9 was observed maximally in dilute acid hydrolysate of wheat straw as reducing sugar content decreased from 1.090 mg/ml to 0.180 mg/ml. the increasing trend in content of protein after inoculation with NA9 was reported maximally in concentrated acid hydrolysate of eucalyptus leaf litter as protein content increased from 0.340 mg/ml to 0.680 mg/ml.

Cost effective production of optically pure lactic acid from lignocellulosic sugars is commercially attractive. Environmentally isolated bacterial strains were used to produce lactic acid by utilising sugars from hydrolysate of rice straw, wheat straw and eucalyptus leaf litter obtained after pre-treatment with acid and alkali at 50ºC without agitation after inoculation with NA9 and DGN2 bacterial isolates. The maximum yield of lactic acid was reported from concentrated acid hydrolysate of eucalyptus leaf litter after inoculation with NA9 i.e. 5.5 g/l. This is concluded that NA9 (*Bacillus licheniformis*) is promising strain for lactic acid production and eucalyptus leaf litter biomass is promising substrate for lactic acid production.

INTRODUCTION

Lignocellulosic biomass may be considered as non-productive raw materials, whose collection cost and downstream processing for reuse is more than its economic value and therefore is not brought into use. These non productive raw materials could be valuable if appropriate methods are applied. Currently, more research is focussed on chemicals produced from lignocellulosic biomass (Okano *et al.,* 2010). Lignocellulosic biomass accumulated in the form of agricultural biowastes every year in large amount. The lignocellulosic material consists of cellulose, hemicellulose and lignin fractions. Cellulose and hemicellulose are sugar rich fractions which can be widely use in bacterial and fungal fermentation process, since microorganisms can use these carbohydrates for their growth and production of some compounds such as ethanol, food additives, organic acids and enzymes. The fermentation systems *i.e*. solid state and submerged have been used to produce industrial compounds from lignocelluloses to solve environmental problems caused by their disposal (Musatto *et al.,* 2010). Earlier, fermentative productions of LA from biomass have been studied. However, these studies suggested that the production process using lignocellulosic biomass was more complicated than that for starch-based material and glucose. They can be cheaply utilized as substrate by microorganisms for producing organic acids like lactic acid, formic acid and acetic acid.

Lactic acid is an organic acid which is under huge demand for production of Polylactic acid polymers (PLA) and in food, Pharmaceutical and Chemical Industries. Lactic acid is either formed through chemical or microbial route via fermentative mode. Lactic acid can potentially be produced from cheap lignocellulosic materials such as agricultural biowaste, industrial solid waste, and forestry waste (Lynd *et al.,* 1991, 1999; Wyman, 2003). Lactic acid is produced by Lactic acid bacteria and also by fungi. Generally, LA exists in stereo-isomers, the L (+) and the D (−) form (Fig 1). In late 1940s and early 1950s, it was used as a raw source for synthesis of biodegradable plastics (Vickroy, 1985).

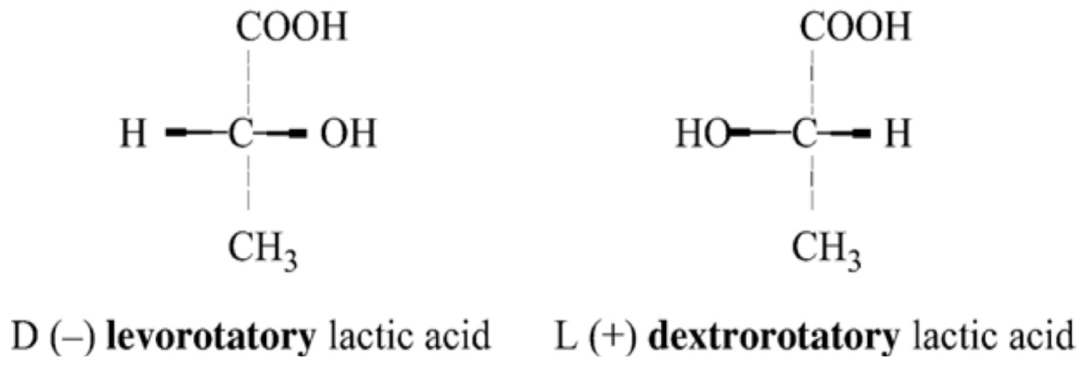

Fig 1: D (-) Lactic acid and L (+) Lactic acid, two optically active sterioisomers (Reddy *et al*., 2008**)**

The present study was focussed on isolation and screening of thermophilic lactic acid bacteria that produces lactic acid and biochemical characterisation of lactic acid producing bacteria. Various lignocellulosic biomasses (wheat straw, rice straw and eucalyptus) were used as potential substrates for the efficient production and estimation of lactic acid using bacterial isolates.

REVIEW OF LITERATURE

Lactic acid is an organic acid (2-hydroxypropanoic acid) is naturally produced acid, which has wide applications in pharmaceutical industry, cosmetic industry, in production of oxygenated chemicals, special chemical intermediates and plant growth regulators (Oshiro *et al*., 2009, Singhvi *et al*.,2010 and Tashiro *et al*., 2011).The review of literature focuses on the lactic acid history, its uses & properties and synthesis mode of lactic acid, lactic acid producing microorganisms, lignocellulosic biomass as substrate and pre-treatment of lignocellulosic biomass for separation of its constituents that are related to lactic acid formation.

2.1 History of lactic acid

The isolation of lactic acid was noted to have occurred in 1789, when acid was separated from sour milk reported by Scheele. The name, lactic acid was derived from the sugar i.e. lactose. The microorganism first used to ferment the acid was *Sreptococcus lactis*. In 1789, Lavoisier named this component acide lactique, which became the origin for the naming of lactic acid. However, Pasteur in his work discovered that it was not a milk component, but a fermentative metabolite that was secreted by certain microbes. Lactic acid was the first acid to be produced at an industrial level in 1980 (Zadow, 1992).

2.2 Uses

The most widely occurring organic acid is lactic acid. Generally, the acid has plentiful applications in beverage, pharmaceutical and food industries. However new applications include areas like biodegradable plastics and medical health. The demand of lactic acid is rising almost day by day. The important uses of lactic acid are described (Narayanan *et al.,* 2004).

2.2.1 Food Industry

Lactic acid has been classified as has GRAS for use in food industries as food additive. L (+) lactic acid, which is one of the optical stereoisomers and is mostly used in food and dairy industries due to the presence of L (+) Lactate dehydrogenase enzyme in humans (Datta *et al.,* 1995; Wee *et al.,* 2006; Reddy G. *et al.,*2008).

1) Lactic acid is used in preparation of margarine, butter, yogurts etc..

2) LA is used as a preserving agent for olives and pickled vegetables.

3) It is used jams and jellies as gelling agent.

4) Calcium-lactate salt is added to milk and other energy drink as mineral supplement.

5) LA and its salts can enhance the shelf life of food items like sausages, jams, poultry, fish etc.

2.2.2 Chemical Industry

Lactic acid, organic acid, is used as an acidulent in leather tan industry and in small scale activities like pH balancing, used in food packaging, terminating agent for phenol formaldehyde resins, solder flux, adhesive formulations, in electroplating and electro polishing baths, detergent builders etc. Lactic acid esters like ethyl lactates can be used in the form of green solvents. These have non-toxic and degradable, high boiling constituents (Dimerci *et al.,* 1993).

2.2.3 Pharmaceutical Industry

Recently, lactic acid has attracted substantial consideration as a precursor for poly-lactic acid, which can replace plastic derived from petroleum because of its good biodegradable and biocompatible properties (Jem *et al.,* 2010; Lasprilla *et al.,* 2012).The formulation of ointments, lotions, anti-acne solutions and dialysis applications are other main applications in this field. Ca-lactate salt can be used as therapy for calcium deficiency (Wee *et al.,* 2006).

2.3 Synthesis of Lactic Acid

Two potential ways for the synthesis of Lactic acid are chemical synthetic method and microbial fermentation method. The main problem with chemically synthesized acid is that racemic end products are obtained. From fermentation mode of synthesis either D (-) or L (+) acid is obtained (Iyer *et al.,* 2000; Gruber *et al.,* 2006). By using petrochemical sources by chemical synthetic method racemic mixture of lactic acid is obtained but an optically pure L (+) or D (–) lactic acid can be only be obtained by microbial fermentation of renewable resources (Hofvendahl *et al.,* 2000) (Fig 2).

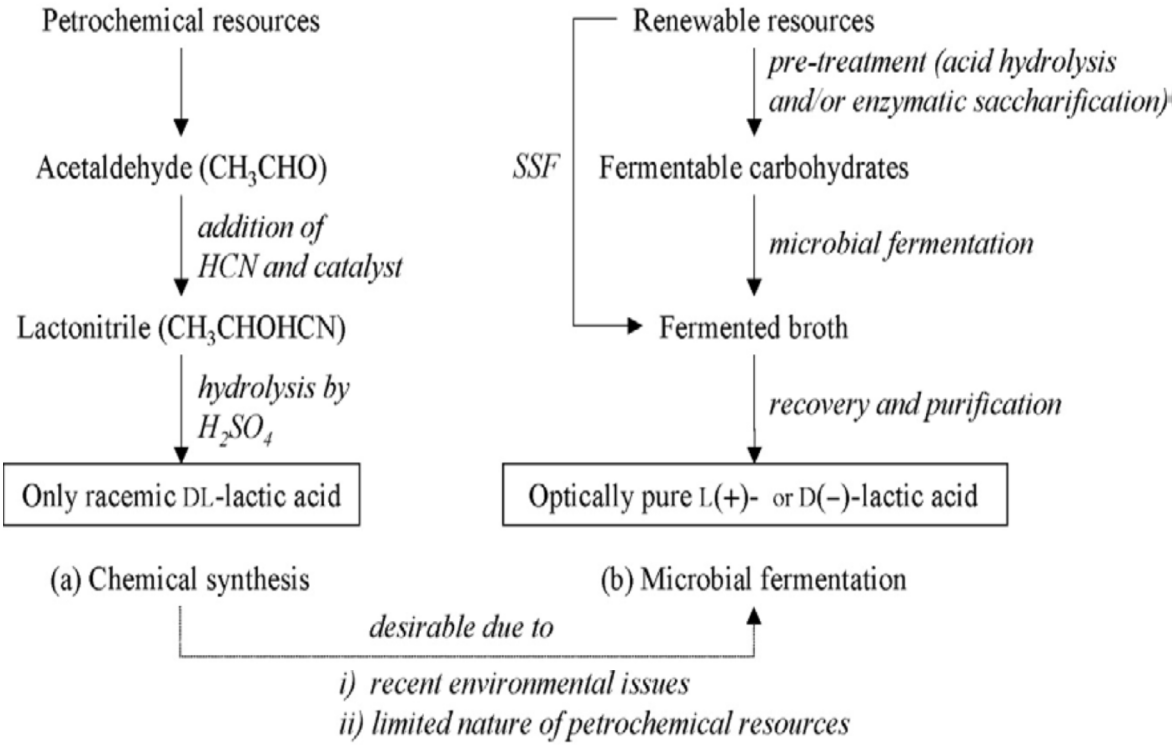

Fig 2: Manufacturing methods of Lactic Acid – Chemical Synthetic (a); Microbial Fermentation method (b) (Wee *et al.,* 2006)

2.3.1 Chemical synthesis

The chemical synthesis mode of Lactic acid production is based on lactonitrile (Narayanan *et al.,* 2004). In this method, HCN is added to solution of acetaldehyde to produce lactonitrile, takes place in liquid phase at high pressure. The lactonitrile is retrieved and purified by distillation process to get pure lactonitrile. Then, it is hydrolyzed to lactic acid, to produce ammonium salt and lactic acid. Then, esterification of lactic acid is done by methanol for production of methyl lactate, which is further purified by distillation and hydrolyzed by water under acid catalyst to produce lactic acid and methanol, which is recycled.

Chemical reaction:

CH_3CHO + $HCNCH_3$ ⟶ $CHOHCN$
Acetaldehyde Cyanide Lactonitrile

$2CH_3CHOHCN + 2H_2O + H_2SO_4 \rightarrow CH_3CHOHCOOH + (NH_4)_2SO_4$
Lactonitrile Lactic acid Ammonium sulphate

$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$
Lactic acid Methanol Methyl lactate

$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$
Methyl lactate Lactic acid Methanol

2.3.2 Microbial Fermentation

Optically pure lactic acid can be obtained by carbohydrate fermentation depending on the microbial strain being used in process. Microbial fermentation is advantageous over chemical synthesis because first it yields optically pure lactic acid and second it uses cheap raw material. There are two modes of lactic acid production through microbial fermentation depending upon microorganism (Iyer *et al.,* 2000; Gruber *et al.,* 2006)

Homolactate fermentation

By homofermentative method, lactic acid bacteria yield pure or almost pure (90%) lactic acid. They consume and metabolize the hexose or pentose sugar via Pentose Phosphate Pathway. The major end product is only lactic acid.
Examples: *Streptococci, Enterococcus faecalis, Bacillus sp., Lactococcus lactis*

Heterolactate fermentation

By Heterofermentative mode of synthesis of lactic acid, lactic acid bacteria produce 1 molecule of lactate along with1 molecule of carbon dioxide (or acetic acid, formic acid). They utilise Phosphoketolase Pathway for lactic acid production.
Examples: *Leuconostoc sp., Lactobacillus brevis, Lactobacillus fermentum*

Depending upon the choice of sugar residue or microorganism to be used, there are two pathways for lactic acid production (Fig.3)

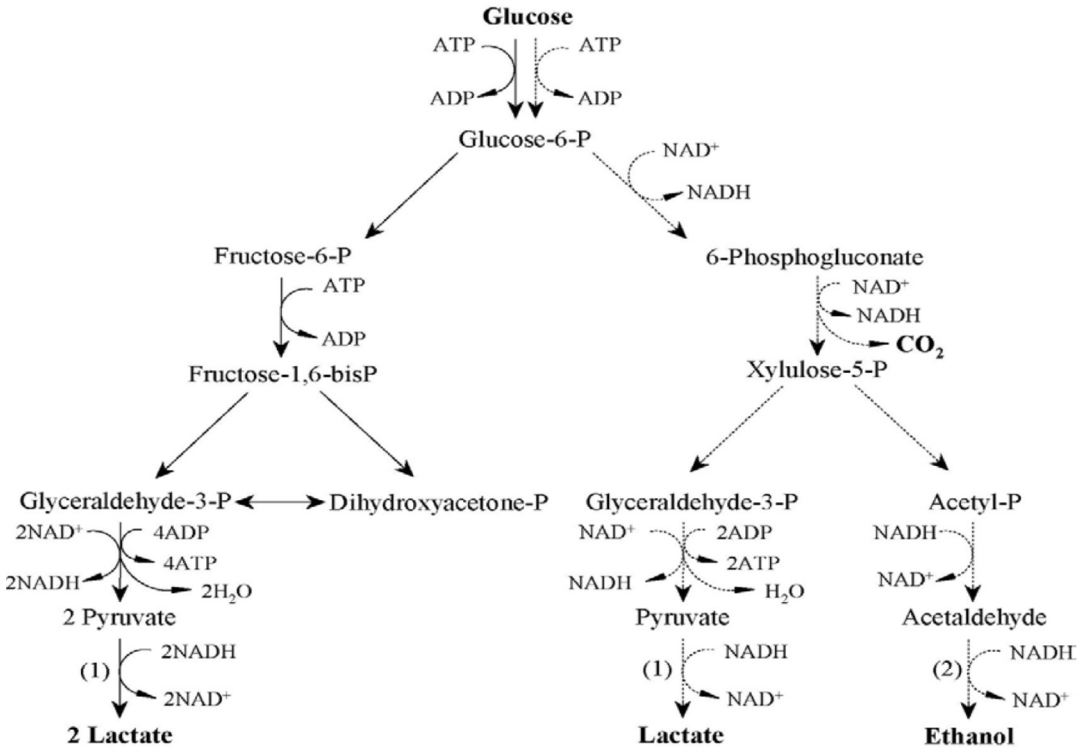

Fig 3: Metabolic pathways of homofermentative (solid line) and heterofermentative (dotted line) lactic acid bacteria; (1), lactate dehydrogenase; (2), alcohol dehydrogenase (Young Jung Wee *et al.,* 2006)

2.4 Lactic acid producing Microorganisms

Microorganisms that have the capability to produce lactic acid belong to bacteria and fungi (Litchfield *et al.,* 1996). Microorganism used for production of lactic acid are listed in Table 1.Although most lactic acid production was done with lactic acid bacteria (LAB), and also with a filamentous fungus, such as *Rhizopus*, utilizes glucose to produce lactic acid aerobically (Zhou *et al.,* 1999; A. Tay *et al.,* 2002). *Rhizopus* species i.e. *R. oryzae* and *R. arrhizus* have α-amylase enzyme activity, which makes them able to convert starch directly to L (+) lactic acid (Park *et al.,* 1998; Yuzi Oda *et al.,* 2002). Fermentation process using fungal culture has some advantages in regards that *R. oryzae* require a simple medium and produces L (+)-lactic acid. In fungal fermentation, the one limitation is of the low production rate. The lower product yield obtained from fungal fermentation is due to the formation of products such as fumaric acid and ethanol.

Recently, microbes used in the commercial production of lactic acid have become almost countermeasure, most of the lactic acid producing bacteria that are used, belongs to the

Lactobacillus. Berry *et al.* reported the production of lactic acid by batch culture of *L. rhamnosus.* Schepers *et al.* utilised *L. helveticus* for producing lactic acid from lactose and concentrated cheese whey. Fu and Mathews reported the lactic acid production using lactose as a substrate by *L. plantarum* batch mode of fermentation. The strains of *L. amylophilus* were oftenly used for the direct conversion of starch into lactic acid as they have amylase activity (Vishnu *et al.,* 2000; Naveena *et al.,* 2004; M. Altaf *et al.,* 2005). In past, mesophilic lactic acid bacteria (LAB) are used to produce LA while recently *Lactobacillus* (*Lb.*) sp. is used in industrial LA production (Litchfield *et al.,* 2000). Mesophilic strains are not acceptable for the industrial lactic acid production because of the high contamination risks but by using thermophilic strains, LA can be produced under unsterile condition that decreases cost for LA fermentation. For these reason, thermophilic *Bacillus* (*B.*) *coagulans* has been used as an efficient lactic acid producer. *Bacillus* strains have several advantages such as simple nutritional requirements (Heriban *et al.,* 1993; Payot *et al.,* 1999) as well as spore forming capability that elucidate preservation of stock cultures and inoculation of fermentation media (Kotani *et al.,* 2000).

Table 1: Microorganisms used in lactic acid production

Microorganism	Substrate	Reference
Fungi		
Rhizopus oryzae	Corn starch	Hang *et al*.,1989
Rhizopus arrhizus	Starch	Kristoficova *et al*.,1991
Rhizopus oryzae	Potato pulp	Yuzi *et al*., 2002
Rhizopus oryzae	Xylose	Ronald H. *et al.,* 2006
Bacteria		
Lactobacillus debruckeii	Xylose	Hofvendahl *et al.,* 2000
Lactobacillus debruckeii	Glucose	Hofvendahl *et al.,* 2000
Bacillus sp	Sugarcane bagasse	Patel *et al.,* 2004
Bacillus coagulans	Wheat straw	Mass *et al.,* 2008
Bacillus subtilis	Cellobiose	Garcia *et al.,* 2009
Bacillus coagulans	Xylose	Walton *et al.,* 2010
Bacillus subtilis	Glucose	Gao *et al.,* 2012
Bacillus coagulans	Tapioca starch	Kedong *et al.,* 2013
Bacillus coagulans	Sweet sorghum bagasse	Mark *et al*., 2016
Bacillus sp	Corn stover hydrolysate	Ting Jiang *et al.,* 2016

2.5 Raw material used for Lactic Acid production

The high yield of lactic acid can be attainable through using cheap raw materials are required because industrial users mostly require lactic acid at high quantity and at a low cost. Raw materials mostly used for production of lactic acid should have the following characteristics: cheap, low levels of contaminants, high yield, little or no by-product formation, ability to be fermented with little or no pre-treatment (Vickroy *et al.,* 1985).In past, lactic acid production by fermentation process is done by using food feedstock such as starches and sugars which caused food shortages and price increases. Therefore, now use of lignocellulosic biomass offers a non-competitive, cost-appealing, and environmentally friendly substrate for the production of lactic acid. Lignocellulosic biomass which comprises of agricultural, forestry and agro-industrial biowastes are in plenty, renewable and reasonable energy sources. Such lignocellulosic wastes include a variety of materials such as sugarcane bagasse, waste papers; brewer's spent grains, switch grass, rice straw, wheat straw, eucalyptus leaf litter, mango leaf litter, bamboo leaf litter. Lignocellulose wastes are compiled in huge amount, causing environmental problems. However, they can be used for the production of a number of products such as ethanol, food additives, organic acids, enzymes and others. Therefore, besides the environmental problems caused by their compilation, the non-use of these wastes caused a loss of valuable sources (Musatto *et al.,* 2010). The major components of lignocellulosic materials are cellulose, hemicellulose and lignin which are closely and tightly associated with each other and constitute the cellular complex of the biomass (Fig. 4).

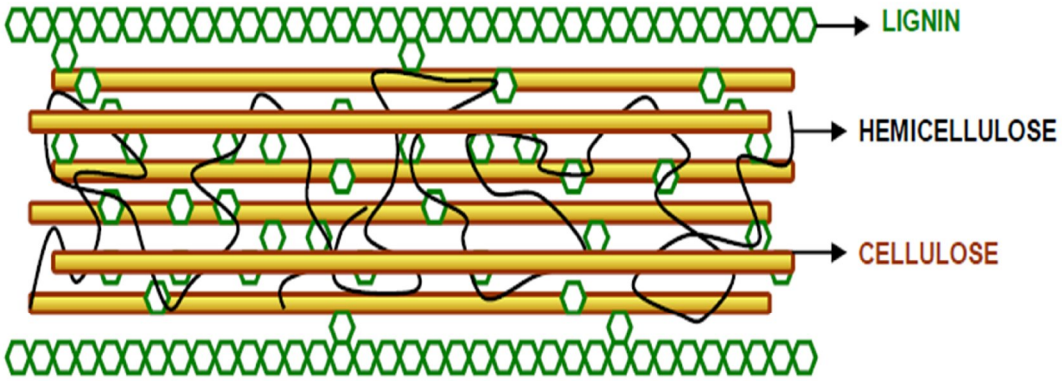

Fig 4: Lignocellulosic structure showing Cellulose, Hemicellulose and Lignin (Musatto *et al.,* 2010)

Cellulose

The most abundant organic compound, on the earth composed of repeated units of cellobiose (two glucose rings joined via a β-1, 4 glycosidic linkages), is cellulose. It is a high molecular weight and linear homopolymer which are linked together by hydrogen and Vanderwaal bond. Cellulose is found in both crystalline and amorphous form, which is found in lignocellulosic biomass (Musatto *et al.,* 2010).

Hemicelluose

The second most abundant natural polymer, which is linear or branched heterogenous polymer, hemicellulose, made up of five different sugars which are L-Arabinose, D-galactose, D-glucose, D-mannose and D-xylose where two sugars i.e. D-xylose and L-arabinose are pentose sugars while rest of three sugars are hexoses. According to main sugar remains, hemicellulose has different classifications- xylans, mannans, glucans, arabinoxylans, xyloglucans. Xylan is the common type of polysaccharide in hemicellulose family consists of mainly D-xylopyranose which is linked together by β-1, 4 linkages **(**Mod *et al.,* 1981; Ademark *et al.,* 1998; Musatto *et al.,* 2010).

Lignin

Lignin is the third most abundant biopolymer which is very composite molecule consists of phenylpropane units linked in 3D structures. The phenyl propane units are p-coumaryl alcohol, nconiferyl alcohol and sinapyl alcohol. Lignin is providing rigidiness and cohesion to the material cell wall and form phyiso-chemical barrier against microbial attack (Musatto *et al.,* 2010). Lignin has molecular weight of around 20,000. Lignins are mostly resistant to chemical and enzymatic degradation due to its molecular configuration. The composition percentage of cellulose, hemicelluloses, and lignin content of such biomasses fall in the range of 30–50%, 15– 35% and 10–20% respectively (Kaparaju *et al.,* 2009).Table 2 gives the percentage of cellulose , hemicelluloses and lignin in different lignocellulosic biomass.

Table 2: Percentage (%) of cellulose, hemicelluloses and lignin in different lignocellulosic biomass

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Poplar wood	44	32	21	Meng *et al.*,2012
Corn Stover	43	24	11	Zeng *et al*., 2011
Sugarcane Bagasse	42	25	20	Kim & Day *et al*., 2011
Sweet sorghum	45	27	21	Kim & Day *et al*., 2011
Wheat straw	35	29	21	Naik *et al*., 2010
Rye straw	35	30	19	Sanchez,2009
Banana waste	13	15	14	Sanchez,2009
Sponge gourd fibers	66	17	15	Guimaraes *et al*., 2009
Newspaper	40	25	18	Prasad *et al*., 2007
Leaves	15	80	-	Prasad *et al*., 2007
Nut shells	25	25	30	Prasad *et al*., 2007
Eucalyptus wood	45	15	26	Emmet *et al*.,2003
Switch grass	45	31	12	Sun *et al*., 2002
Hardwood	40	24	18	Malharbe & Cloete, 2002
Softwood	45	25	25	Malharbe & Cloete, 2002
Grasses	25	25	-	Malharbe & Cloete, 2002
Cotton gin waste	78	16	-	Raveendran *et al*., 1995

Table 3: Bacteria involved in lactic acid production

Substrate	Lactic acid producing microorganism	Amount of lactic acid produced (%)	Mode of lactic acid production	References
Sweet sorghum juice	***Bacillus coagulans***	80	Homo fermentative	Mark S. Ou *et al*., 2015
Cellobiose	***Bacillus subtilis***	82	Homo fermentative	Garcia *et al*., 2009
Cellobiose	***Lactobacillus delbrueckii***	90	Hetero fermentative	Adsul *et al*., 2007
Food waste	***Lactobacillus manihotivorus***	19.5	Hetero fermentative	Ohkouchi *et al.* ,2006
Xylose	***Bacillus coagulans***	60	Homo fermentative	Patel *et al*., 2006
Starch	***S. bovis***	14.2	Hetero fermentative	Junya *et al*., 2004
Wheat flour	***Lactobacillus amylophilus***	90	Homo fermentative	Vishnu *et al*., 2002
Glucose	***Bacillus coagulans***	56	Homo fermentative	Simisker *et al*., 2002
Starch	***Lactobacillus amylovorus***	96.2	Homo fermentative	Zhang *et al.*, 1991
Starch	***Lactobacillus amylophilus***	86	Homo fermentative	Vishnu *et al.*, 2000

2.6 Pre-treatment technologies for lignocellulosic biomass

Lignocellulosic biomass is attractive biomass because of affordable price and high polysaccharide content; however, it is not easy to use lignocellulosic resources for ethanol and other chemicals products. An effective pre-treatment is given as it decreases the need of getting the particle size of biomass particles to be small, preserving the pentose (hemicellulose) fractions, preventing the formation of degradative materials that inhibit the growth of fermentative microorganism, minimizing the energy demands and limiting the cost of the downstream processing steps, capital costs and biomass costs (Lynd *et al.*,1996; Wyman, 1995, 1996, 1999; Delgenes *et al.*, 1996;Palmqvist and Hagerdal, 2000; Ladisch *et al.*,1983). Various pre-treatment methods have been used for hydrolysis of hemicellulosic content of lignocellulosic biomass.

Acid hydrolysis

Acid hydrolysis pre-treatment for lignocellulosic feedstock has been developed as early in 19^{th} century. It can be categorized in two general methods- concentrated acid and dilute acid.

Concentrated acid hydrolysis

The processes based on concentrated acids are costlier and sometimes caused operational technical problems (Sun *et al.,* 2002; Galbe *et al.,* 2002). Mostly preferred acids as catalyst are sulphuric acid (H_2SO_4), hydrochloric acid (HCl) and trifluoroacetic acid (TFA). Concentrated acids mostly hydrolyse cellulose and hemicellulose and operate at low/medium temperature and pressure (Ogier *et al.,* 1999).

Dilute acid hydrolysis

Dilute sulphuric acid processes for pre-treatment generally operate at 0.5-1.5% and temperature between 121-160ºC have been favoured because of giving reasonably high sugar yields from hemicelluloses (Galbe *et al.*,2002).H_2SO_4,HCl and H_3PO_4 are usually employed acids for this process. Addition of CO_2 to aqueous solution forms carbonic acid which is suitable for hydrolysis condition under pH of mild conditions at high temperature and pressure.

Hydrothermal pre-treatment

Water, steam and heat are used in hydrothermal process. Before enzymatic method, hydro-thermolysis is used as pre-treatment method for lignocellulosic biomass. Hydrothermal method mainly includes hot water with steam explosion between 150ºC and 230ºC (Garrote *et al.,* 1999).

Autohydrolysis

Autohydrolysis process of pre-treatment uses compressed liquid hot water with temperature above 200ºC and pressure above saturation point to hydrolyze hemicelluloses in some few minutes. In this process, recovery rate is very high and no use of catalyst is required. Autohydrolysis process has almost same mechanism as a dilute acid hydrolysis process because in both processes catalyst is being used is Hydronium ion (H_3O^+). In autohydrolysis method, water is only reactive species to react with substrate where Hydronium ion does auto-ionization that leads to depolymerisation of hemicellulose by autohydrolysis of glycosidic linkages and acetyl groups (Carrasco *et al.,* 1989). Autohydrolysis results in high yield of saccharides from hydrolysis and low yield of by-products (Lamptey *et al.,* 1985; Mok *et al.,* 1992and Allen *et al.,* 1996).

Steam explosion

In this method, heating of biomass done using high pressure steam around 20-50 Bar, 210-290ºC for small time- interval. Under high pressure, steam condenses and which wet the material which is driven out through nozzle from reactor. When there is decline in raised pressure, condensed moisture evaporates and there is desegregation of lignocellulosic biomass takes place by breaking down molecular linkages (Carrasco *et al.,* 1989).

Wet oxidation

In wet oxidation pre-treatment, auto-hydrolysis process proceed in presence of oxygen or air which acts as catalysts (Mcginnins *et al.,* 1983) at reduced temperature and shorter reaction. However, not oxygen but sodium carbonate is mostly employed as catalysts for wet oxidation method.

Alkaline treatment

Alkaline pre-treatment increases the cellulose digestibility of lignocellulosic biomass. Based on catalyst being used in process, alkaline pre-treatment can be further classified into two groups (a) pre-treatment that uses sodium(Na), potassium(K) or calcium hydroxide ($Ca(OH)_2$) (b) pre-treatments which uses ammonia. This method is more effective for lignin solublization.

Lime and sodium hydroxide pre-treatment

Na and lime are most common catalysts which used in concentration about 0.05-0.15 g alkali per g biomass directly to biomass (Wyman *et al.,* 2005). Parayo et al., 1996 reported the alkali treatment at temperature 30-130ºC and its reaction time is 10 min -18 hours. To increase the efficiency of this treatment, ultrasonic assisted extraction has been done to get high yield of hemicelluloses (Sun *et al.,* 2002). Use of low temperature and pressure and low operational cost is main attractive point of this process

Alkaline peroxide treatment

Addition of oxidative agent (air or H_2O_2) to alkaline pre-treatment, greatly enhance the performance of treatment by removing lignin. Hydrogen peroxide treatment has following parameters: concentration is 1-3%; reaction time is 45 min to 5 hours; temperature is being to follow is 30-80ºC and optimal pH is 11.5 (Gould *et al.,* 1984;Sun *et al.,*2005; Cara *et al.,*2006 and Yanez *et al.,*2006). This pre-treatment method is complemented with direct oxidative effect over lignin and seems to have fast kinetics at low temperature thus show technical and economical advantage over lime and sodium hydroxide catalyzed pre-treatment.

Ammonia Recycling Percolation (ARP)

Aqueous ammonia mostly used in column reactor having biomass in ARP. ARP contains following parameters: concentration of ammonia- 2.5-20%; reaction time upto 90 min; temperature-140-170ºC; solid conc- 15-30% (w/w) and percolation rate – 5ml/min (Sun *et al.,* 2002; Mosier *et al.,* 2005 and Kim *et al.,* 2008). ARP process gives highly effective delignification (60-85%). ARP can also solublize hemicelluloses (40-60%)

MATERIALS AND METHODS

3.1 Sample collection for isolation of lactic acid producing microbes

Screening and characterization of lactic acid producing microorganisms were done from samples collected from different locations. Samples of dairy waste and decaying wood were collected from India Bakery, Patiala and Thapar University, Patiala respectively. Samples were characterized for their physio-chemical properties and bacterial isolates were screened for their lactic acid producing capability. Screened bacterial isolates were further used for morphological and bio-chemical characterization.

3.2 Isolation of lactic acid producing microbes

Dairy products samples and decaying wood samples were collected from different locations. About 1 g of sample was weighed and inoculated in 50 ml nutrient broth(Appendix 1) for the isolation of bacterial strains and was incubated at 37ºC for 24 hours under shaking conditions (120 rpm). For isolation of pure culture, the serial dilutions of all samples were done in saline (0.85%) and were plated on NA plates. These plates were incubated at 37ºC for 24 hours.

3.3 Screening of thermophilic lactic acid producing microbes (Lidan *et al.,* 2013*)*

The colonies obtained on nutrient agar were picked and inoculated in enrichment broth (Appendix I) and incubated at 50ºC without agitation. The serial dilutions of suspensions were done which were obtained above and plated on agar plates containing 10g/l xylose, 5 g/l yeast extract, 10 g/l agar and 0.2 g/l bromocresol green. The plates then incubated at 50ºC for 24-48 hours till the appearance of colonies with clear color change from green to yellow not occurred. The single colony was picked up with sterilised loop and cultivated in modified nutrient media (Appendix 1) and incubated at 50ºC for till the O.D. reached at 0.6. Same procedure was done with already isolated bacterial strains.

3.4 Estimation of lactic acid

The estimation of lactic acid was done as described below; broth was taken in oakridge tubes and spun at 8000 rpm for 10 minutes. Then the supernatant was transferred to a fresh beaker and the solution was heated upto its temperature reached to 80ºC. Then, $Ca(OH)_2$ having concentration of 5% was added to it drop wise till the pH of supernatant becomes 7. The broth was then filtered using filter paper and the filtrate was discarded. The

precipitate obtained was dissolved in beaker using less volume of 0.1N HCl. The dissolution of the precipitate was done using double distilled water for titration against 0.1N NaOH (standardized) using phenolphthalein as indicator. The standard curve was plotted using 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 g/l stock solution of lactose for estimation of lactic acid (Fig. 5).

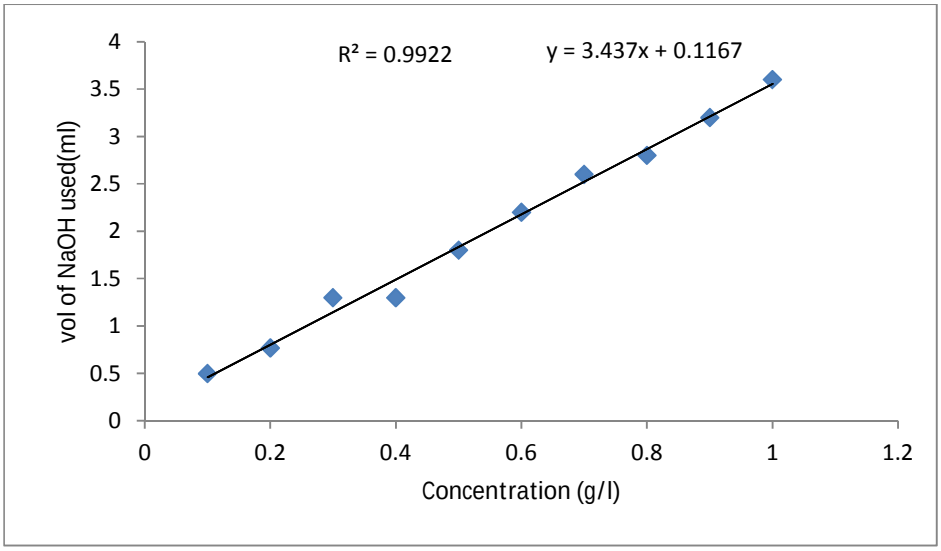

Fig 5: Standard curve of lactic acid

Amount of lactic acid comes from z ml of fermentation broth so the strength of lactic acid that is actually produced was (X*y)*90*1000/ (1000*z) g/l

X= Normality of sodium hydroxide being used

y= volume of NaOH being used by lactic acid

90= Gram equivalent weight of lactic acid

z= ml of broth used

3.5 Morphological and biochemical characterization of bacterial isolates

The biochemical and morphological characterization of all bacterial strains was done by conventional biochemical and physiological tests described in Microbiolgy: a laboratory manual, 5th edition (James G. Cappuccino; Natalite Sherman)

3.5.1 Gram staining

The slide was cleaned with double distilled water and was air dried. The bacterial smear was prepared by putting the water droplet at the centre of slide and loop of bacteria from plate was transferred to it and was spread it gently. The bacterial smear was air dried and then smear was fixed by slight heating. Heating results in coagulation of cellular proteins due to which cells sticks to slide surface. After that, slide was flooded with primary stain that is crystal violet for 1 min and was gently washed with water to remove excess stain. Slide was flooded with gram iodine for 1 min and washed gently with water. Further, it was flooded with decolourising agent for 5s and quickly washed under tap water. Then, counter stain, safranin, was added for 1 min and washed under tap water to remove excessive stain. The slide was air dried and observed under microscope at different magnifications.

3.5.2 Catalase test

Slide was cleaned up with water and air dried. A drop of hydrogen peroxide (3%) was taken at the centre of slide. A loop of bacteria was taken aseptically in laminar flow chamber with the help of sterilised loop and was put onto the drop of hydrogen peroxide on the slide and observed the results.

Observations:

1. Bubbling or foaming occurs in the drop- Catalase positive

2. Bubbling or foaming does not occur in the drop- Catalase negative

3.5.3 Nitrate reduction test

Nitrate broth medium (Appendix I) was used to test the ability of bacteria to convert nitrates to nitrites as they can produce the enzyme nitrate reductase. The main component of nitrate broth medium is nitrate. The ingredients of nitrate broth medium were weighed and dissolved in 100 ml of distilled water and pH was adjusted to 7.2.The broth is

distributed into test tubes (approx. 10ml) and broth tubes were autoclaved at 121ºC at 15 psi pressure for 15 minutes. The tubes were allowed to cool at room temperature. The bacteria is inoculated aseptically in broth tubes and incubated at 37ºC for 48 hours. After that, 0.5 ml each of nitrate reagent solution A (Appendix II) and nitrate reagent solution B (Appendix II) were added into each test tube and shaken well and colour change was observed after 15 minutes.

Observation:

1. Red colour produced: Nitrate reduction positive

2. Red colour not produced: Nitrate reduction negative

3.5.4 Methyl red test

MR test was done to check the ability of bacteria to form acid like lactic acid, acetic acid or formic acid by utilizing any monosaccharide sugar. MR broth medium (Appendix I) ingredients were weighed and dissolved in 100 ml distilled water and pH was adjusted to 6.9. The broth was distributed into test tubes (approx. 10ml) and autoclaved at 121ºC at 15 psi pressure for 15 minutes. The test bacteria were inoculated aseptically in broth tubes with the help of sterilised loop and incubated at 37ºC for 24 to 48 hours in an incubator. Alcoholic solution of methyl red (Appendix II) was dropped into each test tube.

Observations:

1. Red colour produced: MR positive

2. Red colour not produced: MR negative

3.5.5 Urease test

Urease test was done to check the capability of bacteria to hydrolyze urea to ammonia with the help of the enzyme ‘urease’. The ingredients of urea agar medium (Appendix I) were weighed except urea and dissolved in 90 ml distilled water and pH was adjusted to 6.9. The test tubes, urea agar media (except urea) were sterilised at 121ºC for 15 minutes. 20% urea solution was prepared and was sterilised by membrane filtration apparatus as urea cannot be sterilised by heat as it degraded upon heating. 10 ml of sterilised urea solution was added in 90 ml urea agar media and equally distributed into test tubes. The test tubes were kept in slanting position as to get urea agar slants. The bacteria was

inoculated aseptically in a laminar flow chamber into the slants with the help of flamed needle and incubated at 37ºC for 24 hours.

Observations:

1. Pink colour produced: Urease positive

2. Pink colour not produced: Urease negative

3.5.6 Voges-Proskauer test

Voges- Proskauer test was done to test the ability of bacteria to convert glucose into acetylmethylcarbinol (acetoin). The ingredients of VP broth media (Appendix II) were weighed and dissolved in 100 ml distilled water and pH was adjusted to 6.9. The broth is distributed into test tubes (approx 10ml) and sterilised at 121ºC (15 psi pressure) for 15 minutes. The test bacteria were inoculated aseptically using sterilised loop. The inoculated broth tubes were incubated at 37ºC for 72 hours in an incubator. The VP solution 1 (Appendix II) was dropped into each test tube (0.2 ml). Further, VP solution 2 (Appendix II) was dropped into each test tube (0.6 ml) and mixed gently and allowed to stand for 30 minutes to 2 hours.

Observations:

1. Rose pink colour produced: VP positive

2. Rose pink colour not produced: VP negative

3.5.7 Carbohydrate fermentation test

The test was done to test the ability of bacteria to ferment carbohydrates mainly sugars. The ingredients of carbohydrate broth media (Appendix I) were weighed for 100 ml and dissolved in 100 ml of distilled water and pH was adjusted to 6.8 using 0.1 N HCl or 0.1 N NaOH. The broth was distributed into test tubes (approx. 10ml). Durham tube was put in inverted position into broth in each test tube. The test tubes were sterilised at 121ºC (15 psi pressure) for 15 minutes. The bacteria were inoculated into the broth with the help of flame sterilised loop. The inoculated broths were incubated at 37ºC for 24 hours.

Observations:

1. If colour of broth changes to yellow and gas accumulates in Durham tube then bacteria is fermentative for carbohydrate and aerogenic

2. If colour of broth changes to yellow but no gas accumulates in Durham tube, then to fermentative for carbohydrate and anaerogenic

3. If colour of broth does not change, then bacteria is Non-Fermentative for carbohydrate

3.5.8 Starch hydrolysis test

Starch hydrolysis test was done to check the ability of bacteria to hydrolyse starch as to check can they produce enzyme 'α- amylase'. The ingredients of starch agar medium (Appendix I) were weighed and dissolved in 100ml of distilled water and pH was adjusted to 7.2. The flask containing starch agar media were sterilised at 121ºC at 15 psi pressure for 15 minutes. In warm molten condition, starch agar was poured aseptically into petri dishes and allowed to cool at 37ºC for about 1 hour. The inoculation of the test bacteria was done aseptically inside a laminar flow chamber onto plates. The inoculated plates were incubated at 37ºC for 24 to 48 hours till the colonies of bacteria were become visible. The plates were flooded with lugol's iodine solution (Appendix II).

Observations:

1. Transparent clear zones formed around colonies of bacteria: Starch hydrolysis positive

2. Transparent clear zones not formed around colonies of bacteria: Starch hydrolysis negative

3.6 Optimization of lactic acid production from Lignocellulosic biomass waste

3.6.1 Sample collection and processing of agricultural waste and leaf litter biomass

Agricultural waste biomass from wheat and rice straw and leaf litter biomass from *Eucalyptus globules* were collected from Thapar University campus and nearby villages of Patiala, Punjab. Samples were washed to remove debris, dried, grounded and sieved to particle size of 0.5 mm. Sieved samples were preserved in air tight containers at room temperature.

3.6.2 Estimation of reducing sugar in biomass sample

In biomass sample, reducing sugars were estimated using DNS method as per given in protocol given by Miller, 1959. Pre-treated biomass sample i.e. rice straw, wheat straw and eucalyptus leaf litter before and after inoculation with NA9 and DGN2 around 0.1 ml was taken and put in test tube containing 500 µl potassium phosphate buffer (50 mM) and volume was made upto 1 ml using distilled water. To this mixture, 3 ml of DNS reagent (Appendix II) was added and incubated at 100ºC in water bath for 10 min and were allowed to cool at room temperature and spun at 11,000 rpm for 10 minutes. The absorbance was recorded at 540 nm. The standard graph was plotted using 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml glucose, to calculate the amount of reducing sugar present in sample (Fig. 6).

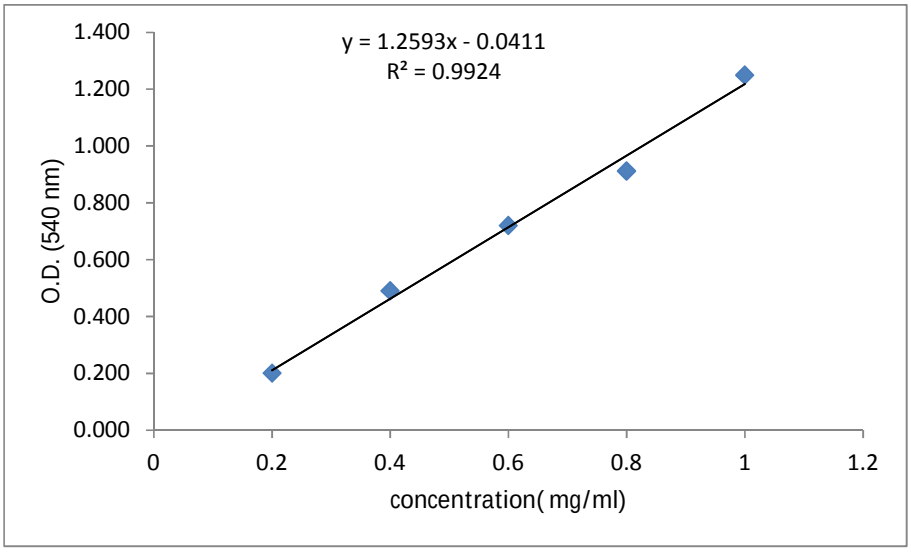

Fig 6: Standard curve for reducing sugar assay

3.6.3 Protein estimation by Folin-Lowry method (Lowry *et al.*, 1951*)*

To study the protein concentration of bacterial isolates, overnight grown culture were taken (0.6 O.D. at 600 nm). Bovine Serum Albumin (BSA) standard solutions were prepared in the range of concentrations 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml. From these solutions and bacterial isolates, 200µl samples were taken in test tubes. 2 ml of reagent C was added in each test tube [prepared by (a) 2% sodium carbonate in 0.1 N NaOH (b) 1% sodium potassium tartrate and 0.5% cupric sulphate pentahydrate ($CuSO_4.5H_2O$). The analytical reagent was prepared by adding 1 ml of (b) in 50 ml of (a)] and solutions were mixed well. The final volume was made 5 ml using distilled water. The solutions were incubated at room temperature for 10 min. Then 200 µl of Folin-Ciocalteau reagent solution (1N) was added in each test tube and incubated for 30 min in dark condition. The absorbance of solution was measured at 660 nm. The absorbance of bacterial samples was checked and protein concentration was determined from the standard curve (Fig. 7).

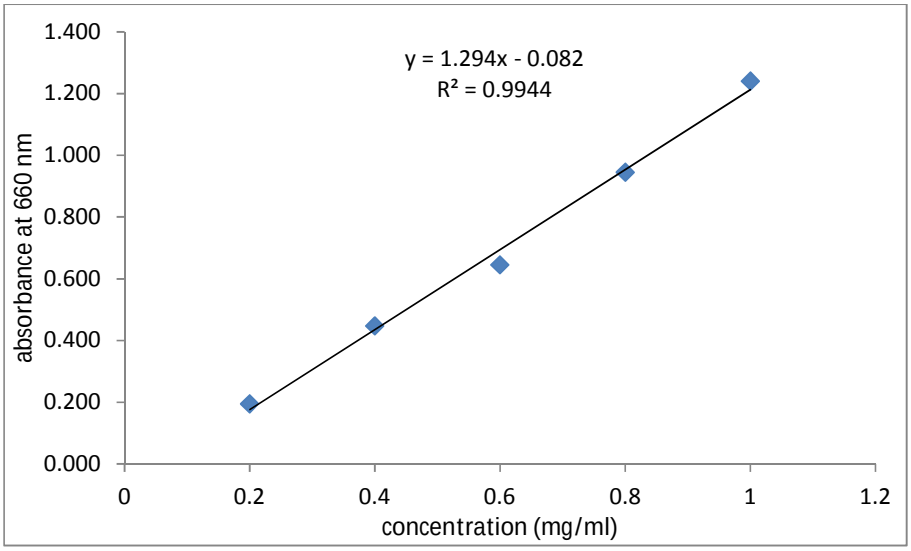

Fig 7: Standard curve of Folin-Lowry assay

3.6.4 Pre-treatment of agricultural biomass

Different lignocellulosic substrates i.e. wheat straw, rice straw and eucalyptus stalk were taken. Different pre-treatments i.e. with conc. Sulfuric acid, 0.6N sulfuric acid and 0.4N alkaline treatment were applied on these three different lignocellulosic biomasses i.e. rice straw, wheat straw and eucalyptus leaf litter for doing the comparative study on the production of lactic acid.

A) Acid Hydrolysis

1) Pre-treatment with concentrated sulfuric acid

10% of each lignocellulosic substrate was taken in 250 ml conical flask and 2% conc. H_2SO_4 was added to each flask and volume was made up using double distilled water and was autoclaved at 121°C for 60 minutes. Upon autoclaving, the flasks were cooled and were transferred to centrifuge bottles for centrifugation at 4500 rpm for 15 minutes at 25°C. The supernatant was taken. The pH for lignocellulosic biomass was maintained at 7 by adding NaOH.

2) Pre-treatment with 0.6N sulfuric acid

10% of each lignocellulosic substrate was taken in 250 ml conical flask and 2% 0.6N H_2SO_4 was added to each flask and volume was made up using double distilled water and was autoclaved at 121ºC for 60 minutes and were transferred to centrifuge bottles for centrifugation at 4500 rpm for 15 minutes at 25ºC. The supernatant was taken. The pH for lignocellulosic biomass was maintained at 7 by adding NaOH.

B) Alkali Hydrolysis

1) Pre-treatment with 0.4N alkaline solution

10% of each lignocellulosic substrate was taken in 250 ml conical flask and 2% 0.4N NaOH was added to each flask and volume was made up using double distilled water and was autoclaved at 100ºC for 60 minutes. Upon autoclaving, the flasks were cooled and were transferred to centrifuge bottles for centrifugation at 4500 rpm for 15 minutes at 25ºC. The supernatant was taken. The pH for lignocellulosic biomass was maintained at 7 by adding HCl.

Conditioning of media

The conditioned wheat straw hydrolysate, rice straw hydrolysate and eucalyptus leaf litter hydrolysate were taken having the desired pH 7.0. From each hydrolysate, 120 ml of hydrolysate was taken. The hydrolysate media was prepared by adding ammonium sulphate and peptone i.e. 1%, 0.1% respectively to each flask and autoclaved at 121ºC for 20 minutes. Upon autoclaving, the NA 9 and DGN2 was inoculated to each flask and kept at 50ºCwithout agitation and the growth was monitored at 24 hours, 48 hours and 72 hours. Lactic acid, produced from different substrates i.e. wheat straw hydrolysate, rice straw hydrolysate and eucalyptus leaf litter hydrolysate, was estimated as described in **3.4**.

RESULTS AND DISCUSSION

4.1 Screening and characterization of lactic acid producing microorganisms

Seven lactic acid producing bacteria were isolated from dairy waste and decaying wood samples and eight previously isolated bacterial strains were also checked for production of lactic acid. These microbes were screened for their efficiency for growth on medium containing xylose, as carbon source. In pentose phosphate pathway, xylose gets converted to pyruvate which is formed as an intermediate and gets converted to lactic acid (Reddy *et al.,* 2008) at thermophilic temperature of 50ºC (Payot *et al.,* 1999) and growth was monitored by using UV-VIS spectrophotometer (Table 4). The maximum growth was observed after 24 hours in strain DGN1 followed by DGB, NA15, DGS3, NA9, T8, DGN2 and DGS3. The order of growth after 48 hours was DGN1>DGB> T8> NA15> NA9> DGS3> DGN2> DGS1 (Fig.8)

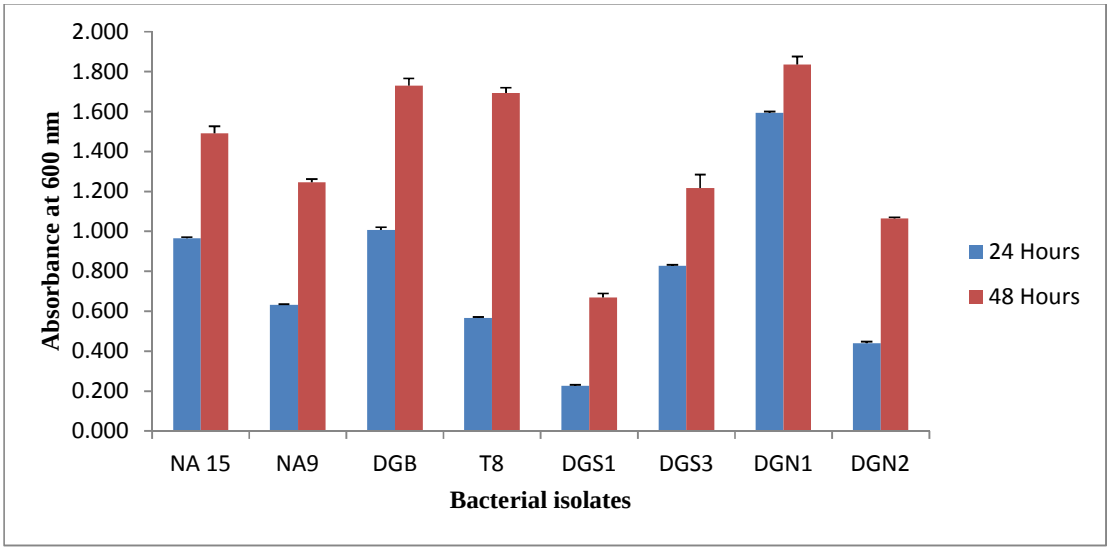

Fig 8: Growth of bacterial isolates after 24 and 48 hours of incubation at 50ºC

Table 4: Growth profile of bacterial isolates after 24hrs and 48hrs at 50°C

	Absorbance at 600 nm after 24 hrs				**Absorbance at 600 nm after 48 hours**			
Bacterial isolates	**R1**	**R2**	**R3**	**Mean ± SE**	**R1**	**R2**	**R3**	**Mean ± SE**
Bacillus subtilis (NA15)	0.968	0.972	0.954	0.965 ± 0.005	1.562	1.458	1.449	1.490 ± 0.036
***Bacillus licheniformis (*NA9)**	0.632	0.625	0.637	0.631 ± 0.003	1.239	1.224	1.276	1.246 ± 0.015
***Bacillus licheniformis* (DGB)**	1.035	0.997	0.986	1.006 ± 0.015	1.765	1.654	1.768	1.729 ± 0.038
T8	0.567	0.574	0.558	0.566 ± 0.005	1.678	1.654	1.745	1.692 ± 0.027
DGS1	0.227	0.22	0.233	0.227 ± 0.004	0.645	0.708	0.653	0.669 ± 0.020
DGS3	0.821	0.837	0.823	0.827 ± 0.005	1.213	1.334	1.108	1.218 ± 0.065
DGN1	1.599	1.578	1.6	1.592 ± 0.007	1.89	1.756	1.86	1.835 ± 0.041
DGN2	0.433	0.456	0.434	0.441 ± 0.008	1.067	1.054	1.072	1.064 ± 0.005

Out of total 15 bacterial isolates, eight isolates (DGN1, DGB, NA15, DGS3, NA9, T8, DGN2 and DGS3) grew best on media containing xylose (Table 5) and were studied for morphological, physiological and biochemical parameters and were further tested for lactic acid production.

Table 5: Samples screened on media containing xylose as sole carbon source (visual observation)

S. No.	**Samples**	**Growth on media containing xylose**
1	T8	+++
2	*Bacillus subtilis* (NA15)	+++
3	*Bacillus licheniformis* (DGB)	++++
4	NA11	_
5	*Bacillus licheniformis* (NA9)	++
6	NA14	_
7	DGN1	++++
8	DGN2	+
	Bakery waste from different locations	
9	DGS1	+
10	DGS2	_
11	DGS3	++
12	DGS4	_
13	DGS5	_
14	DGS6	_
15	DGS7	_

+: growth; ++: moderate growth; +++: best growth; -: no growth

4.1.1 Screening of lactic acid producing bacteria

Selected bacteria NA9, NA15, T8, DGB, DGS1, DGS3, DGN1 and DGN2 were screened on agar plates containing xylose and bromocresol green and were selected for further studies on the basis of colour change from green to yellow colonies. The colour change is a clear indication of the presence of lactic acid producing bacterium (Fig.9).

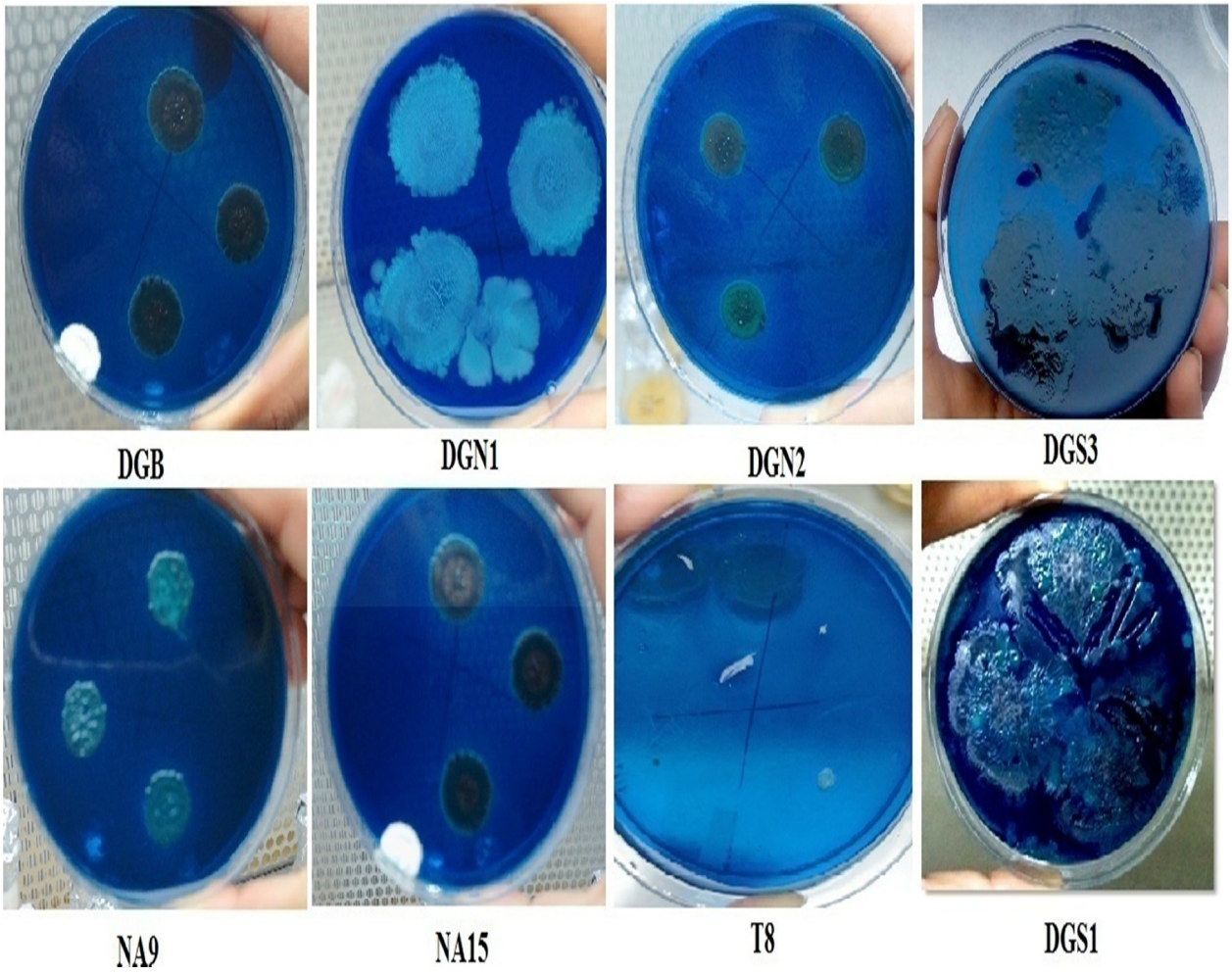

Fig 9: Screening of lactic acid producing microbes on the basis of hydrolysis zone around colonies using bromocresol green staining.

4.2 Production and estimation of lactic acid

The bacterial isolates which showed the conversion of green to yellow colour zone around colonies on bromocresol green containing plates, were selected for further study. These cultures were further grown in modified nutrient media and their absorbance was taken at 600nm (Table 6). After 72 hrs of incubation, lactic acid estimation was done as given in the materials and methods. The maximum concentration of lactic acid was produced by DGN2, (0.432 g/l) followed by NA9, DGS1, DGN1, DGB, DGS3, T8, and NA15 having concentrations 0.403g/l, 0.403g/l, 0.315g/l, 0.228g/l, 0.228g/l, 0.170g/l and 0.170g/l respectively (Fig. 10). From this data NA9, DGN2, DGS1 were identified as potential lactic acid producers.

Table 6: Production of lactic acid by different bacterial isolates

Bacterial isolates	Lactic acid (g/l)
*Bacillus licheniformis (*NA9)	0.403 ± 0.0009
Bacillus subtilis (NA15)	0.170 ± 0.0026
Bacillus licheniformis (DGB)	0.228 ± 0.0103
T8	0.170 ± 0.0015
DGN2	0.432 ± 0.0012
DGS3	0.228 ± 0.0009
DGN1	0.315 ± 0.0010
DGS1	0.403 ± 0.0021

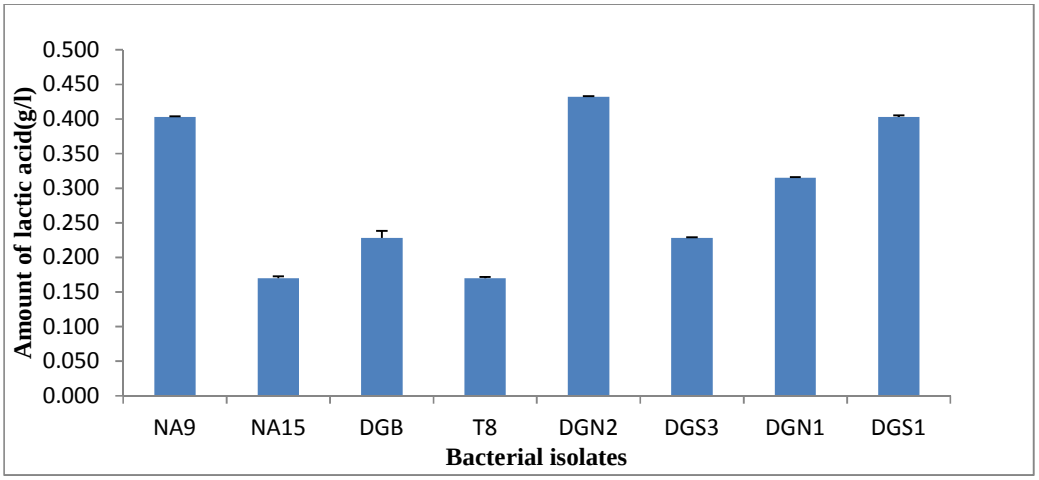

Fig 10: Production of lactic acid by different bacterial isolates

4.3 Morphological and biochemical characterization of bacterial isolates

All bacterial isolates were found to be Gram positive except T8 and DGN1 (Table 7). Out of 8 bacterial isolates, only DGS3 was found to be positive for methyl red test i.e. this bacterial isolate utilised and converted glucose to stable acid like lactic acid, acetic acid through mixed acid pathway (Table 7).

Out of eight bacterial isolates, five bacterial isolates (NA15, T8, DGS1, DGS3 and DGN1) were found to be non-fermentative i.e. they did not have the ability to ferment sugars and other three bacterial isolates i.e. NA9, DGB and DGN2 were found to be fermentative but anaerogenic i.e. they fermented sugars and acid was produced which reduced the pH, changing the colour of bromocresol purple from purple to yellow but there was no production of gas. These isolates were potential strains for consideration because of their sugar fermentative capability which could be tested for lactic acid production using lignocellulosic substrate (Table 7).

NA15, T8, DGS1 and DGN1 were found to be urease positive as they have the enzyme 'urease', which utilised urea from media and converted urea into ammonia and carbon dioxide, presence of ammonia increased the pH changing the colour of phenol red from yellow to pink (Table 7).

For catalase test, NA15, NA9, T8 and DGN1 were found to be positive as they have the ability to degrade hydrogen peroxide and convert it into water with the evolution of oxygen gas with the help of enzyme 'catalase'. This implied these strains had great catalytic efficiency (Table 7).

All eight bacterial isolates were found to be negative for starch hydrolysis test as they did'nt have the enzyme α- amylase for hydrolysis of starch (Table 7).

For VP (Voges- Proskauer) test, NA15, T8, DGS3, DGN1 and DGN2 were found to be positive as they utilised glucose and converted it into neutral end product, acetoin, via the 'butylene glycol pathway' (Table 7). NA15, NA9, DGB, DGS3 and DGN2 have 'nitrate reductase' enzyme which reduced nitrate to nitrite (Table 7).

Table 7: Bio-chemical characterisation of lactic acid producing bacterial isolates

TEST	NA15	NA9	DGB	T8	DGS1	DGS3	DGN1	DGN2
Carbohydrate fermentation	Non-fermentative	Anaerogenic & fermentative	Anaerogenic & fermentative	Non-fermentative	Non-fermentative	Non-fermentative	Non-fermentative	Anaerogenic & fermentative
Urease	+	-	-	+	+	-	+	-
Catalase	+	+	-	-	-	-	+	-
Starch hydrolysis	-	-	-	-	-	-	-	-
Gram staining	+	+	+	-	+	+	-	+
Voges-proskauer	+	-	-	+	-	+	+	+
Nitrate reductase	+	+	+	-	-	+	-	+
Methyl red	-	-	-	-	-	+	-	-

4.4 Estimation of reducing sugar in different lignocellulosic biomass

Wheat straw was reported to have 29% hemicellulose, 35% cellulose and 21% lignin (Naik *et al.,* 2010), rice straw had 24% cellulose 39% hemicellulose and 6% lignin (Chandra *et al.,* 2012) and eucalyptus leaf litter had 45% hemicellulose, 15%, cellulose and 26% lignin (Emmel *et al.,* 2003). In our lab previously, wheat straw was found to have 36% cellulose, 28% hemicellulose and 18% lignin, rice straw was reported to have 43% cellulose, 24% hemicellulose and 9% lignin content and eucalyptus leaf litter biomass had 31% cellulose, 17% hemicellulose and 12% lignin content (Akhtar and Goyal, 2015).

Pre-treatment of different lignocellulosic biomass such as rice straw, wheat straw and eucalyptus leaf litter was done using chemical methods i.e. treatment with concentrated H_2SO_4, 0.6N H_2SO_4, 0.4N dilute NaOH. The hydrolysates was used as substrates in media and inoculated with either NA9 or DGN2 and incubated at 50^{o}C without agitation. The absorbance was taken at 600nm and the culture filtrate was checked for residual concentration of reducing sugar using DNS assay.

1) Acid hydrolysis

A) Pre-treatment with concentrated H_2SO_4

The maximum amount of sugar was utilised from wheat straw hydrolysate by NA9, when compared with control (uninoculated) since the concentration of sugar reduced from 0.403 to 0.242 mg/ml. Also the concentration of sugar reduced to 0.376 mg/ml after inoculation with DGN2. Using rice straw hydrolysate by NA9 lead to reduction in sugar from 0.267 to 0.198 mg/ml and it was reduced to 0.242 mg/ml after inoculation with DGN2. Similarly when eucalyptus leaf litter hydrolysate was used as substrate, inoculation by NA9 leads to reduction in sugar 0.207 to 0.190 mg/ml whereas when inoculated with DGN2, it was reduced to 0.195 mg/ml (Fig. 11, Table 8).

Table 8: Reducing sugar (mg/ml) before and after bacterial inoculation in different media supplemented with lignocellulosic hydrolysates obtained after pre-treatment with concentrated H_2SO_4 of biomass

	Rice straw hydrolysate (mg/ml)	**Wheat straw hydrolysate (mg/ml)**	**Eucalyptus leaf litter hydrolysate (mg/ml)**
Control (uninoculated)	0.267 ± 0.003	0.403 ± 0.004	0.207 ± 0.001
NA 9	0.198 ± 0.002	0.242 ± 0.051	0.19 ± 0.003
DGN2	0.242 ± 0.046	0.376 ± 0.006	0.195 ± 0.042

Reduction in residual concentration of sugar after inoculation with NA9 and DGN2 concluded that hydrolysates obtained after pre-treatment with concentrated H_2SO_4 of wheat straw, rice straw and eucalyptus leaf litter is a good source of easily utilisable carbohydrate such as glucose supporting good bacterial growth.

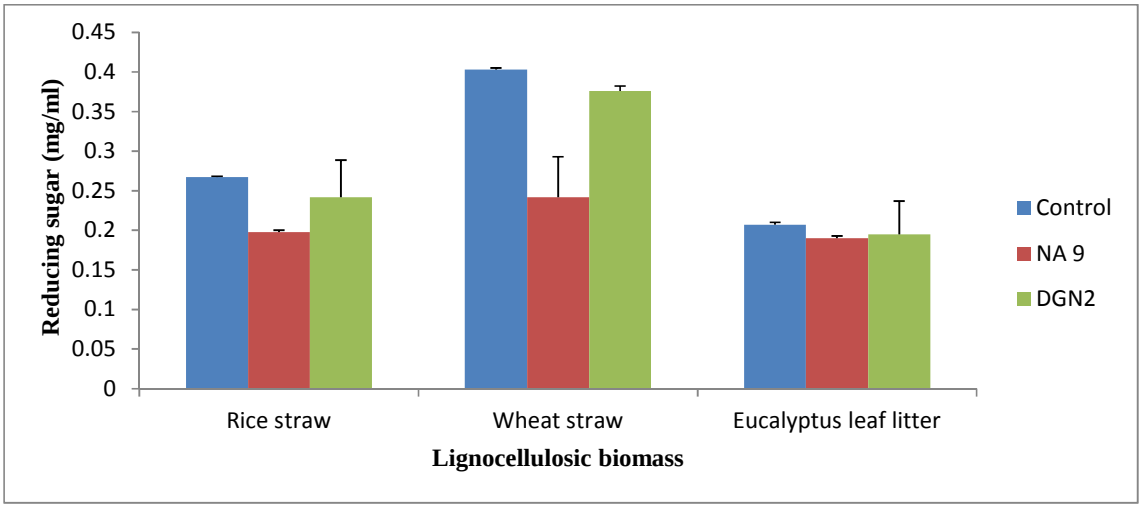

Fig 11: Reducing sugar (mg/ml) before and after bacterial inoculation in different media supplemented with lignocellulosic hydrolysates obtained after pre-treatment with concentrated H_2SO_4 of biomass

B) Pre-treatment with 0.6N H_2SO_4

From pre-treated wheat straw hydrolysate after inoculation with NA9, bacteria utilised maximum amount of sugar when compared with control (uninoculated) as the residual sugar concentration reduced from 1.09 to 0.18 mg/ml. Also the concentration of sugar reduced to 0.652 mg/ml after inoculation with DGN2. The sugar is being utilised from pre-treated rice straw hydrolysate after inoculation with NA9 when compared with control (uninoculated) as the concentration of the sugar reduced from 0.404 to 0.397 mg/ml. Also the concentration of sugar reduced to 0.353 mg/ml after inoculation with DGN2. Similarly when eucalyptus leaf litter hydrolysate was used as substrate, inoculation by NA9 leads to reduction in sugar from 1.306 to 1.081 mg/ml. Also the concentration of sugar reduced to 1.119 mg/ml after inoculation with DGN2 (Fig. 12, Table 9). On an average less amount of sugar was utilised by DGN2 strain as compared to NA9 from different lignocellulosic hydrolysates.

Table 9: Reducing sugar (mg/ml) before and after bacterial inoculation in different media supplemented with lignocellulosic hydrolysates after pre-treatment with 0.6N H_2SO_4 of biomass

	Rice straw hydrolysate (mg/ml)	**Wheat straw hydrolysate (mg/ml)**	**Eucalyptus leaf litter hydrolysate (mg/ml)**
Control (uninoculated)	0.404 ± 0.003	1.090 ± 0.004	1.306 ± 0.001
NA 9	0.397 ± 0.006	0.180 ± 0.026	1.081 ± 0.012
DGN2	0.353 ± 0.004	0.652 ± 0.001	1.119 ± 0.055

After inoculation with bacterial strains in lignocellulosic hydrolystaes, a decreasing trend in the reducing sugar value could be seen. This suggests that some amount of sugar is being utilised by the bacterial isolates for their multiplication and production of the useful product i.e. lactic acid.

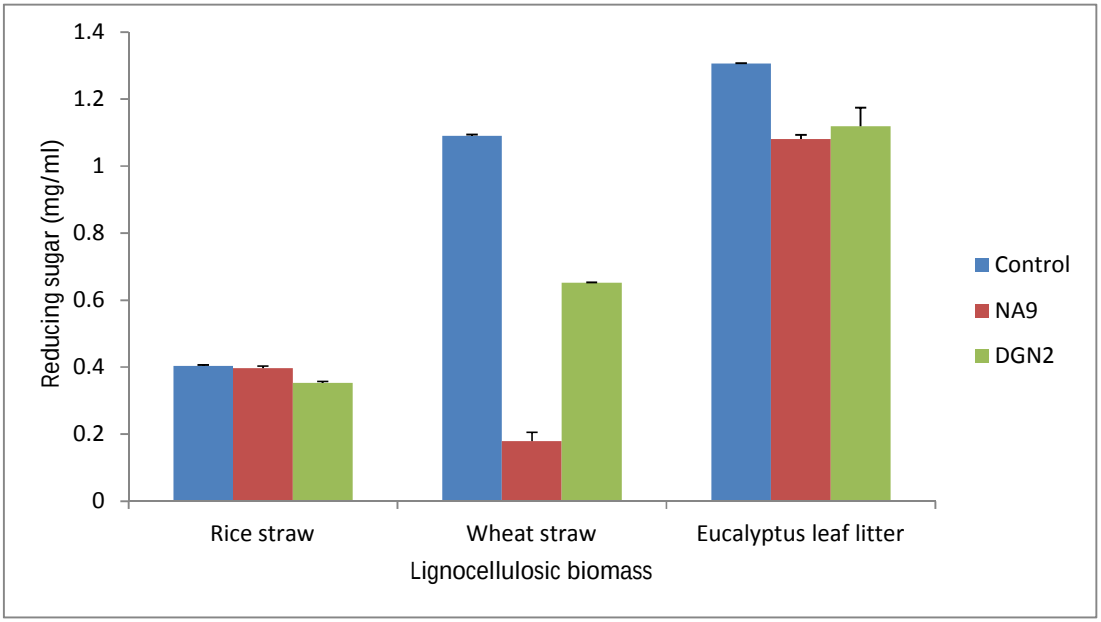

Fig 12: Reducing sugar (mg/ml) present before and after bacterial inoculation in media supplemented with lignocellulosic hydrolysates after pre-treatment with 0.6N H_2SO_4 of biomass

2) Alkali Hydrolysis

A) Pre-treatment with 0.4N NaOH

The maximum amount of sugar is being utilised from pre-treated wheat straw hydrolysate after inoculation with NA9 when compared with control (uninoculated) since the concentration of the sugar reduced from 0.979 to 0.533 mg/ml. Also the concentration of sugar reduced to 0.662 mg/ml after inoculation with DGN2. The sugar is being utilised from pre-treated rice straw hydrolysate after inoculation with NA9 when compared with control (uninoculated) as the concentration of the sugar reduced from 0.565 to 0.422 mg/ml. Also the concentration of sugar reduced to 0.396 mg/ml after inoculation with DGN2. When we compared the reducing sugar value obtained, after bacterial inoculation in eucalyptus leaf litter hydrolysate, the maximum amount of sugar is being utilised fromthis hydrolysate by NA9 (0.987 mg/ml) followed by DGN2 (1.042 mg/ml) (Fig. 13, Table 10).

Table 10: Reducing sugar (mg/ml) before and after bacterial inoculation in different media supplemented with lignocellulosic hydrolysates after pre-treatment with 0.4N NaOH of biomass

	Rice straw hydrolysate (mg/ml)	**Wheat straw hydrolysate (mg/ml)**	**Eucalyptus leaf litter hydrolysate (mg/ml)**
Control(uninoculated)	0.565± 0.0043	0.979 ± 0.003	1.321 ± 0.0032
NA 9	0.422 ± 0.006	0.533 ± 0.026	0.987 ± 0.012
DGN2	0.396 ± 0.004	0.662 ± 0.001	1.045± 0.055

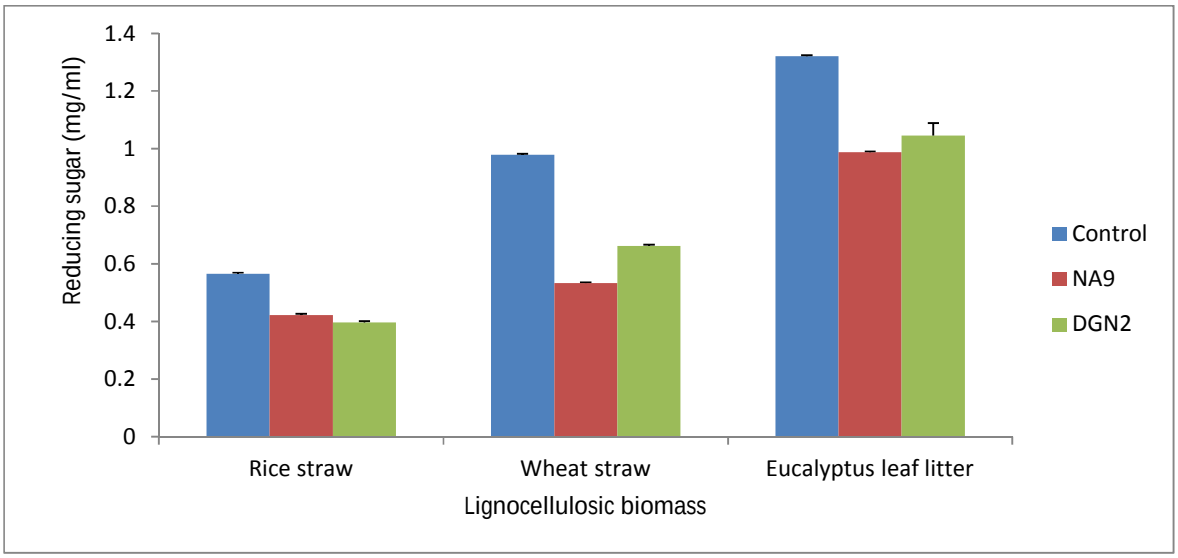

Fig 13: Reducing sugar (mg/ml) before and after bacterial inoculation in different media supplemented with lignocellulosic hydrolysates afterpre-treatment with 0.4N NaOH of biomass

The decreasing trend in the presence of sugar after inoculation could be seen as compared with control (uninoculated) lignocellulosic hydrolysate This suggests that some amount of sugar is being utilised by the bacterial isolates for their multiplication and production of the useful product i.e. lactic acid.

The pre-treated substrates i.e. rice straw hydrolysate, wheat straw hydrolysate and eucalyptus leaf litter hydrolysate when inoculated with bacterial isolates i.e. NA9 and DGN2. The maximum amount of sugar being utilized by NA9 was from wheat straw hydrolysate pre-treated with 0.6N H_2SO_4. Pre-treatment with 0.6N H_2SO_4 method is best

for separating the components of lignocellulosic substrates and for hydrolysing sugar fractions, which are being further utilised by bacterial isolates for their growth.

4.5 Estimation of protein

The protein was estimated in each pre-treated lignocellulosic biomass from the broth before the inoculation with NA9 and DGN2 and also after 48hrs of incubation at 50ºC without agitation. The protein content present in lignocellulosic hydrolysate was measured by using equation obtained through straight line of standard curve (Fig. 7)

1) Acid Hydrolysis

A) Pre-treatment with concentrated H_2SO_4

The increasing trend in the content of protein after inoculation could be seen as compared with control lignocellulosic hydrolysate, suggesting that these substrates are best for their growth as there is more accumulation of protein. Maximum concentration of protein was obtained in pre-treated eucalyptus leaf litter after inoculation with NA9 as compared with control (uninoculated) since protein concentration increased from 0.34 to 0.68 mg/ml followed by rice straw (0.505 mg/ml), wheat straw (0.35 mg/ml). When pre-treated biomass was inoculated with DGN2 bacterial isolates and their protein value was compared with control pre-treated biomass, the maximum concentration was observed in eucalyptus leaf litter (0.405 mg/ml) followed by wheat straw (0.192 mg/ ml) and rice straw (0.39 mg/ml) (Fig 14, Table 11).

Table 11: Protein content (mg/ml) in different lignocellulosic biomass hydrolysates (pre-treated with concentrated H_2SO_4) before and after bacterial inoculation

	Rice straw hydrolysate (mg/ml)	**Wheat straw hydrolysate (mg/ml)**	**Eucalyptus leaf litter hydrolysate (mg/ml)**
Control (uninoculated)	0.32 ± 0.004	0.12 ± 0.003	0.340 ± 0.002
NA 9	0.505 ± 0.05	0.35 ± 0.05	0.680 ± 0.03
DGN2	0.39 ± 0.01	0.192 ± 0.05	0.405 ± 0.00

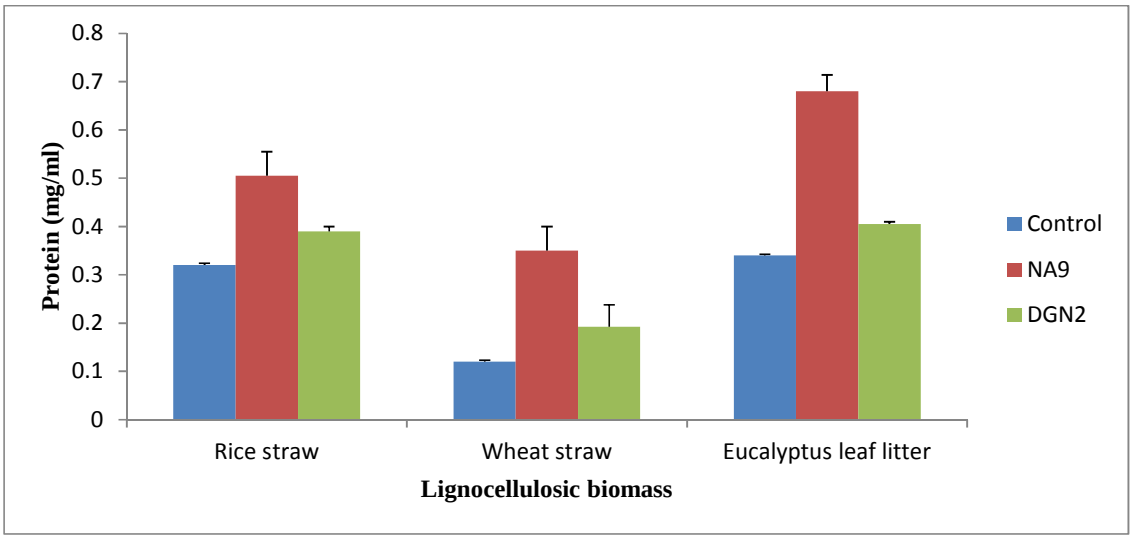

Fig 14: Protein content (mg/ml) in different lignocellulosic biomass hydrolysates (pre-treated with concentrated H_2SO_4) before and after bacterial inoculation

B) Pre-treatment with 0.6N H_2SO_4

The maximum concentration of protein was obtained in pre-treated wheat straw after inoculation with NA9 as compared with native wheat straw as protein concentration increases from 0.3 to 0.854 mg/ml followed by rice straw (0.852 mg/ml), eucalyptus leaf litter (1.306 mg/ml). When DGN2 was inoculated in pre-treated biomass, the maximum concentration of protein was found to be in wheat straw (0.852 mg/ml) followed by eucalyptus leaf litter (1.314 mg/ml) and rice straw (0.627 mg/ml) when compared with native (Fig.15, Table 12). The increasing trend in the concentration of protein after inoculation could be seen as compared with control lignocellulosic hydrolysate, suggesting that these substrates are best for their growth as there is more accumulation of protein.

Table 12: Protein content (mg/ml) in different lignocellulosic biomass hydrolysates (pre-treated with 0.6N H_2SO_4) before and after bacterial inoculation

	Rice straw hydrolysate (mg/ml)	Wheat straw hydrolysate (mg/ml)	Eucalyptus leaf litter hydrolysate (mg/ml)
Control (uninoculated)	0.610 ± 0.006	0.30 ± 0.004	1.130 ± 0.003
NA 9	0.852 ± 0.026	0.854 ± 0.019	1.306 ± 0.052
DGN2	0.627 ± 0.049	0.852 ± 0.028	1.314 ± 0.031

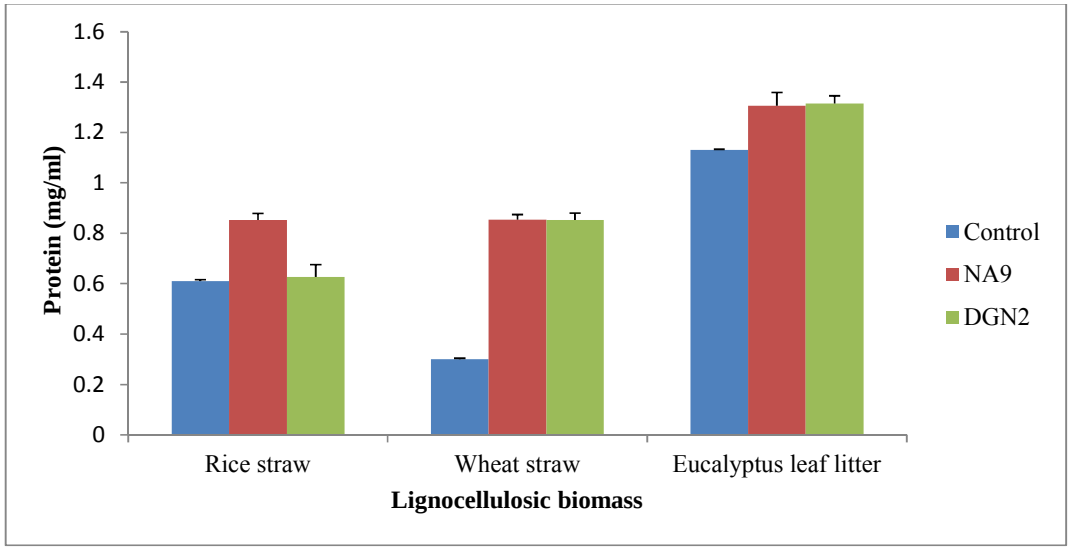

Fig 15: Protein content (mg/ml) in different lignocellulosic biomass hydrolysates (pre-treated with 0.6N H_2SO_4) before and after bacterial inoculation

2) Alkali Hydrolysis

A) Pre-treatment with 0.4N NaOH

The maximum concentration of protein was obtained in pre-treated wheat straw after inoculation with DGN2 as compared with native wheat straw as protein concentration increases from 0.56 to 0.997 mg/ml followed eucalyptus leaf litter (1.367 mg/ml), rice straw (0.85 mg/ml). When pre-treated biomass was inoculated with NA9 bacterial isolate and their reducing sugar value was compared with native pre-treated biomass, the maximum concentration was observed in eucalyptus leaf litter (1.302 mg/ml), wheat straw

(0.866 mg/ml) and rice straw (0.695 mg/ml) (Fig.16, Table 13). This showed that wheat straw is best substrate for good bacterial growth. The increasing trend in the content of protein after inoculation could be seen as compared with native lignocellulosic biomass hydrolysate. This thus ensures that these substrates are best for their growth as there is more accumulation of protein.

Table 13: Protein content in different lignocellulosic biomass hydrolysates (pre-treated with 0.4N NaOH) before and after inoculation with bacteria.

	Rice straw hydrolysate (mg/ml)	**Wheat straw hydrolysate (mg/ml)**	**Eucalyptus leaf litter hydrolysate (mg/ml)**
Control (uninoculated)	0.540 ± 0.004	0.56 ± 0.005	0.998 ± 0.003
NA 9	0.695 ± 0.028	0.866 ± 0.040	1.302 ± 0.048
DGN2	0.850 ± 0.012	0.997 ± 0.006	1.367 ± 0.011

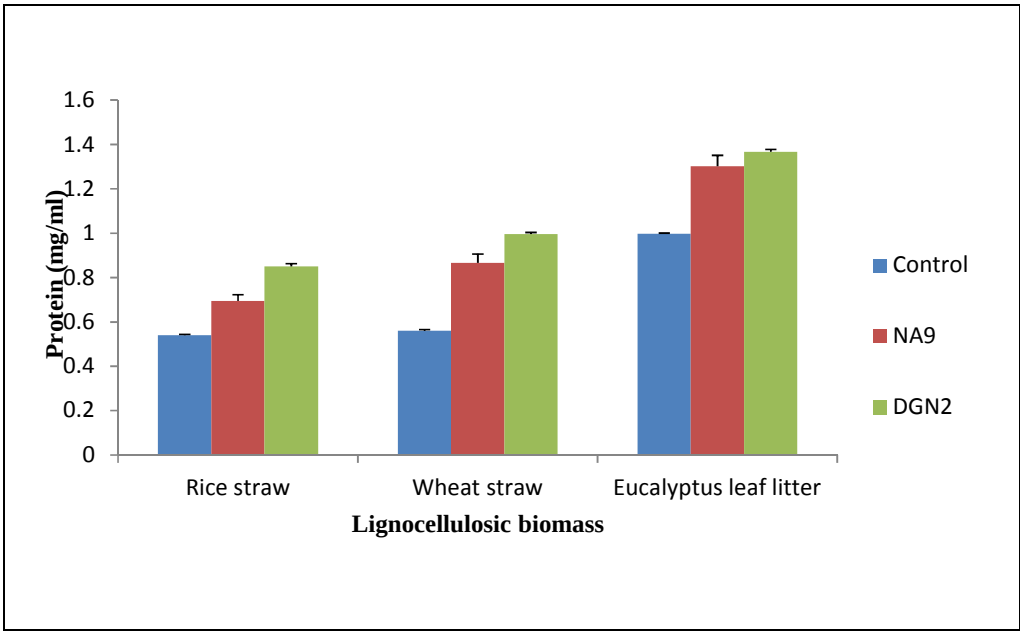

Fig 16: Protein content in different lignocellulosic biomass hydrolysates (pre-treated with 0.4N NaOH) before and after inoculation with bacteria.

4.6 Production of lactic acid from lignocellulosic biomass

Lignocellulosic substrates are rich in hemicellulose, cellulose and lignin content. Three different pre-treatment i.e. concentrated H_2SO_4, 0.6N H_2SO_4 and 0.4N NaOH, were done on lignocellulosic substrates to separate these structural components. Three substrates rice straw, wheat straw and eucalyptus leaf litter were used as substrates for growth of NA9 and DGN2 which are the potential lactic acid producers when xylose was used as a substrate. Hence, these were selected for action on these three different substrates for lactic acid production. Lactic acid was estimated in culture filtrate after 48 hrs of incubation at 50°C without agitation.

1) Acid hydrolysis

A) Pre-treatment with concentrated H_2SO_4

Using NA9 strain, the maximum amount of lactic acid was produced by eucalyptus i.e. 5.5 g/l followed by rice straw and wheat straw i.e. 1.3 g/l and 0.7 g/l respectively. Using DGN2 strain, the maximum amount of lactic acid was produced by eucalyptus leaf litter i.e. 3.25 g/l followed by wheat straw and rice straw i.e. 2.9 g/l and 1 g/l respectively (Fig. 17, Table 14). Eucalyptus leaf litter hydrolysate gave maximum productivity of lactic acid by NA9 and DGN2.

Table 14: Bacterial production of lactic acid from lignocellulosic biomass pre-treated with concentrated H_2SO_4

	NA9 (g/l)	**Absorbance at 600 nm**	**DGN2 (g/l)**	**Absorbance at 600 nm**
Rice straw hydrolysate	1.3	0.95	1	0.78
Wheat straw hydrolysate	0.7	0.652	2.9	0.854
Eucalyptus leaf litter hydrolysate	5.5	1.26	3.25	1.15

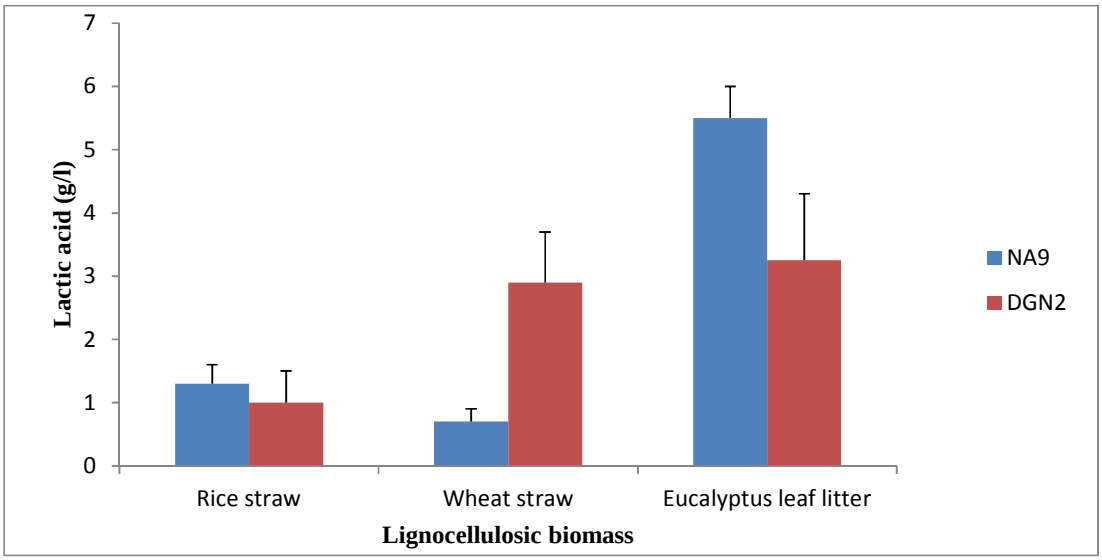

Fig 17: Bacterial production of lactic acid from lignocellulosic biomass pre-treated with concentrated H_2SO_4

B) Pre-treatment with 0.6N H_2SO_4

Using NA9 strain, the maximum amount of lactic acid was produced when eucalyptus leaf litter biomass was used as substratei.e. 2.2 g/l followed by rice straw and wheat straw i.e. 1.05 g/l and 0.62 g/l respectively. Using DGN2 strain, the maximum amount of lactic acid was also produced when eucalyptus leaf litter biomass was used as substrate i.e. 1.81 g/l followed by rice straw and wheat straw i.e. 0.62 g/l and 0.41 g/l respectively (Fig 18, Table 15). Maximum yield of lactic acid was obtained using eucalyptus leaf litter hydrolysate as substrate.

Table 15: Bacterial production of lactic acid from lignocellulosichydrolysates pre-treated with 0.06N H_2SO_4

	NA9 (g/l)	**Absorbance at 600 nm**	**DGN2 (g/l)**	**Absorbance at 600 nm**
Rice straw hydrolysate	1.05	0.751	0.62	0.75
Wheat straw hydrolysate	0.62	0.652	0.41	0.61
Eucalyptus leaf litter hydrolysate	2.20	1.964	1.81	1.45

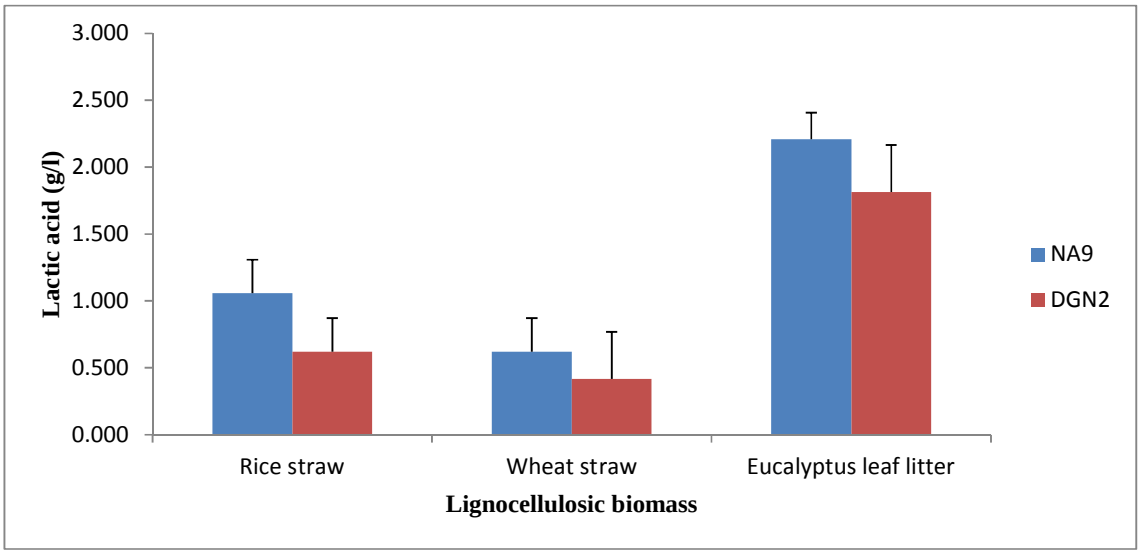

Fig 18: Bacterial production of lactic acid from lignocellulosic biomass pre-treated with 0.6N H_2SO_4

2) Alkali Hydrolysis

A) Pre-treatment with 0.4N NaOH

The maximum amount of lactic acid was produced by eucalyptus leaf litter hydrolysate i.e. 1.17 g/l followed by rice straw hydrolysate and wheat straw hydrolysate i.e. 0.62 g/l and 0.58 g/l respectively, using NA9. Using DGN2 strain, the maximum amount of lactic acid was produced by eucalyptus leaf litter hydrolysate i.e. 1.20 g/l followed by wheat straw hydrolysate and rice straw hydrolysate i.e. 0.61 g/l and 0.30 g/l respectively (Fig 19, Table 16).The different lignocellulosic biomass i.e. rice straw, wheat straw and eucalyptus leaf litter when pre-treated with 0.4N NaOH, eucalyptus leaf litter hydrolysate had maximum potential for lactic acid production as it showed maximum lactic acid productivity when inoculated with DGN2.

Table 16: Bacterial production of lactic acid from lignocellulosic biomass pre-treated with 0.4N NaOH

	NA9 (g/l)	Absorbance at 600 nm	DGN2 (g/l)	Absorbance at 600 nm
Rice straw hydrolysate	0.62	0.78	0.30	0.611
Wheat straw hydrolysate	0.58	0.683	0.61	0.741
Eucalyptus leaf litter hydrolysate	1.17	0.952	1.20	0.855

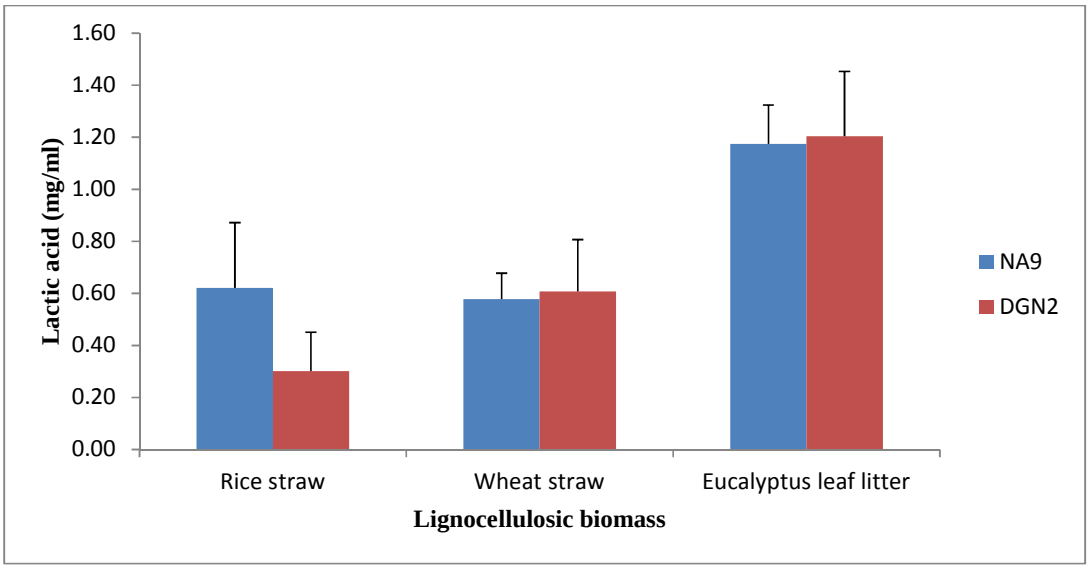

Fig 19: Bacterial production of lactic acid from lignocellulosic biomass pre-treated with 0.4N NaOH

After pre-treatment with three different methods i.e. concentrated H_2SO_4, 0.6N H_2SO_4 and 0.4N NaOH, maximum lactic acid production was shown by eucalyptus leaf litter hydrolysate even after inoculation with bacterial isolates i.e. NA9 and DGN2 followed by wheat hydrolysate and rice hydrolysate. This showed that maximum amount of sugar being realesed from eucalyptus leaf litter biomass when pre-treatment is given. A.B. Moldes *et al,* (2006) reported the yield of lactic acid to be 3.79 g/l using *Lactobacillus pentosus.* Hanson *et al*, (1972) reported the yield of lactic acid from *E. globules* to be 2.7-5.2 g/l. Yen *et al*., (2004) reported the lactic acid productivity from rice straw to be 3.1 g/l.

Hofvendahl *et al*., (1996) reported the lactic acid productivity to be 3.3 g/l. Garde *et al*., (2002) reported 90% yield of lactic acid from wheat straw after acid hydrolysis.

NA9 gave maximum lactic acid productivity using biomass hydrolysates that were pre-treated with either concentrated H_2SO_4, 0.6N H_2SO_4 or 0.4N NaOH and ensures that NA9 had potential for efficient lactic acid production. With acid hydrolysis, xylose concentration was found to be 10- fold higher than other pre-treatment methods since xylose is utilised by bacterium to convert it into lactic acid (Garde *et al*., 2002). As lactic acid was produced from hemicellulosic sugars, these were mostly obtained from acid hydrolysis (Musatto *et al*., 2010).

Conclusions

1. Out of 15 strains, 8 strains i.e. DGN1, DGB, NA15, DGS3, NA9, T8, DGN2 and DGS showed maximum growth in xylose containing media, showing these isolate sutilises carbohydrate through pentose phosphate pathway.
2. Eight bacterial isolates (DGN1, DGB, NA15, DGS3, NA9, T8, DGN2 and DGS) gave positive result on bromocresol green selective media, indicating that they can be used for the production of lactic acid.
3. Three strains DGN2, NA9 and DGS1 were found as the potential lactic acid producers, since they showed maximum titre of lactic acid, which was 0.432 g/l, 0.403g/l, 0.403g/l respectively.
4. Afer pre-treatment of different of lignocellulosic biomas, maximum lactic acid production was reported from Eucalyptus leaf litter (5.5 g/l).
5. Out of the three pre-treatment methods, 0.4 N NaOH treatment yielded highest of protein content i.e. 1.367 g, Eucalyptus leaf litter biomass showed maximum yield of lactic acid, protein content and reducing sugar.
6. Pre-treatment of biomass with concentrated H_2SO_4 was found to be best among the otherperformed pre-treatment methods i.e. with 0.6N H_2SO_4 and 0.4N NaOH.

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APPENDIX I

NUTRIENT BROTH (pH- 7.0)

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

ENRICHMENT BROTH (pH-7.0)

INGREDIENTS	QUANTITY (g/l)
Xylose	10
YE	5

MODIFIED MINERAL SALT MEDIUM (pH- 7.0)

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

NITRATE BROTH MEDIUM (pH- 7.2)

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

MR- VP BROTH (PH- 6.9)

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

UREA AGAR (pH- 6.9)

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

CARBOHYDRATE BROTH (pH- 6.8)

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

STARCH AGAR (pH- 7.2)

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

APPENDIX II

LUGOL'S IODINE SOLUTION

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

METHYL RED SOLUTION

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

NITRATE REAGENT (SOLUTION A)

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

NITRATE REAGENT (SOLUTION B)

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

VP SOLUTION I

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

VP SOLUTION II

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

DNS REAGENT (1%)

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

Volume was made up to 1000 ml