STUDIES ON MICROBIAL PRODUCTION OF LACTIC ACID BY BATCH FERMENTATION

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled **STUDIES ON MICROBIAL PRODUCTION OF LACTIC ACID BY BATCH FERMENTATION**, in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Paper Technology of Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July, 2004 to July, 2011 under the supervision of Dr. Uttam Kumar Ghosh, Assistant Professor, Department of Paper Technology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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ABSTRACT

The present thesis work includes detailed comparative studies between the four standard *Lactobacillus* strains acquired from the culture collection NCIM-National Collection of Industrial Microorganisms, at National Chemical Laboratory Pune India, for their potential of lactic acid production. The study includes liquid state batch fermentation, liquid state fed batch fermentation, studies on batch growth and solid state fermentation. The study not only aims towards the higher production of lactic acid, by the use of carbohydrate rich waste materials such as dairy whey, bagasse, starch, but it also highlights the significance of utilizing inexpensive nitrogen source dried baker's yeast. The solid state fermentation studies have been carried out with an aim to utilize several cheap and abundant agricultural and forestry waste materials such as sugarcane bagasse, wheat bran and pine needles and to investigate their potential to support the lactic acid producing bacterial strains. The solid state fermentation studies till now have involved predominantly fungi, but bacterial solid state fermentation has received lesser attention. Hence the solid state studies for these *Lactobacillus* strains were aimed to determine their comparative lactic acid production performances on the different bed materials with a supporting production media containing pure sugar or partially substituted pure sugar with carbohydrate rich waste materials. Very few studies have been reported on coculture, that present a detailed account of, its performances that were better than the pure strains, in terms of lactic acid production, through batch, fed batch and solid state fermentation methods. The present work involved the production study of coculture with different sources of carbon and nitrogen, pH, salts, temperature, neutralizer and agitation etc. With the aim to reduce the use of costly sugars in the bacterial culture development, modified MRS culture media has been used for the growth studies, utilizing cheap carbohydrate rich waste materials such as acid treated bagasse extract under boiling or autoclaving conditions and dairy whey. The coculture was prepared from strain-1 (*Lactobacillus delbrueckii*) and strain-2 (*Lactobacillus pentosus*). Coculture showed overall maximum lactic acid production 93.57, 115.56, 114.96 and 116.01g/L with pure glucose, lactose, whey substituted glucose and whey substituted lactose respectively. Most suitable pH values for higher production of lactic acid were 6.5, 6.75, 6.0 and 6.75 for strains-1, 2, 3, 4 and coculture respectively. An agitation of 180rpm (during shake flask batch and fed batch fermentations) and a temperature of 37^0 C was found most

suitable for all the strains. However the strain-1 and its coculture, had better production at 40^0 C. The cheap nitrogen source, dried baking yeast, produced higher lactic acid concentrations at its 20 g/L input, which were found competitive with some of the synthetic nitrogen sources such as meat extract. In the fed batch fermentations also, coculture reached overall maximum lactic acid production around 162 g/L. The solid state fermentation studies had the overall maximum lactic acid production of 54.25 g/L with coculture on wheat bran bed material. The batch growth studies indicated maximum cell biomass growth and pH drop with coculture on the modified MRS media as compared to other strains. Hence it can be concluded from the present studies that, coculture can be potentially beneficial for the lactic acid fermentation industries.

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(1) Utilization of agroindustrial waste and dairy waste in solid state production of lactic acid.

(2) Utilization of bagasse hydrolyzate for lactic acid production by fed batch fermentation.

(3) Utilization of cheese whey for lactic acid production by batch and fed batch fermentation.

(4) Comparative studies in batch and fed batch production of lactic acid by utilization of hydrolyzed potato starch.

(5) Studies on fermentative batch production of lactic acid by application of mixed sugar.

(6) Utilization of wheat bran agro-industrial residue as bed material in solid state bacterial production of lactic acid

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CHAPTER-1

INTRODUCTION

Lactic acid (2-hydroxypropanoic acid) has been found by Swedish scientist Sheele in 1780, in sour milk, hence, considered it as milk component but later on, in 1857 Pasteur discovered that it was actually a fermentation metabolite, due to action of microbes on milk[65, 112]. It is a chiral multifunctional organic acid, that is applied as an acidulant, flavouring agent and as preservative, in food and dairy industry and serves as feedstock in production of ecofriendly biodegradable polymer (PLA) and its salts having biomedical uses. Lactic acid fermentation has been applied for the preservation of the vegetables such as carrot and radish. With large scale utilization of polylactate biopolymers, about 20-30% of global production of lactic acid has been used as monomer feedstock for this industry in 2005 [119]. The global demand for lactic acid has been reported to approximately around 130,000 to 150, 000 metric tons per year (1999), and predicted demand of 500,000 metric tons by 2010[112, 119]. Major portion (90%) of the lactic acid production is through fermentation, carried out by various fungi and lactic acid bacteria(LAB), which provide stereo-specific L(+) and D(-) forms of high purity lactic acid, while chemical synthesis provides a racemic mixture DL lactic acid which is expensive to separate. The *Lactobacillus* is the largest genus, which contains about 80 identified species, some of the important ones are *Lactobacillus delbrueckii, L. plantarum, L. helveticus, L. brevis, L. casei* etc. The LAB can be homofermentative (produce more than 85% lactic acid), heterofermentative (produce 50% lactic acid with ethanol and carbon dioxide) and facultatively heterofermentative (produce carbon dioxide and other byproducts only under certain conditions or substrates)[109]. The batch and fed batch production of lactic acid provide higher lactic acid concentrations than the continuous fermentations. Apart from lactic acid, the lactobacilli are used as probiotics and also synthesize nisin.

The current major markets for lactic acid are food and related industries, but the emerging markets for PLA polymer would cause a significant increase in growth of lactic acid consumption. Currently, the worldwide consumption of lactic acid is estimated to be 130,000–-150,000 (metric) tons per year [112]. The lactic acid for food grade use (50 % purity) has price $1.38 /kg, while for application as salts, the lactic acid price is $1.54 /kg [112]. Technical

grade lactic acid with 88% purity costs, $ 1.59 /kg[112]. Lactic acid consumption in chemical applications, which include PLA polymer and new green solvents, such as ethyl lactate, is predicted to grow 19% per year. There are several major manufacturers of fermentative lactic acid, including Purac (Netherlands), Galactic (Belgium), Cargill (USA), and several Chinese companies. In late 1997, Cargill had a joint venture with Dow Chemical for the production of biodegradable polymer, polylactic acid. In early 2002, Nature Works LLC completed the construction of a PLA plant with the production capacity 140 000 (metric) tons of PLA per year [112].

Besides costly pure materials, agro-wastes such as wheat bran, sugarcane straw, orange peel have been used for the fermentative production of different acids and enzymes .The residual starch (60-65%) in fibrous residues of cassava after extraction of starch, has been utilized for lactic acid production [76]. The production media materials amount to about 38% of total cost of lactic acid production. Hence, the use of low cost carbohydrate rich raw materials such as starch, molasses, bagasse hydrolyzates, wheat bran, etc. and nitrogen sources like whey permeate, baker's yeast, corn steep liquor and casein hydrolyzate etc. are beneficial for lactic acid production. Cheese whey has been utilized as carbon source in liquid state production of lactic acid.

Large quantity of agroindustrial waste, sugarcane bagasse (about 8-10 ton/100 ton of sugarcane), are liberated, which are used for power generation, paper making, various types of boards and chemical preparation [92]. The huge quantity of sugarcane should be processed by the sugar or jaggery industries quickly otherwise the post harvest degradation reduces the sucrose content of sugarcane, which can cause apparent loss to the sugar industries and bagasse based fermentation industries. Such abundant amount of bagasse can be utilized as bed material in (SSF) and its acid hydrolyzates have been used as potential carbon sources for lactic acid production. In the present studies the utility of such abundant bagasse as bed material in (SSF) and role of its acid hydrolyzate, as carbon source has been investigated.

The batch and fed batch modes utilize the suspended cell growth systems for lactic acid production, where the free cells are suspended in an agitated liquid production medium. Some of the significant advantages of suspended cell growth are, maintenance of uniformity in

microbial concentration and temperature, while the cell aggregation and sedimentation are avoided. Cell damage, wall growth, contamination and higher cost of equipment and power for agitation, are some of the disadvantages associated with the suspended cell growth. The present work compares the lactic acid production capacities of all the lactobacilli under study under the influence of different parameters. The previous studies on *Lactobacillus* growth mostly utilized synthetic carbon sugars that proved expensive, for the industries requiring large volumes of inoculum, hence bagasse hydrolyzate and whey have also been applied here. Very few of the liquid state production studies have utilized coculture but in the present work the lactic acid production from coculture has been studied in detail. Majority of previous studies only described the advantages of calcium carbonate as neutralizer in lactic acid fermentation, but some of its adverse effects and comparison with sodium hydroxide as neutralizer have been discussed here. Lactic acid production by SSF technology has been investigated predominantly with fungi but a very few studies are available with the bacteria. Thus present study attempts a comprehensive approach in lactic acid production, with lactobacilli, utilizing different bed materials through SSF. The pine needles which are known to cause fire hazards in forests, have been used in preparing bio-composite polymers. Apart from the present study there is hardly any report of pine needles being used as bed material in lactic acid production by SSF.

CHAPTER-2

REVIEW OF LITERATURE

2.1 HISTORICAL BACKGROUND OF LACTIC ACID

The knowledge about lactic acid can be traced long back in history when in 1780, Swedish chemist Carl Wilhelm Sheele, isolated lactic acid for the first time from sour milk, and considered it as one of the components of milk. The origin of the term lactic acid is related to the term, "acide lactique," which was stated by Lavoisier in 1789. In 1857 Louis Pasteur discovered that lactic acid was a metabolite generated by the action of fermentative microorganisms on milk. This finding contradicted the previous view that considered it as a milk component [112]. A rise in industrial production of lactic acid in 1881 was evidenced after, French scientist Fremy, produced lactic acid by fermentation. The lactic acid production at the commercial level was first initiated in 1881, by Avery of USA [108].

2.2 APPLICATIONS OF LACTIC ACID

Lactic acid (α- hydroxy propionic acid), is an organic carboxylic acid that, occurs in the human, animal, plant and microbial systems. It exists in two optically active forms L (+) and D(-). The L(+) form is suitable for the human body hence this form is important in dairy , food and pharmaceutical preparations, while high levels of D(-) are considered harmful for the human consumption, as it may cause acidosis and decalcification[112]. Lactic acid is a multifunctional speciality chemical used as preservative, acidulant, pickling agent flavouring agent and probiotics in food and dairy industries and in production of ecofriendly biodegradable polymer (PLA) having biomedical uses[65, 1] It is odourless, nonvolatile and generally recognized as safe (GRAS) by FDA in US. The shelf-life of packaged poultry and fish products gets enhanced by the addition of aqueous solution of lactic acid. Lactic acid fermentation has been applied for the preservation of the vegetables such as carrot and raddish [46].The esters of lactic acid are used as emulsifying agents in baking foods (stearoyl-2-lactylate, glyceryl lactostearate, glyceryl lactopalmitate) [65].The lactic acid possesses very high reactivity due to the presence of both hydroxyl and carboxyl groups, hence it has the capability as a feed stock for production of various chemicals such as acetaldehyde, acrylic acid, propylene glycol and 2,3 pentandione [67]. The pharmaceutical and cosmetic

applications of the lactic acid include its utilization in topical ointments, lotions, humectants, anti acne solutions, dialysis applications, and calcium lactate can be used for calcium deficiency therapy and as anti caries agent [65].The applications of the lactic acid have been summarized in the Table1.

Table 1 Applications of lactic acid

Chemical industry	Feedstock for Chemical production	Food, dairy And Agriculture	Cosmetic industry	Pharmaceuticals
Cleaning agents	Poly-lactic acid	Acidulant	Moisturizers	Prosthesis
Metal complexing agents	Acetaldehyde		Humectants	Surgical sutures
Neutralizers	Propionic acid	Flavors	Skin lightning agents	Drug delivery
Chiral intermediates	Acrylic acid	Mineral supplement	Antitartar agents	Mineral supplement as calcium lactate
Scale removal agents	Propylene oxide	Pickling	pH regulators	Tablets
Solvents	Ethyl lactate	Silage preparation	Anti acne agents	Dialysis solutions
pH regulators	2,3-pentandione	Antifungal activity	Agents for skin rejuvenation	I.V. solutions

Source [112, 18, 106, 91]

2.3 SYNTHESIS OF LACTIC ACID

The lactic acid (2-hydroxy propanoic acid) obtained through microbial fermentation is preferred over chemical synthesis methods, as it provides steriospecific L(+) and D(-) forms of high purity lactic acid, while chemical synthesis provides a racemic mixture of DL lactic acid which is expensive to separate [6].To reduce high costs involved in the purification of lactic acid isomers in chemical synthesis, 90% of world wide lactic acid production is based on microbial fermentation [2].An optically pure product can be obtained through proper selection of strains of lactic acid bacteria, which produce only one of the isomers of lactic acid, while the production through synthetic route always provides racemic mixture of lactic acid [35, 36]

2.3.1 Chemical Synthesis of Lactic Acid

The manufacture of lactic acid through synthetic pathway started in 1963 in Japan and United States. The major steps in the chemical synthesis of lactic acid include lactonitrile formation (Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile). This reaction occurs in liquid phase at high atmospheric pressures. The crude lactonitrile is recovered and purified by distillation, hydrolyzed by concentrated sulphuric acid or hydrochloric acid followed by esterification and acid hydrolysis to provide lactic acid [65]. The scheme of reactions is given below:

(a) **Addition of Hydrogen Cyanide**

$CH_3CHO + HCN \rightarrow CH_3CHOHCN$

Acetaldehyde Hydrogen cyanide Lactonitrile

(b) **Hydrolysis by H_2SO_4**

$CH_3CHOHCN + H_2O + 1/2\ H_2SO_4 \rightarrow CH_3CHOHCOOH + 1/2\ (NH_4)_2SO_4$

Lactonitrile Sulphuric acid Lactic acid Ammonium sulphate

(c) **Esterification**

$CH_3CHOHCOOH + CH_3OH \rightarrow CH_3CHOHCOOCH_3 + H_2O$

Lactic acid Methanol Methyl lactate

(e) **Hydrolysis by H_2O**

$CH_3CHOHCOOCH_3 + H_2O \rightarrow CH_3CHOHCOOH + CH_3OH$

Methyl lactate Lactic acid Methanol

2.3.2 Microorganisms for Lactic Acid Production

Major portion of the lactic acid production is through fermentation, carried out by various fungi such as *Rhizopus oryzae* and *Rhizopus arrhizus* and lactic acid bacteria (LAB). The LAB consist of several important genera such as *Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Aerococcus and Leuconostoc* etc. [109]. LAB, normally consist of gram positive, cocci except lactobacilli and carnobacteria which have lactic acid as major end product. These are facultatively anaerobic, catalase negative, non motile and non spore forming bacteria. They have high acid tolerance and can survive between 20°C and 45°C.The *Lactobacillus* forms the largest genus containing about eighty identified species, some of the important ones are *Lactobacillus delbrueckii, L. plantarum, L. helveticus, L.brevis and L.casei ,L. acidophilus* etc.

[24,109]. On the basis of the fermentation behavior the LAB have been divided into three well categorized groups:

(1) **Homofermentative:** They provide single product i.e. lactic acid (more than 85%). They usually metabolize glucose by EMP (Embden Meyerhoff- Paranas) or glycolytic pathway. They generate two moles of lactic acid from one mole of glucose and generate two moles of ATP per molecule of glucose. Due to higher generation of lactic acid per mole of glucose the homofermentative lactic acid bacteria are majorly utilized in the commercial production of lactic acid.

(2) **Heterofermentative:** These lactobacilli produce lactic acid to the extent of 50% only with other products such as ethanol, diacetyl, formate, acetoin or acetic acid and carbon dioxide. The initial degradation of glucose is through pentose phosphate pathway, upto xylulose-5-phosphate which forms glyceraldehyde-3 phosphate and acetyl phosphate due to the action of phosphoketolase enzyme.

(3)**Facultatively Heterofermentative**: They produce carbon dioxide and other by products only under certain condition or substrates [109].

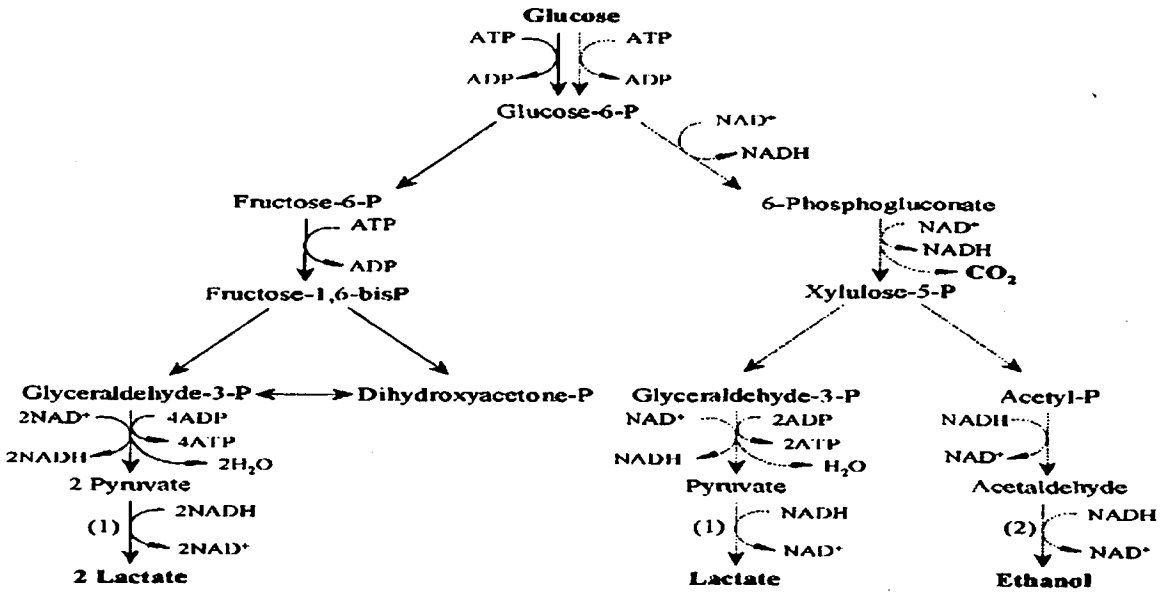


Figure1 Homofermentative (solid line)and heterofermentative (dotted line) metabolic pathways of lactic acid bacteria (1), lactate dehydrogenase; (2), alcohol dehydrogenase Source[112].

Metabolic pathways of homofermentative and heterofermentative LAB are given in Figure 1. The formation of D or L lactic acid from pyruvic acid depends upon the presence of the D or L

(LDH) Lactate dehydrogenase enzymes (EC 1.1.1.27 and EC 1.1.1.28) in the concerned LAB involved in lactic acid production [68]. Age of cultures and pH conditions may influence the L(+)/D(-) ratio in lactic acid product [18].The homofermentative, heterofermentative nature and the sterioisomers of lactic acid produced by some of the lactobacilli have been summarized in the Table 2.

Table 2 Isomeric forms of NAD linked LDH and lactic acid provided by different Lactobacilli through homofermentative or heterofermentative pathways of lactic acid synthesis.

Lactobacillus sp.	Homofermentative	Heterofermentative	Isomeric forms of NAD linked LDH and lactic acid product
Lactobacillus bulgaricus	+	_	L (+)
L.delbrueckii	+	_	D(-)
L. lactis	+	_	D(-)
L. casei	+	_	L(+), D(-)
L. plantarum	+	_	L(+), D(-)
L. brevis	_	+	L(+), D(-)
L. curvatus	_	+	L(+), D(-)
L. fermentum	_	+	L(+), D(-)

Source: [108, 25]

Apart from lactic acid, the lactobacilli are used as probiotics and also synthesize nisin [107, 94]. Lactobacilli are potent producers of wide range of antagonistic primary and secondary metabolites such as, organic acids, diacetyl (flavouring agent), bacteriocins (nisin) and antibiotics [80]. Lactobacilli are also in demand for probiotic properties, as they play an important role in stabilizing the intestinal microflora by checking the colonization of pathogenic microorganisms[102]. The recent research studies indicate that antifungal compounds released from various strains of *Lactobacillus sp.* are helpful in checking the spoilage or pathogenic fungi in food, feed and growing plants. The cell free extracts of *L. plantarum* strains VTT E-78076 (E 76) and VTT E– 79098 (E 98) have been found effective against *Fusarium sp.* fungi [57]. As a remedial measure against the attack of post harvest spoilage fungi (*Penicillium roqueforti* and *Aspergillus flavus*) the lactic acid bacteria are added during silage making. The lactic acid liberated from them, reduces the pH, resulting into

growth inhibition of fungi and pathogenic bacteria, that consequently prevents the contamination of silage. Various antifungal compounds have been identified from different strains of *L. plantarum* such as cyclic dipeptides, phenyl lactic acid and hydroxy fatty acids [91]. The 'Reuterin' is a newly found antibiotic from *L. reuteri* which has broad spectrum inhibitory activity against yeast *Saccharomyces cerevisiae*, gram positive and gram negative bacteria, protozoa and *Trypanosoma cruzi* . Hence reuterin is a broad spectrum antimicrobial agent. It has (i) Antibacterial (ii) Antiviral (iii) Probiotic activities [105].

2.4 LACTIC ACID PRODUCTION THROUGH LIQUID STATE BATCH FERMENTATION

The most frequently used method adopted for the industrial production of the lactic acid is through batch fermentation. The batch fermentation provides higher concentration and yields than the continuous fermentations as there is higher or complete substrate utilization, in batch fermentation where as in the continuous one it has residual concentration of unutilized substrates. Very high dilution rates in the continuous fermentation may cause higher portion of unutilized substrate and a tend towards a wash out condition, for the microbes carrying out the continuous fermentations, subsequently the lactic acid production may be lower than the batch fermentation. A productivity of 6.4 g/L/h has been obtained through cell-recycle repeated batch fermentation[67].This system had additional advantages of 26% of the total requirement of yeast extract as compared to conventional batch fermentation(for obtaining same level of lactic acid) and a maximum cell concentration of 28 g/L that may lead to better productivity. The application of agitation in batch fermentation is necessary for maintenance of uniform cell density and nutrient concentration throughout the fermentation broth, for higher productivity of lactic acid. For this purpose stirred tank reactors with modification of cell recycle have been found better where as the orbital shaker systems are applied in the laboratory level studies. The homofermentative lactic acid bacteria with high yield (above 0.90g/g with glucose) are predominantly utilized in industrial production of lactic acid [112]. Batch production of lactic acid has been studied with *L. plantarum*, on lactose based media and *L. rhamnosus*, on chemically defined media [114, 12].

The inoculum is prepared in a culture media such as MRS (De Mann Rogosa Sharpe Media) which contains rich nitrogen sources, such as proteose peptone, yeast extract and beef extract,that helps in development of high concentration of biomass. The cell density is further

increased through inoculum development process. The production media contains pure carbon sources or carbohydrate rich raw materials, nitrogen source and salts, which are properly mixed and sterilized in *situ* in the reactor vessel. The developed inoculum is added to the cool sterilized media, and the reactor is operated on optimum temperature, agitation and pH. The lactic acid formed during fermentation can be inhibitory towards the bacteria itself [74]. Without pH control of the fermentation broth, yield of lactic acid decreases by 30 – 50%, hence neutralizing agents such as sodium hydroxide, calcium carbonate and ammonium hydroxide are usually added, for higher yield of lactic acid [62]. Hence the reactor systems should have proper pH measurement and control. After due course of batch fermentation the crude lactic acid in the broth is subjected to separation and purification through several downstream processing methods [47].

Table 3 Lactic acid production by various lactobacilli

Lactic acid bacteria	Lactic acid (g/L)	Yield(g/g)	Productivity (g/L.h)
Lactobacillus delbrueckii NCIMB- 8130	90	0.97	3.8
Lactobacillus pentosus ATCC-8041	21.8	0.77	0.80
Lactobacillus plantarum ATCC-21028	41.0	0.97	1.0
Lactobacillus bulgaricus NRRL B-548	38.7	0.90	3.5
Lactobacillus helveticus ATCC-15009	65.5	0.66	2.7
Lactobacillus rhamnosus ATCC-10863	67.0	0.90	2.5
Lactobacillus casei NRRL B-441	82.0	0.91	5.6
Lactobacillus helveticus R-211	66	-	1.4
Lactobacillus casei NRRL B-441	46	-	4.0

Source-[112]

2.4.1 Factors Effecting Production of Lactic Acid in Liquid State Fermentation

There are various process related factors such as carbon sources, nitrogen sources, pH , neutralizing agents, salts, agitation and temperature that determine the production and productivity of lactic acid during liquid state batch fermentation. The effects of these factors have been described below.

2.4.1.1 Application of carbon sources

The lactobacilli have quite high requirement of carbon for lactic acid production. They draw their carbon demand from either pure sugars or from carbohydrate rich waste materials. The pure sugars may be glucose, sucrose, lactose, maltose, xylose and arabinose. The higher production costs incurred with the synthetic sugar sources and abundance of the renewable lignocellulosic agro wastes encourages the use of carbohydrate rich raw materials as carbon sources in lactic acid fermentation. Hence, the use of low cost carbohydrate rich raw materials, like starch, molasses, bagasse hydrolyzates, wheat bran, etc., have been found beneficial in lactic acid production. The selection of organism for lactic acid production is based on the type of carbohydrate pure sugar or raw material to be fermented. *Lactobacillus delbrueckii* subspecies *delbrueckii* is able to ferment disachharides such as sucrose or other hexose sugars but can't be utilized with any of the pentose sugars. *Lactobacillus delbrueckii* subspecies *bulgaricus* is able to use lactose. *Lactobacillus helveticus* is able to use both lactose and galactose. *Lactobacillus amylophylus* and *Lactobacillus amylovirus* are able to ferment starch. *Lactobacillus lactis* can ferment glucose, sucrose and galactose. *Lactobacillus pentosus* have been used to ferment sulfite waste liquor or pentose pure sugars [65]. The desirable traits for a raw material to be used in the industrial production of lactic are low cost, easy availability, abundant quantity, low levels of contaminants, ability to get fermented with least pretreatments, rapid fermentation rate, high lactic acid yields, little or no by-product formation [82]. The cost for raw material possessed 68% of the total cost for lactic acid production from whey permeate and yeast extract using *Lactobacillus bulgaricus*[101,67].

Abundantly found carbohydrate rich waste materials such as dairy whey, sugarcane bagasse (acid hydrolyzed extracts) and starches (directly or either as acid or as enzyme hydrolyzates, from potato, wheat, rice and cassava etc.) have frequently been used as carbon source in lactic acid production. Cheese whey has been utilized as carbon source in liquid state

production of lactic acid [26]. Apart from 5% available lactose, the whey also contains stimulatory substances for growth of the bacterial cells.

Large quantity of agro-industrial waste, sugarcane bagasse (about 8-10 ton/100ton of sugarcane) are liberated, which are used for power generation, paper making, various types of boards and chemical preparation [92].The huge quantity of sugarcane should be processed by the sugar or jaggery industries quickly to prevent the post harvest degradation (that reduces the sucrose content of sugarcane), that might additionally cause loss to the sugar industries and bagasse based fermentation industries [96].Acid hydrolyzates have been used as potential carbon sources for lactic acid production [58].In the present studies the utility of acid hydrolyzed extract from sugarcane bagasse as carbon source has been investigated. The residual starch (60-65%) in fibrous residues of cassava after extraction of starch, has been utilized for lactic acid production [76].The starchy materials have to be hydrolyzed into fermentable sugars (glucose) before fermentation, because they consist mainly of a(1,4)- and a(1,6)-linked glucose [77,36,67]. Amylase-producing *L. amylophilus* and *L. amylovorus* can be applied for the direct fermentation of starchy materials into lactic acid [112].

2.4.1.2Effect of nitrogen sources

Nitrogen sources form an integral part of bacterial nutrition as nitrogen exists as component of important biomolecules such as (nucleic acids) DNA, RNA, enzymes, proteins and amino acids that are essential for the cell growth and metabolism. Nitrogen is required for the synthesis of purines, pyrimidines, amino acids, some of the carbohydrates, lipids and cofactors. Yeast extract, meat extract, peptones etc. act as the synthetic organic nitrogen sources where as the salts such as, ammonium sulphate, ammonium nitrate and nitrite salts constitute the inorganic nitrogen source[119].The amino acids that are essential for growth and existence for lactobacilli consist of arginine, cysteine, tryptophane, histidine, tyrosine, isoleucine, glutamic acid, valine, leucine, methionine, phenyl alanine, proline and threonine etc. Many of these amino acids can be supplied from the nitrogen sources [34]. Yeast extract has been analyzed to contain, 60.2% protein, 12.9% carbohydrates on dry basis. It also contains important vitamins such as 14 mg/kg, thiamine (B_1);35 mg/kg riboflavin(B_2); 420 mg/kg niacin(B_3); 140 mg/kg, pantothenic acid (B_5);17.5 mg/kg, pyridoxine(B_6) ; 0.3 mg/kg, cyanocobalamin (B_{12}); 0.5 mg/kg, biotin and 0.6 mg/kg, folic acid[116].The important amino acids supplied by the yeast extract for lactobacilli are 36.9 g/kg valine; 42.3 g/kg leucine; 41.4

g/kg lysine; 48.6 g/kg alanine; 23.4 g/kg arginine; 93.6 g/kg glutamic acid; 32.4 g/kg phenyl alanine; 7.20 g/kg methionine; 14.4 g/kg threonine; 21.6 g/kg glycine; 17.1 g/kg proline; 15.3 g/kg serine; 10.8 g/kg histidine and 48.6 g/kg aspartic acid [116]. The mixture of amino acids, peptides, and amino acid amides usually stimulates the growth of LAB, to obtain significantly higher growth rates than those available with free amino acids .The mineral components the yeast extract consist of 5.316 g/kg potassium, 2.430 g/kg phosphorus,1.119 g/kg sodium, 1.170 g/kg chloride, 0.171 g/kg calcium and 0.502 g/kg magnesium [116].The presence of mineral components in yeast extract can be helpful for the *Lactobacillus* in the better uptake of sugars and formation of ATP which consequently can be beneficial in lactic acid production. The nitrogen source meat extract contains about 6 to 9% of total nitrogen,9.8-11% peptone like bodies and polypeptides;11.41% proteoses;, 5.552% creatin and creatinin; 0.86-1.84% purine bases; 7.6% other meat bases ; 6.76-8.14% potash;5.54% phosphoric acid and 0.82% ammonia[115].The nitrogen source proteose peptone is an enzymatic digestion product of animal tissues. It is highly nutritious source for the growth of wide range of organisms, hence it is used in the fermentation industry for starter cultures. It contains 10-12.57% total nitrogen (3.4-4.3% amino nitrogen; significant amount of amino acids such as 34.9 g/kg alanine; 35.4 g/kg arginine; 65 g/kg aspartic acid; 3.8 g/kg cysteine; 155.10 g/kg glutamic acid; 34.10 g/kg glycine; 19.8 g/kg histidine; 36.60 g/kg isoleucine; 66.8 g/kg leucine;58.10 g/kg lysine; 16.4 g/kg methionine ;35.3 g/kg phenyl alanine; 71.1 g/kg proline; 43.0 g/kg serine; 34.6 g/kg threonine; 8 g/kg tryptophan;15.9 g/kg tyrosine; 48.2 g/kg valine;0.024% calcium; 0.023% magnesium; 1.40% potassium and 2.70% sodium[21].

From the detailed compositions of the yeast extract , meat extract and proteose peptone it is evident that only yeast extract contains all the three nutrients, vitamins , amino acids and carbohydrates, hence it may prove most beneficial for the growth and lactic acid production by the lactobacilli. Due to their restricted ability to synthesize their own growth factors such as B vitamins and amino acids the lactobacilli require some elements for growth, from carbon and nitrogen sources, in the form of carbohydrates, amino acids, vitamins, and minerals [112, 9]. It has been found that yeast extract although a better nitrogen source, accounts for about 38% of total production medium cost [7,101]. Hence some of the workers have used cheaper nitrogen source such as dried baker's yeast (*Saccharomyces cerevisiae*) as nitrogen source in production of lactic acid [7]. Yeast are a unicellular fungi or plant-like microorganism that exists in or on all living matter i.e. water, soil, plants, air, etc. They are a microbial eukaryote,

associated with ascomycetes that are rich in protein and vitamin B. Their proteins and vitamins components can be beneficial for the better growth of lactobacilli and enhance lactic acid production.

2.4.1.3 Effect of pH and neutralizing agents

The lactic acid fermentation is highly sensitive towards the initial pH and the variations in pH, occurring during the course of fermentation, due to lactic acid production. The hydrogen ion concentration of medium has the maximum influence on the microbial growth. The pH influences the microbial cells in a number of ways such as, functioning of microbial enzymes, the transport of nutrients into the cell, limiting the synthesis of metabolic enzymes responsible for the synthesis of new protoplasm, affecting the RNA and protein synthesis [69]. A pH of 5.5 has been found suitable for using *L. helveticus*, while for *L. casei* the pH range of 6.0-6.5 has been reported optimal, for lactic acid production [27, 42].The survival rate of the *Lactobacillus* has been studied under different pH conditions[28].

Accumulation of higher quantities of the acid product existing in free acid form shows a very low pH, at which inhibition of the acid synthesizing cells takes place. The organic acids such as lactic acid are inhibitory towards bacterial growth as they can chelate essential growth elements like iron, while its undissociated form is lipophilic that could enter the bacterial cell and cause greater inhibition than externally active strong mineral acid[74]. Diffusion of solutes (if polar) through membranes takes place down the electrochemical potential gradient, aided by the carrier proteins that overcome the activation energy required by that solute to enter through the lipid portion of the membrane[41]. During the fermentative production of lactic acid the undissociated form of lactic acid (a strong growth inhibitor) diffuses across the cell membrane. Various theories have been proposed about inhibitory effects of lactic acid on bacterial growth such as (i) Cytosol acidification due to acid influx, (ii) Dissipation of membrane potential and (iii) Accumulation of anions intracellularly [73]. Intracellular accumulation of lactate anions from the diffusion of lactic acid in media can cause loss in water activity and end product inhibition, that impedes the regeneration of NAD^+ for the lactobacilli under the anaerobic conditions when their cells could not regenerate NAD^+ through NADH oxidase. Thus it is suitable for lactobacilli to maintain a proton gradient across the cell membrane, possessing a higher intracellular pH, than that of extra cellular medium. The influx of undissociated form of lactic acid (present in fermentation broth) in the bacterial

cells causes the acidification of cytoplasm and dissipation of proton gradient. The cell in response carries out extrusion of protons to maintain the proton gradient through energy dependent transport process. Hence the extra expenditure of energy on the internal pH maintenance causes growth inhibition and the growth may stop when the pH gradient collapses due to scarcity of catabolic energy meant for preventing the influx of undissociated acid in the cell.

On running a lactic acid fermentation without pH control of the fermentation broth, there is a decline in lactic acid yield by 30 – 50%[62]. Hence neutralizing agents such as sodium hydroxide, calcium carbonate and ammonium hydroxide are usually added, to maintain favourable pH, for higher yield of lactic acid.

Although calcium carbonate has been usually taken as neutralizer for maintenance of pH during the lactic acid fermentation, but previous studies have clearly indicated some of its inhibitory effects (at higher concentrations) on the lactic acid producing strains. The hyphal growth of *Rhizopus oryzae* (for lactic acid production) was totally inhibited while bacterial growth of *Lactobacillus* strains were reduced [62]. Role of calcium ions in phage infection of *Lactobacillus sp.* cells have also been studied [111]. Hence a lowering of the acid production may be evidenced with $CaCO_3$ neutralizer application, at higher concentrations (above 1.5-2%) due to attack of bacteriophage on the actively acid synthesizing cells. The cells keep calcium ions at low level as at higher concentrations calcium ions can bind to proteins and alter their enzymatic properties [90]. Lower concentration of calcium ions has been found to increase the L-LDH enzyme activity, by two folds, in *Lactobacillus helveticus* [85]. Hence higher concentrations of calcium ions may inhibit the lactate dehydrogenase LDH enzyme in lactobacilli and consequently the lactic acid production may drop. Effects of neutralizers such as NaOH, $CaCO_3$ and NH_4OH and their lactate salts on biomass growth and lactic acid production have been reported [37]. In the fungal fermentations for lactic acid production the calcium carbonate neutralizer gets wrapped in the fungi hence it can't function properly. When calcium carbonate is applied as a neutralizer, calcium sulphate can be produced during reconversion of lactate to lactic acid by sulphuric acid, which might cause considerable environmental problems and involve extra costs for removal [119].

2.4.1.4 Effect of salts

The inorganic salts play important roles in lactic acid fermentation. Inorganic salts employed in lactic acid production media mainly are sodium acetate, KH_2PO_4, $MgSO_4$, $ZnSO_4$ and $Fe_2(SO_4)_3$. It has been found that an increase in the phosphate level from 0.1 to 0.6 g/L of KH_2PO_4 reduced the maximum concentration of L-lactic acid from 85 to 71 g/L [119]. In the lactic acid fermentation, the production media contains manganese salt, as Mn^{+2} manganese ion forms a component of lactate dehydrogenase. The effects of sodium acetate and its replacement by other salts on the lactic acid production by *Lactobacillus sakei* has been studied in detail [59]. Some of the salts such as ammonium sulphate, ammonium nitrate, nitrites and ammonium citrate can contribute to the nitrogen requirements of lactobacilli [119].Sodium pyruvate can supply additional pyruvate in the EMP pathway during lactic acid production and enhance lactic acid production, while the sodium ions help in uptake of sugars such as glucose. The maximum lactic acid production has been found with sodium pyruvate as compared with the other salts such as, tri-sodium citrate, sodium succinate, sodium fumarate, sodium gluconate, sodium acetate tri-hydrate and sodium oxaloacetate applied in the production media during lactic acid production with *Lactobacillus sakei* NRIC 1071[59].

2.4.1.5 Effect of agitation

Proper agitation of the fermentation broth provides uniformity in concentration of the nutrients, cell concentration, temperature throughout the media and the bacterial cells do not form aggregates but lie in uniform concentration which may be beneficial for the growth and lactic acid production. Better mixing is provided through agitation to keep the fermentation broth homogeneous, check sedimentation and as well as enhance mass transfer of nutrients [55,87]. In cases of non Newtonian media, such as starch containing ones, do not have constant viscosities, the agitation system should maintain turbulent flow conditions during fermentation, because under such conditions the power consumption is independent of Reynold number and hence of viscosity [55]. The agitation for batch fermentation in the laboratory flasks is provided through incubator shaker systems while the stirred tank bioreactors are equipped with agitator shafts placed inside the vessel. Agitation at a very high rate may give deleterious effects such as, damage to bacterial cells or fungal hyphae and

splashing out of media , resulting in wall growth(from bacteria removed out of the media) and a possible chance of contamination.

2.4.1.6 Effect of temperature

The temperature is also one of the important factors, which influences the activity of metabolic/cell enzymes. Enzymes are most active at optimum temperature and enzymatic reaction proceeds at maximum rate. However, below and above optimal temperature reaction rate is decreased which causes the problems in cell metabolism [69].The optimal temperature for growth of lactic acid bacteria varies between the genera from 20 to 45°C.In fermentations using *L. delbrueckii,* and *L. bulgaricus* a temperature of 45°C, or higher may be maintained *L. helveticus*, and *L.acidophilus* can be used in a temperature range of 37-45°C [69]. A 37°C temperature for lactic acid production using *L. casei* has been found suitable [51]. However, a temperature of 28°C has also been reported optimal for *L. casei* in a separate study [64]. From the above observations, a temperature range of 37-40°C was considered optimal for lactose conversion to lactic acid using bacterial cells, however, a temperature of 37°C was selected for experimentation.

In agreement with previous observations, *Lactococcus lactis* and *L. rhamnosus* exhibited the highest yields and productivities at 33 to 35°C and 41 to 45°C, respectively [36]. Very high temperatures can prove detrimental to bacteria as the cells may get ruptured, or their cellular proteins and nucleic acids get denatured causing cell death. Arrhenious law gives the dependence of specific reaction rate k of chemical or biochemical reactions on absolute temperature (T) and activation energy (E). It is expressed in differential form as, dlnk/dT $=E/RT^2$; or in integrated form k = A exp (-E/RT), where R is gas constant and in case of biological reactions, k (temperature dependent rate constant) gets replaced by growth rate constant (r) and E (Activation energy) by μ, the specific growth rate [75]. Bacterial growth is a complex biological process involving a variety of substrates and enzymes, and hence the Arrhenius law may not adequately describe the effect of temperature on the growth of bacteria. An alternative linear growth relationship that holds good for large number of bacteria, growing between the minimum and optimum growth temperatures has been described, $r^{1/2}= b$ (T - To), where b is the regression coefficient and To is a hypothetical temperature, which is

an intrinsic property of the organism[75]. A plot of lnr against l/T to produces an Arrhenius plot, a curve is obtained instead of a straight line.

2.5 LACTIC ACID PRODUCTION THROUGH FED BATCH FERMENTATION

2.5.1 General principles

Fed batch culture is a batch culture, which substrate nutrients are fed continuously or sequentially without the removal of fermentation broth. The term fed batch culture was first introduced by Yoshida to describe batch cultures which are fed continuously, or sequentially /intermittently, with medium without removal of culture fluid [97]. Initially a fed batch culture is established in batch mode then it may be operated further by utilizing various strategies of feeding [97]. The strategies of feeding include the following: (1)The initial medium for the batch culture is fed in the system leading to an increase in volume.(2) A solution of limiting substrate is added in the system, keeping its concentration same as in batch culture medium, again leading to an increase in volume. (3) A slower rate of feeding (less than first two steps) of a concentrated solution of the limiting substrate in the system, which leads to enhancement in volume.(4) A highly concentrated solution of the limiting substrate is added at the slowest rate, resulting in negligible change in volume. The fed batch systems based on (1) and (2) type of feeding pattern, are termed as variable volume, while the fourth one is called constant volume [97]. The third one gives a culture intermediate within the two extremes of variable and fixed volume fed batch fermentations.

2.5.2 Applications of fed batch fermentation

Fed batch fermentations have been widely used for the production of microbial biomass, baker's yeast, ethanol, organic acids, antibiotic(penicillin, cephalosporin), 3-phenyl lactic acid (antimicrobial), vitamins, enzymes(cellulose, β-amylase), and other compound [81,14, 87]. The pineapple juice waste which contains glucose, sucrose and fructose has been utilized in production of lactic acid both the application of fed batch fermentation with

Lactobacillus delbrueckii subsp. delbrueckii ATCC 9649 [16].The results of pine apple juice fed batch fermentation when compared with the batch one, showed a higher productivity[16]. Fed batch fermentation through *Lactobacillus lactis* has been performed for hyper-production of L-lactic acid.

2.5.3 Advantages and disadvantages of fed batch fermentation

There are several advantages associated with the fed batch production, that are mentioned as: (1) Although the batch production is more frequently used due to its simplicity, but it becomes less suitable, when cell growth is retarded by substrate inhibition during product formation. The operation of such fermentation in fed batch mode gives better cell growth and production. (2) The inhibitory effects due to the high concentration of the limiting substrate (as observed in batch fermentation) can be prevented by maintaining its supply in the medium at lower rates in fed batch fermentation [97]. (3)The steady state of the variable volume fed batch system, successfully maintains the constant concentration of the biomass and the non growth limiting nutrients in medium. The concentration of non growth limiting substrates have significant impact on the product formation and biomass composition, hence the maintenance of its concentration through variable volume fed batch method is important [97]. (4) Fed batch operations generally allow extended operating time, where high cell concentration and better volumetric productivity can be obtained. This aspect is beneficial for the growth associated products such as lactic acid. The important rationale of fed batch operations are the excess of carbon caused inhibition of cell growth and the excess of carbon or nitrogen caused the catabolite repression [110].(5) Fed batch fermentations provide faster substrate depletion and removal of product inhibition and higher product yield. The fast depletion of carbon sources in lactic acid fermentation may cause product inhibition due to the organic acid formations.

Higher input cost of media components such as pure sugars; prior knowledge of nutritional requirements, physiological aspects and productivity of the microorganism; after long time operation, accumulation of non producing or low producing strains may occur, chances of contamination during feeding, are some of the disadvantages of this type of fermentation.

2.5.4 Application of raw materials in fed batch fermentation

Although the fed batch mode of fermentation is capable of utilizing higher doses of substrate and checking the substrate inhibition of microbial cells through controlled feeding, but the input cost of the pure sugar is high. Hence, from the economic point of view, the application of low cost carbohydrate rich waste materials such as, sugarcane bagasse, whey, starchy materials, pineapple wastes and some of the other wastes from dairy, sugar and food process industries, in fed batch fermentation, shall bring down the cost of production.

Whey is the liquid portion of the milk, which is liberated after coagulation of milk proteins as curd, on application of acids or proteolytic enzymes. The coagulation of the milk protein ,casein can be performed either enzymatically with rennet at pH 6.5 or by the application of organic or mineral acids at pH below 5.0 .Cheese whey has a BOD of 38,000 to 46,000 ppm and COD 80 g/L due to the presence of approximately 5% lactose, 0.06% fat, and 0.8 to 1% nitrogenous compounds and amino acids and vitamins[15]. About 10^8 tons per annum of cheese whey produced as dairy by product, can be efficiently utilized as cheap carbon source for microbial preparation of lactic acid [26]. Cheese whey also contains important minerals such as sodium, potassium, calcium, magnesium and zinc etc. which may be helpful in the transport processes and metabolic processes of the *Lactobacillus* strains[30].Such bioconversion of whey to lactic acid can potentially bring down the cost of activated sludge treatment [26, 4]. Lactose sugar contained in whey can be bio-converted into various fermentation products such as lactic acid, ethanol and single cell proteins, through fermentation, proved economical [68]. Cheese whey has been utilized as carbon source in liquid state production of lactic acid [26]. The pineapple juice waste which contains glucose, sucrose and fructose has been utilized in production of lactic acid [16].

The sugarcane bagasse is a lignocellulosic material that is mainly consists of two polysaccharide fractions (cellulose and hemicellulose) and a polyphenolic macromolecule (lignin). Hemicellulose is the second predominant fraction (23-30%), and it is composed of heteroxylans, with a major portion of xylose. Dilute acid hydrolysis pretreatment of sugar cane bagasse generates (hemicellulose hydrolysate) rich in xylose, that can be used for the production of lactic acid [58,72].

2.6 LACTIC ACID PRODUCTION THROUGH SOLID STATE FERMENTATION

2.6.1. General Aspects

Solid-state fermentation (SSF) processes can be defined as, "the growth of microorganisms (mainly fungi), on moist solid materials in the absence of free-flowing water". These processes have been used for the production of food, animal feed, and both pharmaceutical and agricultural products. The porous bed as support material appears to be the best for the growth of fungi but bacteria and yeast have also been used in solid state fermentation.

Substrates that have been traditionally fermented by solid-state include a variety of agricultural products such as rice, wheat, millet barley, grains, beans, corn and soybeans. However, non-traditional substrates which may also be of interest in industrial process development, include an abundant supply of agricultural, forest and food-processing wastes such as wheat bran and soy grits (flakes remaining after extraction of oil).

2.6.2 Operating conditions for SSF

Moisture content: It is a critical factor on SSF processes because this variable has influence on growth and biosynthesis and secretion of different metabolites. A lower moisture content causes reduction in solubility of nutrients of the substrate, low degree of swelling and high water tension. On the other hand, higher moisture levels can cause a reduction in enzyme. Generally, the water content of the substrate oscillates between 30 and 75%. Lower values can induce the sporulation of the microorganism, whereas higher levels can reduce the porosity of the system [32].

Nutrients diffusion: This occurs at an intraparticular level and includes both the diffusion of nutrients toward the cells and the hydrolysis of solid substrates by the microbial enzymes. This last point is an important aspect in SSF because the most part of the substrate is water insoluble (In substrates with a small pore size, the resistance to the intraparticular mass transference increases with the diameter of the particle and the degradation of the substrate occurs mainly at the outer surface [32]. Nutrient diffusion processes are especially important in bacterial and yeast SSF.

Temperature: The increase in temperature in SSF is a consequence of the metabolic activity when the heat removal is not enough.

2.6.3. Applications of SSF

Solid state fermentation has been used in producing wide range of products which are mentioned below:

Mycotoxins, gibberellins, alkaloids, antibiotics, hormones [32].
Organic acid such as citric acid, fumaric acid, itaconic acid, lactic acid and enzymes such as amylases, amyloglucosidase, cellulases, proteases, pectinases, xylanases, glucoamylases[31,32,43,44,45]. Traditional food fermented (Koji,sake, ragi, tempeh), protein, enrichment and single cell protein production, mushrooms production.

2.6.4. Advantages and disadvantages of SSF

Some of the advantages of the solid state fermentation:
1. Small volumes of polluting effluents. 2. Fewer requirements of dissolvents are necessary for product extraction due to their high concentration. 3. Due to the concentrated nature of the substrate, smaller reactors in SSF with respect to SLF can be used to hold the same amounts of substrate. 4. Culture media are often quite simple. 5. Simple design of reactors, with lesser spatial requirements.

Disadvantages associated with the solid state fermentation:

1. Only microorganisms that can grow at low moisture levels can be used.
2. Usually the substrates require pre-treatment (size reduction by grinding, rasping or chopping, homogenization, physical, chemical or enzymatic hydrolysis, cooking or vapour treatment).
3. Biomass determination is very difficult.
4. The solid nature of the substrate causes problems in the monitoring of the process parameters (pH, moisture content, and substrate, oxygen and biomass concentration).
5. Agitation may be a difficulty. 6. Mass transfer limited to diffusion [40].

7. Cultivation times are longer in SSF. Poor heat transfer, without agitation of culture and media distribution chances of contamination by fungi.

2.6.5 Bed materials for solid state fermentation

In SSF, two types of process can be distinguished depending on the nature of the solid phase. In the first and the most used, the solid serves both as a support and a nutrient source. These substrates are heterogeneous water insoluble materials from agriculture or by-products from food industry, which have an amylaceous or ligno-cellulosic nature (grains and grain by-products, cassava, potato, beans and sugar beet pulp).

Besides costly pure materials, large quantity of agroindustrial such as as wheat bran, sugarcane straw, sugarcane bagasse and orange peel have been used for the fermentative production of different acids and enzymes [93,72,112,8,31].
In India huge quantity of sugarcane bagasse, wheat bran and forest waste pine needles is available, which has the potential to be utilized as bed materials in solid state fermentation.

The sugar mills and juice extraction units produce large amount of sugarcane bagasse as agroindustrial waste. India produces over 250 million tones sugarcane per year, engaging about 4.36 million hectares of total cropped area [90]. Brazil produces over 60,000,000 tons of sugarcane bagasse while Asia shares about 44% of sugarcane bagasse [50, 72]. Under the Indian conditions the processing of 100 tons of sugarcane provides 10 tons of sugar and 30-34 tons of bagasse (rest being molasses, press mud and flue gases etc.), out of which 22-24 tons are used in processing while 8-10 tons are still left [92]. Inhalation of sugarcane dust particles in work place can cause various lung problems such as bagassosis. Utilization of sugarcane bagasse as bed material, in solid state fermentation provides an eco-friendly technology for microbial production of various biochemicals such as xylitol, organic acids, amino acids and enzymes [43, 44, 45, 78, 79].

Wheat bran constitutes a significant portion of agro-industrial waste produced in the flour mills. It forms the outer portion of the wheat seed, usually accounts for 14–19% of the grain. It comprises of the outer layers of grain, exocarp, mesocarp and endocarp (rich in minerals), the testa (rich in vitamins and enzymes), the aleurone layer(rich in protein and fats) and the remnants of the starchy endosperm [3]. The wheat bran is an abundant agro-industrial waste as wheat is the second major food crop in India (about one third of the food grain production), with 26.6 million hectares of cropped land (2003-04) under it, having a

productivity of 2,707kg/hectare [104]. The global production of wheat was 585million tons (1999-2001) to which India contributed 12.3% [104].Large quantity of wheat bran (1.6 million tons) is produced in Italy as agricultural by product and investigations have been done on the bioconversion of its ferulic acid (phenolic compound) constituent of cell wall polysaccharides into vanillin utilizing wheat bran in solid state fermentation [93].It has been reported that, wheat bran, whole or hydrolyzed, enhanced the cellulase production in filamentous fungi [99]. It has been reported that, cultivation of wheat as an energy crop, provided wheat starch to European plants, that converted it into ethanol, but utilization of the bran fraction, i.e., both the starch and hemicellulose/cellulose part, should be successful in enhancing the ethanol production [3].Raw starch based media has been applied on wheat bran bed material for lactic acid production by amylolytic lactobacilli .Protease treated wheat bran, was utilized by mixed cultures of *L. delbrueckii* and *L. casei*, to produce lactic acid through simultaneous saccharification and fermentation [43]. Wheat bran consists of approximately about 19% starch, 18% crude protein, 58% non starch carbohydrates with 24% cellulose, 70% arabinoxylans and 6% glucans as major non starch polysaccharides and lignin [99].Wheat bran could be utilized in solid state, liquid state fermentations and animal feeds. The sugars, nitrogenous substances such as proteins, amino acids and various vitamins (niacin, thiamine, riboflavin, vitamin A, vitamin K, vitamin E and folate etc.) and minerals(potassium, phosphorus, magnesium, calcium), in wheat bran, have the potential to support lactobacilli, in growth and lactic acid production [52]. The bran part contains higher quantity of the amino acids (0.25g cysteine, 0.85g arginine, 0.54g lysine, 0.18g histidine and 0.28g tryptophan per 100g wheat bran) than the endosperm [63]. Nutritionally essential amino acids are present in larger quantities in soluble protein fractions of wheat bran such as, albumin (contains large quantities of tryptophane, tyrosine and cysteine) and globulin than in insoluble fraction of prolamine [63]. The amount of proteins in the inner layer of the wheat bran contains much higher amount of proteins than the outer layer and intermediate portions of wheat bran. Outer layer consists of 0.4mg total protein per g, out of which 25% were water soluble; Intermediate fraction has 3.6 mg total protein per g, while the inner layer consists of 156mg protein [42].

The enormous amounts of fallen pine needles, usually found as waste biomass in coniferous forests, have been often associated with devastating forest fires. These fires are one of the dominant ecological events that occur periodically and pose formidable threat for the

animal and plant life in the coniferous forests. Pine needles bear immense potential in the future through their utilization as bed material in solid state fermentative production of value-added chemicals, such as lactic acid.

The pines are coniferous, evergreen, resinous trees belonging to the genus *Pinus* of the family *Pinaceae*, native to northern hemisphere. They are also found in south-east Asia and the Himalayan regions of India. The Chir pine (*Pinus roxburghii*) is predominant in the coniferous forests of the Himalayan regions of Uttarakhand and Himachal in India [49]. The needle-shaped green colored adult pine leaves found in clusters are known as pine needles. They constitute the major portion of the litter fall in coniferous forests [61]. Pine needles have several applications, in addition to their role in returning nutrients to the forest floor. These applications include their use as a mulching material, a supplier of nutrients when added to soil, and as a beverage (with high amounts of vitamins A and C), pine needle wine. Pine needle powder is a rich source of essential oils, vitamin C, and alkaloids. Pine needles also help in biomonitoring of pollution due to absorption of atmospheric polycyclic aromatic hydrocarbons (PAHs) [48,50]. The ethanol extracts of pine needles (*Pinus densiflora*) exhibit, strong antioxidant, antimutagenic, antiproliferative properties and antitumor effects under in vitro and in vivo conditions [56]. Pine needles have also been applied as reinforcing agents in place of the conventional ones in the polymer composites due to several advantages such as cheaper cost, lesser density, non corrosiveness, reduced wear in process equipments, improvement in mechanical properties, biodegradability, and recyclability, etc. [95].Pine needles of length 170 to 250 mm, diameter 0.7 to 1.3mm, or in dried ground particle form (200 μm), containing about 33.37% lignin, 67.29% holocellulose, 2.71% ash, and 15% extractives, have been used in preparation of polymer-based biocomposites with resorcinol formaldehyde matrix, urea formaldehyde resin, and phenol formaldehyde, resulting in improvement of mechanical properties such as tensile strength, compressive strength, etc. [95].

A high risk of forest fires, associated with dried, fallen, pine needles, results in large-scale destruction of fauna and flora and is hazardous to the environment due to air pollution [95]. It has been reported that roughly $50x10^3$ fires per year destroy about, $6x10^5$ hectares of forest per year, in Mediterranean islands (France) and these fires mainly involve Corsican pine (*Pinus nigra ssp laricio*) found abundantly in mountains of Corsica[19]. Consumption of pine needles by cattle, especially during late stages of pregnancy, can cause premature parturition,

in which the calf dies after birth [70]. Entry of tannins from pine needles in the soil was found to inhibit the growth of various beneficial agricultural microbes, causing delayed availability of nutrients in soil [88]. Considering the abundance and all above mentioned hazards and demerits associated with the pine needles, their huge underexploited potential can be utilized beneficially in future as bed material in solid state production of lactic acid. The pine needles are among one of the newer bed materials to be used in solid state fermentative production of lactic acid. It has been used as one of the bed materials in the present studies keeping in view the capability of the lactobacilli to resist the phenolic or alcoholic type of substances that can result from such bed material.

2.7 Batch growth studies on lactic acid bacteria

Batch culture systems for the microorganisms include their growth in a supporting medium in a closed environment inside a batch reactor or a flask operated under optimum conditions of temperature, agitation and pH etc. The bacterial growth in batch culture shows first order kinetics. The graphical plot between the natural log of biomass concentration or cell dry weight and time represents the batch growth curve of the microbe under batch culture. The batch growth curve consists of distinct phases such as (1) Lag phase, there is apparently no cell growth, cells take time to adapt themselves with the conditions in media (2) Log phase, it is the phase where the cells have accelerated growth and they grow at constant maximum rate .Under these conditions the synthesis all the cell constituents increases at a constant rate so that cell population doubles and continues doing so at regular intervals [87]. During this phase the nutrients are in excess and the organism grows at maximum specific growth rate [97]. (3)Stationary phase, the cell growth starts decreasing towards the end of log phase until the net growth becomes zero, due to the depletion of essential nutrients or accumulation of toxic material. The cells remain viable in this phase due to their own storage polymers which can provide substrates [87]. (4) Decline phase, the cell numbers start decreasing because the death rate of the cells exceeds the growth rate. If there are two or more sugars present in the batch culture media, then there will be 2 or more such phases.

Kinetic studies in microbial fermentations are important to determine the occurrence of growth in log phase, biomass production, pH changes affected, product formation etc., for different microbial strains under different set of experimental conditions. Leudking et al., (2000), proposed a mathematical model for batch process of lactic acid fermentation known as

Leudking -Piret Model, which relates rate of product formation bacterial growth rate, and bacterial cell density. This model shows the involvement of growth dependent and independent terms in lactic acid production kinetics [60].

In lactic acid fermentation by *Lactobacillus sp.* the biomass in fermentation broth is reported to exist in three different physiological states (1) Showing growth and acidification (2) Non growing but acid forming (3) Showing neither growth nor acid formation [17]. While studying the batch culture of *Streptococcus sp.* IO-1, it had been observed that the growth of microbes might be classified into three phases, the lag phase, exponential growth phases with sterile cell formation and without sterile cell formation [39].

In majority of the literature regarding the batch growth studies pure sugars have been used as carbon source. The input cost of the pure sugars may be insignificant under the laboratory conditions but it shall be important in the large scale fermentation industries where 100-1000 of liters of inoculum have to be prepared. Hence partial or total substitution of the expensive pure glucose component in the culture media by low cost waste materials such as sugarcane bagasse hydrolyzate and whey etc. can prove economically feasible.

2.8 Industries for Lactic acid production

Some companies such as Musashino (Japan) and Sterling Chemicals, Texas (USA), use chemical synthesis based lactic acid production technology [65]. There are numerous large scale industries which are actively involved in lactic acid production through fermentation. The major global manufacturers of lactic acid include, Galactic (Belgium), Cargill (USA), Purac (Netherlands) and many of the Chinese industries [112]. Cargill and Dow Chemicals have combined venture in poly lactic acid production.

The Nature Works LLC, has established a lactic acid major manufacturing plant in Blair, Nebraska, USA, that became functional from (2002), and has the capacity of producing 180,000 (metric) tons of lactic acid per year[112]. Nature Works LLC had prediction that the PLA market would go up to 500,000 (metric) tons per year worldwide by 2010 [112]. In 1997, Cargill had a joint venture with Dow Chemical for the production of polylactic acid.

Some of the important manufacturers and exporters of lactic acid in India are, Sheliz Overseas pvt limited., Delhi (lactic acid and buffered lactic acid).Kem Light Laboratories pvt. Ltd.-Mumbai, Asha Ram and Sons, Delhi and Krupa corporation, Vadodara Gujrat.

CHAPTER- 3

MATERIALS AND METHODS

3.1 Materials

3.1.1Microbial cultures

The chemicals used in these experiments were of Merck and High media make. Pure cultures of Lactobacilli (1) *L. delbrueckii* (NCIM2025) (2) *L. pentosus* (NCIM 2912) (3) *Lactobacillus sp.*(NCIM 2734) (4) *Lactobacillus sp.* (NCIM2084) were acquired from National collection on industrial microorganisms (NCIM) of National Chemical Laboratory(NCL) Pune(India). The strains were subcultured monthly as per the directions, on MRS agar and kept at 4^0C.A coculture was prepared from the inoculums of strains-1and 2, on MRS culture medium, for attaining predicted benefits in lactic acid production (Appendix-1).

3.1.2 Media used in the experiments

Composition of various media utilized during the batch, fed batch and solid state fermentations have been mentioned

(1)The inoculum for the *Lactobacillus* strains were prepared in MRS (de Mann Rogosa Sharpe) media. Composition of one liter MRS growth medium: 10 g proteose peptone, 5 g yeast extract, 10 g beef extract, 20 g dextrose, 1g Tween 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4$, 2g K_2HPO_4 in distilled water as solvent.

(2)Composition of one liter of synthetic glucose based production media: 60 g glucose,1g sodium acetate, 0.03 g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 9 g yeast extract,0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$

The above media have been used in major portion of the research work. The concentration or the types of any one of the components have been varied in the studies involving their effects, while the other components have been kept the same [53].

(3)Lactic acid production medium for batch fermentation with different levels of glucose

One liter of the production media contained: Various levels of glucose (60 g,80 g, 100 g and 120 g) , 9 g yeast extract,1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.

Conditions of the experiment: A 100 mL medium was provided in each flask for each individual strain, at the initiation of the reaction. Initial pH of the medium was 6.5, that was inoculated with, 1.55g/L inoculum dose of 16 hour old cultures developed in MRS media, and

kept at 37 ^{0}C, 180 rpm for 108 hours incubation period. The pH was controlled by addition of 2% NaOH solution every 12 h. These conditions were also applied for the batch fermentation experiments with, whey substituted glucose WSG, lactose, whey substituted lactose WSL, acid hydrolyzed starch AHS, enzyme hydrolyzed starch EHS, acid treated sugarcane bagasse under boiling or autoclaving treatments, various nitrogen sources and salt applications.

(4)Lactic acid production medium for batch fermentation with different levels of whey substituted glucose WSG

One liter of the production media contained, various levels of whey substituted glucose (WSG) (60 g,80 g, 110 g and 120 g) , 9 g yeast extract,1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.

(5) Lactic acid production medium for batch fermentation with different levels lactose

One liter of the production media contained, various levels of lactose (60 g,80 g, 100 g and 120 g) , 9 g yeast extract,1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.

(6) Lactic acid production medium for batch fermentation with different levels of whey substituted lactose WSL

One liter of the production media composed of various levels of whey substituted lactose WSL(60 g,80 g, 110 g and 120 g) , 9 g yeast extract,1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.

(7)Lactic acid production medium for batch fermentation with different levels of acid hydrolyzed starch AHS

One liter of the production media contained, various inputs of starch (60 g,80 g, 100 g and 120 g) followed by acid hydrolysis and neutralization, 9 g yeast extract,1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.

(8)Lactic acid production medium for batch fermentation with different levels of enzyme hydrolyzed starch EHS

One liter of the production media contained, various inputs of starch (60 g,80 g, 100 g and 120 g) followed by enzymatic hydrolysis, 9 g yeast extract,1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.

(9)Lactic acid production medium for batch fermentation utilizing acid treated sugarcane bagasse under boiling or autoclaving conditions

One liter of the production media contained, Inputs of pure glucose 40 g in1000 mL solution of neutralized extract obtained from boiling or autoclaving of bagasse in acidic conditions, 9 g yeast extract,1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$. The detoxified (Appendix-2) and neutralized extract obtained from 1% sulphuric acid treatment of sugarcane bagasse (taking 1:10 w/v ratio under boiling conditions at atmospheric pressure or autoclaving conditions-121^{0}C and 15psi, for 30 minutes) was used to prepare the production media. such that initial pH was kept at 6.5. The pentoses were estimated by colorimetric method [103]. Glucose was determined by DNS method [83].

(10)Lactic acid production medium for batch fermentation utilizing various nitrogen sources

One liter of the production media contained, 60 g glucose, various doses of any one of the nitrogen sources, meat extract ,proteose peptone and yeast extract (9,10,12 and 15 g) while for baker's yeast the doses of (12,15,20,23 g) , 1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.

(11)Lactic acid production medium for batch fermentation under influence of various initial pH values

One liter of the production media contained, 60 g glucose, 9 g yeast extract;1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$. Initial pH values 6.0, 6.5, 6.75,7.00 have been applied for the medium with 1.55g/L inoculum dose(from 16 hour old cultures developed in MRS media, expressed in cell dry weight), at 37 ^{0}C, 180 rpm for 108 hours incubation period. The pH was controlled by addition of 2% NaOH solution every 12 h.

(12) Lactic acid production medium for batch fermentation under influence of different neutralizers

One liter of the production media contained , 60 g glucose, 9 g yeast extract;1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$. The medium had Initial pH value 6.5, 1.55g/L inoculum dose (from 16 hour old cultures developed in MRS media, expressed in cell dry weight),and was kept at 37 ^{0}C, 180

rpm for 108 hours incubation period. The pH was controlled by addition of 2% NaOH or $CaCO_3$ solution every 12 h.

(13) Lactic acid production medium for batch fermentation under influence of different salts

One liter of the production media contained the following: 60 g glucose, 9 g yeast extract;1 g salt under study(such as sodium pyruvate SP, sodium succinate SS, ferrous ammonium sulphate AS and ammonium citrate AC), 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.

(14) Lactic acid production medium for batch fermentation under influence of different agitation rates or temperatures

One liter of the production media applied for the experiments on agitation rates or temperatures, contained, 60 g glucose, 9 g yeast extract;1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$. The medium had Initial pH value 6.5 and was inoculated with, 1.55 g/L cell dry weight of 16 hour old cultures developed in MRS media. In the agitation experiment an incubation temperature of 37 ^{0}C and agitation rates of 0,150, 180 and 240 rpm were provided for 108 hours. The batch fermentation medium on temperature effects had, initial pH 6.5 and was inoculated with,1.55g/L cell dry weight of the inoculum and kept at 30,34 ,37 and 40 ^{0}C(in separate sets of experiments), 180 rpm for 108 hours incubation period. The pH was controlled by addition of 2% NaOH solution every 12 h.

(15) Lactic acid production medium for fed batch fermentation utilizing glucose or lactose as carbon source

One liter of the production media contained , 130 g (glucose or lactose), 9 g yeast extract;1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$. Different levels such as 2, 4 or 6 g of glucose or lactose were fed in the fermentation medium, from their sterile concentrated stock solution every 12 hours for different sets of experiments, corresponding to each dose. Glucose is fed in a media with glucose carbon source. Similarly lactose is fed in a lactose based media.

Conditions of the experiment:

A 100 mL medium with initial pH 6.5 was provided in each flask and inoculated with 1.55g/L inoculum (of 16 hour old cultures developed in MRS media for each individual strain) at the initiation of the reaction., then kept at 37 ^{0}C, 180 rpm for 108 hours incubation period. The pH was controlled by addition of 2% NaOH solution every 12 h.

(16) Lactic acid production medium for fed batch fermentation with acid hydrolyzed sugarcane bagasse extract or cheese whey as carbon source used in combination with glucose or lactose.

In case of acid hydrolyzed bagasse extract (obtained under autoclaving conditions) 110 g glucose was dissolved in one liter detoxified, neutralized extract along with other components of media such as 9 g yeast extract;1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$.The conditions of acid treatment of bagasse under autoclaving conditions are same as mentioned in (9).

In case of filtered cheese whey studies 80 g glucose or 80 g lactose, was dissolved in one liter whey accompanied with other media components such as 9 g yeast extract;1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$. Five milliliters of sugarcane bagasse extract or cheese whey were fed every 12h.The experiments were carried out with initial pH 6.5, 1.55g/L inoculum dose at 37 ^{0}C, 180 rpm for 108 hours incubation period. The pH was controlled by addition of 2% NaOH solution every 12 h.

(17) Lactic acid production medium for solid state fermentation

One liter of the production media contained , 60,80 and 120 g (glucose or whey lactose mixed glucose), 9 g yeast extract;1 g sodium acetate, 0.03g $MnSO_4.H_2O$, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 and 0.03 g $FeSO_4$. The experiments were carried out with 6g bed material per flask added with 40 mL medium having initial pH 6.5, 1.55g/L inoculum dose, 2% NaOH neutralizer and incubated at 37 ^{0}C, for six days. For the studies with nitrogen sources on wheat bran bed material, 10,12,15 and 20 g/L of meat extract and proteose peptone were taken while for baker's yeast 12,15,20 and 23.33 g/L doses were applied, but other components of medium and the fermentation conditions remained same.

3.2. Methods

The different methods used in the experimental studies have been described herein.

3.2.1. Bacterial cell concentration

The bacterial biomass was expressed as cell dry weight in gram per liter (g/L).Equal volumes of culture broth were filtered through preweighed microporous filter discs that were first washed with 0.85% sterile saline solution prepared in distilled water followed by drying at 85^0 C until attainment of constant weight. The biomass concentration in (g/L), is given by the difference of initial and final weights of the filter paper per volume of the broth passed through the filter (expressed in liter) [87].

3.2.2. Estimation of lactic acid

The lactic acid present in the fermentation broths in liquid state fermentation or extracts from fermented bed materials in case of solid state fermentation, were quantitatively assayed by Kimberly Taylor method [100]. This method utilizes hot concentrated sulphuric acid effects,which include oxidation of lactic acid to acetaldehyde, which subsequently forms a chromogenic complex with p-phenyl phenol in presence of copper. The extracts or the fermented broths were centrifuged at 8000g and their supernatants were used for lactic acid estimation. 0.5 mL of supernatant was added with 3mL of 96% sulphuric acid, followed by heating for ten minutes in boiling water bath for ten minutes, then cooling it to room temperature for about 30 minutes. The cool solution was added with 50 µL 4% copper sulphate and 100 µL p- phenyl phenol (prepared by dissolving 1.5% of the reagent in 95% ethyl alcohol) which provided a chromogenic complex. The absorbance for lactic acid is measured in a UV-VIS double beam spectrophotometer of (Systronics, India) at 570nm [62].

3.2.3. Estimation of sugars by DNS method

This method utilized DNS reagent, which consists of, 3,5 dinitrosalicylic acid, phenol, sodium sulphate, and sodium potassium tartarate [83]. All the components require to be dissolved in 1% sodium hydroxide. A mixture of 3mL test solution and an equivalent volume of the DNS reagent were mixed and heated in boiling water bath for 5 minutes. The absorbance was measured at 575nm in a UV-VIS double beam spectrophotometer (Systronics, India).

3.2.4. Determination of pentoses

A colorimetric method has been developed for quantitative determination of pentose sugars even in the presence of large excess of hexoses [103]. The reagent was prepared by mixing 100 mL glacial acetic acid, 10 mL 5% aqueous acetic acid, 24 mL water 16 mL colorless aniline were mixed and stored in dark glass bottles. Six milliliters of the reagent were mixed in a test tube with 2mL sugar sample to be determined and these test tubes were kept in dark at room temperature for twenty hours, xylose was used as standard. The color developed was measured by spectrophotometrically at 480nm.This method was applied for determining of the pentose from the sugarcane acid treated bagasse extract. But prior to the autoclaving or boiling treatment of bagasse with 1% sulphuric acid the bagasse was subjected to lignin removal by ammonium hydroxide [58].Then delignified bagasse in dried and ground form was used. Subsequent to boiling or autoclaving treatments the extract was detoxified with calcium hydroxide [72].

3.2.5. Hydrolysis of starch

Acid hydrolysis of starch was carried out by the Yins method of [117]. A 100 mL of double distilled water was added with the 450 grams of starchy material this mixture was added with 500 mL boiling water. The boiling conditions were applied for five minutes for gelatinization of starch. Gelatinized starch was cooled down to the room temperature. Water was added to make up the volume to one liter. The slurry was treated with 2N HCl (pH 1.5), followed by autoclaving at 121^0C, 20 minutes. The clear supernatant was collected for reducing sugar estimation and to be used as carbon source in the experiments [117]. As like the acid hydrolysis of starch, the gelatinization step was similar. The gelatinized starch was added with 1M$CaCl_2$ to make up a concentration, 0.45mM and pH 6.9. Alpha amylase was added in the solution and the mixture was left for two hour incubation at 70^0C for liquification. In the next step, pH 4.8 and temperature 60^0C were applied with the addition of glucoamylase. The hydrolyzed starch was cooled to the room temperature and filtered. The resulting filtrate containing sugars, was used for the experiments.

The hydrolysis of starch can be tested by a qualitative rapid method [40].One drop of starch solution undergoing hydrolysis is added on a slide with one drop of iodine which provided indication of stage of starch hydrolysis, blue color (indicating the presence of

starch), reddish brown(indicating degradation of starch) and colorless (indicating complete hydrolysis of starch).

3.2.6. Lactose estimation

Lactose content of the cheese whey was estimated by colorimetric method given by Nickerson [66]. 5 mL of whey sample was prepared by prior treatment with zinc acetate–phosphotungstic acid reagent for removal of proteins and sodium hydroxide. This was further added with 5 mL of glycine–NaOH buffer and 0.5 mL each of methylamine–HCl and sodium sulfite solution followed by thorough mixing and kept at 65°C in a water bath for 25 min. The sample mixture was cooled immediately in an ice-water bath for 2 min and the absorbance was measured at 540 nm. The absorbance of the sample was taken at 540 nm on a UV-VIS double beam Systronics 118, spectrophotometer [68]. The lactose from cheese whey was estimated by the above method.

3.2.7. Pentosan assay in bed materials

Pentosans in the wood and pulp can be determined by TAPPI (Technical Association of Pulp and Paper Industry-member of American National Standard Institute) method T223 cm-01 based on the principle of conversion of pentosans to furfural (in the distillate) by boiling with HCl. The amount of furfural is quantified colorimetrically. Based on a similar principle, a spectrophotometric method for determination of the pentosan content in the non-wood (agricultural/forest residues) or woody materials has been mentioned in the TM1-A11 test method of the laboratory manual of Central Pulp and Paper Research Institute (CPPRI), Saharanpur, U.P., India. Absorbance of the distillate in the receiver resulting from acid digestion of 3 g OD (oven dry) sample in 300 mL 13.5% HCl (at boiling condition) contained in a round-bottomed flask connected with pentosan apparatus, (Appendix-17) was measured at 280 nm. During digestion the acid level in the round-bottomed flask was maintained by addition of HCl drops through a separating funnel at the top of the flask. Pentosan percentage was evaluated by the following expression

Pentosan (%) = Absorbance at 280 nm x Dilution x 1.563 x 0.5 x

100/151 x OD weight of the sample (1)

3.2.8. Estimation of lignin content in bed materials

Lignin was quantified as per the TM1-A-7 test method mentioned in the laboratory manual of CPPRI (Laboratory manual 2001),having similar principal to the T222 om-06 TAPPI standard method for Klason lignin (insoluble lignin) estimation. It consists of addition of 2 mL 72% sulphuric acid to 1 g OD sample, followed by addition of 13 mL of the same acid in the same beaker that was placed in a water bath at 20°C with continuous stirring for 2 hours. The contents of the beaker were filtered through a G2 crucible that weighed (W_1). The crucible along with the residues was dried overnight in an oven at 105 °C and weighed (W_2). The acid-insoluble lignin was evaluated by the following expression:

Acid insoluble lignin, % = (W_2-W_1) x 100/OD weight of the sample (2)

The filtrate from the G_2 crucible was kept for soluble lignin estimation, which was measured spectrophotometrically at 280 nm. Acid-soluble lignin can be evaluated through the following expression

Acid soluble lignin, % =Absorbance at 280 nm x Dilution x

100/20/1000 x OD weight of the sample (3)

3.2.9. Estimation of holocellulose content in pine needles

This method quantifies the total carbohydrate content of woody or non-woody materials, as per the TM1-A-9 test method mentioned in the laboratory manual of CPPRI is analogous to Goncalve's method[29]. 5 g of ground OD material (passed through mesh size 40) was placed in a 250 mL Erlenmeyer flask with 10 mL distilled water, followed by the addition of 1.5 g of sodium chlorite and 0.5 mL of acetic acid. The mixture was incubated in a flask for one hour at 70 °C in a water bath. The supernatant was transferred to a tared crucible (W_1). The contents of the conical flask were filtered in a tared crucible, followed by washing of the residue with acetone and oven drying at 105 °C for two hours. The crucible with dried contents weighed (W_2).Holocellulose % can be calculated by the following expression

Holocellulose, % = (W_2-W_1) x 100/ OD weight of the sample (4)

3.3 Solid state fermentation process

The inoculum preparation for the *Lactobacillus* strains under study was performed in standard MRS culture media. A loop full of pure culture obtained from the National Chemical Laboratory, Pune (India) were transferred from MRS agar stabs to the 50 mL MRS media contained in the Earlenmayer flasks, under aseptic conditions. In case of the coculture a loop full each from strain-1 and strain-2 were transferred to the MRS media. All the inoculated flasks were screw capped tightly and then kept in an incubator shaker at 37 ^{0}C, 180 rpm for 14 hours. The biomass concentration of each culture was determined in form of cell dry weight.

The particle size fractions of the dried bed material were determined through a set of vibratory screens arranged vertically in order of decreasing mesh sizes, 1680, 1204, 710, 500, 420 and 150μ.

Each of the conical flasks containing 6 g bed of wheat bran were added uniformly with 40 mL of the production medium which were subsequently autoclaved for sterilization. The volumes of the inoculum for cultures of different strains of lactobacilli were adjusted such that their final biomass concentration before inoculation, becomes 2 g/L. These were inoculated in different conical flasks, containing cool sterilized media in the bed and added with 1.0 mL, 2%NaOH neutralizer, uniformly on it and the flasks were tightly screw capped(with sterile cotton packing at the bottom and top portion of the screw cap, immediately, under aseptic conditions. The inoculated flasks were kept for incubation, at 37 ^{o}C under static conditions in an incubator for six days. Control flasks containing all other components of the production media except the carbon source were supplied to the bed, and the resulting growth of the bacterial strains on the bed indicated the capacity of the bed materials to support the bacterial growth.

After the incubation period each conical flask was added with 50mL of distilled water and shaken for two hours at 200rpm, for better mixing and liberation of acid from the bed. The acid was extracted by passing the mixture through a muslin cloth where the solid bed particles are removed and the pH of the extract collected were recorded in a digital pH meter. The extracts were centrifuged at 8000rpm and the supernatants were used for lactic acid estimation.

Then the extracts, were assayed for lactic acid by Kimberley Taylor method [62,100]. The absorbance for lactic acid was measured with a UV-VIS double beam Systronics 118 spectrophotometer at 570 nm.

3.4.Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed using a LEO-435VP microscope to obtain clearly magnified view of the growth of *Lactobacillus* cells on the bed material. Very small portions of dried wheat bran beds(after inoculation and six days incubation except the control raw material), dried at 60 °C for 30 hours, were placed on the stubs and subjected to gold coating and observed at 15 kV.

3.5. FT-IR analysis

Fourier transform infrared spectroscopy (FT-IR) was performed using a Nicolet-6000 spectrophotometer to ascertain the presence of some of the functional groups associated with the bed material. Thoroughly washed (by distilled water) and dried wheat bran was used for FTIR. It was mixed with potassium bromide and pressed under vacuum. Transmittances were measured over a range near 500 to 4000 cm^{-1}.

The data shown in the research work is the average of three replicates of each experiments. The graphical plots are drawn around the mean values. The standard deviations have been mentioned in some of the experiments. The student's t-test, ANOVA, coefficient of variation (C.V.) and correlation coefficient have been applied in some of the experiments.

CHAPTER-4

RESULTS AND DISCUSSIONS

4.1 Lactic Acid Production by Liquid State Batch Fermentation

Liquid state batch fermentation has been performed with all the four *Lactobacillus* pure strains and the coculture. Various factors affecting the lactic acid production such as carbon sources (pure sugars and raw materials), nitrogen sources, pH, neutralizer, salts, agitation and temperature have been studied.

4.1.1Effect of carbon sources

The carbon sources under study consisted of pure glucose, lactose, partly whey substituted glucose, partly whey substituted lactose, acid hydrolyzed starch and enzyme hydrolyzed starch.

4.1.1.1 Effect of glucose

Various doses of glucose 60 g/L, 80 g/L, 100 g/L and 120 g/L have been applied as carbon source along with yeast extract and salts in the production medium for lactic acid fermentation by the pure strains and the coculture of lactobacilli. Different levels of glucose were applied in medium for four sets of experiments corresponding to each dose.

Table4 shows a rising trend of lactic acid concentration for each strain with increasing inputs of glucose till 100 g/L. All the strains attain their individual maximum lactic acid production at 100 g/L. A drop in the lactic acid concentration is observed in Table4 for all the *Lactobacillus* strains at 120 g/L due to inhibition caused by high concentration of glucose. Table4 indicates that coculture of strain 1 and 2 achieved the highest overall lactic acid production 93.57 g/L. It also attained the maximum lactic acid production values for each dose of glucose input, and was closely followed by strain-1 which had the next overall maximum 92.82 g/L of lactic acid. As the substrate concentration is increased to 100 g/L, the trend for lactic acid concentration was increasing, but further increase in sugar concentration (120g/L), caused substrate inhibition, since lactic acid concentration of all the strains dropped, in comparison to the lactic acid produced at 100 g/L glucose input.

Table4. The concentration values of lactic acid in batch fermentation produced by the different strains of lactobacilli on glucose based production media.

Bacterial strains	Glucose 60g/L		Glucose 80g/L		Glucose 100g/L		Glucose 120g/L	
	t (h)	LA (g/L)	t (h)	LA (g/L)	t (h)	LA (g/L)	t (h)	LA (g/L)
L. delbrueckii (Strain-1)	36	51.33	36	72.85	36	92.82	36	81.17
L.pentosus (Strain-2)	36	29.53	36	67.71	36	88.31	36	72.31
Coculture of strains-1, 2	36	53.24	36	73.68	36	93.57	36	84.57
Lactobacillus sp. (Strain-3)	36	41.31	36	69.58	36	89.12	36	74.02
Lactobacillus sp. (Strain-4)	36	46.24	36	71.20	36	91.56	36	75.63

LA - Lactic acid concentration (g/L); t-Time for attainment of lactic acid concentration (h); Conditions - Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Excessively high amount of glucose and the levels of ATP affect the activity of several enzymes of EMP pathway (Appendix-3,4), leading to reduced lactic acid production [71, 11].It has been proven through ANOVA test (Appendix-13) that variation in levels of glucose inputs bears a significant effect on lactic acid production by the different pure cultures and coculture of lactobacilli. The lactic acid production data(Table4) with 100 g/L glucose level showed least value,(2.2487) for the coefficient of variation C.V., and also least variance(standard deviation^2) value of 4.19, which indicated that at this level, the lactobacilli under study exhibit most uniform and efficient lactic acid production. However, the drop in lactic acid production at 120 g/L glucose input provided highest variance (21.1978) and C.V.(5.9377), clearly indicated non-uniform and less consistent lactic acid production by the lactobacilli and hence signified the substrate inhibition, which has least effect in case of coculture and strain-1. Coculture performed better lactic acid production than its component strains-1 and 2. Thus coculture and strain-1 may serve best in the glucose based fermentative production of lactic acid.

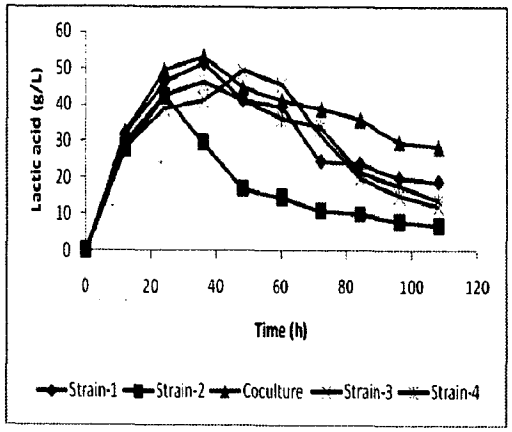


Figure 2a Lactic acid production by the *Lactobacillus* strains at 60 g/L glucose input

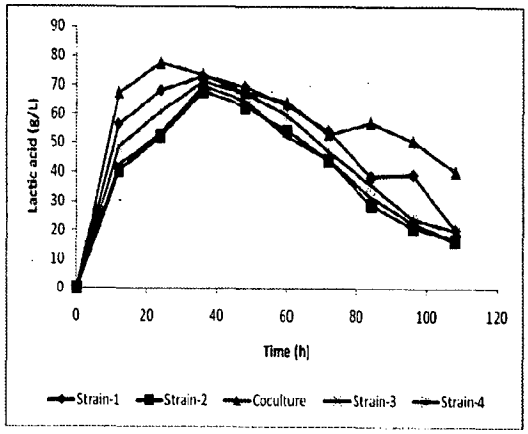


Figure 2b Lactic acid production by the *Lactobacillus* strains at 80 g/L glucose input

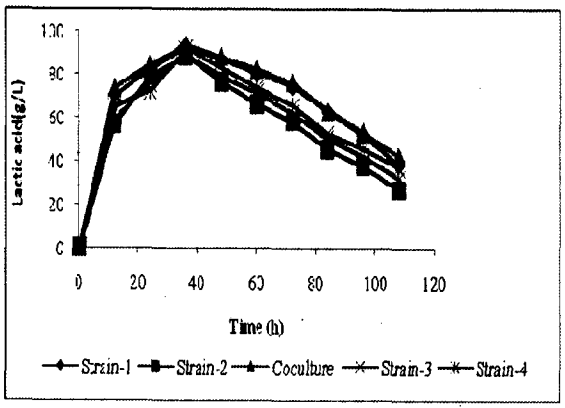


Figure 2c Lactic acid production by the *Lactobacillus* strains at 100 g/L glucose input

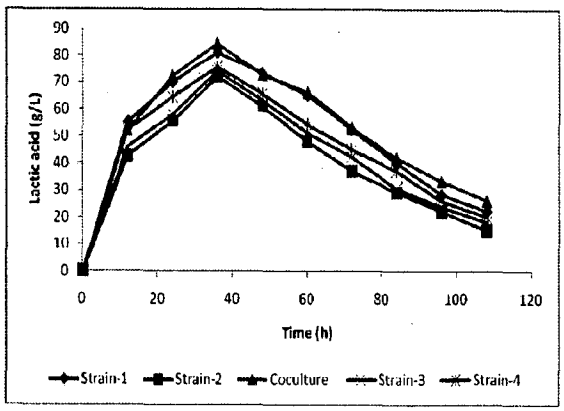


Figure 2d Lactic acid production by the *Lactobacillus* strains at 120 g/L glucose input

Figure 2a and Table 4 indicate that, at 60 g/L, the lowest dose of glucose, bacterial strains attained their individual maximum lactic acid production, at different times. Strain-3 attains the highest lactic acid production at 48 hours at 60 g/L glucose (Figure 2a) but attains maximum lactic acid production, earlier (36 hours) for all the other higher doses of glucose input, hence glucose input greater than 60 g/L are better suited for strain-3. Figures 2a, 2b, 2c and 2d and Table4 indicate the lowest lactic acid production by strain-2 (*Lactobacillus pentosus*) for each dose of the glucose input. This was due to the fact that strain-2, preferred pentose sugar utilization over the hexose sugars, such as glucose. From Figures2a, 2b, 2c and 2d, the bacterial strains show fluctuations (rises, drops) in lactic acid production values, due to the different responses of different strains of lactobacilli towards the pH drop caused due to

accumulation of free lactic acid product. Very high concentration of lactic acid leads to the influx of H^+ ions, in the cells, which in turn have to pump out these extra H^+ ions coupled with the expenditure of ATP, in order to maintain the intracellular pH. But this wasteful utilization of ATP, leads to growth inhibition which can bring down the lactic acid production levels [13]. The periodic addition of the neutralizer converts the free acid in lactate salt form and raises the pH, thus under the favourable conditions of pH, the bacterial cells again increase their lactic acid production. Moreover, a reported loss of 30-40% in lactic acid production due to product inhibition, through reduction of phosphofructokinase and lactate dehydrogenase enzyme activities(Appendix-4) by H^+ ions (in absence of neutralizer), could be avoided [62,98,71]. Sodium ions from the neutralizer may also help in sugar transport in the cell, which may increase the lactic acid production [89].

Table5. The weights and productivities of lactic acid in batch fermentation by the different strains of lactobacilli

Bacterial strains	Glucose 60g/L		Glucose 80g/L		Glucose 100g/L		Glucose 120g/L	
	W (g/g)	PLA (g/L/h)	W (g/g)	P LA (g/L/h)	W (g/g)	PLA (g/L/h)	W (g/g)	P LA (g/L/h)
L. delbrueckii (Strain-1)	0.86	2.0095	0.9106	4.695	0.9282	5.8416	0.6764	4.603
L.pentosus (Strain-2)	0.4922	1.7712	0.8645	3.385	0.8831	4.7300	0.6025	3.5533
Coculture of strains-1, 2	0.87	2.2329	0.921	5.6216	0.9357	6.1216	0.7047	4.9208
Lactobacillus sp. (Strain-3)	0.6885	1.6145	0.8697	3.5258	0.8912	4.9441	0.6168	3.834
Lactobacillus sp. (Strain-4)	0.77	1.7795	0.89	4.0625	0.9156	5.3983	0.6303	4.3591

PLA- Maximum productivity of lactic acid (g/L/h); W- Weight of lactic acid per unit weight of sugar input (g/g)

Table5 shows a rise in lactic acid productivity for each of the bacterial strains with increasing inputs of glucose till 100 g/L. All the strains attain their individual maximum lactic acid productivity at 100 g/L. A drop in the lactic acid productivity is observed in Table5 for all the *Lactobacillus* strains at 120 g/L due to inhibition by high dose of substrate (glucose) input. Coculture had the highest overall productivity, even higher than its component strains. It also

produced highest weight of lactic acid per unit weight of glucose input. Hence, due to these properties coculture should be the organism of choice for glucose based batch production of lactic acid.

4.1.1.2 Effect of whey substituted glucose

The portions of lactose sugar from whey, contributed 30,40,45 and 50 g/L of sugar in the media, while 30,40,65 and 70 g/L of pure glucose had been added, to provide a total sugar level of 60,80,110 and 120 g/L, in form of whey substituted glucose (WSG), as carbon source in production media. Various doses of whey substituted glucose have been applied as carbon source along with yeast extract and salts in the production medium for lactic acid fermentation by the pure strains and the coculture of lactobacilli. Different levels of whey substituted glucose (WSG), were applied in medium for four sets of experiments corresponding to each dose.

Table 6. The concentration values of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing various doses of whey substituted glucose application (WSG) as carbon source.

Bacterial strains	(WSG) 60g/L		(WSG) 80g/L		(WSG) 110g/L		(WSG) 120g/L	
	t (h)	LA (g/L)	t (h)	LA (g/L)	t (h)	LA (g/L)	t (h)	LA (g/L)
L. delbrueckii (Strain-1)	36	44.87	36	63.63	36	92.33	36	110.10
L .pentosus (Strain-2)	36	30.35	36	65.97	36	85.91	36	91.80
Coculture of strains-1, 2	36	48.37	36	76.89	36	104.64	36	114.96
Lactobacillus sp. (Strain-3)	36	36.31	36	59.26	36	65.01	36	85.92
Lactobacillus sp. (Strain-4)	36	40.39	36	68.98	36	88.11	36	95.11

LA - Lactic acid concentration (g/L); t-Time for attainment of lactic acid concentration (h); Conditions - Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 6 shows a rising trend of lactic acid concentration with increasing inputs of whey substituted glucose (WSG) up to 120 g/L. All the strains attained their individual maximum lactic acid production at 120 g/L. There is no drop in the lactic acid concentration, due to high dose of sugar application, (substrate inhibition) as evidenced in Table4 for all the

Lactobacillus strains at 120 g/L. Probably the stimulatory effect of whey components (proteins, amino acids, mineral salts) leads to better growth of cells, which in turn were capable of utilizing of high level of sugar (120 g/L) input, successfully without any inhibition or decline in acid production [15,112, 30]. Table 6 indicates that coculture achieved highest overall lactic acid production 114.96 g/L. Coculture also attained the maximum lactic acid production values for each dose of glucose input(Appendix-1), closely followed by strain-1, which has the next overall maximum 110.10 g/L of lactic acid. Coculture performs better production than its component strains. Thus coculture and strain-1 may serve best in the WSG based fermentative production of lactic acid.

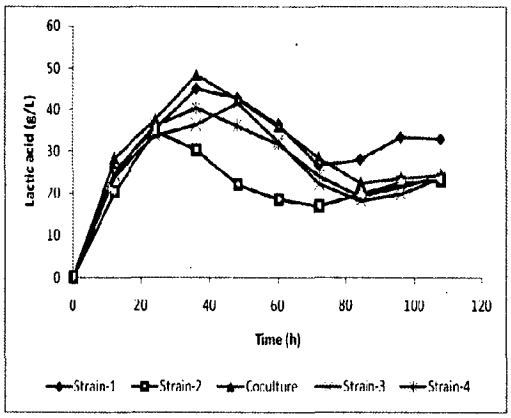


Figure 3a Lactic acid production by the *Lactobacillus* strains at 60 g/L WSG input

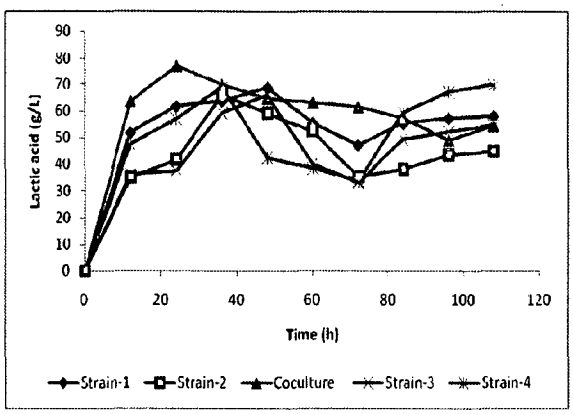


Figure 3b Lactic acid production by the *Lactobacillus* strains at 80 g/L WSG input

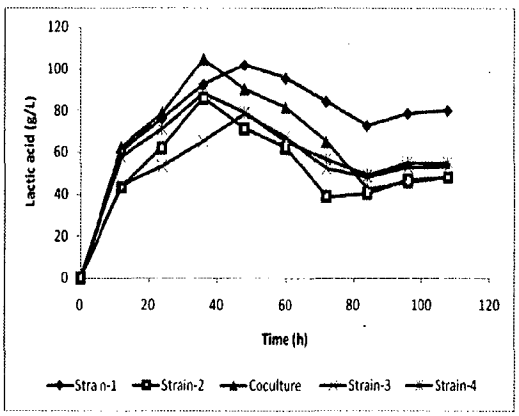


Figure 3c Lactic acid production by the *Lactobacillus* strains at 110 g/L WSG input

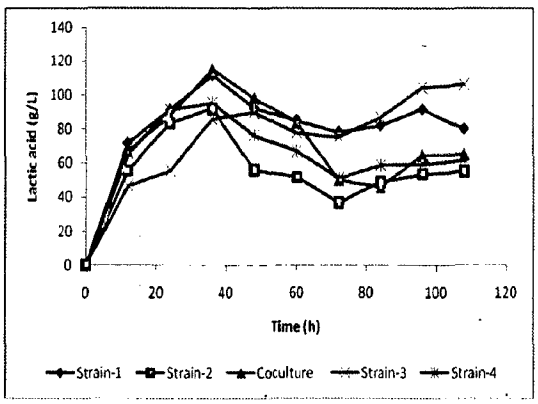


Figure 3d Lactic acid production by the *Lactobacillus* strains at 120 g/L WSG input

Figure 3a and Table 6 indicate that, at 60 g/L, lowest dose of glucose, the bacterial strains attain their individual maximum lactic acid production at different times. Figures 3a,

3b, 3c and 3d and data in Table 6 indicated low lactic acid production by strain-2 (*Lactobacillus pentosus*) the for each dose of the glucose input, which may be due to the fact that strain-2, predominantly has preference for pentose sugars. The fluctuations in the lactic acid production from the *Lactobacillus* strains observed from Figures3a, 3b, 3c and 3d, are possibly, due to the different responses of these strains towards the varying pH drop that occur during the course of fermentation, due to accumulation of free lactic acid product. The addition of the neutralizer removed the free acid and raised the pH again, up to a value, where the bacterial cells again enhanced their lactic acid production. A steep rise in lactic acid production from the strains is observed in Figures3a,3b, 3c and 3d, after a sharp decline, is possibly, due to the changeover in their sugar consumption(preferential sugar uptake) from glucose to lactose and a possible diauxic growth[84, 89].

Table 7. Maximum weights and productivities of lactic acid in batch fermentation, by the different strains of lactobacilli in a production medium, containing various levels of whey substituted glucose (WSG) as carbon source.

Bacterial strains	(WSG) 60g/L		(WSG) 80g/L		(WSG) 110g/L		(WSG) 120g/L	
	W (g/g)	PLA (g/L/h)	W (g/g)	P LA (g/L/h)	W (g/g)	PLA (g/L/h)	W (g/g)	P LA (g/L/h)
L. delbrueckii (Strain-1)	0.7478	2.1130	0.7954	4.3125	0.9249	5.0260	0.9300	5.5067
L.pentosus (Strain-2)	0.5058	1.6990	0.8246	2.9140	0.7810	3.606	0.7650	4.6200
Coculture of strains-1, 2	0.8061	2.370	0.9611	5.2974	0.9512	5.1940	0.9580	5.5825
Lactobacillus sp. (Strain-3)	0.6052	2.018	0.7407	3.0225	0.5910	3.6700	0.7160	3.8700
Lactobacillus sp. (Strain-4)	0.6731	2.0650	0.8622	4.775	0.8010	4.810	0.7926	5.4573

PLA- Maximum productivity of lactic acid (g/L); W - Weight of lactic acid per unit weight of sugar input (g/g)

Table 7 shows a rise in lactic acid productivity for each of the bacterial strains with increasing inputs of WSG up to 120 g/L. All the strains attain their individual maximum lactic acid productivity at 120 g/L.

The coculture had the highest overall productivity, which was even higher than its component strains. It also produced highest weight of lactic acid per unit weight of WSG input. Hence, due to these properties the coculture should be preferred in WSG based fermentative production of lactic acid.

4.1.1.3 Effect of lactose

Various doses of lactose 60 g/L, 80 g/L, 100 g/L and 120 g/L have been applied in the production medium as carbon source along with yeast extract and salts for lactic acid fermentation by the pure strains and coculture of lactobacilli for lactic acid fermentation. Different levels of lactose were applied in medium for four sets of experiments corresponding to each dose.

Table 8. The maximum concentration values of lactic acid produced in batch fermentation by the different strains of lactobacilli in a production medium containing various doses of pure lactose as carbon source.

Bacterial strains	Lactose 60g/L		Lactose 80g/L		Lactose 100g/L		Lactose 120g/L	
	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)
L. delbrueckii (Strain-1)	36	52.05	36	72.88	36	93.00	36	112.84
L.pentosus (Strain-2)	36	48.95	36	67.96	36	83.60	36	98.60
Coculture of strains-1, 2	36	52.65	36	74.60	36	95.21	36	115.56
Lactobacillus sp. (Strain-3)	36	50.16	36	70.02	36	90.10	36	104.85
Lactobacillus sp. (Strain-4)	36	50.93	36	71.12	36	90.42	36	108.90

LA Max.- Maximum lactic acid concentrations obtained(g/L);t-Time for attainment of maximum lactic acid concentration (h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 8 shows a rise in lactic acid concentration for each bacterial strain with increasing inputs of lactose up to 120 g/L. All the strains attained their individual maximum lactic acid production at 120 g/L. The higher values of lactose based lactic acid production (Table 8) and absence of high dose sugar inhibition, in comparison with those obtained with pure glucose in

Table 4, may possibly be due to better uptake of lactose through one or more mechanisms, the lac operon(lactose permease), lactose galactose dependent antiport or phosphoenol pyruvate dependent phosphotransferase system in lactobacilli [23,89,113]. Table 8 indicates that the coculture of strain 1 and 2 achieved the highest overall lactic acid production 115.56 g/L, and also attained the maximum lactic acid production values for all the lactose inputs. It was closely followed by strain-1 which has the next overall maximum 112.84 g/L of lactic acid. Coculture performed better production than its component strains. Thus the coculture and the strain-1 may serve best in the lactose based fermentative production of lactic acid.

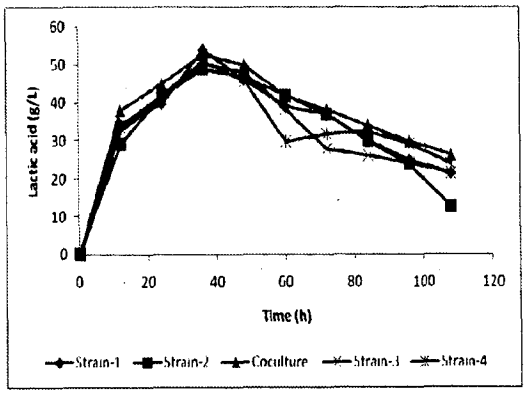


Figure 4a Lactic acid production by the *Lactobacillus* strains at 60 g/L lactose input

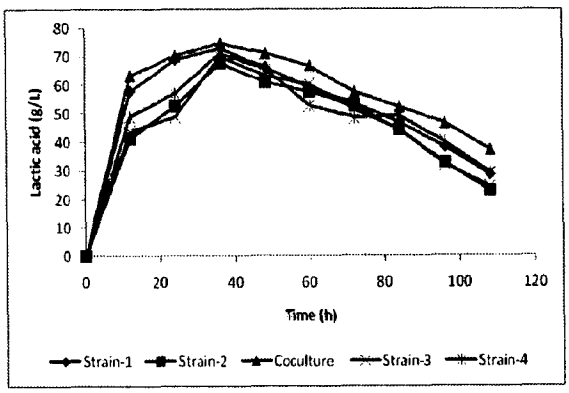


Figure 4b Lactic acid production by the *Lactobacillus* strains at 80 g/L lactose input

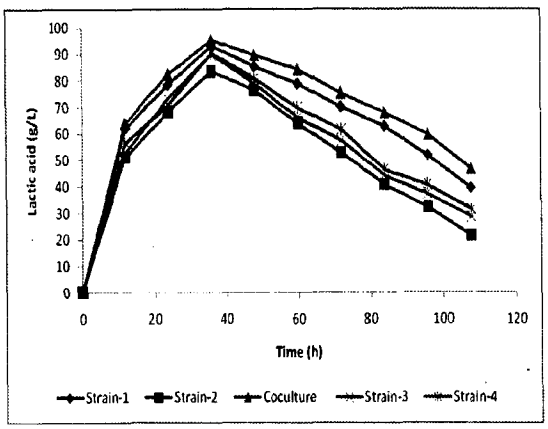


Figure 4c Lactic acid production by the *Lactobacillus* strains at 100 g/L lactose input

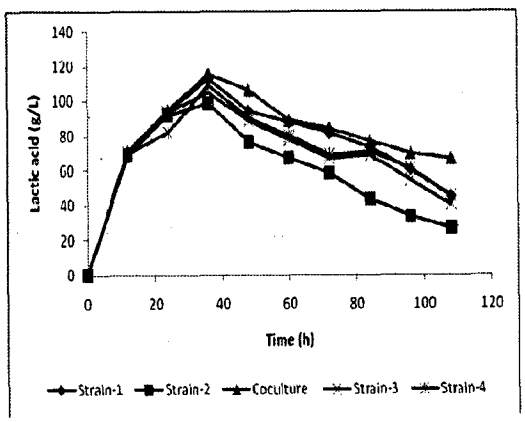


Figure 4d Lactic acid production by the *Lactobacillus* strains at 120 g/L lactose input

Figures 4a, 4b, 4c and 4d and Table 8 indicate that, all the strains attained their maximum individual production in 36 hours with all the doses of lactose applied. It is evident from Table 8 and Figures 4a through 4d that, strain-2 (*Lactobacillus pentosus*) showed the lowest lactic acid production for all the doses of the lactose input, possibly due to the fact that strain-2 is primarily a pentose sugar consumer. It can be observed from Figures 4a, 4b, 4c and 4d, that the lactic acid concentrations from coculture exhibit, lesser fluctuations, as compared with strain-3 and strain-4, which display sharp fluctuations in lactic acid production. This indicated that the coculture had more stable production in the varying pH environment during the course of lactose based fermentation, while the production trend of strain-3 and strain-4 showed more variations in response to such pH values. The periodic addition of the sodium hydroxide neutralizer removes the free acid and restores favorable pH, again for better growth and production, hence losses in lactic acid production are averted [62,119].

Table 9. The maximum weights and productivities of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing various doses of pure lactose as carbon source

Bacterial strains	Lactose 60g/L		Lactose 80g/L		Lactose 100g/L		Lactose 120g/L	
	$W_{Max.}$ (g/g)	PLA (g/L/h)	$W_{Max.}$ (g/g)	P LA (g/L/h)	$W_{Max.}$ (g/g)	PLA (g/L/h)	$W_{Max.}$ (g/g)	P LA (g/L/h)
L. delbrueckii (Strain-1)	0.8675	2.8580	0.9218	4.8050	0.9301	5.1800	0.9403	5.890
L.pentosus (Strain-2)	0.8158	2.4230	0.8495	3.4580	0.8360	4.2766	0.8216	5.7366
Coculture of strains-1, 2	0.8835	3.1550	0.9470	5.2641	0.9521	5.3390	0.9630	5.9650
Lactobacillus sp. (Strain-3)	0.8360	2.6830	0.8905	3.655	0.9010	4.4408	0.8737	5.7508
Lactobacillus sp. (Strain-4)	0.8488	2.7980	0.9001	4.0625	0.9042	4.7275	0.9075	5.8433

PLA- Maximum Volumetric Productivity of Lactic acid (g/L/h); $W_{Max.}$-Maximum weight of lactic acid per unit weight of sugar input(g/g)

Table 9 shows a rise in lactic acid productivity for each of the bacterial strains with increasing inputs of lactose till 120 g/L where all the strains attain their individual maximum

lactic acid productivity. Coculture has the highest overall productivity of 5.9650 g/L/h. It also produces highest weight of lactic acid per unit weight of lactose input. Hence, it should be a preferred organism for lactose based lactic acid fermentations. High productivity values of lactic acid(Table 9), shown by all the pure strains and coculture indicate that the galactose formed along with glucose through the hydrolysis of lactose by β-galactosidase, is also consumed efficiently for lactic acid production, in the EMP pathway (like glucose), after being converted to glucose-6-phosphate [11].

4.1.1.4 Effect of whey substituted lactose

The portions of lactose sugar from whey, contributed 30,40,45 and 50 g/L of sugar in the media, while 30,40,65 and 70 g/L of pure lactose had been added, to provide a total sugar level of 60,80,110 and 120 g/L, in form of whey substituted lactose (WSL), as carbon source in production media. Different levels of (WSL), were applied in medium for four sets of experiments corresponding to each dose.

Table10. The maximum concentration values of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing various doses of whey substituted lactose (WSL) as carbon source

Bacterial strains	(WSL) 60g/L		(WSL) 80g/L		(WSL) 110g/L		(WSL) 120g/L	
	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)
L. delbrueckii (Strain-1)	36	52.47	36	73.75	36	102.85	36	113.75
L.pentosus (Strain-2)	36	49.56	36	69.40	36	96.58	36	105.84
Coculture of strains-1, 2	36	53.01	36	75.16	36	104.72	36	116.01
Lactobacillus sp. (Strain-3)	36	51.04	36	71.24	36	98.83	36	108.65
Lactobacillus sp. (Strain-4)	36	51.92	36	72.01	36	99.94	36	109.85

LA Max.- Maximum lactic acid concentrations obtained(g/L);t-Time for attainment of maximum lactic acid concentration(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

The lactose content in whey was 4.98%. Whey substituted lactose along with yeast extract and salts are added in the production medium for lactic acid production by the pure strains and the coculture of lactobacilli.

Table10 shows enhancement in lactic acid concentration for each bacterial strain with increasing inputs of whey substituted lactose (WSL) up to the highest input 120 g/L. All the strains attained their individual maximum lactic acid production at 120 g/L. There is no drop in the lactic acid concentration, due to high dose of sugar application (substrate inhibition) as evidenced in Table4 for all the *Lactobacillus* strains at 120 g/L. Probably the stimulation by the whey component containing various proteins, amino acids and vitamins leads to growth stimulation of cells, which in turn were capable of utilizing of high level of sugar (120 g/L) input, successfully without any inhibition or decline in acid production [15]. Better cell growth enhances lactic acid production because the lactic acid fermentation is both growth and non growth associated one [60]. Table10 indicates that the coculture of strain 1 and 2 achieved the highest overall lactic acid production 116.01 g/L.

Coculture also attained the maximum lactic acid production values for each concentration of (WSL) input closely followed by strain-1, which has the next overall maximum 113.75 g/L of lactic acid. The coculture performs better production than its component strains. Thus the coculture and strain-1, may serve best in the WSL based fermentative production of lactic acid. Table 10, showed higher values of lactic acid production with WSL as compared to Table 6 with WSG (whey substituted glucose) for all the bacterial strains because the glucose is known for its role in inhibiting the lac-operon, through lowering of cyclic AMP levels, required for binding of CRP to promoter so that RNA polymerase can attach with DNA for mRNA synthesis prior to β-galactosidase and lactose permease formation[84,89].This consequently lowers the uptake and utilization of the whey lactose meant for lactic acid production in WSG.

Figure 5a and Table 10 indicate that, at 60 g/L, the lowest dose of WSL, bacterial strains attained their individual maximum lactic acid production at 36 hour except strain-3, which does it in 48 hours. This suggested that doses of WSL above 60 g/L are more effective on the strain-3 in reducing the time of attainment of maximum lactic acid concentration.

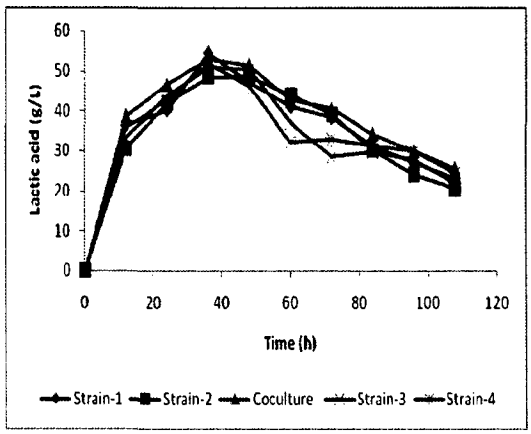


Figure 5a Lactic acid production by the *Lactobacillus* strains at 60 g/L WSL input

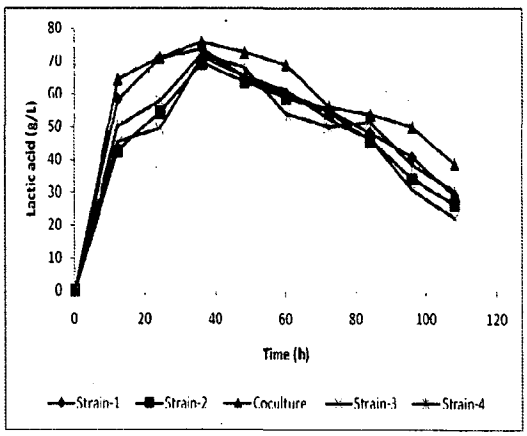


Figure 5b Lactic acid production by the *Lactobacillus* strains at 80 g/L WSL input

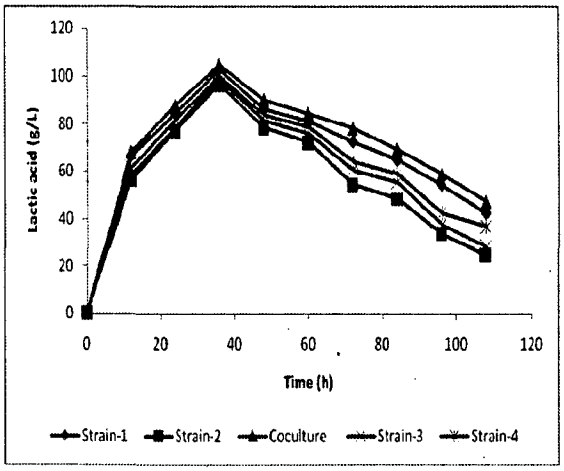


Figure 5c Lactic acid production by the *Lactobacillus* strains at 110 g/L WSL input

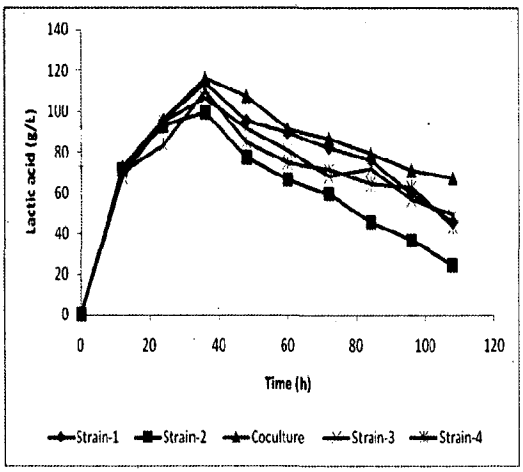


Figure 5d Lactic acid production by the *Lactobacillus* strains at 120 g/L WSL input

Figures 5a, 5b, 5c and 5d and data in Table 10 indicated lowest lactic acid production by strain-2 (*Lactobacillus pentosus*) the for each dose of the WSL input, as it is majorly a pentose consumer. The fluctuations in the lactic acid production from the *Lactobacillus* strains-2 and 3, were higher than other strains, with all the doses of WSL inputs, as observed from the Figures 5a,5b, 5c and 5d. This showed lesser stability in their lactic acid production under varying conditions of pH during fermentation.

Table11. The maximum weights and productivities of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing various doses of whey substituted lactose (WSL) as carbon source

Bacterial strains	(WSL) 60g/L		(WSL) 80g/L		(WSL) 110g/L		(WSL) 120g/L	
	$W_{Max.}$ (g/g)	PLA (g/L/h)	$W_{Max.}$ (g/g)	P LA (g/L/h)	$W_{Max.}$ (g/g)	PLA (g/L/h)	$W_{Max.}$ (g/g)	P LA (g/L/h)
L. delbrueckii (Strain-1)	0.8745	3.0350	0.9218	4.8600	0.9350	5.5192	0.9479	5.9875
L.pentosus (Strain-2)	0.8260	2.5458	0.8675	3.5500	0.8780	4.6941	0.8820	5.9538
Coculture of strains-1, 2	0.8835	3.2350	0.9395	5.3700	0.9520	5.6675	0.9667	6.0538
Lactobacillus sp. (Strain-3)	0.8506	2.7708	0.8905	3.7600	0.8985	4.8800	0.9054	5.6316
Lactobacillus sp. (Strain-4)	0.8650	2.9716	0.9001	4.1683	0.9085	5.1108	0.9154	5.8341

PLA- Maximum productivity of lactic acid (g/L/h)); $W_{Max.}$-Maximum weight of lactic acid per unit sugar input (g/g)

A rising trend in lactic acid productivity is exhibited by all the bacterial strains in response to increasing inputs of WSL till 120 g/L (Table 11). All the strains attain their individual maximum lactic acid productivity at 120 g/L lactose. Coculture has the highest overall productivity value 6.0538 g/L/h. It also produces highest weight of lactic acid per unit weight of WSL input. Hence, it should be a preferred organism for WSL based lactic acid fermentations.

4.1.1.5 Effect of acid hydrolyzed starch

Various doses of soluble potato starch used for acid hydrolysis 60, 80, 100 and 120 g/L. The neutralized acid hydrolyzates corresponding to each dose of starch have been applied in the production medium as a carbon source for lactic acid production by the pure strains and the coculture.

Table 12. The values of maximum concentration of lactic acid in batch fermentation attained by the different strains of lactobacilli in a production medium containing various doses of acid hydrolyzed starch (AHS) as carbon source

Bacterial strains	Starch (AHS) 60g/L		Starch (AHS) 80g/L		Starch (AHS) 100g/L		Starch (AHS) 120 g/L	
	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)
L. delbrueckii (Strain-1)	48	48.76	48	66.25	48	88.90	48	109.60
L.pentosus (Strain-2)	48	39.61	48	50.83	48	73.40	48	93.75
Coculture of strains-1, 2	48	50.81	48	71.58	48	91.60	48	112.13
Lactobacillus sp. (Strain-3)	48	44.61	48	60.03	48	78.30	48	98.32
Lactobacillus sp. (Strain-4)	48	41.25	48	56.76	48	75.60	48	100.27

LA Max.- Maximum lactic acid concentrations (g/L); t-Time for attainment of maximum lactic acid concentration (h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 12, shows an increasing trend of lactic acid concentration from all the strains with the increasing doses of starch. Coculture showed maximum overall production of 112.13 g/L, closely followed by strain-1 with 109.60 g/L. There is no inhibition due to high dose of substrate concentration as it was observed with glucose as carbon source in Table 1. The maximum lactic acid production for each strain mentioned in Table 12 at 120 g/L is lesser than those in Table 10, obtained with WSL as carbon source. This may be due to presence of

oligosaccharides (more resistant to hydrolysis), besides glucose from starch hydrolysis, which may not be readily utilized by the strains as carbon source and moreover there are no stimulatory substances for bacteria in starch as like whey [118].

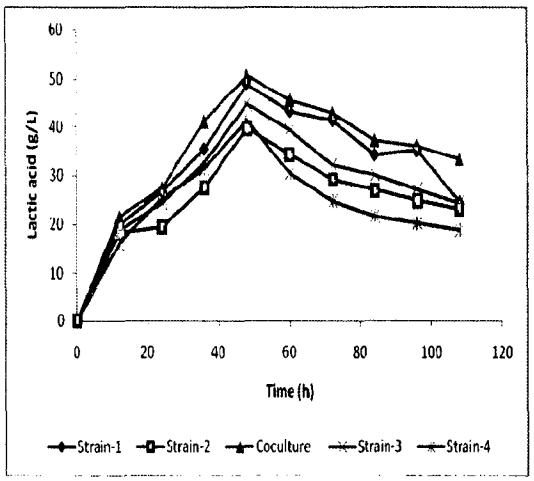


Figure 6a Lactic acid production by the *Lactobacillus* strains at 60 g/L starch input followed by acid hydrolysis

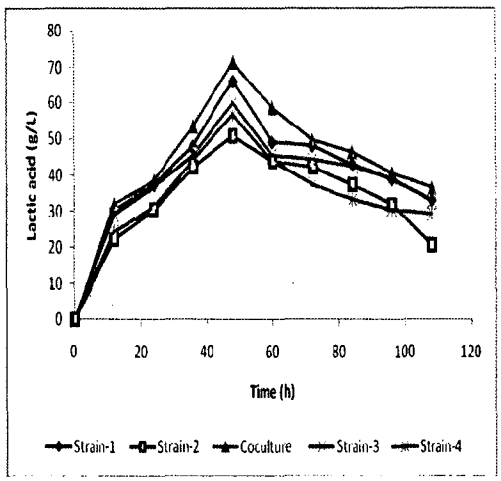


Figure 6b Lactic acid production by the *Lactobacillus* strains at 80 g/L starch input followed by acid hydrolysis

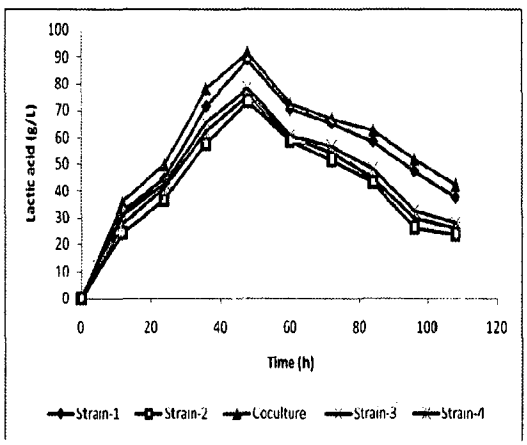


Figure 6c Lactic acid production by the *Lactobacillus* strains at 100 g/L starch input followed by acid hydrolysis

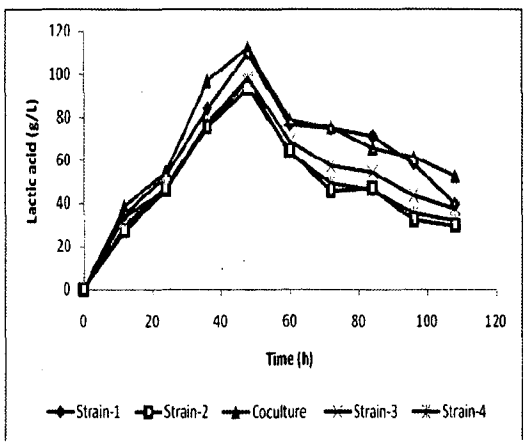


Figure 6d Lactic acid production by the *Lactobacillus* strains at 120 g/L starch input followed by acid hydrolysis

Figures 6a, 6b, 6c and 6d and Table 12, show that the maximum lactic acid concentration is attained by all the strains at 48 hours instead of 36 hours as seen in majority of previous values with other carbon sources. Figures 6a, 6b, 6c and 6d show that the with AHS as carbon source the strain-3 shows lesser fluctuations in lactic acid production while the

strains-1,2,4 and coculture show more fluctuations. The variation in pH increases, hence the fluctuations also increase after 60 h of fermentation.

Table 13. The maximum weights and productivities of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing various doses of acid hydrolyzed starch (AHS) as carbon source

Bacterial strains	Starch (AHS) 60g/L		Starch (AHS) 80g/L		Starch (AHS) 110g/L		Starch (AHS) 120g/L	
	$W_{Max.}$ (g/g)	PLA (g/L/h)	$W_{Max.}$ (g/g)	P LA (g/L/h)	$W_{Max.}$ (g/g)	PLA (g/L/h)	$W_{Max.}$ (g/g)	P LA (g/L/h)
L. delbrueckii (Strain-1)	0.8128	1.646	0.8281	2.5375	0.8890	2.6791	0.9133	2.9033
L.pentosus (Strain-2)	0.6602	1.5133	0.6978	1.8633	0.7340	2.0340	0.7813	2.3008
Coculture of strains-1, 2	0.8468	1.7991	0.8947	2.6716	0.9160	2.9825	0.9344	3.2200
Lactobacillus sp. (Strain-3)	0.7345	1.5483	0.7504	2.4341	0.7873	2.5891	0.8193	2.8383
Lactobacillus sp. (Strain-4)	0.6875	1.330	0.7095	2.0308	0.7560	2.2941	0.8355	2.4758

PLA- Maximum Productivity of Lactic acid (g/L/h); $W_{Max.}$-Maximum weight of lactic acid per unit sugar input (g/g)

Table 13, shows a rise in lactic acid productivity for each of the bacterial strains with increasing inputs of AHS till 120 g/L. All the strains attain their individual maximum lactic acid productivity at 120 g/L lactose. Coculture has the highest overall productivity value 3.220 g/L/h. It also produces highest weight of lactic acid per unit weight of AHS input. Hence, it should be a preferred organism for AHS based lactic acid fermentations. The maximum values of productivity found at 120 g/L dose of AHS in Table 10 are comparatively lesser than those found in Tables 5, 7,9 and 11 with other carbon sources, hence AHS is less suitable as carbon source for all the strains under study.

4.1.1.6 Effect of enzyme hydrolyzed starch application

Various doses of soluble potato starch used for enzymatic hydrolysis 60, 80, 100 and 120 g/L. The hydrolyzates corresponding to each dose of starch have been applied in the production medium as a carbon source for lactic acid production by the pure strains and the coculture.

Table14. The maximum concentration values of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing various doses of enzyme hydrolyzed potato starch EHS as carbon source.

Bacterial strains	Starch (EHS) 60g/L		Starch (EHS) 80g/L		Starch (EHS) 100g/L		Starch (EHS) 120g/L	
	T (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)
L. delbrueckii (Strain-1)	48	52.04	48	69.21	48	90.10	48	112.37
L.pentosus (Strain-2)	48	43.95	48	59.39	48	78.07	48	101.54
Coculture of strains-1, 2	48	53.25	48	73.38	48	92.80	48	114.33
Lactobacillus sp. (Strain-3)	48	48.64	48	67.37		85.06	48	103.67
Lactobacillus sp. (Strain-4)	48	46.85	48	64.06	48	82.01	48	105.29

LA Max.- Maximum lactic acid concentrations (g/L);t-Time for attainment of maximum lactic acid concentration (h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

All the strains, exhibit an increasing trend of lactic acid concentration in Table 14, with the enhanced levels of EHS application. Coculture showed maximum overall production of 114.33 g/L, closely followed by strain-1 with 112.37 g/L. There is no inhibition due to high dose of substrate concentration as it was observed with glucose as carbon source in Table 1. The maximum lactic acid production for each strain mentioned in Table14 at 120 g/L (EHS) is more than those in Table 12, obtained with AHS as carbon source. It has been reported that the acid or enzymatic hydrolysis of starch produce oligosaccharides (dextrins), some fraction of which are more susceptible (having α-1,4 glycosidic bonds) while the other fraction is resistant

to hydrolysis (having α-1,6 glycosidic bonds) which on further course liberates maltotriose, maltose and glucose[118]. Hence, lower lactic acid production with AHS indicates the formation of higher amount of resistant oligosaccharides which may not be readily converted to glucose or directly utilized as carbon source [89].

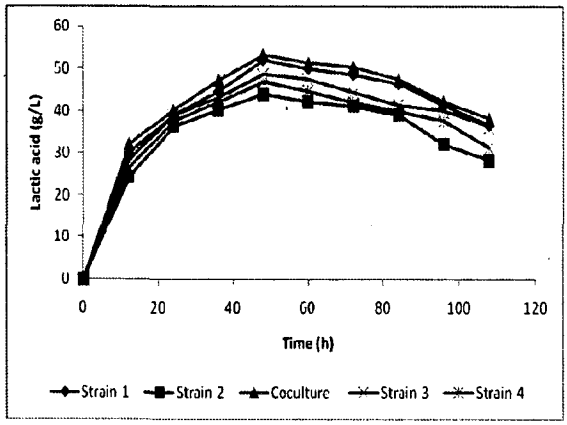


Figure 7a Lactic acid production by the *Lactobacillus* strains at 60 g/L starch input followed by enzymatic hydrolysis

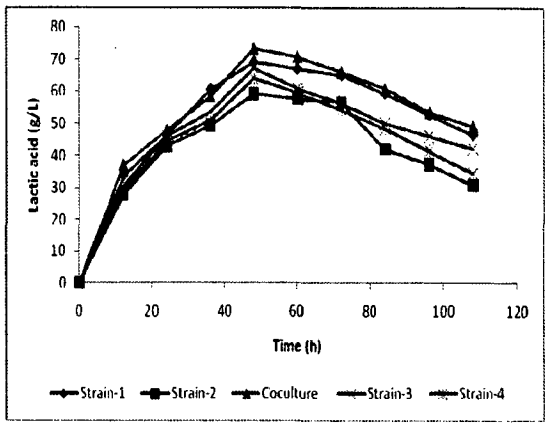


Figure 7b Lactic acid production by the *Lactobacillus* strains at 80 g/L starch input followed by enzymatic hydrolysis

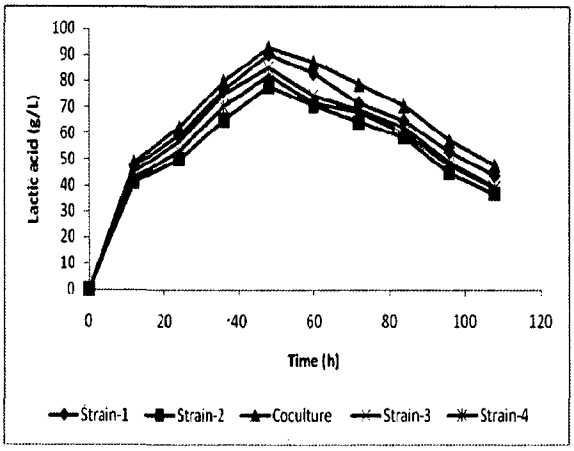


Figure 7c Lactic acid production by the *Lactobacillus* strains at100 g/L starch input followed by enzymatic hydrolysis

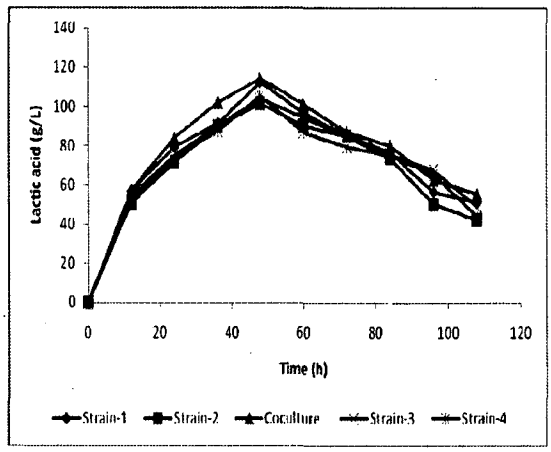


Figure 7d Lactic acid production by the *Lactobacillus* strains at 120 g/L starch input followed by enzymatic hydrolysis

Figures 7a, 7b, 7c and 7d and Table 14, show that the maximum lactic acid concentration is attained by all the strains at 48 hours instead of 36 hours as seen in majority of previous values with other carbon sources. Figures 7a, 7b, 7c and 7d show that with EHS as carbon source strain-1,strain-3 and coculture showed lesser fluctuations in lactic acid production while the strains-2 and 4 show more fluctuations. The variation in pH increases,

hence the fluctuations in response also increase after 60 h of fermentation. The productions of all the *Lactobacillus* strains including coculture with AHS and EHS were greater than 19g/L obtained with *Rhizopus oryzae* directly utilizing potato starch containing waste stream [38].

Table15. The maximum weights and productivities of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing various doses of enzyme hydrolyzed starch (EHS)

Bacterial strains	Starch (EHS) 60g/L		Starch (EHS) 80g/L		Starch (EHS) 110g/L		Starch (EHS) 120g/L	
	$W_{Max.}$ (g/g)	PLA (g/L/h)	$W_{Max.}$ (g/g)	P LA (g/L/h)	$W_{Max.}$ (g/g)	PLA (g/L/h)	$W_{Max.}$ (g/g)	P LA (g/L/h)
L. delbrueckii (Strain-1)	0.8673	2.5040	0.8651	2.8066	0.9010	3.9400	0.9364	4.7083
L.pentosus (Strain-2)	0.7326	2.0258	0.7423	2.3350	0.7807	3.4400	0.8461	4.2241
Coculture of strains-1, 2	0.8875	2.6791	0.9172	3.0858	0.9280	4.0800	0.9527	4.7741
Lactobacillus sp. (Strain-3)	0.8106	2.3550	0.8421	2.5758	0.8506	3.7641	0.8639	4.5310
Lactobacillus sp. (Strain-4)	0.7808	2.236	0.8007	2.4450	0.8201	3.5325	0.8774	4.3416

PLA- Maximum productivity of lactic acid (g/L); $W_{Max.}$-Maximum weight of lactic acid per unit sugar input (g/g)

Table 15, shows a rising trend in lactic acid productivity for each of the bacterial strains with increasing inputs of EHS till 120 g/L. All the strains attained their individual maximum lactic acid productivity at 120 g/L lactose. Coculture has the highest overall productivity value 4.7741 g/L/h. It also produces highest weight of lactic acid per unit weight of EHS input. Hence, it should be a preferred organism for EHS based lactic acid fermentations. The maximum values, of productivity found at 120 g/L dose of EHS in Table 15 is higher than those found in Tables 13 with AHS. The lower productivity with AHS may be possible due to several reasons such as, presence of higher portion of resistant oligosaccharides, formation of longer chain oligomers, for the uptake of which there may be lack of suitable permease enzyme in lactobacilli under study, high energy expenditure in consuming longer chain

oligomers which lead to reduction in the growth rate and productivity [118, 89, 84]. Hence EHS is more suitable as carbon source than AHS, for all the strains under study.

4.1.1.7 Effect of acid treated and boiled sugarcane bagasse extract

The detoxified (Appendix-2), neutralized extract obtained from 1% sulphuric acid treatment of sugarcane bagasse (taking 1:10 w/v ratio under boiling conditions at atmospheric pressure for 30 minutes) was used to prepare the production media, such that initial pH was kept at 6.5.The main sugar components obtained from the acid hydrolyzate of dried, ground sugarcane bagasse were: Xylose , 4.56 g/L; arabinose, 0.54 g/L and glucose, 1.01 g/L. Pure glucose input of 40 g/L was also supplied apart from the sugars in bagasse hydrolyzate to be used as carbon source for lactic acid production by pure strains and coculture of lactobacilli through batch fermentation.

Table16. The maximum lactic acid production(LA max) and productivities of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing acid treated and boiled bagasse extract and glucose as carbon sources.

Bacterial strains	Acid treated and boiled extract of sugarcane bagasse as carbon source			
	t(h)	LA max (g/L)	pH	P LA (g/L/h)
L. delbrueckii (Strain-1)	84	31.68±1.45	5.56	0.3772
L.pentosus (Strain-2)	84	26.00±0.96	5.64	0.3095
Coculture of strains-1, 2	84	36.98±1.63	5.34	0.4335
Lactobacillus sp. (Strain-3)	84	36.21±1.51	5.36	0.4315
Lactobacillus sp. (Strain-4)	60	34.00±1.34	5.44	0.5667

PLA- Productivity of Lactic acid (g/L/h); t- time to attain LA max (h);
LA max-Maximum lactic acid concentration (g/L); ±indicates standard deviations from three trials

Table 16 shows that coculture attained the maximum lactic acid production 36.98 g/L, closely followed by strain-3, which provided a maximum lactic acid production of 36.21 g/L. The productivity of coculture was overall highest but strain-3 also closely followed the highest productivity. Coculture and strain-3, performed better than all other strains, in production medium (containing, extract of sugarcane bagasse under acid treatment and

boiling conditions) and glucose, as carbon sources. Strain-1 produced lesser amount of lactic acid (Table 16) in comparison to strain-3, strain-4 and coculture because the major portion of the sugars in the hydrolyzate are pentoses such as xylose, arabinose etc., but strain-1, is specific for hexose consumption [65,112].

4.1.1.8 Effects of application of acid treated and autoclaved sugarcane bagasse extract

The detoxified, neutralized extract obtained from 1% sulphuric acid treatment of sugarcane bagasse (taking 1:10 w/v ratio under autoclaving conditions121°C and 15psi pressure for 30 minutes) was used to prepare the production media, such that initial pH was kept at 6.5.

Table17. The maximum values of lactic acid production (LA max), and productivity of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing acid treated and autoclaved bagasse extract as carbon source

Bacterial strains	Acid treated and autoclaved extract of sugarcane bagasse as carbon source			
	t(h)	LA max (g/L)	pH	P LA (g/L/h)
L. delbrueckii (Strain-1)	48	40.37±1.20	5.22	0.8410
L.pentosus (Strain-2)	48	50.01±1.72	5.03	1.0418
Coculture of strains-1, 2	48	51.86±1.89	4.92	1.0800
Lactobacillus sp. (Strain-3)	48	49.84±1.68	4.98	1.03800
Lactobacillus sp. (Strain-4)	60	38.61±1.51	5.26	0.6434

PLA- Productivity of Lactic acid (g/L/h);t-time to attain LA max (h);LA max-Maximum lactic acid concentration (g/L) , ±indicates standard deviations from three trials.

The main sugar components obtained from the acid hydrolyzate of dried, ground sugarcane bagasse were: Xylose , 11.62 g/L; arabinose, 1.24 g/L and glucose, 2.85 g/L. Pure glucose input of 40 g/L was also supplied apart from the sugars in bagasse hydrolyzate to be used as carbon source for lactic acid production by pure strains and coculture of lactobacilli through batch fermentation.

Table 17, shows that the coculture attained the maximum lactic acid production, 51.86 g/L, closely followed by strain-2, which provided a maximum lactic acid production of 50.01 g/L. The productivity of the coculture was the overall highest but the strain 2 also closely followed the highest productivity. Coculture and strain-2, performed better than all other strains in a production medium containing acid treated and autoclaved sugarcane bagasse extract and glucose as carbon sources. It has been reported that more than70% of pentoses are released from hemicellulose fraction when acid hydrolysis of sugarcane bagasse is performed at temperatures between 100-160° C [72].

Hence, Table 16 values of lactic acid concentration were found to be lesser than those in Table 17 because the high pressure and high temperature (121°C) autoclaving treatment of bagasse liberated more amount of xylose and glucose sugar as compared with boiling treatment. This higher level of pentose, enhanced the lactic acid production in strain-2, which prefers pentose sugars.

4.1.2 Effect of nitrogen sources

Various doses 9,10,12,15 g/L of nitrogen sources such as meat extract, proteose peptone and yeast extract and for cheap nitrogen source baker's yeast the doses of12,15,20,23 g/L have been applied in the production medium for lactic acid fermentation by the pure strains and the coculture in different batch experiments.

4.1.2.1 Effect of nitrogen sources on strain-1

Different nitrogen sources were used with strain-1 for lactic acid production.

Table18. The maximum concentration and productivity values of lactic acid in batch fermentation, produced by strain-1, of lactobacilli with various doses of nitrogen sources

Strain-1	LA Max.	t (h)	Dose (g/L)	P (g/L/h)	$P_{Max.}$ (g/L/h)
Meat extract	45.31	36	12	1.2586	2.2758
Proteose peptone	48.40	36	12	1.3444	2.3506
Baker's yeast	45.20	36	20	1.2555	2.1092
Yeast Extract	52.93	36	10	1.4702	2.6583

LA Max.- Maximum lactic acid concentration (g/L); P- Volumetric productivity; $P_{Max.}$ - Maximum volumetric productivity(g/L/h); t-time to attain LA Max. (h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table18 shows that yeast extract had the maximum production of lactic acid at lowest dose, with strain-1, followed by the proteose peptone. The lactic acid fermentation is associated with growth and the yeast extract possesses the stimulatory amino acids, vitamins and relatively higher quantity of proteins, that are important for the growth of bacteria hence it enhances the lactic acid production[60,21,116]. The cheap nitrogen source baker's yeast (the parent source of yeast extract) required a higher dose 20 g/L to provide reasonably high lactic acid production, which should be beneficial in lowering the cost incurred on the nitrogen sources [7]. The application of yeast extract also provides highest values of volumetric productivity and maximum productivity of lactic acid.

4.1.2.2 Effect of nitrogen sources on strain-2

Different nitrogen sources were used with strain-1 for lactic acid production.

Table19. The maximum concentration values of lactic acid in batch fermentation produced by strain-2 of lactobacilli in a production medium containing various doses of nitrogen sources

Strain-2	LA Max.	t (h)	Dose (g/L)	P (g/L/h)	$P_{Max.}$ (g/L/h)
Meat extract	35.08	24	12	1.4619	2.2333
Proteose peptone	38.89	60	12	0.6481	2.2525
Baker's yeast	36.24	36	20	1.8120	2.1675
Yeast Extract	44.86	24	10	1.8691	2.3166

LA Max.- Maximum lactic acid concentration (g/L); P- Volumetric productivity; $P_{Max.}$ - Maximum volumetric productivity(g/L/h); t-time to attain LA Max. (h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table19 shows that yeast extract had the maximum production 44.86 g/L lactic acid at lowest dose,10 g/L, with strain-2, followed by proteose peptone. The lactic acid fermentation is growth dependent and the yeast extract provides the stimulatory amino acids and vitamins that are important for the growth of bacteria hence it is able to increase the lactic acid production [116, 65]. The cheap nitrogen source baker's yeast required a higher dose 20 g/L to provide third higher lactic acid production of 36.24 g/L, which should be beneficial in lowering the expenses on the nitrogen sources [7]. The application of yeast extract with higher amount of proteins (60.2%) and carbohydrates (12.9%), provides highest values of volumetric productivity and maximum productivity of lactic acid [116]. Strain-2 produced lactic acid at a higher rate with baker's yeast than proteose peptone and meat extract, hence it provided a higher productivity of 1.8120 g/L/h .

4.1.2.3 Effect of nitrogen sources on Coculture

Table20. The maximum concentration values of lactic acid in batch fermentation produced by the coculture of lactobacilli in a production medium containing various doses of nitrogen sources

Coculture	LA Max.	t (h)	Dose (g/L)	P (g/L/h)	$P_{Max.}$ (g/L/h)
Meat extract	48.96	48	12	1.0200	2.3816
Proteose peptone	50.01	60	12	0.8335	2.4960
Baker's yeast	48.36	48	20	1.0075	2.4366
Yeast Extract	55.40	36	10	1.5388	2.7133

LA Max.- Maximum lactic acid concentration (g/L); P- Volumetric productivity; $P_{Max.}$ - Maximum volumetric productivity(g/L/h); t-time to attain LA Max. (h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 20 shows that yeast extract had the maximum production of 55.40 g/L, lactic acid at lowest dose, with coculture, followed by proteose peptone. The lactic acid fermentation is associated with growth and yeast extract possesses the stimulatory amino acids and vitamins that help in the bacterial growth, hence it enhances the lactic acid production [60]. The cheap nitrogen source baker's yeast (the parent source of yeast extract) required a higher dose 20 g/L to provide reasonably high lactic acid production, which should be beneficial in bringing down the cost incurred on the nitrogen sources[6,7]. The application of yeast extract also provides highest values of volumetric productivity and maximum productivity of lactic acid. Coculture also provided higher productivity with meat extract and baker's yeast as it takes 48 hours to attain the respective maximum values of lactic acid production. This duration is lesser than 60 hours required for proteose peptone.

4.1.2.4 Effect of nitrogen sources on strain-3

Table21. The maximum concentration values of Lactic acid in batch fermentation produced by strain-3 of lactobacilli in a production medium containing various doses of nitrogen sources

Strain-3	LA Max.	t (h)	Dose (g/L)	P (g/L/h)	$P_{Max.}$ (g/L/h)
Meat extract	43.33	60	12	0.7221	2.2508
Proteose peptone	46.22	48	12	0.9629	2.3033
Baker's yeast	44.28	48	20	0.9225	2.2750
Yeast Extract	50.01	48	10	1.0418	2.3558

LA Max.- Maximum lactic acid concentration (g/L); P- Volumetric productivity;
$P_{Max.}$ - Maximum volumetric productivity(g/L/h); t-time to attain LA Max. (h);
Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table21 shows that yeast extract had the maximum production 50.01 g/L, lactic acid at lowest dose, with strain-3, followed by the proteose peptone. The cheap nitrogen source baker's yeast required a higher dose 20 g/L to provide reasonably high lactic acid production of 44.28 g/L which is higher than 43.33 g/L with meat extract. The productivity of the baker's yeast is also higher than meat extract (Table 21).This can be beneficial in lowering the cost incurred on the nitrogen sources in lactic acid fermentation with strain-3. The application of yeast extract also provides highest values of volumetric productivity and maximum productivity of lactic acid. The meat extract has lower nitrogen content (6-9%), as compared with that of proteose peptone (10-12.5%), which affects the bacterial growth resulting in to lesser lactic acid production and productivity (Table 21) [21,115].

4.1.2.5 Effect of nitrogen sources on strain-4

Table 22. The maximum concentration values of lactic acid produced in batch fermentation by the strain-4 of lactobacilli in a production medium containing various doses of nitrogen sources

Strain-4	LA Max.	t (h)	Dose (g/L)	P (g/L/h)	$P_{Max.}$ (g/L/h)
Meat extract	42.60	36	12	1.1833	2.1980
Proteose peptone	44.72	48	12	0.9316	2.3366
Baker's yeast	43.85	36	20	1.2180	2.2966
Yeast Extract	47.86	36	10	1.3294	2.4583

LA Max.- Maximum lactic acid concentration (g/L); P- Volumetric productivity; $P_{Max.}$ - Maximum volumetric productivity(g/L/h); t-time to attain LA Max. (h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table22 shows that yeast extract had the maximum production 47.86 g/L lactic acid at lowest dose, with strain-4, followed by the proteose peptone. The lactic acid fermentation is associated with growth and the yeast extract possesses the stimulatory amino acids and vitamins that are important for the growth of bacteria hence it enhances the lactic acid production [34, 60, 86]. The cheap nitrogen source baker's yeast required a higher dose 20 g/L to provide reasonably high lactic acid production of 43.85g/L which is higher than 42.60 g/L with meat extract. The productivity of the baker's yeast is also higher than meat extract (Table 18). This may be possible as the baker's yeast is the parent source of yeast extract, hence contains same amino acids, vitamins, proteins and carbohydrates like yeast extract [116]. The acidic pH of the production media, high temperature and pressure during autoclaving could have played their role in liberation of the amino acids through hydrolysis of these proteins, which in further course benefit the bacterial strain in growth and production. The application of yeast extract also provides highest values of volumetric productivity and maximum productivity of lactic acid.

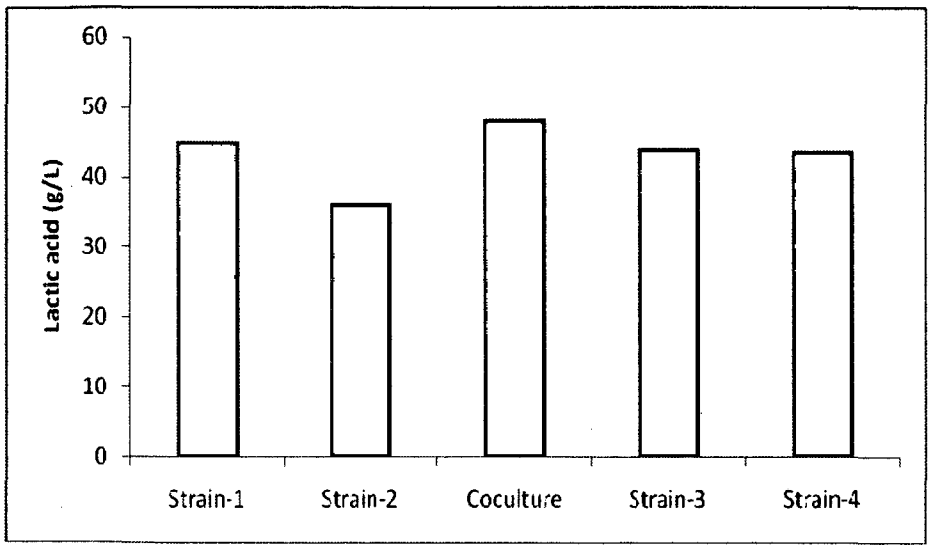


Figure 8 The performance of the cheap nitrogen source Baker's yeast with different strains of lactobacilli in lactic acid production.

Figure 8 summarizes the performances of the different strains of lactobacilli with baker's yeast in terms of lactic acid production. The highest lactic acid production, is with the coculture. It has reasonably high lactic acid production with the strains-1,3 and 4.Hence baker's yeast can serve as the profitable nitrogen source and can lower the cost of fermentations and give better competitive level of production.

Comparative data of lactic acid production and productivities from these *Lactobacillus* strains, utilizing, the same level of each of the four nitrogen sources(meat extract, proteose peptone, dried baker's yeast and yeast extract) have been provided in Appendix-5.

4.1.3 Effect of pH

pH value is an important parameter that affects the lactic acid production during the course of fermentation. Different values of initial pH values have been studied for the *Lactobacillus* strains, in the same glucose based production media also containing yeast extract as nitrogen source and salts.

Table 23. The concentration values of lactic acid in batch fermentation by the different strains of lactobacilli under different initial pH values of the production medium.

Bacterial strains	pH 6.0		pH 6.5		pH 6.75		pH 7.00	
	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)
L. delbrueckii (Strain-1)	36	49.31	36	51.75	36	45.67	36	36.41
L.pentosus (Strain-2)	24	40.47	24	42.48	24	46.13	24	30.46
Coculture of strains-1, 2	36	52.56	36	53.28	36	55.61	36	46.87
Lactobacillus sp. (Strain-3)	48	51.85	48	49.57	48	46.87	48	34.33
Lactobacillus sp. (Strain-4)	36	48.89	36	46.26	48	44.18	36	32.26

LA Max.- Maximum lactic acid concentrations (g/L); t- Time for attainment of maximum lactic acid concentration (h); Conditions- 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 23 data for lactic acid production , clearly indicate that, the strains-3,4 attained their maximum lactic acid production at the initial pH 6.0, while the maxima for strain-2 and coculture occurred with initial pH 6.75. Strain-1 had its highest production at an initial pH value of 6.5. Table 23 shows that neutral pH was unsuitable, as the lactic acid production dropped for all the strains because slightly acidic pH in media, supplies H^+ ions for sugar transport in the cell through H+ coupled symport [89]. Figures 9, 10 and 11 exhibit the variations in the lactic acid concentrations under the influence of different initial pH values.

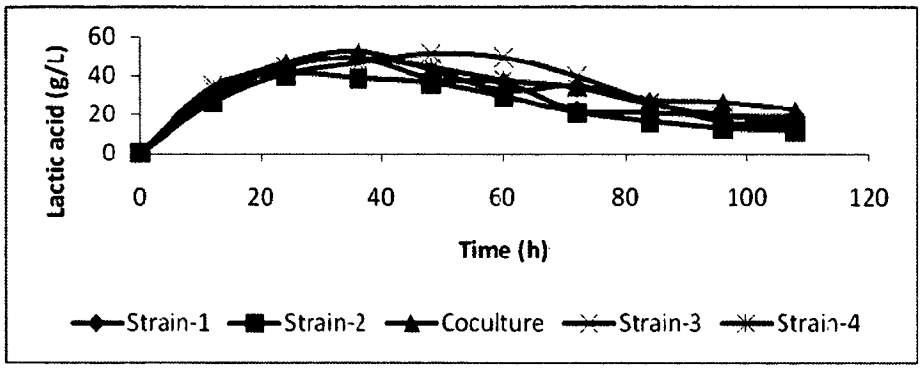


Figure 9 The production of lactic acid by different strains of lactobacilli in production media with the initial pH 6.0.

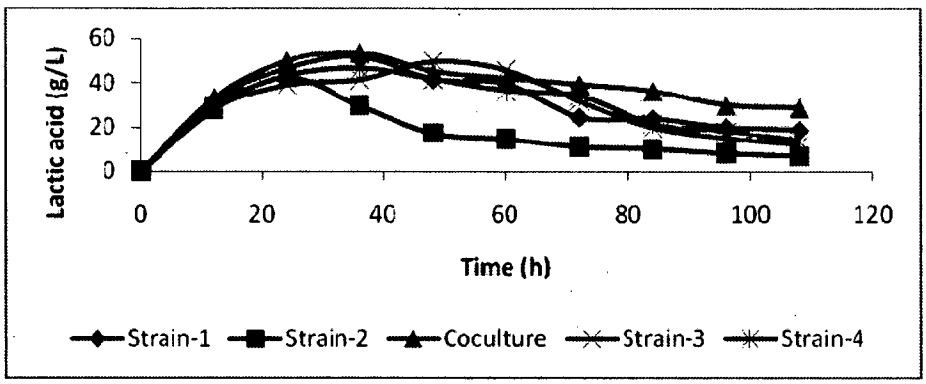


Figure 10 The production of lactic acid by different strains of lactobacilli in production media with the initial pH 6.5.

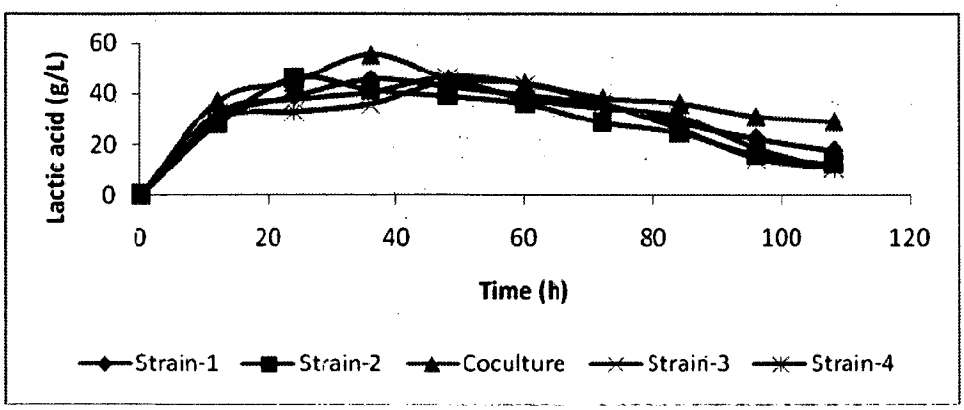


Figure 11 The production of lactic acid by different strains of lactobacilli in production media with the initial pH 6.75.

Strain-3 and strain-4 showed lesser fluctuations in the production, under their most suitable pH6.0 (Figure 9) while they showed more fluctuations in production at the other pH values. The difference of initial pH at 6.75 and the actual prevailing pH values during fermentation for the strains is more so more fluctuations in lactic acid production are evident in Figure 11.It has been shown through ANOVA test (Appendix-14), that effect of applying different initial pH values of the production medium were significant at 5% level in terms of lactic acid production.

Table 24. The maximum weights and productivities of lactic acid in batch fermentation by the different strains of lactobacilli under different initial pH values of the production medium.

Bacterial strains	pH 6.0		pH 6.5		pH 6.75		pH 7.00	
	$W_{Max.}$	PLA (g/L/h)	$W_{Max.}$	P LA (g/L/h)	$W_{Max.}$	PLA (g/L/h)	$W_{Max.}$	P LA (g/L/h)
L. delbrueckii (Strain-1)	0.8218	2.4133	0.8625	2.6675	0.7611	2.5175	0.6068	2.3071
L.pentosus (Strain-2)	0.6745	2.2016	0.7080	2.3266	0.7688	2.3533	0.5076	1.8341
Coculture of strains-1, 2	0.8760	2.6141	0.8880	2.7375	0.9268	3.0750	0.7812	2.5412
Lactobacillus sp. (Strain-3)	0.8641	2.9308	0.8263	2.3566	0.7812	2.6975	0.5721	2.2546
Lactobacillus sp. (Strain-4)	0.8148	2.7466	0.7710	2.4575	0.7363	2.4700	0.5376	2.1435

PLA- Maximum productivity of lactic acid (g/L/h); $W_{-Max.}$-Maximum weight of lactic acid produced per unit weight of initial sugar input (g/g)

Table 24 shows that the maximum lactic acid productivity and maximum weight of lactic acid W, of strains-3 and 4 were at the initial pH 6.0, while the maxima for strain-2 and coculture occurred with initial pH 6.75. The strain-1 had its highest lactic acid productivity at an initial pH value of 6.5. Table 24 shows that the initial pH 7.0 was not suitable, as the lactic acid productivity dropped for all the strains. The pH drops with the progress of lactic acid fermentation. Hence, 2% NaOH has been applied every 12 h to restore the pH.

4.1.4 Effect of Neutralizer

The pH value is an important parameter that affects the lactic acid production during the course of fermentation. Lactic acid fermentation involves control on pH during fermentation through neutralizers such as calcium carbonate or sodium hydroxide to prevent the lowering of pH in the fermentation broth. This is essential because the lactic acid synthesizing bacteria get inhibited at lower pH values due to accumulation of the free acid product. Although calcium carbonate has been usually taken as neutralizer for maintenance of pH during the lactic acid fermentation, but in the present studies as well as some previous studies , some of its inhibitory effects has been observed (at or above 2% concentration)[62]. Hence calcium carbonate has been compared with sodium hydroxide as neutralizer at 2% level, for the effects on lactic acid production by the *Lactobacillus* strains in a glucose based production media along with yeast extract and salts.

4.1.4.1 Effect of application of 2% sodium hydroxide as neutralizer

Table 25. Lactic acid production by the different strains of lactobacilli in a production medium containing 60 g/L of pure glucose as carbon source and yeast extract as nitrogen source, other salts and 2% NaOH neutralizer.

t (h)	*L.delbrueckii* Strain-1	*L. pentosus* Strain-2	Coculture of strain-1 and 2	*Lactobacillus sp.*Strain-3	*Lactobacillus sp.*Strain-4
	LA (g/L)	LA (g/L)	LA (g/L)	LA (g/L)	LA (g/L)
0	0.00	0.00	0.00	0.00	0.00
12	31.96	27.82	32.56	28.30	29.50
36	51.42	29.46	53.44	41.40	46.64
60	39.45	14.60	41.57	45.72	36.28
84	24.08	10.10	36.10	19.81	21.40
108	18.81	6.96	28.48	12.21	13.70

LA-Lactic acid concentration (g/L); t-time (h); Conditions- 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 25 and Figure 12, show that the highest production in lactic acid is attained by strain-1,4 and coculture in 36 hours. The Strain-2 and strain-3 attain their maximum lactic acid production in 24 hours and 48 hours respectively (Figure 12).

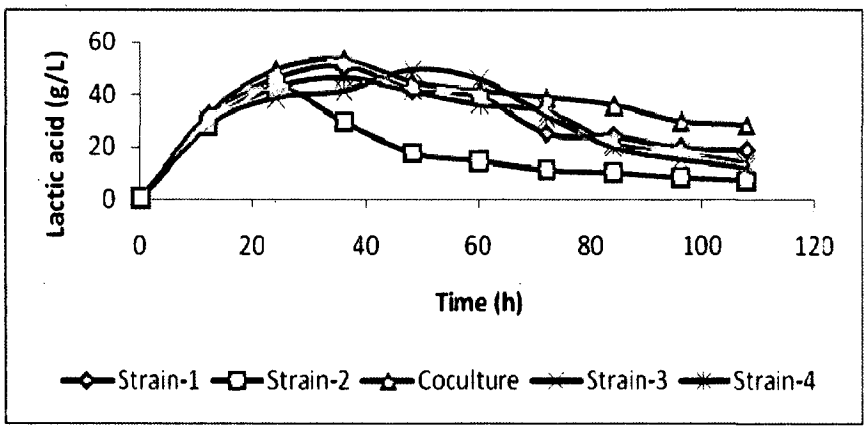


Figure 12 Lactic acid production with 2%NaOH neutralizer

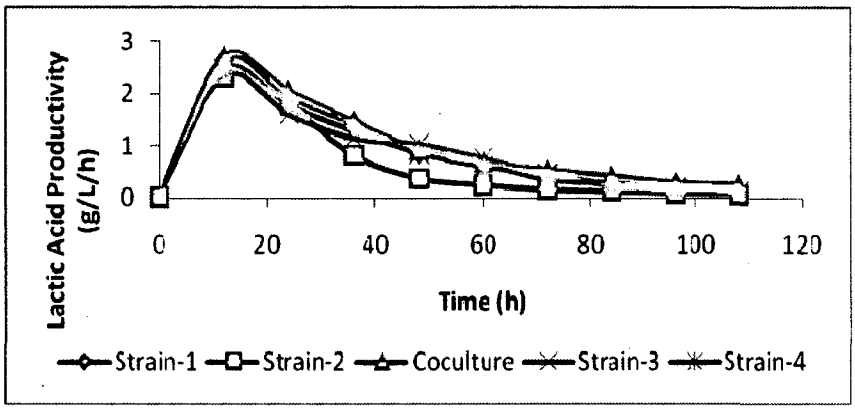


Figure 13 Lactic acid productivity with 2%NaOH neutralizer

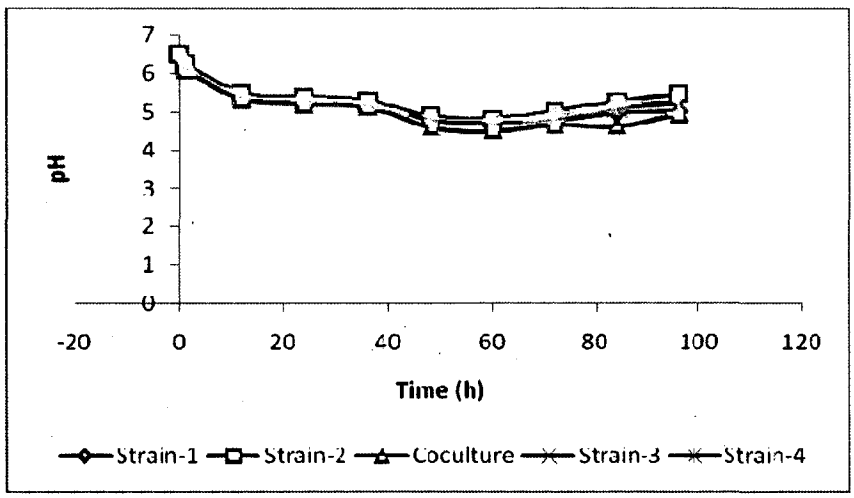


Figure 14 Variation of pH on application of 2%NaOH neutralizer

Coculture had overall maximum lactic acid production(Table 25) and the highest productivity(Figure13), which was closely followed by strain-1.The lactic acid production values for strain-1, coculture ,strain-3 and strain-4 in Table 25 were found higher in comparison with the maximum values of lactic acid concentration of these strains available in Table 26 with the calcium carbonate as neutralizer. It may be possible that besides neutralization of the free lactic acid, accumulated during the fermentation, some of the sodium ions also help in the sugar (glucose) uptake in these *Lactobacillus* strains, hence could have contributed towards their higher lactic acid production and productivity. Transport of sugars in the cell through sodium ion coupled symport has been described [89, 90]. Figure 13 shows a gradual decline in productivity in presence of 2%NaOH as neutralizer. Figure 14 clearly indicates that in presence of 2%NaOH the coculture attained the maximum decline in pH while strain-2 had the least drop in pH values.

4.1.4.2 Effect of application of 2% calcium carbonate as neutralizer

Table 26. Lactic acid production by the different strains of lactobacilli in a production medium containing 60 g/L of pure glucose as carbon source and yeast extract as nitrogen source, other salts and 2% $CaCO_3$ neutralizer

t (h)	*L.delbrueckii* Strain-1	*L. pentosus* Strain-2	Coculture of strain-1 and 2	*Lactobacillus sp.*Strain-3	*Lactobacillus sp.*Strain-4
	LA (g/L)	LA (g/L)	LA (g/L)	LA (g/L)	LA (g/L)
0	0.00	0.00	0.00	0.00	0.00
12	20.46	29.51	24.34	19.86	21.14
36	27.98	44.36	32.56	33.60	31.25
60	38.79	45.68	44.95	37.36	32.11
84	24.03	27.33	32.55	26.61	18.89
108	12.09	14.76	19.88	11.76	9.86

LA-Lactic acid production (g/L); t-time(h); Conditions- 2% $CaCO_3$ added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 26 and Figure 15 show comparatively higher production of the lactic acid by strain-2 than strain-1 , coculture and other strains. Maximum lactic acid production is attained by strain-2,3 and 4 in 48 hours (Figure 15), while for strain-1 and coculture it occurs in 60 hours (Table 26). The Table 26, Figure 15 and 16 indicate that strain-2, has the maximum lactic acid production and productivity amongst all strains with $CaCO_3$. Figure 17 showed that strain-2

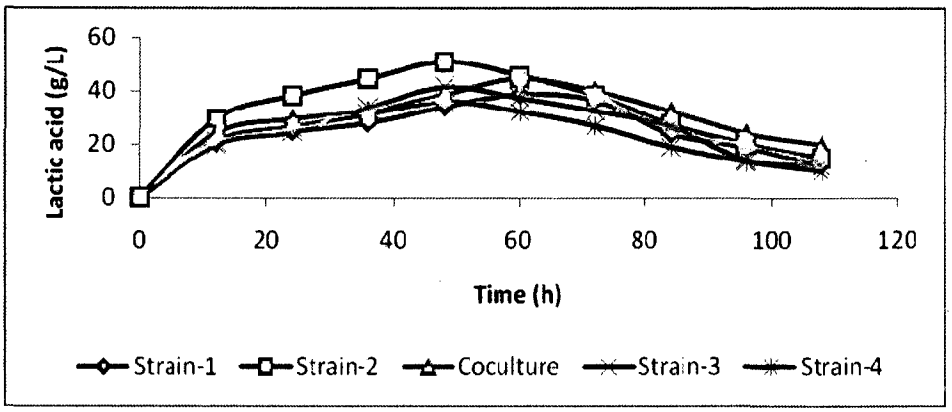


Figure 15 Lactic acid production with 2% $CaCO_3$ neutralizer

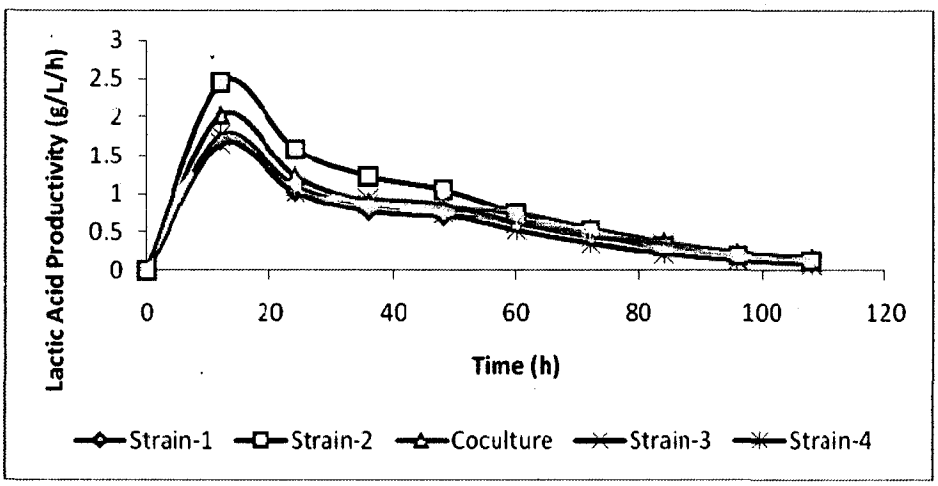


Figure 16 Lactic acid productivity with 2% $CaCO_3$ neutralizer

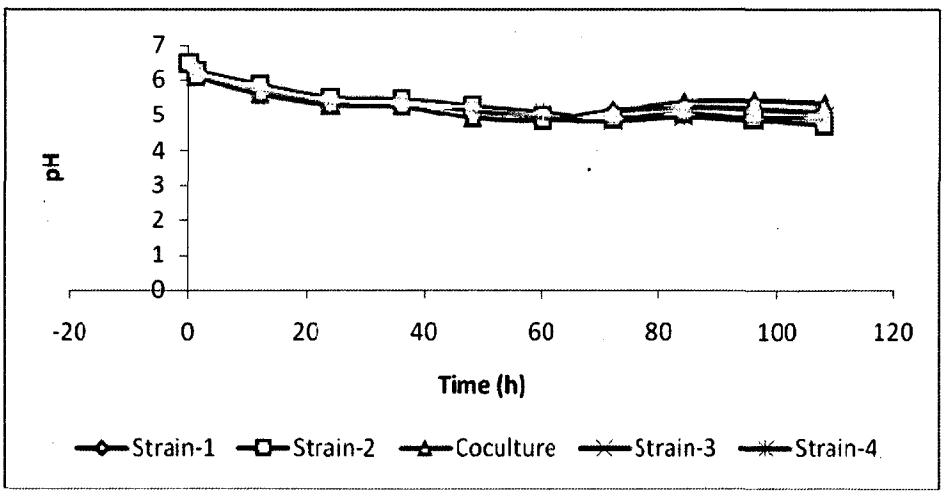


Figure 17 Variation of pH on application of 2% $CaCO_3$ neutralizer

achieved maximum drop in the pH values while coculture had the least drop with 2% $CaCO_3$ neutralizer while this was just the opposite with NaOH neutralizer, as per Figure 14. The decline in the production of lactic acid in calcium carbonate neutralizer can be due to the reduction in synthesis and activity of the bacterial lactate dehydrogenase enzyme LDH, by calcium ions or the higher concentration of calcium ions increases risk of cell lysis due to phage attack [85,111]. The LDH activity is reported to increase two folds in low concentration of Ca^{2+} but its supply from $CaCO_3$ neutralizer may reduce the activity of LDH, which results to reduction in lactic acid production [85]. Calcium carbonate has been reported for its growth inhibition effects on some of *Lactobacillus* strains which ultimately can lead to decline in lactic acid production [62].

4.1.5 Effect of salts

Different salts affect transport and metabolism of many sugars that are important for lactobacilli in lactic acid production. Hence various salts such as sodium pyruvate (SP), sodium succinate (SS), ferrous ammonium sulphate (AS) and ammonium citrate (AC) have been applied separately in glucose based production medium with yeast extract and other salts.

Table 27. The maximum concentration values of lactic acid in batch fermentation by the different strains of lactobacilli in a production medium containing different salts.

Bacterial strains	Sodium Pyruvate-SP		Sodium Succinate- SS		Ferrous Ammonium Sulphate-FAS		Ammonium Citrate- AC	
	t	LAMax	t	LA Max.	t	LA Max.	t	LA Max.
L. delbrueckii (Strain-1)	72	41.45	72	54.77	60	54.31	60	52.41
L.pentosus (Strain-2)	24	36.56	24	38.60	24	46.49	24	50.87
Coculture of (Strains-1, 2)	48	53.57	72	42.54	24	56.58	72	51.08
Lactobacillus sp. (Strain-3)	48	54.59	48	51.08	96	49.96	36	50.57
Lactobacillus sp. (Strain-4)	24	48.29	24	42.66	48	49.91	48	51.16

LA Max.-Maximum lactic acid production(g/L); t-time(h); Conditions- 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 28. The maximum weights and productivities of lactic acid in batch fermentation by the different strains of lactobacilli in a glucose based production medium

Bacterial strains	Sodium Pyruvate- SP		Sodium Succinate- SS		Ferrous Ammonium Sulphate-FAS		Ammonium Citrate- AC	
	W Max.	PLA (g/L/h)	W Max.	P LA (g/L/h)	W Max.	PLA (g/L/h)	W Max.	P LA (g/L/h)
L. delbrueckii (Strain-1)	0.6908	0.5756	0.9128	0.7606	0.9051	0.9051	0.8735	0.8735
L.pentosus (Strain-2)	0.6160	1.5233	0.6433	1.6083	0.7748	1.9371	0.8478	2.1196
Coculture of strains-1, 2	0.8931	1.1604	0.7090	0.5908	0.9431	2.3579	0.8513	0.7094
Lactobacillus sp. (Strain-3)	0.9098	1.1373	0.8513	1.0641	0.8326	0.5204	0.8428	1.4047
Lactobacillus sp. (Strain-4)	0.8048	2.0120	0.7110	1.7775	0.8318	1.0397	0.8526	1.0658

PLA- Maximum productivity of lactic acid (g/L); W.Max.-Maximum weight of lactic acid per unit weight of initial sugar input (g/g)

Table 27 shows that, among pure strains, strain-1 provided maximum lactic acid production 54.77 g/L with SS, closely followed by, strain-3, 54.59g/L lactic acid ,with SP and again with that of strain-1, 54.31g/L lactic acid utilizing FAS. However FAS provided overall highest lactic acid production (Table 27), productivity and weight (Table 28) with coculture (56.58 g/L) .The maximum productivity with SS was given by strain-4 (Table 28). Table 27 data suggest SP to be the best suitable salt for the strain-3(54.59 g/L lactic acid) closely followed by coculture (53.57 g/L lactic acid) while the maximum productivity with SP, is given by strain-4 (Table 28). Analogous to strain-3, highest lactic acid production in comparison to other salts has been reported for *L. sakei*-NRIC 1071 with SP [59]. As per the data in (Table 27), the AC benefitted all the strains, however strain-1 gave highest lactic acid production (52.41 g/L), followed by individual maximum values achieved by strain-2 and 4, while the maximum productivity was provided by strain-2(Table 28). The FAS and AC also contribute towards nitrogen requirements of the strains for enzyme synthesis and better growth resulting in high lactic acid production [119]. The sodium ions from SS and SP, may help in glucose uptake in the cells for better lactic acid production [80,89].

4.1.6 Effect of agitation

The effects of agitation on the lactic acid production through batch fermentation were studied at static, 150,180 and 240 rpm.

Table 29.The maximum concentration values of lactic acid in batch fermentation by the different strains of lactobacilli in a glucose based production medium

Bacterial strains	Static condition		150 rpm		180 rpm		240 rpm	
	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)
L. delbrueckii (Strain-1)	48	29.31	36	38.85	36	51.38	36	18.76
L.pentosus (Strain-2)	36	23.42	24	31.48	24	42.60	60	19.53
Coculture of strains-1, 2	48	32.66	36	42.76	36	53.47	36	20.82
Lactobacillus sp. (Strain-3)	60	25.01	48	34.25	48	49.50	48	16.55
Lactobacillus sp. (Strain-4)	48	26.87	36	36.87	36	46.24	36	17.79

LA Max.- Maximum lactic acid concentrations (g/L); t-Time for attainment of maximum lactic acid concentration (h); Conditions- 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C

Table 29 shows that all the bacterial strains follow an increasing trend of lactic acid production with an increase in agitation rate till 180 rpm. The high values of lactic acid production attained at 150 rpm and 180 rpm (Table 29) due to enhanced uniformity in, temperature, microbial cell suspension, prevention of cell aggregation and sediment formation [87,97]. After that the lactic acid production drastically falls. This may be either due to cell damage or at high rate of agitation turbulent conditions prevail and splashing may take place where bacterial biomass is thrown out of the production media and may get collected as wall growth [87]. This removal of biomass from media can cause decline in production. Coculture attained the highest overall lactic acid production and is closely competed by the strain-1(Table 29). Coculture achieved the highest lactic acid production for

all the agitation rates. Table 29 suggests reduction in time of attainment of maximum individual lactic acid production for all the strains at higher agitation rates,150 rpm and 180 rpm, as compared to the static condition.

4.1.7 Effect of temperature

Table 30. The maximum productions of lactic acid in batch fermentation by the different strains of lactobacilli in a glucose based production medium at different temperatures

Bacterial strains	30^0C		34^0C		37^0C		40^0C	
	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)	t (h)	LA Max. (g/L)
L. delbrueckii (Strain-1)	48	30.81	36	43.66	36	51.85	36	55.78
L. pentosus (Strain-2)	36	24.76	24	34.85	24	42.64	24	34.57
Coculture of strains-1, 2	36	34.68	36	45.52	36	53.44	36	56.83
Lactobacillus sp. (Strain-3)	60	28.38	48	38.76	48	49.60	48	38.56
Lactobacillus sp. (Strain-4)	36	25.55	24	35.64	24	42.85	24	35.94

LA Max.-Maximum values of lactic acid production (g/L); t-Time for attainment of maximum lactic acid concentration (h); Conditions- 2% NaOH added /12 h, 1.55g/L inoculum at 180 rpm

It can be observed from Table 30, that all the bacterial strains follow an increasing trend of lactic acid production with an increase in temperature up to 37^0C, but beyond this, the lactic acid production falls at 40^0C for all the pure strains, except strain-1and its coculture. Strain-1, is thermophilic in nature and is reported to produce lactic acid up to 45^0C [69]. The increase of temperature up to 37^0C provided the necessary heat energy required for growth and metabolic activity of enzymes in the strains. Table 30, shows that 37^0C is the optimal temperature for the strains-2,3,4 and any temperature above or below this gives lower production of lactic acid, as fall in reaction rate has been reported due to reduction in enzyme activity on either sides of optimum temperature[69]. Excessively high temperatures can kill the bacterial cells due to denaturation of the enzymes, proteins and the nucleic acids and can lead to cell lysis. It is evident from the Table30, that the increase of temperature up to34^0C

and shortened the time to reach the maximum lactic acid production. A linear relationship has been reported between the square root of growth rate constant of bacteria and the temperature, hence the growth of lactobacilli and production of lactic acid are affected by temperature [75].

4.1.8 Utilization of different carbon sources in *Lactobacillus* growth media

Comparative batch growth studies of four *Lactobacillus* pure strains, Strain-1, *L. delbrueckii* (NCIM2025); Strain-2, *L. pentosus* (NCIM 2912); Strain-3 *Lactobacillus sp.*(NCIM2734); Strain-4, *Lactobacillus sp.* (NCIM2084) and Co-culture of first two strains, have been carried out on liquid MRS (De Mann Rogosa Sharpe culture media) with different carbon sources such as acid hydrolyzate of bagasse under boiling condition, acid hydrolyzate of bagasse under autoclaving conditions and filtered cheese whey substituting the glucose component at180 rpm and 37^0 C temperature. Comparative batch growth studies on pure glucose based MRS medium, with the above mentioned pure strains and coculture of lactobacilli, has been provided in Appendix-6.

Table31. Biomass growth (CDW)) and pH drops associated with *Lactobacillus sp.* pure strains and coculture in modified MRS medium containing acid extract of bagasse obtained under boiling condition, partly substituting glucose as carbon source

T (h)	*L.delbrueckii* Strain-1		*L. pentosus* Strain-2		*Coculture* of strain-1 and 2		*Lactobacillus sp.*Strain-3		*Lactobacillus sp.*Strain-4	
	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH
0	0.5	6.50	0.5	6.50	0.5	6.50	0.5	6.50	0.5	6.50
3	2.28	5.24	1.81	5.28	2.86	5.21	2.12	5.24	2.01	5.25
6	6.86	5.18	5.61	5.22	7.12	5.16	6.14	5.20	6.82	5.21
9	10.6	5.15	8.89	5.17	9.98	5.14	11.21	5.17	11.69	5.19
12	12.01	5.12	10.96	5.14	12.46	5.11	13.96	5.15	14.24	5. 17
15	12.10	5.10	11.04	5.12	13.26	5.09	15.16	5.14	16.49	5.16
18	14.4	5.07	11.48	5.09	13.31	5.07	15.22	5.11	16.56	5.13
21	16.80	5.05	14.45	5.07	13.76	5.05	11.83	5.07	12.45	5.10
24	16.89	5.03	14.52	5.04	17.96	5.01	6.86	5.04	8.87	5.08
27	14.00	5.00	11.20	5.02	18.01	4.98	4.75	5.02	6.25	5.04

CDW- Cell dry weight; Conditions-Initial pH 6.5, inoculum 0.5 g/L, at 37° C and 180 rpm

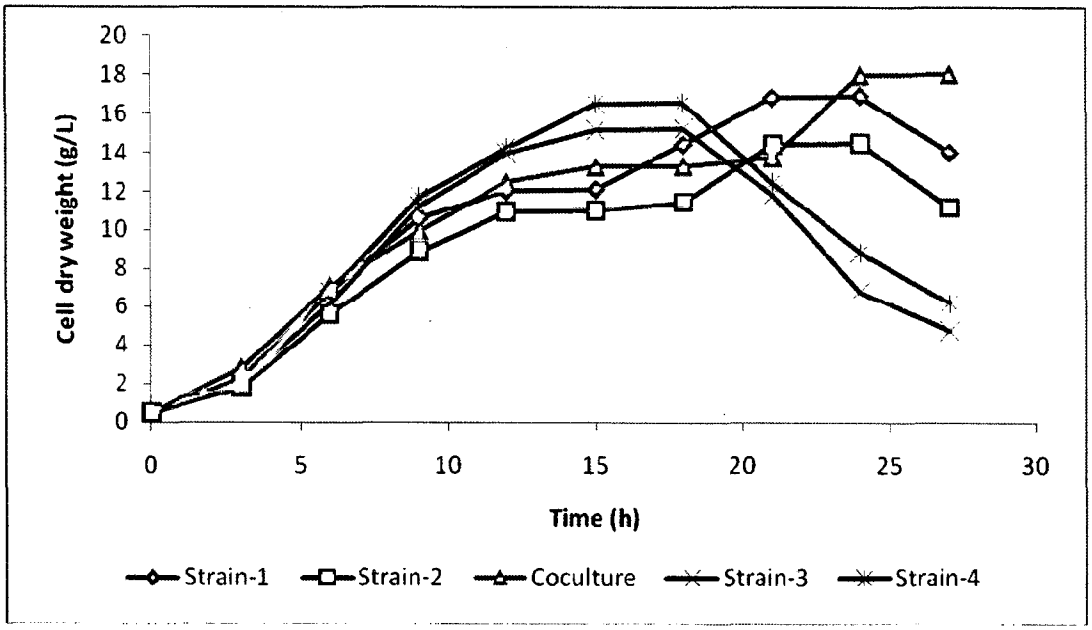


Figure 18 Batch growth of *Lactobacillus* strains with sugarcane bagasse acid extract under acidic boiling conditions

Table 32. Biomass growth (CDW) and pH drops associated with *Lactobacillus sp.* pure strains and coculture in modified MRS medium containing acid hydrolyzate of bagasse treated under autoclaved condition, partly substituting glucose as carbon source

Time (h)	*L. delbrueckii* Strain-1		*L. pentosus* Strain-2		*Coculture* Of strain-1 and 2		*Lactobacillus sp.*Strain-3		*Lactobacillus sp.*Strain-4	
	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH
0	0.5	6.50	0.5	6.50	0.5	6.50	0.5	6.50	0.5	6.50
3	1.98	5.20	1.56	5.24	2.46	5.18	1.88	5.22	1.96	5.23
6	6.20	5.17	5.69	5.19	7.34	5.14	6.10	5.17	6.62	5.18
9	10.01	5.14	9.20	5.12	10.89	5.11	9.68	5.13	10.56	5.16
12	12.41	5.12	11.29	5.10	12.86	5.09	13.76	5.11	13.43	5.14
15	12.49	5.11	13.52	5.08	12.93	5.08	15.85	5.07	16.66	5.11
18	14.40	5.09	13.59	5.07	14.82	5.06	15.91	5.04	16.56	5.09
21	15.56	5.06	14.25	5.05	17.85	5.04	12.48	5.02	12.45	5.07
24	16.89	5.04	16.21	5.02	17.94	5.01	7.69	5.01	5.98	5.04
27	16.96	4.99	16.40	4.98	15.26	4.96	5.66	4.95	4.26	5.00

CDW- Cell dry weight; Conditions-Initial pH 6.5,inoculum 0.5 g/L, at 37° C and 180 rpm

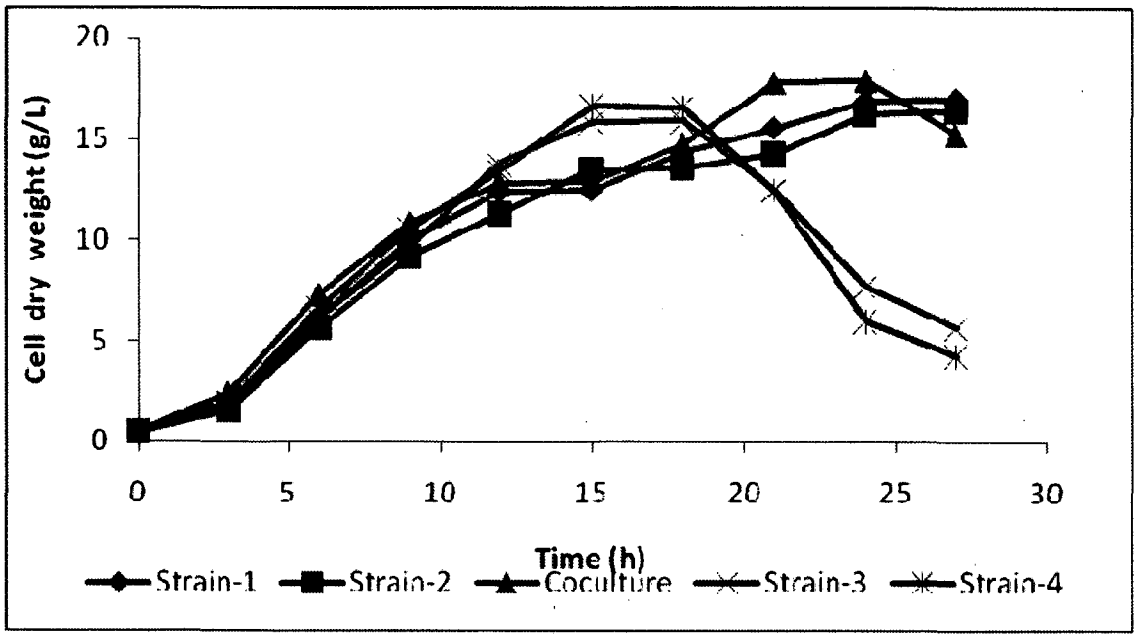


Figure 19 Batch growth of *Lactobacillus* strains with sugarcane bagasse extract under acidic treatment and autoclaved conditions

Table 33.Biomass growth (cell dry weight) and pH drops associated with *Lactobacillus sp.* pure strains and coculture in modified MRS medium containing cheese whey, partly substituting glucose as carbon source

T (h)	*L.delbrueckii* Strain-1		*L. pentosus* Strain-2		*Coculture* of strains-1 and 2		*Lactobacillus sp.*Strain-3		*Lactobacillus sp.*Strain-4	
	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH
0	0.5	6.50	0.5	6.50	0.5	6.50	0.5	6.50	0.5	6.50
3	2.27	5.04	1.68	5.06	2.76	4.86	1.99	4.89	2.09	5.02
6	7.21	4.94	5.69	4.96	7.88	4.75	6.67	4.78	6.91	4.92
9	10.12	4.70	8.96	4.75	11.46	4.61	9.51	4.72	10.87	4.86
12	12.89	4.66	11.24	4.70	13.64	4.57	12.28	4.70	11.89	4.81
15	12.96	4.58	12.08	4.67	13.72	4.54	12.37	4.64	12.78	4.78
18	15.02	4.54	12.11	4.64	15.86	4.50	14.36	4.58	12.83	4.74
21	16.99	4.50	16.48	4.61	17.98	4.47	15.99	4.40	16.68	4.70
24	17.04	4.48	16.66	4.56	18.09	4.44	16.06	4.36	16.71	4.68
27	13.96	4.46	11.16	4.50	14.98	4.38	12.10	4.34	13.01	4.64

CDW- Cell dry weight; Conditions-Initial pH 6.5, inoculum 0.5 g/L, at 37° C and 180 rpm

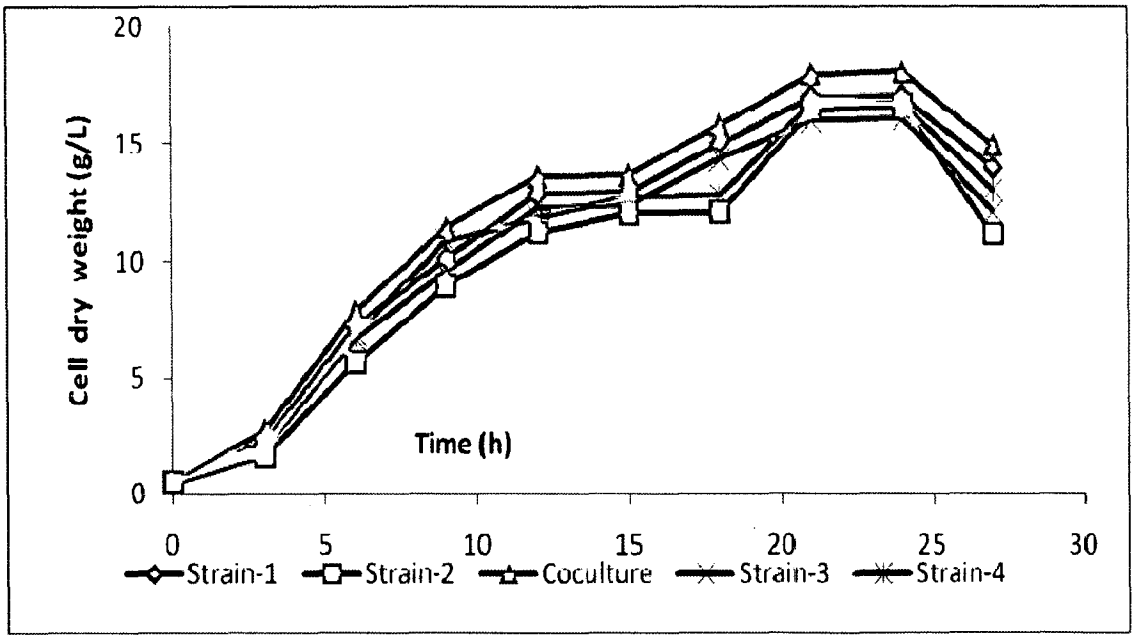


Figure 20 Batch growth of *Lactobacillus* strains with whey lactose

Tables 31, 32 and 33 and Figures18, 19 and 20, show that strains 1,2 and coculture have a diauxic growth pattern in all the three carbon sources while strains-2 and 3 showed diauxic growth with cheese whey source only. When media consists of two sugars (like glucose and lactose here) the bacteria which consume these sugars preferentially one after the other show, two log phases as visible in Figures 20, this phenomenon is called diauxy [89, 84]. This suggested that strains-2 and 3 utilize the sugars simultaneously (in first two cases) while the other strains utilize the different sugars preferentially hence showed a diauxic growth. Strain2, *L. pentosus*, is majorly pentose utilizing bacteria hence it gave lower pH values (Table32) with acid treated, autoclaved, detoxified and neutralized sugarcane bagasse extract (hemicellulose hydrolyzate), which is rich in pentoses like xylose [72,58,112]. Coculture attained highest biomass growth expressed as cell dry weight and also achieved the best pH drop with all the three carbon sources. Hence, it proves that, coculture can attain high cell dry weight and can withstand low pH conditions MRS media with different kind of mixed carbon sources.

4.2 Fed batch fermentation

Fed batch fermentation experiments were performed at high initial sugar (glucose or lactose) concentration in the production medium (which may be inhibitory in case of batch fermentation). Either glucose or lactose, based production media is taken with yeast extract and salts and effects of feeding 2g, 4g and 6g glucose or lactose in the production media have been studied.

4.2.1. Effect of feeding glucose

Table 34. The values of maximum productions, corresponding productivities and maximum weights of lactic acid in fed batch fermentation by the different strains of lactobacilli in a glucose feeding of 2 g

Bacterial strains	t (h)	LA Max. (g/L)	PLA (g/L/h)	$W_{Max.}$ (g/g)
L. delbrueckii (Strain-1)	96	132.96	1.3852	0.9363
L.pentosus (Strain-2)	96	120.22	1.2523	0.8466
Coculture of strains-1, 2	96	135.43	1.4108	0.9537
Lactobacillus sp. (Strain-3)	96	122.41	1.2750	0.8620
Lactobacillus sp. (Strain-4)	84	75.93	0.9040	0.5347

LA Max.- Maximum lactic acid production (g/L); PLA- Productivity of lactic acid corresponding to LA Max (g/L/h); $W_{Max.}$-Maximum weight of lactic acid per unit weight of sugar input(g/g); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table34 shows, that for periodic feeding of 2 g glucose on a production media with high initial content of glucose, did not inhibit the cells and bring down the lactic acid production level. Fed batch fermentations are used because of fast substrate depletion (lesser substrate inhibition) and removal of product inhibition (Appendix-3,4) usually found in organic acid production. In such cases the fed batch production be successful in better lactic acid production. Coculture had the overall maximum lactic acid production, weight and the highest productivity which is closely followed by strain-1, but strain-4 attained lowest lactic acid concentration. Strain-1, *L. delbrueckii* , (Table 34) has higher lactic acid production than *L. delbrueckii* NRRL B445 (23.1 g/L) under fed batch fermentation [112].

Table 35. The values of maximum productions, corresponding productivities and maximum weights of lactic acid in fed batch fermentation by the different strains of lactobacilli in a glucose feeding of 4 g

Bacterial strains	t(h)	LA Max. (g/L)	PLA (g/L/h)	$W_{Max.}$(g/g)
L. delbrueckii (Strain-1)	96	145.39	1.5140	0.9202
L.pentosus (Strain-2)	96	130.13	1.3555	0.8236
Coculture of strains-1, 2	96	151.71	1.5803	0.9602
Lactobacillus sp. (Strain-3)	96	139.09	1.4489	0.8803
Lactobacillus sp. (Strain-4)	96	117.71	1.2262	0.7451

LA Max.- Maximum lactic acid production (g/L); PLA- Productivity of lactic acid corresponding to LA Max (g/L/h); $W_{Max.}$.Maximum weight of lactic acid per unit weight of sugar input(g/g); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 35 lactic acid concentration data show that for periodic feeding of 4 g glucose on a media with high initial content of sugar did not inhibit the cells and bring down the lactic acid production level. Coculture had the overall maximum lactic acid production, weight and the highest productivity, which is closely followed by the strain1. Strain 4 had the lowest production among all the strains.

Table 36. The values of maximum productions, corresponding productivities and maximum weights of lactic acid in fed batch fermentation by the different strains of lactobacilli in a glucose feeding of 6 g

Bacterial strains	t(h)	LA Max.(g/L)	PLA (g/L/h)	$W_{Max.}$ (g/g)
L. delbrueckii (Strain-1)	108	154.36	1.4292	0.8672
L.pentosus (Strain-2)	108	138.51	1.2825	0.7781
Coculture of strains-1, 2	108	162.36	1.5033	0.9121
Lactobacillus sp. (Strain-3)	108	147.44	1.3652	0.8283
Lactobacillus sp. (Strain-4)	108	130.57	1.2089	0.7335

LA Max.- Maximum lactic acid production (g/L); PLA- Productivity of lactic acid corresponding to LA Max (g/L/h); $W_{Max.}$.Maximum weight of lactic acid per unit weight of sugar input(g/g); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 36 shows that for periodic feeding of 6 g glucose on a media with high initial content of sugar did not inhibit the cells and bring down the lactic acid production level. Coculture had the overall maximum lactic acid production, weight and the highest productivity which is closely followed by the strain1. Strain-4 improved its lactic acid production at this dose to 130.57 g/L.

4.2.2. Effect of feeding lactose

Table 37. The values of maximum productions, corresponding productivities and maximum weights of lactic acid in fed batch fermentation by the different strains of lactobacilli in a lactose feeding of 2 g

Bacterial strains	t (h)	LA Max. (g/L)	PLA (g/L/h)	$W_{Max.}$ (g/g)
L. delbrueckii (Strain-1)	96	134.32	1.3990	0.9328
L.pentosus (Strain-2)	96	129.65	1.3505	0.9003
Coculture of strains-1, 2	84	140.227	1.6693	0.9875
Lactobacillus sp. (Strain-3)	108	135.50	1.2546	0.9281
Lactobacillus sp. (Strain-4)	84	130.34	1.5516	0.9178

LA Max-Maximum lactic acid production (g/L); PLA- Productivity of lactic acid corresponding to LA Max (g/L/h); $W_{Max.}$-Maximum weight of lactic acid per unit weight of sugar input(g/g); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table37 data show that for periodic feeding of 2 g lactose on a media with high initial content of sugar did not inhibit the cells and bring down the lactic acid production level. Coculture had the overall maximum lactic acid production, weight and the highest productivity, which is closely followed by strain-1. Strain-4 had higher lactic acid production at this dose as compared to the glucose feeding on same dose. The coculture of strain-1 and 2 has higher lactic acid production as compared to a coculture of *L. lactis* and *L. casei* (47.0 g/L) reported for fed batch fermentation [112].

Table38. The values of maximum productions, corresponding productivities and maximum weights of lactic acid in fed batch fermentation by the different strains of lactobacilli in a lactose feeding of 4 g

Bacterial strains	t(h)	LA Max. (g/L)	PLA (g/L/h)	$W_{Max.}$ (g/g)
L. delbrueckii (Strain-1)	96	148.75	1.5490	0.9415
L.pentosus (Strain-2)	84	135.26	1.610	0.8783
Coculture of strains-1, 2	96	154.47	1.6109	0.9776
Lactobacillus sp (Strain-3)	84	136.05	1.6190	0.8534
Lactobacillus sp (Strain-4)	96	140.78	1.4660	0.8910

LA Max -Maximum lactic acid production (g/L); PLA- Productivity of lactic acid corresponding to LA Max (g/L/h); $W_{Max.}$-Maximum weight of lactic acid per unit weight of sugar input(g/g); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 38 data, show that for, periodic feeding of 4g lactose on a media with high initial content of sugar did not inhibit the cells and bring down the lactic acid production level. Coculture had the overall maximum lactic acid production, weight that is closely competed by strain-1. Strain-4 improved its lactic acid production at this dose to 140.78 g/L.

Table39. The values of maximum productions, corresponding productivities and maximum weights of lactic acid in fed batch fermentation by the different strains of lactobacilli in a lactose feeding of 6 g

Bacterial strains	t(h)	LA Max. (g/L)	PLA (g/L/h)	$W_{Max.}$(g/g)
L. delbrueckii (Strain-1)	96	158.42	1.6502	0.9210
L.pentosus (Strain-2)	96	145.35	1.514	0.8450
Coculture of strains-1, 2	96	161.64	1.6837	0.9396
Lactobacillus sp. (Strain-3)	108	148.46	1.3746	0.8340
Lactobacillus sp. (Strain-4)	96	152.37	1.5871	0.8858

LA Max- Maximum lactic acid production by each strain (g/L); PLA- Productivity of lactic acid corresponding to LA Max (g/L/h); $W_{Max.}$-Maximum weight of lactic acid per unit weight of sugar input(g/g); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 39 showed that for periodic feeding of 6 g glucose on a media with high initial content of sugar did not inhibit the cells and hence there was no decline of the lactic acid production level. Coculture had the overall maximum lactic acid production, weight and the highest productivity which is closely followed by strain-1. Strain-4 improved its lactic acid production at this dose to 152.37 g/L.

4.2.3 Utilization of acid treated and autoclaved sugarcane bagasse extract in fed batch fermentation

The detoxified and neutralized extract obtained from 1% sulphuric acid treatment of sugarcane bagasse under autoclaving conditions for 30 minutes was used to prepare the production media with other components, such that initial pH was kept at 6.5.

Table 40. The values of maximum lactic acid production with corresponding productivity of lactic acid in fed batch fermentation by the different strains of lactobacilli in a production medium containing acid treated and autoclaved bagasse extract and glucose as carbon sources with yeast extract and other salts

Bacterial strains	Carbon inputs in the Production Medium			
	Glucose 110 g/L in 1Lmedium prepared with bagasse extract +5 mL of bagasse extract fed every 12 hours			
	t(h)	LA Max. (g/L)	pH	P LA (g/L/h)
L. delbrueckii (Strain-1)	60	101.09±1.76	4.38	1.6860
L.pentosus (Strain-2)	96	96.42±1.43	4.64	1.0050
Coculture of strains-1,2	60	110.16±1.92	4.15	1.8360
Lactobacillus sp. (Strain-3)	96	112.68±2.10	3.90	1.1740
Lactobacillus sp. (Strain-4)	84	71.22±1.20	5.42	0.8478

LA Max-Maximum lactic acid production (g/L) ± standard deviations from three trials PLA-Productivity of lactic acid corresponding to LA Max (g/L/h); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

The detoxified, neutralized extract obtained from 1% sulphuric acid treatment of sugarcane bagasse was same as it had been taken for Table 14 for batch fermentation. The main sugar components obtained from the detoxified(Appendix-2) and neutralized acid hydrolyzate of dried, ground sugarcane bagasse were: Xylose , 11.62 g/L; arabinose, 1.24 g/L and glucose, 2.85 g/L.

Table 40 data, show that for periodic feeding of 5 mL sugarcane bagasse extract on a media with high initial content of sugar did not inhibit the cells and bring down the lactic acid .production level. Strain-3 had the overall maximum lactic acid production, followed closely by coculture, but the coculture had highest productivity.Strains-1,2,3 and coculture showed better lactic acid production in fed batch fermentation utilizing a mixture of bagasse hydrolyzate and glucose(Table 40),as against their values 92.82, 88.31,89.12 and 93.57 g/L obtained in batch fermentation with pure glucose (Table 4), which is in agreement with the similar finding in case of lactic acid production by *Lactobacillus delbrueckii* with pineapple juice through fed batch fermentation[16].In the present experiment fed batch production of lactic acid utilizing bagasse hydrolyzate is higher (101.09 g/L) in, *L. delbrueckii,* than its production with molasses, 90 g/L[112].Strain-4, again showed lower lactic acid production. Application of pentose utilizing lactobacilli, *L. pentosus* in coculture has been beneficial in the present experiment using hydrolyzed bagasse extract in terms of highest productivity of 1.8360 g/L/h. The use of such a coculture is supported by a study that reports total utilization of substrate (hemicellulose hydrolyzate from wheat straw) by a coculture of *L. brevis* and *L. pentosus* [112].

4.2.4 Utilization of whey in fed batch fermentation

Table41. The values of maximum lactic acid production and corresponding productivity of lactic acid in fed batch fermentation by the different strains of lactobacilli in a production medium containing filtered cheese whey and glucose as carbon source, yeast extract and other salts

Bacterial strains	Carbon inputs in the Production Media			
	Glucose 80 g/L in 1Lmedium prepared with whey +5 mL of whey fed every 12 hours			
	t(h)	LA max (g/L)	pH	P LA (g/L/h)
L. delbrueckii (Strain-1)	48	105.16±2.25	4.56	2.1908
L.pentosus (Strain-2)	48	90.95±1.92	4.74	1.8948
Coculture of strains-1, 2	48	108.32±2.44	4.48	2.2567
Lactobacillus sp. (Strain-3)	48	92.84±1.86	4.68	1.9342
Lactobacillus sp. (Strain-4)	48	82.10±1.70	4.92	1.7105

LA Max-Maximum lactic acid production (g/L) ± standard deviations from three trials; PLA- Productivity of lactic acid corresponding to LA Max (g/L/h); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 41 data show, that for periodic feeding of 5mL of filtered whey on a media with high initial content of sugar did not inhibit the cells and bring down the lactic acid production level. The stimulatory substances and the lactose component (4.98%) in whey may have possibly helped in the growth and production of bacteria [57]. The coculture had the overall maximum lactic acid production, lowest pH and the highest productivity which is closely followed by the strain-1. Strain -4, showed the least lactic acid production at this dose. All the pure strains and coculture (Table 41) under fed batch fermentation utilizing whey mixed glucose, show higher production and productivity of lactic acid as compared to 66 g/L lactic acid and 1.4 g/L/h from *L. helveticus* R 211 with whey [112].

4.2.5 Effects of application of whey in fed batch fermentation with lactose

Table 42. The values of maximum lactic acid production and corresponding productivity of lactic acid in fed batch fermentation by the different strains of lactobacilli in a production medium containing filtered cheese whey and pure lactose as carbon sources yeast extract and other salts

Bacterial strains	Carbon inputs in the Production Media			
	Lactose 80 g/L in 1L medium prepared with whey +5 mL of whey fed every 12 hours			
	t(h)	LA max (g/L)	pH	P LA g/L/h)
L. delbrueckii (Strain-1)	48	118.23±2.86	3.82	2.4631
L.pentosus (Strain-2)	48	95.78±1.89	4.69	1.9954
Coculture of strains-1, 2	48	121.92±1.52	3.78	2.5400
Lactobacillus sp. (Strain-3)	48	98.44±1.09	4.57	2.0508
Lactobacillus sp. (Strain-4)	48	91.09±1.67	4.76	1.8970

LA Max-Maximum lactic acid production (g/L) ± standard deviations from three trials; PLA-Productivity of lactic acid (g/L/h); t- Time for attainment of LA Max.(h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm

Table 42 data, indicate that feeding of 5mL of filtered whey on a media with high initial content of lactose did not cause substrate inhibition and bring down the lactic acid production. Application of cheese whey containing lactose, fat, proteins, vitamin and mineral ions, may have possibly helped in the growth of bacteria, sugar uptake and production of lactic acid[15, 112]. The coculture had the overall maximum lactic acid production, lowest pH and the highest productivity which is closely followed by the strain-1(Table 42). Application of whey feeding on whey mixed lactose media proved to be better than whey feeding on whey mixed glucose because all the pure strains and the coculture in first case (Table 42) have higher lactic acid production than the later (Table 41).This happened primarily because glucose suppresses the lactose uptake by inhibiting the lac operon [89].

4.3 Lactic Acid Production by Solid State Fermentation

The experimental studies have been carried out with the pure strains and the coculture for solid state fermentative production of the lactic acid.

4.3.1 Bed materials for solid state fermentation

Three bed materials have been utilized for the solid state fermentation studies for lactic acid production by pure strains and coculture of lactobacilli bed materials: (1) Sugarcane bagasse (2) Wheat bran and (3) Pine needles

4.3.1.1Composition of major constituents of the bed materials

The chemical composition of the bed material can influence the growth of bacteria and production of lactic acid hence the major chemical constituents and their percentages have been mentioned in the following tables.

Table43. Composition of major constituents of dry ground sugarcane bagasse

Analyzed constituents of wheat bran	Weight %
Holocellulose	69.09±1.72
Pentosan	25.68±1.56
Lignin	20.06±1.21
Ash	2.1±0.85

Results expressed as mean ± standard deviation, based on the three trials

Table44. Composition of major constituents of dry wheat bran

Analyzed constituents of wheat bran	Weight %
Holocellulose	44.06±1.52
Pentosan	31.02±1.28
Klason Lignin	4.85±0.98
Starch	32.24±1.31

Results expressed as mean ± standard deviation, based on the three trials

Table45. Composition of major constituents of ground oven-dried pine needles.

Analyzed constituents of pine needles	Weight %
Holocellulose	64.12 ± 2.68
Pentosan	14.12 ± 1.76
Lignin	27.79 ± 1.8
Ash	3.24 ± 0.09

Results expressed as mean ± standard deviation, based on the three trials

4.3.1.2 Particle size fractions of the bed materials

The particle size fractions of the bed material play an important role in distribution of production media, liquid holding capacity, inoculums distribution, gaseous exchange and mass transfer etc.. Hence the particle size influences growth and production of lactobacilli under study. The Particle Size Fractions of the bed materials used have been mentioned below.

Table 46. Particle size fractions of dried, ground sugarcane bagasse bed material obtained by sieving through vertically arranged screens in order of diminishing pore sizes in a vibratory shaker operated for ten minutes.

Mesh size (microns)	Weight% of the particles retained
1680	0.00
1204	0.007132
710	0.4022
500	17.298
420	19.1713
150	37.6365
Fine residual particles	25.4842

Table 47. Particle size fractions of dried, wheat bran bed material obtained by sieving through vertically arranged screens in order of diminishing pore sizes in a vibratory shaker operated for 10 minutes.

Mesh size (microns)	Weight% of the particles retained
1680	0.2288
1204	5.0691
710	24.5528
500	46.4736
420	14.5358
150	9.0984
Fine residual particles	0.2702

Table 48. Particle size fractions of dried, ground pine needle bed material obtained by sieving through vertically arranged screens in order of diminishing pore sizes in a vibratory shaker operated for 10 minutes.

Mesh size (microns)	Weight% of the particles retained
1204	3.63
750	11.57
710	9.82
500	17.85
420	26.61
210	7.02
150	7.43
Fine residual particles	16.07

4.3.2 Effect of Carbon Sources in Solid State Fermentation

4.3.2.1 Effect of glucose in solid state fermentation on sugarcane bagasse bed material

Table 49. Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6g bed of sugarcane bagasse with production medium containing various doses of glucose

Bacterial strains	Glucose 60g/L		Glucose 80g/L		Glucose 120g/L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L. delbrueckii (Strain-1)	3.80	8.25	3.61	33.00	3.41	21.00
L.pentosus (Strain-2)	4.06	5.85	3.68	27.00	3.78	6.86
Coculture of first two strains	3.70	24.67	3.46	45.50	3.73	17.85
Lactobacillus sp. (Strain-3)	4.92	4.40	4.24	4.10	4.06	8.63
Lactobacillus sp. (Strain-4)	3.92	4.13	3.89	4.40	4.20	9.01

LA- Lactic acid in extract*(g/L); Conditions1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

Table 49 shows that, the *Lactobacillus* strains-1,2 and co-culture, achieved maximum lactic acid production with, 80 g/L pure glucose, as carbon source in the production media. The values in Table 49 show highest lactic acid production of 45.50 g/L (pH 3.46) for the coculture followed by significant production values from strain-1, 33 g/L (pH 3.61) and strain-2, 27 g/L (pH 3.68). It also indicates that at 120 g/L pure glucose application, the bacterial strains-1,2 and co-culture, experienced inhibition due to high sugar level leading to reduction of lactic acid production. Even after decline in lactic acid production at 120g/L glucose treatment, the strain-1, *Lactobacillus sp.* (NCIM2025) exhibits maximum lactic acid production, 21 g/L, closely followed by coculture, 17.85 g/L, clearly indicates their capability to withstand the high level sugar inhibition and carry out reasonable level of lactic acid

production. Although values for 120 g/L glucose application showed a drastic reduction in lactic acid production for strains-1,2 and coculture, corresponding pH values of some of the strains remained quite low which indicates possible formation of some other acidic byproducts when the lactic acid production was inhibited due to high sugar level(substrate inhibition) explained in (Appendix-3,4). It can also be seen from Table 49 that the coculture showed better acid production than its constituent strains-1 and 2 at 60 and 80 g/L doses of glucose. Thus, application of coculture may prove better at the lower doses of glucose in the production media on a bed of sugarcane bagasse.

4.3.2.2 Effect of whey substituted glucose in solid state fermentation on sugarcane bagasse bed material

Table 50. Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6g bed of sugarcane bagasse with production medium containing various doses of whey lactose and glucose

Bacterial strains	Glucose 30g/ L +WL30g/ L TS 60g/ L		Glucose 40g/L + WL 40g/L TS 80g/L		Glucose 70g/L + WL 50g/L TS 120g/L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L.delbrueckii (Strain-1)	4.06	10.00	3.64	24.25	3.69	14.50
L.pentosus (Strain-2)	3.68	6.75	3.78	10.00	3.62	23.50
Coculture of first two strains	3.72	8.76	3.71	10.75	3.56	32.00
Lactobacillus sp. (Strain-3)	5.60	3.78	3.75	5.00	4.94	3.50
Lactobacillus sp. (Strain-4)	5.40	2.25	3.66	16.25	4.80	9.76

WL-Whey lactose; TS-Total sugar; Conditions1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

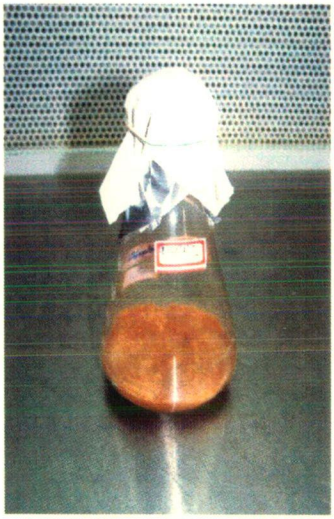
Table 50 shows that strain numbers 1, 3 and 4 gave maximum values for lactic acid production at 80g/L total sugar level in whey substituted glucose media as 24.25 g/L, 5 g/L and 16.25 g/L, respectively, whereas for strain-2 and coculture the maximum values were 23.5

g/L and 33 g/L, respectively at 120 g/L total sugar level with rest of the strains being inhibited at the same total sugar level. Thus, the observations contradict the findings in Table 49, where all the bacterial strains including the coculture were inhibited at 120g/L dose of pure glucose.

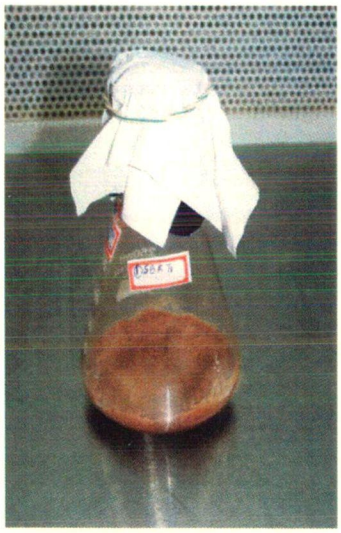
Table 50 also indicates that, apart from optimum dose of glucose, 40 g/L, (at 80 g/L total sugar level) with whey substituted media, the acid production of strains- 1, 3 and 4 got adversely affected at the lower or higher concentration levels. Coculture, exhibited lactic acid production of 32 g/L, at 120 g/L total sugar level, which was higher than that of the constituent strains-1 and 2, without any significant sugar dose inhibition and possesses the dual advantage of utilizing hexose sugar and pentose sugars. The pentose sugars such as xylose, might possibly be liberated due to temperature, pressure and slightly acidic pH conditions during autoclaving, from the (hemicelluloses) pentosan fraction of bed material (27.68% under dry conditions) besides consuming lactose sugar from whey substitute[112,72].

A maximum lactic acid production of 249 mg/gds (gram dry fermented substance) has been reported for *L. delbrueckii* action on inert sugarcane bagasse bed with starch hydrolyzate in 5 days, but in the present studies with whey addition on bagasse bed the *L. delbrueckii* in the coculture produced 266.66 mg/gds lactic acid while singly it liberated 202.1mg/gds lactic acid [43]. Hence, this observation signifies the importance of utilization of coculture, whey substitution and non inert sugarcane bagasse bed.

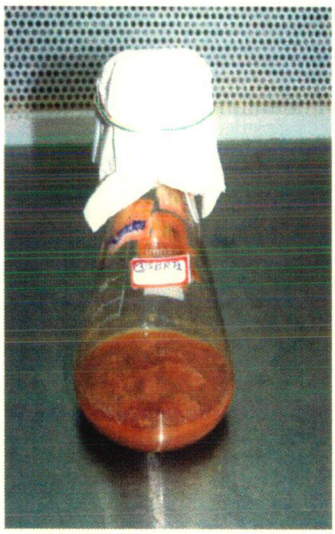
It has been reported that, in a liquid state fermentation experiment to produce lactic acid through *L.bulgaricus* by substitution of 5% lactose containing cheese whey in the production media up to 50, 66.6, 75, 80 and 99% by volume the highest lactic acid production of 20.8 g/L was achieved [26]. In the present study the whey substituted glucose media bed of sugarcane bagasse produced a maximum of 32g/L lactic acid (Table50) by coculture, although the conditions (biomass, nutrients and neutralizer) in solid state production are not as uniform as in liquid state fermentation.



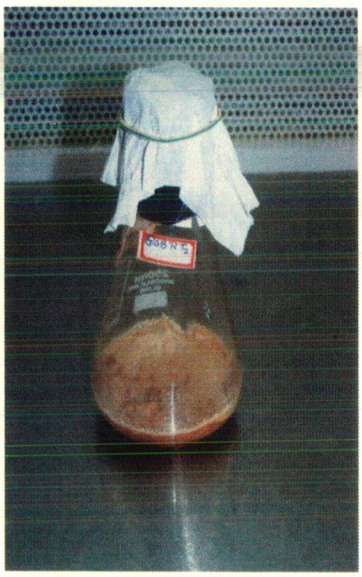
21(a) Strain -1



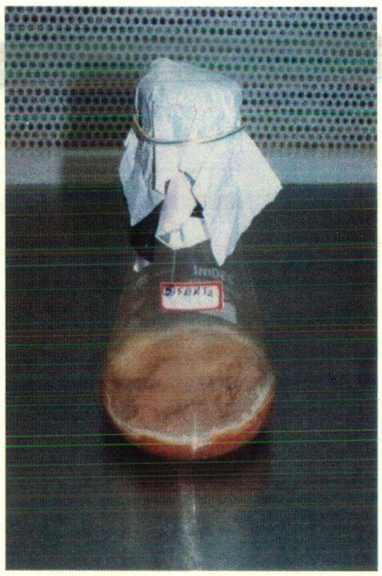
21(b) Strain -2



21(c) Coculture



21(d) Strain -3



21(e) Strain-4

Figure21 Growth of lactobacilli on solid bed of sugarcane bagasse with whey substituted glucose based production media at 120g/L total sugar level.

Figure21 shows the growth patterns of different strains of lactobacilli on the sugarcane bagasse bed with whey substituted glucose based production media containing 120g/L total sugar.

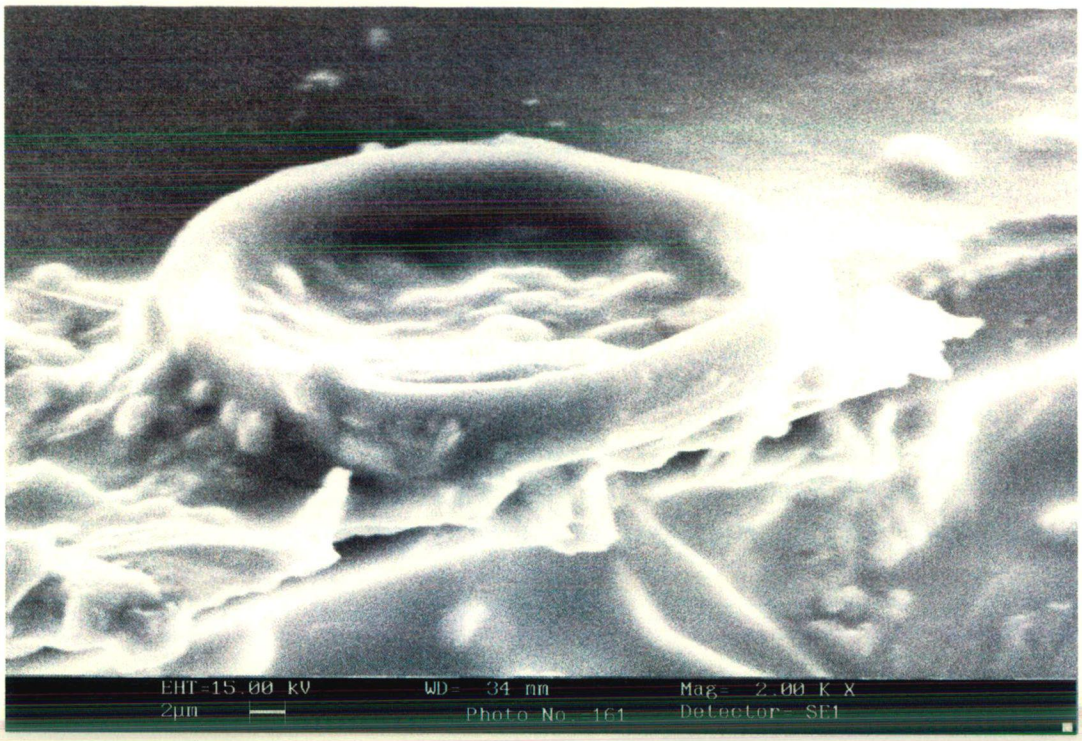


Figure 22 Growth of *L. delbrueckii* (NCIM2025) on solid bed of sugarcane bagasse with whey substituted glucose based production media at 60g/l total sugar level.

It is evident from the SEM micrograph (Figure22) that, dense biomass growth of *L. delbrueckii* (strain-1) on a bed of sugarcane bagasse has been due to the application of whey which, apart from lactose, contains different amino acids and proteins that encourage cell growth. Hence at 60 g/L total sugar level with whey mixed glucose as carbon source, strain-1, *L. delbrueckii*, showed highest lactic acid production of 10.0 g/L .This observation is supported by the fact that lactic acid production is reported to be related to growth of lactic acid bacteria [60].

4.3.2.3 Effects of pure glucose application on wheat bran bed material

Table 51. Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6g bed of wheat bran with production medium containing various doses of glucose

Bacterial strains	Glucose 60g/L		Glucose 80g/L		Glucose 110g/L		Glucose 120g/L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L.delbrueckii (Strain-1)	4.01	41.24	3.62	50.01	3.82	45.10	3.98	41.25
L.pentosus (Strain-2)	5.28	11.00	4.48	23.25	4.64	20.05	5.94	15.01
Coculture of first two strains	4.08	39.01	3.56	54.25	3.70	47.31	3.96	40.00
Lactobacillussp. (Strain-3)	5.24	15.50	4.42	25.50	5.46	12.14	5.74	9.75
Lactobacillus sp. (Strain-4)	5.36	11.50	4.01	30.75	3.89	21.26	5.98	6.00

LA- Lactic acid in extract*(g/L); Conditions 1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

The results in Table51 reveal that maximum acid production were attained by all the *Lactobacillus* strains including co-culture of the first two strains, at 80 g/L dose of pure glucose containing, synthetic production media. The results of lactic acid production at higher concentration of pure glucose treatment(120 g/L) in production media indicated that, the acid synthesizing ability of strain-1 and co-culture were reduced due to inhibition at high sugar concentration in comparison to their own maximum production values attained at 80 g/L glucose treatment, while a significant decline in the lactic acid production of strain- 2, 3 and 4 were observed, indicating their higher susceptibility to inhibition due to higher sugar concentration. Compared to all the strains under different levels of glucose treatments, co-culture in Table 51 showed the maximum lactic acid production 54.25 g/L at pH 3.56 (at a glucose level of 80 g/L) closely followed by strain-1, 50.01 g/L at pH 3.62. Co-culture showed better acid production (lower pH and higher lactic acid concentration) than its constituent

strains -1 and 2 with first two doses of glucose in Table 51, while it drops to 40 g/L which is a slightly behind that of strain-1(41.25 g/L) at 120 g/L dose of pure glucose, which highlights the fact that amongst all strains, the strain-1 is best suitable (against inhibition) for the lactic acid production at high concentration of glucose. Strain-2, *L. pentosus* has lowest concentration 25.25g/L (pH 4.36) of lactic acid since it is primarily pentose sugar utilizer but the supply of glucose as carbon source, may have reduced the lactic acid production by it.

4.3.2.4 Effect of whey substituted glucose treatments on wheat bran bed material

Table52. Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6g bed of wheat bran with production medium containing various doses of whey lactose and glucose

Bacterial strains	Glucose 30g/ L +WL30g/ L TS 60g/ L		Glucose 40g/ L +WL40g/ L TS 80g/ L		Glucose 65g/ L +WL45g/ L TS 110g/ L		Glucose 70g/ L +WL50g/ L TS 120g/ L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L.delbrueckii (Strain-1)	4.10	39.10	3.96	41.68	3.82	44.01	3.72	48.76
L.pentosus (Strain-2)	5.78	8.56	4.96	14.68	4.66	19.61	4.38	25.01
Coculture of first two strains	4.28	28.60	3.75	45.00	3.65	49.41	3.56	54.25
Lactobacilluss p. (Strain-3)	5.26	15.00	4.72	18.05	4.46	22.15	4.36	25.16
Lactobacillus sp. (Strain-4)	5.37	10.00	4.66	19.67	4.40	23.61	4.24	29.85

WL-Whey lactose; TS-Total sugar; LA- Lactic acid in extract*(g/L); Conditions1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

In Table 52 coculture again exhibited highest lactic acid production, 54.25 g/L (pH 3.56) in comparison to other strains and its constituent strains-1 and 2 in Table 51 at 120 g/L

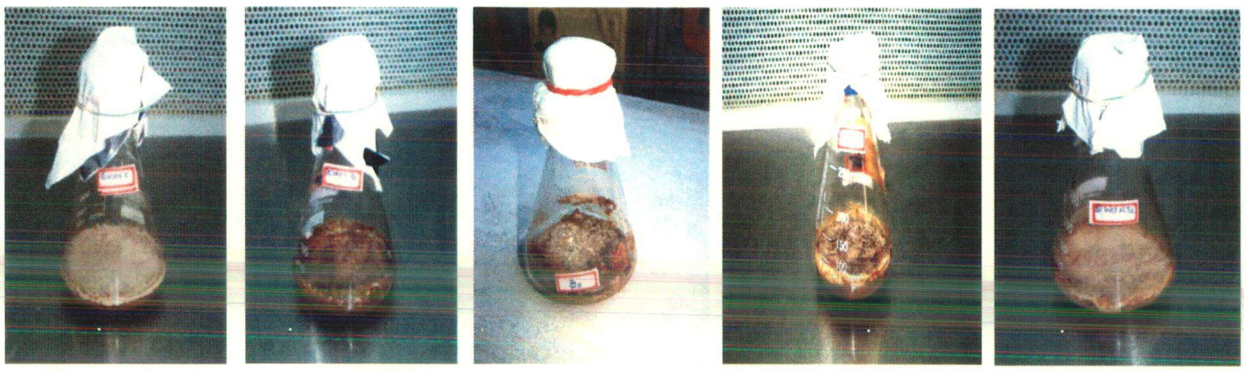
dose of whey substituted glucose. In Table 52 all the strains followed gradually increasing trend of lactic acid production with the increase in the levels of glucose and whey substitution. All the strains attained their highest acid yields with the highest level of sugar (120 g/L) in Table 52, while the same sugar level (pure glucose) in Table 51, witnessed an inhibition, for all these *Lactobacillus* strains.

All the pure strains and coculture of lactobacilli under study show good compatibility with the whey substituted glucose, which is clearly evident from the highest values of lactic acid production in Table 52,that were marginally lesser than their maximum values observed in Table 51(in pure glucose). This observation may be attributed due to several possible reasons such as lesser glucose inhibition of their lac operon (reported in several lactobacilli), by glucose, presence of other reported lactose uptake systems(phosphoenolpyruvate dependent phosphotransferase system lac-PTS, coupled transport symport with H^+, lactose galactose antiport), capability to utilize pentose sugars (that might have been liberated during high temperature, pressure and acidic conditions during autoclaving)or higher cell numbers (available for sugar utilization) due to stimulatory substances from whey[5,98,113].

A closer comparison between Table 51 and Table 52, showed that the co-culture attained a maximum production of 54.25 g/L lactic acid at 80 g/L pure glucose level, while the same lactic acid concentration (54.25 g/L) had been achieved with 120 g/L whey substituted glucose. This fact proved that co-culture could efficiently utilize higher doses of whey substituted glucose, without any significant inhibition, and provide highest lactic acid production that matches with the production level obtained with pure sugar glucose as sole carbon source. Hence coculture can serve better in lactic acid production industries based on the utilization of inexpensive carbon sources such as whey.

In the present studies, addition of production media containing whey (120g/L), on wheat bran bed, inoculated with *L. delbrueckii* pure strain provided 347.93 mg/gds of lactic acid,

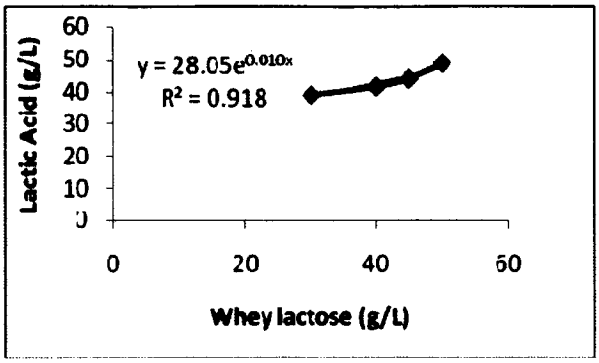
which is much higher in comparison to 249 mg/gds lactic acid produced by *L. delbrueckii* on inert washed sugarcane bagasse[43]. Hence, in the present study these observations highlighted the significance of utilizing, wheat bran as bed material, coculture instead of pure one at 120g/L whey substitution, and a non-inert bed for lactic acid production.



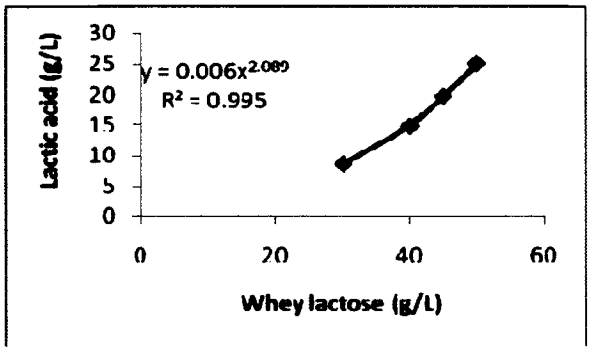
23(a) **23(b)** **23(c)** **23(d)** **23(e)**

Figure23 Growth of lactobacilli on wheat bran bed material

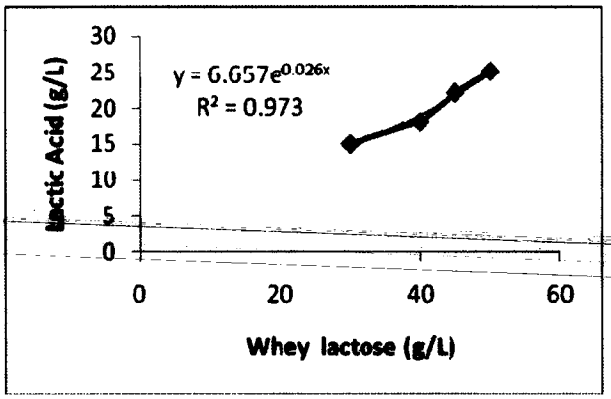
The frames 23(a), 23 (b), 23(d), 23 (e) and 23 (c) depict the growth of lactic acid yielding pure strains- 1,2,3,4 and coculture, utilizing whey substituted glucose at 120g/L level as carbon source.



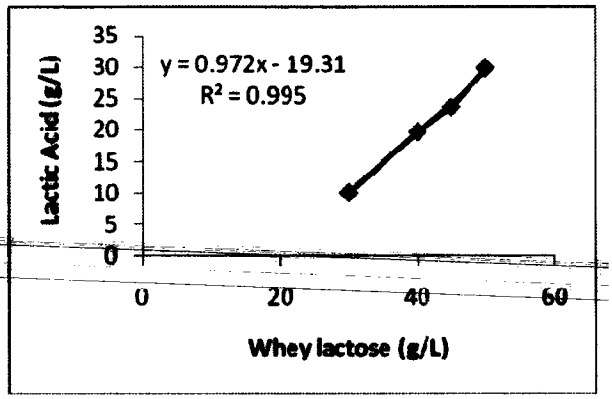
24(a)



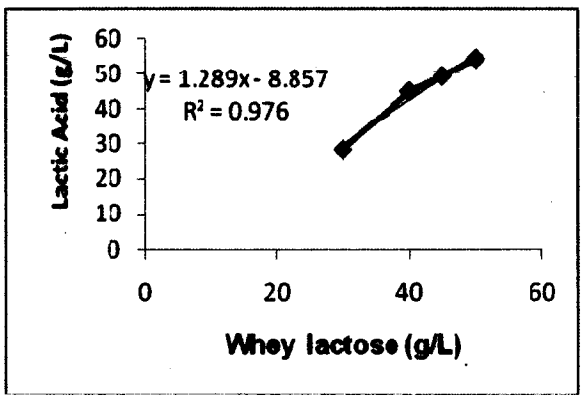
24(b)



24(c)



24(d)



24(e)

Figure24 Mathematical relations between whey lactose input and lactic acid output.

Plots 24(a),24(b), 24(c), 24(d) and 24(e) denote the mathematical relationship between the whey lactose input and lactic acid output of each of strains-1 through 4 and co-culture in solid state fermentation utilizing a wheat bran bed after six days of incubation.

Data Analysis

The values of Karl Pearson's coefficient of variation CV (Appendix-7), for the lactic acid concentration data in Table51(57.33,34.99, 48.89 and 67.54) and Table 52(58.19,46.08,39.78 and 33.90) corresponding to four different levels of sugar inputs(60,80,110 and 120 g/L), showed a least CV value 33.90 at 120 g/L total sugar level with whey-substitution indicated, that this treatment provided most consistent lactic acid concentration values from different strains, with least variation. This uniformity in the lactic acid production values at 120 g/L dose of whey-substituted glucose and the least CV further indicated that this treatment level was most successful in enhancing the lactic acid production of all the strains under study. All of the pure cultures and coculture of *Lactobacillus* strains under study attained their maximum concentration of lactic acid at this level (Table 52).

The CV value of 34.99 was obtained with 80 g/L pure glucose application, where all strains including coculture attained their maximum lactic acid concentration, indicated higher consistency among the lactic acid production values. A very high CV value, 67.54 at 120 g/L pure glucose input indicated comparatively lesser consistency and higher disparity in lactic acid production (Table 51) by the bacterial strains under study, as a consequence of sugar inhibition (Appendix-3). Very high CV thus suggests that the corresponding input level of pure glucose (120 g/L) was not beneficial for production of lactic acid by the strains under study.

The correlation analysis between the concentrations of lactic acid produced in bed and input of lactose from whey (Table 52) provided positive correlation coefficient(γ) values such as 0.9482,0.9891,0.9765,0.9975 and 0.9883 for strains-1,2,3,4 and coculture respectively (Appendix-9). This suggests interdependence of lactic acid produced in bed material on the whey lactose input as carbon source and indicates existence of very strong positive correlation between these variables.

The t- test analysis when applied on the sets of lactic acid concentration data in Table 51 between levels 80 g/L and 120 g/L of pure glucose input provided1.4521as calculated value of t(Appendix-11), which was lesser than the table value of t (2.306) at eight degrees of freedom at 5% level of significance hence it is accepted that the increase in glucose input from 80 to 120 g/L was not effective in increasing the lactic acid output, which also indicated the

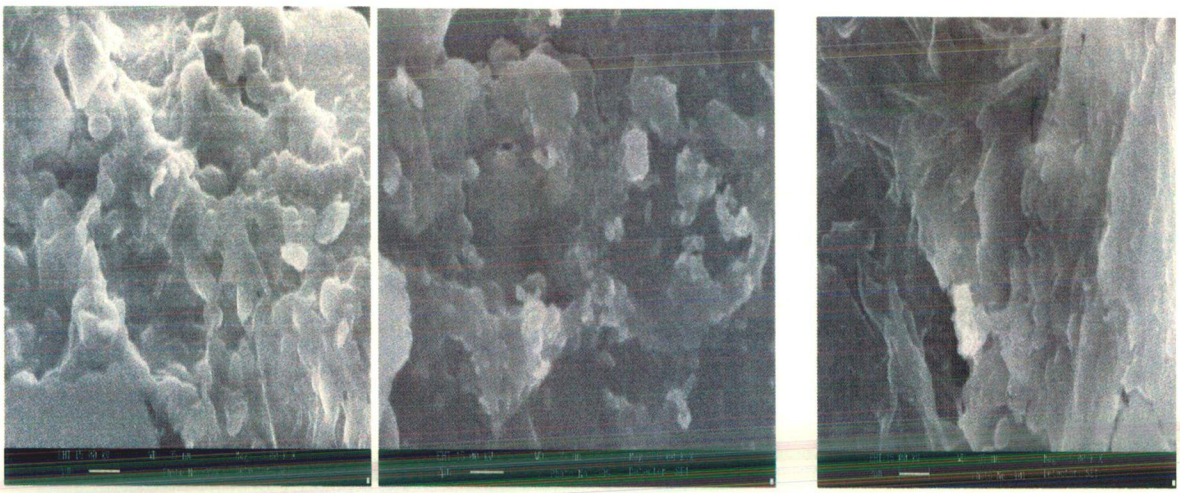
occurrence of inhibition, resulting due to higher dose of sugar. Hence the 120 g/L pure glucose input as sole carbon source has not proven beneficial for the lactic acid production. The t- test analysis carried out on the lactic acid concentration data by different bacterial strains (Table 52), between 80 g/L and 120 g/L levels of whey mixed glucose input provided 0.9853 as calculated value of t (Appendix-11), which was lesser than the table value of t (2.306) at eight degrees of freedom at 5% level of significance hence it is accepted that the rising sugar inputs along with whey lactose did not cause any significant sugar inhibition affecting lactic acid production. The above statistical inference is further complemented by the fact that whey is known to contain stimulatory constituents for the lactobacilli, which are helpful in their growth and production [15,30]. More generalized approach, ANOVA analysis (Appendix-15) was applied with Table 52 data, which analyses whether, the inputs of incremental doses of the whey mixed glucose (all doses) and the difference in *Lactobacillus* strains, causes significant changes in lactic acid production. Since both the calculated values of F, that related to variation in strains and the one related to all the dose variations of whey mixed glucose were greater than their respective table values of F, 3.84 and 4.86 respectively. Therefore it can be inferred that application of the incremental doses of whey mixed glucose on the different pure strains and coculture, produces significant difference in lactic acid production at 5% level of significance.

Taking the whey lactose input as independent variable and lactic acid produced (by each individual bacterial strain) as dependent variable the predicted mathematical relations between these variables for each bacterial strain and the coculture has been mentioned below. The above plots 24(a), 24(b), 24(c),24(d) and 24(e) show predicted, possible mathematical relationships (between whey lactose doses applied and their lactic acid output responses) for strains-1 through 4 and coculture which are found to follow exponential, power, exponential, linear and linear functions respectively.

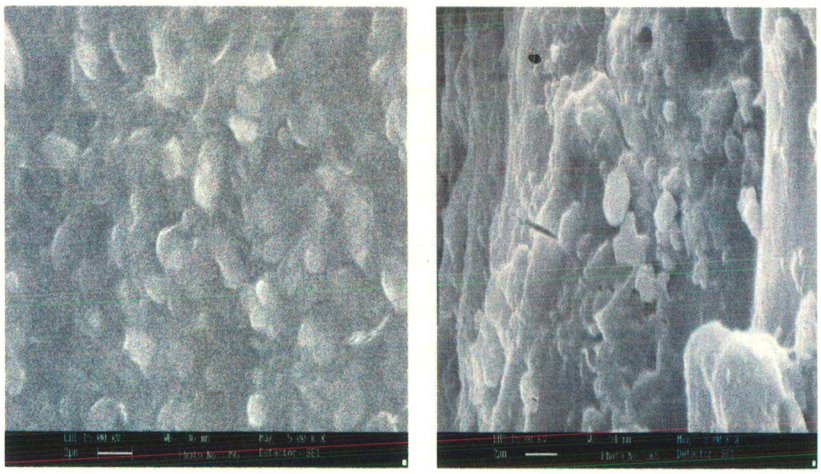
Scanning Electron Micrographs of Bacterial Growth on the bed of Wheat Bran

Groups of lactobacilli cells of strains-1,2, 3, 4, and co-coculture are observed in the SEM micrographs Figure25: 25(a), 25(b), 25(c),25(d), and 25(e), as fused growth, distinct capsular rod-shaped bacterial cells or seen to form aggregates adhering with the bed material at 120 g/L whey-substituted glucose media application (under 5000X magnification). Figure 25(b), 25(c)

for strain-2,and strain-3 show lesser cell population, hence provided minimum lactic acid concentrations of 24.01 g/L and 25.16 g/L respectively (mean values in Table 52). Figure 25(e) for co-culture indicated isolated capsular cells and also fused growth of bacteria very closely aggregated to each other provided the highest lactic acid production of 54.25 g/L mean value (mentioned in Table 52).



25(a) 25(b) 25(c)



25(d) 25 (e)

Figure 25. SEM micrographs showing the growth of lactobacilli on solid bed of wheat bran.

Micrographs 25(a), 25(b), 25(c), 25(d), and 25(e) show the growth of strains-1, 2, 3, 4, and coculture, respectively, at 5000X magnification, on wheat bran bed fed with a production media containing whey lactose (50 g/L), combined with glucose (70 g/L) at 120 g/L total sugar level, after six days incubation. The aggregates as well as separate rod shaped lactobacilli

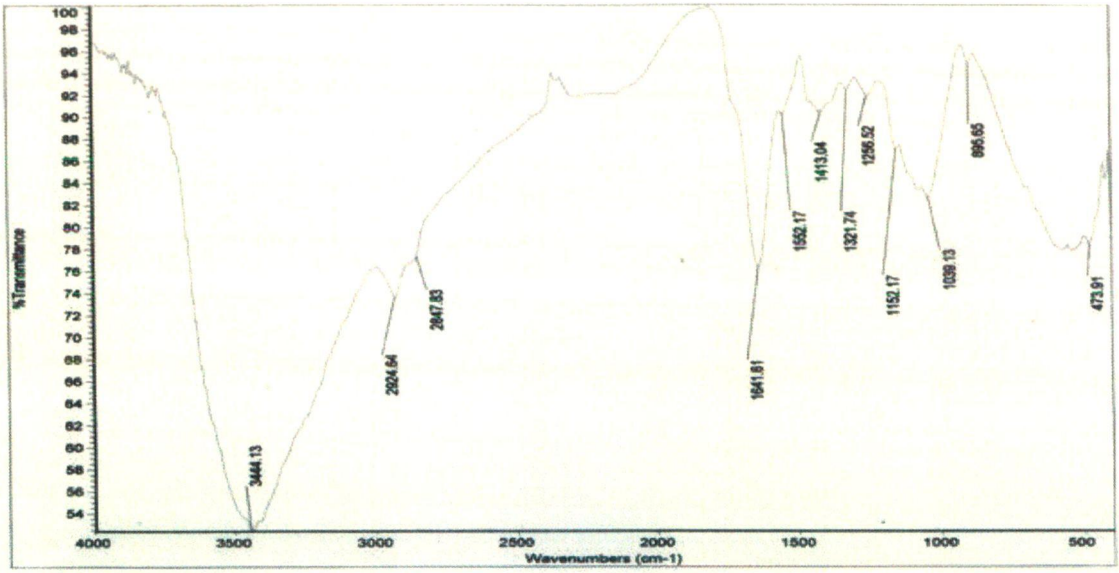


Figure 26 FT-IR spectra of a wheat bran bed sample in dried state

4.3.2.5 Effect of pure glucose application on pine needles bed material

Table 53 Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6g bed of pine needles with production medium containing various doses of glucose

Bacterial strains	Glucose 60g/L		Glucose 80g/L		Glucose 120g/L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L.delbrueckii (Strain-1)	4.22	35.97	4.08	43.87	4.42	32.56
L.pentosus (Strain-2)	6.10	3.37	5.89	8.72	5.34	13.075
Coculture of first two strains	4.20	36.71	3.90	45.10	4. 48	34.84
Lactobacillus sp. (Strain-3)	5.32	13.87	5.88	21.87	6.02	5.74
Lactobacillus sp. (Strain-4)	5.92	7.00	4.46	26.15	5.98	4.56

LA- Lactic acid in extract*(g/L); Conditions1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

In Table 53, the *Lactobacillus* strains 1, 4, and co-culture attained the maximum individual lactic acid concentrations of 43.87 g/L, 26.15 g/L, and 45.10 g/L, respectively, at 80

g/L dose of pure glucose as carbon source, while strains-2 and 3 reached 13.075 g/L and 13.87 g/L at 120 g/L and 60 g/L pure glucose levels, respectively, in the production media. Thus, co-culture achieved the highest lactic acid production among all the strains with 80 g/L pure glucose application. The lowest dose of glucose, 60 g/L proved to be the most suitable for strain-3, where it attained its maximum individual concentration of lactic acid. Inhibition due to high sugar level (120 g/L pure glucose) was evidenced in Table 53, where a sharp decline in lactic acid production occurred in all the bacterial strains, except, strain-2. Although strain- 2 could grow and utilize glucose (hexose) but being primarily a pentose sugar consumer, it was not significantly inhibited by higher dose of glucose (hexose). It may, additionally, consume some of the pentose sugars available, due to hemicellulose degradation from pine needle bed, during autoclaving; hence it showed its highest lactic acid concentration of 13.075 g/L at 120 g/L pure glucose. The higher production of lactic acid at 120 g/L glucose treatment by strain-1 *Lactobacillus sp.* (NCIM2025) and co-culture as per Table 53 indicated their better capability to withstand the high level sugar inhibition better than other strains and carry out a reasonable level of lactic acid production. Among all the bacterial strains at the lowest glucose level (60 g/L) in Table 53, co-culture showed highest lactic acid formation 36.71 g/L (pH 4.20), closely followed by strain-1 with 35.97 g/L (pH 4.22) lactic acid. In Table 53, co-culture showed better acid production (lower pH) than its constituent strains-1 and 2 at 60 and 80 g/L doses of glucose; thus its application may prove better at the lower and the optimum doses of glucose.

4.3.2.6 Effects of whey substituted glucose application on pine needle bed material

In Table 54, all the bacterial strains and co-culture attained their individual maximum values for lactic acid production at 120 g/L total sugar level, in whey-substituted glucose media, where co-culture displayed the highest lactic acid production of 44.88 g/L. The increase in lactic acid production with increase in total sugar level, given in Table 54, indicated no significant sugar inhibition at 120 g/L total sugar level. This observation contradicted the trend of inhibition observed in Table 53, at 120 g/L pure glucose. This may be due to the presence of nitrogenous compounds and vitamins in whey, which stimulate more cell growth, leading to better utilization of sugars. In Table 54, co-culture exhibited the highest lactic acid production at 120 g/L total sugar level, which was higher than that of the constituent strains-1 and 2. Co-culture was more beneficial due to the dual advantage of utilizing disaccharides (lactose) and monosaccharides hexose (glucose), as well as pentose sugars (possibly liberated during

autoclaving, from the mean value 14.12% of pentosan fraction of pine needle bed material, as mentioned in Table 45).

Table 54. Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6g bed of pine needles with production medium containing various doses of whey lactose and glucose

Bacterial strains	Glucose 30g/ L +WL30g/ L TS 60g/ L		Glucose 40g/ L +WL40g/ L TS 80g/ L		Glucose 70g/L +WL50g/ L TS 120g/ L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L .delbrueckii (Strain-1)	4.18	37.21	4.14	39.46	3.98	43.67
L.pentosus (Strain-2)	5.98	5.19	5.86	8.02	4.81	22.08
Coculture of first two strains	4.41	27.77	4.12	40.84	3.93	44.86
Lactobacillussp. (Strain-3)	6.20	1.28	5.90	7.89	5.04	18.02
Lactobacillus sp. (Strain-4)	5.92	5.73	5.18	10.02	4.45	26.45

WL-Whey lactose; TS-Total sugar; LA- Lactic acid in extract*(g/L); Conditions1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

In Table 54 co-culture showed the highest pH drop (3.93), closely followed by strain-1, pH 3.98. Each strain including co-culture attained its lowest pH values with 120 g/L total sugar level, which is in accordance with their highest lactic acid production values in Table54.

The slightly acidic pH accompanied with high temperature (100 - 120 ^{0}C) and pressure conditions during autoclaving might have possibly degraded the acetylated xylans from the bed material into xylan and acetic acid .The breakdown of hemicellulose may have been due to the auto-hydrolysis or dilute acid hydrolysis reactions, possible during autoclaving. Hydronium ions required for both the processes may either be present initially or could be generated later through the autoionization of water. These subsequently depolymerize hemicelluloses, by hydrolysis of glycosidic linkages and acetyl groups [53]. In the second

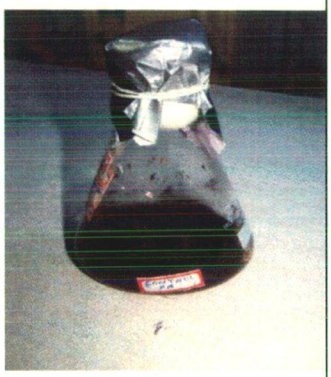
stage of hydrolysis, hydronium ions from the generated acetic acid have greater impact on hydrolysis than those available from auto-ionization. These catalyze the hydrolysis of links between hemicelluloses and lignin [53, 54].

The auto-hydrolysis may convert hemicelluloses into soluble monosaccharides or oligosaccharides, with reduced amount of byproducts [20]. Higher lactic acid production values by some strains also reflected their ability to withstand the low pH (acidic conditions), as in case of strain-1 and co-culture, in Table54.

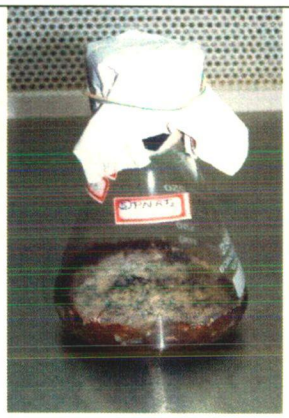
At 120 g/L total sugar level all the strains and co-culture provided higher lactic acid production in Table 54 than Table 53 (with pure glucose). This indicated stimulatory action of proteins (α-lactoglobulin, β-lactoglobulin, serum albumin etc.) and amino acids (high amount of cysteine, methionine) and minerals from whey, also important for the nutritional requirement of lactobacilli [34, 30,89]. Hence these factors possibly resulted in higher growth of bacterial biomass, that enhanced sugar uptake, resulting in higher lactic acid production.

Particle size fractions of the pine needles bed material

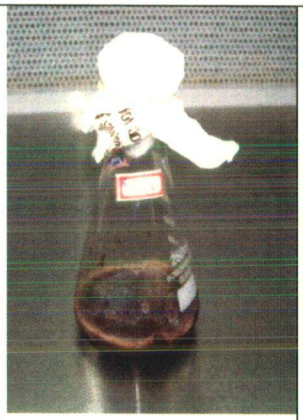
The particle size of the bed material exerts a significant impact on growth and production of microorganisms, in solid state fermentation. The surface area, liquid holding capacity, and the gaseous exchange depend upon the size of bed material particles. In the present study (Table 48), the size of the ground pine needles had particle sizes lesser than 150 μm and up to 1204 μm. A major portion (by weight) of powdered pine needle particles used here was greater than 420 μm, followed by those of greater than 500 μm and lesser than 150 μm. If the bed is composed predominantly of very small sized particles, it has large surface area but reduced mass transfer.



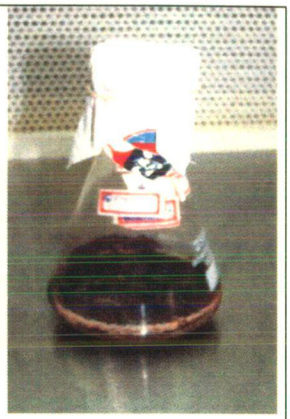
27(a)



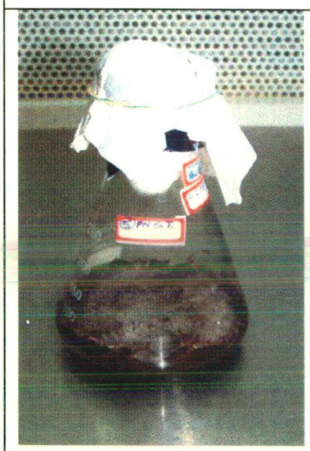
27(b)



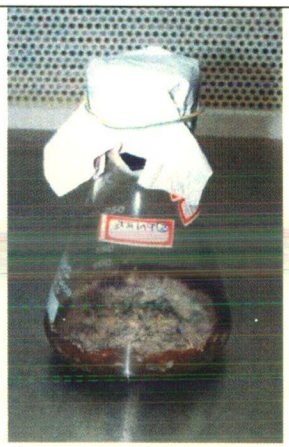
27(c)



27(d)



27(e)



27(f)

Figure 27 Growth of lactobacilli on solid bed of pine needles. Frames 27(b), 27(c), 27(d), 27(e), and 27(f) show the growth of strains-1, 2, 3, 4, and coculture on pine needle bed fed with a production media containing whey lactose (50 g/L), combined with glucose (70 g/L) at 120 g/L total sugar level, incubated for six days, while 27(a) shows control bed with no inoculation.

Figure27 shows the growth patterns of different strains of lactobacilli on the pine needle bed, added with whey substituted glucose based production media containing 120 g/L total sugar level where they attain their individual maximum lactic acid production values as observed in Table 54. Frames 27(b), 27(c), 27(d), 27(e), and 27(f) represent the growth of strains-1, 2, 3, 4, and coculture, while 7(a) shows a non-inoculated bed of powdered pine needles. Among these, the strains-1, 2, 4, and coculture displayed greyish white colored, dense growth over the bed, as observed in Figure 27: 27(b), 27(c), 27(e), and 27(f) due to higher utilization of the vitamin and nitrogenous substances from whey, as compared to that of strain-3, seen in Figure 27(d). The lesser growth of strain 3 observed in 27(d), consequently, resulted in its least lactic acid production of 18.02 g/L, among all the strains, as mentioned in Table 54.

Data Analysis

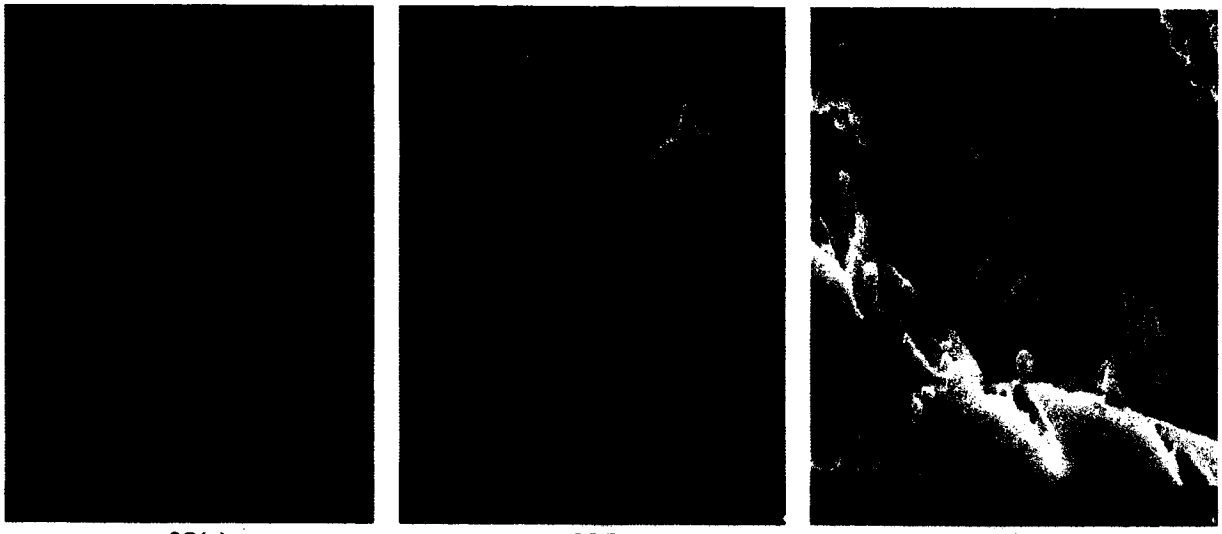
The values of Karl Pearson's coefficient of variation CV (Appendix-8) for the lactic acid concentration data in Table53 (73.53, 47.31 and 71.87) and Table54 (92.76,72.75,35.93) , corresponding to different levels of sugar treatments(60,80 and 120 g/L), suggests that 120 g/L dose of whey-substituted glucose (with least CV), had most consistent lactic acid concentration values from different strains, with least variation. The uniformity in the production values at 120 g/L dose of whey-substituted glucose further indicated that this treatment level was most successful in enhancing the lactic acid production of all the strains under study. All of these strains attained their maximum concentration of lactic acid at this level (Table 54). The next higher value of CV was obtained with 80 g/L pure glucose application, (where only three out of five strains attained their maximum lactic acid concentration), indicated lesser consistency among the production values from different strains. A very high CV value at 120 g/L pure glucose input level indicated comparatively lesser consistency and higher disparity in lactic acid production by the bacterial strains under study, as a consequence of inhibition in lactic acid production (Table 53), due to highest dose of sugar application. Very high CV thus suggests that the corresponding input level of pure glucose (120 g/L) ceased to be beneficial for production of lactic acid. The correlation analysis (Appendix-10) between the concentration of lactic acid from bed and input of lactose from whey (Table 12) provided positive values of correlation coefficient (γ),such as (0.9851,0.9332, 0.9927,0.9472 and 0.9563) for strains-1,2,3,4 and coculture, which suggests strong interdependence and positive correlation between these variables. The t- test analysis (Appendix-12) carried out on the lactic acid production data (Table 54), by different bacterial strains showed lower calculated value of t than the table value of t at eight degrees of freedom and 5% level of significance, which justified the fact that the rising sugar inputs along with whey lactose did not have any significant sugar inhibition affecting lactic acid production. The above statistical finding is further complemented by the fact that whey is known to contain stimulatory constituents for the lactobacilli, which are helpful in their growth and production.

ANOVA analysis (Appendix-16) was applied with Table 54 data, which helps in analyzing whether, the inputs of incremental doses of the whey mixed glucose (all doses) and the difference in *Lactobacillus* strains, pure cultures and coculture, used for present study causes significant changes in lactic acid production. Since both the calculated values of F, one

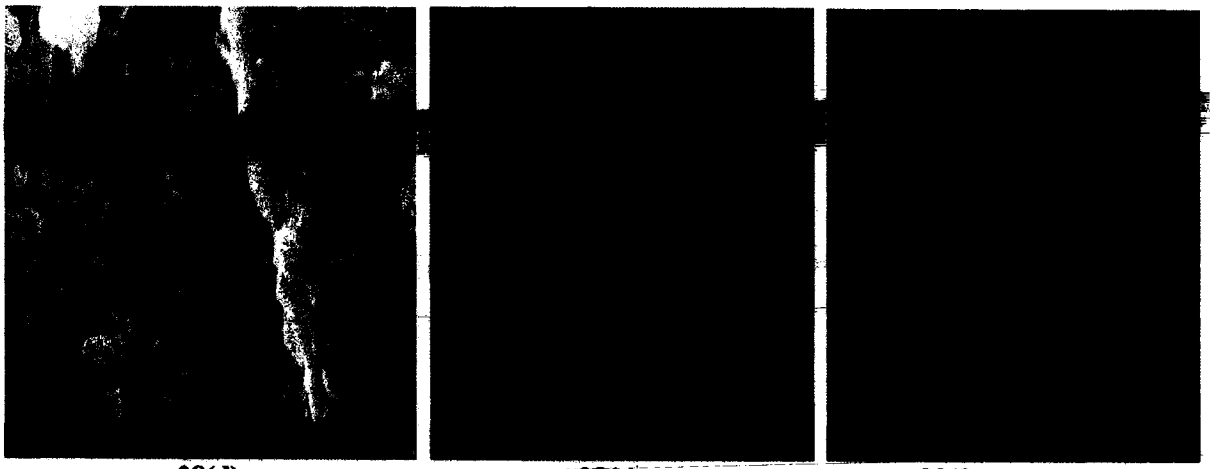
related to variation in strains and the other one related to all the dose variations of whey mixed glucose were greater than their respective table values of F, 3.26 and 3.49(at 5% level) and 5.41 and 5.95 (at 1% level), respectively. Therefore it can be inferred that application of the incremental doses of whey mixed glucose on the different pure strains and coculture, produces significant difference in lactic acid production at 5% level of significance.

Scanning electron micrographs of bacterial growth on the solid bed

The SEM micrographs in Figure28: 28(a), 28(b), 28(c), 28(d), and 28(e), exhibited clusters of *Lactobacillus* strains-1,2, 3, 4, and co-coculture, fused growth and some distinct capsular rod-shaped bacterial cells, while others formed aggregates adhering with the bed material at 120 g/L whey-substituted glucose media application (under 5000X magnification). Figure 28(c) for strain-3, again shows lesser cell population, hence it provided the least lactic acid production of 18.02 g/L mean value (mentioned in Table 54). Figure 28(e) for co-culture indicated dense growth of bacteria very closely aggregated to each other provided the maximum lactic acid production of 44.86 g/L mean value (mentioned in Table 54). The aggregates as well as separate rod shaped lactobacilli cells adhering to the bed material can be observed in the SEM micrographs in Figure28. This clearly indicates that the present bacterial strains bear the capacity to grow on the bed material despite the possibility of the release of inhibitory phenolic substances from the pine needle bed material during autoclaving.



28(a) 28(b) 28(c)



28(d) 28(e) 28(f)

Figure 28 SEM micrographs showing the growth of Lactobacilli on solid bed of pine needles. Frames 28(a), 28(b), 28(c), 28(d), and 28(e) show the growth of the strains-1, 2, 3, 4, and coculture, respectively, at 5000X magnification, on pine needle bed fed with a production media containing whey lactose (50 g/L), combined with glucose (70 g/L) at 120 g/L total sugar level, after six days incubation, while 28(f) shows pine needles in bed at 20X magnification.

The aggregates as well as separate rod shaped lactobacilli cells adhering to the bed material can be observed in the SEM micrographs in Figure 28. This clearly indicates that the present bacterial strains bear the capacity to grow on the pine needles bed material despite the possibility of the release of inhibitory phenolic substances from the pine needle bed material during autoclaving.

The FT-IR analysis of a dried ground pine needle bed sample shown in Figure29 indicated peaks corresponding to different functional groups associated with various compounds predicted to be present in the pine needle bed material. These compounds could be liberated by degradation reactions, under high temperature and mild acidic conditions prevalent during autoclaving, and thereby affect the lactic acid producing bacterial strains under study.

The peak around 1378 cm^{-1} could be attributed to C-H bending vibrations in cellulose and hemicellulose, while the peak near 1165 signifies glycosidic linkages and showed C-O, C-O-C, stretching, and C-OH bending vibrations in arabinoxylans of hemicelluloses[54].The peak detected at 2921 cm^{-1} indicates a symmetric C-H stretching vibration. The spectral region near peak observed at 1730 cm^{-1} suggests C=O stretching vibrations due to presence of carboxyl group [22]. The region detected around absorption band near 3420 to 3530 cm^{-1} indicated O-H stretching vibrations in guaiacyl and syringyl rings (which form monomer units in lignin) under intramolecular hydrogen bonding, while the peak near 1326 cm^{-1} may be attributed to C-O stretching of phenol [54]. The absorption peak around 3413 cm^{-1} indicates the presence of O-H stretching of phenolic hydroxyl group.

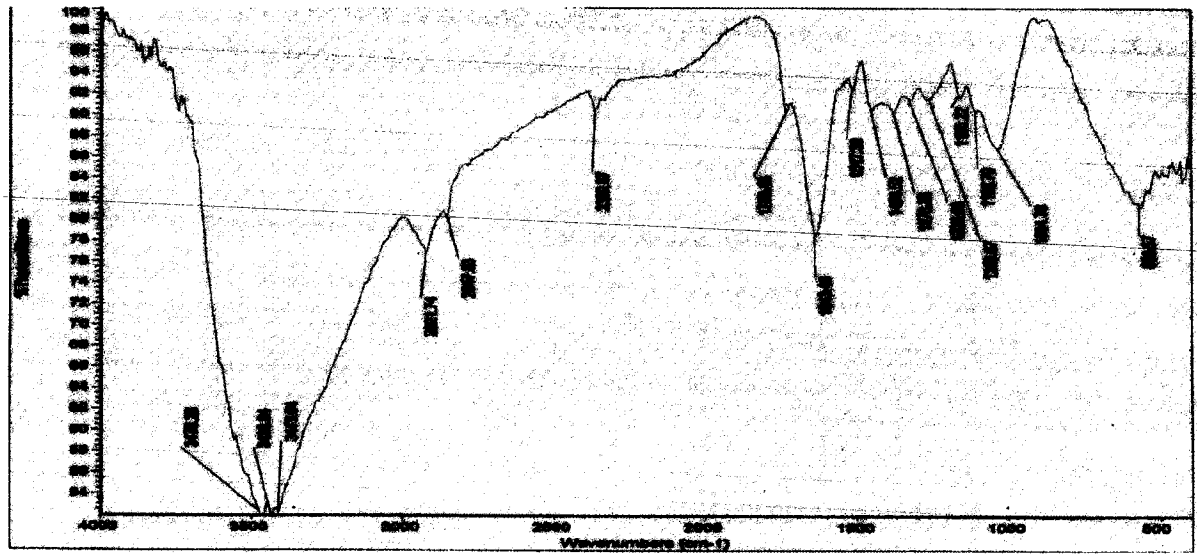


Figure 29. FT-IR spectra of a ground pine needle bed sample in dried state

The FTIR studies of the dry pine needles indicated about three peaks within the range 3420 to 3530 cm^{-1}, as shown in Figure29, out of which only one remained near 3429 cm^{-1} after autoclaving, while the other two disappeared. This clearly suggests the degradation of lignin in the bed material and release of phenolic compounds (from the bed material during the

autoclaving process) that might have affected the growth and production of the present lactic acid producing strains.

4.3.3 Effect of Nitrogen Sources in Solid State Fermentation Utilizing Wheat Bran Bed Material

The effect of different nitrogen sources at different doses, on solid state lactic acid production were studied utilizing wheat bran bed material by *Lactobacillus* strains and coculture.

Table 55. Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6 g wheat bran bed with glucose based production medium containing various doses of meat extract

Bacterial strains	[ME] 10 g/L		[ME] 12 g/L		[ME] 15 g/L		[ME] 20 g/L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L. delbrueckii (Strain-1)	4.98	18.36	3.61	41.83	3.82	29.68	5. 78	9.89
L .pentosus (Strain-2)	5.64	9.68	4.94	21.06	5.41	13.61	5.90	5.81
Coculture of first two (Strains)	3.65	26.19	3.56	43.75	3.70	35.41	5.62	10.76
Lactobacillus sp. (Strain-3)	4.94	20.23	3.74	33.67	3.64	27.48	5. 84	7.71
Lactobacillus sp(Strain-4)	5.72	12.76	4.92	21.46	4.96	18.77	6.14	4.09

[ME] Meat extract; LA- Lactic acid in extract*(g/L); Conditions1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

The data observed in Table 55 reveal that coculture and strain-1 attain their highest values of lactic acid concentration 43.75 g/L and 41.83 g/L respectively at a dose of 12 g/L meat extract as nitrogen source. Table 55 results also indicate that all the strains and coculture attain their maximum lactic acid production at 12 g/L dose of meat extract because meat extract contains 6-9% total nitrogen, purine bases, polypeptides ,potash and phosphoric acid required for growth and metabolism of lactobacilli[112, 115]. At higher doses of meat extract 15 and 20 g/L, a decline in lactic acid production has been observed probably due to the fact

excess growth of bacterial biomass due to higher dose of the nitrogen source deviates the metabolic activities from acid synthesis towards growth.

Table 56. Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6 g wheat bran bed with, glucose based production medium containing various doses of proteose peptone

Bacterial strains	[PP] 10 g/L		[PP] 12 g/L		[PP] 15 g/L		[PP] 20 g/L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L.delbrueckii (Strain-1)	4.95	22.46	3.59	42.31	3.80	31.44	5. 60	11.04
L. pentosus (Strain-2)	5.67	10.12	4.78	22.79	5.15	14.37	5.78	6.93
Coculture of first two strains	3.66	26.31	3.53	45.08	3.64	36.84	5.63	12.52
Lactobacillus sp. (Strain-3)	4.93	21.57	3.67	35.42	3.78	28.01	5.64	10.61
Lactobacillus sp. (Strain-4)	5.60	14.08	4.76	23.50	4.89	17.80	6.10	4.86

[PP] Proteose peptone; LA- Lactic acid in extract*(g/L); Conditions1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

The data in Table56 suggest that the proteose peptone nitrogen source provides a higher value of lactic acid concentration for strain-1 and co-culture 42.31 g/L and 45.08 g/L than those in Table 55 at 12 g/L of nitrogen source because the nitrogen content in the proteose peptone is,10-12.5%, which is higher than that of meat extract[21]. Moreover It contains significant amount of amino acids such as alanine; arginine; methionine cysteine; aspartic acid; glutamic acid; glycine; histidine; isoleucine; leucine; lysine; phenyl alanine; proline; serine; threonine; tryptophan; tyrosine; valine; and minerals such as calcium; magnesium; potassium and sodium that help in growth and enhance lactic acid production and meet the nutritional requirements of lactobacilli[21,89,34].Here also, the maximum lactic acid production has been attained at 12 g/L dose of proteose peptone and a decline in lactic acid concentration similar to that in Table 55, has also been witnessed.

Table 57. Lactic acid production through solid state fermentation by the different strains of lactobacilli on 6 g wheat bran bed with, glucose based production medium containing various doses of baker's yeast

Bacterial strains	[BK] 12 g/L		[BK] 15 g/L		[BK] 20 g/L		[BK] 23.33 g/L	
	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*	pH	LA (g/L)*
L.delbrueckii (Strain-1)	5.82	8.85	5.68	12.29	4.79	23.28	6.20	4.51
L. pentosus (Strain-2)	5.88	6.36	3.76	28.31	5.58	12.18	6.25	3.76
Coculture of first two strains	5.60	12.61	5.54	15.04	3.38	47.56	5.54	15.29
Lactobacillus sp. (Strain-3)	4.98	19.75	3.44	46.41	3.79	27.37	5.58	12.96
Lactobacillus sp. (Strain-4)	5.81	9.06	5.67	10.07	4.93	22.08	5.80	9.38

[BK] Baker's yeast; LA- Lactic acid in extract*(g/L); Conditions1mL 2% NaOH neutralizer, inoculum dose of 2g/L, production medium per flask 40 mL at 37 ^{0}C, initial pH 6.5, and six days incubation period

Dried baker's yeast, used for the strains under study, has been applied previously as an inexpensive source of nitrogen for lactic acid production due to higher cost (about 38% of total cost of production)[7]. Table 57 data show that the highest lactic acid production has been attained by coculture 47.56 g/L(lowest pH 3.38), followed by strain-3, 46.41 g/L at 20 g/L and 15 g/L doses of baker's yeast. Strain-3 achieved its highest lactic acid production 46.41 g/L but at a lower input of (15 g/L) of baker's yeast, indicates lower nitrogen requirement. Dried baker's yeast (Table 57) proved to be reasonably good nitrogen source for the lactobacilli under study, which attained the maximum lactic acid production, 28.31 g/L, 46.41 g/L and 47.56 g/L for strain-2, strain-3 and coculture, this is because of the fact that, baker's yeast is parent source of yeast extract which contains 60.2% protein, important vitamins(thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, cyanocobalamin, biotin and folic acid), amino acids (lysine, arginine, methionine, glycine, valine, leucine, threonine, glycine, serine, aspartic acid and glutamic acid) and mineral components required for growth and lactic acid synthesis[116]. Strain-1 and strain-4 attain their maximum value in lactic acid production with proteose peptone as nitrogen source. Coculture emerged to be the highest

lactic acid producer among all the strains under study that too with the cheaper nitrogen source baker's yeast, hence coculture may play an important role in fermentation industries for lactic acid production. The wheat bran showed its potential as an efficient bed material that can support the bacterial growth through its amino acids and proteins and can provide important pentose or hexose sugars from its hemicelluloses fraction (after autoclaving in slightly acidic media) significant in lactic acid production[63,67,99].

The lower dose 12g/L of the synthetic nitrogen sources served most suitable for lactic acid production. Proteose peptone proved to be better nitrogen source for lactic acid production amongst the present bacterial strains [21]. The superior performance of coculture is evident in terms of lactic acid production with the pure nitrogen sources (at lower doses) but at higher doses of baker's yeast (20 g/L) provided better lactic acid production. Meat extract and proteose peptone showed better compatibility for production with strain-1, coculture and strain-4. Co-culture showed higher acid production (lower pH) than its constituent strains -1 and 2 at 15g/L dose of meat extract and proteose peptone. Strain-2 and strain-3 showed their highest lactic acid production and hence indicated better compatibility with baker's yeast as nitrogen source. Dried baker's yeast provides reasonably good help as low cost nitrogen source, in terms of lactic acid production from strain-3, strain-4 and coculture, which attained their highest acid production [6,7]. The wheat bran showed its potential as a good bed material that can support the growth and production of lactic acid by the different strains of lactobacilli through solid state fermentation technology. This also presents an amicable solution to the problem of solid agro-waste generation during processing.

CHAPTER-5

CONCLUSION

The first section 4.1.1 includes the liquid state batch production of lactic acid under the influence of various pure sugars and carbohydrate rich raw materials. The study includes the comparative effects of the pure sugars glucose and lactose and their combinations with whey at different levels on the *Lactobacillus* strains in terms of lactic acid concentration. With the pure sugar glucose the 100 g/L dose is the most suitable where all the strains attained their individual maximum values of lactic acid production, which were, 92.82,88.31,93.57,89.12 and 91.56 g/L for strains-1,2, coculture, 3 and 4 respectively. This clearly showed that the coculture was the overall major lactic acid producer closely followed by the strain-1. The coculture also attained the highest productivity and weight of lactic acid per unit weight of glucose input. The batch fermentation studies with the lactose indicated no inhibition as it was observed with the glucose at 120 g/L dose where the production of all the strains dropped. The maximum values of lactic acid concentration with lactose were, 112.84,98. 60,115.56,104.85 and 108.90 g/L for strains-1,2,coculture, 3 and 4 respectively at a dose of 120 g/L. Again the coculture was the major producer of lactic acid. The lactic acid concentrations obtained with lactose were clearly higher than the values obtained with glucose for all the strains. Hence lactose served better as carbon source than glucose for attainment of higher lactic acid production without any substrate inhibition.

The studies with whey substitutions in the glucose indicated the removal of inhibition due to high substrate doses of glucose and the lactic acid production of the strains increased. At 120 g/L level of whey substituted glucose the highest lactic acid production were110.10,91.8,114.96,84.85 and 95.11 g/L for strains-1,2,coculture, 3 and 4 respectively. These values were higher than obtained with pure glucose. The combination of whey with pure lactose, was even more beneficial as better concentration of lactic acid were obtained. The concentrations achieved were,113.75, 105.84, 116.01,108.65 and 109.85 g/L for strains-1,2,coculture, 3 and 4 respectively. Among the acid hydrolyzed starch and the enzyme hydrolyzed starch as carbon source, higher lactic acid production and productivity were obtained with enzyme treated starch. Among all the carbon sources tested the whey substituted lactose provided the highest lactic acid production at 120 g/L dose while

coculture maintained highest production and productivity of lactic acid. The acid treated bagasse extract obtained under the autoclaved conditions served better than the boiled condition as a carbon source in combination with reduced amount of glucose. The boiled condition provided 31.68, 26.0,36. 98,36.21 and 34.00 g/L while the autoclaved extract gave 40.37, 50.01,51.86,49.84 and 38.61 g/L lactic acid for strains-1,2,coculture, 3 and 4 respectively. Along with coculture the strain-2 and strain-3 also enhanced their production in autoclaved bagasse extract.

The studies with nitrogen sources showed that yeast extract was the most successful nitrogen source that provided maximum lactic acid production with all the strains at the least input of 10 g/L while proteose peptone , meat extract and the baker's yeast required 12 g/L and 20 g/L dose to get maximum lactic acid production. The cheap nitrogen source bakers yeast is preferable due to lower cost and at higher doses 20 g/L is quite competitive with meat extract in terms of lactic acid production.

In the experiments with effects of pH values the highest lactic acid production by, strain-1 was at 6.5, strain-2 and coculture at 6.75 while, strain-3 and 4 at 6.0. The studies on the neutralizer indicated that the lactic acid production and the productivity values for all the strains were higher with 2% NaOH with the exception of strain-2, which favours 2% calcium carbonate.

. Studies on the salts showed that ferrous ammonium sulphate provided highest lactic acid production with coculture, while sodium pyruvate gives higher values with strain-1,3 and coculture. Sodium succinate was better suited with strain-1and 3, while ammonium citrate had reasonably high production with all the strains.

An agitation of 180 rpm and a 37^0C temperature was the best for all the strains. However strain-1 and coculture required 40 ^{0}C as its most preferable temperature for higher lactic acid production. The fed batch method provided very high production up to 162 g/L. The fed batch studies with whey and lactose gave the highest lactic acid production.

The application of whey and glucose with wheat bran bed material provided the highest lactic acid production of 54.25 g/L, in solid state fermentation followed by that of sugarcane bagasse and pine needles bed materials.

The growth studies with modified MRS media with carbon components from carbohydrate rich raw materials indicated different patterns of the growth of the *Lactobacillus* strains which were related to their sugar consumption. Strain-1,2 and coculture showed a diauxic growth with all the three carbon sources which was due to their preferential consumption of sugars. However, the other two strains-3 and 4, the usual batch growth pattern in the MRS media with two bagasse extracts indicated that they consumed different sugars simultaneously. Strains-3 and 4 also show diauxic growth when whey was used with MRS, indicating a preferential sugar consumption pattern. In all the growth studies the coculture had highest biomass growth and maximum pH drop.

Coculture had shown highest biomass growth, highest acid tolerance, maximum lactic acid production as compared to the pure strains in liquid batch, fed batch and solid state fermentation of lactic acid, utilizing different pure materials or raw material containing media. Hence coculture can be successfully used for the industrial production of lactic acid through fermentation.

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APPENDIX-1

Use of only one type of coculture

The preparation of the coculture with strain-1 (*Lactobacillus delbrueckii*) and strain-2 (*Lactobacillus pentosus*) as component strains, was based on the fermentation studies with the pure culture. The following significant observations, during fermentation studies on lactic acid, from the pure cultures were taken into consideration, for planning the selection of the component strains in above mentioned coculture.

(1) Strain-1provided the highest production, productivity and biomass with the hexose sugars.
(2) Strain-1 showed the highest capability to with stand the most acidic pH values.
(3) Strain-1 *L. delbrueckii* is known to work under higher temperatures (40-45^0 C).
(4) Strain-1produced highest lactic acid concentration in cases of high sugar substrate inhibition or product inhibition.
(5) As pure culture, besides the batch fermentation, strain-1 showed highest overall lactic acid production in fed batch fermentation as well as solid state fermentation but predominantly with the hexose sugar based carbon source in production medium.
(6) Strain-2 works preferably better with pentose sugars but can also provide fairly good lactic acid production with the hexose carbon sources.

The above mentioned facts formed the basis of selection of these two strains-1 and 2 (that complement each other in various aspects of lactic acid production) for the design of coculture used in the present studies. Hence, for wide range applicability of the coculture in lactic acid production utilizing pentose and hexose sugars available from inexpensive lignocellulosic agricultural or forestry waste materials, the strains-1 and 2 were decided as its component strains. Such a coculture can be predicted to perform more efficiently than coculture prepared from other strains with pure or mixed sugars (pentoses and hexoses) in hydrolyzates of lignocellulosic materials at higher temperatures under more acidic conditions.

APPENDIX-2

Removal of toxicity of furfural and hydroxy methyl furfural resulting from boiling and acid treatment of sugar cane bagasse

The agricultural materials such as corn cobs, rice hulls or sugarcane bagasse when treated with hot hydrochloric acid or sulphuric acid liberate pentoses from the acid hydrolysis of pentosans to further form furfural.

For obtaining sugars through acid hydrolysis of sugarcane bagasse (free of lignin), the delignification of bagasse by 10% NH_4 OH has been used.

For removal of toxic substances following steps were performed in sequential order.

(1) Bagasse drying at 80^0 C till constant weight and dry to small particles for better reaction.

(2) 10% NH_4 OH was used at 100ml/g (material) at 25^0 C for 24 hours. The lignin was librated in the soluble fraction while the solid fraction was washed with [illegible] water 2-3 times and dried 80^0 C for 24 hours [58].

(3) Acid hydrolysis takes place with 1% H_2SO_4 (1:10 w/v ratio) at 121^0 C, 15psi in an autoclave for 30 minutes.

(4) Removal of toxic materials by raising the pH value of the sugarcane bagasse acid hydrolyzate to 10 with calcium hydroxide followed by decrease in pH to 6.5 by using H_2SO_4. The precipitate resulting from the treatment were removed by centrifugation at 5000 rpm for 20 minutes. The pH variation was done firstly to precipitate the toxic compound and later brought down to 6.5 to set the pH of the production medium such that the acetate or acidic molecules acquire unprotonated form which is less toxic to bacteria [72].

APPENDIX-3

Effect of high substrate concentration (sugars) on the enzymes of EMP path way

The substrate inhibition due to application of high dose of sugars such as glucose during the batch fermentative production of lactic acid occurs due to the impacts on several components of EMP path way which reduced the lactic acid production. In the metabolic path ways the enzymes catalyzing the irreversible reactions are potential side of control. The hexokinase (HK) converts the glucose to glucose 6 phosphate, phosphofructo kinase (PFK) converts fructose-6-phosphate to fructose 1,6 bisphosphate and pyruvate kinase (PK) converts phosphoenolpyruvate to pyruvate. According to the study of Papagianni et al. (2007), in the case of lactic acid production with excess glucose it showed that an optimum level of glucose leads to highest value of the specific activities of the key enzymes of glycolysis path way such as PFK, PYK and LDH. However very high dose of glucose resulted into decline of PFK activity and the glycolytic and lactate flux. Very high dose of glucose leads to accumulation of unphosphorylated intracellular glucose and lowers the amount of phosphorylated sugars, which indicate the inhibition PFK enzyme.

The phosphorylated sugars such as FBP-fructose1,6 bisphosphate, activate PYK and LDH enzymes and direct the flux towards lactate production. However the lower levels of FBP (due to the predominance of unphosphorylated sugars inside the cell) cause the inhibition of PFK and inactivate LDH (which converts the pyruvic acid to lactic acid). Hence very high doses of sugar substrate lower the lactic acid production ADP and ATP play important roles influencing several steps in EMP path way because they are substrate and product of kinases and inhibitors of dehydrogenases. A high level ATP lowers the activity of PFK by binding at the allosteric site which causes a conformational change in the substrate binding site by reducing its affinity towards the substrate fructose6 phosphate. PFK is also inhibited by H ions, so very high production of free lactic acid can cause inhibition of glycolysis which consequently lowers the lactic acid production.

Hexokinase-HK is inhibited by glucose 6 phosphate which is in equilibrium with fructose6 phosphate. The glucose induces a large conformational change in HK, where the glucose gets surrounded by the closure of cleft between two lobes of the enzyme HK where it

adds the phosphoryl group from ATP to form glucose6 phosphate. It may be possible that too much of glucose entry in the cell may not be favorable in the formation of glucose 6 phosphate through the closure of cleft.

The regulatory enzymes of the EMP are activated by ADP, AMP and Pi, but they get inhibited by ATP, hence increase or decrease of glycolysis is governed by decrease or increase of the ratio of [ATP]/[ADP][Pi].

In nutshell it can be summarized that the activity of following enzymes of EMP path- way increase or decrease according to the amounts of certain compounds given below.

1. The higher amount of fructose1, 6 bisphosphate and AMP activates PK and while higher amounts of ATP or alanine reduce the activity of PK [98,71].
2. The higher amount of fructose 1, 6 bisphosphate and ADP activates PFK and while higher amounts of ATP or H^+ reduce the activity of PFK[11, 71, 98].
3. The higher amount of glucose 6 phosphate and fructose 6 phosphate bring down the activity of HK [98].
4. The activity of LDH is enhanced by an increase in the concentration of fructose 1, 6 bis phosphate and other phosphorylated sugars [71].

APPENDIX-4

In the fermentative production of lactic acid there are two types of inhibition are frequently observed.

1. Product inhibition 2. Substrate inhibition

1.Product inhibition

During the lactic acid production accumulation of large amount of free acid form of lactic acid (in absence of neutralizers) inhibits the growth of the cells and consequently the production of lactic acid is lowered. The H ions from the free acid when present in high concentration in the media enter the cells, acidify the cytosol and affect some of the enzymes in the metabolism. The extra H ions inside the cytosol have to be pumped out with the expenditure of ATP. This extra utilization of ATP for maintenance of intracellular pH can cause growth inhibition [13]. Since lactic acid production is growth associated, hence, the growth inhibition of cells may lead to decline in lactic acid production. To check such an inhibition from the product lactic acid the neutralizers such as NaOH, $CaCO_3$ or NH_4OH are added periodically in the fermentation growth to convert the free lactic acid to its salt form otherwise without neutralizers, a drop of 30-50% in lactic acid production without pH control has been reported [62].

Effect of product inhibition on the bacterial system

The accumulation of free lactic acid in the fermentation broth lowers the pH and this variation in pH values effects the functioning of microbial cells. It influences the functioning of enzymes, transport of nutrients in cell, synthesis metabolic enzymes, RNA and protein synthesis. High concentration of H^+ ions due to higher amount of free lactic acid (product) accumulation may enter the bacterial cell and are reported to lower the activity(inhibits), the phosphofructokinase PFK, enzyme of the glycolysis pathway ,as a result of which, lesser amount of fructose 1.6 bis phosphate(FBP) is generated[98]. The higher amount of FBP, is known to increase the activity of (LDH),lactate dehydrogenase enzyme, required for the conversion of pyruvate to lactate in glycolysis [71]. Hence, the LDH activity may not increase due to the lower level of FBP(as a result of product inhibition of PFK) which

consequently can lower the lactic acid production. The influx of undissociated form of lactic acid product in the cell can cause acidification of cytosol, dissipation of proton gradient and impede the generation of NAD+.

2. Substrate inhibition

If the doses of the carbohydrate sources in the lactic acid production media are increased the production level of lactic acid is enhanced but at very high level of substrate application the lactic acid production gets inhibited due to the effect of these excess quantities of substrate on the enzymes of the lactic acid synthesis path way.

Events related to substrate inhibition

Very high doses of substrate (sugars) bring down the lactic acid production. The excess of glucose substrate enters the cells and effects the important enzymes (PFK-phosphofructokinase, LDH-lactate dehydrogenase, PYK-pruvate kinase) of the glycolysis (EMP pathway), which leads to the reduction in lactic acid synthesis. The amount of ATP, ADP, AMP, Pi and the phosophorylated sugars available from the sugar substrate, also affect the activity of key enzymes of the EMP pathway, thus these factors also regulate the enzyme activity which in turn controls the lactic acid production.

APPENDIX - 5

Table 58 Lactic acid production and productivity values obtained from different pure strains and coculture of lactobacilli at 72 h of batch fermentation with same level of nitrogen sources.

Bacterial Strains	Meat extract		Proteose Peptone		Baker's Yeast		Yeast Extract	
	LA (g/L)	P (g/L/h)	LA (g/L)	P (g/L/h)	LA (g/L)	P (g/L/h)	LA (g/L)	P (g/L/h)
L.delbrueckii Strain-1	36.64	0.5088	39.47	0.5481	32.87	0.4565	43.59	0.6054
L. pentosus Strain-2	33.36	0.4633	35.64	0.4950	28.34	0.3936	38.78	0.5386
Coculture Of strain-1 and 2	40.03	0.5597	42.86	0.5952	35.77	0.4968	47.32	0.6572
*Lactobacillus sp.*Strain-3	37.22	0.5169	39.10	0.5430	32.12	0.4461	42.37	0.5884
*Lactobacillus sp.*Strain-4	35.07	0.4870	37.86	0.5258	33.39	0.4637	40.02	0.5558

LA-Lactic acid concentration(g/L); P-Productivity(g/L/h); Conditions- Initial pH 6.5, 2% NaOH added /12 h, 1.55g/L inoculum at 37 ^{0}C and 180 rpm, utilizing 10 g/L of the nitrogen source in the production medium

The Tables 18,19,20,21 and 22 showed the comparison of maximum values lactic acid production (by any one of the pure strains or coculture), corresponding to the application of different doses of the nitrogen sources in the production medium. However the Table58, provides comparative lactic acid production and productivity values obtained from different pure strains and coculture of lactobacilli at 72 hours of batch fermentation utilizing 10 g/L (same dose)for all the four nitrogen sources (mentioned above) in the production medium. Hence, due to the lower lactic acid production (Table58), obtained from the cheap nitrogen source, dried baker's yeast, its higher dose, 20 g/L was applied in the previous Tables 18,19,20,21 and 22. The 20g/L consequently raised the lactic acid production from inexpensive nitrogen source, dried baker's yeast.

APPENDIX - 6

Table 59 Batch growth studies on pure glucose based MRS medium for all the pure cultures and coculture

T (h)	*L.delbrueckii* Strain-1		*L. pentosus* Strain-2		*Coculture* Of strain-1 and 2		*Lactobacillus sp.* Strain-3		*Lactobacillus sp.* Strain-4	
	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH	CDW (g/L)	pH
0	0.5	7.0	0.5	7.0	0.5	7.0	0.5	7.0	0.5	7.0
1.50	0.59	6.59	0.52	6.75	0.64	6.47	0.55	6.70	0.56	6.64
3	3.08	5.30	2.31	5.50	3.86	5.24	2.42	5.44	2.51	5.35
6	8.46	5.08	5.96	5.22	9.87	5.01	6.50	5.17	7.01	5.12
9	13.01	4.88	9.95	5.07	14.85	4.80	11.21	5.02	11.56	4.96
12	16.25	4.81	12.96	4.96	17.85	4.68	14.02	4.90	15.03	4.86
15	17.27	4.59	14.85	4.71	18.57	4.56	15.75	4.67	16.61	4.61
18	17.88	4.56	15.44	4.65	18.95	4.52	16.24	4.61	17.27	4.59
21	18.01	4.54	15.65	4.63	19.10	4.48	16.64	4.59	17.46	4.56
24	16.37	4.51	14.26	4.59	17.48	4.45	15.12	4.56	15.91	4.54
27	12.92	4.45	10.53	4.57	14.40	4.40	11.44	4.54	12.38	4.50

CDW- Cell dry weight; Conditions-Initial pH 7.0,with no neutralizer at 37° C and 180 rpm

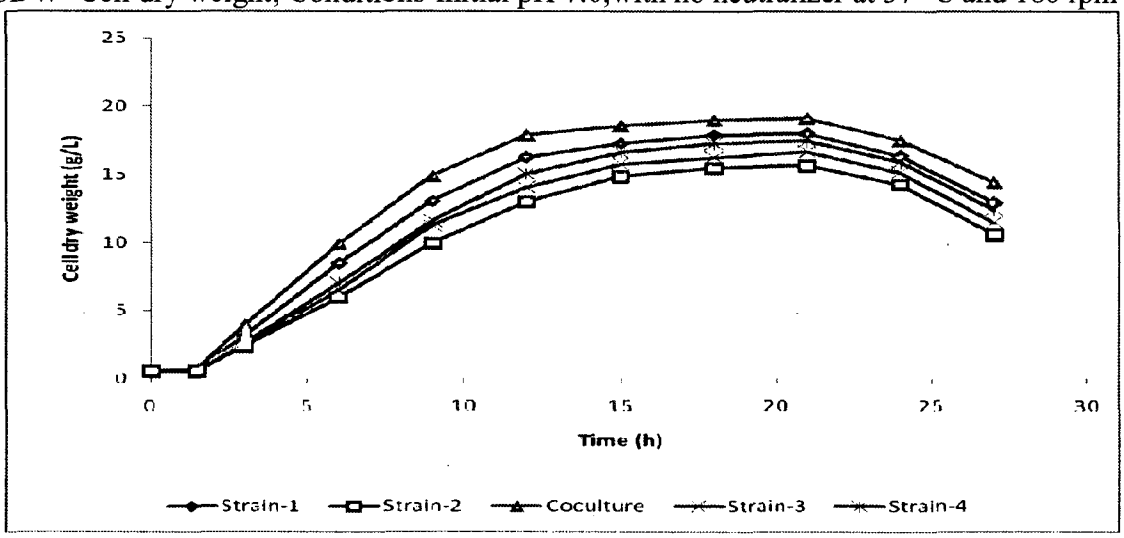

Figure30 Batch growth curves of the pure strains and coculture of lactobacilli on pure glucose based MRS medium

The maximum value for the specific growth rate μ for strains-1,2,3,4 and coculture (Table 59) are 0.6061,0.5102,0.5257,0.5802 and 0.6814 h^{-1} respectively by using $\mu = 2.303/t \log_{10} (N_t / N_0)$. Where N_t and N_0 are number of bacterial cells proportionate to their cell dry weight at t time and zero time.

APPENDIX -7

Calculation of Karl Pearsons coefficient of variation C.V. for the lactic acid production by the pure strains and coculture of lactobacilli used in the present studies in solid state fermentation.

The coefficient of variation is given by the following expression

C.V. = Standard Deviation (S.D.)/Mean (A) x 100 ---(1); S.D.= [Sum d^2/n] $^{0.5}$

Where d is the deviation from mean and n denotes total number of items

Lesser value of Coefficient of variation, C.V., for a group of data denotes that the group is more stable, more uniform, more homogenous and more consistent.

Calculation of Karl Pearson's coefficient of variation C.V. for the lactic acid production by the pure strains and coculture of lactobacilli used in the present studies on a bed of wheat bran.

1. C.V. for 60 g/L pure glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	41.24	17.59	309.4081
Strain-2	11.00	-12.65	160.0225
Coculture	39.01	15.36	235.9296
Strain-3	15.50	-8.15	66.4225
Strain-4	11.50	-12.15	147.6225
	Sum=118.25		Sum d^2= 919.3992

A=118.25/5=23.65; S.D.= $(919.3992/5)^{0.5}$ = 13.5602

C.V. = (S.D./A)x 100= (13.5602/23.65)x 100=57.33

2. C.V. for 80 g/L pure glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	50.01	13.26	175.8276
Strain-2	23.25	-13.50	182.25
Coculture	54.25	17.50	306.25
Strain-3	23.50	-11.25	126.5625
Strain-4	30.75	-6.00	36.00
	Sum=183.76		Sum d^2=826.89

A=183.76/5=36.75; S.D.= $(826.89/5)^{0.5}$ = 12.8599

C.V. = (S.D./A)x 100= (12.8599/36.75)x 100=34.99

3. C.V. for 110 g/L pure glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	45.10	15.91	253.1281
Strain-2	20.05	9.14	83.5396
Coculture	47.31	18.12	328.3344
Strain-3	12.14	17.05	290.7025
Strain-4	21.26	7.93	62.8849
	Sum=145.96		Sum d^2=1018.5895

A=145.96/5=29.19; S.D.= $(1018.5895/5)^{0.5}$ = 14.2729

C.V. = (S.D./A)x 100= (14.2729/29.192)X 100=48.89

4. C.V. for 120 g/L pure glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	41.00	18.65	347.8225
Strain-2	15.01	7.34	53.8756
Coculture	40.00	17.65	311.5225
Strain-3	9.75	12.60	158.7600
Strain-4	6.00	16.35	267.3225
	Sum=111.76		Sum d^2=1139.3031

A=111.76/5=22.35; S.D.= $(1139.3031/5)^{0.5}$ = 15.095

C.V. = (S.D./A)x 100= (15.0950/22.35)x 100=67.5393

5. C.V. for 60 g/L whey mixed glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	39.10	18.85	355.3225
Strain-2	8.56	-11.69	136.6561
Coculture	28.60	8.35	69.7225
Strain-3	15.00	-5.25	27.5625
Strain-4	10.00	-10.25	105.0625
	Sum=101.26		Sum d^2=694.3261

A=101.26/5=20.25; S.D.= $(694.3261/5)^{0.5}$ = 11.7841

C.V. = (S.D./A)x 100= (11.7841/20.25)x 100=58.19

6. C.V. for 80 g/L whey mixed glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	41.68	13.860	192.0996
Strain-2	14.68	-13.136	172.5544
Coculture	45.00	17.184	295.2898
Strain-3	18.05	-9.766	95.2576
Strain-4	19.67	-8.146	66.3573
	Sum=139.08		Sum d^2=821.4687

A=139.08/5=27.81; S.D.= $(821.4687/5)^{0.5}$ = 12.8177

C.V. = (S.D./A)x 100= (12.8177/27.81)x 100=46.08

7. C.V. for 110 g/L whey mixed glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	44.01	12.25	150.0625
Strain-2	19.61	12.15	147.6225
Coculture	49.41	17.65	311.5225
Strain-3	22.15	9.61	92.3521
Strain-4	23.61	8.15	66.4225
	Sum=158.79		Sum d^2=767.9821

A=158.79/5=31.75; S.D.= $(767.9821/5)^{0.5}$ = 12.6331

C.V. = (S.D./A)x 100= (12.6331/31.75)x 100=39.77

8. C.V. for 120 g/L whey mixed glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	48.76	12.154	147.7197
Strain-2	25.01	11.596	134.4672
Coculture	54.25	17.644	311.3107
Strain-3	25.16	11.446	131.0109
Strain-4	29.85	6.756	45.6435
	Sum=183.03		Sum d^2=770.1520

A=183.03/5=36.60; S.D.= $(770.1520/5)^{0.5}$ = 12.4108

C.V. = (S.D./A)x 100= (12.4108/36.60)x 100=33.90

Sugar Levels (g/L)	C.V. at Pure Glucose Application	C.V. at Whey mixed Glucose Application
60	57.34	58.19
80	34.99	46.08
110	48.89	39.77
120	67.54	33.90

Inference

Among pure glucose applications, the 80 g/L dose with least CV (34.99) gives the most uniform consistent lactic acid production. However among overall sugar treatments the 120 g/L whey mixed glucose treatment gives least C.V. (33.90), which indicated, most uniform lactic acid production values obtained from all the *Lactobacillus* strains at this dose, where all the strains perform efficiently. Hence, such a sugar treatment, on a bed of wheat bran can have wide industrial applicability for fermentative production of lactic acid by different strains of lactobacilli.

APPENDIX-8

Calculation of Karl Pearson's coefficient of variation C.V. for the lactic acid production by the pure strains and coculture of lactobacilli used in the present studies on a bed of pine needles.

1. C.V. for 60 g/L pure glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	35.97	16.59	275.23
Strain-2	3.37	-16.01	256.32
Coculture	36.71	17.33	300.33
Strain-3	13.87	-5.51	30.36
Strain-4	7.00	-12.38	153.26
	Sum=96.92		Sum d^2=1015.5046

A=96.92/5=19.38; S.D.= $(1015.5046/5)^{0.5}$ = 14.2513; C.V.= (S.D./A)X 100= (14.2513/19.38)x 100=73.54

2. C.V. for 80 g/L pure glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	43.87	14.73	2.16.97
Strain-2	8.72	-20.42	416.97
Coculture	45.10	15.96	254.72
Strain-3	21.87	7.27	52.85
Strain-4	26.15	2.99	8.94
	Sum=145.71		Sum d^2=950.4529

A=145.71/5=29.142; S.D.= $(950.4529/5)^{0.5}$ = 13.7833;C.V. = (S.D. /A) x 100= (13.7833/29.142)x 100=47.31

3. C.V. for 120 g/L pure glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	32.56	14.41	207.65
Strain-2	13.07	-5.08	25.81
Coculture	34.84	16.69	278.55
Strain-3	5.74	-12.41	154.01
Strain-4	4.56	-13.59	184.68
	Sum=90.77		Sum d^2=850.708

A=90.77/5=18.154; S.D.= $(850.708/5)^{0.5}$ = 13.0438

C.V. = (S.D./A)x 100= (13.0438/18.154)x 100=71.86

4. C.V. for 60 g/L whey mixed glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	37.21	21.77	473.93
Strain-2	5.19	-10.25	105.06
Coculture	27.77	12.33	152.03
Strain-3	1.28	14.16	200.50
Strain-4	5.73	9.71	94.28
	Sum=77.18		Sum d^2=1025.80

A=77.18/5=15.44; S.D.= $(1025.80/5)^{0.5}$ = 14.32

C.V. = (S.D./A)x 100= (14.32/15.44)x 100=92.76

5. C.V. for 80 g/L whey mixed glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	39.46	18.21	331.60
Strain-2	8.02	-13.23	175.03
Coculture	40.84	19.59	383.76
Strain-3	7.89	-13.36	178.49
Strain-4	10.02	11.23	126.11
	Sum=106.23		Sum d^2=1194.99

A=106.23/5=21.25; S.D.= $(1194.99/5)^{0.5}$ = 15.46

C.V. = (S.D./A)x 100= (15.46/21.25)x 100=72.75

6. C.V. for 120 g/L whey mixed glucose application

Lactobacillus Strains	Lactic Acid X (g/L)	d=X-A	d^2
Strain-1	43.67	12.65	160.02
Strain-2	22.08	-8.94	79.92
Coculture	44.86	13.84	191.55
Strain-3	18.02	-13.00	169.00
Strain-4	26.45	-4.57	20.88
	Sum=155.08		Sum d^2=621.37

A=155.08/5=31.02; S.D.= $(621.37/5)^{0.5}$ = 11.1478

C.V. = (S.D./A)x 100= (11.1478/31.02)x 100=35.93

Summary of C.V. values

Sugar Levels (g/L)	C.V. at Pure Glucose Application	C.V. at Whey mixed Glucose Application
60	73.54	92.76
80	47.31	72.75
100	71.86	35.93

Inference

Among pure glucose applications, the 80 g/L dose with least CV (47.31) gives the most uniform consistent lactic acid production. However, among overall sugar treatments the 120 g/L whey mixed glucose treatment gives least C.V. (35.93), which suggests most uniform lactic acid production values obtained from all the *Lactobacillus* strains at this dose where all the strains perform efficiently. Hence, such a sugar treatment on a bed of pine needles, can have wide industrial applicability for fermentative production of lactic acid by different strains of lactobacilli.

APPENDIX-9

Correlation Analysis for lactic acid production on wheat bran bed material

The Correlation analysis depicts the strength or Degree of relationship between variables. When some definite connection exists between 2 or more groups or series of data a correlation is said to be existing between them. The measurement of correlation is called Correlation coefficient (γ) given by

$$(\gamma) = \sum dX \times dY / [\sum dX^2 \times \sum dY^2]^{1/2}$$

Where X and Y are the two variables

In the present correlation analysis, the X variable represents the amounts of whey lactose applied, while the Y variable represents the lactic acid production with respect to any one pure strain or coculture of lactobacilli.

1. Correlation Analysis for lactic acid production by strain-1 on a bed of wheat bran

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
39.1	-4.2875	18.3826	30	-11.25	126.5625	48.2343
41.68	-1.7075	2.9155	40	-1.25	1.5625	2.1344
44.01	0.6225	0.3875	45	3.75	14.0625	2.3343
48.76	5.3725	28.8638	50	8.75	76.5625	47.0093
$\sum Y=173.55$		$\sum(dY)^2$ =50.5494	$\sum X=165$		$\sum(dX)^2$ =218.75	$\sum(dX).(dY)$ =99.7123

$A_Y=173.55/4=43.3875$; $A_X=165/4=41.25$

$(\gamma) = \sum dX \times dY / [\sum dX^2 \times \sum dY^2]^{1/2} = 99.7125/[218.75 \times 50.5494]^{1/2} = 0.9482$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for strain-1 on wheat bran bed material.

2. Correlation Analysis for lactic acid production by strain-2 on a bed of wheat bran

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
8.56	-8.405	70.6440	30	-11.25	126.5625	94.5563
14.68	-2.285	5.2212	40	-1.25	1.5625	2.8563
19.61	2.645	6.9960	45	3.75	14.0625	9.9188
25.01	8.045	64.7220	50	8.75	76.5625	70.3938
$\sum Y=67.86$		$\sum(dY)^2$ =147.5832	$\sum X=165$		$\sum(dX)^2$ = 218.75	$\sum(dX).(dY)$ =177.7252

$A_Y=67.86/4=16.9650$; $A_X=165/4=41.25$

$(\gamma) = \sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2} = 177.7252/[218.75 \times 147.5832]^{1/2} = 0.9891$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for strain-2 on wheat bran bed material.

3.Correlation Analysis for lactic acid production by coculture on a bed of wheat bran

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
28.60	-15.715	246.9612	30	-11.25	126.5625	176.7938
45.00	0.6850	0.46923	40	-1.25	1.5625	-0.85625
49.41	5.0950	25.9590	45	3.75	14.0625	19.1062
54.25	9.9350	98.7042	50	8.75	76.5625	86.9313
$\sum Y=177.26$		$\sum(dY)^2$ = 372.09	$\sum X=165$		$\sum(dX)^2$ = 218.75	$\sum(dX).(dY)$ =281.9750

$A_Y=177.26/4=44.3150$; $A_X=165/4=41.25$

$(\gamma) = \sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2} = 281.9750/[218.75 \times 372.09]^{1/2} = 0.9883$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for coculture on wheat bran bed material.

4. Correlation Analysis for lactic acid production by strain-3 on a bed of wheat bran

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
15.00	-5.09	25.9081	30	-11.25	126.5625	57.2625
18.05	-2.04	4.1616	40	-1.25	1.5625	2.55
22.15	2.06	4.2436	45	3.75	14.0625	7.725
25.16	5.07	25.7049	50	8.75	76.5625	44.3625
$\sum Y=80.36$		$\sum(dY)^2 =$ 60.0182	$\sum X=165$		$\sum(dX)^2$ =218.7500	$\sum(dX).(dY)$ =111.900

$A_Y=80.36/4=20.09$; $A_X=165/4=41.25$

$(\gamma) = \sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2} = 111.9/[218.75 \times 60.0182]^{1/2} = 0.9765$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for strain-3 on wheat bran bed material.

5. Correlation Analysis for lactic acid production by strain-4 on a bed of wheat bran

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
10.00	-10.78	116.2084	30	-11.25	126.5625	121.275
19.67	-1.11	1.2321	40	-1.25	1.5625	1.3875
23.61	2.83	8.0089	45	3.75	14.0625	10.6125
29.85	9.07	82.2649	50	8.75	76.5625	79.3625
$\sum Y=83.13$		$\sum(dY)^2 =$ 207.7143	$\sum X=165$		$\sum(dX)^2 =$ 218.75	$\sum(dX).(dY)$ =212.6375

$A_Y=83.13/4=20.78$; $A_X=165/4=41.25$

$(\gamma) = \sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2} = 212.6375/[218.75 \times 207.7143]^{1/2} = 0.9975$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for strain-4, on wheat bran bed material.

APPENDIX-10

Correlation Analysis for lactic acid production on pine needles bed material

The Correlation analysis depicts the strength or Degree of relationship between variables. When some definite connection exists between 2 or more groups or series of data a correlation is said to be existing between them. The measurement of correlation is called Correlation coefficient (γ) given by

$(\gamma) = \sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2}$;Where X and Y are the two variables

In the present correlation analysis, the X variable represents the amounts of whey lactose applied, while the Y variable represents the lactic acid production with respect to any one pure strain or coculture of lactobacilli.

1.Correlation Analysis for lactic acid production by strain-1 on a bed of pine needles

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
37.21	-2.90	8.41	30	-10	100	29
39.46	-0.65	0.42	40	0	0	0
43.67	3.56	12.67	50	10	100	35.6
$\sum Y=120.34$		$\sum(dY)^2$ =21.50	$\sum X=120$		$\sum(dX)^2$ =200	$\sum(dX).(dY)$ =64.60

$A_Y=120.34/3=40.11$; $A_X=120/3=40$

$(\gamma) = \sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2}$ =64.60/[200x 21.5]1/2= 0.98514

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for strain-1 on a bed of pine needles.

2.Correlation Analysis for lactic acid production by strain-2 on a bed of pine needles

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
5.19	-6.57	43.16	30	-10	100	65.70
8.02	-3.76	14.13	40	0	0	0
22.08	10.32	106.50	50	10	100	103.20
$\sum Y=35.29$		$\sum(dY)^2$ =163.79	$\sum X=120$		$\sum(dX)^2$ =200	$\sum(dX).(dY)$ =168.90

$A_Y=35.29/3=11.76$; $A_X=120/3=40$

$(\gamma) = \sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2} = 168.90/[200 \times 163.79]^{1/2} = 0.9332$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for strain-2 on a bed of pine needles.

3.Correlation Analysis for lactic acid production by Coculture on a bed of pine needles

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
27.77	-10.05	101	30	-10	100	100.5
40.84	3.02	9.12	40	0	0	0
44.86	7.04	49.56	50	10	100	70.40
$\sum Y=113.47$		$\sum(dY)^2$ =159.68	$\sum X=120$		$\sum(dX)^2$ =200	$\sum(dX).(dY)$ =170.90

$A_Y=113.47/3=37.82$; $A_X=120/3=40$

$(\gamma) = \sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2} = 170.90/[159.68 \times 200]^{1/2} = 0.95632$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for coculture on a bed of pine needles.

4.Correlation Analysis for lactic acid production by strain-3 on a bed of pine needles

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
1.28	-7.78	60.53	30	-10	100	77.8
7.89	-1.17	1.37	40	0	0	0
18.02	8.96	80.28	50	10	100	89.6
$\sum Y=27.19$		$\sum(dY)^2$ =142.18	$\sum X=120$		$\sum(dX)^2$ =200	$\sum(dX).(dY)$ =167.40

$A_Y=27.19/3=9.06$; $A_X=120/3=40$

$(\gamma)=\sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2}=167.40/[142.18 \times 200]^{1/2}=0.9927$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for strain-3, on a bed of pine needles.

5.Correlation Analysis for lactic acid production by strain-4 on a bed of pine needles

Lactic Acid(Y)	$dY=Y-A_Y$	$(dY)^2$	Whey Lactose(X)	$dX=X-A_X$	$(dX)^2$	(dX). (dY)
5.73	-8.34	69.56	30	-10	100	83.40
10.02	-4.05	16.40	40	0	0	0
26.45	12.38	153.26	50	10	100	123.80
$\sum Y=42.20$		$\sum(dY)^2$ =239.22	$\sum X=120$		$\sum(dX)^2$ =200	$\sum(dX).(dY)$ =207.20

$A_Y=42.20/3=14.07$; $A_X=120/3=40$

$(\gamma)=\sum dX \times dY/[\sum dX^2 \times \sum dY^2]^{1/2}=207.20/[200 \times 239.22]^{1/2}=0.9472$

Inference

Hence, a very high degree of positive correlation exists between lactic acid production and application of whey lactose for strain-4, on a bed of pine needles.

APPENDIX-11

t- Test Analysis

t test is applied on samples with small sample size less than 30 and have different mean values. The t value for independent samples 1 and 2, with sample size n_1 and n_2 having mean values X_{M1} and X_{M2} is given by the following expression

$$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$$

$$X_{M1}= \sum X_1/ n_1 \; ; \; X_{M2}= \sum X_2/ n_2$$

Where S is the combined standard deviation of the samples 1 and 2

$$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$$

$$S=[\sum(dX_1)^2 +\sum(dX_2)^2]^{1/2}/(n_1+ n_2-2)^{1/2}$$

dX_1 and dX_2 are the deviations from the mean values in samples 1 and 2. X_1 and X_2 denote the data of sample 1 and 2.

Degrees of freedom= $n_1+ n_2-2$

The value of t obtained by the calculation when matched with table value of t at particular degrees of freedom and given level of significance, can be used to investigate the truth of a null hypothesis about a particular trait affecting both the samples. In the present study the t test has been applied on 2- samples showing lactic acid production values (corresponding to any two levels of whey mixed glucose applications) on wheat bran or pine needles used as bed materials. Comparison of the calculated value of t with the table value of t shall be helpful to investigate the null hypothesis that application of increasing doses (two levels) of sugar has no inhibitory effect (substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli.

1. t-Test for two groups of lactic acid production data corresponding to 80 g/L and 60 g/L levels of whey mixed glucose applied on wheat bran bed material

The lactic acid production values for sample1 are, 41.68, 14.68, 45.00,18.05 and 19.67 at (80 g/L) level; The lactic acid production values for sample2 are, 39.10,8.56,28.60,15.0,10.0 at (60 g/L)

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$;Degrees of freedom=5+5-2=8

$X_{M1}=41.68+14.68+ 45.00+18.05+ 19.67 /5 =27.82$

$X_{M2}=39.10+8.56+28.60+15.0+10.0 /5=20.25$

$\sum(X_1- X_{M1})^2 =(41.68-27.82)^2+(14.68-27.82)^2+(45-27.82)^2+(18.05-27.82)^2+(19.67-27.82)^2$

$\sum(X_1- X_{M1})^2=821.4687$

$\sum(X_2- X_{M2})^2= (39.1-20.25)^2+(8.56-20.25)^2+(28.6-20.25)^2+(15-20.25)^2+ (10-20.25)^2$

$\sum(X_2- X_{M2})^2=694.3261$

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [821.4687+694.3261]^{1/2}/(5+ 5-2)^{1/2}$

S=13.7649

$t= (27.82-20.25)/13.7649 \times [5x5/ 5+5]^{1/2}$

t= 0.8695 (calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore, it is accepted that, application of increasing doses (two levels) of sugar has no inhibitory effect(substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli at 5% level of significance.

2. t-Test for two groups of lactic acid production data corresponding to 110 g/L and 80 g/L levels of whey mixed glucose applied on wheat bran bed material

The lactic acid production values for sample1 are, 44.01, 19.61, 49.41, 22.15and 23.61 at (110 g/L) level; The lactic acid production values for sample2 are, 41.68, 14.68, 45.00, 18.05 and 19.67 at (80 g/L)

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$;Degrees of freedom=5+5-2=8

$X_{M1}=44.01+ 19.61+ 49.41+22.15+ 23.61 /5 =31.76$

$X_{M2}=41.68+ 14.68+ 45.00+ 18.05 + 19.67/5=27.82$

$\sum(X_1- X_{M1})^2= (44.01-31.76)^2+(19.61-31.76)^2+(49.41-31.76)^2+(22.15-31.76)^2+ (23.61-31.76)^2$

$\sum(X_1- X_{M1})^2=767.9821$

$\sum(X_2- X_{M2})^2 =(41.68-27.82)^2+(14.68-27.82)^2+(45-27.82)^2+(18.05-27.82)^2+(19.67-27.82)^2$

$\sum(X_2- X_{M2})^2=821.4687$

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [767.9821+821.4687]^{1/2}/(5+ 5-2)^{1/2}$

$S=14.0954$

$t= (31.76-27.82)/14.0954 \times [5x5/ 5+5]^{1/2}$

t= 0.4419 (calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore, it is accepted that, application of increasing doses (two levels) of sugar has no inhibitory effect (substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli at 5% level of significance.

3. t-Test for two groups of lactic acid production data corresponding to 120 g/L and 110 g/L levels of whey mixed glucose applied on wheat bran bed material

The lactic acid production values for sample1 are, 48.76, 25.01, 54.25, 25.16 and 29.85 at (120 g/L); The lactic acid production values for sample2 are, 44.01, 19.61, 49.41, 22.15and 23.61 at (110 g/L) level

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$;Degrees of freedom=5+5-2=8

X_{M1}=48.76+ 25.01+54.25+ 25.16 + 29.85 /5=36.60

X_{M2}=44.01+ 19.61+ 49.41+22.15+ 23.61 /5 =31.76

$\sum(X_1- X_{M1})^2 =(48.76-36.60)^2+(25.01-36.60)^2+(54.25-36.60)^2+(25.16-36.60)^2+(29.85-36.60)^2$

$\sum(X_1- X_{M1})^2$=770.152

$\sum(X_2- X_{M2})^2= (44.01-31.76)^2+(19.61-31.76)^2+(49.41-31.76)^2+(22.15-31.76)^2+ (23.61-31.76)^2$

$\sum(X_2- X_{M2})^2$=767.9821

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [770.152+767.9821]^{1/2}/(5+ 5-2)^{1/2}$

S=13.8660

$t= [(36.60-31.76)/13.866] \times [5x5/ 5+5]^{1/2}$

t= 0.5519(calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore, it is accepted that, application of increasing doses (two levels) of sugar has no inhibitory effect(substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli at 5% level of significance.

4. t-Test for two groups of lactic acid production data corresponding to 120 g/L and 60 g/L levels of whey mixed glucose applied on wheat bran bed material

The lactic acid production values for sample1 are, 48.76, 25.01, 54.25, 25.16 and 29.85 at (120 g/L) level; The lactic acid production values for sample2 are, 39.10, 8.56, 28.60,15.0,10.0 at (60 g/L) level

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$;Degrees of freedom=5+5-2=8

X_{M1}=48.76+ 25.01+54.25+ 25.16 + 29.85 /5=36.60

X_{M2}=39.10+8.56+28.60+15.0+10.0/5 =20.25

$\sum(X_1- X_{M1})^2 =(48.76-36.60)^2+(25.01-36.60)^2+(54.25-36.60)^2+(25.16-36.60)^2+(29.85-36.60)^2$

$\sum(X_1- X_{M1})^2$=770.152

$\sum(X_2- X_{M2})^2= (39.1-20.25)^2+(8.56-20.25)^2+(28.6-20.25)^2+(15-20.25)^2+ (10-20.25)^2$

$\sum(X_2- X_{M2})^2$=694.3261

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [770.152+694.3261]^{1/2}/(5+ 5-2)^{1/2}$

S=13.52995

$t= [(36.60-20.25)/13.52995] \times [5x5/ 5+5]^{1/2}$

t= 1.9106 (calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore, it is accepted that, application of increasing doses (two levels) of sugar has no inhibitory effect(substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli at 5% level of significance.

5. t-Test for two groups of lactic acid production data corresponding to 120 g/L and 80 g/L levels of whey mixed glucose applied on wheat bran bed material

The lactic acid production values for sample1 are, 48.76, 25.01, 54.25, 25.16 and 29.85 at (120 g/L) level; The lactic acid production values for sample2 are, 41.68, 14.68, 45.00,18.05 and 19.67 at (80 g/L) level

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$;Degrees of freedom=5+5-2=8

X_{M1}=48.76+ 25.01+54.25+ 25.16 + 29.85 /5=36.60

X_{M2}=41.68+ 14.68+ 45.00+18.05+19.67/5 =27.82

$\sum(X_1- X_{M1})^2 =(48.76-36.60)^2+(25.01-36.60)^2+(54.25-36.60)^2+(25.16-36.60)^2+(29.85-36.60)^2$

$\sum(X_1- X_{M1})^2$=770.152

$\sum(X_2- X_{M2})^2 =(41.68-27.82)^2+(14.68-27.82)^2+(45-27.82)^2+(18.05-27.82)^2+(19.67-27.82)^2$

$\sum(X_2- X_{M2})^2$=821.4687

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [770.152+821.4687]^{1/2}/(5+ 5-2)^{1/2}$

S=14.1050

$t= [(36.60-27.82)/14.1050] \times [5x5/ 5+5]^{1/2}$

t= 0.9842 (calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore, it is accepted that, application of increasing doses (two levels) of sugar has no inhibitory effect(substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli at 5% level of significance.

6. t-Test for two groups of lactic acid production data corresponding to 120 g/L and 80 g/L levels of pure glucose applied on wheat bran bed material

The lactic acid production values for sample1 are, 41.00, 15.01, 40.00, 9.75 and 6.00 at (120 g/L) level; The lactic acid production values for sample2 are, 50.01, 23.25, 54.25, 25.50 and 30.75 at (80 g/L) level

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$;Degrees of freedom=5+5-2=8

$X_{M1}=41+ 15.01+40+ 9.75 + 6.00 /5=22.35$

$X_{M2}=50.01+ 23.25+ 54.25+ 25.50 + 30.75/5 =36.75$

$\sum(X_1- X_{M1})^2 = (41-22.35)^2+(15.01-22.35)^2+(40-22.35)^2+(9.75-22.35)^2+(6-22.35)^2$

$\sum(X_1- X_{M1})^2=1139.3031$

$\sum(X_2- X_{M2})^2 =(50.01-36.75)^2+(23.25-36.75)^2+(54.25-36.75)^2+(25.5-36.75)^2+(30.75-36.75)^2$

$\sum(X_2- X_{M2})^2=826.8901$

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [1139.3031+826.8901]^{1/2}/(5+ 5-2)^{1/2}$

$S=15.6772$

$t= [(36.75-22.35)/15.6772] \times [5x5/ 5+5]^{1/2}$

t=1.4523 (calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore, it is accepted that, application of increasing doses (from 80 to 120 g/L) of sugar does not increase the lactic acid production by the pure cultures and coculture of lactobacilli at 5% level of significance.

APPENDIX-12

1. t-Test for two groups of lactic acid production data corresponding to 80 g/L and 60 g/L levels of whey mixed glucose applied on pine needles bed material

The lactic acid production values for sample1 are, 39.46,8.02,40.84,7.89 and 10.02 at (80 g/L) level; The lactic acid production values for sample2 are, 37.21,5.19,27.77,1.28,5.73 at (60 g/L)

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$

$X_{M1}=39.46+8.02+40.84+7.89+ 10.02/5 =21.25$

$X_{M2}=37.21+5.19+27.77+1.28+5.73/5=15.14$

$\sum(X_1- X_{M1})^2 =(39.46-21.25)^2+(8.02-21.25)^2+(40.84-21.25)^2+(7.89-21.25)^2+(10.02-21.25)^2$

$\sum(X_1- X_{M1})^2=1194.99$

$\sum(X_2- X_{M2})^2= (37.21-15.14)^2+(5.19-15.14)^2+(27.77-15.14)^2+(1.28-15.14)^2+ (5.73-15.14)^2$

$\sum(X_2- X_{M2})^2=1025.80$

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2] ^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [1194.99+1025.80] ^{1/2}/(5+ 5-2)^{1/2}$

S=16.7212

$t= (21.25-15.14)/16.7212 \times [5x5/ 5+5]^{1/2}$

t= 0.5494 (calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore the hypothesis that that application of increasing doses (two levels) of sugar has no inhibitory effect(substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli is accepted at 5% level of significance.

2. t-Test for two groups of lactic acid production data corresponding to 120 g/L and 80 g/L levels of whey mixed glucose applied on pine needles bed material

The lactic acid production values for sample1 are, 43.67,22.08,44.86,18.02 and 26.45 at (120g/L) level; The lactic acid production values for sample2 are, 39.46,8.02,40.84,7.89,10.02 at (80 g/L)

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$

$X_{M1}=43.67+22.08+44.86+18.02 +26.45 /5 =31.02$

$X_{M2}=39.46+8.02+40.84+7.89+10.02 /5=21.25$

$\sum(X_1- X_{M1})^2 =(43.67-31.02)^2+(22.08-31.02)^2+(44.86-31.02)^2+(18.02 -31.02)^2+(26.45 -31.02)^2$

$\sum(X_1- X_{M1})^2=621.37$

$\sum(X_2- X_{M2})^2= (39.46-21.25)^2+(8.02-21.25)^2+(40.84-21.25)^2+(7.89-21.25)^2+ (10.02-21.25)^2$

$\sum(X_2- X_{M2})^2=1194.99$

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [621.37+1194.99]^{1/2}/(5+ 5-2)^{1/2}$

S=15.0680

$t= [(31.02-21.25)/15.068] \times [5x5/ 5+5]^{1/2}$

t= 1.025 (calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore the hypothesis that that application of increasing doses (two levels) of sugar has no inhibitory effect(substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli is accepted at 5% level of significance.

3. t-Test for two groups of lactic acid production data corresponding to 80 g/L and 60 g/L levels of whey mixed glucose applied on pine needles bed material

The lactic acid production values for sample1 are, 43.67,22.08,44.86,18.02 and 26.45 at (120g/L) level; The lactic acid production values for sample2 are, 37.21,5.19,27.77,1.28,5.73 at (60 g/L)

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$

$X_{M1}=43.67+22.08+44.86+18.02+26.45/5=31.02$ $X_{M2}=37.21+5.19+27.77+1.28+5.73/5=15.14$

$\sum(X_1- X_{M1})^2 =(43.67-31.02)^2+(22.08-31.02)^2+(44.86-31.02)^2+(18.02 -31.02)^2+(26.45 -31.02)^2$

$\sum(X_1- X_{M1})^2=621.37$

$\sum(X_2- X_{M2})^2= (37.21-15.14)^2+(5.19-15.14)^2+(27.77-15.14)^2+(1.28-15.14)^2+ (5.73-15.14)^2$

$\sum(X_2- X_{M2})^2=1025.80$

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [621.37+1025.80]^{1/2}/(5+ 5-2)^{1/2}$

S=14.35

$t= [(31.02-15.44)/14.35] \times [5x5/ 5+5]^{1/2}$

t= 1.7166 (calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore the hypothesis that that application of increasing doses (two levels) of sugar has no inhibitory effect(substrate inhibition) on lactic acid production by the pure cultures and coculture of lactobacilli is accepted at 5% level of significance.

4. t-Test for two groups of lactic acid production data corresponding to 120 g/L and 80 g/L levels of pure glucose applied on pine needles bed material

The lactic acid production values for sample1 are, 32.56,13.07,34.84,5.74 and 4.56 at (120g/L) level; The lactic acid production values for sample2 are, 43.87,8.72,45.10,21.87and 26.15 at (80 g/L)

$X_{M1}= \sum X_1/ n_1$; $X_{M2}= \sum X_2/ n_2$; $n_1=5$ and $n_2=5$

$X_{M1}=32.56+13.07+34.84+5.74 +4.56 /5 =18.15$

$X_{M2}=43.87+8.72+45.10+21.87+ 26.15 /5=29.14$

$\sum(X_1- X_{M1})^2 =(32.56-18.15)^2+(13.07-18.15)^2+(34.84-18.15)^2+(5.74 -18.15)^2+(4.56 -18.15)^2$

$\sum(X_1- X_{M1})^2=850.70$

$\sum(X_2- X_{M2})^2= (43.87-29.14)^2+ (8.72-29.14)^2+(45.10-29.14)^2+(21.87-29.14)^2+ (26.15-29.14)^2$

$\sum(X_2- X_{M2})^2=950.4529$

$t= (X_{M1} - X_{M2})/S \times [n_1 \times n_2/ n_1+ n_2]^{1/2}$

$S= [\sum(X_1- X_{M1})^2 +\sum(X_2- X_{M2})^2]^{1/2}/(n_1+ n_2-2)^{1/2}$

$S= [850.70+950.4529]^{1/2}/(5+ 5-2)^{1/2}$

$S=15.004$

$t= [(29.14-18.15)/15.004] \times [5x5/ 5+5]^{1/2}$

t= 1.158(calculated value) at 8 degrees of freedom

t=2.306 (Table value of t at 8 degrees of freedom and 5% level of significance)

Since the calculated value of t is lesser than its table value, therefore the null hypothesis that that application of increasing doses (from 80 to 120 g/L) of sugar has not increased lactic acid production by the pure cultures and coculture of lactobacilli is accepted at 5% level of significance.

APPENDIX-13

ANOVA Test for effect of glucose

ANOVA has been applied on the lactic acid production data resulting from the different inputs of pure glucose as carbon source, corresponding to the different pure cultures and coculture under study, utilizing liquid state batch fermentation

Inputs of Pure glucose	Strain-1 LA	Strain-2 LA	Coculture LA	Strain-3 LA	Strain-4 LA	Total
60	51.33	29.53	53.24	41.31	46.24	$221.65=\sum X_1$
80	72.85	67.71	73.68	69.58	71.20	$355.02=\sum X_2$
100	92.82	88.31	93.57	89.12	91.56	$455.38=\sum X_3$
120	81.17	72.31	84.57	74.02	75.63	$387.70=\sum X_4$
Total	∑Strain-1 =298.17	∑Strain-2 =257.86	∑Coculture =305.06	∑Strain-3 =274.03	∑Strain-4 =284.63	

LA Denotes lactic acid concentration (g/L); X_1, X_2, X_3 and X_4 represent the levels of inputs
$\sum X$ shows the sum of lactic acid productions in a row corresponding to a particular input level

(A) **Calculation of sum of squares**

1. **Sum of squares of all items** = $(51.33)^2+$
$(72.85)^2+(92.82)^2+(81.17)^2+(29.53)^2+(67.71)^2+(88.31)^2+(72.31)^2+$

$(53.24)^2+ (73.68)^2+(93.57)^2+(84.57)^2+(41.31)^2+(69.58)^2+(89.12)^2+(74.02)^2+(46.24)^2+$

$(71.20)^2+ (91.56)^2 + (75.63)^2= 107,080.6751$

2. **Sum of values all items**(T)=1419.75

3. **Total number of items** (N) = 4+4+4+4+4=20

4. **Total sum of squares SST**= Sum of squares of all items-Correction factor $(T^2)/N$

SST=$107,080.6751-[(1419.75)^2/20]=6296.1720$

5.**Sum of squares between the columns (SSC)**= $(\sum Strain\text{-}1)^2/N_{C1}+(\sum Strain\text{-}2)^2/N_{C2}+(\sum Coculture)^2/N_{CC}+(\sum Strain\text{-}3)^2/N_{C3}+(\sum Strain\text{-}4)^2/N_{C4}$

N_{C1}, N_{C2}, N_{CC}, N_{C3} and N_{C4} ; Represent the number of items in each of the column.

Here, $N_{C1}= N_{C2}= N_{CC}= N_{C3} = N_{C4}=4$

Thus, SSC= $[298.17^2+257.86^2+305.06^2+274.03^2+284.63^2]/4 - (1419.75)^2/20$

SSC=101,141.3525-100,784.5031=356.8493

6. **Sum of squares between the rows (SSR)** = $(\sum X_1)^2/N_{R1}+(\sum X_2)^2/N_{R2}+(\sum X_3)^2/N_{R3}+(\sum X_4)^2/N_{R4}$

Here, $N_{R1}= N_{R2}= N_{R3}= N_{R4} = 5$;

N_R Represents the number of items in each of row

Thus, SSR= $[221.65^2+355.02^2+455.38^2+387.70^2]/5 - 1419.75^2/20$

SSR=106,570.0315-100,784.5030=5785.5285

7. **Sum of square of error (SSE)**=SST-[SSC+SSR]

SSE=6296.1720-[356.8493+5785.5285]=153.8085

(B)**Degrees of freedom (df)**

1. df for Total variance=CxR-1=5x4-1=19
2. df for variance between columns=C-1=5-1=4
3. df for variance between rows=R-1=4-1=3
4. df for residual variance = (C-1)(R-1) =(5-1) (4-1)=12

Where C and R are number of columns and rows

(C) **Calculation of mean squares MS**

1. MS between columns (MSC)=SSC/C-1=356.8493/5-1 =89.2123
2. MS between rows (MSR) = SSR/R-1 = 5785.5285/4-1 =1928.5095
3. MS error (MSE) =SSE/(C-1)(R-1) =153.8085/12 =12.8173

ANOVA Table

Sources of variation	Sum of squares	(d.f.) degrees of freedom	Mean Squares	F- Calculated value	F- Table Value 5% level
Between columns	(SSC) 356.8493	(C-1) 4	MSC 89.2123	F=MSC/MSE =89.2123/12.8173 =6.9603	3.26
Between rows	(SSR) 5785.5285	(R-1) 3	MSR 1928.5095	F=MSR/MSE =1928.5095/12.8173 =150.46	3.49
SS residual	(SSE) 153.8085	(C-1) (R-1) 4x3=12	MSE 12.8173		
Total	6296.1720	19			

Inference

F ratio of the columns in the ANOVA table, concerned with the strains of lactobacilli is higher than its table value at 5% (3.26). F ratio of the rows, concerned with the treatment levels of pure glucose is higher than its table value at 5% (3.49).From the above data of F values for columns and rows it can be inferred that differences in the lactic acid production, concerned with application of different levels of pure glucose and the difference in *Lactobacillus* strains, are significant at 5% level. An excessively high value of F ratio for the rows (concerned with input levels of pure glucose), as compared to the table values, indicates that the differences in glucose input level plays a predominant role in causing changes in lactic acid production.

APPENDIX-14

ANOVA Test for effect of initial pH

ANOVA has been applied on the lactic acid production data resulting from the different inputs of levels of initial pH, applied to the different pure cultures and coculture under study, utilizing liquid state batch fermentation

Variations in initial pH	Strain-1 LA	Strain-2 LA	Coculture LA	Strain-3 LA	Strain-4 LA	Total
pH 6.00	49.31	38.61	52.56	46.91	48.89	236.28=$\sum X_1$
pH 6.50	51.75	29.53	53.28	41.40	46.26	222.22=$\sum X_2$
pH 6.75	45.67	41.46	55.61	40.45	35.80	218.99=$\sum X_3$
pH 7.00	36.41	34.88	46.87	29.56	32.26	179.98=$\sum X_4$
Total	$\sum$Strain-1 =183.14	$\sum$Strain-2 =144.48	$\sum$Coculture =208.32	$\sum$Strain-3 =158.32	$\sum$Strain-4 =163.21	

LA Denotes lactic acid concentration (g/L);X_1, X_2, X_3 and X_4 represent the levels of initial pH;$\sum X$ shows the sum of lactic acid productions in a row corresponding to a particular initial pH value

(A) **Calculation of sum of squares**

1. **Sum of squares of all items** =

$(49.31)^2+(51.75)^2+(45.67)^2+(36.41)^2+(38.61)^2+(29.53)^2+(41.46)^2+(34.88)^2+$

$(52.56)^2+(53.28)^2+(55.61)^2+(46.87)^2+(46.91)^2+(41.40)^2+(40.45)^2+(29.56)^2+(48.89)^2+$

$(46.26)^2+(35.80)^2+(32.26)^2=37,986.9331$

2. **Sum of values all items**(T)=857.47

3. **Total number of items** (N) = 4+4+4+4+4=20

4. **Total sum of squares SST**= Sum of squares of all items-Correction factor $(T^2)/N$

$SST=37,986.9331-[(857.47)^2/20]=1224.1930$

5. **Sum of squares between the columns (SSC)**= $(\sum\text{Strain-1})^2/N_{C1}+(\sum\text{Strain-2})^2/N_{C2}+(\sum\text{Coculture})^2/N_{CC}+(\sum\text{Strain-3})^2/N_{C3}+(\sum\text{Strain-4})^2/N_{C4}$

N_{C1}, N_{C2}, N_{CC}, N_{C3} and N_{C4} ; Represent the number of items in each of the column.

Here, $N_{C1}= N_{C2}= N_{CC}= N_{C3} = N_{C4}=4$

Thus, SSC= $[183.14^2+144.48^2+208.32^2+158.32^2+163.21^2]/4 - 857.47^2/20$

SSC=37,378.6697-36,762.7400=615.9296

6. **Sum of squares between the rows (SSR)** = $(\sum X_1)^2/N_{R1}+(\sum X_2)^2/ N_{R2}+(\sum X_3)^2/ N_{R3} + (\sum X_4)^2/ N_{R4}$

Here, $N_{R1}= N_{R2}= N_{R3}= N_{R4} = 5$;

N_R Represents the number of items in each of row

Thus, SSR= $[236.28^2+222.22^2+218.99^2+179.98^2]/5 - (857.47)^2/20$

SSR=37,111.87746-36,762.74005=349.13

7. **Sum of square of error(SSE)**=SST-[SSC+SSR]

SSE=1224.1930-[615.9296+349.13]=259.1334

(B)**Degrees of freedom (df)**

1. df for Total variance=CxR-1=5x4-1=19
2. df for variance between columns=C-1=5-1=4
3. df for variance between rows=R-1=4-1=3
4. df for residual variance = (C-1)(R-1) = (5-1) (4-1) =12

Where C and R are number of columns and rows

(C) **Calculation of mean squares MS**

1. MS between columns (MSC)=SSC/C-1=615.9296/5-1 =153.9894
2. MS between rows (MSR) = SSR/R-1 = 349.13/4-1 =116.3766
3. MS error (MSE) =SSE/(C-1)(R-1) = 259.1334/(5-1) (4-1) =21.5945

ANOVA Table

Sources of variation	Sum of squares	d.f.	Mean Squares	F- Calculated value	F- Table Value 5% level
Between columns	(SSC) 615.9296	(C-1) 4	MSC 153.9894	F=MSC/MSE =7.1375	3.26
Between rows	(SSR) 349.13	(R-1) 3	MSR 116.3766	F=MSR/MSE =5.3903	3.49
SS residual	(SSE) 249.133	(C-1) (R-1) 4x3=12	MSE 21.594		
Total	1224.1930	19			

Inference

F ratio of the columns, concerned with the strains of lactobacilli is higher than its table value at 5% (3.26). F ratio of the rows, concerned with the application of different initial pH values, is higher than its table value at 5% (3.49).From the above data of F values for columns and rows it can be inferred that differences in the lactic acid production, concerned with the difference in of initial pH values and differences in *Lactobacillus* strains, are significant at 5% level.

APPENDIX-15

ANOVA Test for effect of whey mixed glucose on wheat bran bed material

ANOVA has been applied on the lactic acid production data resulting from the different inputs of whey mixed glucose corresponding to the different pure cultures and coculture under study utilizing wheat bran as bed material .

Inputs of Whey mixed glucose	Strain-1 LA	Strain-2 LA	Coculture LA	Strain-3 LA	Strain-4 LA	Total
60	39.10	8.56	28.60	15.00	10.00	$101.26=\sum X_1$
80	41.68	14.68	45.00	18.05	19.67	$139.08=\sum X_2$
110	44.01	19.61	49.41	22.15	23.61	$158.79=\sum X_3$
120	48.76	25.01	54.25	25.16	29.85	$183.03=\sum X_4$
Total	∑Strain-1 =173.55	∑Strain-2 =67.86	∑Coculture =177.26	∑Strain-3 =80.36	∑Strain-4 =83.13	

LA Denotes lactic acid concentration (g/L); X_1, X_2, X_3 and X_4 represent the levels of inputs; $\sum X$ shows the sum of lactic acid productions in a row corresponding to a particular input level

(A) Calculation of sum of squares

1. **Sum of squares of all items**

$=(39.1)^2+(41.68)^2+(44.01)^2+(48.76)^2+(8.56)^2+(14.68)^2+(19.61)^2+(25.01)^2+$

$(28.6)^2+(45.0)^2+(49.41)^2+(54.25)^2+(15.0)^2+(18.05)^2+(22.15)^2+(25.16)^2+(10.0)^2+$

$(19.67)^2+(23.61)^2+(29.85)^2= 20,715.9699$

2. **Sum of values all items**(T)=582.16

3. **Total number of items** (N) = 4+4+4+4+4=20

4. **Total sum of squares SST=** Sum of squares of all items-Correction factor $(T^2)/N$

SST=20,715.9699-[$(582)^2$/20]=3770.4566

5. **Sum of squares between the columns (SSC)=** $(\sum Strain\text{-}1)^2/N_{C1}+(\sum Strain\text{-}2)^2/ N_{C2}+(\sum Coculture)^2/ N_{CC}+ (\sum Strain\text{-}3)^2/ N_{C3}+ (\sum Strain\text{-}4)^2/ N_{C4}$

N_{C1}, N_{C2}, N_{CC}, N_{C3} and N_{C4} ; Represent the number of items in each of the column.

Here, $N_{C1}= N_{C2}= N_{CC}= N_{C3} = N_{C4}=4$

Thus, SSC= [173.55^2+67.86^2+177.26^2+80.36^2+83.13^2]/4 - 582.16^2/20

SSC=19,878.50255-16,945.51325=2932.9893

6. **Sum of squares between the rows (SSR)** = $(\sum X_1)^2/N_{R1}+(\sum X_2)^2/ N_{R2}+(\sum X_3)^2/ N_{R3}+ (\sum X_4)^2/ N_{R4}$

Here, $N_{R1}= N_{R2}= N_{R3}= N_{R4} = 5$;

N_R Represents the number of items in each of row

Thus, SSR= [101.26^2+139.08^2+158.79^2+183.03^2]/5 - 582.16^2/20

SSR=17,662.21580-16,945.51325=716.70255

7. **Sum of square of error(SSE)**=SST-[SSC+SSR]

SSE=3770.4566-[2932.9383+716.7025]=120.7582

(B)Degrees of freedom (df)

1. df for Total variance=CxR-1=5x4-1=19
2. df for variance between columns=C-1=5-1=4
3. df for variance between rows=R-1=4-1=3
4. df for residual variance =(C-1)(R-1)=(5-1) (4-1)=12

Where C and R are number of columns and rows

(C) **Calculation of mean squares MS**

1. MS between columns (MSC)=SSC/C-1=2932.9893/5-1 =733.2473

2. MS between rows (MSR) = SSR/R-1 = 716.70255/4-1 =238.9008

3. MS error (MSE) =10.0632

ANOVA Table

Sources of variation	Sum of squares	d.f.	Mean Squares	F- Calculated value	F- Table Value 5% level
Between columns	(SSC) 2932.9893	(C-1) 4	MSC 733.2473	F=MSC/MSE =733.2473/10.0631 =72.86	3.26
Between rows	(SSR) 716.7025	(R-1) 3	MSR 238.9008	F=MSR/MSE =238.9008/10.0631 =23.7401	3.49
SS residual	(SSE) 120.7581	(C-1) (R-1) 4x3=12	MSE 10.0631		
Total	3770.4499	19			

Inference

F ratio of the columns, concerned with the strains of lactobacilli is higher than its table value at 5% (3.26). F ratio of the rows, concerned with the treatment levels of whey glucose is higher than its table value at 5% (3.49).From the above data of F values for columns and rows it can be inferred that differences in the lactic acid production, concerned with the variation in *Lactobacillus* strains and levels of whey mixed glucose applied on wheat bran bed ,are significant at 5% level of significance.

APPENDIX-16

ANOVA Test for effect of whey mixed glucose on pine needles bed material

ANOVA has been applied on the lactic acid production data resulting from the different inputs of whey mixed glucose corresponding to the different pure cultures and coculture under study utilizing pine needles as bed material .

Inputs of Whey mixed glucose	Strain-1 LA	Strain-2 LA	Coculture LA	Strain-3 LA	Strain-4 LA	Total
60	37.21	5.19	27.77	1.28	5.73	77.18=$\sum X_1$
80	39.46	8.02	40.84	7.89	10.02	106.23=$\sum X_2$
120	43.67	22.08	44.86	18.02	26.45	155.08=$\sum X_3$
Total	∑Strain-1 =120.34	∑Strain-2 =35.29	∑Coculture =113.47	∑Strain-3 =27.19	∑Strain-4 =42.20	

LA Denotes lactic acid concentration (g/L););X_1, X_2, X_3 and X_4 represent the levels of inputs ;$\sum X$ shows the sum of lactic acid productions in a row corresponding to a particular input level

(A) **Calculation of sum of squares**

1. **Sum of squares of all items**

$=(37.21)^2+(39.46)^2+(43.67)^2+(5.19)^2+(8.02)^2+(22.08)^2+(27.77)^2+(40.84)^2+$

$(44.86)^2+(1.28)^2+(7.89)^2+(18.02)^2+(5.73)^2+(10.02)^2+(26.45)^2$

$= 11,103.4723$

2. **Sum of values all items** (T)=338.49

3. **Total number of items** (N) = 3+3+3+3+3=15

4. **Total sum of squares SST**= Sum of squares of all items-Correction factor $(T^2)/N$

$SST=11,103.4723-|(338.49)^2/15|=3465.107$

5. **Sum of squares between the columns (SSC)**= $(\sum Strain\text{-}1)^2/N_{C1}+(\sum Strain\text{-}2)^2/N_{C2}+(\sum Coculture)^2/N_{CC}+(\sum Strain\text{-}3)^2/N_{C3}+(\sum Strain\text{-}4)^2/N_{C4}$

N_{C1}, N_{C2}, N_{CC}, N_{C3} and N_{C4} ; Represent the number of items in each of the column.

Here, $N_{C1}= N_{C2}= N_{CC}= N_{C3} = N_{C4}=3$

Thus, SSC= $[120.34^2+35.29^2+113.47^2+27.19^2+42.20^2]/3 - [(338.49)^2/15]$

SSC=10,374.2255-7638.3653=2735.8602

6. **Sum of squares between the rows (SSR)** = $(\sum X_1)^2/N_{R1}+(\sum X_2)^2/N_{R2}+(\sum X_3)^2/N_{R3}+(\sum X_4)^2/N_{R4}$

Here, $N_{R1}= N_{R2}= N_{R3}= N_{R4} = 5$; N_R Represents the number of items in each of row

Thus, SSR= $[77.18^2+106.23^2+155.08^2]/5 - [(338.49)^2/15]$

SSR=8258.2743-7368.3653=619.9090

7. **Sum of square of error (SSE)**=SST-[SSC+SSR]

SSE=3465.107-[2735.8602+619.9090]=109.3378

(B) **Degrees of freedom (df)**

1. df for Total variance=CxR-1=5x3-1=14
2. df for variance between columns=C-1=5-1=4
3. df for variance between rows=R-1=3-1=2
4. df for residual variance=(C-1)(R-1)=(5-1) (3-1)=8

Where C and R are number of columns and rows

(C) **Calculation of mean squares MS**

1. MS between columns (MSC)=SSC/C-1=2735.8602/5-1 =683.9650
2. MS between rows (MSR) = 619.90904/3-1 = 309.9545
3. MS error (MSE) =13.6672

ANOVA Table

Sources of variation	Sum of squares	d.f.	Mean Squares	F- Calculated value	F- Table Value 5% level
Between columns	(SSC) 2735.8602	(C-1) 4	MSC 683.9650	F=MSC/MSE =50.0442	3.84
Between rows	(SSR) 619.9090	(R-1) 2	MSR 309.9542	F=MSR/MSE =22.6786	4.46
SS residual	(SSE) 109.3376	(C-1) (R-1) 4x2=8	MSE 13.6672		
Total	3465.41068	14			

Inference

F ratio of the columns, concerned with the different strains of lactobacilli is higher than its table value at 5% (3.84). F ratio of the rows, concerned with the treatment levels of whey glucose is higher than its table value at 5% (4.46).From the above data of F values for columns and rows it can be inferred that differences in the lactic acid production, due to the variation in *Lactobacillus* strains and levels of whey mixed glucose applied on pine needles bed, are significant at 5% level.

APPENDIX-17

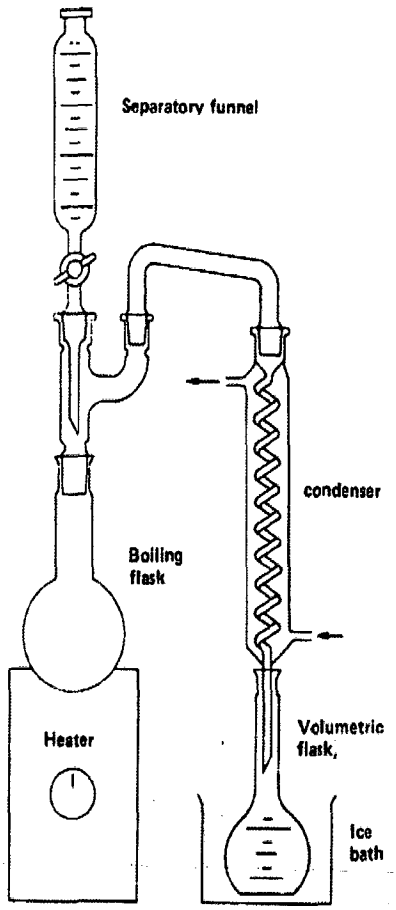

Figure 31 Distillation apparatus for determination of pentosan

Lignocellulosic materials used as bed materials in solid state fermentation contain certain amount of noncellulosic carbohydrates known as hemicelluloses, which mainly contain pentosans. The distillation apparatus shown in Figure 31, consists of a round bottom boiling flask placed over an electric heater, a graduated separating funnel, a condenser, a receiving volumetric flask (placed in ice bath) joint by two way and three way connecting tubes. All connections utilize ground glass joints. Pentosans are converted to in boiling in HCl to furfural which is collected in the distillate and stored in the volumetric flask for spectrophotometric analysis.